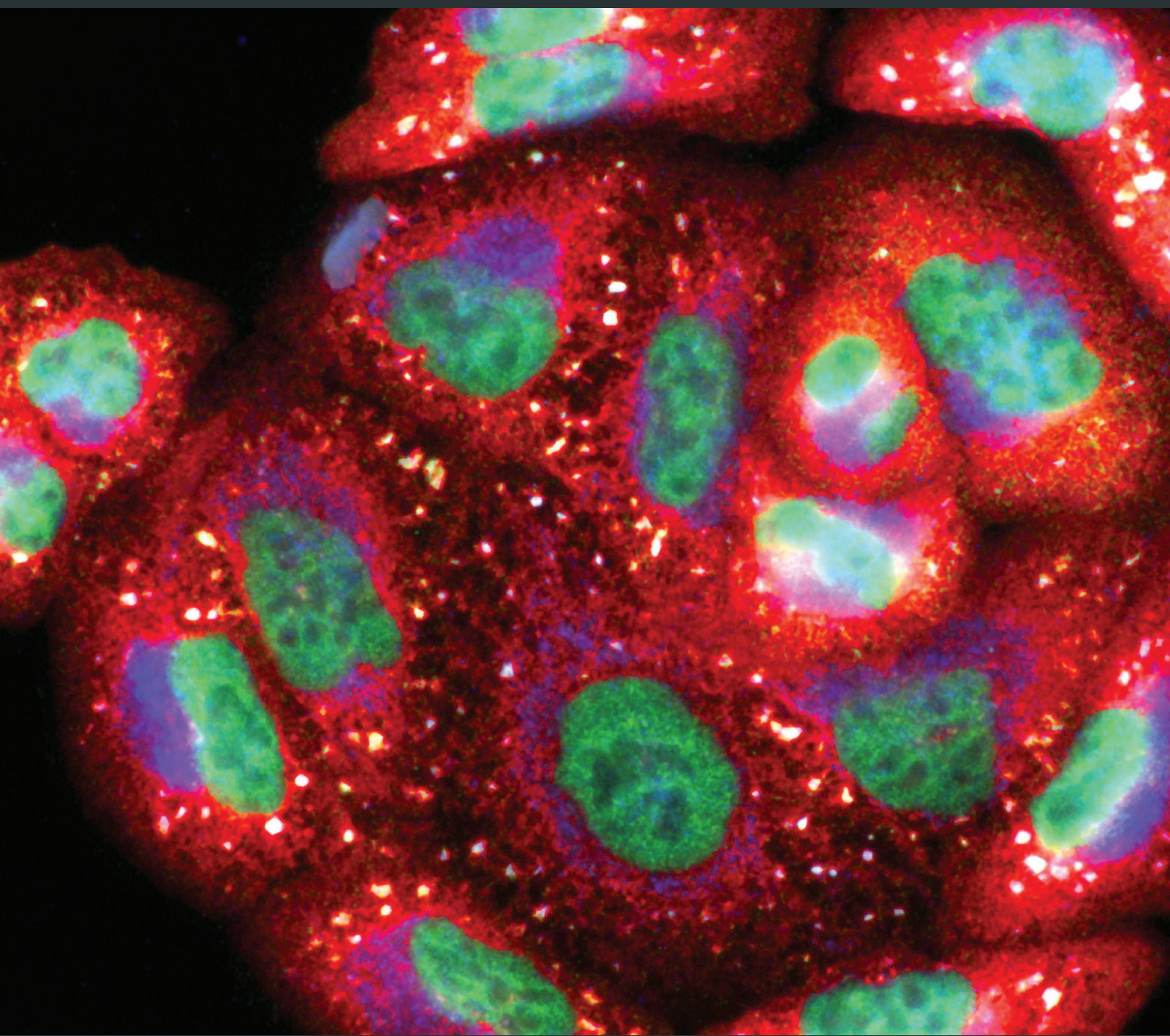
Oxidative Medicine and Cellular Longevity

Plant-Derived Antioxidants in Disease Prevention

Guest Editors: Renata Szymanska, Pavel Pospisil, and Jerzy Kruk



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Editorial Board

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 1920208, 2 pages
http://dx.doi.org/10.1155/2016/1920208

Editorial

Plant-Derived Antioxidants in Disease Prevention

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Received 1 December 2016; Accepted 1 December 2016

Plant-derived antioxidants are a large group of natural products with reducing or radical-scavenging capacity. Due to their potent preventive, as well as therapeutic actions, these compounds receive a great deal of attention by not only scientists but also pharmacologists and physicians.

Maintenance of redox homeostasis plays a central role in health and disease prevention. Oxidative stress is generated by unbalance between reactive oxygen species (ROS) and antioxidants. Excess of ROS leads to degradation of lipids, proteins and nucleic acids and thus may lead to oxidative damage of cells and in a consequence to overexpression of oncogenes, mutagens formation, induction of atherogenic activity, or inflammation. Oxidative stress is suggested to play a major role in pathogenesis of cardiovascular diseases, neurodegeneration, cancers, immune disorders, diabetes, aging, and others. Plants, especially dietary fruits and vegetables, are a rich source of antioxidants. It is postulated that antioxidants show health benefits through direct reduction of oxidative stress. In the body, antioxidant network works in concert through several different mechanisms: ROS scavenging, termination of lipid peroxidation, or chelating of metals. Despite the fact that broad knowledge of antioxidant structures, properties, and biological actions has been gathered, many aspects required clarification and further studies. Relatively little is known about cellular mechanisms of their therapeutic potential, interactions with other compounds, appropriate dosage, and effectiveness of a treatment (especially their effect in randomized clinical trials). Furthermore, the bioactiveness of a large number of natural compounds remains unknown. Well-known antioxidants, as well as those newly discovered, raise hopes for their use in the prevention and treatment of the above-mentioned diseases.

In this special issue, an attempt has been made to gather articles that update our understanding about the role of plant-derived antioxidants in disease prevention. These reports filled the gaps in the field of antioxidants research, allow better understanding of their action, and facilitate their future usage in diseases prevention and treatment. The special issue complies sixteen (16) excellent articles including four (4) reviews, eleven (11) research papers, and one (1) clinical study, which show current and recent developments in plant-derived antioxidant research.

The review by S. Perrone et al. summarizes the latest knowledge about the role of plant-derived xanthophyll, lutein, as an antioxidant and anti-inflammatory agent in animal model and human, with special focus on newborns. An interesting review by Y. Zhao et al. describes mechanism of action and the clinical status of ligustrazine (tetramethylpyrazine), a natural compound from *Ligusticum wallichii*, which is extensively used in Chinese medicine for treatment of such diseases as cardiovascular disorders, diabetes, cancers, or liver injury. S. Ojha et al.'s review is focused on the role on plant-derived compounds for counteracting cisplatin-induced nephrotoxicity. Cisplatin is a commonly used chemotherapeutic, which very often induces organ toxicity, in particular of kidneys and ears. Thus, the authors describe the most suitable plant-originated compounds that are able to combat cisplatin-induced nephrotoxicity. A nice

"methodological" review provided by A. M. Pisoschi et al. gathers the information about analytical methods which can be applied for measurements of antioxidants and antioxidant capacity of plant-derived products. This article describes the antioxidant extraction procedures and discusses advantages and disadvantages of commonly used analytical methods for antioxidant detection.

ROS play a major role in pathogenesis of numerous diseases. It is believed that application of exogenous antioxidants is a promising strategy to suppress oxidative stress associated with those disorders or *via* other still not recognized mechanisms. In this special issue most of research articles show that plant-derived compounds (or their extracts) acts successfully as suppressors of oxidation and can be used in the future as therapeutic agents. The published papers show data obtained either on (1) cell lines or (2) on the animal models. The research article by P.-H. Li et al. shows that ethyl acetate and dichloromethane extracts of medicinal plant *Equisetum ramosissimum* inhibit the growth of human malignant melanomas. Authors in *in vitro* experiments tried also to explain the mechanism of their action, which probably is connected with caspase induction. Likewise, J. Ferreira Campos et al. evaluated the chemical profile of ethanol extract of *Senna velutina* leaves and showed its cytotoxic effect against leukemic cells. Next, *Plinia peruviana* (*Jaboticaba*) fruit peels, which is a rich source of phenolic compounds and well-known antioxidant, were examined by H. S. Pitz et al.'s group. They have reported that a hydroalcoholic extract of this Brazilian plant has a positive potential during the wound healing. In turn, B. Zhou et al. have explored the effect of soy oligopeptides in UVB-induced acute photodamage of human skin. Topically applied soy oligopeptides showed protective effects by increased Bcl-2 protein expression and decreased CDPs-positive cells, sunburn cells, apoptotic cells, p53, and Bax proteins expression in epidermis layer of UVB-irradiated foreskin.

R. H. Oliveira Lopes et al. conducted the research on animal models. They treated hyperlipidemic Wistar rats with hydroethanolic extract of *Curatella americana* leaves. They have demonstrated that, as compared to the control, this extract contains compounds that have ability to lower cholesterol and triglycerides levels in tested rats. The study of M. Xu et al. showed prophylactic effect of resveratrol on the incidence of portal vein system thrombosis in rat fibrosis model. The protective effects of resveratrol, among others, were connected with decrease in platelet aggregation, ROS production, and increase of nitric oxide synthesis. P. P. de Toledo Espinodola et al. group have also evaluated the antihyperlipidemic effects of plant extract. They tested root water extract of guavira (*Campomanesia adamantium*) on lipid profile of hyperlipidemic Wistar rats. The extract, rich in phenolic compounds (mostly gallic and ellagic acids), had ability to lower lipid peroxidation and improve lipid serum level in animals. R. M. Perez-Gutierrez et al. isolated six novel flavonoids from the bark of *Eysenhardtia polystachya*. They tested their activity on streptozotocin-induced diabetic mice and found that newly discovered phenolics are potent antioxidants and can have therapeutic effects in diabetes. M. Kluknavsky et al. examined the effect of (−)-epicatechin on locomotor activity and hypertension in commonly used animal model of hypertension, spontaneously hypertensive rats. (−)-Epicatechin treatment prevented from hypertension and reduced locomotor hyperactivity which was, among others, the results of increase of nitric oxide synthase and total antioxidants capacity in the blood. H. Li et al. studied the protective effect of astragalan, an acidic polysaccharide isolated from medicinal plant, *Astragalus membranaceus*, against neurotoxin 6-hydroxydopamine that can cause parkinsonism. Experiments, which were performed on *Caenorhabditis elegans* models, have shown that *Astragalus* polysaccharide shows a great therapeutic potential in neurodegeneration treatment. R. C. L. Affonso et al. investigated whether aqueous extract of coffee bean residual press cake improved the skin wound healing. They found that coffee extracts significantly reduced wound area size on the inflammatory phase.

The article provided by X. Li et al. describes the results of clinical trial combined with preclinical studies on rat model. A clinical trial experiments confirmed the association of oxidants/antioxidants unbalance with coronary chronic total occlusion. They examined 399 patients at age of 80–89. The preclinical studies encompass rat treatment with a polysaccharide isolated from endothelium corneum gigeriae galli. In both cases the authors have measured the level of superoxide dismutase 3, nitric oxide, endothelial nitric oxide synthase, and malondialdehyde. The obtained results have shown that the tested polysaccharide could be used as a potential therapeutic agent for coronary chronic total occlusion in very old patients.

Taken altogether, the data presented in this special issue cover a series of topics addressing the role of plant-derived antioxidants in different oxidative stress-related diseases prevention. We believed that the papers published in this special issue enrich our understanding of a physiological action of natural products but also provide promising perspectives on their future usage as therapeutic agents. We are sure that all the information provided in this issue will be of broad interest.

Acknowledgments

We would like to thank all the authors, reviewers, and editorial staff who contributed to the preparation of this special issue.

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Pavel Pospisil
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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9130976, 36 pages
http://dx.doi.org/10.1155/2016/9130976

Review Article

Antioxidant Capacity Determination in Plants and Plant-Derived Products: A Review

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Received 26 June 2016; Revised 24 September 2016; Accepted 10 October 2016

Academic Editor: Jerzy Kruk

The present paper aims at reviewing and commenting on the analytical methods applied to antioxidant and antioxidant capacity assessment in plant-derived products. Aspects related to oxidative stress, reactive oxidative species' influence on key biomolecules, and antioxidant benefits and modalities of action are discussed. Also, the oxidant-antioxidant balance is critically discussed. The conventional and nonconventional extraction procedures applied prior to analysis are also presented, as the extraction step is of pivotal importance for isolation and concentration of the compound(s) of interest before analysis. Then, the chromatographic, spectrometric, and electrochemical methods for antioxidant and antioxidant capacity determination in plant-derived products are detailed with respect to their principles, characteristics, and specific applications. Peculiarities related to the matrix characteristics and other factors influencing the method's performances are discussed. Health benefits of plants and derived products are described, as indicated in the original source. Finally, critical and conclusive aspects are given when it comes to the choice of a particular extraction procedure and detection method, which should consider the nature of the sample, prevalent antioxidant/antioxidant class, and the mechanism underlying each technique. Advantages and disadvantages are discussed for each method.

1. Introduction

Metabolism implies oxidative processes vital in cell survival. In the course of molecular oxygen stepwise reduction, a series of reactive oxygenated species occur [1–3]. Reactive species may be oxygenated/nitrogenated free radicals defined as chemical species possessing an unpaired electron in the valence shell (superoxide anion radical $O_2^{\bullet -}$, hydroxyl $HO^{\bullet}$, hydroperoxyl $HO_2^{\bullet}$, peroxyl $ROO^{\bullet}$, alkoxyl $RO^{\bullet}$, nitric oxide $NO^{\bullet}$, peroxynitrite $ONOO^-$, and nitrogen dioxide NO_2) or neutral molecules (H_2O_2 or HClO) [4–7].

Free radicals generated in aerobic metabolism are involved in a series of regulatory processes such as cell proliferation, apoptosis, and gene expression. When generated in excess, free radicals can counteract the defense capability of the antioxidant system, impairing the essential biomolecules in the cell by oxidizing membrane lipids, cell proteins, carbohydrates, DNA, and enzymes. Oxidative stress results in cytotoxic compounds occurrence (malonyl dialdehyde, 4-hydroxynonenal) and alters the oxidant-antioxidant balance (redox homeostasis) that characterizes normal cell functioning [2–4].

With respect to alteration in the protein structure, amino acid oxidation, free radical-induced cleavage, and cross-linking due to reaction with lipid peroxidation products may occur [8]. In nucleic acids, structural alterations imply generation of base-free sites, deletions, oxidation of bases, frame shifts, strand breaks, DNA-protein cross-links, and chromosomal arrangements. The peroxyl radicals and the Fenton-generated OH radicals can induce the oxidation not only of purine and pyrimidine bases but also of the deoxyribose moiety [9, 10]. Regarding influences that involve sugar chemistry, oxygenated free radicals which resulted in early glycation stages have been proven to be contributors to glycoxidative damage: glycolaldehyde that results in the initial stages of nonenzymatic glycosylation is noncyclizable

and may undergo tautomerization, yielding enediols that are easily subject to autooxidation. This step is initiated and propagated by superoxide radical. α- and β-dicarbonyls may also result during this glycolaldehyde autooxidation [11]. Peroxidation of lipids means primarily the attack to the fatty acid's chain by a radical, which abstracts a hydrogen atom from a methylene group, with polyunsaturated fatty acids being the most susceptible to undergo this process. $OH^{\bullet}$, as one of the most active radical species, and $HO_2^{\bullet}$ attack lipid substrates (L-H), yielding the corresponding lipid radicals $L^{\bullet}$. The attack on polyunsaturated fatty acids by singlet oxygen can yield lipid peroxides [12, 13].

In recent studies, it has been repeatedly asserted that oxidative stress not only is not limited to free radical-induced damage on biomolecules but also involves perturbation of cellular redox status, which has been described as "a disruption in redox signaling and control"; hence the antioxidant system implies more than mere free radical capture [14–17].

Oxidative stress-induced pathology includes cancer [18, 19], cardiovascular disease [20], neural disorders [21], Alzheimer's disease [22], mild cognitive impairment [23], Parkinson's disease [24], alcohol induced liver disease [25], ulcerative colitis [26], atherosclerosis [27], and aging [28].

The antioxidant action mechanism cannot be understood without describing the model lipid peroxidation in cell membranes or foodstuffs, a radical mechanism that these biomolecules undergo, with initiation, propagation, and chain termination stages, which is promoted by heat, light, and ionizing radiation or by metal ions or metalloproteins [29–31].

Initiation:

$$\mathrm{LH} + \mathrm{R}^{\bullet} \longrightarrow \mathrm{L}^{\bullet} + \mathrm{RH} \tag{1}$$

LH is the lipid substrate, $R^{\bullet}$ is the initiating oxidizing radical, and $L^{\bullet}$ is the allyl radical endowed with high reactivity.

Propagation:

$$\begin{aligned} \mathrm{L}^{\bullet} + \mathrm{O}_2 &\longrightarrow \mathrm{LOO}^{\bullet} \\ \mathrm{LOO}^{\bullet} + \mathrm{LH} &\longrightarrow \mathrm{L}^{\bullet} + \mathrm{LOOH} \end{aligned} \tag{2}$$

So, during this step, the lipid peroxyl radicals $LOO^{\bullet}$ act as chain carriers, further oxidizing the lipid substrate and generating lipid hydroperoxides (LOOH), which can decompose into alcohols, aldehydes, alkyl formates, ketones, hydrocarbons, and radicals such as lipid alkoxyl radical $LO^{\bullet}$ [3, 32].

Branching:

$$\begin{aligned} \mathrm{LOOH} &\longrightarrow \mathrm{LO}^{\bullet} + \mathrm{HO}^{\bullet} \\ 2\mathrm{LOOH} &\longrightarrow \mathrm{LOO}^{\bullet} + \mathrm{LO}^{\bullet} + \mathrm{H}_2\mathrm{O} \end{aligned} \tag{3}$$

The decay of lipid hydroperoxides often takes place in the presence of transition metal ions, generating lipid peroxyl and lipid alkoxyl radicals:

$$\begin{aligned} \mathrm{LOOH} + \mathrm{M}^{n+} + \mathrm{H}^{+} &\longrightarrow \mathrm{LO}^{\bullet} + \mathrm{M}^{(n+1)+} + \mathrm{H}_2\mathrm{O} \\ \mathrm{LOOH} + \mathrm{M}^{(n+1)+} + \mathrm{OH}^{-} &\longrightarrow \mathrm{LOO}^{\bullet} + \mathrm{M}^{n+} + \mathrm{H}_2\mathrm{O} \end{aligned} \tag{4}$$

Termination implies the combination of radicals to form nonradical chemical species:

$$\begin{aligned} \mathrm{LO}^{\bullet} + \mathrm{LO}^{\bullet} &\longrightarrow \text{nonradical products} \\ \mathrm{LOO}^{\bullet} + \mathrm{LOO}^{\bullet} &\longrightarrow \text{nonradical products} \\ \mathrm{LO}^{\bullet} + \mathrm{LOO}^{\bullet} &\longrightarrow \text{nonradical products} \end{aligned} \tag{5}$$

Antioxidants can act as chain breakers, scavenging chain initiating radicals like hydroxyl, alkoxyl, or peroxyl, quenching singlet oxygen, decomposing hydroperoxides, and chelating prooxidative metal ions [13, 33]. Epidemiological studies confirm that the incidence of oxidative stress-related conditions is lowered by the consumption of fruits and vegetables rich in compounds possessing high antioxidant activity [18, 34–37]. Foods containing antioxidants and antioxidant nutrients play an important role in prevention.

Chain breaking antioxidants able to scavenge radical species are called primary antioxidants. Secondary antioxidants are singlet oxygen quenchers, peroxide decomposers that yield nonradical species, oxidative enzyme (e.g., lipoxygenase) inhibitors, UV radiation absorbers, or compounds that act by metal chelating [38–40].

Natural antioxidants constitute the essential part in the cell's defense mechanisms and they can be *endogenous or exogenous*.

Endogenous antioxidants can be nonenzymatic, such as glutathione, alpha-lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, metallothionein, l-carnitine, melatonin, albumin, and antioxidant enzyme cofactors, or enzymatic, such as superoxide dismutase, catalase, glutathione peroxidases, thioredoxins, and peroxiredoxins. Peroxiredoxins regulate cytokine-induced peroxide levels and mediate cell signal transduction [41].

Enzymatic antioxidants at their turn are grouped within the primary and secondary defence systems. The primary defence is formed by three crucial enzymes capable of preventing the occurrence or neutralizing free radicals: glutathione peroxidase, which donates two electrons that reduce peroxides, catalase that decomposes hydrogen peroxide into water and molecular oxygen, and superoxide dismutase that turns superoxide anions into hydrogen peroxide [13, 41]. The secondary enzymatic defense comprises glutathione reductase and glucose-6-phosphate dehydrogenase. Glutathione reductase turns glutathione into its reduced form, thus recycling it. Glucose-6-phosphate reforms reductive NADPH [42, 43]. Although these two enzymes do not directly neutralize free radicals, they promote the endogenous antioxidants' activity [13]. It has been assessed that enzymatic antioxidants act by decomposing free radicals and in this case damaging oxidative species are converted into hydrogen peroxide and water, while nonenzymatic antioxidants are mainly chain breakers. For instance, it has been reported that tocopherol disrupts a radical oxidation chain after five reactions [44].

Apart from the endogenous, enzymatic, and nonenzymatic antioxidants previously discussed, there are also *exogenous*, diet-sourced antioxidants [40, 43], represented by carotenoids, tocopherols, vitamin D, phenolic acids, flavonoids, or ascorbic acid, as well as high-molecular weight

metabolites such as tannins. For this second category, the source is represented by foodstuffs, pharmaceuticals, and food supplements. They are important in counteracting the reactive oxygenated species, when the endogenous compounds are not able to ensure thorough protection [40, 43, 45–47].

The intake of antioxidants from diet is always meant to counterpart the organism's antioxidant defense. Enzymic natural antioxidants in food (superoxide dismutase, glutathione peroxidase, and catalase) can be inactivated during processing.

Particularly plant-sourced low-molecular weight antioxidants such as glutathione and ascorbate are synthesized within the chloroplast stroma and the cytosol in the presence of reduced coenzyme molecules (NADPH) acting as the final electron source [48]. These low-molecular weight antioxidants, the cell's redox buffer, are involved in plant growth and development, as they are able to modulate processes from mitosis and cell elongation to senescence and death [49, 50]. Commercial synthetic antioxidants with phenolic structure such as BHA, BHT, and TBHQ are added to foodstuffs to prevent lipid rancidity [51] and the difference in structure transduces itself in antioxidant capacity difference [38].

Although review papers have been previously published on antioxidant activity in plants, the present paper provides a novel way of gathering and also critically and comparatively presenting these aspects. The section devoted to critical and conclusive aspects provides the reader with an original discussion over extraction techniques and their comparison, as well as methods' performances (in a way that has not been systematized until now), with the following aspects concerned: sample, mechanism underlying the method, working parameters, and detection.

2. Antioxidant Extraction Procedures

Extraction techniques aim not only at extracting the active biocompounds from the plant sample but also at imparting selectivity and optimizing sensitivity of the applied analytical methodology due to the increase of the concentration of the compound of interest. The biocompound is more easily detected and separated from other matrix components, and the assay becomes independent on the variable matrix characteristics [52].

Classical extraction techniques are based on the extractive potential of various solvents, using heating or mixing. The main shortcomings of conventional extraction are long extraction times, the need for high purity expensive solvents, evaporation of solvents in significant amounts, reduced selectivity, and, finally, the thermal decomposition in the case of thermolabile substances [53]. These problems can be solved by nonconventional extraction techniques that are mainly regarded as "green techniques," as they use less toxic chemicals, safer solvents, which are characterized by better energy efficiency and minimum by-product amounts [54].

An important goal is represented by high extraction efficiency and efficacy. Efficiency was defined as the yield of extraction, whereas efficacy represents the potential to induce bioactivity and the ability to produce an effect. Therefore, a selection of the most appropriate extraction method is required in each case, as it was proven that various techniques applied on the same plant material employing the same solvent can lead to different extraction efficiencies. Moreover, it has been confirmed that the most convenient method in this regard requires standardization to attain reproducibility [55].

2.1. Conventional Techniques. Soxhlet extraction was first applied only for lipid extraction, but its use has been extended for extracting active principles. The solvent is heated, vaporized, and condensed and extracts the interest compound(s) by contact with the sample-containing thimble. When the solvent in the extraction chamber reaches the overflow level, the solution in the thimble-holder is aspirated by a siphon and returns in the distillation flask. Significant extraction yields can be reached, with a small solvent amount. It can be applied in batch at small scale, but it can be converted into a continuous extraction set-up on medium or large scale [56].

Maceration is applied to obtain essential oils and bioactive compounds. The plant material is ground to improve the surface area. The solvent is then added and allowed to stand at ambient temperature for several days, and the mixture is subject to frequent stirring until dissolution. The damped material is then pressed and then the liquid is purified by filtration or decantation [54, 56].

Hydrodistillation (as water, water/steam, and direct steam distillation) is applied to the extraction of bioactive compounds and essential oils from plants, generally prior to dehydration, and does not imply the use of organic solvents [57]. Hot water and steam isolate the bioactive compounds from the plant tissue. Consequently, cool water condenses the vapor mix of water and oil. The condensed mixture reaches the separator, where oil and biocompounds are isolated from water [58]. Hydrodistillation involves three main steps, hydrodiffusion, hydrolysis, and thermal decomposition, with the risk being represented by the decay of thermolabile substances [54, 56].

Infusions are prepared by shortly macerating the raw plant material with either cold or boiling water. It is often mentioned that concentrated infusions are the result of a modified percolation or maceration procedure [56].

Percolation is a recognized procedure applied for the preparation of tinctures and fluid extracts and makes use of a cone-shaped vessel opened at both ends (percolator). The solid material is moistened with an adequate amount of the appropriate solvent (menstruum) and left for about 4 h. Solvent amount is necessary, until the percolate represents about three-quarters of the quantity corresponding to the final product. The marc is then pressed and the eliminated liquid is added to the percolate. Solvent is again added to get the required volume, and the liquid mixture is clarified by filtration or by decanting [56].

In the *decoction* process, the crude plant material is subject to boiling in an appropriate water amount, for a well-defined period, followed by cooling and then straining or filtering. This approach is adequate for the extraction of hydrosoluble, thermostable components, being popular for obtaining Ayurvedic extracts [56].

Cold pressing or expression consists in pressing or grinding fruits or seeds by using a press. Oil release is possible due to crushing or breaking of essential oil glands in the peel. Olive, peanut, sunflower, and citrus oils are obtained through cold pressing, which results in preserving flavor, aroma, and nutritional value.

Aqueous Alcoholic Extraction by Fermentation. The formed ethanol enables extraction of the active principles from the material and also contributes to preserving the product's qualities. In Ayurveda, this method is not standardized, but, with progresses in the fermentation technology, standardization would be of use for obtaining herbal drug extracts [56].

Vortex apparatus is commonly used to mix the interest plant sample with the dilutant. It is applied for dissolution, namely, in aqueous environment and polar solvents, of samples of plants to yield a fluid and homogeneous solution subject to analysis. As in the case of other techniques like shaking or sonication, it can be followed by centrifugation, with use of the supernatant.

2.2. Nonconventional (Modern) Techniques

Supercritical Fluid Extraction (SFE). Critical point was defined as the temperature and pressure above which distinction between gas and liquid phases does not exist [59]. In supercritical state, gas and liquid properties are not individualized, and supercritical fluid properties are tunable by temperature and pressure modification. Supercritical fluids (SCFs) possess both gas-like properties (diffusion, viscosity, and surface tension) and liquid-like density and solvation power [60]. The advantages are constituted by reduction of extraction time when compared to conventional methods, complete extraction by repeatable refluxes, better selectivity in comparison to common liquid solvents due to solvation power, enhanced transport properties exhibited near the critical point, and hence high extraction yields [55]. CO_2 use does not imply high costs. It operates at room temperature, so it is adequate for thermosensitive compounds; smaller samples can be extracted compared with conventional solvent extraction. It is characterized by facility of coupling with chromatographic procedures and reutilization of SCF [54, 56]. Disadvantages may be represented by polarity limitations of carbon dioxide, which can be minimized by the use of organic solvents, or inert gases (Ar) [56].

Solid Phase Microextraction (SPME). SPME employs a sorbent, which usually coats the surface of small fibers, for the isolation and concentration of target compounds from the sample and is applied to quantitative assay of analytes (essentially flavor compounds) in aqueous or gaseous phase.

Microwave-Assisted Extraction (MAE). Microwaves interact with the dipoles of polar and polarizable matrixes [61, 62]. As the forces of electric and magnetic field components swiftly modify their orientation, polar molecules also adopt orientation in the changing field direction, and heat is generated. So, ionic conduction and dipole rotation are the mechanisms underlying the conversion of electromagnetic energy to heat [54, 63]. The components of the sample absorb microwave energy in conformity with their dielectric constants [64]. When the plant material is found in a solvent transparent to microwaves, the elevated vapour pressure causes rupture of the cell wall of the substrate and frees the content into solvent [55]. Separation of solute molecules from the sample matrix at increased temperature and pressure is followed by diffusion of solvent molecules across the sample matrix and transfer of solute molecules from the sample matrix to the solvent. Microwave-assisted extraction is characterized by rapid heating to reach the temperature required for extracting bioactive principles [65], enhanced extraction yields, very good recovery and selectivity, and minimum equipment size and solvent use [54, 66].

Ultrasound-Assisted Extraction (UAE). Ultrasound waves with frequencies comprised between 20 kHz and 100 MHz induce compression and expansion as they pass through the extractable plant matrix, producing cavitation. The energy produced can promote the conversion of kinetic energy into thermal one, inducing heating of the bubble contents. In solid plant samples, ultrasounds enable compound leaching from the plant materials [67]. The mechanism implies wave diffusion across the cell wall and rinsing of the cell's content after breaking the walls [68]. The physical, chemical, and mechanical forces induced by the collapse of bubbles result in the disruption of membranes to enable the release of extractable compounds and to facilitate penetration of the solvent into cell material [69, 70]. Rapidity, intensified mass transfer, low solvent amounts, high extraction yields and throughput, and reduced temperature gradients characterize this technique [54]. Nevertheless, high ultrasound energy may result in cell membrane impairment due to free radical generation, but the deletions can be resealed by aggregation of lipid vesicles [71].

Pulsed-Electric Field (PEF) Extraction. Living cells are suspended in an electric field, and an applied potential crosses the membrane. The electric potential induces molecule separation according to the molecular charge. At values greater than 1 V for the transmembrane potential, the electrostatic repulsion between the charged molecules results in pore generation in the membrane and produces dramatic permeability increase, and yield is optimized [54, 72]. The efficacy of the pulsed-electric field extraction depends on field strength, energy input, pulse number, temperature, and matrix characteristics [73]. Pulsed-electric field extraction is also appliable as pretreatment before carrying out traditional extraction [74]. It can be employed before grape skins maceration, minimizing maceration time and imparting stability to anthocyanins and polyphenols [75].

Enzymatic Treatment. Enzymes used are cellulase, α-amylase, and pectinase, which act by breaking the cellular wall, with subsequent hydrolysis of the structural polysaccharides and lipids [76, 77]. Enzyme-assisted aqueous extraction and enzyme-assisted cold pressing are the main techniques applied [78]. Enzyme amount, particle size of the material, solid to moisture ratio, and hydrolysis time influence the performances [79]. Enzyme-assisted cold pressing is the

most proper for extracting biocompounds from oilseeds as nontoxic procedure, which does not involve flammable liquids. The oils extracted are richer in fatty acids and phosphorus than hexane-extracted ones [80]. The enzyme-assisted aqueous extraction is environmental-friendly [81]. In enzyme-assisted cold pressing, biocatalysts hydrolyse the seed cell wall, because the polysaccharide-protein colloid is not present, as happens in the enzyme-assisted aqueous extraction [82].

Pressurized Liquid Extraction (PLE). PLE implies exerting an elevated pressure to the remaining liquid solvent above the boiling point. High pressure values favor the extraction process, which is easily prone to automation. Pressurized liquid extraction benefits much shorter extraction times and lower solvent requirements, when compared to conventional Soxhlet extraction. At elevated temperatures and pressures, the extraction performances are improved by the increased analyte solubility and mass transfer rate, as well as by the diminished viscosity and low surface tension of solvents [54, 83].

3. Analytical Methods Applied to Antioxidant Content and Antioxidant Capacity Assessment in Plant Extracts: Classification and Principles

The investigation of performant analytical methods aiming to assess the antioxidant capacity in plants and plant extracts remains a constant goal and a series of classifications have been proposed. Antioxidant measurement techniques were classified as methods based on the inhibition of low-density lipoprotein oxidation estimation and the ones relying on the quantification of the free radical scavenging capacity [84].

Considering the mechanism underlying the antioxidant–oxidant reaction, the methods were also divided in hydrogen atom transfer (HAT) and single electron transfer (SET) techniques. HAT-based methods measure the capacity of an antioxidant to trap free radicals by hydrogen donation, while SET methods rely on the one electron transfer reductive ability of an antioxidant compound versus a radical species [85]. ORAC, TRAP, and chemiluminescence are hydrogen atom transfer-based methods, whereas FRAP and CUPRAC are single electron transfer methods [85]. DPPH and TEAC methods were regarded as methods using both hydrogen and single electron transfer, as the radicals in these cases can be scavenged by either electron reduction or radical quenching that involves hydrogen transfer [85, 86]. DPPH scavenging, TEAC assay, ferric reducing antioxidant power, $OH^{\bullet}$ scavenging, the phosphomolybdenum method, and beta-carotene linoleate bleaching are applied *in vitro*, while the lipid peroxidase, catalase, and glutathione peroxidase activity assays are techniques used *in vivo* [18]. The analytical response is also recorded as per reference to a standard antioxidant: Trolox, gallic acid, ascorbic acid, caffeic acid, and so forth.

The main chemical processes underlying antioxidant activity assay (a–d) and lipid oxidation status evaluation (e) are detailed in Table 1. The latter are presented, as they can constitute the basis for antioxidant screening: the assays can be performed by following the prevention of peroxidation products generation in the presence of antioxidants, measured against a control. The determinations may involve hydroperoxide, conjugated diene, or thiobarbituric acid reactive substances assay. The antioxidant effect is expressed as percent of lipid peroxidation inhibition. The group of techniques involving low-density lipoprotein peroxidation inhibition by antioxidants is also classified as belonging to HAT methods, as the reaction between the antioxidant and the peroxyl radicals (such as AAPH-initiated) involves hydrogen transfer.

In Table 2, the methods are classified following the detection mode, with principle description for each technique.

4. Significant Analytical Applications to Plant and Plant Extracts

4.1. Chromatography

4.1.1. Planar Techniques. Thin layer chromatograms of the methanolic extract of *Bergia suffruticosa* (used as bone and sore healer) proved antiradical activity by bleaching $DPPH^{\bullet}$. This free radical scavenging activity was assigned to the high tannin and phenolic amounts [90]. A recently developed TLC–$DPPH^{\bullet}$ assay allowed for the swift detection of the antioxidant potential of nine out of ten tested polyphenols (except for apigenin 7-O-glucoside), present in five analysed plant species: *Hypericum perforatum L.*, *Matricaria recutita L.*, *Achillea millefolium L.*, *Thymus vulgaris L.*, and *Salvia officinalis L.* By LC–MS, the presence of compounds previously identified by TLC was confirmed. Four other compounds (caffeic acid and apigenin in St. John wort and apigenin and apigenin 7-O-glucoside in sage) have been identified. Their presence was not revealed by TLC and it has been stated that their low level in the plant samples could be the reason [91].

Sonneratia caseolaris (astringent and antiseptic) extracts were tested for their antioxidant composition: column chromatography with a Diaion HP-20 column and successive elution with methanol and acetone was first applied. The chlorophyll-free eluate was separated into 5 fractions by C18 column chromatography, with methanol and acetone for elution. The methanol-eluted fraction containing DPPH positive spots was then applied to a silica gel presqualene column with n-hexane-acetone–methanol eluents, resulting in eight fractions. The first compound was obtained after precipitation from the fraction corresponding to the n-hexane-acetone 1 : 1 eluate. One acetone-eluted fraction also yielded a precipitate, which after washing with methanol resulted in the second compound. The structures of the isolated compounds were assessed by one-dimensional and two-dimensional NMR and mass spectroscopy. Moreover, both showed positive (discolored) spots with a reddish purple background on the thin layer chromatogram, using a 0.02% (w/v) methanolic solution of DPPH as spray reagent. Luteolin and luteolin-7-O-β-glucoside were identified as the two bioactive antioxidant and anti-inflammatory compounds [92].

Table 1: The main chemical mechanisms underlying antioxidant activity (a–d) and lipid oxidation (e).

(a) Hydrogen atom transfer (HAT)

Corresponding method of assay	Mechanistic description
TRAP (Total Radical Trapping Antioxidant Parameter) assay ORAC (Oxygen Radical Absorbance Capacity) assay Beta carotene/crocin bleaching method Inhibition of induced low-density lipoprotein peroxidation assay Chemiluminescence quenching, due to luminol-derived radicals scavenging by antioxidants	$ArOH + X^{\bullet} \longrightarrow ArO^{\bullet} + XH$ An antioxidant (e.g., phenolic compound ArOH) directly interacts with a free radical ($X^{\bullet}$), yielding a phenolic radical species derived from the antioxidant molecule $ArO^{\bullet}$, and a neutral species XH. The antioxidant facility to follow HAT mechanism is correlated with low bond-dissociation enthalpy [117]. The presence of dihydroxy functionality imparts good hydrogen donation abilities, correlatable with low bond-dissociation enthalpy values [118].

(b) Single electron transfer (SET)

Corresponding method of assay	Mechanistic description
DMPD (N,N-dimethyl-p-phenylenediamine) method FRAP (ferric reducing antioxidant power) assay CUPRAC (cupric reducing antioxidant capacity) method PFRAP (potassium ferricyanide reducing power) method	$ArOH + X^{\bullet} \longrightarrow ArOH^{\bullet+} + X^{-}$ SET assays rely on the capacity of an antioxidant ArOH to reduce the radical species $X^{\bullet}$ by electron donation, which is accompanied by the color change of the radical solution. Low adiabatic ionization potentials are correlated with good electron transfer abilities [117]. Extended delocalization and electron conjugation result in low ionization potentials [118]. Also, pH increase (deprotonation) favors electron transfer.

(c) Mixed HAT and SET

Corresponding method	Mechanistic description
DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging method	Hydrogen atom transfer and sequential proton-loss electron transfer (SPLET), also designated proton-coupled electron transfer (PCET) [119, 120], were both confirmed as being thermodynamically favorable. A SPLET mechanism involving the antioxidant ArOH and the radical $ROO^{\bullet}$ was represented as [121] $ArOH \longrightarrow ArO^{-} + H^{+}$ $ArO^{-} + ROO^{\bullet} \longrightarrow ArO^{\bullet} + ROO^{-}$ $ROO^{-} + H^{+} \longrightarrow ROOH$ or coupling the second and third steps as [122] $ArOH \longrightarrow ArO^{-} + H^{+}$ $ArO^{-} + X^{\bullet} + H^{+} \longrightarrow ArO^{\bullet} + XH$
TEAC (Trolox Equivalent Antioxidant Capacity) method	During the first step the phenolic antioxidant dissociates into its corresponding anion ArO^{-} and a proton, and subsequently the ions which resulted in the first step react with the free radical, yielding a radical form of the phenolic antioxidant $ArO^{\bullet}$ and a neutral molecule XH [122]. Proton transfer can also occur following electron transfer, as in single electron transfer-proton transfer mechanism (SET-PT) [122]: $ArOH + X^{\bullet} \longrightarrow ArOH^{\bullet+} + X^{-}$ $ArOH^{\bullet+} \longrightarrow ArO^{\bullet} + H^{+}$ During the first step a phenolic antioxidant reacts with the free radical $X^{\bullet}$, yielding a cationic radical $ArOH^{\bullet+}$ derived from the phenolic compound and the anionic form of the radical X^{-}. This first step has been reported as thermodynamically significant step. In the second step the cationic radical form of the antioxidant $ArOH^{\bullet+}$ decomposes into a phenolic radical $ArO^{\bullet}$ and a proton [122].

(d) Chelation power of antioxidants

Corresponding method	Mechanistic description
Tetramethylmurexide (TMM) assay	Free Cu(II) or Zn(II) which is not complexed by phenolics (e.g., tannins) is bound to tetramethylmurexide (TMM). The complexation with TMM is assessed at 482 nm for Cu(II) and at 462 nm for Zn(II) [123].

(d) Continued.

Corresponding method	Mechanistic description
Ferrozine assay	Free Fe(II) that is not complexed by phenolics (e.g., tannins) is bound to ferrozine. The complexation of divalent iron with ferrozine is assessed at 562 nm [123].

(e) Oxidation of lipids

Corresponding method	Mechanistic description
Peroxide value assessment	Lipid autoxidation results in generation of hydroperoxides, determined iodometrically or colorimetrically [119].
Conjugated diene assay	Fatty acids autoxidation yields conjugated dienes, assessed by UV absorbance at 234 nm [119].
Anisidine assay	Secondary lipid oxidation yields p-anisidine-reactive aldehydes (alkenals, alkadienals, and malondialdehyde), the resulted Schiff base being determined at 350 nm [119].
Thiobarbituric acid reactive substances	Malondialdehyde and unsaturated aldehydes (alkenals and alkadienals) react with thiobarbituric acid; the reaction product is determined photocolorimetrically at 532 nm [119].

High performance thin layer chromatography combined with densitometry was applied for caffeic acid quantitation in *Plantago lanceolata*. The best eluent composition was determined: in first step of development, the mobile phase contained hexane, diisopropyl ether, and formic acid 90% (6.0 : 4.0 : 0.5) v/v. In the second and third steps, a mixture of hexane, diisopropyl ether, dichloromethane, formic acid 90%, and propan-2-ol (6.0 : 4.0 : 2.0 : 1.0 : 0.1) v/v was employed. The application of this HPTLC technique with area measurements at 320 nm led to a caffeic acid amount equal to 99.3 μg/g of dried plant, with RSD of 3.19% [93].

HPTLC [94] was also used for the screening and quantitation of phytochemicals present in *Scoparia dulcis*, known for many health benefits [95] (see Table 3). After application of the anisaldehyde-sulphuric acid visualization reagent, the spotted plate was exposed to UV radiation (254 and 366 nm) and multicolored bands at various intensities were noticed. On the TLC plates, the presence of phenolics (flavonoids) and terpenoids has been revealed [94].

The antioxidant capacity of essential oils obtained from the seed and whole plant of *Coriandrum sativum* was assessed, and HPTLC was applied to assess significant phytomarkers. The *in vitro* determined antioxidant capacity was greater than the one corresponding to various extracts of this Ayurvedic plant. The chromatographic profile showed linalool and geranyl acetate as main phytoconstituents of the analysed samples. The HPTLC system was based on a TLC scanner, an autosampler connected to a nitrogen cylinder, a UV scanner, and visualizer. The limits of detection and quantification were obtained as 0.4 and 1.2 ng/mL for linalool and 0.6 and 1.4 ng/mL for geranyl acetate, revealing sensitivity. The precision was proven by the result of minimum six replicate analyses, with a coefficient of variability of 0.07% [96].

4.1.2. Column Techniques

(1) Gas Chromatography. The composition of various extracts of *Merremia borneensis* was assessed by GC-MS, showing the presence of flavonoids, terpenoids, alkaloids, and glycosides in the analysed organic crude extracts [97]. The qualitative analysis of bioactive compounds present in *Datura metel* was performed in crude extracts by GC/MS, revealing abundancy of high-molecular weight components such as polyphenols, flavonoids, triterpenoids, and hydrocarbons. The phenolic level was expressed as gallic acid equivalents, with chloroform having the best extractive potential, followed by methanol, butanol, ethyl acetate, and hexane. It has been concluded that the chloroform crude extract had the highest phenolics amount and its potential as antibiotic has been stated [98].

Essential oils from the aerial parts of *Ajuga bracteosa* and *Lavandula dentata* obtained by hydrodistillation were analysed by GC and GC/MS. 47 and 48 biocomponents were identified for the two analysed plants, respectively. The oils contained high amounts of oxygenated monoterpenes (34 to 51%). Borneol (20.8%) and hexadecanoic acid (16.0%) were the major compounds present in the oil of *A. bracteosa, which also contained* aliphatic acids (30.3%). Camphor (12.4%), *trans*-pinocarveol (7.5%), and β-eudesmol (7.1%) were prevalent in *Lavandula dentata oil*. The antioxidant activity of the oil extracts was confirmed by DPPH$^{\bullet}$ scavenging assay [99].

(2) Liquid Chromatography. The rapid and resolution-high determination of six bioactive flavonoids present in the pericarp of *Citri reticulata* has been performed by liquid chromatography/electrospray ionization coupled with mass spectrometry. The chromatographic system used a C18 column and a 0.1% formic acid/acetonitrile mobile phase with a gradient elution. Naringin, hesperidin, nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, tangeritin, and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone were assessed by the above-mentioned chromatographic technique and were also investigated for their antiproliferative activities by Cell Counting Kit-8 Assay. In the cultivars analysed, hesperidin presented the highest content, ranging from 50.137 to 100.525 mg/g. The levels of nobiletin, tangeritin, and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone were higher in the peel of *Citrus reticulata* "Chachi" than in other cultivars. With respect to the antiproliferative activity against A549 and HepG2 cells, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone has been proven to be the most effective [100].

Table 2: Illustration of the main principles and detection mechanisms in antioxidant activity measurement.

Method for antioxidant capacity assay	Principles underlying the analytical techniques	Detection modes	Ref.
	Chromatographic techniques		
Thin layer chromatography	The stationary phase is a thin layer of silica gel, aluminium oxide, or cellulose which covers a support of glass, plastic, or aluminium foil. The mobile phase moves by capillarity.	Migration of analytes takes place at different rates due to various repartition coefficients	[91]
High performance thin layer chromatography	It relies on the same principle as conventional TLC but uses a stationary phase with smaller particle size.	Separation performed with improved resolution versus TLC	[93, 94]
Gas chromatography	Separation is based on the repartition between a liquid stationary phase and a gas mobile phase.	Flame ionization, thermal conductivity, or mass spectrometry detection	[124]
Liquid chromatography	Separation is based on the repartition between a solid stationary phase and a liquid mobile. phase	Mass spectrometry or electrochemical detection	[106]
High performance liquid chromatography	Separation is based on the repartition between a solid stationary phase and a liquid mobile phase with distinct polarities at high flow rate and pressure of the mobile phase.	UV-VIS (diode array), fluorescence, mass spectrometry, or electrochemical detection	[108]
	Spectrometric techniques		
DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging method	Antioxidant reaction with the nitrogenated radical, followed by absorbance diminution at 515–518 nm.	Photocolorimetry	[125, 126]
TEAC (Trolox Equivalent Antioxidant Capacity) method	Antioxidant reaction with $ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid cation radical) generated by $K_2S_2O_8$, followed by blue solution absorbance diminution at 734 nm.	Photocolorimetry	[127]
DMPD (N,N-dimethyl-p-phenylenediamine) method	Reduction of $DMPD^{\bullet+}$ by antioxidants, in the presence of $FeCl_3$, with subsequent absorbance decrease at 505 nm.	Photocolorimetry	[128]
FRAP (ferric reducing antioxidant power) method	Reduction of the Fe^{3+}-TPTZ (2,4,6-tripyridyl-s-triazine) complex, by sample antioxidants, with absorbance taken at 593 nm.	Photocolorimetry	[129]
PFRAP (potassium ferricyanide reducing power) method	Reduction of potassium ferricyanide by antioxidants, yielding potassium ferrocyanide. The latter reacts with ferric trichloride, and the resulted ferric ferrocyanide blue colored complex is measured at maximum absorbance of 700 nm.	Photocolorimetry	[130]
CUPRAC (cupric reducing antioxidant capacity) method	Cu(II)-neocuproine complex reduction to Cu(I) – bis (neocuproine) chelate, with absorbance recorded at 450 nm.	Photocolorimetry	[131, 132]
Phosphomolybdenum assay	Mo (VI) is reduced Mo (V) by the antioxidants in the sample with generation of a green phosphate/Mo (V) complex at acidic pH, determined at 695 nm.	Photocolorimetry	[133]
Lipid peroxidation activity assay	Antioxidants delay lipid hydroperoxide generation caused by lipoxygenase. The absorbance is measured at 234 nm.	UV absorbance	[106, 134]
	Antioxidants delay radical-induced malonyl dialdehyde generation, as decomposition product of endoperoxides of unsaturated fatty acids, in the presence of thiobarbituric acid. The absorbance is measured at 535 nm.	Photocolorimetry	[106, 135]
	Antioxidants delay conjugated dienes generation as a result of peroxidation of lipid components. The absorbance is measured at 234 nm.	UV absorbance	[85]

Table 2: Continued.

Method for antioxidant capacity assay	Principles underlying the analytical techniques	Detection modes	Ref.
Superoxide radical scavenging activity assay	Antioxidants are subject to reaction with a substrate solution containing xanthine sodium salt and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride. Xanthine oxidase is used as biocatalyst and the absorbance increase was monitored at 505 nm.	Photocolorimetry	[136]
	Superoxide anions are generated in a solution containing nitroblue tetrazolium, NADH and phenazine methosulfate. The absorbance taken at 560 nm decreases in the presence of antioxidants, pointing towards superoxide anion scavenging activity.	Photocolorimetry	[137]
Beta carotene bleaching method	Linoleic acid is oxidized by reactive oxygen species. The generated oxidation products such as lipid peroxyl radicals initiate β-carotene oxidation and, consequently, its decolorization. Antioxidants delay the discoloration rate, with absorbance measured at 434 nm.	Photocolorimetry	[138, 139]
Xanthine oxidase inhibition assay	Xanthine is used as substrate that yields uric acid as product of XOD-catalyzed reaction. Allopurinol is used as xanthine oxidase inhibitor. Absorbance is measured at 293 nm.	Photocolorimetry	[140]
Superoxide dismutase method	It is assessed in an erythrocyte lysate in the presence of pyrogallol. The enzyme inhibits the autooxidation of the hydroxylated compound, with absorbance read at 420 nm.	Photocolorimetry	[141]
Catalase activity assay	It is measured in an erythrocyte lysate in the presence of H_2O_2. The rate of H_2O_2 decomposition is assessed at 240 nm.	Photocolorimetry	[142]
Ferrous ion chelating activity assay	Antioxidants react with ferrous salt (e.g., $FeCl_2$). Ferrozine as Fe(II) chelator yields a violet complex with absorbance read at 562 nm. The reaction is hindered in the presence of antioxidants that act by chelation, and the result is a decrease of the color of the ferrozine-Fe^{2+} complex, as chelators other than ferrozine act as competing agents for the metal ion.	Photocolorimetry	[143, 144]
ORAC (Oxygen Radical Absorbance Capacity) assay	Antioxidants scavenge the peroxyl radicals, induced by 2,2′-azobis-(2-amidino-propane) dihydrochloride (AAPH) decomposition, slowing the fluorescent decay of fluorescein or phycoerythrin.	Fluorimetry	[145–147]
HORAC (Hydroxyl Radical Antioxidant Capacity) assay	Antioxidants quench OH radicals formed in a Fenton-like system.	Fluorimetry	[148]
TRAP (Total Radical Trapping Antioxidant Parameter) assay	The rate of peroxyl radical generation by 2,2′-diazobis-2-amidinopropane dihydrochloride (ABAP) is quantified through the fluorescence diminution of the protein R-phycoerythrin.	Fluorescence	[149, 150]
Horseradish peroxidase-luminol-hydrogen peroxide chemiluminescent assay	Horseradish peroxidase catalyses luminol oxidation by H_2O_2 with light emission. Light emission is quenched by antioxidants.	Chemiluminescence	[151]
Electrochemical techniques			
Cyclic voltammetry	The potential is linearly swept in a triangular waveform.	The analytical signal is represented by the intensity of the cathodic/anodic peak	[152, 153]
Differential pulse voltammetry	Potential voltage pulses are superimposed on the potential scan, which is performed linearly or stairstep-wise.	First current sampling before applying the pulse and the second towards the end of the pulse period	[154, 155]

Table 2: Continued.

Method for antioxidant capacity assay	Principles underlying the analytical techniques	Detection modes	Ref.
Square-wave voltammetry	A square wave is superimposed on the potential staircase sweep.	Current intensity recorded at the end of each potential change	[155, 156]
Amperometry	The potential of the working electrode is maintained at a constant value versus the reference electrode.	Current intensity generated by the oxidation/reduction of an electroactive analyte	[157]
Biamperometry	The reaction of the antioxidant with the oxidized form of a reversible indicating redox couple in an electrochemical cell containing two identical electrodes.	The current flowing between two identical working electrodes at a constant small applied potential difference	[158–160]
Potentiometry	The analytical signal represented by the potential change is the result of the variation of an ionic species concentration. The antioxidants react with the oxidized form of a redox couple, altering the concentration ratio between the oxidized form and the reduced form.	Potential change after reaction of antioxidants with an indicating redox couple	[161]

Chromatography followed by electrochemical detection proved its viability in the assessment of onion (*Allium cepa*), parsley (*Petroselinum crispum*) roots and leaves, celery (*Apium graveolens*) roots, and leaves of dill (*Anethum graveolens*) extracts, relying on the antioxidant compounds' specific oxidation. It has been confirmed that the method is characterized by sensitivity and simplicity of detection, since no additional instrumentation (reagent pump or secondary detector) is necessary. In comparison to the results obtained using reversed-phase chromatographic separation with online postcolumn DPPH scavenging detection, HPLC-ED provided much richer chromatographic profiling of celery leaves extracts. At elevated electrooxidation potential values higher than 700 mV, compounds that are electroactive contribute to HPLC-ED detection but are missed in the postcolumn DPPH scavenging [101].

The HPLC chromatograms of *Carissa opaca* various fractions proved the presence of orientin, isoquercetin, myricetin, and apigenin endowed with antioxidant activity. The antibacterial, antitumoral, and anticarcinogenic potential of these flavonoid-rich fractions of *Carissa opaca* has also been confirmed in this study [102].

Eleven Algerian medicinal plants were subject to analysis for their antioxidant capacity and phenolic profile. The HPLC results revealed that the hydroxycinnamic acid derivatives were the predominant phenolics of the extracts endowed with best antioxidant activity (*Anthemis arvensis* and *Artemisia campestris*). Nevertheless, it was stated that in this case the correlation between the antioxidant activity of analysed extracts and their phenolic composition is very difficult to be described by statistical tools. It was assumed that this difficulty may result not only from the fact that total phenolics do not include all the antioxidants but also from the synergism and structure interaction among the antioxidants, which does not always involve concentration influence. For instance, samples such as *Artemisia arborescens* and *Oudneya africana*, with close concentration values of total phenolics, exhibited varying antioxidant activity. On the whole, the antioxidant activity and flavonoids concentration did not correlate significantly in comparison to hydroxycinnamic acids and hydroxybenzoic acids. *Artemisia campestris* was assessed as the most powerful inhibitor of radical-induced red blood cells hemolysis, more active than caffeic acid, more than three times more active than ascorbic acid, and two times more active than α-tocopherol. The UV spectra were obtained in the range of 220–600 nm and the amounts of phenolics in the extracts were assessed from the calibration curves developed at the absorption maxima of each phenolic class [103].

Methanolic extracts of the leaves of *Rosmarinus officinalis* were assessed by HPLC for their radical scavenging antioxidant activities. The identified compounds, namely, carnosol, carnosic acid, and rosmarinic acid, varied as depending on the geographical regions and season. The chromatographic system involved a C18 column and a mobile phase composed of methanol and acetic acid/acetonitrile, with gradient elution. The highest content of carnosic acid was obtained in the samples harvested from Mersin; the highest rosmarinic acid level was assigned to Canakkale-originating samples (14.0–30.4 mg/g). For all extracts, the carnosol content ranged from 5.4 to 25.5 mg/g, and the carnosic acid level ranged from 3.8 to 115.8 mg/g [104].

The phenolic ingredients in samples of 24 cereal grains were analysed by HPLC, relying on the peak area of maximum absorption wavelength. The chromatographic setup was comprised of a C18 column, a mobile phase with an elution gradient between solution A (acetic acid-water and methanol) and solution B (methanol and acetic acid-water solution), and a photodiode array detector. Gallic acid, kaempferol, quercetin, galangin, and cyanidin 3-glucoside were found in high amounts in these cereals [105].

HPLC was also applied along with LC-MS for the estimation of polyphenolic compounds from bitter cumin. The amount of phenolic compounds (μg/g dry weight) was estimated by comparing the peak areas (at 254 nm) of the samples with that of standards, proving the prevalence of caffeic acid: 500.0 μg/g dry weight [106].

Table 3: Significant examples of total antioxidant capacity assessment in plants.

Number	Analysed products (extracts)	Compounds determined	Applied analytical technique	Health benefits as they appear in the cited studies	Ref.
(1)	Leaves from cherry tree, peach tree, plum tree, olive tree, pear tree, apple tree, pistachio, and chestnut	(i) Total phenols (ii) Nonflavonoids phenol (iii) Total antioxidant capacity	(i) DPPH assay (ii) FRAP assay	Used in pharmaceutical purposes and also act as natural pesticides and beverage ingredients	[162]
(2)	Leaf extracts from six *Vitis vinifera* L. varieties	(i) Total phenols (ii) Flavonoids, nonflavonoids, and flavanols (iii) Total antioxidant capacity	(i) HPLC (ii) DPPH assay (iii) FRAP assay	Antimicrobial activity	[163]
(3)	Tropical herbs: *Momordica charantia*, *Centella asiatica*, and *Morinda citrifolia*	(i) Catechin (ii) Total antioxidant capacity	(i) HPLC (ii) DPPH assay (iii) FRAP assay	Inhibitors of pancreatic lipase activity	[164]
(4)	Edible and medicinal *Acacia albida* organs (leaves and bark)	(i) Polyphenols (ii) Total antioxidant capacity	(i) HPLC (ii) DPPH assay (iii) ABTS assay	Traditionally used to treat colds, flu, fever, tooth decay, vomiting, diarrhea, urinary disorders, malaria, and inflammation	[165]
(5)	*Citrus* fruits	Total antioxidant capacity	(i) HPLC free radical scavenging detection (ii) DPPH assay (iii) ABTS assay		[166]
(6)	*Salvia* sp. and *Plantago* sp.	(i) Total phenolic content (ii) Total antioxidant capacity	(i) UV-Vis fingerprint (ii) DPPH assay	Helpful in preventing different diseases	[167]
(7)	*Ajuga iva* (leaf extracts)	(i) Total phenolic content (ii) Total flavonoids (iii) Total antioxidant capacity	(i) DPPH assay (ii) FRAP assay	Diuretic, cardiac tonic, and hypoglycemic	[168]
(8)	*Filipendula vulgaris*	(i) Total phenolic content (ii) Total antioxidant capacity	(i) DPPH assay (ii) ABTS assay	(i) Antibacterial activity (ii) Fights against inflammatory diseases, rheumatoid arthritis, and gout	[169]
(9)	*Asphodelus aestivus* Brot.	Total antioxidant capacity	(i) FRAP assay (ii) DPPH assay (iii) ABTS assay	(i) Are used against hemorrhoids, nephritis, burns, and wounds (ii) Gastroprotective effect against ethanol-induced lesions	[170]
(10)	*Melia azedarach* (Chinaberry) (bark extract)	Total antioxidant capacity	DPPH assay	Antimicrobial agents in various infectious diseases	[171]
(11)	Bitter bean, *Parkia speciosa*	(i) Total phenolic constituents (ii) Total antioxidant capacity	(i) HPLC (ii) Folin-Ciocalteu method (iii) DPPH assay (iv) ABTS assay	(i) Antibacterial effects on kidney, ureter, and urinary bladder (ii) Diuretic and relaxing properties (iii) Seed extracts were reported to possess hypoglycemic, anticancer, and antiangiogenic activities	[172]
(12)	*Brassica oleracea* L.	(i) Glucosinolates (ii) Total phenolic constituents (iii) Ascorbic acid (iv) Total antioxidant capacity	(i) HPLC (ii) Folin-Ciocalteu method (iii) DPPH assay	(i) Neutralizes carcinogens (ii) Attenuates cancer cell division (iii) Accelerates the atrophy of cancer cells with damaged DNA	[116]

Table 3: Continued.

Number	Analysed products (extracts)	Compounds determined	Applied analytical technique	Health benefits as they appear in the cited studies	Ref.
(13)	Grape pomace seed and skin extracts	(i) Total phenols (ii) Total anthocyanins (iii) Total tannins (iv) Total antioxidant capacity	(i) HPLC MS (ii) DPPH assay (iii) TEAC assay (iv) ABTS assay (v) Folin-Ciocalteu method	Limit the oxidation of nucleic acids, proteins, and lipids, which may initiate degenerative diseases	[173]
(14)	*Diplotaxis simplex* (Brassicaceae) (flower, leaf, and stem extracts)	(i) Total phenols, flavonoids, and proanthocyanidins (ii) Total antioxidant capacity	ORAC assay	Anti-inflammatory activity	[174]
(15)	Cereal grains (24 cereal grains from China)	(i) Total phenolic constituents (ii) Total antioxidant capacity	(i) FRAP assay (ii) TEAC assay (iii) HPLC (iv) Folin-Ciocalteu method	Reduces the risk of cardiovascular diseases and reduces type II diabetes, ischemic stroke, and some cancers	[105]
(16)	Some cereals and legumes	(i) Total phenolic constituents (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay (iii) FRAP assay	(i) Reduces the incidence of age-related chronic diseases (ii) Reduces heart diseases and some types of cancer	[175]
(17)	*Clusia fluminensis* Planch. & Triana	(i) Flavonoids content (ii) Total antioxidant capacity	(i) Photometric assay based on aluminum chloride complex formation (ii) DPPH assay	(i) Antifungicidal activity (ii) Protection against cardiovascular diseases	[176]
(18)	Bitter cumin (*Cuminum nigrum* L.)	(i) Total phenolic constituents (ii) Total antioxidant capacity	(i) HPLC (ii) DPPH assay	(i) Antibacterial activity (ii) Reduces risk of cancer and cardiovascular diseases	[106]
(19)	Essential oils of *Cynanchum chinense* and *Ligustrum compactum*	Total antioxidant capacity	(i) DPPH assay (ii) ABTS assay	(i) Anticonvulsant (ii) Antitumor (iii) Antimicrobial	[177]
(20)	*Caspicum annum* L. grossum sendt.; *Rosmarinus officinalis*	(i) Total phenolic constituents (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) ABTS assay		[178]
(21)	*Diospyros bipindensis* (Gürke)	(i) Plumbagin, canaliculatin, ismailin, betulinic acid, and 4-hydroxy-5-methyl-coumarin (ii) Total antioxidant capacity	(i) HPLC, NMR, and MS analyses (ii) DPPH assay (iii) ABTS assay (iv) ORAC assay	Anti-inflammatory and antimicrobial activities	[179]
(22)	*Carissa opaca* fruits	Total flavonoids content	HPLC	(i) Antibacterial activity (ii) Anticancer activity (iii) Antitumoral activity	[102]
(23)	*Artemisia capillaris herba*	(i) Total phenolic constituents (ii) Total antioxidant capacity	(i) HPLC MS (ii) DPPH assay (iii) β-carotene bleaching method	(i) Cholagogic, antipyretic, anti-inflammatory, and diuretic in jaundice (ii) Used against inflammation of the liver and cholecyst	[114]
(24)	*Lantana camara* (various parts: leaf, root, fruit, and flower)	(i) Total phenolic constituents (ii) Total antioxidant capacity	(i) DPPH assay (ii) Folin-Ciocalteu method	Used against itches, cuts, ulcers, rheumatism, eczema, malaria, tetanus, and bilious fever	[180]

Table 3: Continued.

Number	Analysed products (extracts)	Compounds determined	Applied analytical technique	Health benefits as they appear in the cited studies	Ref.
(25)	Grape extracts	(i) Total phenolic constituents (ii) Total anthocyanins (iii) Tannins (iv) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) Binding with polyvinylpyrrolidone (iii) ABTS assay		[181]
(26)	*Scutellaria baicalensis* radix	Total antioxidant capacity	DPPH assay	Used in hepatitis and inflammation of the respiratory and gastrointestinal tract	[182]
(27)	*Lycium* species	(i) Total phenolic constituents (ii) Total antioxidant capacity	(i) HPLC (ii) DPPH assay	Diuretic, antipyretic, tonic, aphrodisiac, hypnotic, hepatoprotective, and emmenagogic	[107]
(28)	Dried fruits consumed in Algeria (prunes, apricots, figs, and raisins)	(i) Total phenolic constituents (ii) Total anthocyanins (iii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay (iii) Phosphomolybdenum method	Reduce the risk of cancer and heart disease	[183]
(29)	*Rubus grandifolius* Lowe (leaves, flowers, and berries)	(i) Total antioxidant capacity (ii) Total phenolic constituents	(i) DPPH assay (i) ABTS assay (iii) FRAP assay (iv) HPLC	Acts as astringent and as remedy for diabetes and is depurative and diuretic and relieves sore throat	[184]
(30)	Red pitaya (*Hylocereus polyrhizus*) seed	(i) Total antioxidant capacity (ii) Total phenolic constituents (iii) Flavonoids content	(i) DPPH assay (ii) Folin-Ciocalteu method (iii) HPLC		[185]
(31)	Cornelian cherry, Japanese persimmon, and cherry laurel	(i) Total phenolic content (ii) Total flavonoids content (iii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay (iii) FRAP assay (iv) CUPRAC assay	Able to provide prevention of diseases	[186]
(32)	*Inula crithmoides* L.	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay	Antibacterial, antifungal, and cytotoxic	[187]
(33)	*Lycium intricatum* Boiss.	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) HPLC (iii) DPPH assay (iv) ABTS assay (v) FRAP assay	Decreases the risk of diseases such as cancer, neurodegenerative disorders, and cardiovascular diseases	[188]
(34)	*Millingtonia hortensis* Linn. parts (leaves, stem, root, and flower)	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay	Reduces risks of diabetes, cancer, and cardiovascular diseases	[189]
(35)	*Ononis natrix*	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay	Antimicrobial activities	[190]
(36)	*Citrus grandis* Osbeck	Total antioxidant capacity	DPPH assay		[191]
(37)	*Sorbus torminalis* (L.) Crantz (wild service tree) fruits	(i) Total phenolic content (ii) Total flavonoids content (iii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) ABTS assay (iii) DPPH assay	Used in treatment of cardiac diseases and Alzheimer's disease	[192]

Table 3: Continued.

Number	Analysed products (extracts)	Compounds determined	Applied analytical technique	Health benefits as they appear in the cited studies	Ref.
(38)	*Rosmarinus officinalis*	(i) Total phenolic content (ii) Total antioxidant capacity	(i) HPLC (ii) DPPH assay (iii) TEAC assay		[104]
(39)	*Sapindus mukorossi* Gaertn.	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay	Fights against heart disease, aging, diabetes mellitus, and cancer	[193]
(40)	11 medicinal Algerian plants	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) HPLC (iii) ABTS assay (iv) TEAC assay	Antitumoral, anticancer, analgesic, diuretic, analgesic, and so forth	[103]
(41)	Six *Teucrium arduini* L. populations	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) FRAP assay (iii) ABTS assay (iv) DPPH assay	Hypoglycemic, antipyretic, antiulcerative, and antibacterial	[194]
(42)	*Vitex agnus-castus* (*Vitex AC*)	Total antioxidant capacity	(i) ABTS assay (ii) DPPH assay (iii) FRAP assay (iv) CUPRAC assay	Cytotoxic activities against various types of cancer cells	[195]
(43)	*Andrographis paniculata*	(i) Total antioxidant capacity (ii) Total phenolic content (iii) Total andrographolides concentration	(i) DPPH assay (ii) FRAP assay (iii) CUPRAC assay (iv) HPLC-DAD (v) LC-MS/MS (vi) GC-MS	(i) Treats dyspepsia, influenza, dysentery, malaria and respiratory infections (ii) Antidote for snakebites and poisonous stings (iii) Active in cytotoxicity tests against cancer cell lines	[111]
(44)	*Hypericum perforatum* L., *Matricaria recutita* L., *Achillea millefolium* L., *Thymus vulgaris* L., and *Salvia officinalis* L.	(i) Total antioxidant capacity (ii) Total phenolic content	(i) Thin layer chromatography (ii) LC MS (iii) DPPH assay	Anti-inflammatory, antiviral, antimicrobial, antiallergic, anticancer, antiulcer, and antidiarrheal	[91]
(45)	*Celastrus paniculatus* Willd.	Total antioxidant capacity	(i) DPPH assay (ii) FRAP assay (iii) TEAC assay (iv) GC MS	Calmant	[196]
(46)	Cerrado Brazilian fruits	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) ABTS assay	Chemopreventive effects	[197]
(47)	Buckwheat (*Fagopyrum esculentum* Moench)	(i) Total phenolic content (ii) Total antioxidant capacity	(i) HPLC (ii) DPPH assay		[198]
(48)	Green and black tea infusions, herbal infusions, and fresh fruit extracts	Total antioxidant capacity	Potentiometric and flow injection		[161]
(49)	Cocoa beans (raw, preroasted, and roasted)	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay (iii) ABTS assay		[199]
(50)	Rapeseed and its products	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Silver nanoparticle-based method (ii) Folin-Ciocalteu method (iii) DPPH assay (iv) FRAP assay		[200]

Table 3: Continued.

Number	Analysed products (extracts)	Compounds determined	Applied analytical technique	Health benefits as they appear in the cited studies	Ref.
(51)	Edible plants (broccoli, cauliflower, strawberry, tomato, potato, and corn)	Total antioxidant capacity	Cyclic voltammetry		[201]
(52)	Herb extracts from the Labiatae family	Total antioxidant capacity	(i) DPPH assay (ii) Amperometric	Antioxidant in food industry	[202]
(53)	Indian mushrooms (*Agaricus bisporus*, *Hypsizygus ulmarius*, and *Calocybe indica*)	(i) Total phenolic content (ii) Total antioxidant capacity	(i) DPPH assay (ii) FRAP assay (iii) Folin-Ciocalteu method (iv) Cyclic voltammetry	Provides health benefits and protection against degenerative diseases	[203]
(54)	Three types of algae: *Spirulina subsalsa* and *Selenastrum capricornutum* (both cultivated) and (powdered) *Spirulina maxima*	Total antioxidant capacity	(i) Amperometric using the enzymatic biosensor with superoxide dismutase (ii) Cyclic voltammetry	Antiaging potential	[204]
(55)	Buckwheat sprouts (roots obtained from dark- and light-grown)	Total antioxidant capacity	(i) TEAC assay (ii) Cyclic voltammetry		[205]
(56)	Tea infusions	(i) Total phenolic content (ii) Total antioxidant capacity	(i) HPLC (ii) Cyclic voltammetry	Reduce blood glucose level	[206]
(57)	*Coriandrum sativum*	Antioxidant terpenes	HPTLC	digestive, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic, and anticarcinogenic	[96]
(58)	*Scoparia dulcis*	Flavonoids and terpenoids	HPTLC	Antibacterial, antifungal, antiherpetic, anti-inflammatory, antiseptic, antispasmodic, antiviral, cytotoxic, emmenagogic, emollient, febrifuge, and hypotensive	[95]
(59)	*Acacia confusa*	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay	Used for wound healing and antiblood stasis	[207]
(60)	Teas and herbal infusions	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay (iii) FRAP assay (iv) ABTS assay (v) Polarographic		[208]
(61)	Extra virgin oils	Total phenolic content	Voltammetric		[209]
(62)	Selected wines	(i) Total phenolic content (ii) Total antioxidant capacity	(i) Folin-Ciocalteu method (ii) DPPH assay (iii) Differential pulse voltammetry		[210]
(63)	Fruits (raspberry, strawberry, and berry fruit) and vegetables (carrot, tomato, and rhubarb)	Antioxidant capacity	Differential pulse voltammetry		[211]

The profile and quantitative analysis of compounds present in *Lycium* species was performed using HPLC with diode array detection: *p*-coumaric acid, chlorogenic acid, and rutin were identified by their retention times and UV spectra versus those of the standards. Other benzoic and hydroxycinnamic acids, flavonoids, and anthocyanin derivatives were identified by UV spectra and quantified by using gallic acid, *p*-coumaric acid, rutin, and cyanidin-3-glycoside, respectively, as standards. Phenolic acid derivatives confirmed their prevalence and presence in the highest amounts in all analysed extracts. Butanolic extracts of *Lycium barbarum* and *Lycium ruthenicum* were characterized by the highest level of benzoic and hydroxycinnamic acid derivatives, which was in accordance with the most enhanced antiradical activity of these extracts [107].

HPLC with diode array detection and ion trap MS was applied to assess dose response and metabolism of anthocyanins present in strawberry. Pelargonidin 3-glucoside was the main anthocyanin present in strawberry, and this anthocyanin and three of its metabolites (detected as monoglucuronides) were excreted and assessed in urine after ingestion. One prevalent monoglucuronide form was detected in urine in masses 10-fold higher than the other two monoglucuronide forms. It was assessed that anthocyanins from strawberries present a linear dose response over ranges of 15–60 mmol. The 24 h urinary recoveries were much more elevated than those reported for most of the other anthocyanins and it has been concluded that pelargonidin-based anthocyanins may be more efficiently absorbed than other anthocyanins [108].

18 phenolic compounds have been analysed by HPLC-MS in harvested and commercial 50% methanolic extracts of *Ocimum basilicum*. In the extracts obtained from harvested samples, rutin (665.052 mg/100 g dried plant) and caftaric acid (1595.322 mg/100 g dried plant) were determined in the largest amount. Commercial samples contained hydroxycinnamic acid derivates, dihydroxybenzoic acid, flavonols, and flavonoid glycosides [109].

The determination of rosmarinic acid content of *Salvia maxima* and *Salvia verde* was carried out by HPLC. Methanol was employed for the extraction of the *Salvia* samples; then filtration (on a 0.45 mm PTFE filter) was performed before injection in the LC-DAD-ESI/MS setup. The mobile phase was comprised of 0.1% (v/v) formic acid and acetonitrile, with the application of linear gradient. The content of phenolics in the analysed samples was assessed through interpolation of the peak area using the calibration curve developed per reference to the rosmarinic acid peak and retention time. The results obtained, as rosmarinic acid equivalent content, ranged from 103 ± 2 μg/g fresh material for *S. maxima* to 174 ± 2 μg/g fresh material for *Salvia verde*, with a limit of detection of 3.4×10^{-7} mol L^{-1} [110].

The application of a series of chromatographic techniques (HPLC-DAD, LC-MS/MS, and GC-MS) led to the successful detection of antioxidant purine alkaloids (caffeine, theobromine, and theophylline) and indole alkaloids (harmine, harmane, harmol, yohimbine, brucine, and strychnine) in *Andrographis paniculata* and in dietary supplements containing this plant. This Ayurveda plant is used for healing purposes (see Table 2), hence the interest in structure and potential toxicity elucidation. Purine and indole alkaloids assessment by HPLC-DAD, LC-MS/MS, and GC-MS showed lower concentration of these components in roots of 50.71 ± 0.36 mg/g d.m. in comparison to the leaves of 78.71 ± 0.48 mg/g d.m. In addition, three bioactive diterpenoids were determined by HPLC-DAD and GC-MS methods with good selectivity, accuracy (recovery > 91.5%), and precision (RSD < 5.0%) [111].

The analysis of phenolic synthetic antioxidants BHA, BHT, and TBHQ in edible oils was carried out by HPLC with UV-VIS detection at 280 nm on the basis of peak area ratios. The mobile phase was composed of methanol and 0.01 mol L^{-1} monosodium phosphate, with gradient elution. BHA content ranged between 20.1 μg g^{-1} in rapeseed oil and 55.9 μg g^{-1} in sesame oil. BHT was only found in blend oil at a level of 21.4 μg g^{-1}. TBHQ amount ranged between 25.4 μg g^{-1} in rapeseed oil and 47.2 μg g^{-1} in corn oil [112].

A number of 19 phenolic compounds were determined by HPLC, during the ripening of cumin seeds. The phenolic compounds were analysed by *Reversed-Phase* High Performance *Liquid Chromatography* with an UV-VIS multiwavelength detection. The separation was performed on a Hypersil ODS C18 column at ambient temperature. The mobile phase comprised acetonitrile and water with 0.2% H_2SO_4. The flow rate was established at 0.5 mL/min and gradient elution was applied. Rosmarinic acid was the main phenolic acid found in the unripe seeds. Then, p-coumaric acid was confirmed as the prevalent phenolic in half ripe and full ripe seeds [113]. HPLC analysis of *Artemisia capillaris* extracts proved that the main compounds imparting antioxidant capacity were chlorogenic acid, 3,5-dicaffeoylquinic acid, and 3,4-dicaffeoylquinic acid [114].

The HPLC profile of methanolic extracts of *Spathodea campanulata* revealed antioxidant potential of this traditionally used plant against malaria and inflammation due to the presence of bioactive compounds such as verminoside (10.33%) and 1-O-(E)-caffeoyl-beta-gentiobiose (6.58%) [115]. Glucosinolates from broccoli were analysed by HPLC after enzymatic desulfation. The HPLC system included a Spherisorb ODS-2 column, and the water/acetonitrile mixture was used for gradient elution of samples. Glucoraphanin, precursor of the most active antioxidant glucosinolate found in broccoli, was assessed as the prevalent compound: 14.06 to 24.17 μmol/g [116]. HPLC chromatographic assay of the methanolic extract of *Bambusa textilis* McClure indicated active antiradical fractions, as presented in Figure 1 [87].

4.2. Spectrometric Techniques

4.2.1. Studies Based on Nonenzyme Assays. The antioxidant activity of *Acacia confusa* bark extracts was determined by free radical scavenging against DPPH. The total phenolic content was assessed according to the Folin-Ciocalteu method, using gallic acid as a standard. The scavenging activity exhibited against the DPPH free radical diminished in the following order: 3,4,5-trihydroxybenzoic acid = 3,4-dihydroxybenzoic acid = 3,4-dihydroxybenzoic acid ethyl

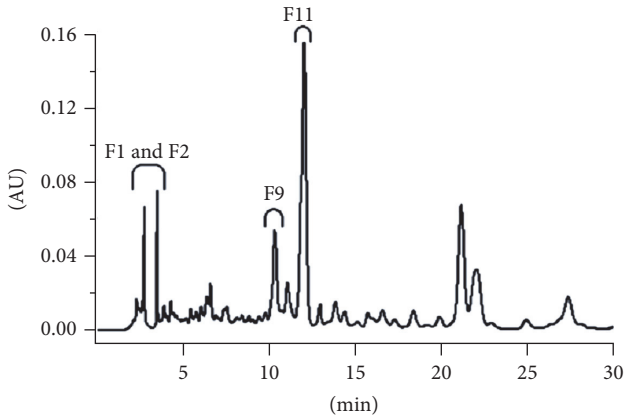

Figure 1: HPLC chromatogram (at 330 nm) of the methanolic extract of *Bambusa*, with illustration of the antioxidant fractions F1, F2, F9, and F11 [87].

ester > 4-hydroxy-3-methoxybenzoic acid > 3-hydroxy-4-methoxybenzoic acid > 4-hydroxybenzoic acid = benzoic acid. It has been stipulated that this trend is due to the presence of catechol moieties in 3,4,5-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, and 3,4-dihydroxybenzoic acid ethyl ester, which impart antioxidant activity [207].

Fruits of *Lycium* species were subject to sequential extraction with petroleum ether, ethyl acetate, methanol, n-butanol, and water in a Soxhlet extractor. All the extracts were analysed for their scavenging potential towards the free DPPH$^{\bullet}$ radical by *in vitro* method. The composition of each extract was also studied for the Folin-Ciocalteu reactive species. It was stressed out that the butanol extracts of both species (*Lycium barbarum* and *Lycium ruthenicum*) were endowed with the highest scavenging potential (smallest IC_{50}). A linear relationship (correlation) was established between the total phenol content (Folin-Ciocalteu assay) and the radical scavenging potential [107].

A research dedicated to the antioxidant composition investigation and antioxidant capacity determination in *Ocimum basilicum* showed that the scavenging effect against the DPPH radical was proportional to the phenolic content, to which flavonoids and caffeic acid derivatives contribute. The DPPH scavenging activity proper to harvested samples (26.55 and 22.43%, resp.) was greater than the one obtained for commercial ones (12.05 and 11.24%), considering the one of BHT as 94.77% [109].

Six spice plant samples, namely, onion (*Allium cepa*), parsley (*Petroselinum crispum*) roots and leaves, celery (*Apium graveolens*) roots and leaves, and leaves of dill (*Anethum graveolens*), were subject to analysis for the total phenolic amount and the antioxidant activity assessed by DPPH scavenging. The celery leaves exhibited the highest total phenolic content, namely, 1637.1 mg gallic acid equivalents/100 g, and the highest radical scavenging activity against DPPH [101].

The antioxidant properties of methanol extracts of 15 broccoli samples were estimated by DPPH$^{\bullet}$ and OH$^{\bullet}$ radical inhibition. These activities ranged from 1.49 μmol Trolox/g DW to 3.34 μmol Trolox/g DW. The sample endowed with the highest DPPH$^{\bullet}$ radical scavenging activity also possessed the highest phenolic and glucosinolate contents, including glucoraphanin [116].

In another study, the antioxidant activity of *Clusia fluminensis* extracts was assessed, exploiting the scavenging of the stable free radical DPPH. The flavones and flavonols content was also determined in order to test the potential correlation with the antioxidant capacity. No significant differences were revealed between the total flavonoid contents of *Clusia fluminensis* in acetone and methanol extracts, respectively. The acetone extract was endowed with the highest antioxidant activity (with almost 2 times smaller EC_{50} value than the one proper to methanol extract) and highest flavonoid level. Hence, it has been asserted that acetone is an efficient solvent for antioxidant extraction. It has been also suggested that the substances with best antioxidant activity in Clusiaceae fruits possess intermediate polarity [176].

The antioxidant activity of 52 wine samples was assessed spectrophotometrically and expressed as the amount of wine able to engender 50% decolorization of the DPPH radical solution per reference to the control (EC_{50}). The obtained average values of EC_{50} were 20.1 μL for red and 98.4 μL for white dry wines. The highest EC_{50} of red dry wines, 26.9 μL (illustrating the lowest antioxidant capacity), was inferior to the one proper to white wines with the most reduced antioxidant capacity, 56.4 μL. It was inferred that, regarding DPPH radical scavenging, red wines are around 5 times stronger than white wines, despite the absence of statistically significant differences between the grape varieties studied, as well as among different wine regions [210].

The total phenolic amount and antioxidant potential expressed by the IC_{50} values (concentration causing a 50% DPPH inhibition) were assessed in the seeds of cumin at different ripening stages. At full ripening stage, for which the highest level of total phenolics was determined (17.74 and 25.15 mg GAE/g DW), the antioxidant capacity also attained its peak, with the smallest values of IC_{50}, 6.24 and 42.16 μg/mL, respectively, for maceration and Soxhlet methods applied for extraction [113].

Another study was performed to assess the antioxidant and antimicrobial potential of methanol (100 and 80%) aqueous extracts of pumelo fruits albedo (*Citrus grandis* Osbeck). The antioxidant and antibacterial activity of both crude extracts and isolated compounds were determined using DPPH scavenging and paper disc diffusion method. The 100% methanol extract was steeped in water at different pH values and subject to partitioning with ethyl acetate yielding basic, acidic, neutral, and phenolic fractions. The neutral fraction revealed the highest antioxidant potential and antibacterial efficacy [191].

The antioxidant activities of *Artemisia capillaris* extracts in different organic solvents (*n*-hexane, ethyl acetate, acetone, and methanol) were tested. Methanol extracts of *Artemisia capillaris herba* possessed the highest phenolic content and were endowed with the strongest antioxidant power, when compared to the other solvent extracts; namely, the scavenging potential of three extracts exhibited against the DPPH radical varied as follows: ethyl acetate extracts < acetone extracts < methanol extracts, corresponding to the inhibition

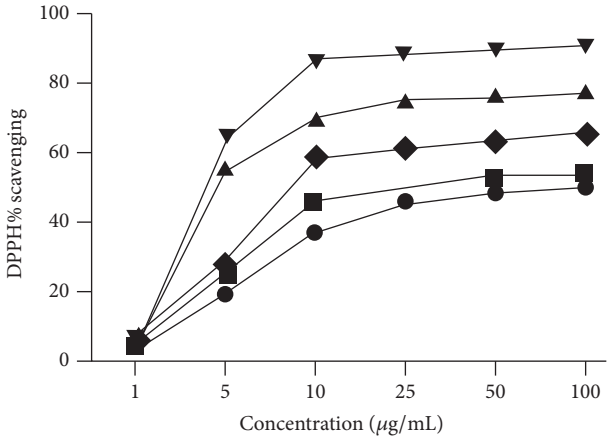

Figure 2: DPPH radical scavenging activity of *Sonchus asper* extracts in different solvents at various concentrations. Each value stands for the mean ± SD ($n = 3$) of hexan, ethyl acetate, chloroform, and methanol crude extracts of the whole plant and ascorbic acid [88].

percentages of 35.2, 57.1 and 91.1% at a dose of 200 ppm, respectively. The scavenging potential of 100 ppm methanol extract (90.8%) equaled the one proper to 100 ppm BHT (90.5%) and was very close to the one of α-tocopherol (92.3%). The methanolic extract also exhibited the strongest antioxidant activity in the β-carotene bleaching system [114].

The antioxidant potential of nonpolar (hexane, ethyl acetate, and chloroform) and polar (methanol) *Sonchus asper* crude extracts was assessed by DPPH radical scavenging. Methanol extract showed the highest scavenging potential (smallest IC_{50}) followed by chloroform, ethyl acetate, and hexane extracts, as revealed in Figure 2 [88].

The antiradicalic activity against DPPH and the superoxide anion scavenging activity (in a riboflavin-light-nitro blue tetrazolium chloride system) were determined in the case of methanolic extracts of *Bergia suffruticosa*, bone, and sore healer. The whole plant extracts proved dose-increasing scavenging activities versus $DPPH^{\bullet}$ and superoxide, with EC_{50} values of 13.1 μg and 139.4 μg, respectively. The Fe^{3+} to Fe^{2+} reducing ability was also proven to be dose-dependent, reaching a maximum for 300 μg extract [90].

In another paper, the antioxidant profiling and antioxidant activities of dried fruits have been assessed, namely, prunes, apricots, raisins, and figs [183]. The highest concentration of carotenoids was present in apricots and figs (10.7 and 10.8 mg β carotene equivalents/100 g). Raisins possessed the highest total phenolic concentration (1.18 g gallic acid equivalents/100 g) and proanthocyanidins (17.53 mg cyanidin equivalents/100 g). Figs presented the highest flavonoid (105.6 mg quercetin equivalents/100 g) and anthocyanin (5.9 mg/100 g) amount. The antioxidant activities were also assessed. The apricot aqueous extract had the best reducing power, Agen prune presented the highest antioxidant activity furnished by the phosphomolybdenum method, and the raisin extract in ethanol showed the best $DPPH^{\bullet}$ quenching capacity [183].

A study based on ABTS scavenging ability proves that rosemary extract exhibits an antioxidant capacity equivalent to the one of BHA and superior to the one proven by BHT. The *chile ancho* extract exhibits a lower antioxidant capacity when compared to rosemary and both BHT and BHA. In the case of rosemary, due to the preponderance of ethanol in the extraction mixture, the polyphenol amount increases, being the most elevated for an ethanol : water ratio of 75 : 25. The rosemary extract is rich in rosmarinic acid, carnosic acid, and carnosol. In the case of *chile ancho*, the maximum extracted polyphenol amount is obtained for an ethanol : water ratio of 50 : 50. The *chile ancho* extract contains flavonoids (luteolin, quercetin), carotenoids, ascorbic acid, and capsaicinoids [178].

The phenolic content and antioxidant activity of *Vitis vinifera* extracts were followed under different storage conditions. The total phenolic content was determined by Folin-Ciocalteu method and the antioxidant capacity by the scavenging ability versus the ABTS cation radical. The extract proved stable for up to one year at storage in darkness as a hydroalcoholic solution at 4°C or as a freeze-dried powder at 25°C. The total phenolic content was found constant at different pH values (3.0, 5.0, 7.0, and 9.0) for up to 400 days, while the antioxidant capacity diminished at pH values greater than 5.0. The thermal treatment (at 121°C for 15 minutes, at different pH values) neither decreased nor increased the ABTS radical cation-scavenging activity. Nevertheless, it was found that the total phenolic content increased after heating at all pH values tested [181].

Another study was dedicated to the assessment of the antioxidant potency of phenolic substances from wild Algerian medicinal plants by chemical and biological methods. *Anthemis arvensis* and *Artemisia campestris* had the highest phenolics amounts (115.2 and 103.4 mg/g DW, resp.) and the most enhanced antioxidant power assessed by $ABTS^{\bullet+}$ decolorization (0.726 and 0.573 mmol TEAC/g DW, resp.). They also promoted an enhanced delay of free radical-induced red blood cells hemolysis, even when compared to caffeic acid, the reference antioxidant endowed with the most effective inhibition capacity [103].

Both lipophilic and hydrophilic components of 24 cereal grains from China were spectrophotometrically assessed. For water-soluble fractions of the analysed grains, the FRAP values varied from 0.87 ± 0.08 to 114.69 ± 2.15 μmol Fe(II)/g DW. Black rice exhibited the highest FRAP value, followed by organic black rice, purple rice, and organic black millet. With respect to the fat-soluble fractions, the FRAP values ranged between 4.27 ± 0.19 and 21.91 ± 1.27 μmol Fe(II)/g, with red rice, buckwheat, organic black rice, and brown rice exhibiting the most elevated FRAP values. The TEAC values (relying on the ability of antioxidants to scavenge $ABTS^{\bullet+}$) ranged between 0.18 ± 0.01 and 25.28 ± 1.07 μmol Trolox/g DW for the water-soluble fractions, with black rice, organic black rice, buckwheat, and red glutinous rice exhibiting the highest values. For fat-soluble fractions, the TEAC values varied from 0.06 ± 0.04 to 5.22 ± 0.29 μmol Trolox/g. The antioxidant capacities of cereals showed a significant correlation between the FRAP value and the TEAC values. A strong correlation between antioxidant capacity and total phenolic content

(Folin-Ciocalteu) was also obtained, indicating that phenolic compounds mainly contribute to the antioxidant capacities of these cereals [105].

The total antioxidant capacity and phenolic content were assessed for 70 medicinal plant infusions. *Melissae folium* infusions exhibited a ferric reducing antioxidant power greater than 20 mmole Fe(II)/L and a phenol antioxidant coefficient greater than 3. The DPPH radical scavenging ability of *Melissae folium* phenolics was close to that of catechin. With respect to ABTS radical cation scavenging, *Melissae folium* phenolics exhibited superior efficacy in comparison to Trolox and vitamin C [212].

Species belonging to Malvaceae family (*Sidastrum micranthum* (A. St.-Hil.) Fryxell, *Wissadula periplocifolia* (L.) C. Presl, *Sida rhombifolia* (L.) E. H. L., and *Herissantia crispa* L. (Brizicky)) were investigated for the total phenolic content, DPPH radical scavenging activity, and Trolox equivalent antioxidant capacity. The antioxidant activity of the crude extract, aqueous and organic phases and isolated flavonoids, kaempferol 3,7-di-O-α-L-rhamnopyranoside (lespedin), and kaempferol 3-O-β-D-(6″-E-p-coumaroyl) glucopyranoside (tiliroside) was assessed. A firm correlation was noticed between total polyphenol content and antioxidant activity of the crude extract of *Sidastrum micranthum* and *Wissadula periplocifolia*; this was not the case for *Sida rhombifolia* and *Herissantia crispa*. The ethyl acetate phase exhibited the best total phenolics content, as well as antioxidant capacity in DPPH and TEAC assays, followed by the chloroform phase. To lespedin, present in the ethyl acetate phase of *W. periplocifolia* and *H. crispa*, no significant antioxidant activity has been ascribed (IC_{50}: DPPH: 1,019.92 ± 68.99 mg/mL; TEAC: 52.70 ± 0.47 mg/mL); tiliroside, isolated from *W. periplocifolia*, *H. crispa*, and *S. micranthum*, had small IC_{50} (1.63 ± 0.86 mg/mL), proving better antioxidant capacity as provided by TEAC method [213].

The antioxidant properties of *Diospyros bipindensis* (Gürke, used in Baka traditional medicine against respiratory diseases) stembark were assessed by ABTS, DPPH, and ORAC assays. The antioxidant properties that contribute to the bioactivity of the plant extract were mainly imparted by ismailin [179].

During the investigation of the antioxidant potentials of some cereals and pseudocereals, polyphenol dry matter extracts from seeds of buckwheat, rice, soybean, amaranth, and quinoa (obtained with 1.2 M HCl in 50% methanol/water) showed better inhibition of lipid peroxidation than the ones extracted with 50% methanol/water and were close to the antioxidant activity of BHT at concentration of 0.2 mg/mL. The antioxidant activities of seed extracts determined by $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging, and β-carotene bleaching proved strong correlation with the total polyphenols assessed by Folin-Ciocalteu assay. It has been concluded that proteins do not significantly contribute to the samples' antioxidant activity and that buckwheat followed by quinoa and amaranth are the most proper as cereal substitutes [214].

A series of foods usually consumed in Italy were analysed for their antioxidant capacity, by TEAC, TRAP (relying on the protective action of antioxidants, over the fluorescence diminution of R-phycoerythrin in a monitored peroxidation reaction), and FRAP. Among vegetables, spinach had the highest antioxidant capacity in the TEAC and FRAP assays, followed by peppers, while asparagus had the greatest antioxidant capacity in the TRAP assay. Among fruits, berries (blackberry, redcurrant, and raspberry) possessed the highest antioxidant capacity in all assays. With respect to beverages, coffee had the greatest total antioxidant activity regardless of the technique, followed by citrus juices. As for oils, soybean oil had the highest antioxidant capacity, followed by extra virgin olive oil, whereas peanut oil proved less effective [215].

The *in vitro* antioxidant activity of wines has been investigated by a series of determinations such as ORAC, ABTS, DPPH, and DMPD quenching. Also, the total phenolic index was assessed for the 41 samples subject to analysis. Red wines necessitated solid phase extraction to discriminate three main fractions. ABTS, DPPH, and ORAC provided the same reactivity ranking of the analysed fractions: fraction 2 (flavan-3-ol and anthocyanins) showed the best activity followed by fractions 1 (phenolic acids) and 3 (flavonols). Nevertheless, a much reduced reactivity of fraction 2 components (anthocyanins and flavanols) towards $DMPD^{\bullet+}$ was noticed which is consistent with the lower correlation with the total phenolic index and the smaller difference (red versus white wines) in comparison to the other methods' results [216].

4.2.2. Studies Relying on Both Enzyme-Based and Nonenzyme Assays. The antioxidant activity of the ethyl acetate and *n*-butanol extracts, along with that of seven flavonol glycosides isolated from *Dorycnium hirsutum* aerial parts, was assessed using the DPPH method and the lipoxygenase assay. With respect to the inhibition activity towards the DPPH radical, kaempferol 3-O-α-L-rhamnopyranoside possessed the highest antioxidant activity (1.226 Trolox equivalents). The lipoxygenase assay has been also performed, in the presence of linoleic acid and commercial lipoxygenase at pH = 6.80, and the hydroperoxide generation was monitored at 235 nm. It was proven that kaempferol 3-(4″-O-acetyl)-O-α-L-rhamnopyranoside-7-O-α-L-rhamnopyranoside exhibited the best antioxidant protection activity in the lipid peroxidation system. The butanolic extract proved less active than the ethyl acetate residue, which confirmed the higher flavonoid level assessed in the latter [217].

The scavenging properties of the DPPH radical and the xanthine oxidase inhibition activity were determined for methanol extracts of *Lantana camara* obtained from different plant parts. The absorbance diminution of the DPPH solution was measured at 517 nm, and the XO inhibition assay was based on uric acid production estimated according to the absorbance increase at 290 nm. Allopurinol (XO inhibitor) was used as positive control. The DPPH assay showed that the leaf extract had the best antioxidant activity, and the highest phenolic content was also found in the leaves: 245.50 ± 3.54 mg gallic acid/g [180].

The *in vitro* antioxidant capacities of methanolic extracts (80%) prepared from *Cornus mas* L., *Diospyros kaki* L., and *Laurocerasus officinalis* Roem were tested by a series of recognized methods: DPPH, superoxide radical scavenging, FRAP,

CUPRAC, metal-chelating capacity, β-carotene bleaching test in a linoleic acid emulsion system, and TEAC [186]. For the superoxide radical scavenging, the extract was subject to reaction with a substrate solution containing sodium xanthine and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride. Xanthine oxidase was used as biocatalyst and the absorbance increase was monitored at 505 nm. Gallic acid was used as reference phenolic [143, 186]. The Folin-Ciocalteu assay of total phenolic content involves sample reaction with a mixture of phosphomolybdate and phosphotungstate in the presence of sodium carbonate 20%. Absorbance readings of the blue molybdenum-tungsten complex formed in the presence of reducing phenolics are taken at 765 nm after incubation, with gallic acid as [218]. *Diospyros kaki* yielded the best results, except for the β-carotene bleaching assay. Also, good correlation was obtained between the phenolic profile and antioxidant activity. No metal-chelating activity was shown [186].

The isolation and characterization of bioactive polyphenolic compounds from bitter cumin (condiment and also stimulant, carminative, and astringent in traditional Ayurvedic medicine) were performed, by various spectrophotometric determinations, to assess their scavenging activities. The bitter cumin seed extract showed enhanced antioxidant activity at μg amounts, trapping effectively $DPPH^{\bullet}$, lipid peroxyl, hydroxyl, and superoxide anion radicals. Superoxide anions were generated in the samples containing nitroblue tetrazolium, nicotinamide adenine dinucleotide-reduced, and phenazine methosulphate. The absorbance taken at 560 nm decreased in the presence of cumin extracts, pointing towards superoxide anion scavenging activity. The measurement of lipid peroxidation activity in rat liver microsomes was performed incubating microsomal protein with ferrous sulphate and ascorbic acid, yielding malonyl dialdehyde, which led to the assessment of TBARS in the presence of thiobarbituric acid with readings taken at 535 nm. The presence of cumin antioxidants led to the decrease of microsomal lipid peroxidation, assessed in the presence of soybean lipoxygenase and linoleic acid. The absorbance due to lipid hydroperoxides was measured at 234 nm. Cumin phenolics decreased lipid peroxidation and proved radical scavenging ability in Fenton OH-initiated DNA damage [106].

Twelve methanolic extracts from rosemary leaves harvested from different locations of Turkey at four different times of the year were analysed for their radical scavenging capacities and antioxidant activities by applying different techniques: DPPH radical scavenging activity, Trolox equivalent antioxidant capacity, and reversing H_2O_2-induced erythrocyte membrane lipid peroxidation. Human erythrocyte superoxide dismutase and catalase activities, after *in vitro* incubation with the extracts, were also tested in order to check altered enzymatic efficiency. *Rosmarinus officinalis* samples were collected from three different locations, namely, Canakkale (southern Marmara region, the coolest climate), Izmir (Aegean region, moderately hot), and Mersin (Eastern Mediterranean region, the hottest), on four different intervals as follows: December 2003 (denoted as C-S1, I-S1, and M-S1, resp.), March (C-S2, I-S2, and M-S2), June (C-S3, I-S3, and M-S3), and September 2004 (C-S4, I-S4, and M-S4). TEAC values ranged between 11.7 and 5.3 mmol Trolox/kg FW, with Canakkale–March samples having the highest value (11.7 mmol/kg). With respect to the H_2O_2-forced human erythrocyte membrane lipid peroxidation test, most of rosemary extracts acted efficiently except for C-S2, I-S1, IS2, I-S3, and I-S4. I-S2 and I-S3 even exhibited prooxidant activity and caused an increase of MDA. The M series together with C-S4 have been proven to be the most active antioxidants in the *in vitro* test. M-S4 extract showed closest effect to the three reference antioxidants: BHT, vitamin C/vitamin E mixture, and quercetin. All extracts caused significant increases in SOD activity, except C-S1, I-S1, I-S4, and M-S4. M series extracts did not affect CAT activity, while C and I series significantly decreased CAT activity.

All extracts proved high ability to quench the free radicals (DPPH and ABTS) and to inhibit malonyl dialdehyde formation. On the whole, the obtained results revealed that the plants harvested in September possess higher levels of active constituents and superior antioxidant activities in comparison to those harvested in other year seasons. Plants harvested from the Izmir region had lower total phenol and active constituent levels and hence smaller antioxidant activity. These differences were assigned to the various compositions in bioactive compounds characterizing the plants harvested from different locations and in various year seasons: the rosmarinic acid contents in the Izmir and Mersin samples increased in summer, with a maximum in September (30.4 mg g^{-1}), and decreased in December, attaining a minimum in March (0.4 mg g^{-1}). In the Canakkale samples, the rosmarinic acid level raised progressively from December to September. As in the case of rosmarinic acid, the levels of carnosol peaked in September and then diminished stepwise, attaining a minimum in March. For carnosic acid, seasonal differences were not as significant as for rosmarinic acid and carnosol: all September samples possessed higher levels of carnosic acid. Mersin samples exhibited the highest and lowest carnosic acid levels in September and March, respectively [104].

111 samples of yerba mate from three (Southeast, South Central, and Metropolitan Area of Curitiba) regions of the Brazilian state of Parana were subject to analysis of the total phenolic content (TPC) by near infrared spectroscopy. Multivariate calibration models were developed to assess the TPC from the NIR spectra using partial least squares regression. Namely, the characteristic spectral signal of phenolic groups (4.670 cm^{-1}) led to the development of multivariate models for quantifying total phenolics. The reference experimental results and those predicted by the partial least squares regression model obtained for the 26 samples ranged between 27.28 and 44.55 mg g^{-1} [219].

In another study, the antioxidant action of different flavonoids (quercetin, glabridin, red clover extract, and the isoflavones mixture Isoflavin Beta) was investigated to assess the appropriateness as topical formulation against free radicals-induced damage. Horseradish peroxidase catalyses luminol oxidation to 3-aminophthalate by H_2O_2, followed by light emission at 428 nm, at pH 7.40, in 0.1 M phosphate buffer.

The reduction of chemiluminescent signal in the presence of antioxidants takes account on their antioxidant potential. All samples proved their capacity to inhibit oxidative damage, as depending on quercetin and beta isoflavin level. The highest chemiluminescence inhibition was noticed for glabridin and dry red clover extract [220].

4.3. Electrochemical Techniques

4.3.1. Linear Sweep Techniques: Cyclic Voltammetry. The antioxidant capacity of dry extracts of green tea, black tea, rosemary, and coffee, acerola and açaì, was assessed voltammetrically at a glassy carbon electrode. Methanol proved better efficacy in extracting antioxidant principles. The antioxidant capacities given by anodic area on the voltammogram and expressed as mg ascorbic acid equivalents ranked as follows: green tea > black tea > rosemary > arabica coffee > herb tea > acerola > quality tea > açaì [221].

A cyclic voltammetric study of the electrooxidation of phenolic compounds present in wine revealed a pivotal influence of the structure on the propensity to undergo oxidation. Compounds with an orthodiphenol group (catechin, epicatechin, quercetin, and gallic, caffeic, and tannic acids) and morin possess the greatest antioxidant potential and are oxidized at low potentials at about 400 mV. Ferulic acid, transresveratrol, and malvin as well as vanillic and p-coumaric acids that have a solitary phenol moiety in many cases close to a methoxy function are less electrooxidizable. Anthocyanins determine the existence of a peak at 650 mV. It was also assessed that phenolics that imparted a first peak in the 370–470 mV range on the voltammogram can be secondly oxidized at around 800 mV: catechin and epicatechin by reason of the meta-diphenol groups on the A-ring, quercetin owing to the -OH group on the C-ring, and gallic acid due to the third -OH group placed next to the orthodiphenol moiety oxidized during the first wave [222].

A voltammetric electronic tongue system composed of an array of modified graphite-epoxy composites and a gold microelectrode was applied in the qualitative and quantitative analysis of polyphenols in wine. Samples were analysed by cyclic voltammetry and did not necessitate sample pretreatment. The analytical responses were processed by discrete wavelet transform in order to compress and extract the essential features of the voltammetric analytical responses. External test subset samples results correlated well with the ones furnished by the Folin-Ciocalteu assay and UV absorbance polyphenol index (I_{280}) for amounts from 50 to 2400 mg L^{-1} gallic acid equivalents [223].

BHA, BHT, and TBHQ in edible oil samples were assessed by first derivative voltammetry at glassy carbon electrode modified with gold nanoparticles. First derivative pretreatment was applied to the linear sweep voltammetric signals, aiming at minimizing noise influence. The values of the peak potentials of 0.273, 0.502, and 0.622 V allowed discrimination between TBHQ, BHA, and BHT with detection limits as low as 0.039, 0.080, and 0.079 $\mu g\,mL^{-1}$ for the three aforementioned analytes. BHA content ranged between 19.3 $\mu g\,g^{-1}$ in rapeseed oil and 56.6 $\mu g\,g^{-1}$ in sesame oil. BHT was only found in blend oil at a level of 20.7 $\mu g\,g^{-1}$. TBHQ level ranged between 26.8 $\mu g\,g^{-1}$ in rapeseed oil and 48.4 $\mu g\,g^{-1}$ in corn oil. Interference studies indicated that the determination of all three compounds in commercial edible oil samples was not significantly affected by the common interferents, 2-fold ascorbic acid and 10-fold vitamin E; phthalate and citric acid concentrations exerted no significant influence on the peak currents of the three synthetic food antioxidants: ascorbic acid peak appeared at 0.437 V and did not hinder the analytical signals of the three analytes. Vitamin E and phthalate and citric acid did not show peaks in the potential range of 0.10–0.80 V. Metal ions (K^+, Na^+, Ca^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+}) and some of the most significant anions (Cl^-, I^-, $SO_3{}^{2-}$, $SO_4{}^{2-}$, $NO_3{}^-$, $CO_3{}^{2-}$, $PO_4{}^{3-}$, and CH_3COO^-) did not interfere also up to a 100-fold increase in their concentrations [112].

4.3.2. Differential Pulse Voltammetry. Sesamol and lignans contents in sesame, tahina, and halva were assessed by polarography and stripping voltammetry. Differential pulse polarography used a capillary hanging mercury drop electrode. In cathodic stripping, sesamol reacts forming a reduced derivative, which is oxidized, yielding a cyclic voltammetric peak. The cathodic stripping voltammetric assessment involved preconcentration (when sesamol accumulates to the electrode-surface at −1650 mV) and scanning (when analyte stripping takes place in the potential range of −1650 mV to −2250 mV). Using these electrochemical methods, sesamol was assessed at levels of 0.26–0.32 mg/100 g oil in three varieties of sesame, 10.98–12.33 mg/100 g oil in tahina, and 4.97–9.12 mg/100 g oil in halva [224].

The antioxidant activity of flavonoids (catechin, quercetin, dihydroquercetin, and rutin) was voltammetrically assessed at a glassy carbon working electrode, and the measured redox potentials were correlated to the antioxidant activity results. Differential pulse measurements relied on molecular oxygen cathodic reduction, at 50 mV s^{-1}, in the 0–800 mV potential range and for an optimal pulse amplitude of 10 mV. The antioxidant activity coefficient was assessed considering O_2 reduction current in the presence of added antioxidants and the limiting O_2 current in the absence of antioxidants. To check reversibility and electrooxidation mechanism, cyclic voltammetric assay was performed for the tested flavonoids (1–10 $\mu mol\,L^{-1}$ in phosphate buffer 0.025 M, pH = 6.86) in the potential range of 0–1000 mV, at 50 mV s^{-1}. All flavonoids presented reversible peaks in the range of 300 mV–400 mV. The intensity of the CV oxidation peak (assigned to the deprotonation of the catechol moiety, namely, of the 3′-OH electron-donating group) increases with the dihydroquercetin concentration. The peak intensity also depended linearly on the square root of the scan rate, so it was concluded that the oxidation of this flavonoid was limited by mass transfer. Both the easiness of electron transfer reflected by the redox potentials (in CV) and the antioxidant activity (assessed by DPV) correlated with the following trend of increase: rutin–dihydroquercetin–catechin–quercetin [225].

A DNA-modified carbon paste voltammetric biosensor functioned on the basis of DNA layer oxidative insults,

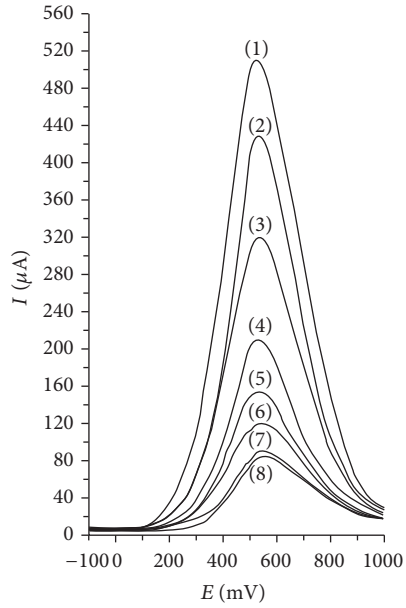

Figure 3: Differential pulse voltammograms at a Pt working electrode for different ascorbic acid concentrations (mM): 20 (1), 15 (2), 10 (3), 5 (4), 2.5 (5), 1.25 (6), 0.625 (7), and 0.31 (8); pulse amplitude, 75 mV, pulse period, 125 ms, and potential scan rate, 50 mV/s [89].

induced by Fenton $OH^{\bullet}$ radicals. The electrooxidation of the left unimpaired adenine bases can give an oxidation product able to catalyse NADH oxidation [226, 227]. Antioxidants scavenged hydroxyl radicals and the current which emerged from NADH oxidation increased as a result of a larger number of unoxidized adenine molecules [226]. Ascorbic acid has been proven to be the most effective antioxidant, as it determined the most significant electrocatalytic current increase. Among the analysed beverages, the greatest antioxidant capacity value was exhibited by lemon flavour, namely, $480 \pm 20\,\mu$M, calculated as per reference to ascorbic acid standard [226].

Differential pulse voltammetry a Pt working electrode allowed for ascorbic acid quantitation in fruit juices, based on its electrooxidation, as presented in Figure 3 [89].

4.3.3. Square Wave Voltammetry. Square wave voltammetry at disposable screen-printed carbon electrodes was developed for the polyphenol antioxidant screening in freshly squeezed blackcurrant and strawberry juices from various cultivars and at different maturity stages. Prior to the electrochemical assessment, the anthocyanins and nonanthocyanins were separated by solid phase extraction. It was proven that the charges passing to 500 and 1000 mV correlated well with the antioxidant activity, as well as with anthocyanin and ascorbate levels. The use of disposable screen-printed sensors was able to surpass the shortcoming of electrode deactivation due to fouling by the polymeric film formed through coupling of phenoxyl radicals during electrooxidation. Nevertheless, it was asserted that phenolics other than anthocyanins, with low formal oxidation potentials, should be quantified on the basis of sensor cumulative responses at 300 mV. Blackcurrant juices possessed high oxidation peaks at low potentials (<400 mV) and so enhanced antioxidant capacities [228].

The phenolic content of extra virgin olive oils was assessed by an array of 12 voltammetric electrodes: five lanthanide bis-phthalocyanines-based sensors, six polypyrrole-based sensors, and one unmodified carbon paste electrode. Apart from the peaks related to phthalocyanine, a peak due to the redox process associated with the polyphenolic fraction was also present as a shoulder in the domain of 300–500 mV (assigned as first peak). The oxidation of lanthanide bis-phthalocyanines complexes occurs at 0.55 V in KCl electrolyte and at 0.66 V in the oil extract, so the antioxidant features of polyphenols render the oxidation of phthalocyanine more difficult. The electrocatalytic efficacy of phthalocyanines enables a more facile oxidation of phenols: for the extract with the highest polyphenol content, when using a bare carbon paste electrode, the peak assigned to polyphenols appeared at 0.68 V and at 0.5 V when using a praseodymium bis-phthalocyanine electrode. The polyphenol content of the extracts ranged between 403.06 mg kg^{-1} and 990.25 mg kg^{-1} [209].

Square wave voltammetry and cyclic voltammetry at different pH values was performed to investigate the antioxidant capacity of cashew nut shell liquid components, such as cardol, cardanol, and tert-butylcardanol which were characterized by lower oxidation potentials in comparison to BHT (Epa = 0.989 V). Cardol possessed the smallest Epa (0.665 V) and hence best antioxidant potential among the tested compounds, followed by tert-butylcardanol (Epa = 0.682 V) and cardanol (Epa = 0.989 V). A linear shift of the peak potential to more negative values with the pH increase was noted for all antioxidant compounds. Increasing the pH of the electrolyte resulted in a nonlinear decrease of the peak currents, so the highest values were obtained at pH = 2.0 [229].

4.3.4. Stripping Voltammetry. Adsorptive stripping voltammetry has been applied for the determination of caffeic acid at a Pb film electrode. The working electrode was obtained in situ on a glassy carbon basis. The analyte accumulates by adsorption on the lead film electrode and is subsequently electrooxidized during the stripping step. The analytical signal depended linearly on caffeic acid concentration in the range of 1×10^{-8} to 5×10^{-7} M with a detection limit of 4×10^{-9} M in acetate buffer pH = 4.0. By operating in the square wave voltammetric mode, this technique proved viable in the determination of caffeic acid in the herbs of *Plantago lanceolata*. The caffeic acid amount obtained by the developed voltammetric method was 107.4 μg/g of dried plant with RSD of 2.95% [93].

4.3.5. Hydrodynamic Techniques: Amperometry. The determination of flavonoids and ascorbic acid in grapefruit (*Citrus paradisi*, antioxidant, antiallergic, and anticarcinogenic) peel and juice has been performed by capillary electrophoresis with electrochemical detection. Hydrodynamic voltammetric measurements aimed at optimizing operational parameters. An applied potential of +0.95 V (versus SCE) was chosen

for an optimum signal to noise ratio and a pH of the running buffer of 9.0, because at this value a rapid and efficient separation was obtained between the five flavonoids. 60 mM was the best buffer concentration, with higher values negatively influencing the detection limit. Hesperidin, naringin, hesperidin, naringenin, rutin, and ascorbic acid were separated and quantified in grapefruit juice by capillary electrophoresis with electrochemical detection, comparing the migration times with those of the standards. Hesperidin, naringin, and ascorbic acid were assessed in grapefruit peel extract, with good peak repeatabilities and recoveries [230].

A sequential injection method with amperometric detection was developed for the total antioxidant capacity assessment in commercial instant ginger infusion beverages. The method relied on the decrease of the cathodic current of $ABTS^{\bullet+}$ at a glassy carbon electrode in phosphate buffer, pH = 7.0, after reaction with antioxidants in the sample. The total antioxidant capacity ranged between 0.326 ± 0.025 and 1.201 ± 0.023 mg gallic acid equivalents/g sample [231].

A laccase-based amperometric biosensor allowed for the optimized determination of phenolic content in tea infusions. The enzyme from *Trametes versicolor* was immobilized by entrapment within polyvinyl alcohol photopolymer fixed onto disposable graphite screen-printed electrodes. An oxidation peak was noticed at 270 mV only in the presence of hydroquinone, the enzyme substrate. The amperometric responses of the biosensor were registered for three tested diphenols under the optimal experimental conditions, 0.1 M acetate buffer at pH 4.70, 30°C, and −300 mV. The highest sensitivity was obtained for catechol 18.82 ± 0.76 nA μM^{-1}, whereas the lowest one was obtained for resorcinol 0.110 ± 0.002 nA μM^{-1}. The equivalent phenol content was comprised between 4.0 and 109.2 mg caffeic acid equivalents/L sample for orange leaves and palo azul infusion, respectively [232].

A laccase-based biosensor aimed at polyphenols determination from *in vitro Salvia* cultures. The biosensor was developed by drop casting 3 mL of laccase solution and stabilization with 0.1% Nafion solution on a screen-printed carbon electrode. Chronoamperometric measurements were performed in 0.1 M phosphate buffer, pH 4.50, at −30 mV versus an Ag/AgCl reference. The results, as rosmarinic acid equivalent content (chosen as standard, as it has been identified as the major phenolic in the samples analysed, by chromatography and MS screening), ranged from 97.8 ± 8.2 μg/g fresh material for *Salvia maxima* to 162.2 ± 11.3 μg/g fresh material for *Salvia verde*, with a limit of detection of 4.2×10^{-7} M. The biosensor retains more than 85% of its initial analytical response up to 90 days. The Michaelis-Menten kinetic apparent constant of 8.3×10^{-6} M revealed the enzyme affinity for the substrate [110].

A tyrosinase-based biosensor was prepared by enzyme immobilization on single wall carbon nanotubes screen-printed electrodes modified with iron(II) phthalocyanine. The electrochemical behavior of the biosensor was studied with optimization of the parameters: the cyclic voltammograms in catechin solution do not present peaks related to phthalocyanine but exhibit only the peak related to the reduction of the o-quinone that resulted from tyrosinase-catalysed catechin oxidation. This peak points to the retaining of the catalytic activity of tyrosinase after immobilization. The maximum analytical response was obtained at 0.15 V, pH = 7.0, in phosphate buffer solution. The amperometric signal at increasing catechin concentrations was registered, with a sensitivity of 0.937 $\mu A\ \mu M^{-1}$ and a 0.89 μM detection limit. The polyphenol content of green tea samples, as mg catechin, ranged from 19 ± 1.45 to 98 ± 3.86 [233].

A superoxide dismutase-based biosensor allowed for the amperometric antioxidant activity determination in aromatic herbs, olives, and fruits. Superoxide dismutase was immobilized between a cellulose acetate membrane and a dialysis membrane. Enzymic xanthine oxidation yields superoxide radical anion and the disproportionation of the latter by superoxide dismutase results in molecular oxygen and hydrogen peroxide occurrence [234]:

$$\begin{aligned} \text{xanthine} + H_2O + O_2 &\longrightarrow \text{uric acid} + 2H^+ + O_2^{\bullet-} \\ 2O_2^{\bullet-} + 2H^+ &\longrightarrow H_2O_2 + O_2 \end{aligned} \quad (6)$$

Finally, the electrochemical response correlatable to the superoxide radical concentration is imparted by H_2O_2 oxidation at the Pt anode and 650 mV. As the electroactive superoxide anion radicals are trapped by antioxidants in the sample, the amperometric signal diminishes. The highest antioxidant capacity was exhibited by sage as herb and by medlar, among analysed fruits [234].

4.3.6. Biamperometry. This method relies on recording at a small potential difference, the current intensity between two identical working electrodes, found in a solution where a reversible redox couple (Fe^{3+}/Fe^{2+}, I_2/I^-, $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$) is present. The analyte reacts with the indicating redox couple: the selectivity of the technique depending on the specificity of the reaction involving the oxidized form of the redox pair and the antioxidant [158].

Particularly in $DPPH^{\bullet}$/DPPH biamperometry, antioxidants react with $DPPH^{\bullet}$ (radical form) decreasing its concentration and generating DPPH (reduced form). The $DPPH^{\bullet}$ reduction at one electrode gives rise to a cathodic current proportional with the concentration of the radical form, whereas the oxidation of DPPH at the other electrode yields an anodic current proportional with the molecular form concentration. When employed working conditions are as such, for the radical form concentration to be smaller than the one proper to the molecular form, cathodic current is limited by the lower concentration of $DPPH^{\bullet}$ radical in the indicating mixture. $DPPH^{\bullet}$/DPPH biamperometry was employed in the analysis of tea, wine, and coffee using glassy carbon electrodes [158]. $ABTS^{\bullet+}$/ABTS biamperometry enables analysis of juices, tea, and wine [159], as well as wines and spirits with excellent sensitivity, 0.165 nA/μM Trolox [160].

Samples of Brazilian woods and oak (*Quercus* sp.) extracts were subject to ceric reducing antioxidant capacity (CRAC) analysis at a boron-doped diamond film electrode, relying on Ce^{4+}/Ce^{3+} redox couple. Chronoamperometric determinations enabled quantification of the decrease of

Ce^{4+} concentration, which was caused by its reduction by antioxidants present in the sample. The following variation of the antioxidant activity of analysed extracts was observed: oak (*Quercus* sp.) (1.73) > cabreuva-vermelha (*Myroxylon balsamum*) (1.05) > cabreuva (*Myrocarpus frondosus*) (0.90) > imbuia (*Octea porosa*) (0.71) > pequi (*Caryocar brasiliense*) (0.31) [235].

The ceric reducing antioxidant capacity assay was also exploited given its direct electron transfer facility for determining the antioxidant capacity of eight antioxidant compounds. The developed technique was based on observing the decrease of Ce^{4+} concentration after its reaction with antioxidants. The following trend of variation of antioxidant capacities resulted from this comparative investigation, which relied on chronoamperometric measurements: tannic acid > quercetin > rutin > gallic acid ≈ catechin > ascorbic acid > butylated hydroxyanisole > Trolox. The results were consistent with those furnished by the applied conventional FRAP assay [236].

Vitamin C assessment relied on the oxidation of the analyte at acidic pH by I_2/I^- employed as oxidizing agent. The biamperometric detection of the amount of iodine consumed allowed for the assessment of vitamin C with a linear range of analytical response comprised between 5×10^{-5} and 5×10^{-4} M: a 1.08% RSD ($n = 10$; $c = 2.5 \times 10^{-4}$ M) and high throughput of 60 samples h^{-1} [237].

4.3.7. Potentiometric Assay. The analytical signal represented by the potential change is the result of the variation of an ionic species concentration. A flow injection potentiometric method was developed to rapidly and reproducibly evaluate the antioxidative ability characteristic for several aqueous plant extracts. This potentiometric technique relies on recording the potential shift in the ferricyanide/ferrocyanide mediator system in the presence of antioxidants, which react with the oxidized form of the redox pair, modifying the concentration ratio between the oxidized and reduced forms. The developed potentiometric method used as detector a Pt electrode transducer based on logarithmic dependence, which provided the antioxidant activity of several hydrosoluble antioxidants (ascorbic acid, pyrocatechol, pyrogallol, caffeic acid, chlorogenic acid, gallic acid, tannic acid, uric acid, l-cysteine, and Trolox). The total antioxidant activity of aqueous fruit extracts was comprised between 0.066 ± 0.002 ascorbic acid equivalents (for lemon) and 0.490 ± 0.001 (for orange). The tea infusions' values ranged between 3.60 ± 0.1 ascorbic acid equivalents for Dolche vita and 18.0 ± 0.2 for Sweet osman [161].

A linear potentiometric response versus ascorbic acid at an iodine-modified platinum electrode was obtained between 1.0×10^{-5} and 1.0×10^{-3} M in model solutions. The study also assessed the contribution of ascorbic acid to the total antioxidant capacity of aqueous extracts of hips, hop cones, and lemon juice, namely, 26.0, 0.16, and 15%, respectively [238].

In Table 3, a synoptic view of relevant examples of total antioxidant capacity assessment in plants is given.

5. Critical Perspective and Conclusive Aspects Regarding Performances of Extraction and Detection Mode

As previously discussed in this review, the first step is represented by the choice of the adequate extraction method and solvent, and here the nature of the sample and contained active principles, as well as the analytical technology subsequently applied, should be considered. Detailed discussions devoted to the choice of the proper solvent as well as to the extraction methodology are given in specific cases.

Ethanol, methanol, and water, due to their polarity, favor the extraction of polar substances such as phenolics and flavonoids. Owing to its low toxicity and being an organic solvent, ethanol is employed with high frequency. Methanol toxicity hinders somehow its use. Nonpolar solvents like ether, as well as solvents endowed with low polarity (chloroform, ester, acetone, etc.), are proper for particular cases and have limited availability [18]. Phenolic compounds from *M. pubescens* were extracted by maceration employing comparatively solvents with various polarities. It has been revealed that the use of aqueous methanol (50%), aqueous ethanol (50%), and aqueous acetone (50%) imparted the highest antioxidant capacity values. The aqueous methanol (50%) and the aqueous ethanol (50%) extracts possessed the highest polyphenol content [239]. The differences between two techniques applied to phenolic extraction from *Ouratea lucens* and *Acomastylis rossii*, namely, conventional sonication (shaker) bath and homogenizer method, revealed a better performance of the latter. Sonication efficacy depends on the cell wall disruption during grinding, and the better homogenizer performances were attributed to efficacious cell wall breakdown [240].

Three genotypes of horseradish roots were subject to extraction employing conventional solvent, as well as Soxhlet extraction. The solvents endowed with the most potent extractive power were ethanol and ethanol/water mixture. The total phenolic content obtained in Soxhlet extracts proved superior to the one obtained by conventional solvent extraction. Nevertheless, the $DPPH^{\bullet}$ scavenging potential was not increased. So, in this case, by applying Soxhlet extraction, compounds other than antioxidants can take part in the extraction [241]. Soxhlet extraction performances were compared to those of maceration in the case of cumin seeds, and it was concluded that the greatest polyphenols and flavonoid contents were obtained following Soxhlet extraction, whereas employing maceration resulted in better antiradical activity [113].

Vortex extraction led to superior performances when compared to sonication and shaking, for phenolics extraction from oregano leaves, as proved by the results of total phenolic content assay and scavenging activity determination by $DPPH^{\bullet}$. Its efficacy has been proven to be solvent dependent, with best performances being obtained with acetone : water [242].

The comparative analysis of both conventional and nonconventional extraction techniques applied on the same plant material (*Quercus infectoria* extract) revealed that the supercritical CO_2 extraction resulted in lower extraction yield

in comparison to Soxhlet conventional technique, though the antioxidant capacity and selectivity with respect to total phenolics given by supercritical CO_2 extraction has been proven to be higher than the one imparted by Soxhlet extraction [243].

Various extraction methods (refluxing, sonication bath, ultrasonic homogenizer, and microwave) were applied to the aerial roots of *Rhaphidophora aurea*. The ultrasonic and microwave assisted extraction gave maximum efficiency reduced the costs and the time, limited solvent use, and resulted in a good yield compared to the other investigated techniques. It has been concluded from this comparative investigation that the ultrasonic homogenizer extraction method can be regarded as standard technique for ethyl acetate and ethanol extraction, whereas microwave assisted extraction has been proven to be the most appropriate when water is used as solvent [244].

Grape resveratrol extraction performances were investigated by liquid-liquid extraction, solid-liquid extraction, pressurized liquid extraction-solid phase extraction, and supercritical carbon dioxide extraction and in all situations the yield can be improved by postharvest ultrasonication, fungal pathogens, *in vitro* $AlCl_3$ treatment, and UV-C radiation [245].

The health benefits of plant-derived products have been already stated: the lipid oxidation delaying ability led to preventing the occurrence of mutagenic and carcinogenic lipid peroxides and aldehydes. Moreover, spices and herbs have been used for years for flavour, aroma, and color preserving [246]. Dried fruits constitute a rich source of antioxidant phytochemicals (namely, phenolics and carotenoids) and have been recently incorporated in fruit-based functional foods. Nevertheless, elaborated studies are still required for the validation of dried fruits benefits [247]. Investigating improved ways for isolation of active phytocompounds, and employing chemometrics in establishing the effective combinations of spices or herb-sourced antioxidants is an increasing trend, in view of the steady high quality requirements that led to constant optimization of analytical techniques [246].

With respect to the analytical methodology, the development of experimental conditions of the working protocols (that should be tuned to the nature of the sample/target compound(s)) and also the interpretation of the results should be carefully considered. A plethora of analytical methods were applied to total antioxidant capacity in plant extracts, yet difficulties may arise when it comes to choosing the most adequate method. It was stressed out that conditions, nature of substrate, and concentration of analysed antioxidants should be as close as possible to those encountered in food or biological media [248]. So, the term antioxidant activity is tightly related to the context of particular reaction conditions [44].

In the case of the radical scavenging antioxidants, the antioxidant-radical reactivity (both rate constant and stoichiometry), antioxidant localization, mobility in reaction media, stability of the antioxidant-derived radical, interrelation with other antioxidants, and metabolism should be measured to thoroughly understand their dynamic action as antioxidants. For instance, vitamin E has been proven to be an efficacious radical scavenger, but it was asserted that it possesses weak activity against lipid peroxidation by lipoxygenase. Carotenoids are not efficient in radical trapping but they inhibit single oxygen-induced oxidation [249]. In the presence of transition metal ions, phenolic radical scavengers can even induce oxidative damage to lipids, acting as prooxidants through the aryloxy radical. The latter is formed during the reaction of the phenolics with Cu(II) and can attack the lipid substrate [44], following a radicalic mechanism similar to the one described in Section 1.

The choice of the appropriate method should be grounded on the biomolecules targeted during the oxidation process (lipids, proteins, and nucleic acids). A viable antioxidant protocol necessitates the quantification of more than one property relevant to either foods or biological systems. Antioxidant standardization is required to diminish the discrepancies that may result from only one technique applied to antioxidant assessment [250].

A comparative discussion of the detection mechanism should consider the following aspects. Gas chromatography allows separation and determination of a precise amount of certain antioxidants in different media. HPLC as well as gas chromatography coupled to various detectors also results in detecting individual, particular antioxidant compounds (ascorbic acid, tocopherols, flavonoids, phenolic acids, etc.). These methods require qualification; they are laborious but benefit from most accurate and efficacious separation and quantitation [13, 251]. HPLC can provide limits of detection and quantification of 0.4 and 1.2 ng/mL such as for the case of linalool, 0.6 and 1.4 ng/mL, and geranyl acetate, respectively [96].

With respect to the methods relying on optical detection, the antioxidant activity is exerted by various mechanisms, so the results furnished by a technique that relies solely on one mechanism may not take account on the actual antioxidant activity value. There are assays referring to various oxygenated/nitrogenated species, methods appliable to both lipophilic and hydrophilic antioxidants, and techniques relying on either hydrogen transfer or single electron transfer [248]. It has been assessed that the ones involving peroxyl radical trapping are the most extensively applied (TRAP, ORAC, beta-carotene bleaching, and chemiluminescence) as this radical species is prevalent in biological media. Nevertheless, these methods relying on hydrogen atom transfer should be corroborated with assays relying on single electron transfer (such as ABTS quenching). On the other hand, single electron transfer-based methods rely on slow reactions that are sensitive to ascorbic acid, uric acid, and polyphenols. Secondary reactions are likely to occur, which may lead to interferences [85, 252]. Among the spectrometric *in vitro* methods, their frequency of application decreases in the order: DPPH scavenging > hydroxyl radical scavenging > superoxide dismutase activity > beta-carotene linoleate bleaching. Recently, reliable results have been obtained by combining *in vitro* method (DPPH assay) with high performance liquid chromatography. Such a DPPH-HPLC online assay especially for natural-sourced antioxidants requires minimum sample preparation [253]. Moreover, it allows hampering of the drawbacks of the simple DPPH technique that uses offline colorimetric

detection and for which the small changes in absorbance cannot be quantified [254]. With respect to the *in vivo* techniques, the lipid peroxidase assay is the most frequently used, followed by catalase and glutathione peroxidase [18, 251]. The *in vivo* antioxidant capacity assay uses biological media [255], so the antioxidants usually undergo absorption, transport, distribution, and retention in the biological fluids, cells, and tissues. As during these steps many alterations can occur (e.g., biotransformation during enzymatic conjugation), the experimental protocol has to be cautiously carried out [256, 257]. The discussion *in vivo* versus *in vitro* is of vital importance in these types of assays, as the antioxidant capacity of plants and phytocompounds is influenced by various parameters *in vivo*, such as gut absorption, metabolism, bioavailability, and the presence of other antioxidants or transition metal ions [50], and screening by *in vitro* assays should be complemented by *in vivo* efficacy testing [258]. Moreover, it has been stressed out that once the efficacy of a phytocompound is proven *in vivo*, the mechanisms of action should be subject to analysis *in vitro* to avoid discrepancies that may occur when validating *in vitro* confirmed methods *in vivo* [50].

When it comes to the chemiluminescent assay, the major shortcoming is represented by light emission from other sources [13, 251]. The ORAC technique with fluorescent detection relies on a mechanism regarded as biologically pertinent, as it takes account on the contribution of both lipophilic and hydrophilic antioxidants [85].

Nevertheless, in ORAC assay, only the antioxidant activity against peroxyl radicals is measured, disregarding the other reactive oxygenated species. It has been also asserted that antioxidant molecules present in foodstuffs exert numerous functions, some not related to the capacity to trap free radicals. Also, it has been considered that the values of antioxidant capacity do not give account on all the effects of particular bioactive principles [259]. The values of antioxidant capacity furnished by *in vitro* studies cannot be extrapolated to *in vivo* effects, and the clinical trials testing benefits of dietary antioxidants may result in mixed results. Moreover, it was mentioned that ORAC values expressed as Trolox equivalents give account on both the inhibition time and the extent of oxidation inhibition. Novel versions of the ORAC assay have been developed, which use other substrates as reference (e.g., gallic acid), which makes data comparison not an easy task. These considerations led to ORAC withdrawal from the online catalog of United States Department of Agriculture [259]. So, if ORAC assay is applied as a complementary assay instrument in connection with other analytical techniques, data comparison has to be performed using the same standard, clearly defined units of expressing the results, distinguishing between dried and fresh foods and juices or other processed foods. Measuring *in vitro* antioxidant properties remains useful, as far as the benefits are *also* related to what happens outside human body.

The type of the prevalent antioxidant class present or the reference antioxidant chosen plays important roles with respect to the correlation of results obtained by different spectrometric methods: in the case of guava fruit methanol extract, the ABTS, DPPH, FRAP, and ORAC methods gave close results. The antioxidant activity of methanol extract exhibited good correlation with ascorbic acid or total phenolics, with best correlation being shown by FRAP results with both ascorbic acid and total phenolic content. On the other hand, ABTS, DPPH, and FRAP results for methanolic extracts were negatively correlated with total carotenoids. The antioxidant capacity of dichloromethane extract (that took account on lipophilic antioxidants) was low, compared to antioxidant activity of methanol extract. So, hydrophilic ascorbic acid and phenolics have been proven to be the main contributors to antioxidant capacity of guava fruit [260]. In Folin-Ciocalteu total phenol assay, it has been assessed that some nonphenolic compounds (e.g., ascorbic acid) that can transfer electrons to phosphomolybdic/phosphotungstic complex in alkaline media may interfere with the results. This shortcoming can be minimized by extraction with 95% methanol applied to plant tissue [261].

Several correlations have been reported between the results obtained at the application of $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and FRAP and total polyphenol content assays as follows: positive correlation between TPC-$DPPH^{\bullet}$ (0.8277), TPC-$ABTS^{\bullet+}$ (0.8835), and TPC-FRAP (0.9153) [262]. Also, correlations have been established between *the antioxidant capacity values reported to different standard antioxidants*: the $ABTS^{\bullet+}$ antioxidant capacities of basil in 57% ethanol extract were 47.27 ± 2.16 mg Trolox equivalents/g DW or 31.17 ± 1.42 mg ascorbic acid equivalents/g DW (white holy basil) and 65.86 ± 5.51 mg Trolox equivalents/g DW or 43.43 ± 3.63 mg ascorbic acid equivalents/g DW (red holy basil). The $DPPH^{\bullet}$ values of 57% ethanol extract of basil were also expressed as per reference to different standards: 5.41 ± 0.04 mg Trolox equivalents/g DW or 4.59 ± 0.03 mg ascorbic equivalents/g DW (white holy basil) and 6.23 ± 0.19 mg Trolox equivalents/g DW or 5.28 ± 0.16 mg ascorbic equivalents/g DW (red holy basil) [263]. The higher values furnished by $ABTS^{\bullet+}$ assay were explained by the fact that compounds which have $ABTS^{\bullet+}$ scavenging activity may not be endowed with $DPPH^{\bullet}$ scavenging potential [264]. Moreover, it was found that some products of $ABTS^{\bullet+}$ scavenging reaction may exert a higher antioxidant capacity than the antioxidants initially present in the reaction medium and can react with $ABTS^{\bullet+}$ [265].

The results of total antioxidant capacity assay of yerba mate (*Ilex paraguariensis*) ethanol extracts evaluated by $DPPH^{\bullet}$ method were expressed as ascorbic acid equivalents or Trolox equivalents (in mass percentage, g% dry matter), trying to facilitate a comparative assessment: 12.8–23.1 g Trolox equivalents % dry matter and 9.1–16.4 g ascorbic acid equivalents % dry matter [266].

Rapidity, lower cost, simpler instrumentation, and oxidation potential value of each particular sample component evaluated with the same accuracy (irrespective of the antioxidant potency, in conditions of efficient peak separation) are reported advantages of electroanalysis versus spectrophotometry. It was asserted that, in $DPPH^{\bullet}$ photometry, the absorbance variations can be subject to more inaccuracy, whereas in voltammetry deviations in the peak potential value smaller than ±3 mV were noticed. Therefore, in photocolorimetry, at low antioxidant capacity values, the results may be prone to a greater uncertainty [267]. Moreover, in such spectrophotometric assays, there can be compounds

other than antioxidants that can contribute to the measured analytical signal at the respective wavelength.

Nevertheless, in the case of samples rich in various phenolic classes and possessing different electrooxidation potentials (e.g., wines), Q_{500} value as analytical signal does not give account on less oxidizable components, such as phenolics with more elevated oxidation potentials (e.g., 800 mV) [268, 269]. Step voltammetric methods like differential pulse or square wave voltammetry have improved resolution versus linear sweeping methods (cyclic technique), as charging current is minimized [269]. In differential pulse voltammetric polyphenol assessment, the results exhibited a tight correlation with the antioxidant activities furnished by photocolorimetry [154]. Excellent sensitivity is obtained at caffeic acid stripping voltammetric assessment operating in square wave mode: a detection limit of 4×10^{-9} mol/L in acetate buffer, pH = 4.0 [93]. Amperometric biosensors have the advantage of enzyme specificity, accuracy, and fastness imparted by electrochemical detection. The drawback of difficultly electrooxidizing high molecular mass antioxidants at fixed potential can be solved by changing the biocatalyst or by the use of mediators that diminish the working potential [269, 270]. Biamperometric techniques provide enhanced selectivity that depends on the specificity of the reaction involving the antioxidant and the oxidized form of the redox couple [158, 271]. The potential value imposed should be strictly controlled, since an increase of the latter could lead to interference of electroactive compounds other than antioxidants that might react at the electrode [272].

Employing minimum two analytical techniques (relying on different mechanisms) and applying three sample dilutions are generally recommended [273, 274]. Hence, a rigorous evaluation of antioxidant capacity should not be restricted to a simple antioxidant test and should consider the variability factors that influence the final value: for instance, the consistency between phenolic content and antioxidant capacity assessed, as well as the techniques' performances, has been proven to be dependent on the nature of the sample, its composition and pH (the latter imparting the existence of protonated or deprotonated form of biocompounds), extraction, and analytical method applied. It was concluded that several test procedures may be necessary to ensure viable antioxidant activity results [3].

Researches focusing on free radicals, plant extracts, plant-derived antioxidants in foodstuffs, and biological media should be accompanied by validation of biological markers meant to define the efficacy of antioxidant compounds in diet [275]. The comparative evaluation of antioxidant potentials reported by different laboratories should consider the significant differences in sample pretreatment, extraction, and final value expression mode [276].

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

References

[1] V. R. Winrow, P. G. Winyard, C. J. Morris, and D. R. Blake, "Free radicals in inflammation: second messengers and mediators of tissue destruction," *British Medical Bulletin*, vol. 49, no. 3, pp. 506–522, 1993.

[2] B. Poljsak, D. Šuput, and I. Milisav, "Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 956792, 11 pages, 2013.

[3] M. Antolovich, P. D. Prenzler, E. Patsalides, S. McDonald, and K. Robards, "Methods for testing antioxidant activity," *Analyst*, vol. 127, no. 1, pp. 183–198, 2002.

[4] R. Shenoy and A. Shirwaikar, "Anti inflammatory and free radical scavenging studies of Hyptis suaveolens (Labiatae)," *Indian Drugs*, vol. 39, no. 11, pp. 574–577, 2002.

[5] P. Evans and B. Halliwell, "Free radicals and hearing: cause, consequence, and criteria," *Annals of the New York Academy of Sciences*, vol. 884, pp. 19–40, 1999.

[6] T. P. A. Devasagayam and P. C. Kesavan, "Radioprotective and antioxidant action of caffeine: mechanistic considerations," *Indian Journal of Experimental Biology*, vol. 34, no. 4, pp. 291–297, 1996.

[7] A. V. Badarinath, K. Mallikarjuna Rao, C. Madhu Sudhana Chetty, S. Ramkanth, T. V. S. Rajan, and K. Gnanaprakash, "A review on In-vitro antioxidant methods: comparisions, correlations and considerations," *International Journal of PharmTech Research*, vol. 2, no. 2, pp. 1276–1285, 2010.

[8] V. Lobo, A. Phatak, and N. Chandra, "Free radicals and functional foods: Impact on human health," *Pharmacognosy Reviews*, vol. 4, pp. 118–126, 2010.

[9] M. Dizdaroglu, P. Jaruga, M. Birincioglu, and H. Rodriguez, "Free radical-induced damage to DNA: mechanisms and measurement," *Free Radical Biology and Medicine*, vol. 32, no. 11, pp. 1102–1115, 2002.

[10] M. Valko, M. Izakovic, M. Mazur, C. J. Rhodes, and J. Telser, "Role of oxygen radicals in DNA damage and cancer incidence," *Molecular and Cellular Biochemistry*, vol. 266, no. 1-2, pp. 37–56, 2004.

[11] L. Benov and A. F. Beema, "Superoxide-dependence of the short chain sugars-induced mutagenesis," *Free Radical Biology and Medicine*, vol. 34, no. 4, pp. 429–433, 2003.

[12] B. Halliwell and S. Chirico, "Lipid peroxidation: its mechanism, measurement, and significance," *The American Journal of Clinical Nutrition*, vol. 57, no. 5, pp. 715–724, 1993.

[13] M. Carocho and I. C. F. R. Ferreira, "A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives," *Food and Chemical Toxicology*, vol. 51, no. 1, pp. 15–25, 2013.

[14] C. López-Alarcón and A. Denicola, "Evaluating the antioxidant capacity of natural products: a review on chemical and cellular-based assays," *Analytica Chimica Acta*, vol. 763, pp. 1–10, 2013.

[15] D. P. Jones, "Redefining oxidative stress," *Antioxidants and Redox Signaling*, vol. 8, no. 9-10, pp. 1865–1879, 2006.

[16] J. W. Finley, A.-N. Kong, K. J. Hintze, E. H. Jeffery, L. L. Ji, and X. G. Lei, "Antioxidants in foods: state of the science important to the food industry," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 13, pp. 6837–6846, 2011.

[17] A. M. Pisoschi and A. Pop, "The role of antioxidants in the chemistry of oxidative stress: a review," *European Journal of Medicinal Chemistry*, vol. 97, pp. 55–74, 2015.

[18] M. N. Alam, N. J. Bristi, and M. Rafiquzzaman, "Review on in vivo and in vitro methods evaluation of antioxidant activity," *Saudi Pharmaceutical Journal*, vol. 21, no. 2, pp. 143–152, 2013.

[19] V. L. Kinnula and J. D. Crapo, "Superoxide dismutases in malignant cells and human tumors," *Free Radical Biology and Medicine*, vol. 36, no. 6, pp. 718–744, 2004.

[20] U. Singh and I. Jialal, "Oxidative stress and atherosclerosis," *Pathophysiology*, vol. 13, no. 3, pp. 129–142, 2006.

[21] K. Sas, H. Robotka, J. Toldi, and L. Vécsei, "Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders," *Journal of the Neurological Sciences*, vol. 257, no. 1-2, pp. 221–239, 2007.

[22] M. A. Smith, C. A. Rottkamp, A. Nunomura, A. K. Raina, and G. Perry, "Oxidative stress in Alzheimer's disease," *Biochimica et Biophysica Acta*, vol. 1502, no. 1, pp. 139–144, 2000.

[23] I. Guidi, D. Galimberti, S. Lonati et al., "Oxidative imbalance in patients with mild cognitive impairment and Alzheimer's disease," *Neurobiology of Aging*, vol. 27, no. 2, pp. 262–269, 2006.

[24] J. L. Bolton, M. A. Trush, T. M. Penning, G. Dryhurst, and T. J. Monks, "Role of quinones in toxicology," *Chemical Research in Toxicology*, vol. 13, no. 3, pp. 135–160, 2000.

[25] G. E. Arteel, "Oxidants and antioxidants in alcohol-induced liver disease," *Gastroenterology*, vol. 124, no. 3, pp. 778–790, 2003.

[26] B. S. Ramakrishna, R. Varghese, S. Jayakumar, M. Mathan, and K. A. Balasubramanian, "Circulating antioxidants in ulcerative colitis and their relationship to disease severity and activity," *Journal of Gastroenterology and Hepatology*, vol. 12, no. 7, pp. 490–494, 1997.

[27] J. M. Upston, L. Kritharides, and R. Stocker, "The role of vitamin E in atherosclerosis," *Progress in Lipid Research*, vol. 42, no. 5, pp. 405–422, 2003.

[28] D.-H. Hyun, J. O. Hernandez, M. P. Mattson, and R. de Cabo, "The plasma membrane redox system in aging," *Ageing Research Reviews*, vol. 5, no. 2, pp. 209–220, 2006.

[29] H. Yin, L. Xu, and N. A. Porter, "Free radical lipid peroxidation: Mechanisms and analysis," *Chemical Reviews*, vol. 111, no. 10, pp. 5944–5972, 2011.

[30] R. J. Hamilton, "The chemistry of rancid foods," in *Rancidity in Foods*, J. C. Allen and R. J. Hamilton, Eds., pp. 1–20, Applied Science Publishers, London, UK, 1983.

[31] J. Kanner, J. B. German, and J. E. Kinsella, "Initiation of lipid peroxidation in biological systems," *Critical Reviews in Food Science and Nutrition*, vol. 25, no. 4, pp. 317–364, 1987.

[32] K. H. Cheeseman and T. F. Slater, "An introduction to free radical biochemistry," *British Medical Bulletin*, vol. 49, no. 3, pp. 481–493, 1993.

[33] D. Martysiak-Zurowską and W. Wenta, "A comparison of ABTS and DPPH methods for assessing the total antioxidant capacity of human milk," *Acta Scientiarum Polonorum Technologia Alimentaria*, vol. 11, no. 1, pp. 83–89, 2012.

[34] E. B. Rimm, A. Ascherio, E. Giovannucci, D. Spiegelman, M. J. Stampfer, and W. C. Willett, "Vegetable, fruit, and cereal fiber intake and risk of coronary heart disease among men," *Journal of the American Medical Association*, vol. 275, no. 6, pp. 447–451, 1996.

[35] E. B. Rimm, M. B. Katan, A. Ascherio, M. J. Stampfer, and W. C. Willett, "Relation between intake of flavonoids and risk for coronary heart disease in male health professionals," *Annals of Internal Medicine*, vol. 125, no. 5, pp. 384–389, 1996.

[36] M. V. Eberhardt, C. Y. Lee, and R. H. Liu, "Antioxidant activity of fresh apples," *Nature*, vol. 405, no. 6789, pp. 903–904, 2000.

[37] K. Ganesan, K. S. Kumar, and P. V. S. Rao, "Comparative assessment of antioxidant activity in three edible species of green seaweed, *Enteromorpha* from Okha, northwest coast of India," *Innovative Food Science and Emerging Technologies*, vol. 12, no. 1, pp. 73–78, 2011.

[38] A. M. Pisoschi, *Food Additives and Ingredients-Structures, Properties, Uses*, Elisavaros Edition, Bucharest, Romania, 2012.

[39] R. Stan, *Food Additives—Natural and Synthetic Products*, Printech, Bucharest, Romania, 2007.

[40] E. A. Shalaby and S. M. M. Shanab, "Antioxidant compounds, assays of determination and mode of action," *African Journal of Pharmacy and Pharmacology*, vol. 7, no. 10, pp. 528–539, 2013.

[41] K. Rahman, "Studies on free radicals, antioxidants and co-factors," *Clinical Invervention in Aging*, vol. 2, pp. 219–236, 2007.

[42] P. E. Gamble and J. J. Burke, "Effect of water stress on the chloroplast antioxidant system," *Plant Physiology*, vol. 76, no. 3, pp. 615–621, 1984.

[43] D. V. Ratnam, D. D. Ankola, V. Bhardwaj, D. K. Sahana, and M. N. V. R. Kumar, "Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective," *Journal of Controlled Release*, vol. 113, no. 3, pp. 189–207, 2006.

[44] D. Gupta, "Methods for determination of antioxidant capacity, a review," *International Journal of Pharmaceutical Sciences and Research*, vol. 6, pp. 546–566, 2015.

[45] P. Rajendran, N. Nandakumar, T. Rengarajan et al., "Antioxidants and human diseases," *Clinica Chimica Acta*, vol. 436, pp. 332–347, 2014.

[46] S. Chand and R. Dave, "In vitro models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: an overview," *African Journal of Microbiology Research*, vol. 3, pp. 981–996, 2009.

[47] S. Ahmad, M. A. Arshad, S. Ijaz, U. Khurshid, F. Rashid, and R. Azam, "Review on methods used to determine antioxidant activity," *International Journal of Multidisciplinary Research and Development*, vol. 1, pp. 35–40, 2014.

[48] R. G. Alscher, J. L. Donahue, and C. L. Cramer, "Reactive oxygen species and antioxidants: relationships in green cells," *Physiologia Plantarum*, vol. 100, no. 2, pp. 224–233, 1997.

[49] C. H. Foyer and G. Noctor, "Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses," *The Plant Cell*, vol. 17, no. 7, pp. 1866–1875, 2005.

[50] D. M. Kasote, S. S. Katyare, M. V. Hegde, and H. Bae, "Significance of antioxidant potential of plants and its relevance to therapeutic applications," *International Journal of Biological Sciences*, vol. 11, no. 8, pp. 982–991, 2015.

[51] M.-H. Yang, H.-J. Lin, and Y.-M. Choong, "A rapid gas chromatographic method for direct determination of BHA, BHT and TBHQ in edible oils and fats," *Food Research International*, vol. 35, no. 7, pp. 627–633, 2002.

[52] R. M. Smith, "Before the injection—modern methods of sample preparation for separation techniques," *Journal of Chromatography A*, vol. 1000, no. 1-2, pp. 3–27, 2003.

[53] M. D. Luque de Castro and L. E. García-Ayuso, "Soxhlet extraction of solid materials: an outdated technique with a promising innovative future," *Analytica Chimica Acta*, vol. 369, no. 1-2, pp. 1–10, 1998.

[54] J. Azmir, I. S. M. Zaidul, M. M. Rahman et al., "Techniques for extraction of bioactive compounds from plant materials: a

review," *Journal of Food Engineering*, vol. 117, no. 4, pp. 426–436, 2013.

[55] A. Gupta, M. Naraniwal, and V. Kothari, "Modern extraction methods for preparation of bioactive plant extracts," *International Journal of Applied and Natural Sciences*, vol. 1, pp. 8–26, 2012.

[56] S. S. Handa, "An overview of extraction techniques for medicinal and aromatic plants," in *Extraction Technologies for Medicinal and Aromatic Plants Scientific*, S. S. Handa, S. P. S. Khanuja, G. Longo, and D. D. Rakesh, Eds., chapter 1, pp. 21–52, United Nations Industrial Development Organization and the International Centre for Science and High Technology, Trieste, Italy, 2008.

[57] P. S. Vankar, "Essential oils and fragrances from natural sources," *Resonance*, vol. 9, no. 4, pp. 30–41, 2004.

[58] L. V. Silva, D. L. Nelson, M. F. B. Drummond, L. Dufossé, and M. B. A. Glória, "Comparison of hydrodistillation methods for the deodorization of turmeric," *Food Research International*, vol. 38, no. 8-9, pp. 1087–1096, 2005.

[59] J. Inczedy, T. Lengyel, and A. M. Ure, "Supercritical fluid chromatography and extraction," in *Compendium of Analytical Nomenclature: Definitive Rules 1997*, Blackwell Science, Oxford, UK, 3rd edition, 1998, http://chemanalytica.com/book/novyy_spravochnik_khimika_i_tekhnologa/02_analiticheskaya_khimiya_chast_I/4690.

[60] M. Sihvonen, E. Järvenpää, V. Hietaniemi, and R. Huopalahti, "Advances in supercritical carbon dioxide technologies," *Trends in Food Science and Technology*, vol. 10, no. 6-7, pp. 217–222, 1999.

[61] J. J. R. Paré, J. M. R. Bélanger, and S. S. Stafford, "Microwave-assisted process (MAP™): a new tool for the analytical laboratory," *TrAC Trends in Analytical Chemistry*, vol. 13, pp. 176–184, 1994.

[62] M. Letellier and H. Budzinski, "Microwave assisted extraction of organic compounds," *Analusis*, vol. 27, no. 3, pp. 259–270, 1999.

[63] T. Jain, "Microwave assisted extraction for phytoconstituents—an overview," *Asian Journal of Research in Chemistry*, vol. 2, pp. 19–25, 2009.

[64] S. Ahuja and D. Diehl, "Sampling and sample prepration," in *Comprehensive Analytical Chemistry*, S. Ahuja and N. Jespersen, Eds., vol. 47, chapter 2, pp. 15–40, Elsevier, Oxford, UK, 2006.

[65] G. Cravotto, L. Boffa, S. Mantegna, P. Perego, M. Avogadro, and P. Cintas, "Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves," *Ultrasonics Sonochemistry*, vol. 15, no. 5, pp. 898–902, 2008.

[66] A. Alupului, I. Calinescu, and V. Lavric, "Microwave extraction of active principles from medicinal plants," *U.P.B Scientific Bulletin, Series B: Chemistry and Materials Science*, vol. 74, no. 2, pp. 129–142, 2012.

[67] M. C. Herrera and M. D. Luque De Castro, "Ultrasound-assisted extraction for the analysis of phenolic compounds in strawberries," *Analytical and Bioanalytical Chemistry*, vol. 379, no. 7-8, pp. 1106–1112, 2004.

[68] T. J. Mason, L. Paniwnyk, and J. P. Lorimer, "The uses of ultrasound in food technology," *Ultrasonics Sonochemistry*, vol. 3, no. 3, pp. S253–S260, 1996.

[69] M. G. Cares, Y. Vargas, L. Gaete, J. Sainz, and J. Alarcon, "Ultrasonically assisted extraction of bioactive principles from Quillaja Saponaria Molina," *Physics Procedia*, vol. 3, pp. 169–178, 2009.

[70] A. H. Metherel, A. Y. Taha, H. Izadi, and K. D. Stark, "The application of ultrasound energy to increase lipid extraction throughput of solid matrix samples (flaxseed)," *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 81, no. 5-6, pp. 417–423, 2009.

[71] A. Bouakaz, "Sonoporation: concept and mechanisms," in *Therapeutic Ultrasound*, J.-M. Escoffre and A. Bouakaz, Eds., vol. 10, pp. 175–189, Springer, Basel, Switzerland, 2016.

[72] M. Savova, H. J. Bart, and I. Seikova, "Enhancement of mass transfer in solid-liquid extraction by pulsed electric field," *Journal of the University of Chemical Technology and Metallurgy*, vol. 40, pp. 251–255, 2005.

[73] V. Heinz, S. Toepfl, and D. Knorr, "Impact of temperature on lethality and energy efficiency of apple juice pasteurization by pulsed electric fields treatment," *Innovative Food Science and Emerging Technologies*, vol. 4, no. 2, pp. 167–175, 2003.

[74] N. López, E. Puértolas, S. Condón, I. Álvarez, and J. Raso, "Effects of pulsed electric fields on the extraction of phenolic compounds during the fermentation of must of Tempranillo grapes," *Innovative Food Science and Emerging Technologies*, vol. 9, no. 4, pp. 477–482, 2008.

[75] N. López, E. Puértolas, S. Condón, J. Raso, and I. Alvarez, "Enhancement of the extraction of betanine from red beetroot by pulsed electric fields," *Journal of Food Engineering*, vol. 90, no. 1, pp. 60–66, 2009.

[76] A. Rosenthal, D. L. Pyle, and K. Niranjan, "Aqueous and enzymatic processes for edible oil extraction," *Enzyme and Microbial Technology*, vol. 19, no. 6, pp. 402–420, 1996.

[77] R. K. Singh, B. C. Sarker, B. K. Kumbhar, Y. C. Agrawal, and M. K. Kulshreshtha, "Response surface analysis of enzyme assisted oil extraction factors for sesame, groundnut and sunflower seeds," *Journal of Food Science and Technology*, vol. 36, no. 6, pp. 511–514, 1999.

[78] S. Latif and F. Anwar, "Physicochemical studies of hemp (*Cannabis sativa*) seed oil using enzyme-assisted cold-pressing," *European Journal of Lipid Science and Technology*, vol. 111, no. 10, pp. 1042–1048, 2009.

[79] K. Niranjan and P. Hanmoungjai, "Enzyme-aided aquous extraction," in *Nutritionally Enhanced Edible Oil Processing*, N. T. Dunford and H. B. Dunford, Eds., AOCS, Champaign, Ill, USA, 2004.

[80] H. Domínguez, M. J. Núñez, and J. M. Lema, "Enzyme-assisted hexane extraction of soya bean oil," *Food Chemistry*, vol. 54, no. 2, pp. 223–231, 1995.

[81] M. Puri, D. Sharma, and C. J. Barrow, "Enzyme-assisted extraction of bioactives from plants," *Trends in Biotechnology*, vol. 30, no. 1, pp. 37–44, 2012.

[82] J. Concha, C. Soto, R. Chamy, and M. E. Zúñiga, "Enzymatic pretreatment on rose-hip oil extraction: hydrolysis and pressing conditions," *Journal of the American Oil Chemists' Society*, vol. 81, no. 6, pp. 549–552, 2004.

[83] E. Ibañez, M. Herrero, J. A. Mendiola, and M. Castro-Puyana, "Extraction and characterization of bioactive compounds with health benefits from marine resources: macro and micro algae, cyanobacteria, and invertebrates," in *Marine Bioactive Compounds: Sources, Characterization and Applications*, M. Hayes, Ed., pp. 55–98, Springer, 2012.

[84] L. D. Mello and L. T. Kubota, "Biosensors as a tool for the antioxidant status evaluation," *Talanta*, vol. 72, no. 2, pp. 335–348, 2007.

[85] R. L. Prior, X. Wu, and K. Schaich, "Standardized methods for the determination of antioxidant capacity and phenolics

in foods and dietary supplements," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 10, pp. 4290–4302, 2005.

[86] A. Jiménez, A. Selga, J. L. Torres, and L. Julià, "Reducing activity of polyphenols with stable radicals of the TTM series. Electron transfer versus H-abstraction reactions in flavan-3-ols," *Organic Letters*, vol. 6, no. 24, pp. 4583–4586, 2004.

[87] J. Wang, Y.-D. Yue, F. Tang, and J. Sun, "TLC screening for antioxidant activity of extracts from fifteen bamboo species and identification of antioxidant flavone glycosides from leaves of *Bambusa. textilis* McClure," *Molecules*, vol. 17, no. 10, pp. 12297–12311, 2012.

[88] R. A. Khan, M. R. Khan, S. Sahreen, and M. Ahmed, "Evaluation of phenolic contents and antioxidant activity of various solvent extracts of *Sonchus asper* (L.) Hill," *Chemistry Central Journal*, vol. 6, no. 1, article 12, 2012.

[89] A. M. Pisoschi, A. Pop, G. P. Negulescu, and A. Pisoschi, "Determination of ascorbic acid content of some fruit juices and wine by voltammetry performed at pt and carbon paste electrodes," *Molecules*, vol. 16, no. 2, pp. 1349–1365, 2011.

[90] S. Anandjiwala, H. Srinivasa, J. Kalola, and M. Rajani, "Free-radical scavenging activity of *Bergia suffruticosa* (Delile) Fenzl," *Journal of Natural Medicines*, vol. 61, no. 1, pp. 59–62, 2007.

[91] W. Jesionek, B. Majer-Dziedzic, and I. M. Choma, "Separation, identification, and investigation of antioxidant ability of plant extract components using TLC, LC–MS, and TLC–DPPH$^{\bullet}$," *Journal of Liquid Chromatography and Related Technologies*, vol. 38, no. 11, pp. 1147–1153, 2015.

[92] S. K. Sadhu, F. Ahmed, T. Ohtsuki, and M. Ishibashi, "Flavonoids from *Sonneratia caseolaris*," *Journal of Natural Medicines*, vol. 60, no. 3, pp. 264–265, 2006.

[93] K. Tyszczuk, A. Skalska-Kamińska, and A. Woźniak, "Voltammetric method using a lead film electrode for the determination of caffeic acid in a plant material," *Food Chemistry*, vol. 125, no. 4, pp. 1498–1503, 2011.

[94] W. Wankhar, S. Srinivasan, and S. Rathinasamy, "HPTLC analysis of Scoparia dulcis Linn (Scrophulariaceae) and its larvicidal potential against dengue vector Aedes aegypti," *Natural Product Research*, vol. 29, no. 18, pp. 1757–1760, 2015.

[95] S. M. F. Freire, A. J. S. Emim, A. J. Lapa, C. Souccar, and L. M. B. Torres, "Analgesic and antiinflammatory properties of *Scoparia dulcis* L. Extracts and glutinol in rodents," *Phytotherapy Research*, vol. 7, no. 6, pp. 408–414, 1993.

[96] K. Singh, R. Rani, P. Bansal, S. Medhe, and M. M. Srivastava, "Antioxidant activity of essential oil of *Coriandrum sativum* and standardization of HPTLC method for the estimation of major phytomarkers," *Journal of Analytical Chemistry*, vol. 70, no. 2, pp. 220–224, 2015.

[97] M. A. Hossain, M. D. Shah, and M. Sakari, "Gas chromatography-mass spectrometry analysis of various organic extracts of *Merremia borneensis* from Sabah," *Asian Pacific Journal of Tropical Medicine*, vol. 4, no. 8, pp. 637–641, 2011.

[98] M. A. Hossain, K. M. ALsabari, A. M. Weli, and Q. Al-Riyami, "Gas chromatography–mass spectrometry analysis and total phenolic contents of various crude extracts from the fruits of *Datura metel* L," *Journal of Taibah University for Science*, vol. 7, no. 4, pp. 209–215, 2013.

[99] R. A. Mothana, M. S. Alsaid, S. S. Hasoon, N. M. Al-Mosaiy, A. J. Al-Rehaily, and M. A. Al-Yahya, "Antimicrobial and antioxidant activities and gas chromatography mass spectrometry (GC/MS) analysis of the essential oils of *Ajuga bracteosa* Wall. ex Benth. and *Lavandula dentata* L. growing wild in Yemen," *Journal of Medicinal Plants Research*, vol. 6, no. 15, pp. 3066–3071, 2012.

[100] E.-H. Liu, P. Zhao, L. Duan et al., "Simultaneous determination of six bioactive flavonoids in Citri Reticulatae Pericarpium by rapid resolution liquid chromatography coupled with triple quadrupole electrospray tandem mass spectrometry," *Food Chemistry*, vol. 141, no. 4, pp. 3977–3983, 2013.

[101] M. Stankevičius, I. Akuåeca, I. Jákobsone, and A. Maruška, "Comparative analysis of radical scavenging and antioxidant activity of phenolic compounds present in everyday use spice plants by means of spectrophotometric and chromatographic methods," *Journal of Separation Science*, vol. 34, no. 11, pp. 1261–1267, 2011.

[102] S. Sahreen, M. R. Khan, R. A. Khan, and N. A. Shah, "Estimation of flavoniods, antimicrobial, antitumor and anticancer activity of *Carissa opaca* fruits," *BMC Complementary and Alternative Medicine*, vol. 13, article 372, 2013.

[103] A. Djeridane, M. Yousfi, B. Nadjemi, N. Vidal, J. F. Lesgards, and P. Stocker, "Screening of some Algerian medicinal plants for the phenolic compounds and their antioxidant activity," *European Food Research and Technology*, vol. 224, no. 6, pp. 801–809, 2007.

[104] O. Yesil-Celiktas, G. Girgin, H. Orhan, H. J. Wichers, E. Bedir, and F. Vardar-Sukan, "Screening of free radical scavenging capacity and antioxidant activities of *Rosmarinus officinalis* extracts with focus on location and harvesting times," *European Food Research and Technology*, vol. 224, no. 4, pp. 443–451, 2007.

[105] G.-F. Deng, X.-R. Xu, Y.-J. Guo et al., "Determination of antioxidant property and their lipophilic and hydrophilic phenolic contents in cereal grains," *Journal of Functional Foods*, vol. 4, no. 4, pp. 906–914, 2012.

[106] V. Ani, M. C. Varadaraj, and K. Akhilender Naidu, "Antioxidant and antibacterial activities of polyphenolic compounds from bitter cumin (*Cuminum nigrum* L.)," *European Food Research and Technology*, vol. 224, no. 1, pp. 109–115, 2006.

[107] M. Kosar, A. Altintas, N. Kirimer, and K. H. C. Baser, "Determination of the free radical scavenging activity of Lycium extracts," *Chemistry of Natural Compounds*, vol. 39, no. 6, pp. 531–535, 2003.

[108] C. Carkeet, B. A. Clevidence, and J. A. Novotny, "Anthocyanin excretion by humans increases linearly with increasing strawberry dose," *The Journal of Nutrition*, vol. 138, no. 5, pp. 897–902, 2008.

[109] D. Benedec, L. Vlase, D. Hanganu, and I. Oniga, "Antioxidant potential and polyphenolic content of Romanian *Ocimum basilicum*," *Digest Journal of Nanomaterials and Biostructures*, vol. 7, no. 3, pp. 1263–1270, 2012.

[110] S. A. V. Eremia, G.-L. Radu, and S.-C. Litescu, "Monitoring of rosmarinic acid accumulation in sage cell cultures using laccase biosensor," *Phytochemical Analysis*, vol. 24, no. 1, pp. 53–58, 2013.

[111] M. Kurzawa, A. Filipiak-Szok, E. Kłodzińska, and E. Szłyk, "Determination of phytochemicals, antioxidant activity and total phenolic content in Andrographis paniculata using chromatographic methods," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 995-996, pp. 101–106, 2015.

[112] X. Lin, Y. Ni, and S. Kokot, "Glassy carbon electrodes modified with gold nanoparticles for the simultaneous determination of three food antioxidant," *Analytica Chimica Acta*, vol. 765, pp. 54–62, 2013.

[113] I. B. Rebey, S. Kefi, S. Bourgou et al., "Ripening stage and extraction method effects on physical properties, polyphenol composition and antioxidant activities of cumin (*Cuminum*

cyminum L.) Seeds," *Plant Foods for Human Nutrition*, vol. 69, no. 4, pp. 358–364, 2014.

[114] H.-C. Seo, M. Suzuki, M. Ohnishi-Kameyama et al., "Extraction and identification of antioxidant components from *Artemisia capillaris* herba," *Plant Foods for Human Nutrition*, vol. 58, no. 3, pp. 1–12, 2003.

[115] P. K. Boniface, M. Singh, S. Verma et al., "RP-HPLC-DAD method for the identification of two potential antioxidant agents namely verminoside and 1-*O*-(*E*)-caffeoyl-*β*-gentiobiose from *Spathodea campanulata* leaves," *Natural Product Research*, vol. 29, no. 7, pp. 676–680, 2015.

[116] J. Borowski, A. Szajdek, E. J. Borowska, E. Ciska, and H. Zieliński, "Content of selected bioactive components and antioxidant properties of broccoli (*Brassica oleracea* L.)," *European Food Research and Technology*, vol. 226, no. 3, pp. 459–465, 2008.

[117] A. A. Al-Amiery, A. A. H. Kadhum, H. R. Obayes, and A. B. Mohamad, "Synthesis and antioxidant activities of novel 5-chlorocurcumin, complemented by semiempirical calculations," *Bioinorganic Chemistry and Applications*, vol. 2013, Article ID 354982, 7 pages, 2013.

[118] M. Leopoldini, T. Marino, N. Russo, and M. Toscano, "Antioxidant properties of phenolic compounds: H-atom versus electron transfer mechanism," *Journal of Physical Chemistry A*, vol. 108, no. 22, pp. 4916–4922, 2004.

[119] B. D. Craft, A. L. Kerrihard, R. Amarowicz, and R. B. Pegg, "Phenol-based antioxidants and the *in vitro* methods used for their assessment," *Comprehensive Reviews in Food Science and Food Safety*, vol. 11, no. 2, pp. 148–173, 2012.

[120] D. Huang, O. U. Boxin, and R. L. Prior, "The chemistry behind antioxidant capacity assays," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 6, pp. 1841–1856, 2005.

[121] E. Klein and V. Lukeš, "DFT/B3LYP study of the substituent effect on the reaction enthalpies of the individual steps of single electron transfer-proton transfer and sequential proton loss electron transfer mechanisms of phenols antioxidant action," *Journal of Physical Chemistry A*, vol. 110, no. 44, pp. 12312–12320, 2006.

[122] A. Urbaniak, M. Molski, and M. Szeląg, "Quantum-chemical calculations of the antioxidant properties of *trans-p*-coumaric acid and *trans*-sinapinic acid," *Computational Methods in Science and Technology*, vol. 18, no. 2, pp. 117–128, 2012.

[123] M. Karamać, "Chelation of Cu(II), Zn(II), and Fe(II) by tannin constituents of selected edible nuts," *International Journal of Molecular Sciences*, vol. 10, no. 12, pp. 5485–5497, 2009.

[124] L. S. Al Hashmi, M. A. Hossain, A. M. Weli, Q. Al-Riyami, and J. N. AlSabahi, "Gas chromatography-mass spectrometry analysis of different organic crude extracts from the local medicinal plant of *Thymus vulgaris* L," *Asian Pacific Journal of Tropical Biomedicine*, vol. 3, no. 1, pp. 69–73, 2013.

[125] W. Brand-Williams, M. E. Cuvelier, and C. Berset, "Use of a free radical method to evaluate antioxidant activity," *LWT—Food Science and Technology*, vol. 28, no. 1, pp. 25–30, 1995.

[126] P. Molyneux, "The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity," *Songklanakarin Journal of Science and Technology*, vol. 26, pp. 211–219, 2004.

[127] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, and C. Rice-Evans, "Antioxidant activity applying an improved ABTS radical cation decolorization assay," *Free Radical Biology and Medicine*, vol. 26, no. 9-10, pp. 1231–1237, 1999.

[128] V. Fogliano, V. Verde, G. Randazzo, and A. Ritieni, "Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines," *Journal of Agricultural and Food Chemistry*, vol. 47, no. 3, pp. 1035–1040, 1999.

[129] I. F. F. Benzie and J. J. Strain, "The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': The FRAP assay," *Analytical Biochemistry*, vol. 239, no. 1, pp. 70–76, 1996.

[130] M. Oyaizu, "Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine," *The Japanese Journal of Nutrition and Dietetics*, vol. 44, no. 6, pp. 307–315, 1986.

[131] R. Apak, K. Güçlü, M. Özyürek, and S. E. Karademir, "Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 26, pp. 7970–7981, 2004.

[132] R. Apak, K. Güçlü, M. Özyürek, and S. E. Çelik, "Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay," *Microchimica Acta*, vol. 160, no. 4, pp. 413–419, 2008.

[133] P. Prieto, M. Pineda, and M. Aguilar, "Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E," *Analytical Biochemistry*, vol. 269, no. 2, pp. 337–341, 1999.

[134] S. Shobana and K. A. Naidu, "Antioxidant activity of selected Indian spices," *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 62, no. 2, pp. 107–110, 2000.

[135] J. A. Buege and S. D. Aust, "Microsomal lipid peroxidation," *Methods in Enzymology*, vol. 52, pp. 302–310, 1978.

[136] R. Jayabalan, P. Subathradevi, S. Marimuthu, M. Sathishkumar, and K. Swaminathan, "Changes in free-radical scavenging ability of kombucha tea during fermentation," *Food Chemistry*, vol. 109, no. 1, pp. 227–234, 2008.

[137] M. Nishikimi, N. A. Rao, and K. Yagi, "The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen," *Biochemical and Biophysical Research Communications*, vol. 46, no. 2, pp. 849–854, 1972.

[138] S. Ercisli, M. Akbulut, O. Ozdemir, M. Sengul, and E. Orhan, "Phenolic and antioxidant diversity among persimmon (*Diospyrus kaki* L.) genotypes in Turkey," *International Journal of Food Sciences and Nutrition*, vol. 59, no. 6, pp. 477–482, 2008.

[139] K. U. Yilmaz, S. Ercisli, Y. Zengin, M. Sengul, and E. Y. Kafkas, "Preliminary characterisation of cornelian cherry (*Cornusmas* L.) genotypes for their physico-chemical properties," *Food Chemistry*, vol. 114, no. 2, pp. 408–412, 2009.

[140] T. Noro, Y. Oda, T. Miyase, A. Ueno, and S. Fukushima, "Inhibitors of xanthine oxidase from the flowers and buds of Daphne genkwa," *Chemical and Pharmaceutical Bulletin*, vol. 31, no. 11, pp. 3984–3987, 1983.

[141] J. M. McCord and I. Fridovich, "Superoxide dismutase. An enzymic function for erythrocuprein," *The Journal of Biological Chemistry*, vol. 244, no. 22, pp. 6049–6055, 1969.

[142] H. Aebi, "Catalase in vitro," *Methods in Enzymology*, vol. 105, pp. 121–126, 1984.

[143] T. Guo, L. Wei, J. Sun, C.-L. Hou, and L. Fan, "Antioxidant activities of extract and fractions from Tuber indicum Cooke & Massee," *Food Chemistry*, vol. 127, no. 4, pp. 1634–1640, 2011.

[144] C. Soler-Rivas, J. C. Espín, and H. J. Wichers, "An easy and fast test to compare total free radical scavenger capacity of

foodstuffs," *Phytochemical Analysis*, vol. 11, no. 5, pp. 330–338, 2000.

[145] R. L. Prior, G. Cao, A. Martin et al., "Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of vaccinium species," *Journal of Agricultural and Food Chemistry*, vol. 46, no. 7, pp. 2686–2693, 1998.

[146] G. Cao, H. M. Alessio, and R. G. Cutler, "Oxygen-radical absorbance capacity assay for antioxidants," *Free Radical Biology and Medicine*, vol. 14, no. 3, pp. 303–311, 1993.

[147] B. Ou, D. Huang, M. Hampsch-Woodill, J. A. Flanagan, and E. K. Deemer, "Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 11, pp. 3122–3128, 2002.

[148] B. Ou, M. Hampsch-Woodill, J. Flanagan, E. K. Deemer, R. L. Prior, and D. Huang, "Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 10, pp. 2772–2777, 2002.

[149] A. Ghiselli, M. Serafini, G. Maiani, E. Azzini, and A. Ferro-Luzzi, "A fluorescence-based method for measuring total plasma antioxidant capability," *Free Radical Biology and Medicine*, vol. 18, no. 1, pp. 29–36, 1995.

[150] D. D. M. Wayner, G. W. Burton, K. U. Ingold, and S. Locke, "Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins," *FEBS Letters*, vol. 187, no. 1, pp. 33–37, 1985.

[151] S. R. J. Maxwell, O. Wiklund, and G. Bondjers, "Measurement of antioxidant activity in lipoproteins using enhanced chemiluminescence," *Atherosclerosis*, vol. 111, no. 1, pp. 79–89, 1994.

[152] A. W. Bott, "Practical problems in voltammetry 2. Electrode capacitance," *Current Separations*, vol. 12, pp. 10–13, 1993.

[153] S. Chevion, M. A. Roberts, and M. Chevion, "The use of cyclic voltammetry for the evaluation of antioxidant capacity," *Free Radical Biology and Medicine*, vol. 28, no. 6, pp. 860–870, 2000.

[154] M. Šeruga, I. Novak, and L. Jakobek, "Determination of polyphenols content and antioxidant activity of some red wines by differential pulse voltammetry, HPLC and spectrophotometric methods," *Food Chemistry*, vol. 124, no. 3, pp. 1208–1216, 2011.

[155] S. P. Kounaves, "Voltammetric techniques," in *Handbook of Instrumental Techniques for Analytical Chemistry*, F. A. Settle, Ed., chapter 37, Prentice Hall, Upper Saddle River, NJ, USA, 1997.

[156] J. Sochor, J. Dobes, O. Krystofova et al., "Electrochemistry as a tool for studying antioxidant properties," *International Journal of Electrochemical Science*, vol. 8, no. 6, pp. 8464–8489, 2013.

[157] P. Jakubec, M. Bancirova, V. Halouzka et al., "Electrochemical sensing of total antioxidant capacity and polyphenol content in wine samples using amperometry online-coupled with microdialysis," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 32, pp. 7836–7843, 2012.

[158] S. Milardovic, D. Ivekovic, V. Rumenjak, and B. S. Grabaric, "Use of DPPH•/DPPH redox couple for biamperometric determination of antioxidant activity," *Electroanalysis*, vol. 17, pp. 1847–1853, 2005.

[159] S. Milardovic, I. Kereković, R. Derrico, and V. Rumenjak, "A novel method for flow injection analysis of total antioxidant capacity using enzymatically produced $ABTS^{\bullet+}$ and biamperometric detector containing interdigitated electrode," *Talanta*, vol. 71, no. 1, pp. 213–220, 2007.

[160] S. Milardovic, I. Kerekovic, and V. Rumenjak, "A flow injection biamperometric method for determination of total antioxidant capacity of alcoholic beverages using bienzymatically produced $ABTS^{\bullet+}$," *Food Chemistry*, vol. 105, pp. 1688–1694, 2007.

[161] L. K. Shpigun, M. A. Arharova, K. Z. Brainina, and A. V. Ivanova, "Flow injection potentiometric determination of total antioxidant activity of plant extracts," *Analytica Chimica Acta*, vol. 573-574, pp. 419–426, 2006.

[162] N. Gougoulias, "Evaluation of antioxidant activity and polyphenol content of leaves from some fruit species," *Oxidation Communications*, vol. 38, pp. 35–45, 2015.

[163] V. Katalinic, S. S. Mozina, I. Generalic, D. Skroza, I. Ljubenkov, and A. Klancnik, "Phenolic profile, antioxidant capacity, and antimicrobial activity of leaf extracts from six vitis vinifera L. Varieties," *International Journal of Food Properties*, vol. 16, no. 1, pp. 45–60, 2013.

[164] N. G. Sahib, A. A. Hamid, N. Saari, F. Abas, M. S. P. Dek, and M. Rahim, "Anti-pancreatic lipase and antioxidant activity of selected tropical herbs," *International Journal of Food Properties*, vol. 15, no. 3, pp. 569–578, 2012.

[165] S. Karoune, H. Falleh, M. S. A. Kechebar et al., "Evaluation of antioxidant activities of the edible and medicinal Acacia albida organs related to phenolic compounds," *Natural Product Research*, vol. 29, no. 5, pp. 452–454, 2015.

[166] H. Zhang, W. Xi, Y. Yang et al., "An on-line HPLC-FRSD system for rapid evaluation of the total antioxidant capacity of Citrus fruits," *Food Chemistry*, vol. 172, pp. 622–629, 2015.

[167] L. Vicaş, A. Teuşdea, S. Vicaş et al., "Assessment of antioxidant capacity of some extracts for further use in therapy," *Farmacia*, vol. 63, no. 2, pp. 267–274, 2015.

[168] M. Makni, A. Haddar, W. Kriaa, and N. Zeghal, "Antioxidant, free radical scavenging, and antimicrobial activities of *Ajuga iva* leaf extracts," *International Journal of Food Properties*, vol. 16, no. 4, pp. 756–765, 2013.

[169] M. Pukalskiene, P. R. Venskutonis, and A. Pukalskas, "Phytochemical composition and antioxidant properties of Filipendula vulgaris as a source of healthy functional ingredients," *Journal of Functional Foods*, vol. 15, pp. 233–242, 2015.

[170] A. Peksel, S. Imamoglu, N. A. Kiymaz, and N. Orhan, "Antioxidant and radical scavenging activities of *Asphodelus aestivus* Brot. extracts," *International Journal of Food Properties*, vol. 16, no. 6, pp. 1339–1350, 2013.

[171] M. Zahoor, M. Ahmed, S. Naz, and M. Ayaz, "Cytotoxic, antibacterial and antioxidant activities of extracts of the bark of Melia azedarach (China Berry)," *Natural Product Research*, vol. 29, no. 12, pp. 1170–1172, 2015.

[172] H.-J. Ko, L.-H. Ang, and L.-T. Ng, "Antioxidant activities and polyphenolic constituents of bitter bean Parkia speciosa," *International Journal of Food Properties*, vol. 17, no. 9, pp. 1977–1986, 2014.

[173] I. Ky and P. Teissedre, "Characterisation of mediterranean grape pomace seed and skin extracts: polyphenolic content and antioxidant activity," *Molecules*, vol. 20, no. 2, pp. 2190–2207, 2015.

[174] S. Oueslati, A. Ellili, J. Legault et al., "Phenolic content, antioxidant and anti-inflammatory activities of Tunisian *Diplotaxis simplex* (Brassicaceae)," *Natural Product Research*, vol. 29, no. 12, pp. 1189–1191, 2015.

[175] T. M. Djordjevic, S. S. Šiler-Marinkovic, and S. I. Dimitrijevic-Brankovic, "Antioxidant activity and total phenolic content in some cereals and legumes," *International Journal of Food Properties*, vol. 14, no. 1, pp. 175–184, 2011.

[176] M. C. A. da Silva and S. R. Paiva, "Antioxidant activity and flavonoid content of Clusia fluminensis Planch. & Triana," *Anais da Academia Brasileira de Ciências*, vol. 84, no. 3, pp. 609–616, 2012.

[177] L. Yu, J.-X. Ren, H.-M. Nan, and B.-F. Liu, "Identification of antibacterial and antioxidant constituents of the essential oils of Cynanchum chinense and Ligustrum compactum," *Natural Product Research*, vol. 29, no. 18, pp. 1779–1782, 2015.

[178] A. Monroy-Vasquez, A. Totosaus, L. R. G. Gonzales, K. A. de la Fuente Salazar, and I. Garcia-Martinez, "Antioxidantes I. Chile ancho (*Caspicum annum* L. grossum sendt.) y romero (*Rosmarinus officinalis* L.) como fuentes naturales de antioxidantes," *Ciencia y Tecnologia*, vol. 6, pp. 112–116, 2007.

[179] I. Cesari, M. Hoerlé, C. Simoes-Pires et al., "Anti-inflammatory, antimicrobial and antioxidant activities of *Diospyros bipindensis* (Gürke) extracts and its main constituents," *Journal of Ethnopharmacology*, vol. 146, no. 1, pp. 264–270, 2013.

[180] B. Mahdi-Pour, S. L. Jothy, L. Y. Latha, Y. Chen, and S. Sasidharan, "Antioxidant activity of methanol extracts of different parts of *Lantana camara*," *Asian Pacific Journal of Tropical Biomedicine*, vol. 2, no. 12, pp. 960–965, 2012.

[181] D. Amendola, D. M. De Faveri, and G. Spigno, "Grape marc phenolics: extraction kinetics, quality and stability of extracts," *Journal of Food Engineering*, vol. 97, no. 3, pp. 384–392, 2010.

[182] D. Wozniak, A. Drys, and A. Matkowski, "Antiradical and antioxidant activity of flavones from *Scutellariae baicalensis radix*," *Natural Product Research*, vol. 29, no. 16, pp. 1567–1570, 2015.

[183] S. Ouchemoukh, S. Hachoud, H. Boudraham, A. Mokrani, and H. Louaileche, "Antioxidant activities of some dried fruits consumed in Algeria," *LWT—Food Science and Technology*, vol. 49, no. 2, pp. 329–332, 2012.

[184] S. C. Gouveia-Figueira and P. C. Castilho, "Phenolic screening by HPLC-DAD-ESI/MSn and antioxidant capacity of leaves, flowers and berries of Rubus grandifolius Lowe," *Industrial Crops and Products*, vol. 73, pp. 28–40, 2015.

[185] L. Adnan, A. Osman, and A. A. Hamid, "Antioxidant activity of different extracts of red pitaya (*Hylocereus polyrhizus*) seed," *International Journal of Food Properties*, vol. 14, no. 6, pp. 1171–1181, 2011.

[186] E. Celep, A. Aydın, and E. Yesilada, "A comparative study on the *in vitro* antioxidant potentials of three edible fruits: cornelian cherry, Japanese persimmon and cherry laurel," *Food and Chemical Toxicology*, vol. 50, no. 9, pp. 3329–3335, 2012.

[187] A. Bucchini, D. Ricci, F. Messina, M. C. Marcotullio, M. Curini, and L. Giamperi, "Antioxidant and antifungal activity of different extracts obtained from aerial parts of Inula crithmoides L.," *Natural Product Research*, vol. 29, no. 12, pp. 1173–1176, 2015.

[188] B. Abdennacer, M. Karim, M. Yassine, R. Nesrine, D. Mouna, and B. Mohamed, "Determination of phytochemicals and antioxidant activity of methanol extracts obtained from the fruit and leaves of Tunisian Lycium intricatum Boiss," *Food Chemistry*, vol. 174, pp. 577–584, 2015.

[189] A. Kumari and R. A. Sharma, "Estimation of total phenolic, flavonoidal content and evaluation of antioxidant activity of methanolic extract of Millingtonia hortensis Linn," *World Journal of Pharmacy and Pharmaceutical Sciences*, vol. 3, pp. 1646–1655, 2014.

[190] B. Mhamdi, F. Abbassi, and C. Abdelly, "Chemical composition, antioxidant and antimicrobial activities of the edible medicinal Ononis natrix growing wild in Tunisia," *Natural Product Research*, vol. 29, no. 12, pp. 1157–1160, 2015.

[191] M. S. Mokbel and T. Suganuma, "Antioxidant and antimicrobial activities of the methanol extracts from pummelo (Citrus grandis Osbeck) fruit albedo tissues " *European Food Research and Technology*, vol. 224, no. 1, pp. 39–47, 2006.

[192] G. Hasbal, T. Yilmaz-Ozden, and A. Can, "Antioxidant and anti-acetylcholinesterase activities of *Sorbus torminalis* (L.) Crantz (wild service tree) fruits," *Journal of Food and Drug Analysis*, vol. 23, no. 1, pp. 57–62, 2015.

[193] R. Singh and N. Kumari, "Comparative determination of phytochemicals and antioxidant activity from leaf and fruit of *Sapindus mukorrossi* Gaertn.—a valuable medicinal tree," *Industrial Crops and Products*, vol. 73, pp. 1–8, 2015.

[194] D. Šamec, K. Durgo, J. Grúz et al., "Genetic and phytochemical variability of six teucrium arduini L. populations and their antioxidant/prooxidant behaviour examined by biochemical, macromolecule- and cell-based approaches," *Food Chemistry*, vol. 186, pp. 298–305, 2015.

[195] Q. V. Vuong, N. Zammit, B. R. Munro, S. Murchie, M. C. Bowyer, and C. J. Scarlett, "Effect of drying conditions on physicochemical and antioxidant properties of *Vitex agnus-castus* leaves," *Journal of Food Processing and Preservation*, vol. 39, no. 6, pp. 2562–2571, 2015.

[196] N. Arora and S. Pandey-Rai, "GC-MS analysis of the essential oil of Celastrus paniculatus Willd: seeds and antioxidant, anti-inflammatory study of its various solvent extracts," *Industrial Crops and Products*, vol. 61, pp. 345–351, 2014.

[197] V. R. De Souza, P. A. P. Pereira, F. Queiroz, S. V. Borges, and J. De Deus Souza Carneiro, "Determination of bioactive compounds, antioxidant activity and chemical composition of Cerrado Brazilian fruits," *Food Chemistry*, vol. 134, no. 1, pp. 381–386, 2012.

[198] I. Sedej, M. Sakač, A. Mandić, A. Mišan, V. Tumbas, and J. Čanadanović-Brunet, "Buckwheat (*Fagopyrum esculentum* Moench) grain and fractions: antioxidant compounds and activities," *Journal of Food Science*, vol. 77, no. 9, pp. C954–C959, 2012.

[199] C. Summa, F. C. Raposo, J. McCourt et al., "Effect of roasting on the radical scavenging activity of cocoa beans," *European Food Research and Technology*, vol. 222, no. 3-4, pp. 368–375, 2006.

[200] A. Szydłowska-Czerniak, A. Tułodziecka, and E. Szłyk, "A silver nanoparticle-based method for determination of antioxidant capacity of rapeseed and its products," *Analyst*, vol. 137, no. 16, pp. 3750–3759, 2012.

[201] S. Chevion, M. Chevion, P. B. Chock, and G. R. Beecher, "Antioxidant capacity of edible plants: extraction protocol and direct evaluation by cyclic voltammetry," *Journal of Medicinal Food*, vol. 2, no. 1, pp. 1–10, 1999.

[202] M. S. Cosio, S. Buratti, S. Mannino, and S. Benedetti, "Use of an electrochemical method to evaluate the antioxidant activity of herb extracts from the Labiatae family," *Food Chemistry*, vol. 97, no. 4, pp. 725–731, 2006.

[203] D. R. Babu and G. N. Rao, "Antioxidant properties and electrochemical behavior of cultivated commercial Indian edible mushrooms," *Journal of Food Science and Technology*, vol. 50, no. 2, pp. 301–308, 2013.

[204] L. Campanella, E. Martini, and M. Tomassetti, "Antioxidant capacity of the algae using a biosensor method," *Talanta*, vol. 66, no. 4, pp. 902–911, 2005.

[205] D. Zielinska, W. Wiczkowski, M. K. Piskula, and M. K. Piskuła, "Evaluation of photochemiluminiscent, spectrophotometric and cyclic voltammetry methods for the measurement of the

antioxidant capacity: the case of roots separated from buckwheat sprouts," *Polish Journal of Food and Nutrition Sciences*, vol. 58, pp. 65–72, 2008.

[206] A. Pekal, P. Drózdz, M. Biesaga, and K. Pyrzynska, "Polyphenolic content and comparative antioxidant capacity of flavoured black teas," *International Journal of Food Sciences and Nutrition*, vol. 63, no. 6, pp. 742–748, 2012.

[207] Y.-T. Tung, J.-H. Wu, Y.-H. Kuo, and S.-T. Chang, "Antioxidant activities of natural phenolic compounds from Acacia confusa bark," *Bioresource Technology*, vol. 98, no. 5, pp. 1120–1123, 2007.

[208] S. Gorjanović, D. Komes, F. T. Pastor et al., "Antioxidant capacity of teas and herbal infusions: polarographic assessment," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 38, pp. 9573–9580, 2012.

[209] M. L. Rodríguez-Méndez, C. Apetrei, and J. A. de Saja, "Evaluation of the polyphenolic content of extra virgin olive oils using an array of voltammetric sensors," *Electrochimica Acta*, vol. 53, no. 20, pp. 5867–5872, 2008.

[210] F. M. A. Lino, L. Z. de Sá, I. M. S. Torres et al., "Voltammetric and spectrometric determination of antioxidant capacity of selected wines," *Electrochimica Acta*, vol. 128, pp. 25–31, 2014.

[211] E. M. Ervin and J. K. Kariuki, "Effect of extraction method on antioxidant determination in produce by differential pulse voltammetry," *International Journal of Electrochemical Science*, vol. 9, no. 11, pp. 6235–6245, 2014.

[212] V. Katalinic, M. Milos, T. Kulisic, and M. Jukic, "Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols," *Food Chemistry*, vol. 94, no. 4, pp. 550–557, 2006.

[213] A. M. Fernandes de Oliveira, L. S. Pinheiro, C. K. S. Pereira et al., "Total phenolic content and antioxidant activity of some malvaceae family species," *Antioxidants*, vol. 1, no. 1, pp. 33–43, 2012.

[214] S. Gorinstein, O. J. M. Vargas, N. O. Jaramillo et al., "The total polyphenols and the antioxidant potentials of some selected cereals and pseudocereals," *European Food Research and Technology*, vol. 225, no. 3-4, pp. 321–328, 2007.

[215] N. Pellegrini, M. Serafini, B. Colombi et al., "Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays," *Journal of Nutrition*, vol. 133, no. 9, pp. 2812–2819, 2003.

[216] M. S. Fernández-Pachón, D. Villaño, M. C. García-Parrilla, and A. M. Troncoso, "Antioxidant activity of wines and relation with their polyphenolic composition," *Analytica Chimica Acta*, vol. 513, no. 1, pp. 113–118, 2004.

[217] L. Pistelli, C. Noccioli, M. Martera et al., "Antioxidant flavonol glycosides from Dorycnium hirsutum," *Chemistry of Natural Compounds*, vol. 42, no. 3, pp. 281–284, 2006.

[218] V. L. Singleton and J. A. Rossi Jr., "Colorimetry of total phenolics with phosphomolibdic-phosphotungtic acid reagents," *American Journal of Enology and Viticulture*, vol. 16, pp. 144–158, 1965.

[219] C. N. T. Frizon, G. A. Oliveira, C. A. Perussello et al., "Determination of total phenolic compounds in yerba mate (Ilex paraguariensis) combining near infrared spectroscopy (NIR) and multivariate analysis," *LWT—Food Science and Technology*, vol. 60, no. 2, pp. 795–801, 2015.

[220] S. R. Georgetti, R. Casagrande, V. M. Di Mambro, A. E. C. S. Azzolini, and M. J. V. Fonseca, "Evaluation of the antioxidant activity of different flavonoids by the chemiluminescence method," *AAPS PharmSciTech*, vol. 5, no. 2, pp. 111–115, 2003.

[221] L. Campanella, E. Martini, G. Rita, and M. Tomassetti, "Antioxidant capacity of dry vegetal extracts checked by voltammetric method," *Journal of Food, Agriculture and Environment*, vol. 4, no. 1, pp. 135–144, 2006.

[222] P. A. Kilmartin, H. L. Zou, and A. L. Waterhouse, "A cyclic voltammetry method suitable for characterizing antioxidant properties of wine and wine phenolics," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 4, pp. 1957–1965, 2001.

[223] X. Cetó, J. M. Gutiérrez, M. Gutiérrez et al., "Determination of total polyphenol index in wines employing a voltammetric electronic tongue," *Analytica Chimica Acta*, vol. 732, pp. 172–179, 2012.

[224] O. Tokusoglu, S. Koçak, and S. Aycan, "The contents of sesamol and related lignans in sesame, tahina and halva as determined by a newly developed polarographic and stripping voltametric analysis," *Grasas y Aceites*, vol. 60, no. 2, pp. 119–124, 2009.

[225] E. I. Korotkova, O. A. Voronova, and E. V. Dorozhkob, "Study of antioxidant properties of flavonoids by voltammetry," *Journal of Solid State Electrochemistry*, vol. 16, no. 7, pp. 2435–2440, 2012.

[226] M. F. Barroso, N. de-los-Santos-Álvarez, M. J. Lobo-Castañón et al., "DNA-based biosensor for the electrocatalytic determination of antioxidant capacity in beverages," *Biosensors and Bioelectronics*, vol. 26, no. 5, pp. 2396–2401, 2011.

[227] N. de-los-Santos-Álvarez, P. de-los-Santos-Álvarez, M. J. Lobo-Castañón, R. López, A. J. Miranda-Ordieres, and P. Tuñón-Blanco, "Electrochemical oxidation of guanosine and adenosine: two convergent pathways," *Electrochemistry Communications*, vol. 9, no. 8, pp. 1862–1866, 2007.

[228] J. Giné Bordonaba and L. A. Terry, "Electrochemical behaviour of polyphenol rich fruit juices using disposable screen-printed carbon electrodes: towards a rapid sensor for antioxidant capacity and individual antioxidants," *Talanta*, vol. 90, pp. 38–45, 2012.

[229] F. J. N. Maia, C. D. S. Clemente, T. M. B. F. Oliveira et al., "Electrochemical and computational studies of phenolic antioxidants from cashew nut shell liquid," *Electrochimica Acta*, vol. 79, pp. 67–73, 2012.

[230] T. Wu, Y. Guan, and J. Ye, "Determination of flavonoids and ascorbic acid in grapefruit peel and juice by capillary electrophoresis with electrochemical detection," *Food Chemistry*, vol. 100, no. 4, pp. 1573–1579, 2007.

[231] S. Chan-Eam, S. Teerasong, K. Damwan, D. Nacapricha, and R. Chaisuksant, "Sequential injection analysis with electrochemical detection as a tool for economic and rapid evaluation of total antioxidant capacity," *Talanta*, vol. 84, no. 5, pp. 1350–1354, 2011.

[232] P. Ibarra-Escutia, J. J. Gómez, C. Calas-Blanchard, J. L. Marty, and M. T. Ramírez-Silva, "Amperometric biosensor based on a high resolution photopolymer deposited onto a screen-printed electrode for phenolic compounds monitoring in tea infusions," *Talanta*, vol. 81, no. 4-5, pp. 1636–1642, 2010.

[233] I. M. Apetrei, G. Bahrim, and M. L. Rodriguez-Mendez, "Electrochemical study of poliphenols with amperometric tyrosinase based biosensors," *Romanian Biotechnological Letters*, vol. 17, pp. 7684–7693, 2012.

[234] L. Campanella, A. Bonanni, G. Favero, and M. Tomassetti, "Determination of antioxidant properties of aromatic herbs, olives and fresh fruit using an enzymatic sensor," *Analytical and Bioanalytical Chemistry*, vol. 375, no. 8, pp. 1011–1016, 2003.

[235] R. de Queiroz and L. A. Ferreira, "Electrochemical determination of the antioxidant capacity of Brazilian woods as alternative materials for the aging of cachaça," *Brazilian Journal of Food Technology*, pp. 27–33, VII BMCFB, 2009.

[236] R. D. Q. Ferreira and L. A. Avaca, "Electrochemical determination of the antioxidant capacity: the ceric reducing/antioxidant

capacity (CRAC) assay," *Electroanalysis*, vol. 20, no. 12, pp. 1323–1329, 2008.

[237] M. Cheregi and A. F. Danet, "Flow injection determination of L-ascorbic acid in natural juice with biamperometric detection," *Analytical Letters*, vol. 30, no. 14, pp. 2625–2640, 1997.

[238] I. F. Abdullin, E. N. Turova, G. K. Ziyatdinova, and G. K. Budnikov, "Potentiometric determination of ascorbic acid: estimation of its contribution to the total antioxidant capacity of plant materials," *Journal of Analytical Chemistry*, vol. 57, pp. 353–355, 2002, (Translated from *Zhurnal Analiticheskoi Khimii*, vol. 57, no. 4, pp. 418–421, 2002).

[239] H. Metrouh-Amir, C. M. M. Duarte, and F. Maiza, "Solvent effect on total phenolic contents, antioxidant, and antibacterial activities of *Matricaria pubescens*," *Industrial Crops and Products*, vol. 67, pp. 249–256, 2015.

[240] S. D. Torti, M. D. Dearing, and T. A. Kursar, "Extraction of phenolic compounds from fresh leaves: a comparison of methods," *Journal of Chemical Ecology*, vol. 21, no. 2, pp. 117–125, 1995.

[241] L. Tomsone, Z. Kruma, and R. Galoburda, "Comparison of different solvents and extraction methods for isolation of phenolic compounds from horseradish roots (Armoracia rusticana)," *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, vol. 6, pp. 903–908, 2012.

[242] A. Karimi, B. Min, C. Brownmiller, and S.-O. Lee, "Effects of extraction techniques on total phenolic content and antioxidant capacities of two oregano leaves," *Journal of Food Research*, vol. 4, no. 1, pp. 112–123, 2015.

[243] M. N. Hasmida, A. R. Nur Syukriah, M. S. Liza, and C. Y. Mohd Azizi, "Effect of different extraction techniques on total phenolic content and antioxidant activity of quercus infectoria galls," *International Food Research Journal*, vol. 21, no. 3, pp. 1039–1043, 2014.

[244] P. Arulpriya and P. Lalitha, "Evaluation of different extraction methods for optimization of extraction of aerial roots of Rhaphidophora aurea entwined over two diverse host trees," *International Journal of ChemTech Research*, vol. 5, no. 5, pp. 2173–2176, 2013.

[245] A. Frum, "Resveratrol extraction and analysis methods from different plant parts," *Journal of Agroalimentary Processes and Technologies*, vol. 21, pp. 95–101, 2015.

[246] M. E. Embuscado, "Spices and herbs: natural sources of antioxidants—a mini review," *Journal of Functional Foods*, vol. 18, pp. 811–819, 2015.

[247] S. K. Chang, C. Alasalvar, and F. Shahidi, "Review of dried fruits: phytochemicals, antioxidant efficacies, and health benefits," *Journal of Functional Foods*, vol. 21, pp. 113–132, 2016.

[248] A. Karadag, B. Ozcelik, and S. Saner, "Review of methods to determine antioxidant capacities," *Food Analytical Methods*, vol. 2, no. 1, pp. 41–60, 2009.

[249] E. Niki and N. Noguchi, "Evaluation of antioxidant capacity. What capacity is being measured by which method?" *IUBMB Life*, vol. 50, no. 4-5, pp. 323–329, 2000.

[250] E. N. Frankel and A. S. Meyer, "The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants," *Journal of the Science of Food and Agriculture*, vol. 80, no. 13, pp. 1925–1941, 2000.

[251] J. M. C. Gutteridge, "Lipid peroxidation and antioxidants as biomarkers of tissue damage," *Clinical Chemistry*, vol. 41, no. 12, pp. 1819–1828, 1995.

[252] V. Sartor, P. T. Henderson, and G. B. Schuster, "Radical cation transport and reaction in RNA/DNA hybrid duplexes: effect of global structure on reactivity," *Journal of the American Chemical Society*, vol. 121, no. 48, pp. 11027–11033, 1999.

[253] X. Dai, Q. Huang, B. Zhou, Z. Gong, Z. Liu, and S. Shi, "Preparative isolation and purification of seven main antioxidants from Eucommia ulmoides Oliv. (Du-zhong) leaves using HSCCC guided by DPPH-HPLC experiment," *Food Chemistry*, vol. 139, no. 1–4, pp. 563–570, 2013.

[254] L.-Q. Sun, X.-P. Ding, J. Qi et al., "Antioxidant anthocyanins screening through spectrum-effect relationships and DPPH-HPLC-DAD analysis on nine cultivars of introduced rabbiteye blueberry in China," *Food Chemistry*, vol. 132, no. 2, pp. 759–765, 2012.

[255] J. Liu, L. Jia, J. Kan, and C.-H. Jin, "In vitro and in vivo antioxidant activity of ethanolic extract of white button mushroom (*Agaricus bisporus*)," *Food and Chemical Toxicology*, vol. 51, no. 1, pp. 310–316, 2013.

[256] E. Niki, "Assessment of antioxidant capacity *in vitro* and *in vivo*," *Free Radical Biology and Medicine*, vol. 49, no. 4, pp. 503–515, 2010.

[257] M. Oroian and I. Escriche, "Antioxidants: characterization, natural sources, extraction and analysis," *Food Research International*, vol. 74, pp. 10–36, 2015.

[258] D. Krishnaiah, R. Sarbatly, and R. Nithyanandam, "A review of the antioxidant potential of medicinal plant species," *Food and Bioproducts Processing*, vol. 89, no. 3, pp. 217–233, 2011.

[259] http://www.ars.usda.gov/services/docs.htm?docid=15866.

[260] K. Thaipong, U. Boonprakob, K. Crosby, L. Cisneros-Zevallos, and D. Hawkins Byrne, "Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts," *Journal of Food Composition and Analysis*, vol. 19, no. 6-7, pp. 669–675, 2006.

[261] E. A. Ainsworth and K. M. Gillespie, "Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent," *Nature Protocols*, vol. 2, no. 4, pp. 875–877, 2007.

[262] A. B. Ribeiro, E. G. Bonafé, B. C. Silva et al., "Antioxidant capacity, total phenolic content, fatty acids and correlation by principal component analysis of exotic and native fruits from Brazil," *Journal of the Brazilian Chemical Society*, vol. 24, no. 5, pp. 797–804, 2013.

[263] W. Wangcharoen and W. Morasuk, "Antioxidant capacity and phenolic content of holy basil," *Songklanakarin Journal of Science and Technology*, vol. 29, no. 5, pp. 1407–1415, 2007.

[264] M. F. Wang, Y. Shao, J. G. Li et al., "Antioxidative phenolic compounds from Sage (*Salvia officinalis*)," *Journal of Agricultural and Food Chemistry*, vol. 46, pp. 4869–4873, 1998.

[265] M. J. T. J. Arts, G. R. M. M. Haenen, H.-P. Voss, and A. Bast, "Antioxidant capacity of reaction products limits the applicability of the Trolox Equivalent Antioxidant Capacity (TEAC) assay," *Food and Chemical Toxicology*, vol. 42, no. 1, pp. 45–49, 2004.

[266] V. G. Hartwig, L. A. Brumovsky, R. M. Fretes, and L. Sanchez Boado, "A novel procedure to measure the antioxidant capacity of yerba maté extracts," *Ciencia e Tecnologia de Alimentos*, vol. 32, no. 1, pp. 126–133, 2012.

[267] J. F. Arteaga, M. Ruiz-Montoya, A. Palma, G. Alonso-Garrido, S. Pintado, and J. M. Rodríguez-Mellad, "Comparison of the simple cyclic voltammetry (CV) and DPPH assays for the determination of antioxidant capacity of active principles," *Molecules*, vol. 17, no. 5, pp. 5126–5138, 2012.

[268] O. Makhotkina and P. A. Kilmartin, "The phenolic composition of Sauvignon blanc juice profiled by cyclic voltammetry," *Electrochimica Acta*, vol. 83, pp. 188–195, 2012.

[269] A. M. Pisoschi, C. Cimpeanu, and G. Predoi, "Electrochemical methods for total antioxidant capacity and its main contributors determination: a review," *Open Chemistry*, vol. 13, no. 1, pp. 824–856, 2015.

[270] A. M. Pisoschi, "Biosensors as bio-based materials in chemical analysis: a review," *Journal of Biobased Materials and Bioenergy*, vol. 7, no. 1, pp. 19–38, 2013.

[271] A. M. Pisoschi and G. P. Negulescu, "Methods for total antioxidant activity determination: a review," *Biochemistry & Analytical Biochemistry*, vol. 1, no. 1, article 106, 2012.

[272] A. M. Pisoschi, M. C. Cheregi, and A. F. Danet, "Total antioxidant capacity of some commercial fruit juices: electrochemical and spectrophotometrical approaches," *Molecules*, vol. 14, no. 1, pp. 480–493, 2009.

[273] C. Hengst, S. Werner, L. Müller, K. Fröhlich, and V. Böhm, "Determination of the antioxidant capacity: influence of the sample concentration on the measured values," *European Food Research and Technology*, vol. 230, no. 2, pp. 249–254, 2009.

[274] R. Apak, S. Gorinstein, V. Böhm, K. M. Schaich, M. Özyürek, and K. Güçlü, "Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report)," *Pure and Applied Chemistry*, vol. 85, no. 5, pp. 957–998, 2013.

[275] O. I. Aruoma, "Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods," *Mutation Research*, vol. 523-524, pp. 9–20, 2003.

[276] J. Pérez-Jiménez, S. Arranz, M. Tabernero et al., "Updated methodology to determine antioxidant capacity in plant foods, oils and beverages: extraction, measurement and expression of results," *Food Research International*, vol. 41, no. 3, pp. 274–285, 2008.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 4910829, 9 pages
http://dx.doi.org/10.1155/2016/4910829

Clinical Study

Unbalanced Oxidant-Antioxidant Status: A Potential Therapeutic Target for Coronary Chronic Total Occlusion in Very Old Patients

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Received 14 July 2016; Revised 6 November 2016; Accepted 10 November 2016

Academic Editor: Renata Szymanska

Unbalanced oxidant and antioxidant status played an important role in myocardial infarction. The present study was a clinical trial combined preclinically with targeted agent against cardiovascular injuries and ischemia in vivo model. We tried to confirm the association of unbalanced oxidant and antioxidant status with coronary chronic total occlusion (CTO) in 399 very old patients (80~89 years) and investigated the potential therapeutic value of purified polysaccharide from endothelium corneum gigeriae galli (PECGGp). We analyzed levels of circulating superoxide dismutase 3 (SOD3), nitric oxide (NO), endothelial nitric oxide synthase (eNOS), and malondialdehyde (MDA) in very old patients with coronary CTO. Levels of SOD3, NO, eNOS, and MDA in the cardiac tissue were measured in myocardial infarction rats. Levels of SOD3, eNOS, and NO were lowered ($p < 0.001$) and levels of MDA were increased ($p < 0.001$). PECGGp treatment increased levels of SOD3, eNOS, and NO ($p < 0.01$) in cardiac tissue, while decreasing levels of MDA ($p < 0.01$). PECGGp may suppress unbalanced oxidant and antioxidant status in infarcted myocardium by inhibiting levels of MDA and elevating NO, eNOS, and SOD3 levels. PECGGp could be considered as a potential therapeutic agent for coronary CTO in very old patients.

1. Introduction

The relationship between impairment of nitric oxide (NO) signaling pathway and myocardial infarction risk has been identified [1–3]. Decreased NO bioactivity and elevated reactive oxygen species levels contributed to impairment of coronary arteries [4]. Thus, NO regulation can be a novel therapeutic target for protecting against myocardial infarction and congestive heart failure [5, 6].

The genetic deficiency of NOS can cause heart failure [5]. Enhanced external counterpulsation treatment for coronary heart disease patients inhibited the development of atherosclerotic lesions by stimulating NOS and NO signaling pathways [7, 8]. NOS in the myocardium had displayed novel molecular targets by which NO regulated nitroso-redox balance. NOS could be a treatment option in patients with heart diseases [9, 10].

Intervention via suppression of reactive oxygen species generation or enhancement of endogenous antioxidant enzymes may limit the infarct size and attenuate myocardial dysfunction [11–13]. Elevating MDA levels in patients with coronary heart disease impaired NO production and MDA levels were remarkably elevated in congestive heart failure patients [14–17]. The studies suggested MDA concentrations were associated with thin-cap fibroatheroma, complex atherosclerotic plaque, and atherosclerotic plaque instability and they are the main cause of myocardial infarction. Anti-MDA could be useful for developing potential antiatherosclerosis vaccine [18].

Superoxide dismutase (SOD) can regulate reactive oxygen species levels and significantly increase in the NO bioactivity under oxidative stress. The expression of the antioxidant enzyme SOD reduced cardiovascular injury and played a vital role in antisuperoxide formation, antioxidative stress damage, and artery angiogenesis. Oxidative stress by elevating reactive oxygen species had been involved in atherosclerosis and heart failure by inhibiting bioactivity of NO in the vascular walls [19–22]. SOD was a major

antioxidative enzyme in the walls of arteries and heavily damaged in coronary heart disease patients. The decreasing activity of SOD contributed to a reduction in NO bioavailability and led to high levels of oxidative stress in coronary heart disease patients. The decreased NO bioavailability may promote development of coronary artery atherosclerosis [23, 24]. Gene transfer of SOD promoted aortic endothelial repair and prevented atherogenesis. SOD had been considered as a main modulator of NO bioactivity and may have the potential therapeutic effects in preventing or reversing cardiovascular damage and ischemic heart failure. However, a novel natural SOD activator under oxidative stress is even more worthy [25, 26].

Patients with coronary heart disease who underwent primary percutaneous coronary intervention were more often of older age. The Occluded Artery Trial and The Synergy between Percutaneous Coronary Intervention With Taxus and Cardiac Surgery (SYNTAX) trial have demonstrated that primary percutaneous coronary intervention does not decrease the incidence of major adverse cardiac events and may lead to ischemic injury to the myocardium with increasing the rates of recurrent myocardial infarction and repeating coronary revascularizations in the patients with coronary chronic total occlusion (CTO). Coronary artery bypass graft surgery was more invasive than primary percutaneous coronary intervention and was executed in older patients with more severe coronary heart disease [27–30].

Our findings suggested that intracoronary infusion of human umbilical cord mesenchymal stem cells ameliorated left ventricular ejection fraction and decreased infarct size remarkably in very old patients with coronary CTO [31, 32]. However, primary percutaneous coronary intervention procedures took longer and had the risks of radiation skin injury and acute kidney injury. Primary percutaneous coronary intervention procedures of coronary CTO involved major risk factors for artery dissection and perforation of artery dissection and cardiac tamponade was a serious complication of primary percutaneous coronary intervention [28]. Therefore, further studies are needed to evaluate the potential novel noninvasive therapy for coronary CTO in very old patients. The present study aimed to demonstrate the association of unbalanced oxidant and antioxidant status with coronary CTO in very old patients and investigate potential therapeutic and preventive values of purified polysaccharide from endothelium corneum gigeriae galli (PECGGp).

2. Subjects and Methods

2.1. Study Population. The study was approved by Xuzhou Medical University and University Affiliated Hospital Ethical Review Board according to the Chinese law and regulations, and informed consent was obtained from the participants according to the Declaration of Helsinki. We studied 399 consecutive very old patients who underwent coronary angiography (232 men; 84.7 ± 5.10 years) from 1 January 2003 to 31 December 2011. The inclusion criteria included (1) age ≥ 80 years, (2) at least single vessel disease or one coronary CTO, and (3) symptomatic angina and/or ischemic heart disease.

Exclusion criteria were (1) recent use of antioxidant supplement, (2) uncontrolled hypertension, (3) acute myocardial infarction, (4) cardiopulmonary resuscitation, (5) severe ventricular arrhythmias, (6) acute coronary syndrome, (7) cardiogenic shock, (8) atrial fibrillation, (9) acute heart failure, (10) stroke, (11) immune-mediated diseases, (12) acute and chronic liver diseases, (13) hematologic disorders, (14) malignant tumours, (15) severe renal impairment, (16) acute and chronic inflammatory diseases, (17) severe iodinated contrast material reactions, (18) hyperthyroidism, (19) severe obstructive lung disease, (20) dialysis, (21) serious anemia, and (22) severe peripheral arterial disease.

2.2. Study Protocol. The patients were categorized as having control (CON) group ($n = 65$), single vessel disease (SVD) group ($n = 114$), multivessel disease (MVD) without CTO group ($n = 93$), MVD with one CTO group ($n = 71$), and MVD with multiple CTO group ($n = 56$). Coronary artery lesion with a diameter stenosis of <50% was included in CON group. SVD was defined as just one coronary artery stenosis ≥70%. MVD was defined as > one major coronary artery, stenosis of ≥70% or left main stenosis of ≥50%. The diagnosis of CTO was based on a total occlusion in a non-infarct-related artery, collateralization of the distal vessel, 100% luminal diameter stenosis with thrombolysis in myocardial infarction flow grade 0, and duration of obstruction for ≥3 months. The stump of the CTO can be defined as tapered. The duration was estimated from the last showing CTO in very old patients who underwent coronary angiograms, myocardial infarction, or acute coronary syndrome, or exertional angina and the presence of coronary collateral vessels on angiography [30, 33]. The left ventricular ejection fraction data were collected from 2-dimensional echocardiogram. The left ventricular ejection fraction was classified as follows: grade I (≥55%); grade II (40% to 54%); grade III (30% to 39%); and grade IV (<30%) [34].

2.3. Coronary Artery Angiography and Echocardiographic Studies. The patients received 300 mg aspirin loading dose along with 600 mg clopidogrel after the collection of blood samples. Coronary angiography was done through the femoral and or radial arteries by antegrade approach. Angiography of bilateral arteries was performed in some patients. Coronary angiographic analyses were performed with the dedicated coronary bifurcation computer system (Qangio® XA, 7.3, MEDIS, Medical Imaging System BV, Leiden, Netherlands). The bifurcation lesions were divided into 4 fragments: the central bifurcation segment, the distal segment of main vessel, the proximal segment main vessel, and the side branch [35–37]. Coronary artery angiograms were analyzed quantitatively by 2 independent cardiologists with experience and blind to the identities and clinical characteristics of all subjects. Discrepancies were resolved by consensus of a third cardiologist. According to the American Society of Echocardiography Standards, a complete Doppler echocardiography study was carried out with electrocardiograms synchronized by two independent cardiologists who were blinded to the clinical trial data of all subjects as described [31].

2.4. Measures of Plasma NO and eNOS. The patients' blood was drawn into the heparinized test tubes, centrifuged (1000 ×g for 10 min at 4°C), and stored at −70°C. The nitrates were reduced to nitrites by nitrate reductase and the plasma levels of NO were quantified using UV spectrophotometry (545 nm) followed by a PicoGreen measurement. The results were expressed as μmol/L [38].

eNOS levels were expressed as pg/mL and the range of this assay was 0.156 to 1000 pg/mL. EDTA-anticoagulated blood samples were obtained at fasting. The blood samples were centrifuged at 1200 rpm for 10 minutes at 4°C and stored at −80°C for assays. Each blood sample was analyzed duplicately, and the overall intra-assay coefficient of variation was best calculated to be 3.6%. All blood samples were measured in the central laboratory and the plasma concentrations of eNOS were assayed by Sigma's Sandwich ELISA kit according to the manufactures' instructions [39].

2.5. Determination of MDA and SOD3 Levels. Plasma MDA concentrations were determined by a high performance liquid chromatography system with fluorometric detection (Shimadzu, Japan) and excitation was performed at 532 nm. MDA in the samples was reacted with a solution of 2-thiobarbituric acid by incubating for two hours at 60°C. Plasma MDA levels were expressed as nmol/L [40]. The blood samples for SOD3 assays were centrifuged at 3000 rpm for 15 minutes. The plasma SOD3 activity was determined by using enzyme-linked immunosorbent assay according to the manufacturer's instructions. The assay kit for SOD3 was provided by RANSOD-Randox, UK. The results from the assay were expressed as U/mL [41].

2.6. Preparation and Purification of Polysaccharide from Endothelium Corneum Gigeriae Galli. Endothelium corneum gigeriae galli was prepared and purified according to our published procedures [42]. Briefly, endothelium corneum gigeriae galli was grinded into powders and the powders were defatted to form pretreated powders. The pretreated powders of 10 g were drawn out using distilled water and the supernatants were deproteinated and centrifugated to afford water solution endothelium corneum gigeriae galli. The endothelium corneum gigeriae galli was purified with diethylaminoethyl cellulose 52 chromatography and Sephadex® G-100. The water solution of endothelium corneum gigeriae galli was added to column of diethylaminoethyl cellulose 52. The carbohydrates eluting from the column were determined using the phenol-sulfuric acid method. One water polysaccharide fraction was collected and further purified to obtain purified polysaccharide from endothelium corneum gigeriae galli (PECGGp).

2.7. Evaluation of Cardioprotective Activities of PECGGp. According to our published procedures [42], adult male Sprague-Dawley rats were randomized into 5 groups (each n = 10): Group A (control group), Group B (PECGGp-untreated model group), Group C (positive control group), Group D (PECGGp low-dose group), and Group E (PECGGp high-dose group). Group A was administrated gastric gavage of 0.9% NaCl (25 mL/per kg body weight) every day. Group B was given gastric gavage of 0.9% NaCl (25 mL/per kg body weight) every day. Group C was treated by gastric gavage with propranolol 30 mg/kg/day. Group D and E were given gastric gavage with daily doses of PECGGp (80 and 240 mg/kg), respectively. After 14 days, all rats were treated subcutaneously with isoproterenol (5 mg/kg/day) for two consecutive days to develop myocardial infarction. Left ventricular ejection fraction was assessed using nuclear magnetic resonance imaging.

2.8. Immunohistochemistry Assessments of SOD3, NO, eNOS, MDA, Syndecan-1, and Nuclear Factor Erythroid-2-Related Factor 2 (Nrf2) in Postinfarct Rat Myocardium. The homogenate of rat myocardium was dissociated in ice cold homogenization buffer (Phosphate buffer, 0.05 M pH 7.4) by laboratory homogenate machine. NO and MDA levels were determined by the reduction of nitrate and colorimetric methods using thiobarbituric acid. eNOS and SOD3 activities were assessed with xanthine oxidase and chemical chromatometry methods according to the manufacturer's instructions [42]. Cardiac tissue cyclic GMP levels were measured by an enzyme immunoassay test-system (R&D Systems, Inc. Minneapolis, MN). Myocardium samples were reacted with a rabbit Nrf2 polyclonal antibody (1 : 100; Santa Cruz Biotechnology, Inc). Immunohistochemistry for syndecan-1 was performed on myocardium sections and the sections were stained with antibodies specific to syndecan-1 (BD Bioscience).

2.9. Statistical Analysis. All data were expressed quantitatively as the mean ± standard deviation. Paired Student's t-test for each pair of data and one-way analysis of variance was applied to compare the means. The P value of less than 0.05 was considered statistically significant. A statistical program for analysis (SPSS 13.0, SPSS Inc, Chicago, Illinois, USA) was used for all statistical analyses.

3. Results

3.1. Basic Clinical Characteristics of Patients. The basic clinical characteristics of patients were similar in very old patients (Table 1). 76 patients experienced heart failure of New York Heart Association Functional Class III-IV. The medical histories were similar and consistent with respect to the very old patients. The very old patients were discharged from the hospital on aspirin, clopidogrel, long-acting oral nitrates, ACEI/AT II blockers, beta blockers, and statins.

3.2. Plasma Levels of Circulating NO, eNOS, MDA, and SOD3 in Very Old Patients with Coronary Stenosis. The plasma levels of NO, eNOS, and SOD3 were decreased markedly in MVD with one CTO group when compared with MVD without CTO group and further reduced in MVD with multiple CTO group when compared with MVD with one CTO group ($P < 0.001$). Plasma levels of circulating MDA were significantly elevated in MVD with one CTO group compared to MVD without CTO group and further increased in MVD with multiple CTO when compared with MVD with one CTO group ($P < 0.001$) (Table 2).

Table 1: Baseline characteristics of very old patients with coronary stenosis.

	CON	SVD	MVD without CTO	MVD with one CTO	MVD with multiple CTO
	$n = 65$	$n = 114$	$n = 93$	$n = 71$	$n = 56$
Age, years	85.9 ± 4.1	83.1 ± 3.1	86.2 ± 5.8	85.7 ± 5.1	87.7 ± 4.0
Male/ female	36/29	64/50	57/36	41/30	34/22
Hypertension	39/26	59/55	61/32	45/26	37/19
Hyperlipidemia	35/30	60/54	52/41	34/37	30/26
Smoking	55/10	72/42	82/11	64/7	50/6
Diabetes mellitus	45/20	70/44	53/40	43/28	36/20
Myocardial infarction	0/0	0/0	0/0	50/21	44/12
Smoker	48/17	90/24	82/11	61/10	50/6
Heart failure	0/0	0/0	7/3	23/10	25/8
Medications					
Aspirin	12/8	64/50	57/36	41/30	34/22
Beta blockers	0/0	67/47	59/34	45/26	37/19
ACEI/AT II blockers	0/0	65/49	57/36	41/30	34/22
Clopidogrel	0/0	63/51	60/33	50/21	36/20
Long-acting oral nitrates	0/0	69/45	59/34	41/30	34/22
Statins	0/0	64/50	57/36	40/31	36/20

CON: control; CTO: chronic total occlusion; MVD: multivessel disease; SVD: single vessel disease.

Table 2: Levels of Circulating NO, eNOS, MDA, and SOD3 in very old patients with coronary stenosis.

	CON	SVD	MVD without CTO	MVD with one CTO	MVD with multiple CTO
NO (μmol/L)	61.2 ± 16.3	60.7 ± 17.1	51.4 ± 16.0	39.8 ± 15.9*	19.6 ± 14.2**
eNOS (pg/mL)	70.3 ± 25.1	71.5 ± 24.9	59.2 ± 22.4	42.6 ± 21.3*	30.0 ± 19.6**
MDA (nmol/L)	2.3 ± 1.4	2.0 ± 1.5	4.3 ± 1.7	6.1 ± 2.0*	9.9 ± 2.9**
SOD3 (U/mL)	19 ± 3.2	18.9 ± 4.1	15.2 ± 2.4	12.0 ± 2.1*	6.1 ± 1.9**

MDA: malondialdehyde; NO: nitric oxide; eNOS: endothelial nitric oxide synthase; SOD3: superoxide dismutase 3.
$^{*}P < 0.001$ (MVD without CTO/MVD with one CTO). $^{**}P < 0.001$ (MVD with one CTO/MVD with multiple CTO).

3.3. Levels of NO, eNOS, MDA, SOD3, and New York Heart Association Functional Class of The Patients. The plasma levels of NO, eNOS, and SOD3 were decreased markedly in III group when compared with II group and further reduced in IV group when compared with III group ($P < 0.001$). Plasma levels of circulating MDA were significantly elevated in III group compared to II group and further increased in IV group when compared with III group ($P < 0.001$) (Table 3).

3.4. Levels of NO, eNOS, MDA, SOD3, and Left Ventricular Ejection Fraction of the Patients. The plasma levels of NO, eNOS, and SOD3 were decreased markedly in left ventricular ejection fraction group (30–39%) when compared with left ventricular ejection fraction group (40–55%) ($P < 0.001$) and further reduced in left ventricular ejection fraction group (<30%) when compared with left ventricular ejection fraction group (30–39%) ($P < 0.001$). Plasma levels of circulating MDA were significantly elevated in left ventricular ejection fraction group (30–39%) compared to left ventricular ejection fraction group (40–55%) and further increased in left ventricular ejection fraction group (<30%) when compared with left ventricular ejection fraction group (30–39%) ($P < 0.001$) (Table 4).

3.5. Effects of PECGGp on NO, eNOS, SOD3, MDA Syndecan-1, and Nrf2 Levels in Rat Cardiac Tissue of Infarcted Myocardium. In the rats with PECGGp-untreated model group, levels of NO, eNOS, and SOD3 in the cardiac muscle tissue were markedly decreased when compared with control group ($P < 0.01$), whereas levels of MDA in the cardiac muscle tissue were markedly elevated when compared with control group. Left ventricular ejection fraction was decreased significantly in Group B when compared with control group ($P < 0.001$).

The levels of NO, eNOS, and SOD3 were increased markedly in Group D when compared with Group B ($P < 0.05, 0.01$) and further increased in Group E when compared with Group D ($P < 0.01$). The levels of MDA were significantly declined in Group D compared to Group B and further decreased in Group E when compared with Group D ($P < 0.01$). Left ventricular ejection fraction was increased markedly in Group D when compared with Group B ($P < 0.05$) and further increased in Group E when compared with Group D ($P < 0.01$) (Table 5). The results indicated that PECGGp treatment had an effect of antioxidative damage and restored the antioxidative capacity in cardiac tissue of infarcted myocardium and the patient completely recovered from heart failure.

Table 3: Levels of NO, eNOS, MDA, SOD3, and New York Heart Association Functional Class of The Patients.

	I	II	III	IV
NO (μmol/L)	50.9 ± 16.3	52.0 ± 18.0	33.4 ± 15.7*	18.1 ± 13.5**
eNOS (pg/mL)	63.1 ± 27.8	60.9 ± 23.7	41.2 ± 19.5*	22.7 ± 11.3**
MDA (nmol/L)	2.9 ± 1.8	2.8 ± 1.9	5.1 ± 2.0*	8.0 ± 2.7**
SOD3 (U/mL)	20 ± 3.9	19.1 ± 4.0	11.9 ± 2.9*	7.0 ± 2.3**

MDA: malondialdehyde; NO: nitric oxide; eNOS: endothelial nitric oxide synthase; SOD3: superoxide dismutase 3.
* $P < 0.001$ (II/III). ** $P < 0.001$ (III/IV).

Table 4: Levels of NO, eNOS, MDA, SOD3, and LVEF of the patients.

LVEF (%)	≥55	40–55	30–39	<30
NO (μmol/L)	59.8 ± 16.9	57.0 ± 14.1	30.7 ± 12.5*	17.0 ± 10.0**
eNOS (pg/mL)	69.1 ± 25.1	67.0 ± 23.8	47.2 ± 20.1*	26.7 ± 17.6**
MDA (nmol/L)	2.7 ± 1.9	2.9 ± 2.0	6.0 ± 2.3*	8.9 ± 2.8**
SOD3 (U/mL)	21.1 ± 3.9	19.9 ± 3.7	10.1 ± 3.1*	6.2 ± 2.9**

LVEF: left ventricular ejection fraction; MDA: malondialdehyde; NO: nitric oxide; eNOS: endothelial nitric oxide synthase; SOD3: superoxide dismutase 3.
* $P < 0.001$ (40–55%/30–39%). ** $P < 0.001$ (30–39%/<30%).

Cyclic GMP levels were significantly ($P < 0.001$) higher in PECGGp-treated group (2.7 ± 0.13 pmol/mg) compared to PECGGp-untreated group (0.53 ± 0.41 pmol/mg). The analysis showed higher expression of Nrf2 protein in PECGGp-treated group (35%, 17.40 ± 12.31) compared to PECGGp-untreated group (11%, 4.92 ± 4.57) ($P < 0.001$). Shedding of syndecan-1 was found to significantly increase in PECGGp-untreated group when compared with PECGGp-treated group ($P < 0.001$).

4. Discussion

Study showed that CTO was a frequent finding in very old patients, and primary percutaneous coronary intervention and coronary artery bypass graft surgery were used to treat CTO [43, 44]. The coronary artery disease patients who underwent primary percutaneous coronary intervention were mainly older adults. Results of the trials showed that primary percutaneous coronary intervention did not reduce the rate of major adverse cardiovascular events and may result in myocardial ischemic injury with elevating the rates of myocardial infarction recurrence and repeating coronary artery revascularizations in CTO patients. Coronary artery bypass graft surgery was more expensive and more invasive than primary percutaneous coronary intervention and performed in older patients with more severe coronary heart disease [27–30]. The results demonstrated that the long-term risk of cardiovascular death and adverse clinical outcome after coronary artery bypass grafting surgery were worse in older patients than in young patients [44]. Coronary angioplasty procedures took longer and had the risks of radiation-induced skin injury and acute renal failure. Coronary angioplasty procedures of coronary CTO involved important risk factors for coronary artery dissection and coronary perforation and pericardial tamponade [28]. Therefore, our study evaluated the strong potential as novel noninvasive therapy in very old patients with coronary CTO. It investigated the association of oxidant/antioxidant imbalance with coronary CTO in very old patients and demonstrated PECGGp as potential preventive and therapeutic agent.

The study showed that dysfunction of the NO signaling pathway increased risk of myocardial infarction. Increased reactive oxygen species levels inhibited NO bioactivity and production and led to coronary arterial injury. Cardiovascular disease often suppressed NO signaling pathway and augmented myocardial infarction risk [1–5]. Upregulation of NO levels can be a potential novel therapeutic target for inhibiting cardiac infarction and chronic congestive heart failure [5, 6]. The findings showed that reactive oxygen species inhibited the eNOS expression and activity in myocardium and the absence of eNOS led to myocardial infarction. Endothelial and myocyte overexpression of eNOS decreased atherosclerosis and influenced the pathophysiology of postmyocardial infarction. Cardiac myocyte-specific eNOS transgenic overexpression decreased oxidative stress and infarct area and improved cardiac function after coronary artery ligation. The clinical translation of potential regulator of eNOS expression may offer a potential new therapy for preventing myocyte imbalance between production and clearance of reactive oxygen species [9].

Our study has suggested that the levels of NO and eNOS were decreased markedly in MVD with one CTO group and further reduced in MVD with multiple CTO group. In the rats with PECGGp-untreated model group, levels of NO, eNOS, and SOD3 in cardiac tissue of infarcted myocardium were markedly decreased, whereas levels of MDA in the cardiac muscle tissue were markedly elevated. PECGGp increased the levels of NO and eNOS in cardiac tissue of infarcted myocardium and restored left ventricular ejection fraction, indicating key protective roles of NO and eNOS in myocardial infarction and heart failure. Our data showed that PECGGp had the potential for the prevention and therapy of coronary CTO in very old patients with heart failure.

Increased MDA levels contributed to enhancing the production of free radical and reduced the antioxidant activity. The suppression of reactive oxygen species generation

Table 5: Assessments of NO, eNOS, MDA, SOD3, and LVEF in postinfarct rat myocardium.

	Group A	Group B	Group C	Group D	Group E
LVEF (%)	55 ± 5.1	30 ± 3.0	56 ± 5.3	41 ± 4.9*	57 ± 5.8***
NO (nmol/mg prot)	20.5 ± 1.0	14.0 ± 0.3	21.3 ± 1.1	15.9 ± 0.5**	18.7 ± 0.8***
eNOS (U/mg prot)	15.3 ± 0.7	13.2 ± 0.1	16.1 ± 0.9	14.8 ± 0.6**	16.0 ± 0.7***
MDA(nmol/mg prot)	30.7 ± 23.8	60.3 ± 16.1	29.5 ± 12.9	49.9 ± 15.9*	29.8 ± 14.1***
SOD3 (U/mg prot)	50.9 ± 26.4	20.3 ± 14.7	51.7 ± 15.9	40.2 ± 15.0*	53.1 ± 16.0***

MDA: malondialdehyde; NO: nitric oxide; eNOS: endothelial nitric oxide synthase; SOD3: superoxide dismutase 3.
* P <0.01 (Group B/Group D). ** P < 0.05 (Group B/Group D). *** P < 0.01 (Group D/Group E).

or enhancement of endogenous antioxidant enzymes may decrease myocardial infarct size and improve myocardial dysfunction [11–14]. Several studies demonstrated that SOD3 was the most powerful antioxidant enzyme that defended the ischemic myocardium by inhibiting reactive oxygen species. The vascular SOD3 activity was severely reduced in coronary artery segments with stenoses of patients with coronary artery disease and SOD expression and activity being reduced in coronary atherosclerotic plaque. Further researches may lead to a novel antioxidant agent able either to increase SOD levels or to decrease MDA levels for myocardial infarction and chronic heart failure [19, 23–26]. We discovered that MVD with one CTO significantly elevated plasma levels of MDA and decreased SOD levels, and MVD with multiple CTO further increased MDA and reduced SOD levels. The MDA levels were significantly increased and levels of SOD were decreased markedly in cardiac tissue of infarcted myocardium and left ventricular dysfunction. PECGGp markedly increased levels of SOD and decreased MDA levels in cardiac tissue of infarcted myocardium and improved left ventricular ejection fraction.

It was concluded that expression of SOD3 in vivo did not directly affect atherosclerosis development. SOD3 was a key regulator of NO bioavailability in the blood vessel wall. Low vascular SOD3 expression played an important role in stenosis remodeling after injury, promoting oxidant stress and reduction in eNOS-derived NO bioavailability.

Increased MDA levels contributed to enhancing the production of free radical and reduced the antioxidant activity and elevating MDA levels were the main cause of myocardial infarction [8]. Atherosclerosis was associated with decreased NO bioactivity after MDA-mediated inhibition of the eNOS and SOD3 being critical in protecting NO from degradation. eNOS was a positive regulator for SOD3 expression and the reduction of eNOS contributed to decreased expression of SOD3 [9]. Our observations showed that the levels of NO, eNOS, and SOD3 were significantly decreased and MDA levels were markedly elevated in MVD patients with one CTO and further reduced and increased in MVD patients with multiple CTO. In the rats, levels of NO, eNOS, and SOD3 in cardiac tissue of infarcted myocardium were markedly decreased, whereas levels of MDA in the cardiac muscle tissue were markedly elevated. PECGGp restored the activities of antioxidant enzymes (eNOS and SOD3) and antioxidant (NO) and inhibited MDA in cardiomyocytes. NO generated within myocardium by the eNOS played a pivotal role in reduction of myocardial infarct size. SOD3 was the most powerful antioxidant enzyme that defended the myocardial infarction by inhibiting reactive oxygen species. The protective effects of PECGGp against oxidative injury were likely to be attributed to the upregulation of the endogenous cellular antioxidant system and MDA scavenging activity. The present study suggested that PECGGp had cardioprotective effects against myocardial infarction and heart failure, and the cardioprotective effects may be associated with increment of endogenous antioxidants, sustained antioxidant status in myocardial infarction, elevation of NO, eNOS, and SOD3 levels, and reduction in MDA levels.

Previous reports have shown that SOD3 derived cardiovascular injury recovery by increasing mitogenic signal transduction and reducing inflammation and apoptosis. The present reports have shown that SOD3 derives cardiovascular injury recovery by reducing inflammation and apoptosis.

Cardiovascular disease often suppressed NO signaling pathway and augmented myocardial infarction risk. Increased reactive oxygen species levels inhibited NO bioactivity and production and led to coronary arterial injury. The dysfunction of the NO signaling pathway increased risk of myocardial infarction. Upregulation of NO levels can be a potential novel therapeutic target for inhibiting cardiac infarction and chronic congestive heart failure [16, 17]. Endothelial and myocyte overexpression of eNOS decreased atherosclerosis and influenced the pathophysiology of post-myocardial infarction. The vascular SOD3 activity was severely reduced in coronary artery segments with stenoses of patients with coronary artery disease and SOD3 expression and activity were reduced in coronary atherosclerotic plaque. SOD3 was the most powerful antioxidant enzyme that defended the ischemic myocardium by inhibiting reactive oxygen species. Further researches may lead to a novel antioxidant agent able either to increase SOD3 levels or to decrease MDA levels for myocardial infarction and chronic heart failure [18–22]. PECGGp increased the levels of NO and eNOS in cardiac tissue of infarcted myocardium and restored left ventricular ejection fraction, indicating key protective roles of NO and eNOS in myocardial infarction and heart failure. PECGGp markedly increased levels of SOD3 and decreased MDA levels in cardiac tissue of infarcted myocardium and improved left ventricular ejection fraction. It was known that Ras-Erk pathway and G protein coupled

receptor signal transduction increased SOD3 expression that then increased the healing of the injuries. Our results suggested that PECGGp may suppress unbalanced oxidant and antioxidant status in infarcted myocardium, coronary arteries damage, atherosclerotic plaque, and heart failure development by inhibiting levels of MDA and elevating NO, eNOS, and SOD3 levels. Ras-Erk mitogenic pathway and G protein coupled receptor signal transduction play significant roles in increasing SOD3 expression and further explain our observations of SOD3-mediated effects in injuries [45, 46].

The activation of the signaling pathway molecules upstream and downstream of SOD3 has been studied. The messenger molecule NO exerted its effects by the stimulation of NO sensitive guanylyl cyclase which led to enhanced production of the intracellular messenger cyclic GMP. Cyclic GMP protected endothelial progenitors from oxidative stress. Cyclic GMP levels were significantly elevated in PECGGp-treated group, thus providing evidence that PECGGp markedly increased levels of SOD3 and SOD3 expression elevated cyclic GMP levels in cardiac tissue of infarcted myocardium. The involvement of NO/cyclic GMP pathway in infarcted myocardium was confirmed [47, 48].

The nuclear factor erythroid 2-related factor (Nrf2) as the upstream regulator of SOD3 expression was a major antioxidant transcription factor to mediate the expression of antioxidant genes. Our results suggested that MDA can act via Nrf2 to regulate antioxidant defence gene expression. Nrf2 acted as a molecular sensor of cellular redox homeostasis disturbance and represented a powerful tool in SOD3 expression. SOD3 was regulated by the Nrf2 transcription factor and Nrf2 elevated SOD3 expression in cardiac tissue of infarcted myocardium. MDA affected the stability and the transcriptional functions of Nrf2. Notably, it was suggested that PECGGp activated SOD3 expression via Nrf2 pathway, protecting rat hearts against oxidative damage by decreasing MDA levels [49].

It has also been found that the loss of SOD3 may promote syndecan-1 vulnerable to oxidative stress and lead to syndecan-1 shedding and SOD3 inhibits oxidant-induced shedding of syndecan-1. Our results showed that the SOD3 decreased and shedding of syndecan-1 increased in cardiac tissue of infarcted myocardium. More importantly, The present study demonstrated that MDA contributed to syndecan-1 shedding in infarcted myocardium and that SOD3 protected the hearts against oxidative damage by limiting MDA-induced shedding of syndecan-1 [50].

Our results suggested that PECGGp may suppress unbalanced oxidant and antioxidant status in infarcted myocardium, coronary arteries damage, atherosclerotic plaque and heart failure development by inhibiting levels of MDA and elevating NO, eNOS, and SOD3 levels. These findings suggested that PECGGp may offer a potential new therapy for preventing and treating imbalance between production and clearance of reactive oxygen species in very old patients with MVD, with multiple CTO and heart failure.

5. Conclusion

The present study suggested that PECGGp had cardioprotective effects against myocardial infarction and heart failure, and the cardioprotective effects may be associated with increment of endogenous antioxidants, sustained antioxidant status in myocardial infarction, elevation of NO, eNOS, and SOD3 levels, and reduction in MDA. Therefore, PECGGp might be considered a potential therapeutic agent for preventing or treating CTO and heart failure in very old patients. However, more research is needed to study the clinical efficacy of PECGGp in prevention or treatment of CTO and heart failure, which may provide clinical evidence for a novel strategy in the prevention and therapy of unbalanced oxidant and antioxidant status and CTO in very old patients with heart failure.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Youdong Hu and Fenglin Zhang contributed equally to this article.

References

[1] J. Erdmann, K. Stark, U. B. Esslinger et al., "Dysfunctional nitric oxide signalling increases risk of myocardial infarction," *Nature*, vol. 504, no. 7480, pp. 432–436, 2013.

[2] D. I. Lee, G. Zhu, T. Sasaki et al., "Phosphodiesterase 9A controls nitric-oxide-independent cGMP and hypertrophic heart disease," *Nature*, vol. 519, no. 7544, pp. 472–476, 2015.

[3] J. E. Freedman, "Inherited dysfunctional nitric oxide signaling and the pathobiology of atherothrombotic disease," *Circulation Research*, vol. 114, no. 9, pp. 1372–1373, 2014.

[4] U. Flierl, D. Fraccarollo, J. D. Widder et al., "The nitric oxide donor pentaerythritol tetranitrate reduces platelet activation in congestive heart failure," *PLoS ONE*, vol. 10, no. 4, Article ID e0123621, 2015.

[5] S. Bhushan, K. Kondo, D. J. Polhemus et al., "Nitrite therapy improves left ventricular function during heart failure via restoration of nitric oxide-mediated cytoprotective signaling," *Circulation Research*, vol. 114, no. 8, pp. 1281–1291, 2014.

[6] J. Davignon and P. Ganz, "Role of endothelial dysfunction in atherosclerosis," *Circulation*, vol. 109, no. 23, pp. e27–e28, 2004.

[7] Y. Zhang, X. He, X. Chen et al., "Enhanced external counterpulsation inhibits intimal hyperplasia by modifying shear stress-responsive gene expression in hypercholesterolemic pigs," *Circulation*, vol. 116, no. 5, pp. 526–534, 2007.

[8] U. Laufs, N. Werner, A. Link et al., "Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis," *Circulation*, vol. 109, no. 2, pp. 220–226, 2004.

[9] R. Carnicer, M. J. Crabtree, V. Sivakumaran, B. Casadei, and D. A. Kass, "Nitric oxide synthases in heart failure," *Antioxidants and Redox Signaling*, vol. 18, no. 9, pp. 1078–1099, 2013.

[10] S. J. Greene, M. Gheorghiade, B. A. Borlaug et al., "The cGMP signaling pathway as a therapeutic target in heart failure with preserved ejection fraction," *Journal of the American Heart Association*, vol. 2, no. 6, Article ID e000536, 2013.

[11] M. C. Polidori, K. Savino, G. Alunni et al., "Plasma lipophilic antioxidants and malondialdehyde in congestive heart failure patients: relationship to disease severity," *Free Radical Biology and Medicine*, vol. 32, no. 2, pp. 148–152, 2002.

[12] V. Adams, C. Besler, T. Fischer et al., "Exercise training in patients with chronic heart failure promotes restoration of high-density lipoprotein functional properties," *Circulation Research*, vol. 113, no. 12, pp. 1345–1355, 2013.

[13] D. V. Folden, A. Gupta, A. C. Sharma, S.-Y. Li, J. T. Saari, and J. Ren, "Malondialdehyde inhibits cardiac contractile function in ventricular myocytes via a p38 mitogen-activated protein kinase-dependent mechanism," *British Journal of Pharmacology*, vol. 139, no. 7, pp. 1310–1316, 2003.

[14] K. H. Lim, D. Ko, and J.-H. Kim, "Cardioprotective potential of Korean red ginseng extract on isoproterenol-induced cardiac injury in rats," *Journal of Ginseng Research*, vol. 37, no. 3, pp. 273–282, 2013.

[15] S. Amir, K. Hartvigsen, A. Gonen et al., "Peptide mimotopes of malondialdehyde epitopes for clinical applications in cardiovascular disease," *Journal of Lipid Research*, vol. 53, no. 7, pp. 1316–1326, 2012.

[16] C. R. Díaz-Vélez, S. García-Castiñeiras, E. Mendoza-Ramos, and E. Hernández-López, "Increased malondialdehyde in peripheral blood of patients with congestive heart failure," *American Heart Journal*, vol. 131, no. 1, pp. 146–152, 1996.

[17] H. S. Kim, S. J. Kwack, and B. M. Lee, "Lipid peroxidation, antioxidant enzymes, and benzo[a]pyrene-quinones in the blood of rats treated with benzo[a]pyrene," *Chemico-Biological Interactions*, vol. 127, no. 2, pp. 139–150, 2000.

[18] K. Tajika, K. Okamatsu, M. Takano et al., "Malondialdehyde-modified low-density lipoprotein is a useful marker to identify patients with vulnerable plaque," *Circulation Journal*, vol. 76, no. 9, pp. 2211–2217, 2012.

[19] F. M. Faraci and S. P. Didion, "Vascular protection: superoxide dismutase isoforms in the vessel wall," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 8, pp. 1367–1373, 2004.

[20] M. O. Laukkanen, A. Kivelä, T. Rissanen et al., "Adenovirus-mediated extracellular superoxide dismutase gene therapy reduces neointima formation in balloon-denuded rabbit aorta," *Circulation*, vol. 106, no. 15, pp. 1999–2003, 2002.

[21] J. H. Bräsen, O. Leppänen, M. Inkala et al., "Extracellular superoxide dismutase accelerates endothelial recovery and inhibits in-stent restenosis in stented atherosclerotic Watanabe heritable hyperlipidemic rabbit aorta," *Journal of the American College of Cardiology*, vol. 50, no. 23, pp. 2249–2253, 2007.

[22] A. E. Dikalova, A. T. Bikineyeva, K. Budzyn et al., "Therapeutic targeting of mitochondrial superoxide in hypertension," *Circulation Research*, vol. 107, no. 1, pp. 106–116, 2010.

[23] T. Fukai, R. J. Folz, U. Landmesser, and D. G. Harrison, "Extracellular superoxide dismutase and cardiovascular disease," *Cardiovascular Research*, vol. 55, no. 2, pp. 239–249, 2002.

[24] B. Hornig, U. Landmesser, C. Kohler et al., "Comparative effect of ACE inhibition and angiotensin II type 1 receptor antagonism on bioavailability of nitric oxide in patients with coronary artery disease: role of superoxide dismutase," *Circulation*, vol. 103, no. 6, pp. 799–805, 2001.

[25] S. Agostini, E. Chiavacci, M. Matteucci, M. Torelli, L. Pitto, and V. Lionetti, "Barley beta-glucan promotes MnSOD expression and enhances angiogenesis under oxidative microenvironment," *Journal of Cellular and Molecular Medicine*, vol. 19, no. 1, pp. 227–238, 2015.

[26] U. Landmesser, R. Merten, S. Spiekermann, K. Büttner, H. Drexler, and B. Hornig, "Vascular extracellular superoxide dismutase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation," *Circulation*, vol. 101, no. 19, pp. 2264–2270, 2000.

[27] J. S. Hochman, G. A. Lamas, C. E. Buller et al., "Coronary intervention for persistent occlusion after myocardial infarction," *The New England Journal of Medicine*, vol. 355, no. 23, pp. 2395–2407, 2006.

[28] P. R. Sinnaeve, J. Simes, S. Yusuf et al., "Direct thrombin inhibitors in acute coronary syndromes: effect in patients undergoing early percutaneous coronary intervention," *European Heart Journal*, vol. 26, no. 22, pp. 2396–2403, 2005.

[29] P. B. Shah, "Management of coronary chronic total occlusion," *Circulation*, vol. 123, no. 16, pp. 1780–1784, 2011.

[30] P. Fefer, M. L. Knudtson, A. N. Cheema et al., "Current perspectives on coronary chronic total occlusions: the Canadian multicenter chronic total occlusions registry," *Journal of the American College of Cardiology*, vol. 59, no. 11, pp. 991–997, 2012.

[31] X. Li, Y.-D. Hu, Y. Guo et al., "Safety and efficacy of intracoronary human umbilical cord-derived mesenchymal stem cell treatment for very old patients with coronary chronic total occlusion," *Current Pharmaceutical Design*, vol. 21, no. 11, pp. 1426–1432, 2015.

[32] X. Li, Y. D. Hu, Y. Guo et al., "Mechanisms of improvement of left ventricular function by intracoronary human umbilical cord-derived mesenchymal stem cell infusion in very old patients with coronary chronic total occlusion," *Current Pharmaceutical Design*, vol. 21, no. 26, pp. 3844–3850, 2015.

[33] D. Mukherjee and M. Roffi, "Chronic total occlusions in non-infarct-related arteries," *European Heart Journal*, vol. 33, no. 6, pp. 695–697, 2012.

[34] Y. Morino, T. Kimura, Y. Hayashi et al., "In-hospital outcomes of contemporary percutaneous coronary intervention in patients with chronic total occlusion. insights from the J-CTO registry (Multicenter CTO Registry in Japan)," *JACC: Cardiovascular Interventions*, vol. 3, no. 2, pp. 143–151, 2010.

[35] D. Zhang, B. Xu, D. Yin et al., "Predictors and periprocedural myocardial injury rate of small side branches occlusion in coronary bifurcation intervention," *Medicine (United States)*, vol. 94, no. 25, p. e992, 2015.

[36] A. J. Lansky, G. Dangas, R. Mehran et al., "Quantitative angiographic methods for appropriate end-point analysis, edge-effect evaluation, and prediction of recurrent restenosis after coronary brachytherapy with gamma irradiation," *Journal of the American College of Cardiology*, vol. 39, no. 2, pp. 274–280, 2002.

[37] M. Ferenc, M. Gick, R.-P. Kienzle et al., "Randomized trial on routine vs. provisional T-stenting in the treatment of de novo coronary bifurcation lesions," *European Heart Journal*, vol. 29, no. 23, pp. 2859–2867, 2008.

[38] P. J. M. Best, P. B. Berger, V. M. Miller, and A. Lerman, "The effect of estrogen replacement therapy on plasma nitric oxide and endothelin-1 levels in postmenopausal women," *Annals of Internal Medicine*, vol. 128, no. 4, pp. 285–288, 1998.

[39] J. Y. Guo, T. Yang, X. G. Sun et al., "Ischemic postconditioning attenuates liver warm ischemia-reperfusion injury through Akt-eNOS-NO-HIF pathway," *Journal of Biomedical Science*, vol. 18, no. 1, article 79, 2011.

[40] S. Rohrbach, A. Martin, B. Niemann, and A. Cherubini, "Enhanced myocardial vitamin C accumulation in left ventricular hypertrophy in rats is not attenuated with transition to heart failure," *European Journal of Heart Failure*, vol. 10, no. 3, pp. 226–232, 2008.

[41] X. L. Wang, T. Adachi, A. S. Sim, and D. E. L. Wilcken, "Plasma extracellular superoxide dismutase levels in an Australian population with coronary artery disease," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 18, no. 12, pp. 1915–1921, 1998.

[42] Q. Xiong, Y. Jing, X. Li et al., "Characterization and bioactivities of a novel purified polysaccharide from *Endothelium corneum gigeriae* Galli," *International Journal of Biological Macromolecules*, vol. 78, no. 7, pp. 324–332, 2015.

[43] S. Deb, H. C. Wijeysundera, D. T. Ko, H. Tsubota, S. Hill, and S. E. Fremes, "Coronary artery bypass graft surgery vs percutaneous interventions in coronary revascularization: a systematic review," *The Journal of the American Medical Association*, vol. 310, no. 19, pp. 2086–2095, 2013.

[44] M. Dalén, T. Ivert, M. J. Holzmann, and U. Sartipy, "Coronary artery bypass grafting in patients 50 years or younger: a Swedish nationwide cohort study," *Circulation*, vol. 131, no. 20, pp. 1748–1754, 2015.

[45] J. P. Laurila, M. D. Castellone, A. Curcio et al., "Extracellular superoxide dismutase is a growth regulatory mediator of tissue injury recovery," *Molecular Therapy*, vol. 17, no. 3, pp. 448–454, 2009.

[46] L. E. Laatikainen, M. D. Castellone, A. Hebrant et al., "Extracellular superoxide dismutase is a thyroid differentiation marker down-regulated in cancer," *Endocrine-Related Cancer*, vol. 17, no. 3, pp. 785–796, 2010.

[47] A. Saqib, K. M. Prasad, A. B. Katwal et al., "AAV9-Mediated overexpression of extracellular superoxide dismutase improves recovery from surgical hind-limb ischemia in BALB/c mice," *Journal of Vascular Surgery*, vol. 54, no. 3, pp. 810–818, 2011.

[48] A. M. Curatola, J. Xu, and K. D. Hendricks-Munoz, "Cyclic GMP protects endothelial progenitors from oxidative stress," *Angiogenesis*, vol. 14, no. 3, pp. 267–279, 2011.

[49] H. K. Poonyagariyagorn, S. Metzger, D. Dikeman et al., "Superoxide dismutase 3 dysregulation in a murine model of neonatal lung injury," *American Journal of Respiratory Cell and Molecular Biology*, vol. 51, no. 3, pp. 380–390, 2014.

[50] C. R. Kliment, J. M. Englert, B. R. Gochuico et al., "Oxidative stress alters syndecan-1 distribution lungs with pulmonary fibrosis," *Journal of Biological Chemistry*, vol. 284, no. 6, pp. 3537–3545, 2009.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 1923754, 10 pages
http://dx.doi.org/10.1155/2016/1923754

Research Article

Phytochemical Composition, Antioxidant Activity, and the Effect of the Aqueous Extract of Coffee (*Coffea arabica* L.) Bean Residual Press Cake on the Skin Wound Healing

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Received 12 July 2016; Revised 9 October 2016; Accepted 17 October 2016

Academic Editor: Renata Szymanska

The world coffee consumption has been growing for its appreciated taste and its beneficial effects on health. The residual biomass of coffee, originated in the food industry after oil extraction from coffee beans, called coffee beans residual press cake, has attracted interest as a source of compounds with antioxidant activity. This study investigated the chemical composition of aqueous extracts of coffee beans residual press cake (AE), their antioxidant activity, and the effect of topical application on the skin wound healing, in animal model, of hydrogels containing the AE, chlorogenic acid (CGA), allantoin (positive control), and carbopol (negative control). The treatments' performance was compared by measuring the reduction of the wound area, with superior result ($p < 0.05$) for the green coffee AE (78.20%) with respect to roasted coffee AE (53.71%), allantoin (70.83%), and carbopol (23.56%). CGA hydrogels reduced significantly the wound area size on the inflammatory phase, which may be associated with the well known antioxidant and anti-inflammatory actions of that compound. The topic use of the coffee AE studied improved the skin wound healing and points to an interesting biotechnological application of the coffee bean residual press cake.

1. Introduction

The imbalance between the production of reactive oxidizing agents and the ability of a biological system to counteract the reactive intermediates results in oxidative stress. The reactive oxidizing agents are formed during normal physiological processes or under stress conditions that can damage biological system [1].

The coffee (*Coffea arabica* L.) beans press cake is a residual biomass from the coffee beans oil extraction process, claimed to be rich in bioactive compounds of interest for human health and cosmetics. There is considerable emphasis on the recovery of plant biomass originating from the food industry in order to target it to other industries [2], adding value and reducing eventual environmental damage. In this sense, the large amount of *C. arabica* beans produced in Brazil gives rise to certain amounts of biomass not acceptable by the beverage market, but with an important potential for the development of other products such as cosmetics.

Coffee has been claimed as a functional beverage being an important source of antioxidants in human diet, especially due to the high amounts of phenolic compounds and caffeine. The chemical constituents of arabica coffee involve phenolic compounds and their derivatives (such as chlorogenic acids), alkaloids (specially caffeine), diterpenoid alcohols (such as cafestol and kahweol), carbohydrates, lipids, and volatile and heterocyclic compounds [3]. Chlorogenic acids (CGA) are esters of the *trans*-cinnamic acids [4] whose amounts in green coffees are significantly reduced during the roasting process [5]. In the past decades, polyphenolic compounds

have been proposed as one of the most effective functional ingredients in foods and beverages, with antiaging properties and able to neutralize the effects of oxidative damage to the skin, for example, [6]. Studies with *C. arabica* extracts have revealed a set of important biological activities, for example, antibacterial [7], antiviral [8], anti-inflammatory [9, 10], suppressive activity of metalloproteinase expression [11], and reduction of oxidative damage to macromolecules [12]. Besides phenolic compounds, coffee is well recognized as a rich source of the alkaloids, especially caffeine. Such secondary metabolites have presented relevant biological activities as, for instance, stimulation of the central nervous system, diuretic, and peripheral vasoconstriction [13]. Caffeine is the major alkaloid present in coffee beans and its content is correlated with the quality of the beverage, also contributing to the brew bitterness [14].

Skin serves as a protective barrier against the environment and also plays a fundamental role in the homeostasis maintenance [15]. The loss of the skin integrity results in injury that can lead to serious commitments to the body if not repaired. The tissue lesions trigger intracellular responses that coordinate the restoration of tissue's integrity and homeostasis. The ability to respond to the injury and tissue repair is a fundamental property for multicellular organisms. The cutaneous tissue repair occurs via tissue regeneration, with the recovery of tissue functionality, or the healing, and the restoration of tissue homeostasis [16].

The wound healing is a complex biological process after an injury. This process can be understood into three classic stages: inflammation, proliferation, and remodeling. The inflammatory stage begins immediately after injury of the tissue and lasts for around 3 days with inflammation. Subsequently, there are cellular proliferation, migration of different cell types, angiogenesis, and extracellular matrix components production, especially collagen. The proliferation stage may last from 2 to 10 days and is characterized by the migration and differentiation of various cell types, being the most abundant fibroblasts. The remodeling stage is the third and last stage of repair. It begins 2 to 3 weeks after the injury and lasts for about a year or more. The majority of endothelial cells, myofibroblasts, and macrophages are destroyed by apoptosis and these changes result in wound contraction and scar formation [16]. Inflammation and oxidative stress are closely related at the wound site considering the influx of neutrophils and macrophages producing reactive oxygen and nitrogen. Reactive oxygen and nitrogen species are involved in the redox regulation of cell functions and increasingly viewed as a major upstream component in the signalling cascade involved in inflammatory responses and the stimulation of adhesion molecule and chemoattractant production [15]. Testing by oxygen radical absorbance capacity assay of the coffee berry outperforms common antioxidants such as green tea extract, pomegranate extract, vitamin C, and vitamin E. Polymerase chain reaction microarray analysis on human cultured fibroblasts treated with multiple doses of 0.001% coffee berry revealed upregulation of gene expression for several collagens and connective tissue growth factor and downregulation for metalloproteinases [17].

It is known that the effect of aging causes a temporal delay in wound healing, but not an actual impairment in terms of the quality of healing [18, 19]. Delayed wound healing in the aged is associated with an altered inflammatory response, such as delayed T-cell infiltration into the wound area with alterations in chemokine production and reduced macrophage phagocytic capacity [20]. Retarded reepithelialization, collagen synthesis, and angiogenesis have also been observed in aged mice as compared with young ones [21].

The topical administration of antioxidant compounds on the skin is gaining prominence among dermatologists because of their anti-inflammatory and anticarcinogenic activity [22]. In this context, it has been claimed that wound repair process could be helped by topically administrating antioxidant molecules that may control the oxidative stress [23]. Phenolic compounds have been showed to be active on skin tissues through hampering collagen destruction and collagenase activation [24]. Indeed, CGA has been shown to accelerate the skin wound healing and burn healing due to its antioxidant, free radical scavenging, anti-inflammatory, radioprotective, antiulcerogenic, and analgesic properties [10, 25–29]. Importantly, the effectiveness of polyphenols in the repair of cutaneous tissue is initially determined by their physicochemical properties and the ability to overcome the epidermal barrier to achieve appropriate receptors [6].

In this sense, the working hypothesis of this study is based on the assumption that a biologically compatible extract, that is, the AE of coffee beans residual press cake, is a rich source of polyphenolic compounds that topically administrated on the damaged cutaneous tissue would ameliorate the wound healing process. Thus, the phytochemical profile and the antioxidant activity of the AE of coffee beans (*C. arabica* L.) residual press cake were determined. In a second approach, the *in vivo* effect of topically administrated hydrogels containing AE and chlorogenic acid on the wound healing process was investigated using a skin excision wound model in Swiss albino mice.

2. Material and Methods

2.1. Coffee Samples, Chemicals, and Cell Lines. Samples of green (i.e., nonroasted beans) and roasted coffee beans press cake residual were supplied by Cooxupé (Regional Cooperative of Coffee Growers Ltda, at Guaxupé, Minas Gerais State, southeastern Brazil). Green and roasted coffee bean press cake samples were obtained after mechanically pressing the beans for the extraction of the oil fraction. After extraction, the residual biomass was packed in polyethylene bags, with silica sachets, and stored at −20°C for further analysis. Analytical grade methanol, ethanol, hydrochloric acid, sodium chloride, and hydrogen peroxide were purchased from Vetec (Rio de Janeiro, Brazil). The Folin-Ciocalteu reagent, sodium dodecyl sulphate (SDS), dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium phosphate buffer (TPK), Tween® 20, Bradford reagent, bovine serum albumin (BSA), methionine, riboflavin, nitrotetrazolium blue chloride (NBT), neutral red (NR), and the analytical reference standards (chlorogenic acid, gallic

acid, *p*-hydroxybenzoic acid, ferulic acid, caffeic acid, syringic acid, *p*-coumaric acid, caffeine, theophylline, theobromine, trigonelline, and allantoin) were purchased from Sigma-Aldrich (Missouri, USA). Carbopol 940 NF polymer, disodium ethylenediaminetetraacetic acid (EDTA), and aminomethyl propanol (AMP) were obtained from Pharma Nostra (Campinas, Brazil). Mouse fibroblasts L929 cell line was purchased from Rio de Janeiro Cell Bank (BCRJ), as Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Sigma-Aldrich (Missouri, USA).

2.2. Preparation of the Aqueous Extracts of Green and Roasted Coffee Bean Residual Press Cake. AE extracts of the biomasses in study were obtained according to the cold extraction method from the Brazilian Pharmacopoeia [30], with modifications. Samples (4 g, dry weight) were added of 100 mL EtOH solution 70% (v/v) at room temperature, followed by magnetic stirring during 1 h, protected from the light, and kept 18 h under refrigeration (2–8°C). The extracts were recovered by filtration on cellulose support under reduced pressure, followed by concentration in a rotatory evaporator (60°C, 82 rpm). Dried extracts were resuspended in 10 mL distilled-deionized water, centrifuge (10 min, 4000 rpm, 13.7 cm rotor radius), and the supernatant collected for further analysis.

2.3. Preparation of the Treatments for Topical Application on the Skin Wound Healing. Carbopol hydrogel was prepared containing 1% (w/v) carbopol 940, 0.1% (w/v) EDTA, 0.5% (w/v) aminomethyl propanol, and water (60 mL). The carbopol hydrogels were used as a vehicle base and the treatments were prepared as follows: (1) carbopol base plus 10% AE (v/v) green coffee, (2) carbopol base plus 10% AE (v/v) roasted coffee, (3) carbopol base plus 3% (v/w) chlorogenic acid, (4) carbopol base plus 1% (v/w) allantoin-positive control, and (5) carbopol base-negative control. The chlorogenic acid concentration in the hydrogel (3%, w/v) was chosen according to the content of this compound in the AE of green coffee press cake previously determined by HPLC analysis.

2.4. Determination of Total Phenolic Contents. The total phenolic contents of the AE were determined using the Folin-Ciocalteu reagent, according to Randhir et al. [31]. For that, 1 mL AE was diluted in 9 mL distilled-deionized water. The diluted sample (40 μL) was added to 3.16 mL distilled-deionized water and 200 μL Folin-Ciocalteau reagent, vortexed, stirring for 1 min, after which 600 μL of sodium carbonate solution was added (20%, w/v), vortexed, and incubated for 2 h. The blank solution was prepared as described above, by replacing the test sample by distilled-deionized water. The absorbance was measured at 750 ηm using a UV spectrophotometer (BEL LGS 53, BEL Engineering, Monza, Italy) and the analysis was carried out in triplicate. The results were expressed as mean (mg equivalent gallic acid/g biomass) ± standard error of the mean (sem), based on a calibration curve of gallic acid (100–700 $\mu g \cdot mL^{-1}$, $r^2 = 0.983$, $y = 0.001x$).

Table 1

Time (min)	Eluent A (%, v/v)	Eluent B (%, v/v)
0–5	85	15
5–45	0	100
45–50	85	15

2.5. RP-HPLC Analysis of Phenolic Acids and Alkaloids. The RP-HPLC analysis was adapted from Rodrigues and Bragagnolo [32] that propose simultaneous determination of caffeine and chlorogenic acids. AE aliquots (60 μL) were injected into a liquid chromatograph (HPLC Thermo Scientific UltiMate 3000 RS Dual System) equipped with a reverse-phase column (Thermo Scientific C18, 250 mm × 4.6 mm, Ø 0.5 μm particle, 35°C) and a diode array detector operating at 240 nm, 260 nm, 280 nm, and 320 nm. The alkaloids and phenolic compounds were quantified at 280 nm and 320 nm, respectively, according to previous records. The mobile phase consisted of HCl acidified Milli-Q water (pH 2.3-Eluent A) and methanol (Eluent B). The chromatographic conditions for the gradient were as shown in Table 1.

A flow rate at 1 mL/min and a run time of 50 min were used. Besides, the AE was solubilized in 10 mL 80% methanol and diluted 9 : 1 (v/v) previously to injection. The quantification of phenolic compounds was based on the integration of the peak areas and a calibration curve of chlorogenic acid (detection range 10–400 $\mu g \cdot mL^{-1}$, $r^2 = 0.99$, $y = 0.707x$). Similarly, the amounts of alkaloids were determined based on the integration of the peak areas and calibration curve of caffeine (detection range 100–1000 $\mu g \cdot mL^{-1}$, $r^2 = 0.99$, $y = 0.842x$). The analysis was carried out in triplicate. The results were expressed as mean ($mg \cdot g^{-1}$) ± standard deviation (sd).

2.6. DPPH Radical Scavenging Activity Assay. The AE was firstly freeze-dried, weighted, and resuspended in 95% methanol (v/v) for a final concentration at 10 mg/mL AE dried mass. Antioxidant activity of the green and roasted coffee AE was determined by measuring their scavenging capacity of the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical as previously described and adapted by Molyneux [33]. The (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as positive control and methanol as the negative one. The amount of 28 μL DPPH methanolic solution (70 μM) was incubated with 972 μL sample test (from 1 to 30 μg/mL), for 20 min, at 25°C ($n = 3$). DPPH scavenging activity, which manifests itself as a decrease in absorbance, was measured at 517 nm with a UV-Vis spectrophotometer (UV-2000 Instruterm). The DPPH radical scavenging activity (%) was calculated as follows:

$$\text{Radical scavenging activity\%} = 100 - \left[\text{Abs sample} * \frac{100}{\text{Abs negative control}}\right]. \quad (1)$$

The experiment was carried out in triplicate and data are expressed as mean ± standard deviation (sd).

2.7. In Vitro Cell Viability. The effect of the AE on the cell viability was determined *in vitro* according to the protocol established by ICCVAM [34], with modifications. The method is based on the assessment of cell viability *in vitro* after exposure to the AE, measuring the lysosome's uptake of neutral red dye. For that, mouse L929 fibroblasts were plated in 96-well plates (1×10^4 cells/well) and incubated during 24 h (37°C, 95% humidity, and 5% CO_2). After this period, cells were washed with phosphate buffered saline (PBS) and treated with increasing concentrations of coffee AE (3, 10, 30, 100, 300, 1000, 3000, and 10000 μg/mL), during 24 h (37°C, 95% humidity, and 5% CO_2). The protein denaturing agent SDS, in the same concentrations of the coffee AE, was used as positive control. With the treatment period elapsing, cells were washed with PBS and incubated in the presence of neutral red dye (25 μg/mL DMEM) for 3 h (37°C, 95% humidity, and 5% CO_2). Subsequently, cells were washed with PBS and neutral red dye destaining solution was added (1% acetic acid : 50% ethanol : 49% distilled water, v/v/v). Absorbance was measured at 540 nm using a microplate reader (*SpectraMax Paradigm Multi-Mode Microplate* Reader, Molecular Devices, Sunnyvale, USA). Cell viability was expressed as percentage of growth based on the control cells and concentration of test samples which showed 50% inhibition of cell grow (IC50), expressing the results in mg AE/mL. The results were calculated according to the equation below:

$$\left[\left(C_v\right) = \left[\frac{\left(C_{\text{me}} - B_m\right)}{\left(\text{Ctr}_m - B_m\right)}\right] * 100\right], \tag{2}$$

where C_v is the cell viability (%), C_{me} is the mean absorbance of each concentration of the sample, B_m is the mean absorbance of the blank, and Ctr_m is the mean absorbance of control.

2.8. In Vivo Skin Wound Healing Assays. Swiss albino male mice *(Mus musculus)*, 40–60 g, 9 months old (assay 1), and 2 months old (assay 2), from the Central Biotery of the Federal University of Santa Catarina (Florianópolis, Santa Catarina State, southern Brazil) were adapted to the laboratory conditions prior to the assay, having free access to food and water. The animals were anesthetized using an intraperitoneal injection of 10% ketamine (75 mg/kg) and 2% xylazine (10 mg/kg). The excisional wound model was realized according to Frank and Kämpfer [35]. Briefly, the dorsal surface of the thoracic region was trichotomized and cleaned up with iodized alcohol. A 10 mm diameter skin wound was made with a scissor aid under aseptic conditions. Hydrogels (0.1 g/animal) were applied topically and daily. Carbopol hydrogel (1%, w/v) was used as negative control, as carbopol hydrogel containing allantoin (1%, w/v) was chosen as positive control, because of the recognized antiseptic action and ability to promote cell proliferation and wound healing of that compound [36]. The wound contraction was evaluated and the wound area measured at the end of the experimental period, that is, day 14. The experimental protocol was approved by the Ethics Committee on the use of animals (Federal University of Santa Catarina, PP0957).

2.8.1. Evaluation of Wound Healing 1: Treatment with Hydrogels Containing Green and Roasted Coffee Bean AE. The aim of this experiment was to determine the effect of the topical treatment with AE of green and roasted coffee beans press cake on the skin wound healing. This investigation adopted an *in vivo* assay with 9-month-old mice, in order to verify the wound healing potential of the AE in an animal group with delayed metabolism due to the advanced age. Initially, 24 male, 40–60 g Swiss albino mice were used, randomly divided into 4 groups ($n = 6$) according to the treatments with hydrogel enriched with (1) AE green coffee, (2) AE roasted coffee, (3) allantoin (positive control), and (4) carbopol gel (negative control). Animals were kept in individual cages over a 14-day-long experimental period. Lesions located in the back of the animals were observed and treated daily after the initial surgical procedure to check the reepithelialization process. The wound healing and the efficacy of a treatment were evaluated by assessing the percentage of wound area reduction along the experimental period. The wound area was measured using a digital caliper at the beginning and at the end of the experimental period, calculating the injury reduction percentage. The wound area was recorded and expressed as the percentage reduction in the original wound area according to the equation:

$$\left[\left(W_{\text{cp}}\right) = \frac{\left(W_{d0} - W_{dn}\right)}{W_{d0}} * 100\right], \tag{3}$$

where W_{cp} is the wound contraction percentage (%), W_{d0} the wound area on day "0," and W_{dn} the wound area on day "*n*."

2.8.2. Evaluation of Wound Healing 2: Treatment with Hydrogels Containing Chlorogenic Acid and Green Coffee Bean AE. In a second experimental approach, the effectiveness of the topical administration of hydrogels containing chlorogenic acid or AE of green coffee was determined on the skin wound healing, using Swiss albino mice 2 months old. A total of 96 male, 40–60 g Swiss albino mice were randomly divided into 3 groups ($n = 32$); considering different sampling times (3, 7, and 15 days). Subsequently, each time group was separated into 4 groups ($n = 8$) according to the treatments with hydrogel as follows: (1) AE green coffee, (2) CGA, (3) allantoin (positive control), and (4) carbopol gel (negative control). Animals were kept in individual cages over a 15-day-long experimental period. Lesions were observed and treated daily after the initial surgical procedure to check the reepithelialization process. The skin wound was photographed using a digital camera placed in a standard distance from the lesion, immediately after surgery, and at 3rd, 7th, and 15th days. The wound healing process was evaluated by assessing the size of wound reduction in each sampling time. The wound area was measured through scanned photographic images using the software *ImageJ®*. The results were recorded and expressed as measurement of wound area. Data are presented as means of wound area (cm^2) ± mean standard error (sem).

2.9. Determination of Oxidative Stress Markers. Skin wound samples of the second experimental approach were collected for biochemical analyses following the protocols of Bagdas

et al. [37] and Pereira [38]. Skin wound samples were collected and the animals sacrificed immediately. Samples were washed in 0.9% (w/v) NaCl and immediately frozen at −80°C until use. Tissue samples were cut into pieces and homogenized (ULTRA-TURRAX homogenizer) in a 2 mL potassium phosphate buffer solution (20 mM, pH 7.4) with TWEEN®20 (1%) and NaCl (150 mM). The samples were vortexed for 10 s and centrifuged (4000 rpm, 15 min, 4°C) and the supernatants recovered for further analysis. Protein contents were determined according to the Bradford method [39]. The catalase (CAT) activity was measured spectrophotometrically (λ = 240 nm), following the method described by Pereira [38]. For the test, 25 μL of supernatant was added to 240 μL of potassium phosphate buffer (50 mM, pH 7.0), supplemented with hydrogen peroxide solution (10 mM). The decomposition rate of hydrogen peroxide was measured sequentially in intervals of 20 s, until 10 min. The results were expressed as mmol·min^{-1}/mg protein. The superoxide dismutase (SOD) activity was assessed based on the method of Giannopolitis and Ries [40]. The determination considers the enzyme's ability to inhibit the photoreduction of nitrotetrazolium blue chloride (NBT). The activity was determined adding 25 μL sample to 240 μL work solution in 96-well microplates. The work solution was prepared with 13 mM methionine, 75 μM NBT, 100 nM EDTA, and 2 mM riboflavin in potassium phosphate buffer (50 mM, pH 7.8). The reaction was started by lighting the microplate in a chamber composed of fluorescent tubes (15 W), at room temperature. After 5 min incubation, the end of catalysis was forced by the interruption of light. The content of the blue compound (formazan) formed by the photoreduction of NBT was measured spectrophotometrically at 560 nm. The same reaction was prepared and protected from the light. One unit of SOD is defined as the enzyme activity required for 50% inhibition of NBT photoreduction. To calculate the specific activity of the enzyme, it was considered as the percentage of enzymatic inhibition obtained, the sample volume, and the sample protein concentration (μg protein). The results were expressed as U/μg protein. The enzymatic experiments were realized in triplicate and the data expressed as means ± standard deviation (sd).

2.10. Statistical Analysis. Data were collected and summarized following statistical analysis using one-way ANOVA and Tukey's test. The results were considered statistically significant when $p < 0.05$. The values were expressed as mean ± sd or mean ± sem, as indicated in tables subtitles.

3. Results and Discussions

In this study, AE of residual biomasses from the green and roasted coffee oil industry were chemically characterized and further investigated to determine the effectiveness of hydrogels containing AE and chlorogenic acid on the cutaneous tissue repair process.

3.1. Total Phenolic Contents. Table 2 provides the total phenolic contents of AE of green and roasted coffee press cake showing appreciable and statistically different ($p < 0.05$) amounts of that secondary metabolite in the samples. The highest concentration of phenolic compounds was detected in the green coffee AE. Thus, one could speculate that the extraction of the apolar fraction of the green coffee beans press cake (i.e., oil fraction) allows obtaining a high yield of phenolic compounds in the AE of the residual biomass. It is well known that the phenolic content in coffee beans may vary largely according to the species, variety, degree of maturation, postharvest processing, and roasting. After the harvest and along the coffee bean processing, some phenolic compounds can be isomerized, hydrolyzed, or degraded into low molecular weight compounds. The high temperatures of coffee roasting process degrade part of the phenolic compounds [41]. Indeed, lower amounts ($p < 0.05$) of phenolic compounds were found in the AE of roasted coffee bean sample comparatively to the nonroasted one, proving the negative effect of the roasting process on the contents of those bioactive compounds.

Table 2: Contents of total phenolic compounds (mg gallic acid equivalents·g^{-1} biomass, dry weight) of the AE of green and roasted coffee beans press cake.

Samples	Total phenolic compounds (mg gallic acid equivalent·g^{-1} biomass, dry weight)
Green coffee	35.39 ± 3.69 a
Roasted coffee	24.13 ± 1.45 b

Mean of extractions in triplicate ± sem. Distinct letters denote significative difference. Tukey's test, $p < 0.05$.

3.2. Quantitation of Phenolic Acids and Alkaloids. The use of the liquid chromatography with a gradient elution system containing acidified water and methanol as mobile phase is often in the analysis of phenolic compounds [42]. However, the method adapted in this assay, for simultaneous determination of phenolics and alkaloids, allowed identifying analytes for both groups of compounds. The results revealed chlorogenic acid (11.11 mg·g^{-1}) and caffeine (4.5 mg·g^{-1}) are the majors compounds in the AE of green coffee samples (Table 3). This finding is consistent with previous results for coffee beans as mentioned by several authors [41, 43–45]. Again, considering that phenolic acids and their derivatives can be degraded under high temperatures, one could speculate that the lowest concentration of these acids in the roasted coffee press cake samples results from the thermal treatment of that biomass during the roasting process. The alkaloid contents revealed important amounts of caffeine and trigonelline in both samples, but low concentration of theophylline. The roasted coffee sample showed superior caffeine amounts comparatively to the other studied sample. Theobromine was not detected. Besides, the studied samples seemed to be quite discrepant in their alkaloid contents, suggesting different potentials regarding their biological effects. The large amount of caffeine in the coffee extracts is responsible for several biological activities [46].

3.3. Antioxidant Potential: DPPH Assay. Reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive

Table 3: Concentrations ($mg \cdot g^{-1}$ biomass, dry weight) of phenolic acids and alkaloids of the green and roasted AE of coffee beans determined by RP-HPLC analysis.

*Phenolic acid and alkaloids ($mg \cdot g^{-1}$)	Samples	
	Green coffee	Roasted coffee
	280 nm	
Chlorogenic acid	11.11 ± 0.28 a	1.95 ± 0.31 b
Syringic acid	0.96 ± 0.04 d	0.84 ± 0.08 c
Ferulic acid	1.40 ± 0.29 d	0.92 ± 0.16 c
Protocatechuic acid	1.20 ± 0.003 d	0.21 ± 0.02 d
Hydroxybenzoic acid	0.08 ± 4.1 e	0.05 ± 1.05 d
Caffeine	4.5 ± 0.06 b	5.60 ± 0.08 a
	320 nm	
Caffeic acid	0.01 ± 0.002 e	n.a.
Theophylline	0.01 ± 0.003 e	n.a.
Trigonelline	1.55 ± 0.51 c	1.02 ± 0.14 c

*Mean of injections in triplicate ± sd. Distinct letters in the column are significantly different. Tukey's test, $p < 0.05$. n.a. = not available.

Table 4: Maximum activity of DPPH radical scavenging of AE of green and roasted coffee beans press cake and Trolox (positive control).

Sample (30 $\mu g \cdot \mu L^{-1}$)	Maximum activity (%)*
AE green coffee bean press cake	96.21 ± 1.26
AE roasted coffee beans press cake	95.35 ± 2.20
Trolox	96.36 ± 0.33

*Values are shown as mean ± standard deviation.

sulfur species (RSS) might react with lipid, protein, and DNA to cause inflammation, cancer, and ischemia, for instance. DPPH is a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case of most of the free radicals. DPPH radical, which shows absorption at 517 nm, has been used as a convenient tool for the radical scavenge assay, which is independent of any enzyme activity [33]. The DPPH radical scavenging activity (%) of the AE in study, calculated after 20 min of incubation, revealed a maximum activity achieved with 30 μg AE/mL, similar to the positive control (Trolox) activity (Table 4). The quantitation of phenolic compounds and alkaloids in samples revealed the presence of secondary metabolites related to the antioxidant capacity, for example, caffeine and chlorogenic acids. It is long known that the roasting process degrades phenolic compounds; however the alkaloids are more resistant and stable, and caffeine has been shown to present expressive antioxidant activity [47]. It is important to note that the method herein used for measuring total phenolic compounds, that is, Folin-Ciocalteau, is based on a redox reaction as it can be considered an evaluation of the antioxidant activity [48]. The oxidative balance in physiological systems is regulated by endogenous and exogenous mechanisms in which the excess of free radicals is related to many diseases [49, 50]. The control of the excess of oxidative molecules includes the intake or topical application of exogenous antioxidants or even molecules that can stimulate the endogenous antioxidant [12]. Thus, the antioxidant activity of coffee bean extracts is related to the presence of several natural constituents and compounds formed during processing, for example, caffeine [51], chlorogenic and hydroxycinnamic acids [52–55], and Maillard reaction products such as melanoidins [54, 55].

3.4. Cell Viability Profile. The cell viability decrease was only detected in cells treated with the roasted coffee AE at 3 $mg \cdot mL^{-1}$, with an IC50 value of 4.88 $mg \cdot mL^{-1}$ and a LD50 = 2482.00 $mg \cdot kg^{-1}$ as showed in Figure 1. Although cell viability decreased totally at 10 $mg \cdot mL^{-1}$, the LD50 value characterized the AE as nontoxic, because the cytotoxicity is considered for LD50 values lower than 2000 $mg \cdot kg^{-1}$. Besides, green coffee AE led to no reduction in cell viability in concentrations equal to or lower than 10 mg/mL. Traditionally, *in vitro* determination of cell viability profile and toxic effect of compounds is the first biological assay to perform aiming at a further application in human health, for instance. If the toxicity of the molecule is not proved, it can proceed to the next step, *in vivo* assay. Experimentally, this has been performed by counting viable cells after staining them with a vital dye. The neutral red assay system is a means of measuring living cells through the uptake of the vital neutral red dye [56] as adopted in this study. As noted by Triglia et al. (1991), the neutral red dye passes through the intact cell membrane and becomes concentrated in lysosomes of viable cells. Test agents that damage the cell surface and lysosomal membrane inhibit the incorporation of the red dye and the amount taken up by the cells is, therefore, proportional to the number of viable ones. The neutral red assay was developed by Borenfreund and Puerner [57] and has been utilized extensively to study the toxic effect of a number of compounds on different cell types grown in monolayer cultures, as L929 cells used in this study. Though cell viability reduction showed the roasted coffee extract, all the extracts concentrations tested in this assay showed being noncytotoxic.

3.5. Effect of AE of Green and Roasted Coffee Beans Press Cake on the Wound Healing. The hydrogel containing AE of green coffee showed the best result on the wound reduction (78.20%), being similar to the positive control (70.83%), as can be noted in Figure 2. In its turn, the roasted coffee hydrogel appeared to be less effective in ameliorating the wound healing (53.71%) comparatively to the green coffee AE. A negligible reduction of the wound area was detected for the negative control (23.56%), statistically differing from the other treatments. Thus, it can be assumed that hydrogels enriched with AE of coffee beans press cake improved the wound healing process, revealing the positive effects on the cutaneous tissue regeneration. Indeed, one could argue that the superior performance of the hydrogel containing green coffee extract in the wound healing results from its highest concentration of phenolic compounds according to the phytochemical analysis. Previous *in vivo* assays in mice on the effect of coffee extracts in the skin wound healing process were not found in literature so far. In the present

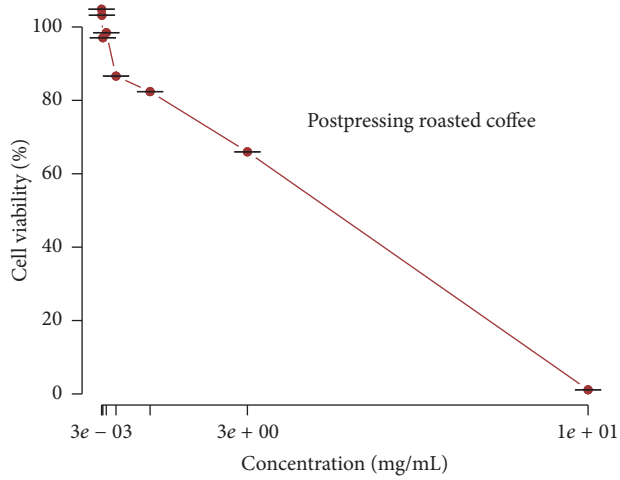

Figure 1: *In vitro* cell viability after 24 h exposure to the AE of roasted coffee press cake. Important reductions of cell viability were detected at AE concentration of 3 mg/mL or higher, as determined through the NRU assay. Results are expressed as mean ± standard deviation (sd).

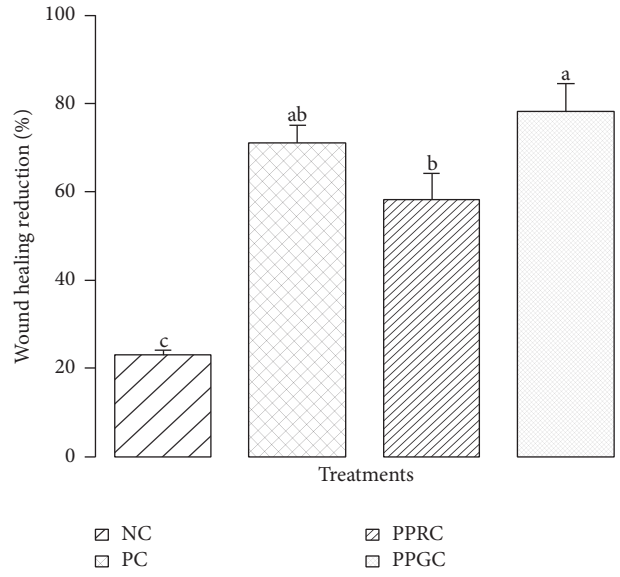

Figure 2: Reduction of the wound area (%) after 14 days of treatment with hydrogels applied daily in mice 9 months old. NC (negative control = 23.56% c), PC (positive control = 70.83% ab), PPGC (postpressing green coffee = 78.20% a), and PPRC (postpressing roasted coffee = 53.71% b). Data are presented as mean ± sem. Distinct letters denote significant differences at $p < 0.05$ (Tukey's test).

study, it is worth mentioning that by using 9-month-old adult mice, typically differing from other experimental approaches where younger animals have been used [58], the elapsing time of 14 days was not enough to achieve full wound healing, although all treatments significantly decreased the wound area comparatively to negative control. In fact, there are substantial differences in the wound healing response between young and adult subjects, generally lasting longer in adults. The adverse effects of aging on wound repair are well known and were offset by treatments that accelerated the wound closure [59, 60]. Previous study by Bagdas et al. [25] reported that systematical CGA application in skin flap surgeries can accelerate healing and flap survival in animal of wound healing delay by diabetic condition. It has been also suggested that topically CGA ointment has a potent wound healing effect on nondiabetics rats [26]. Thus, our results are consistent with previous reports in literature. In this sense, the findings are relevant since it can be assumed that even for adult individuals the treatment of skin lesions with coffee bean press cake extracts is truly efficient to increase the physiological response, ameliorating the cutaneous regeneration process significantly.

3.6. Effect of Chlorogenic Acid on the Wound Healing. The chlorogenic acid hydrogel daily applied on the skin wound appeared to reduce significantly the wound area size in the inflammatory phase (e.g., day 3) which can be associated with its antioxidant and anti-inflammatory activities. Indeed, hydrogel containing CGA resulted (0.57 cm^2 ± 0.06 sem) in a similar effect regarding the positive control (0.55 cm^2 ± 0.06 sem) as can be noted in Table 5. Contrarily, the postpressing green coffee AE assayed under the same conditions did not show the same performance (0.75 cm^2 ± 0.06 sem) at the third day, especially taking into consideration that the wound area at the beginning of the experiment was 0.72 cm^2 ± 0.02 sem. All the treatments achieved similar performance after 7 days, and 15 days was enough to achieve full wound healing. It can be assumed that the physiological responses in young mice are decisive for full wound healing considering the elapsing time of 15 days. The significant differences at the third day can be attributed to the influence of the treatments on the inflammatory response that begins immediately after injury of the tissue and lasts around 3 days. The inflammation helps wound heal with activation of immune system cellular components, the blood coagulation cascade, cytokines, and the oxidative stress [16]. Studies of Moreira et al. [9] showed that AE of green coffee has anti-inflammatory effect due to the presence of anti-inflammatory and antioxidant compounds. The use of chlorogenic acid improving skin wound healing may be derived from its influence on the inflammatory mediators involved in this response and its antioxidant capacity.

3.7. Oxidative Stress Markers. The antioxidant defense system has been developed by the organism as a protective mechanism against ROS formation. Among the most reported endogenous antioxidant systems are the activity of the enzymes SOD and CAT. Wound healing needs a fine balance between the antioxidants activities because ROS are harmful to cells and tissues in cutaneous injury [60]. It is interesting to note the significant increasing of CAT activity in the healed tissues when compared to the unhealed ones (Table 6). Besides, the CAT activity was increased on the tissue treated in all treatment groups along the wound healing process. Probably, the increase can be attributed to the higher ROS levels on the wound bed resulting from the inflammatory

Table 5: Wound area size (cm^2) in the elapsing time of 15 days, with daily application of green coffee press cake and chlorogenic acid hydrogels.

Groups ($n = 8$)	Day 0	Day 3	Day 7	Day 15
GC	0.70 ± 0.02 a	0.75 ± 0.06 ab	0.42 ± 0.04 a	0.002 ± 0.02 a
CGA	0.70 ± 0.02 a	0.57 ± 0.02 a	0.41 ± 0.02 a	0.002 ± 0.02 a
PC	0.72 ± 0.02 a	0.55 ± 0.06 a	0.31 ± 0.04 a	0.00 ± 0.01 a
NC	0.74 ± 0.02 a	0.85 ± 0.06 b	0.42 ± 0.04 a	0.002 ± 0.02 a

Data are presented as means ± sem. Distinct letters in the column denote significant differences (Tukey test, $p < 0.05$). GC = green coffee, CGA = chlorogenic acid, PC = positive control, and CN = negative control.

Table 6: Activity of catalase (CAT) and superoxide dismutase (SOD) in the epithelial tissue of Swiss albino mice according to the treatment with AE of green coffee bean press cake and chlorogenic acid related to controls groups.

Day/treatment	GC	CGA	PC	NC
		CAT ($mmol \cdot min^{-1}$/mg protein)		
Day 0*	164.1 ± 12.6	164.1 ± 12.6	164.1 ± 12.6	164.1 ± 12.6
Day 3	597.5 ± 12.4 b	360.6 ± 12.6 c	909.35 ± 5.8 a	174.7 ± 2.1 d
Day 7	260.7 ± 11.9 d	399.5 ± 19.4 c	846.6 ± 20.1 a	683.7 ± 4.1 b
Day 15	1005.0 ± 16.5 a	506.6 ± 10.7 b	408.7 ± 1.8 c	300.0 ± 12.8 d
		SOD (U/mg protein)		
Day 0*	0.45 ± 0.02	0.45 ± 0.02	0.45 ± 0.02	0.45 ± 0.02
Day 3	1.05 ± 0.08 b	1.07 ± 0.04 b	0.25 ± 0.01 c	2.33 ± 0.02 a
Day 7	0.52 ± 0.02 a	0.29 ± 0.03 b	0.26 ± 0.02 b	0.20 ± 0.03 c
Day 15	0.12 ± 0.01 b	0.17 ± 0.03 b	0.21 ± 0.02 a	0.25 ± 0.05 a

Data are presented as means ± sd. Different letters in the line denote significant differences (Tukey test, $p < 0.05$). *Day 0 = enzymatic activity on the undamaged tissue. GC = green coffee, CGA = chlorogenic acid, PC = positive control, and CN = negative control.

response, cellular signaling pathways to avoid the infection, recruiting different kinds of cells, and promoting cells divisions. The increase in the CAT enzymatic activity upon the green coffee AE and hydrogel containing CGA treatments appeared to be increased on day 3 (inflammation stage), following a reduction at the 7th day during the cell proliferation stage, with a strong augment again until day 15. Interestingly, the positive control group also showed a similar profile of CAT activity, except at the end of the experimental period, on day 15. Importantly, regardless of the treatment the results revealed a superior CAT activity along the wound healing process in comparison to the basal level (day 0), suggesting that CAT activity is positively modulated in damaged cutaneous tissues. Finally, meaningful differences ($p < 0.05$) were detected for each sampling time in all treatments, suggesting that a typical standard of CAT activity seems not to occur.

The SOD assay showed higher activity in the early stage of healing (day 3) for the green coffee AE- and CGA-treated groups, followed by a gradual reduction. Furthermore, contrary to CAT activity, SOD decreased bellow the baseline in all experimental groups at the end of the experiment, suggesting that such an enzymatic response to the oxidative stress seems not to be relevant to the cutaneous cells upon regeneration.

4. Conclusions

The chemical analysis revealed the green coffee is a raw material richer in chlorogenic acid than the roasted one. The present study shows that the daily topical application of chlorogenic acid and coffee beans press cake AE enhanced the skin wound healing in mice model, especially the later one that reduced more quickly the wound area. Taking into consideration that the experiments were performed with plant extracts, that is, chemically complex matrices, it is assumed that the effects herein described eventually result from the synergistic action of their components. We suggest that the highest amount of CGA and caffeine in the extracts of coffee press cake presents beneficial effects on wound repair by its antioxidant potential, enhancing the physiological responses to achieve the wound closure. The formation of reactive oxygen radical species (ROS) can play an important role in delayed wound healing. Antioxidants can help controlling wound oxidative stress related to ROS generation and thus accelerate wound repair.

Finally, to the best of our knowledge, this is the first study investigating the effect of AE of coffee beans press cake on the skin wound healing, showing beneficial effects on wound repair. Antioxidant and radical scavenger activities of coffee beans press cake AE and CGA can improve wound healing to control overexposure of oxidative stress in the wound bed. The coffee beans press cake has shown a lower commercial value; however it might gain value as thought of as a valuable biomass source of bioactive compounds interesting for the human health usage. Indeed, the residual coffee biomasses studied are able to improve the regeneration of damage skin tissue, allowing new product development in the cosmetical and pharmaceutical industries.

Competing Interests

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

Acknowledgments

The authors are thankful to FAPESC (Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina, Brazil) and Cooxupé (Coffee Growers Cooperative) for financial support. The research fellowship from CNPq on behalf of the later author is acknowledged.

References

[1] R. Quek and C. J. Henry, "Influence of polyphenols from lingonberry, cranberry, and red grape on in vitro digestibility of rice," *International Journal of Food Sciences and Nutrition*, vol. 66, no. 4, pp. 378–382, 2015.

[2] G. Laufenberg, B. Kunz, and M. Nystroem, "Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementations," *Bioresource Technology*, vol. 87, no. 2, pp. 167–198, 2003.

[3] V. Brezová, A. Šlebodová, and A. Staško, "Coffee as a source of antioxidants: an EPR study," *Food Chemistry*, vol. 114, no. 3, pp. 859–868, 2009.

[4] M. N. Clifford, "Chlorogenic acids," in *Coffee Chemistry*, R. J. Clarke and R. Macrae, Eds., pp. 153–202, Elsevier Applied Science Publication, London, UK, 1985.

[5] L. C. Trugo and R. Macrae, "A study of the effect of roasting on the chlorogenic acid composition of coffee using HPLC," *Food Chemistry*, vol. 15, no. 3, pp. 219–227, 1984.

[6] A. Ratz-Yko, J. Arct, S. Majewski, and K. Pytkowska, "Influence of polyphenols on the physiological processes in the skin," *Phytotherapy Research*, vol. 29, no. 4, pp. 509–517, 2015.

[7] A. A. P. Almeida, A. Farah, D. A. M. Silva, E. A. Nunan, and M. B. A. Glória, "Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 23, pp. 8738–8743, 2006.

[8] H. Utsunomiya, M. Ichinose, M. Uozaki, K. Tsujimoto, H. Yamasaki, and A. H. Koyama, "Antiviral activities of coffee extracts in vitro," *Food and Chemical Toxicology*, vol. 46, no. 6, pp. 1919–1924, 2008.

[9] D. P. Moreira, M. C. Monteiro, M. Ribeiro-Alves, C. M. Donangelo, and L. C. Trugo, "Contribution of chlorogenic acids to the iron-reducing activity of coffee beverages," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 5, pp. 1399–1402, 2005.

[10] M. D. dos Santos, M. C. Almeida, N. P. Lopes, and G. E. P. de Souza, "Evaluation of the anti-inflammatory, analgesic and antipyretic activities of the natural polyphenol chlorogenic acid," *Biological and Pharmaceutical Bulletin*, vol. 29, no. 11, pp. 2236–2240, 2006.

[11] H.-M. Chiang, T.-J. Lin, C.-Y. Chiu et al., "Coffea arabica extract and its constituents prevent photoaging by suppressing MMPs expression and MAP kinase pathway," *Food and Chemical Toxicology*, vol. 49, no. 1, pp. 309–318, 2011.

[12] C. Hoelzl, S. Knasmüller, K.-H. Wagner et al., "Instant coffee with high chlorogenic acid levels protects humans against oxidative damage of macromolecules," *Molecular Nutrition and Food Research*, vol. 54, no. 12, pp. 1722–1733, 2010.

[13] C. I. Heck and E. G. De Mejia, "Yerba mate tea (Ilex paraguariensis): a comprehensive review on chemistry, health implications, and technological considerations," *Journal of Food Science*, vol. 72, no. 9, pp. R138–R151, 2007.

[14] A. Farah, M. C. Monteiro, V. Calado, A. S. Franca, and L. C. Trugo, "Correlation between cup quality and chemical attributes of Brazilian coffee," *Food Chemistry*, vol. 98, no. 2, pp. 373–380, 2006.

[15] C. L. Baum and C. J. Arpey, "Normal cutaneous wound healing: clinical correlation with cellular and molecular events," *Dermatologic Surgery*, vol. 31, no. 6, pp. 674–686, 2005.

[16] G. C. Gurtner, S. Werner, Y. Barrandon, and M. T. Longaker, "Wound repair and regeneration," *Nature*, vol. 453, no. 7193, pp. 314–321, 2008.

[17] P. Farris, "Idebenone, green tea, and Coffeeberry® extract: new and innovative antioxidants," *Dermatologic Therapy*, vol. 20, no. 5, pp. 322–329, 2007.

[18] A. Gosain and L. A. DiPietro, "Aging and wound healing," *World Journal of Surgery*, vol. 28, no. 3, pp. 321–326, 2004.

[19] K. T. Keylock, V. J. Vieira, M. A. Wallig, L. A. DiPietro, M. Schrementi, and J. A. Woods, "Exercise accelerates cutaneous wound healing and decreases wound inflammation in aged mice," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 294, no. 1, pp. R179–R184, 2008.

[20] M. E. Swift, A. L. Burns, K. L. Gray, and L. A. DiPietro, "Age-related alterations in the inflammatory response to dermal injury," *Journal of Investigative Dermatology*, vol. 117, no. 5, pp. 1027–1035, 2001.

[21] M. E. Swift, H. K. Kleinman, and L. A. DiPietro, "Impaired wound repair and delayed angiogenesis in aged mice," *Laboratory Investigation*, vol. 79, no. 12, pp. 1479–1487, 1999.

[22] A. Nkondjock, "Coffee consumption and the risk of cancer: an overview," *Cancer Letters*, vol. 277, no. 2, pp. 121–125, 2009.

[23] S. D. Fitzmaurice, R. K. Sivamani, and R. R. Isseroff, "Antioxidant therapies for wound healing: a clinical guide to currently commercially available products," *Skin Pharmacology and Physiology*, vol. 24, no. 3, pp. 113–126, 2011.

[24] J.-Y. Bae, J.-S. Choi, Y.-J. Choi et al., "(-)Epigallocatechin gallate hampers collagen destruction and collagenase activation in ultraviolet-B-irradiated human dermal fibroblasts: involvement of mitogen-activated protein kinase," *Food and Chemical Toxicology*, vol. 46, no. 4, pp. 1298–1307, 2008.

[25] D. Bagdas, N. Cinkilic, H. Y. Ozboluk, M. O. Ozyigit, and M. S. Gurun, "Antihyperalgesic activity of chlorogenic acid in experimental neuropathic pain," *Journal of Natural Medicines*, vol. 67, no. 4, pp. 698–704, 2013.

[26] J. Bravo, L. Arbillaga, M. P. de Peña, and C. Cid, "Antioxidant and genoprotective effects of spent coffee extracts in human cells," *Food and Chemical Toxicology*, vol. 60, pp. 397–403, 2013.

[27] N. Cinkilic, S. K. Cetintas, T. Zorlu et al., "Radioprotection by two phenolic compounds: chlorogenic and quinic acid, on X-ray induced DNA damage in human blood lymphocytes in vitro," *Food and Chemical Toxicology*, vol. 53, pp. 359–363, 2013.

[28] A. T. Shimoyama, J. R. Santin, I. D. Machado et al., "Antiulcerogenic activity of chlorogenic acid in different models of gastric ulcer," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 386, no. 1, pp. 5–14, 2013.

[29] N. Yun, J.-W. Kang, and S.-M. Lee, "Protective effects of chlorogenic acid against ischemia/reperfusion injury in rat liver: molecular evidence of its antioxidant and anti-inflammatory

properties," *The Journal of Nutritional Biochemistry*, vol. 23, no. 10, pp. 1249–1255, 2012.

[30] ANVISA, Agência Nacional de Vigilância Sanitária. Farmacopéia Brasileira, 2010, http://www.anvisa.gov.br/hotsite/cd_farmacopeia/index.htm.

[31] R. Randhir, P. Shetty, and K. Shetty, "L-DOPA and total phenolic stimulation in dark germinated fava bean in response to peptide and phytochemical elicitors," *Process Biochemistry*, vol. 37, no. 11, pp. 1247–1256, 2002.

[32] N. P. Rodrigues and N. Bragagnolo, "Identification and quantification of bioactive compounds in coffee brews by HPLC–DAD–MS^n," *Journal of Food Composition and Analysis*, vol. 32, no. 2, pp. 105–115, 2013.

[33] P. Molyneux, "The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity," *Songklanakarin Journal and Science Technology*, vol. 26, no. 2, pp. 211–219, 2004.

[34] ICCVAM. Inter-Agency Coordinating Committee on the Validation of Alternative Methods, *In Vitro Methods for Assessing Acute Systemic Toxicity*, 2001, http://iccvam.niehs.nih.gov/docs/FR/6649686.htm.

[35] S. Frank and H. Kämpfer, "Excisional wound healing: an experimental approach," in *Wound Healing: Methods and Protocols*, L. A. DiPietro and A. L. Burns, Eds., vol. 78 of *Methods in Molecular Medicine*™, pp. 3–15, Humana Press, Totowa, NJ, USA, 2003.

[36] J. R. C. Pereira, "Tratamento de cicatrizes patológicas com a associação de cepalin, heparina e alantoína," *Revista Brasileira de Medicina*, vol. 60, pp. 603–608, 2003.

[37] D. Bagdas, B. C. Etoz, Z. Gul et al., "In vivo systemic chlorogenic acid therapy under diabetic conditions: wound healing effects and cytotoxicity/genotoxicity profile," *Food and Chemical Toxicology*, vol. 81, pp. 54–61, 2015.

[38] A. Pereira, *Avaliação das atividades cicatrizante e antitumoral de extratos provenientes da casca de banana cultivar Prata Anã (Musa spp) [Dissertação]*, Universidade Federal de Santa Catarina, Florianópolis, Brazil, 2010.

[39] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[40] C. N. Giannopolitis and S. K. Ries, "Superoxide dismutases: I. occurrence in higher plants," *Plant Physiology*, vol. 59, no. 2, pp. 309–314, 1977.

[41] A. Farah and C. M. Donangelo, "Phenolic compounds in coffee," *Brazilian Journal of Plant Physiology*, vol. 18, no. 1, pp. 23–36, 2006.

[42] A. Farah, T. de Paulis, L. C. Trugo, and P. R. Martin, "Effect of roasting on the formation of chlorogenic acid lactones in coffee," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 5, pp. 1505–1513, 2005.

[43] M. W. Cheong, K. H. Tong, J. J. M. Ong, S. Q. Liu, P. Curran, and B. Yu, "Volatile composition and antioxidant capacity of Arabica coffee," *Food Research International*, vol. 51, no. 1, pp. 388–396, 2013.

[44] M. C. Monteiro and A. Farah, "Chlorogenic acids in Brazilian *Coffea arabica* cultivars from various consecutive crops," *Food Chemistry*, vol. 134, no. 1, pp. 611–614, 2012.

[45] M. E. D. C. Moreira, R. G. F. A. Pereira, D. F. Dias et al., "Anti-inflammatory effect of aqueous extracts of roasted and green *Coffea arabica* L.," *Journal of Functional Foods*, vol. 5, no. 1, pp. 466–474, 2013.

[46] P. Esquivel and V. M. Jiménez, "Functional properties of coffee and coffee by-products," *Food Research International*, vol. 46, no. 2, pp. 488–495, 2012.

[47] J. A. Vignoli, D. G. Bassoli, and M. T. Benassi, "Antioxidant activity, polyphenols, caffeine and melanoidins in soluble coffee: the influence of processing conditions and raw material," *Food Chemistry*, vol. 124, no. 3, pp. 863–868, 2011.

[48] R. L. Prior, X. Wu, and K. Schaich, "Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 10, pp. 4290–4302, 2005.

[49] J. Bouayed and T. Bohn, "Exogenous antioxidants—double-edged swords in cellular redox state: health beneficial effects at physiologic doses versus deleterious effects at high doses," *Oxidative Medicine and Cellular Longevity*, vol. 3, no. 4, pp. 228–237, 2010.

[50] L. Su, J.-J. Yin, D. Charles, K. Zhou, J. Moore, and L. Yu, "Total phenolic contents, chelating capacities, and radical-scavenging properties of black peppercorn, nutmeg, rosehip, cinnamon and oregano leaf," *Food Chemistry*, vol. 100, no. 3, pp. 990–997, 2007.

[51] T. P. A. Devasagayam, J. P. Kamat, H. Mohan, and P. C. Kesavan, "Caffeine as an antioxidant: inhibition of lipid peroxidation induced by reactive oxygen species," *Biochimica et Biophysica Acta*, vol. 1282, no. 1, pp. 63–70, 1996.

[52] C. Gallardo, L. Jiménez, and M.-T. García-Conesa, "Hydroxycinnamic acid composition and in vitro antioxidant activity of selected grain fractions," *Food Chemistry*, vol. 99, no. 3, pp. 455–463, 2006.

[53] J. Á. Gómez-Ruiz, D. S. Leake, and J. M. Ames, "In vitro antioxidant activity of coffee compounds and their metabolites," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 17, pp. 6962–6969, 2007.

[54] R. C. Borrelli, A. Visconti, C. Mennella, M. Anese, and V. Fogliano, "Chemical characterization and antioxidant properties of coffee melanoidins," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 22, pp. 6527–6533, 2002.

[55] C. Delgado-Andrade, J. A. Rufián-Henares, and F. J. Morales, "Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 20, pp. 7832–7836, 2005.

[56] D. Triglia, S. S. Braa, C. Yonan, and G. K. Naughton, "In vitro toxicity of various classes of test agents using the neutral red assay on a human three-dimensional physiologic skin model," *In Vitro Cellular & Developmental Biology—Animal*, vol. 27, no. 3, pp. 239–244, 1991.

[57] E. Borenfreund and J. A. Puerner, "Toxicity determined in vitro by morphological alterations and neutral red absorption," *Toxicology Letters*, vol. 24, no. 2-3, pp. 119–124, 1985.

[58] R. D. Galiano, J. Michaels V, M. Dobryansky, J. P. Levine, and G. C. Gurtner, "Quantitative and reproducible murine model of excisional wound healing," *Wound Repair and Regeneration*, vol. 12, no. 4, pp. 485–492, 2004.

[59] S. Guo and L. A. Di Pietro, "Factors affecting wound healing," *Journal of Dental Research*, vol. 89, no. 3, pp. 219–229, 2010.

[60] S. Suzuki, Y. Miyachi, Y. Niwa, and N. Isshiki, "Significance of reactive oxygen species in distal flap necrosis and its salvage with liposomal SOD," *British Journal of Plastic Surgery*, vol. 42, no. 5, pp. 559–564, 1989.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 4856761, 10 pages
http://dx.doi.org/10.1155/2016/4856761

Research Article

Astragalus Polysaccharide Suppresses 6-Hydroxydopamine-Induced Neurotoxicity in *Caenorhabditis elegans*

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Received 17 June 2016; Revised 19 September 2016; Accepted 13 October 2016

Academic Editor: Renata Szymanska

Astragalus membranaceus is a medicinal plant traditionally used in China for a variety of conditions, including inflammatory and neural diseases. *Astragalus* polysaccharides are shown to reduce the adverse effect of levodopa which is used to treat Parkinson's disease (PD). However, the neuroprotective effect of *Astragalus* polysaccharides *per se* in PD is lacking. Using *Caenorhabditis elegans* models, we investigated the protective effect of astragalan, an acidic polysaccharide isolated from *A. membranaceus*, against the neurotoxicity of 6-hydroxydopamine (6-OHDA), a neurotoxin that can induce parkinsonism. We show that 6-OHDA is able to degenerate dopaminergic neurons and lead to the deficiency of food-sensing behavior and a shorter lifespan in *C. elegans*. Interestingly, these degenerative symptoms can be attenuated by astragalan treatment. Astragalan is also shown to alleviate oxidative stress through reducing reactive oxygen species level and malondialdehyde content and increasing superoxide dismutase and glutathione peroxidase activities and reduce the expression of proapoptotic gene *egl-1* in 6-OHDA-intoxicated nematodes. Further studies reveal that astragalan is capable of elevating the decreased acetylcholinesterase activity induced by 6-OHDA. Together, our results demonstrate that the protective effect of astragalan against 6-OHDA neurotoxicity is likely due to the alleviation of oxidative stress and regulation of apoptosis pathway and cholinergic system and thus provide an important insight into the therapeutic potential of *Astragalus* polysaccharide in neurodegeneration.

1. Introduction

Parkinson's disease (PD) is one of the most prevalent neurodegenerative disorders characterized by progressive loss of dopaminergic neurons in substantia nigra of the midbrain. The dopaminergic neurodegeneration results in severe dopamine depletion and thus leads to a variety of motor complications, including muscle rigidity, tremors, bradykinesia, and postural instability [1]. In the advanced stages of PD, the emerged cognitive and mental problems, such as dementia, depression, and anxiety, further reduce life quality and increase cost burden [2]. Evidences from clinical and epidemiological investigations have demonstrated that the onset and progression of PD is closely associated with aging process. For instance, PD affects approximately 7–10 million people worldwide, and its annualized incidence rate dramatically increases from 0.041% in individuals of 40–49 years old to 0.428%–1.903% in people above 60 years old [3, 4]. Given the detrimental consequences of PD, development of promising therapeutics represents an urgent social and medical need.

A number of studies have revealed that the cause of PD correlates with certain genetic and environmental risk factors, such as mutations in specific genes and toxin exposure [5]. For example, 6-hydroxydopamine (6-OHDA), a toxic

oxidation metabolite of dopamine, is detected in the brains and urine of patients with PD [6]. This neurotoxin can selectively enter dopaminergic neurons through dopamine or noradrenaline transporter (DAT and NAT, resp.) and then stimulate overproduction of reactive oxygen species (ROS) via enzymatic oxidation and autooxidation [7]. Increased ROS level elicited by 6-OHDA damages cellular proteins and nucleus, leading to dopaminergic neuron death and dysfunction in PD patients [7]. In experimental models, the administration of 6-OHDA is also able to induce a variety of physiological events including neuronal death and behavioral deficit similar to PD.

Caenorhabditis elegans is a relatively simple but powerful animal model in neurobiology field [8]. It has a well-characterized nervous system consisting of 302 neurons, and the neuronal signalling processes such as neurotransmitter formation and release are well conserved. Moreover, exogenous neurotoxin-induced alterations in *C. elegans* are also similar with those in more complex systems. For example, 6-OHDA is capable of degenerating dopaminergic neurons via DAT in both human and *C. elegans* [9]. In addition, genetic manipulation with fluorescent reporter allows for visual inspection of neuronal viability and behavioral performance in the nematodes.

Astragalus membranaceus is a well-known Chinese medicine with antiaging functions recorded in Shen Nong's Classic of Materia Medica (~110 BC). It is widely used against mental and emotional stress, anorexia, fatigue, and general weakness [10]. A number of studies have revealed that the neuroprotective effect of *A. membranaceus* is associated with its polysaccharide fraction. For instance, we have recently found that astragalan, an acidic polysaccharide isolated from *A. membranaceus*, is able to reduce the neurotoxicity of polyglutamine, the critical pathogenic protein in Huntington's disease [11]. *Astragalus* polysaccharide is also shown to protect rat astrocyte cultures against oxidative damage induced by levodopa, which is widely used in PD treatment but shows adverse effects [12], suggesting a neuroprotective potential of the polysaccharide. However, studies on the underpinning mechanisms of neuroprotection of *Astragalus* polysaccharides in PD models are lacking. Therefore, we investigated the protective effect of the *Astragalus* polysaccharide astragalan against 6-OHDA neurotoxicity using *C. elegans* models and attempted to unravel the underlying mechanisms, including its effects on lifespan, ROS and lipid peroxidation levels, antioxidant enzyme activities, apoptosis-related gene expression, and acetylcholinesterase activity.

2. Materials and Methods

2.1. Preparation of Polysaccharides. The dry roots of *Astragalus membranaceus* (Fisch.) Bunge were purchased from Tongrentang Group (Bozhou, China). The polysaccharide astragalan was prepared essentially as described previously [11]. Briefly, the sliced roots were refluxed in ethanol, and the materials were used for isolation of *Astragalus* polysaccharides. The polysaccharides were then fractionated by anion-exchange chromatography on a DEAE-Sepharose Fast Flow (GE Healthcare, Uppsala, Sweden) column eluted with water followed by 0.5 M NaCl solution, and the yield of the acidic polysaccharide obtained from NaCl eluate was 1.56%. Further fractionation of the acidic polysaccharide by gel filtration on a Sepharose 6 Fast Flow column exhibited a distinct single peak (data not shown), indicating the homogeneity of the polysaccharide. Thus, the acidic polysaccharide was used as astragalan in the following experiments. The molecular weight of astragalan was measured using high-performance gel permeation chromatography [13], and its number-average molecular weight (Mn) and weight-average molecular weight (Mw) were 427,906 Da and 482,372 Da, respectively. The polydispersity, a ratio of Mw to Mn, of astragalan was ~1.1, indicating a relatively narrow molecular weight distribution of astragalan. Glycosyl composition of astragalan was measured by gas chromatography after methanolysis [14], and the molar composition of monosaccharides in astragalan was 19.2% arabinose, 6.9% rhamnose, 16.0% galactose, 28.8% glucose, and 29.1% galacturonic acid.

2.2. Nematode and Bacterial Strains. All *Caenorhabditis elegans* and *Escherichia coli* strains were obtained from the *Caenorhabditis* Genetics Centre (University of Minnesota, Minneapolis, MN, USA). The BZ555 (*Pdat-1*:GFP) nematodes were maintained at 20°C on NGM agar plates with *E. coli* OP50 as food. Synchronization was performed using the standard alkaline hypochlorite method as described [15].

2.3. 6-OHDA Exposure and Astragalan Treatment. 6-OHDA (Sigma, St. Louis, MO, USA) was used to induce selective degeneration of dopaminergic neurons in BZ555 nematodes as described previously [16]. Briefly, synchronized L3 larvae were incubated with 50 mM 6-OHDA and 10 mM ascorbic acid in S. medium supplemented with *E. coli* NA22 for 1 h at 20°C. After treatment, the nematodes were washed three times with M9 buffer and then incubated with astragalan at the indicated concentrations in S. medium containing *E. coli* NA22 for 24 h. Then 75 μg/mL 5-fluoro-2′-deoxyuridine was added to inhibit reproduction. After further incubation for 48 h, the nematodes were used for various assays. In dopaminergic neurodegeneration, food-sensing behavior, and survival assays, epigallocatechin-3-gallate (EGCG) (final concentration of 0.05 mM) was used as a positive control.

2.4. Dopaminergic Neurodegeneration Assay. The nematode dopaminergic neurodegeneration was examined as described previously [16]. In brief, after treatment with 6-OHDA and astragalan, the nematodes were washed with M9 buffer, paralyzed by 50 mM sodium azide, and then mounted onto a 2% agar pad on a glass slide. About 30 nematodes were randomly selected in each treatment, and the GFP fluorescence was imaged using an IX51 inverted fluorescence microscope (Olympus, Tokyo, Japan). The fluorescence intensity in head region was estimated using Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

2.5. Food-Sensing Behavior Assay. The food-sensing behavior was carried out as described [16]. Briefly, a 9 cm NGM agar plate was spread with *E. coli* OP50 overnight at 37°C in a ring

with an inner diameter of 1 cm and an outer diameter of 8 cm. After treatment with 6-OHDA and astragalan, the nematodes were washed with M9 buffer and transferred to the center of the NGM agar plate with or without bacterial lawn in a drop of M9 buffer, allowed to sit for 5 min, and then the number of body bends of each nematode was counted microscopically for 1 min. The slowing rate was calculated as follows: slowing rate = $(N_{\text{control}} - N_{\text{food}})/N_{\text{control}}$, where N_{food} and N_{control} represent the numbers of body bends in the plates with and without bacteria lawn, respectively. At least 15 nematodes were scored in each treatment.

2.6. Survival Assay. The survival of *C. elegans* was performed using liquid culture at 20°C as described previously [11]. In brief, the nematodes were treated with 6-OHDA and astragalan as above and further incubated in S. medium containing *E. coli* NA22 (initial OD_{570} of ~0.6) and 100 μg/mL ampicillin for 24 h. The nematodes were then transferred into 96-well plates (about 10 nematodes per well). The day of nematode transfer was set as Day 0 in this assay. The numbers of live and dead nematodes were counted microscopically every 2 days based on their movements.

2.7. Determination of ROS Level. The ROS level in *C. elegans* was determined using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Sigma, St. Louis, MO, USA) as previously described [17]. Briefly, approximately 1000 nematodes treated with 6-OHDA and astragalan were collected, washed, and homogenized in 350 μL of PBS (50 mM, pH 7.8) with 0.1% Tween 20 on ice. The supernatant was collected by centrifugation, and its protein content was determined by BCA assay kit (Thermo Fisher, Waltham, MA, USA). Then 50 μL of the supernatant was transferred into a black 96-well plate and incubated with 50 μL of 100 μM DCFH-DA. The DCF fluorescence was determined in a Fluoroskan Ascent FL microplate reader (Thermo, Waltham, MA, USA) every 10 min for 2 h at an excitation of 485 nm and an emission of 535 nm. The ROS level was calculated as the DCF fluorescence per μg proteins.

2.8. Determination of Antioxidant Enzyme Activity and Malondialdehyde Content. The antioxidant enzyme activities and malondialdehyde (MDA) content were determined as previously described [17]. Briefly, about 2,000 nematodes treated with 6-OHDA and astragalan were collected, washed with M9 buffer, and homogenized in 150 μL of PBS (50 mM, pH 7.8) with 1% Triton X-100 and 1 mM PMSF on ice. The supernatant was collected by centrifugation and then used to determine superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione peroxidase (GPx) activity, and MDA content by the assay kits (Beyotime, Shanghai, China). The protein content of the supernatant was determined by BCA assay kit. The SOD and GPx activities were expressed as U/mg proteins, CAT activity was expressed as U/μg proteins, and MDA content was expressed as nM/mg proteins.

2.9. Quantitative Real-Time PCR. Total RNA of the nematodes with or without 2.0 mg/mL of astragalan treatment was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After reverse transcription, relative quantification of cDNA by real-time PCR was performed using SYBR Green I (BioRad, Hercules, CA, USA) and MyiQ™2 real-time detection system (BioRad, Hercules, CA, USA) as described previously [11]. Data were normalized to BZ555 nematodes without 6-OHDA exposure and astragalan treatment using the geometric mean of *cdc-42*, *pmp-3,* and *Y45F10D.4* as the reference genes. The primers for PCR analysis are listed in Table 1.

2.10. Determination of Acetylcholinesterase Activity. The acetylcholinesterase (AChE) activity of *C. elegans* was determined as previously described [18]. Briefly, approximately 3,000 nematodes with or without 2.0 mg/mL of astragalan treatment were washed twice with M9 buffer and then homogenized in PBS (50 mM, pH 7.8) on ice. The lysate supernatant was collected by centrifugation and then used for determination of AChE activity by the assay kit (Jiancheng Institute of Biotechnology, Nanjing, China). The protein content was determined by BCA assay kit. The AChE activity was expressed as U/mg proteins.

2.11. Statistical Analysis. The statistical analysis was performed primarily by GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was determined by Student's *t*-test or one-way ANOVA followed by Tukey's *post hoc* test. The survival data were analyzed by Kaplan-Meier method and Peto's log-rank test using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). A probability value of $p < 0.05$ was considered to be statistically significant. All experiments were performed at least three times.

3. Results and Discussion

3.1. Astragalan Alleviates 6-OHDA-Induced Neurodegeneration in C. elegans. It is well characterized that *C. elegans* contains eight dopaminergic neurons, including two anterior deirid (ADE) neurons and four cephalic (CEP) neurons in the head and two posterior deirid (PDE) neurons in the posterior lateral position [19]. The transgenic *C. elegans* strain BZ555 constitutively expresses GFP in dopaminergic neurons, and the GFP fluorescence intensity indicates the neuronal viability. Previous studies have shown that 6-OHDA exposure reduces the GFP fluorescence markedly in ADE and CEP neurons and weakly in other dopaminergic neurons of *C. elegans* [20]. Therefore, we used this transgenic model to investigate whether the *Astragalus* polysaccharide astragalan was able to inhibit 6-OHDA-mediated neurodegeneration. As shown in Figure 1, in 6-OHDA-exposed BZ555 nematodes, the GFP fluorescence intensity of ADE and CEP neurons was decreased to 46.8% of the unexposed nematodes ($p < 0.05$), demonstrating the capability of 6-OHDA to impair dopaminergic neurons. When the 6-OHDA-intoxicated nematodes were treated with astragalan at the indicated concentrations (1.0, 2.0, or 4.0 mg/mL), the GFP fluorescence intensities were increased to 77.1%, 84.6%, and 82.9%, respectively ($p < 0.05$),

Table 1: List of primers used for quantitative real-time PCR.

	Forward ($5' \rightarrow 3'$)	Reverse ($5' \rightarrow 3'$)
	Apoptosis-related genes	
egl-1	CTAGCAGCAATGTGCGATGAC	GGAAGCATGGGCCGAGTAG
ced-9	TGCTCAGGACTTGCCATCAC	TTGACTCTCCGATGGACATTCTT
ced-4	AAGTCGAGGATTAGTCGGTGTTG	AGAGCCATTGCGAGTGACTTG
ced-3	TCAACGCGGCAAATGCT	GCCTGCACAAAAACGATTTTC
	Reference genes	
cdc-42	CTGCTGGACAGGAAGATTACG	CTCGGACATTCTCGAATGAAG
pmp-3	GTTCCCGTGTTCATCACTCAT	ACACCGTCGAGAAGCTGTAGA
Y45F10D.4	GTCGCTTCAAATCAGTTCAGC	GTTCTTGTCAAGTGATCCGACA

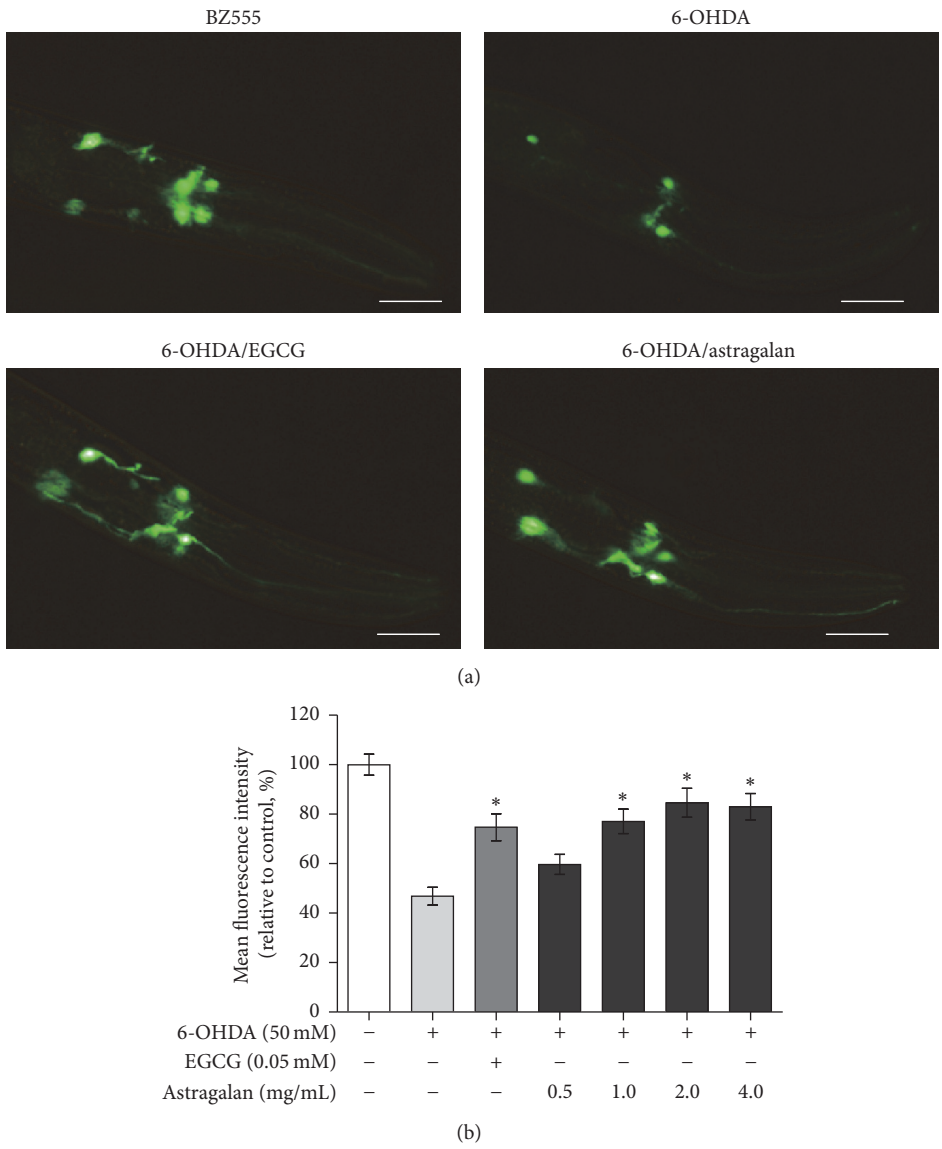

Figure 1: Effect of astragalan on 6-OHDA-induced degeneration of dopaminergic neurons in *C. elegans*. (a) Representative fluorescence images of *Pdat-1*::GFP in dopaminergic neurons were taken from the transgenic *C. elegans* strain BZ555. The nematodes were exposed to 50 mM 6-OHDA for 1 h prior to treatment with astragalan (2.0 mg/mL) or EGCG (0.05 mM). Scale bars, 20 μm. (b) Graphical presentation for fluorescence intensity of *Pdat-1*::GFP in dopaminergic neurons of the nematodes treated with or without astragalan at the indicated concentrations (0.5–4.0 mg/mL). Results are presented as mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus 6-OHDA-exposed nematodes.

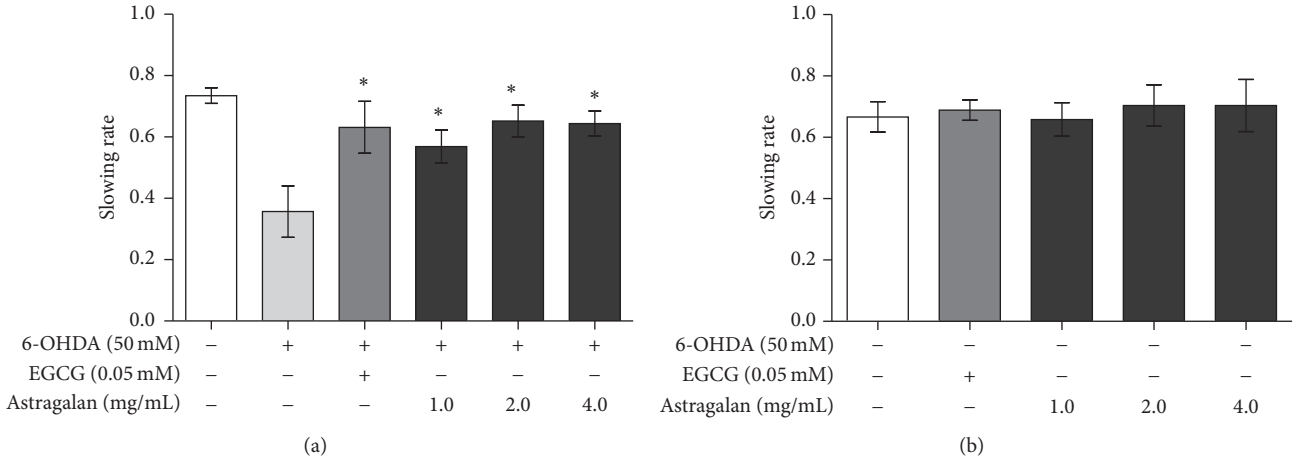

Figure 2: Effect of astragalan on food-sensing behavior in *C. elegans*. (a) The body bends of BZ555 nematodes treated with or without astragalan (1.0–4.0 mg/mL) after 6-OHDA exposure were counted, and the slowing rate was defined as the rate decrease of body bends in the plates with bacteria lawn compared with those without bacteria lawn. (b) The body bends of BZ555 nematodes treated with or without astragalan (1.0–4.0 mg/mL) were counted and used to calculate the slowing rate. Results are presented as mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus 6-OHDA-exposed nematodes.

which are comparable with the action of the positive control EGCG (74.7% at 0.05 mM), a green tea polyphenol reported to prevent 6-OHDA neurotoxicity [21]. These results demonstrate that astragalan is capable of alleviating 6-OHDA-mediated dopaminergic neurodegeneration in *C. elegans* model.

3.2. Astragalan Attenuates 6-OHDA-Induced Food-Sensing Deficit in C. elegans. Under normal conditions, *C. elegans* moves slowly to sense and consume food. Since the dopaminemediated neural circuit regulates the basal slowing response of *C. elegans* to food [16], a reduced dopamine level caused by the dopaminergic neurodegeneration makes the nematodes defective in this food-sensing behavior. Since astragalan is able to suppress 6-OHDA-induced dopaminergic neurodegeneration as shown above, we tested whether it could rescue the deficit of food-sensing performance under 6-OHDA exposure. As shown in Figure 2(a), the slowing rate of BZ555 nematodes was 0.73, while 6-OHDA exposure significantly decreased the slowing rate to 0.35 ($p < 0.05$), indicating a deficit of food-sensing behavior due to dopaminergic neuronal dysfunction. When treated with astragalan at the indicated concentrations (1.0, 2.0, or 4.0 mg/mL), the slowing rate of 6-OHDA-intoxicated nematodes was significantly increased to 0.57, 0.66, and 0.64, respectively ($p < 0.05$). As a control, treatment with EGCG also increased the slowing rate to 0.63 under 6-OHDA exposure. However, treatment with astragalan (1.0–4.0 mg/mL) alone had almost no effect on the slowing rate of BZ555 nematodes (Figure 2(b)), indicating that astragalan *per se* did not affect the viability of dopaminergic neurons under normal condition. Together, these data demonstrate that astragalan is able to rescue dopaminergic neuronal dysfunction mediated by 6-OHDA.

It is known that *Astragalus* polysaccharide has antitumor effect against human tumor cells [22]. Interestingly, however, studies have also shown that *Astragalus* polysaccharide has protective effects on neuronal cells [11, 12]. This paradox is likely due to the distinct but also overlapping mechanisms regulated by the polysaccharide under different disease and stress contexts. The anticancer effect of *Astragalus* polysaccharide is most likely due to its immunomodulatory effect rather than direct cytocidal action [23, 24]. For instance, *Astragalus* polysaccharide has shown therapeutic effect by promoting the secretion of proinflammatory cytokines including interleukin and tumor necrosis factor in S180 sarcoma-bearing mice [23]. On the other hand, the polysaccharide can also protect microglial cells against lipopolysaccharide-stimulated inflammation through suppression of nuclear factor-κB and protein kinase B signalling pathways [25]. Interestingly, recent studies have shown that immunotherapy not only is a successful strategy that mediates tumor regression in patients with metastatic cancer but also holds the promise to treat neurodegenerative diseases [26]. Therefore, the specific roles of *Astragalus* polysaccharide in neurons and cancer cells may depend on different cellular and physiological environment.

3.3. Astragalan Increases the Lifespan of 6-OHDA-Intoxicated C. elegans. It is known that aging contributes to the development of late-onset neurodegenerative diseases, and age-dependent neurodegenerative changes are usually associated with a shortened life expectancy [27]. Therefore, we investigated the effect of astragalan on the lifespan of BZ555 nematodes under 6-OHDA intoxication. As shown in Figure 3, 6-OHDA-exposed nematodes exhibited a shorter mean lifespan (16.8 ± 0.35 d) as compared to that of the unexposed nematodes (19.3 ± 0.48 d) ($p < 0.05$), suggesting the toxic effect of 6-OHDA to accelerate senescence. When the 6-OHDA-exposed nematodes were treated with astragalan at 2.0 mg/mL, the mean lifespan was significantly increased to 19.4 ± 0.64 d ($p < 0.05$), which is similar to the action of EGCG (18.1 ± 0.51 d), indicating the capacity of astragalan

Table 2: Effect of astragalan on ROS level, antioxidant enzyme activities and malondialdehyde content in *C. elegans*.

Treatment	ROS level[a]	MDA content[b]	Antioxidant enzyme activity		
			SOD[c]	CAT[d]	GPx[c]
BZ555	4.55 ± 0.20	12.52 ± 2.63	69.35 ± 2.65	1.53 ± 0.09	17.19 ± 0.24
Astragalan	3.60 ± 0.01[e]	9.64 ± 0.90	72.02 ± 6.56	1.48 ± 0.10	19.15 ± 1.29
6-OHDA	7.97 ± 0.55[e]	16.21 ± 4.43[e]	43.90 ± 3.12[e]	1.34 ± 0.07	12.32 ± 0.33[e]
6-OHDA/Astragalan	4.70 ± 0.10[f]	14.39 ± 2.25[f]	69.33 ± 2.46[f]	1.45 ± 0.10	16.44 ± 0.99[f]

[a]ROS level, DCF fluorescence/μg proteins; [b]MDA content, nM/mg proteins; [c]SOD and GPx activities, U/mg proteins; [d]CAT activity, U/μg proteins; [e]$p < 0.05$ versus BZ555 nematodes without 6-OHDA exposure and astragalan treatment; [f]$p < 0.05$ versus 6-OHDA-exposed BZ555 nematodes.

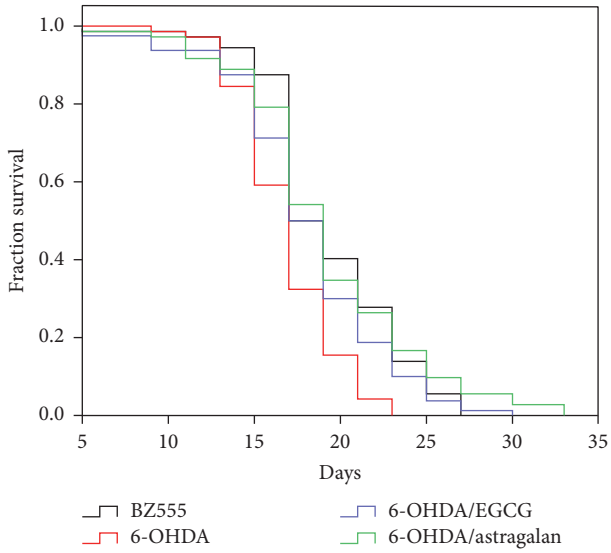

Figure 3: Effect of astragalan on the lifespan of 6-OHDA-intoxicated *C. elegans*. The BZ555 nematodes were treated with or without 2.0 mg/mL of astragalan after exposure to 6-OHDA, and the survival rates were scored every 2 days from the beginning of adulthood until all dead. A representative Kaplan-Meier survival curve from at least three independent experiments is presented.

to delay 6-OHDA-induced senescence. We have previously found that astragalan is able to extend the lifespan of wild-type and transgenic polyglutamine *C. elegans*, and its antiaging activity is associated with the modulation of DAF-16, a major downstream component of the insulin/insulin-like growth factor 1 (IGF-1) signalling pathway related to both lifespan regulation and stress resistance [11]. Recent studies have revealed that manipulation of insulin-related signalling pathways can resist 6-OHDA-mediated neurotoxicity. For example, ginsenoside Rg1 alleviates dopaminergic neuronal injury in 6-OHDA-lesioned rats through IGF-1 receptor, while IGF-1 receptor antagonist JB-1 reduces the neuroprotective effects of ginsenoside Rg1 [28]. Together, these results suggest that regulation of DAF-16 may contribute to the protective effect of astragalan against 6-OHDA neurotoxicity.

3.4. Astragalan Reduces ROS Level and MDA Content and Enhances Antioxidant Enzyme Activities in 6-OHDA-Intoxicated C. elegans. Oxidative stress is known to play an important role in the pathological process of age-related neurodegenerative diseases. For example, the increase of ROS level and oxidized lipids and proteins was observed in the brains of both sporadic and familial PD patients [29]. The oxidation of 6-OHDA generates cytotoxic hydrogen peroxide and other reactive species, leading to dopaminergic neuronal apoptosis [7]. Therefore, antioxidant intake represents a promising strategy against age-related neurodegenerative disorders [30]. Here we tested whether astragalan was able to reduce ROS level in *C. elegans* under 6-OHDA exposure using the DCF method. As shown in Table 2, the ROS level of 6-OHDA-exposed nematodes was significantly increased as compared to that of unexposed nematodes ($p < 0.05$). When the nematodes were cotreated with 6-OHDA and 2.0 mg/mL of astragalan, the ROS level was reduced compared with that of the nematodes exposed to 6-OHDA alone ($p < 0.05$). When BZ555 nematodes were treated with 2.0 mg/mL of astragalan, the ROS level was also reduced ($p < 0.05$). We then further examined the effect of astragalan on the content of MDA, a toxic lipid peroxidation product. As shown in Table 2, 6-OHDA exposure increased the MDA content in BZ555 nematodes, while astragalan was able to reduce the increased MDA content in 6-OHDA-intoxicated nematodes ($p < 0.05$), indicating its ability to inhibit lipid peroxidation. Together, these data demonstrate the antioxidant capacity of astragalan against 6-OHDA intoxication. Since PD is closely associated with oxidative stress, our results suggest an involvement of antioxidant activity in the neuroprotective effect of astragalan. This is supported by previous studies; for instance, a combination of antioxidant creatine and coenzyme Q10 can delay cognitive function decline and reduce plasma phospholipid level in PD patients [31].

The endogenous antioxidant system, including antioxidant enzymes and nonenzymatic antioxidants, is known to scavenge excessive ROS and maintain cellular redox balance. For example, SOD converts superoxide radicals to hydrogen peroxide and oxygen, while CAT and GPx detoxify hydrogen peroxide to water [32]. Inherited and acquired deficiencies of antioxidant system such as a defect in antioxidant enzyme activity lead to an imbalance of cellular redox environment and thus promote a variety of disorders including PD. For example, inhibition of Cu/Zn-SOD expression and activity aggravates 6-OHDA-mediated neuronal apoptosis [33]. Therefore, enhancing antioxidant system function is helpful to reduce oxidative damage in neurodegenerative diseases. In this study, we examined the effect of astragalan on antioxidant enzyme activities and found that 6-OHDA exposure caused a

significant decrease of both SOD and GPx activities in BZ555 nematodes ($p < 0.05$). However, 2.0 mg/mL of astragalan was able to increase SOD and GPx activities in the nematodes with 6-OHDA exposure ($p < 0.05$). It also slightly enhanced CAT activity in 6-OHDA-intoxicated nematodes (Table 2). Interestingly, *Astragalus* polysaccharides are previously shown to scavenge various ROS species *in vitro* and increase the level of nonenzymatic antioxidant glutathione in porcine circovirus type 2 infected cells [34, 35]. Together, these findings indicate that *Astragalus* polysaccharide is capable of enhancing antioxidant system function, and this ability may provide protections against 6-OHDA-induced damage.

3.5. Astragalan Suppresses the Proapoptotic Gene egl-1 Expression in 6-OHDA-Intoxicated C. elegans. In *C. elegans*, several proteins, including CED-4, CED-3, CED-9, and EGL-1, play a critical role in programmed cell death. The mammalian Apaf-1 counterpart CED-4 promotes the proteolytic activation of CED-3, which is the mammalian caspase-1 homolog and acts as the executor in apoptosis process. This activation process can be inhibited by CED-9, a homolog of the mammalian Bcl-2 and Bcl-XL antiapoptotic proteins. The BH3-only domain protein EGL-1 is a nematode counterpart of proapoptotic Bcl-2 family members. It interacts with CED-9-CED-4 complex and promotes the release of CED-4, resulting in the activation of CED-3 [36]. Previous reports have shown that 6-OHDA exposure induces apoptosis-like changes, such as condensed chromatin structures and shrunken cell morphologies, in dopaminergic neurons of *C. elegans* [37]. To investigate whether the aforementioned apoptosis-related proteins were involved in the actions of 6-OHDA and astragalan, we examined the mRNA levels of *ced-4*, *ced-3*, *ced-9,* and *egl-1* using quantitative real-time PCR. As shown in Figure 4, unexpectedly, 6-OHDA exposure did not influence the transcript levels of *ced-4*, *ced-3*, *ced-9,* and *egl-1* in BZ555 nematodes, suggesting that these gene expressions may be not involved in 6-OHDA-mediated neuronal apoptosis in *C. elegans*. Interestingly, another neurotoxin 1-methyl-4-phenylpyridnium ion (MPP^+) that can trigger parkinsonism also destroys *C. elegans* dopaminergic neurons independent of *ced-4* pathway [38]. However, a recent study has shown that 6-OHDA activates the mitochondrial apoptosis pathway including the release of cytochrome c to cytosol and the activation of caspase-3 in rat adrenal phaeochromocytoma PC12 cells [39], indicating that other mechanisms may contribute to 6-OHDA-induced neuronal apoptosis. On the other hand, in 6-OHDA-intoxicated nematodes, treatment with 2.0 mg/mL of astragalan exhibited negligible effects on the mRNA levels of *ced-4*, *ced-3,* and *ced-9* but significantly reduced the mRNA level of *egl-1* as compared to those in the nematodes exposed to 6-OHDA alone, indicating a potential of astragalan in regulating apoptotic pathway.

A large body of evidence has shown that modulation of apoptotic pathway holds promise to delay aging and treat aging-related neurodegenerative disorders. For instance, a decrease of *ced-3* expression by overexpression of the mammalian KIF11 homolog *bmk-1* extends the lifespan of wild-type *C. elegans* [40]. The BH3-only domain protein Bim plays

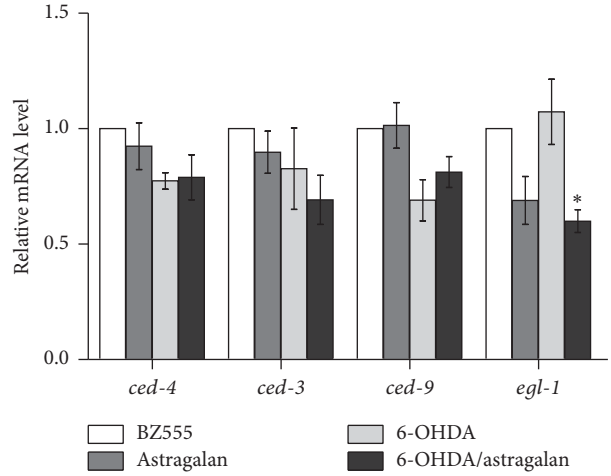

Figure 4: Effect of astragalan on the transcript levels of apoptosis-related genes in *C. elegans*. The mRNA levels of *ced-3*, *ced-4*, *ced-9,* and *egl-1* of BZ555 nematodes with or without 2.0 mg/mL of astragalan treatment under 6-OHDA exposure were performed using quantitative real-time PCR. Data are normalized to the nematodes without 6-OHDA and astragalan treatment and presented as mean ± SEM of three independent experiments. $^*p < 0.05$ versus 6-OHDA-exposed nematodes.

a pivotal role in mouse cerebrovascular degeneration induced by β-amyloid peptide, the main component of amyloid plaques in patients with Alzheimer's disease, while a decrease of Bim expression attenuates the death of cerebral endothelial cells [41]. Therefore, our data suggest that the regulation of proapoptotic gene *egl-1* expression may contribute to the protective effects of astragalan in *C. elegans*. In addition, a number of signalling pathways play important roles in free radical-mediated cell death [42, 43]. Among these pathways that respond to stresses, the MAPK family, including p38, ERK, and JNK, are important regulators in cell survival and death. Recent studies have shown that MAPKs are involved in the antioxidant and antiapoptotic effects of *Astragalus* polysaccharides. For example, *Astragalus* polysaccharides ameliorate doxorubicin-induced oxidative stress and apoptosis by inhibiting p38 MAPK pathway in cultured primary neonatal rat ventricular myocytes [44]. Thus, the MAPK pathway is likely associated with the protective function of astragalan in 6-OHDA-intoxicated *C. elegans*. Nevertheless, other stress-response signalling pathways may also be involved. For example, a sulfated polysaccharide isolated from sea cucumber *Stichopus japonicus* prevents 6-OHDA cytotoxicity through activation of PI3K/Akt signalling pathway in human neuroblastoma SH-SY5Y cells [45]. However, the exact signalling mechanisms underlying the neuroprotective effect of astragalan need further investigation.

3.6. Astragalan Increases Acetylcholinesterase Activity in 6-OHDA-Intoxicated C. elegans. Although PD is characterized by motor impairment and psychiatric disturbance mainly due to nigrostriatal dopaminergic denervation, increasing evidences reveal that a varying degree of degeneration in the cholinergic, serotonergic, and noradrenergic systems also

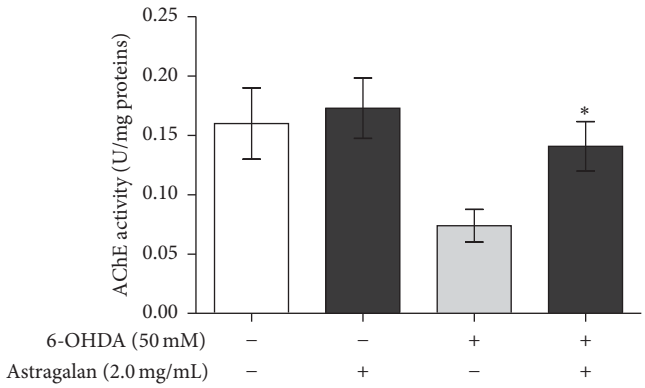

Figure 5: Effect of astragalan on the acetylcholinesterase activity in *C. elegans*. The 6-OHDA-exposed or unexposed BZ555 nematodes were treated with 2.0 mg/mL of astragalan and then used to determine the acetylcholinesterase activities. Results are presented as mean ± SEM of three independent experiments. $^{*}p < 0.05$ versus 6-OHDA-exposed nematodes.

contributes to motor and nonmotor abnormalities [46, 47]. For example, the nigrostriatal dopamine depletion triggers excessive release of excitatory neurotransmitter acetylcholine, leading to dysfunction of the corticobasal ganglia-thalamocortical loop circuits in PD patients [46]. In *C. elegans*, acetylcholine plays an important role in the control of various behaviors, including locomotion, feeding, mating, and egg laying, whereas acetylcholinesterase (AChE) is able to hydrolyze acetylcholine at neuronal junctions [48]. The administration of 6-OHDA has been reported to decrease AChE activity in animal models such as *C. elegans* and rats [7, 18, 49]. Thus, targeting AChE, an indirect indicator of cholinergic system function, provides a helpful strategy to alleviate the behavioral deficit in PD progression. In the present study, we tested whether astragalan regulated AChE activity in BZ555 nematodes. As shown in Figure 5, AChE activity was decreased in BZ555 nematodes after being exposed to 6-OHDA ($p < 0.05$). When the nematodes were cotreated with 6-OHDA and 2.0 mg/mL of astragalan, the decreased AChE activity was significantly elevated ($p < 0.05$). Interestingly, a previous study has shown that *Astragalus* polysaccharide was able to attenuate homocysteine-mediated inhibition of vasorelaxation to acetylcholine [50], suggesting its potential to influence cholinergic system. Together, our results suggest that the restoration of cholinergic system function may be associated with the protective effect of astragalan against 6-OHDA-mediated neuronal dysfunction.

4. Conclusions

In this study, we reveal that *A. membranaceus* polysaccharide astragalan not only attenuates the degeneration of dopaminergic neurons and the deficiency of food-sensing behavior but also increases the lifespan of 6-OHDA-exposed nematodes. We also demonstrate that astragalan is capable of reducing ROS level and inhibiting lipid peroxidation as well as increasing SOD and GPx activities in 6-OHDA-intoxicated nematodes. Further studies indicate that astragalan is able to reduce the transcript level of proapoptotic gene *egl-1* and increase AChE activity in 6-OHDA-exposed nematodes. Together, these findings suggest that the *Astragalus* polysaccharide astragalan can suppress 6-OHDA neurotoxicity through reducing oxidative stress, regulating apoptosis pathway and restoring cholinergic system function.

Competing Interests

The authors declare that there is no conflict of interests.

Authors' Contributions

Haifeng Li and Ruona Shi contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grants 81274048 and 81403081), the National High-Tech R & D Program of China (863 Program; Grant 2014AA022001), the Special Funds of the Central Finance to Support the Development of Local Universities and Colleges, and Guangdong Province Department of Education (Grant 2015KGJHZ022).

References

[1] W. G. Ondo, "Motor complications in Parkinson's disease," *International Journal of Neuroscience*, vol. 121, no. 2, pp. 37–44, 2011.

[2] M. Coelho and J. J. Ferreira, "Late-stage Parkinson disease," *Nature Reviews Neurology*, vol. 8, no. 8, pp. 435–442, 2012.

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

[4] C. Hellqvist and C. Berterö, "Support supplied by Parkinson's disease specialist nurses to Parkinson's disease patients and their spouses," *Applied Nursing Research*, vol. 28, no. 2, pp. 86–91, 2015.

[5] V. Bellou, L. Belbasis, I. Tzoulaki, E. Evangelou, and J. P. A. Ioannidis, "Environmental risk factors and Parkinson's disease: an umbrella review of meta-analyses," *Parkinsonism & Related Disorders*, vol. 23, pp. 1–9, 2016.

[6] J. Bové and C. Perier, "Neurotoxin-based models of Parkinson's disease," *Neuroscience*, vol. 211, pp. 51–76, 2012.

[7] J. Segura-Aguilar and R. M. Kostrzewa, "Neurotoxin mechanisms and processes relevant to Parkinson's disease: an update," *Neurotoxicity Research*, vol. 27, no. 3, pp. 328–354, 2015.

[8] Q. Wang, F. Yang, W. Guo et al., "*Caenorhabditis elegans* in Chinese medicinal studies: Making the case for aging and neurodegeneration," *Rejuvenation Research*, vol. 17, no. 2, pp. 205–208, 2014.

[9] R. Nass, M. K. Hahn, T. Jessen, P. W. McDonald, L. Carvelli, and R. D. Blakely, "A genetic screen in *Caenorhabditis elegans* for dopamine neuron insensitivity to 6-hydroxydopamine identifies dopamine transporter mutants impacting transporter biosynthesis and trafficking," *Journal of Neurochemistry*, vol. 94, no. 3, pp. 774–785, 2005.

[10] J. Fu, Z. Wang, L. Huang et al., "Review of the botanical characteristics, phytochemistry, and pharmacology of *Astragalus membranaceus* (Huangqi)," *Phytotherapy Research*, vol. 28, no. 9, pp. 1275–1283, 2014.

[11] H. Zhang, N. Pan, S. Xiong et al., "Inhibition of polyglutamine-mediated proteotoxicity by *Astragalus membranaceus* polysaccharide through the DAF-16/FOXO transcription factor in *Caenorhabditis elegans*," *Biochemical Journal*, vol. 441, no. 1, pp. 417–424, 2012.

[12] W.-X. Li, S.-G. Sun, H. Yuan, H.-T. Wang, Y.-B. Zhang, and B.-L. Sun, "Time dependency of *astragalus* polysaccharides against systemic injury of free radical in astrocyte culture medium," *Chinese Journal of Clinical Rehabilitation*, vol. 10, no. 11, pp. 59–61, 2006.

[13] P. Newland, B. Bingham, E. Tarelli, and A. H. Thomas, "High-performance gel permeation chromatography of meningococcal polysaccharides," *Journal of Chromatography A*, vol. 483, pp. 406–412, 1989.

[14] Z. Huang, Y. Liu, B. S. Paulsen, and D. Klaveness, "Studies on polysaccharides from three edible species of *Nostoc* (Cyanobacteria) with different colony morphologies: Comparison of monosaccharide compositions and viscosities of polysaccharides from field colonies and suspension cultures," *Journal of Phycology*, vol. 34, no. 6, pp. 962–968, 1998.

[15] S. W. Emmons, M. R. Klass, and D. Hirsh, "Analysis of the constancy of DNA sequences during development and evolution of the nematode *Caenorhabditis elegans*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 76, no. 3, pp. 1333–1337, 1979.

[16] R.-H. Fu, H.-J. Harn, S.-P. Liu et al., "*n*-Butylidenephthalide protects against dopaminergic neuron degeneration and α-synuclein accumulation in *Caenorhabditis elegans* models of Parkinson's disease," *PLoS ONE*, vol. 9, no. 1, article e85305, 2014.

[17] Q. Wang, Y. Huang, C. Qin et al., "Bioactive peptides from *Angelica sinensis* protein hydrolyzate delay senescence in *Caenorhabditis elegans* through antioxidant activities," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 8956981, 10 pages, 2016.

[18] S. Shashikumar, H. Pradeep, S. Chinnu, P. S. Rajini, and G. K. Rajanikant, "Alpha-linolenic acid suppresses dopaminergic neurodegeneration induced by 6-OHDA in *C. elegans*," *Physiology & Behavior*, vol. 151, pp. 563–569, 2015.

[19] S. Suo, S. Ishiura, and H. H. M. Van Tol, "Dopamine receptors in *C. elegans*," *European Journal of Pharmacology*, vol. 500, no. 1–3, pp. 159–166, 2004.

[20] M. L. Tucci, A. J. Harrington, G. A. Caldwell, and K. A. Caldwell, "Modeling dopamine neuron degeneration in *Caenorhabditis elegans*," *Methods in Molecular Biology*, vol. 793, pp. 129–148, 2011.

[21] D. Chen, A. G. Kanthasamy, and M. B. Reddy, "EGCG protects against 6-OHDA-induced neurotoxicity in a cell culture model," *Parkinson's Disease*, vol. 2015, Article ID 843906, 10 pages, 2015.

[22] M. Jin, K. Zhao, Q. Huang, and P. Shang, "Structural features and biological activities of the polysaccharides from *Astragalus membranaceus*," *International Journal of Biological Macromolecules*, vol. 64, pp. 257–266, 2014.

[23] S. Sun, K. Zheng, H. Zhao et al., "Regulatory effect of *astragalus* polysaccharides on intestinal intraepithelial $\gamma\delta$T cells of tumor bearing mice," *Molecules*, vol. 19, no. 9, pp. 15224–15236, 2014.

[24] B. Yang, B. Xiao, and T. Sun, "Antitumor and immunomodulatory activity of *Astragalus membranaceus* polysaccharides in H22 tumor-bearing mice," *International Journal of Biological Macromolecules*, vol. 62, pp. 287–290, 2013.

[25] T. Luo, J. Qin, M. Liu et al., "*Astragalus* polysaccharide attenuates lipopolysaccharide-induced inflammatory responses in microglial cells: regulation of protein kinase B and nuclear factor-κB signaling," *Inflammation Research*, vol. 64, no. 3-4, pp. 205–212, 2015.

[26] C. Dansokho, D. Ait Ahmed, S. Aid et al., "Regulatory T cells delay disease progression in Alzheimer-like pathology," *Brain*, vol. 139, no. 4, pp. 1237–1251, 2016.

[27] C.-W. Hung, Y.-C. Chen, W.-L. Hsieh, S.-H. Chiou, and C.-L. Kao, "Ageing and neurodegenerative diseases," *Ageing Research Reviews*, vol. 9, pp. S36–S46, 2010.

[28] L. Xu, W.-F. Chen, and M.-S. Wong, "Ginsenoside Rg1 protects dopaminergic neurons in a rat model of Parkinson's disease through the IGF-I receptor signalling pathway," *British Journal of Pharmacology*, vol. 158, no. 3, pp. 738–748, 2009.

[29] S. Nikam, P. Nikam, S. K. Ahaley, and A. V. Sontakke, "Oxidative stress in Parkinson's disease," *Indian Journal of Clinical Biochemistry*, vol. 24, no. 1, pp. 98–101, 2009.

[30] S. L. Albarracin, B. Stab, Z. Casas et al., "Effects of natural antioxidants in neurodegenerative disease," *Nutritional Neuroscience*, vol. 15, no. 1, pp. 1–9, 2012.

[31] Z. Li, P. Wang, Z. Yu et al., "The effect of creatine and coenzyme Q10 combination therapy on mild cognitive impairment in Parkinson's disease," *European Neurology*, vol. 73, no. 3-4, pp. 205–211, 2015.

[32] J. M. Matés, "Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology," *Toxicology*, vol. 153, no. 1–3, pp. 83–104, 2000.

[33] S. In, C.-W. Hong, B. Choi, B.-G. Jang, and M.-J. Kim, "Inhibition of mitochondrial clearance and Cu/Zn-SOD activity enhance 6-hydroxydopamine-induced neuronal apoptosis," *Molecular Neurobiology*, vol. 53, no. 1, pp. 777–791, 2016.

[34] X. Pu, X. Ma, L. Liu et al., "Structural characterization and antioxidant activity *in vitro* of polysaccharides from *angelica* and *astragalus*," *Carbohydrate Polymers*, vol. 137, pp. 154–164, 2016.

[35] H. Xue, F. Gan, Z. Zhang, J. Hu, X. Chen, and K. Huang, "*Astragalus* polysaccharides inhibits PCV2 replication by inhibiting oxidative stress and blocking NF-κB pathway," *International Journal of Biological Macromolecules*, vol. 81, pp. 22–30, 2015.

[36] L. del Peso, V. M. González, and G. Núnez, "Caenorhabditis elegans EGL-1 disrupts the interaction of CED-9 with CED-4 and promotes CED-3 activation," *The Journal of Biological Chemistry*, vol. 273, no. 50, pp. 33495–33500, 1998.

[37] R. Nass, D. H. Hall, D. M. Miller III, and R. D. Blakely, "Neurotoxin-induced degeneration of dopamine neurons in *Caenorhabditis elegans*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 5, pp. 3264–3269, 2002.

[38] P. Pu and W. Le, "Dopamine neuron degeneration induced by MPP^+ is independent of CED-4 pathway in *Caenorhabditis elegans*," *Cell Research*, vol. 18, no. 9, pp. 978–981, 2008.

[39] O. J. Olatunji, Y. Feng, O. O. Olatunji, J. Tang, Z. Ouyang, and Z. Su, "Cordycepin protects PC12 cells against 6-hydroxydopamine induced neurotoxicity via its antioxidant properties," *Biomedicine & Pharmacotherapy*, vol. 81, pp. 7–14, 2016.

[40] H. Qian, X. Xu, and L. E. Niklason, "Bmk-1 regulates lifespan in *Caenorhabditis elegans* by activating *hsp-16*," *Oncotarget*, vol. 6, no. 22, pp. 18790–18799, 2015.

[41] K.-J. Yin, C. Y. Hsu, X.-Y. Hu et al., "Protein phosphatase 2A regulates *bim* expression via the Akt/FKHRL1 signaling pathway in amyloid-β peptide-induced cerebrovascular endothelial cell death," *The Journal of Neuroscience*, vol. 26, no. 8, pp. 2290–2299, 2006.

[42] M. Genestra, "Oxyl radicals, redox-sensitive signalling cascades and antioxidants," *Cellular Signalling*, vol. 19, no. 9, pp. 1807–1819, 2007.

[43] Y. Son, S. Kim, H.-T. Chung, and H.-O. Pae, "Reactive oxygen species in the activation of MAP kinases," *Methods in Enzymology*, vol. 528, pp. 27–48, 2013.

[44] Y. Cao, Y. Ruan, T. Shen et al., "*Astragalus* polysaccharide suppresses doxorubicin-induced cardiotoxicity by regulating the PI3k/Akt and p38MAPK pathways," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 674219, 12 pages, 2014.

[45] C. Cui, N. Cui, P. Wang, S. Song, H. Liang, and A. Ji, "Neuroprotective effect of sulfated polysaccharide isolated from sea cucumber *Stichopus japonicus* on 6-OHDA-induced death in SH-SY5Y through inhibition of MAPK and NF-κB and activation of PI3K/Akt signaling pathways," *Biochemical and Biophysical Research Communications*, vol. 470, no. 2, pp. 375–383, 2016.

[46] T. Aosaki, M. Miura, T. Suzuki, K. Nishimura, and M. Masuda, "Acetylcholine-dopamine balance hypothesis in the striatum: an update," *Geriatrics & Gerontology International*, vol. 10, supplement 1, pp. S148–S157, 2010.

[47] N. I. Bohnen and R. L. Albin, "The cholinergic system and Parkinson disease," *Behavioural Brain Research*, vol. 221, no. 2, pp. 564–573, 2011.

[48] M. Jospin, Y. B. Qi, T. M. Stawicki et al., "A neuronal acetylcholine receptor regulates the balance of muscle excitation and inhibition in *Caenorhabditis elegans*," *PLoS Biology*, vol. 7, no. 12, article e1000265, 2009.

[49] T. A. Slotkin, I. T. Ryde, N. Wrench, J. A. Card, and F. J. Seidler, "Nonenzymatic role of acetylcholinesterase in neuritic sprouting: regional changes in acetylcholinesterase and choline acetyltransferase after neonatal 6-hydroxydopamine lesions," *Neurotoxicology and Teratology*, vol. 31, no. 3, pp. 183–186, 2009.

[50] B.-Q. Zhang, S.-J. Hu, L.-H. Qiu et al., "Effects of *Astragalus membranaceus* and its main components on the acute phase endothelial dysfunction induced by homocysteine," *Vascular Pharmacology*, vol. 46, no. 4, pp. 278–285, 2007.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 6949020, 14 pages
http://dx.doi.org/10.1155/2016/6949020

Research Article

(−)-Epicatechin Prevents Blood Pressure Increase and Reduces Locomotor Hyperactivity in Young Spontaneously Hypertensive Rats

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Received 30 May 2016; Revised 8 September 2016; Accepted 4 October 2016

Academic Editor: Renata Szymanska

This study investigated the effects of subchronic (−)-epicatechin (Epi) treatment on locomotor activity and hypertension development in young spontaneously hypertensive rats (SHR). Epi was administered in drinking water (100 mg/kg/day) for 2 weeks. Epi significantly prevented the development of hypertension (138 ± 2 versus 169 ± 5 mmHg, $p < 0.001$) and reduced total distance traveled in the open-field test (22 ± 2 versus 35 ± 4 m, $p < 0.01$). In blood, Epi significantly enhanced erythrocyte deformability, increased total antioxidant capacity, and decreased nitrotyrosine concentration. In the aorta, Epi significantly increased nitric oxide (NO) synthase (NOS) activity and elevated the NO-dependent vasorelaxation. In the left heart ventricle, Epi increased NOS activity without altering gene expressions of nNOS, iNOS, and eNOS. Moreover, Epi reduced superoxide production in the left heart ventricle and the aorta. In the brain, Epi increased nNOS gene expression (in the brainstem and cerebellum) and eNOS expression (in the cerebellum) but had no effect on overall NOS activity. In conclusion, Epi prevented the development of hypertension and reduced locomotor hyperactivity in young SHR. These effects resulted from improved cardiovascular NO bioavailability concurrently with increased erythrocyte deformability, without changes in NO production in the brain.

1. Introduction

Arterial hypertension is a frequent health problem worldwide. Primary hypertension is detectable in children and adolescents and is increasing in prevalence [1]. Attention deficit hyperactivity disorder (ADHD) is one of the most common developmental disorders that affects approximately 5–7% of children and adolescents [2]. The rate of learning disabilities, including ADHD, is significantly higher for children with sustained primary hypertension as compared to children without hypertension [3]. Arterial hypertension, in addition to other mechanisms, was associated with reduced deformability of erythrocytes, which may participate in the development of both arterial hypertension [4] and behavioral changes.

Spontaneously hypertensive rats (SHR) are a commonly used model of human essential hypertension. SHR also serve as an experimental model of ADHD due to their locomotor hyperactivity and reduced anxiety [5, 6]. However, it is not clear whether the pathways involved in the development of hypertension overlap with those involved in the modulation of locomotor activity. Notably, NO serves as vasodilator in the cardiovascular system (CVS) and as neurotransmitter and neuromodulator in the central and peripheral nervous

systems. Therefore, alterations in NO production affect both blood pressure (BP) and behavior. Indeed, neuronal nitric oxide synthase (nNOS) was shown to be involved in various behavioral abnormalities, including ADHD [7, 8].

Oxidative stress is the excessive formation of reactive oxygen species (ROS), especially superoxide ($O_2^{\bullet-}$), to a level exceeding the maximal capacity of the antioxidant defense mechanisms of the organism. Oxidative stress has been found to be involved in many disease states, including hypertension and behavioral/mental disorders, in both rodents and humans [9–11]. Superoxide is produced by various physiological aerobic metabolic processes as well as by several enzymatic pathways. One of the main sources of $O_2^{\bullet-}$ in living organisms is nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase); however, uncoupled nitric oxide synthase (NOS) may also be a significant source of ROS [9]. Furthermore, increased $O_2^{\bullet-}$ production may lead to formation of peroxynitrite, a strong prooxidant, which causes peroxynitrite-related cellular damage [12], observed in various cardiovascular disorders [13, 14].

Over the past two decades, there has been increasing interest in the potential health benefits associated with the consumption of flavanol-containing foods [15, 16]. Several studies have reported that the consumption of flavanol-containing foods was associated with a lower prevalence of cardiovascular diseases [17, 18]. Furthermore, several meta-analyses have confirmed a BP-lowering capacity and antihypertensive effect of flavanol-rich foods derived from cocoa [19, 20]. BP-lowering effect of cocoa-derived products depended on the dose of ingested (−)-epicatechin (Epi) [21].

Epi is absorbed well from the gastrointestinal tract in both humans and rats, detectable in plasma approximately 30 min after ingestion. Epi concentrations peak 2-3 h after ingestion and return to baseline by 6–8 h [22, 23], suggesting that the continuous intake of Epi-containing food is needed to maintain elevated circulation levels. The BP-reducing effect of Epi was shown in models of L-NAME-induced [24], fructose-induced [25], and DOCA-salt hypertension [26]. We have previously observed that Epi reduces BP and improves endothelium-dependent vasorelaxation in adult SHR with fully developed hypertension via improved vascular NO bioavailability [27].

Regarding the role of Epi in the central nervous system (CNS), recent studies have demonstrated that Epi can cross the blood-brain barrier (BBB) and enter the brain [28, 29], which may result in altered CNS function. Studies have also shown that prolonged cocoa flavanol consumption improves cognitive function, blood pressure control, and metabolic profile in elderly subjects [30]. Although the underlying mechanism responsible for the observed effects of cocoa-derived foods on the CNS remains unknown, it may be associated with improved NO bioavailability, vascular function, and/or increased erythrocyte deformability, which all together may improve organ perfusion.

Therefore, the aim of this study was to investigate whether the subchronic treatment of peripubertal SHR with Epi may prevent the development of hypertension and locomotor hyperactivity in this genetic model of hypertension and ADHD. To elucidate the mechanism(s) of Epi action, we investigated superoxide and NO production as well as the gene expression of the p22phox subunit of NADPH oxidase and individual NOS isoforms in the CVS and selected regions of the brain, total antioxidant capacity of plasma, nitrosative damage, the deformability of erythrocytes, and vascular function.

2. Material and Methods

2.1. Animals and Treatment. Young 5-week-old SHR males ($n = 18$) were used. All rats were born in our certified animal facility (Institute of Normal and Pathological Physiology SAS) in order to maintain the same environmental background for all animals. The rats were housed two per cage at constant temperature 22–24°C and humidity (45–60%) with a 12 : 12 h light-dark cycle (lights on from 06.00 a.m. to 06.00 p.m.) and fed a standard pellet diet with tap water *ad libitum*. At the beginning of the experiment (Basal, B), rats were randomly assigned to the control group (Cont, $n = 8$) or a group treated with Epi (Epi, $n = 10$). Epi was administered to rats diluted in the appropriate daily volume of water, in concentration that resulted in a final daily dose of Epi approximately 100 mg/kg body weight/day, for two weeks. Daily volume of water was assessed for each cage of rats prior to starting the experiment and adjusted daily. Average daily drinking volume of rats was 17 ± 0.8 mL/100 g of body weight and Epi did not influence it. Concentrated Epi solution (100 mg/mL) was prepared fresh every day before administration to rats by dilution of Epi in tap water (85°C, 3 min, in water bath). Calculated volume of concentrated Epi solution was added to assessed volume of fresh tap water in the bottles of rats to reach the dose 100 mg/kg body weight/day after drinking out all liquid during 24 h period. Concentration of Epi in bottles was approximately 0.58 mg/mL. If rats drank the given volume of liquid earlier, fresh water was added to the bottle to prevent thirst and/or stress from the lack of water. Epi solutions (both concentrated and diluted in bottles) were protected against the light. Fresh Epi was administered to rats at the end of the light period, as the majority of drinking activity of rodents occurs in the dark (active) period [31]; thus approximately 80% of solution was drunk during the dark period and the rest in the light period. Thermal and time-dependent stability of Epi in water has been shown previously [32].

At the end of the 2-week treatment, the rats were exposed to brief CO_2 anesthesia. Rats were subsequently killed by decapitation, and trunk blood was collected to evaluate erythrocyte deformability, nitrotyrosine concentration, and total antioxidant capacity. Wet mass of the left heart ventricle (LHV) was determined to calculate relative weight (LHV/body weight) in order to ascertain the degree of LHV hypertrophy.

All procedures were performed in accordance with the institutional guidelines and approved by the Department of Animal Wellness, State Veterinary and Food Administration of the Slovak Republic.

2.2. Blood Pressure and Heart Rate. Systolic blood pressure and heart rate (HR) were measured in preconditioned, conscious rats

by noninvasive tail-cuff plethysmography between 08:00 a.m. and 11:00 a.m. as described in detail previously [33]. Each value was calculated as the average of five measurements. BP values were measured repeatedly at the beginning of the experiment (B) and after the seventh, tenth, and fourteenth day of treatment. Body weight (BW) was determined on the same days.

2.3. Open-Field Test. Rat motor activity and anxiety level were measured using the open-field test (OF) between 07:30 a.m. and 10:00 a.m. The open-field apparatus comprised a 100 × 100 cm area with a black floor and black walls (50 cm high) with a virtual central zone (55 × 55 cm) and corners (12.5 × 12.5 cm). The OF was illuminated by warmwhite light at 150 lx. Rats were placed in the centre of the OF; motor activity was recorded and evaluated by ANY-maze video-tracking software (Stoelting, USA) during 10 min trials. The OF area was cleaned with soapy water and dried with paper towels after each trial. The following behavioral parameters were determined: total distance traveled, total time of immobility, central zone distance traveled, and time spent in the central zone and in the corners. Average speed was calculated as the ratio of total distance traveled to time of mobility for a given rat. As anxiety markers, relative central zone distance (calculated as the percentage of central zone distance with respect to total distance traveled) and relative central zone time (calculated as the percentage of time spent in the central zone with respect to total mobility time) were determined [34].

All rats were tested one day before the beginning of the experiment to determine baseline measurements (B) in 5-week-old rats. Rats were randomly assigned to the control or Epi-treated group and tested again two days before the end of the experiment (~7 weeks of age). Thus, OF behavior was determined one day prior to BP measurement to avoid the effect of the OF test on BP level. All cages with rats were placed into a test room with the lighting and environmental conditions described above, approximately 12 h before the test.

2.4. Erythrocyte Deformability, Total Antioxidant Capacity of Plasma, and Nitrotyrosine Concentration. Trunk blood samples were collected in heparinized test tubes and immediately thereafter centrifuged at 850 ×g for 10 min at 4°C to obtain plasma and erythrocytes. Plasma was separated, aliquoted, and stored at −80°C until the time of analysis.

After removing the plasma, the buffy coat and upper 20% of packed red blood cells were removed by aspiration. The remaining erythrocytes were washed three times in manufacturer-formulated Cellpack solution (diluent for Sysmex blood analyser, Sysmex F–820, Japan). The washed erythrocytes were diluted in Cellpack solution (1 : 1, v : v) and adjusted to 30–40% hematocrit. The diluted suspension of erythrocytes was filtered by centrifugation through membrane filters with pores of 5 μm in diameter (Ultra-free-MC SV Centrifugal Filter, Millipore, Germany) at 1400 rpm (Hettich MIKRO 120 centrifuge). Erythrocyte deformability was calculated as the percentage of filtered erythrocytes with respect to the number of erythrocytes counted before centrifugation [35].

The total antioxidant capacity (TAC) of plasma was measured by determining the trolox equivalent antioxidant capacity as described previously by [36]. Quantification was performed using the dose-response curve for the reference antioxidant trolox, which is a water-soluble form of vitamin E. The results are presented as mmol of trolox/L. TAC was determined in six control and six Epi-treated rats.

Concentration of nitrotyrosine in plasma was detected by ELISA using commercially available kit (HK501-02, Hycult Biotech, Uden, Netherlands) according to the manufacturer's protocol. Absorbance of the plasma samples was measured at 450 nm. The nitrotyrosine concentration of samples was determined from the standard curve and expressed in nmol/L.

2.5. Superoxide Production. The production of superoxide ($O_2^{\bullet -}$) was measured in tissue samples of the LHV and thoracic aorta (15–20 mg) by lucigenin-enhanced chemiluminescence (50 μmol/L) using a TriCarb 2910TR liquid scintillation analyser (Perkin Elmer), as described previously [37]. The results are expressed as counts per minute per milligram of tissue (cpm/mg).

2.6. Nitric Oxide Synthase Activity. Total NOS activity was measured in the 20% tissue homogenates of the LHV, aorta, brainstem, and cerebellum by determining [^{3}H]-L-citrulline formation from [^{3}H]-L-arginine (MP Biomedicals, USA) as described previously [33] and expressed as pmol/min/mg of tissue proteins as determined using the Lowry method. NOS activity was determined in six control and eight Epi-treated rats.

2.7. Vascular Function. The vascular reactivity of the aorta was investigated as described previously [38]. Endothelium-dependent vasorelaxant responses were examined in rings precontracted with phenylephrine (3 μmol/L) to produce a stable plateau of contraction. After a contraction plateau had been reached, increasing concentrations of acetylcholine (ACh, 0.001–10 μmol/L) were added cumulatively. When the ACh-induced concentration-relaxation curve was completed, the drugs were washed out (20 min), and the same experiment was repeated after 25 min preincubation with NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μmol/L). After this procedure and a 30 min washout period, the NO donor sodium nitroprusside (SNP, 0.001–10 μmol/L) was added cumulatively to the 3 μmol/L phenylephrine precontracted aortae. The extent of vasorelaxation was expressed as the percentage change with respect to stable phenylephrine-induced contraction.

NO-independent component of endothelium-dependent ACh-induced relaxation was determined as the rest of relaxation present after inhibition of vascular NO production with L-NAME and expressed as the area under the concentration-response curve (AUC), in arbitrary units (a.u.). Endothelium-dependent ACh-induced relaxation mediated by NO (i.e., NO-dependent component) was calculated as the difference in the AUC before L-NAME pretreatment (i.e., total ACh-induced relaxation) and after L-NAME pretreatment (i.e.,

Table 1: Primer pairs used to amplify selected genes.

Genes	Forward (sense) primer	Reverse (antisense) primer	Temp
eNOS	CCC ACA GTC TGG TTG CT	TCA CCG TGC CCA TGA GT	57°C
iNOS	TGG AGG TGC TGG AAG AGT T	GGA GGA GCT GAT GGA GTA GT	57°C
nNOS	CGC TAC GCG GGC TAC AAG CA	GCA CGT CGA AGC GGC CTC TT	60°C
β-actin	AAT CGT GCG TGA CAT CAA AG	ATG CCA CAG GAT TCC ATA CC	57°C
p22phox	CAG GCA TAT ACC CGC TAC CT	TCT GTC ACC CTG TGC TTG AC	60°C

NO-independent relaxation). AUC was calculated from individual concentration-response curves, as it was described in detail previously [33].

2.8. Gene Expression. Expression levels of neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) as well as p22phox (a transmembrane subunit of NADPH oxidase) were investigated by real-time quantitative polymerase chain reaction (RT-qPCR) using a CFX96 Real-Time PCR detection system (Bio–Rad, USA). Total RNA from the brainstem, cerebellum, and LHV samples was isolated using TRIsure reagent (Bioline, United Kingdom) according to the manufacturer's protocol. The amount of total RNA isolated was quantified spectrophotometrically at 260/280 nm using a NanoDrop spectrophotometer (Thermo Scientific, USA).

For reverse transcription (Eppendorf Mastercycler, Germany), 1 μg of total RNA was added to 20 μL of reaction medium using a SensiFAST™ cDNA Synthesis Kit (Bioline, UK) according to the manufacturer's protocol.

The primer pair specifications used to amplify the genes studied (nNOS, iNOS, eNOS, and p22phox, resp.) as well as a housekeeping gene (β-actin) are listed in Table 1. The PCR mixture contained 1.5 μL of template cDNA diluted tenfold, 10 μL SensiFAST mix (SensiFAST SYBR No–ROX kit, Bioline, UK), 1.5 μL of both forward and reverse primers (Metabion, Germany, 4 μmol/L), and 5.5 μL diethylpyrocarbonate-treated water (Sigma–Aldrich, Germany) in a final volume of 20 μL. The thermal cycling conditions were as follows: (1) 50°C for 2 min, (2) 95°C for 2 min, (3) 39 cycles consisting of (a) 95°C for 5 sec, (b) an optimal annealing temperature (depending on the selected primer, see Table 1) for 10 sec, and (c) 72°C for 5 sec for PCR product elongation, and (4) 72°C for 1.5 min. Finally, melt curves for amplicon analyses were constructed at 50–99°C, 10 sec/1°C. Samples were measured using Bio–Rad CFX Manager software (version 2.0) and β-actin as the housekeeping gene. Gene expression was determined in six control and eight Epi-treated rats and expressed as the ratio of gene expression with respect to β-actin levels.

All chemicals used in this study were purchased from Sigma–Aldrich (Germany) and Merck Chemicals (Germany), if not stated differently. Epi was purchased from Sigma (Germany, Cat. no. E1753).

2.9. Statistical Analysis. Results were analysed by unpaired Student t-test or one-way analysis of variance (ANOVA) where appropriate. BP, HR, and BW were analysed by two-way ANOVA (treatment × time). Vascular function was analysed by two-way ANOVA (treatment × ACh concentration). All ANOVA analyses were followed by the Bonferroni *post hoc* test. Values were considered to differ significantly when $p < 0.05$. Data are presented as mean ± standard error of the mean (SEM). Correlations between variables were determined using Pearson's correlation coefficient (r). GraphPad Prism 5.0 (GraphPad Software, Inc., USA) and Statistica 7 (Stat Soft, Inc., USA) were used for the statistical analyses.

3. Results

Two-week Epi treatment had no effect on the increase in BW controlled for age (data not shown). Relative weight of the LHV was similar in the Epi (2.21 ± 0.07 mg/g) and control (2.31 ± 0.06 mg/g) groups. BP was reduced by approximately 18% in Epi-treated rats as compared to controls at the end of treatment (Figure 1(a)). Epi treatment reduced heart rate only on the 10th day of treatment (571.8 ± 17 bpm in control versus 524.8 ± 8.2 bpm in Epi, $p < 0.05$) while only nonsignificant difference (540 ± 19 bpm versus 530 ± 9 bpm) was observed on day 14. In addition, Epi increased erythrocyte deformability by approximately 8% ($p < 0.05$), increased the TAC ($p < 0.05$), and reduced nitrotyrosine concentration in plasma ($p < 0.05$) versus controls (Figures 1(b), 1(c), and 1(d)).

Regarding rat behavior, repeated testing in the open-field at the end of experiment led to habituation of locomotor activity detected as reduction of total distance traveled and increase of total immobility compared to Basal values (Figures 2(a) and 2(b)). Epi administration led to a significant decrease in locomotor activity as represented by total distance traveled (Figure 2(a)), increased immobility (Figure 2(b)), and reduced the average speed of movement (Figure 2(g)) in treated animals as compared to age-matched controls. Epi decreased the distance traveled (Figure 2(c)) and time spent in the central zone (Figure 2(d)). In addition, Epi reduced both relative distance traveled and relative time spent in the central zone (Figures 2(e) and 2(f)). Epi treatment also significantly elevated time spent in the corners (Figure 2(h)) in treated animals as compared to controls.

Epi significantly reduced $O_2^{\bullet -}$ production and increased NOS activity in the LHV (Figures 3(a) and 3(b)). However, Epi failed to affect gene expression levels for individual NOS isoforms (Figures 3(c)–3(e)) and p22phox (Figure 3(f)) in the LHV.

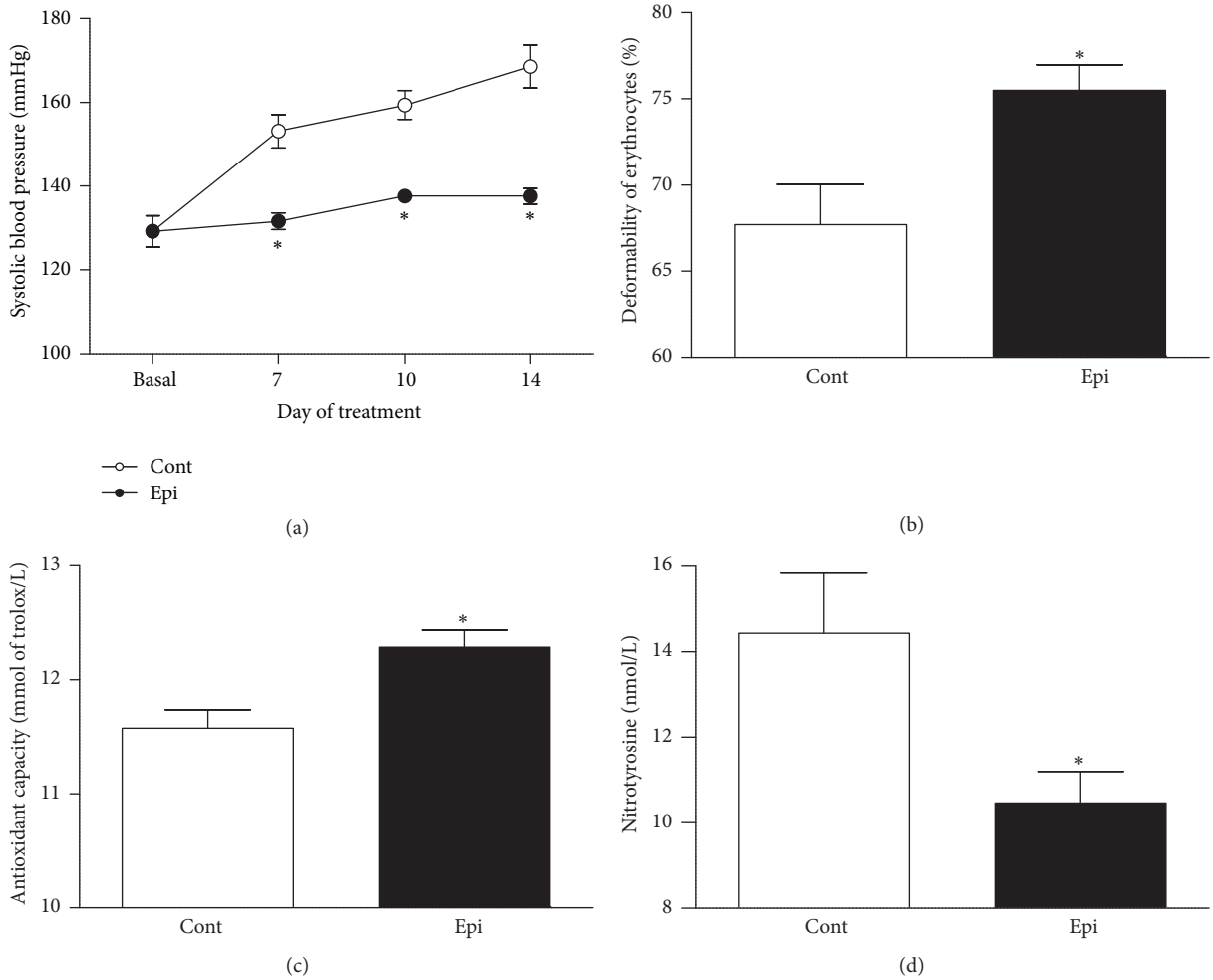

Figure 1: Effect of (−)-epicatechin on systolic blood pressure (a), deformability of erythrocytes (b), total antioxidant capacity of plasma (c), and plasma nitrotyrosine concentration (d) in spontaneously hypertensive rats. $^{*}p < 0.05$ versus Cont group. Values represent mean ± SEM; $n = 6$–8 for Cont and $n = 6$–10 for Epi. Abbreviations: Cont: control group and Epi: (−)-epicatechin-treated group.

In the aorta, Epi significantly reduced $O_2^{\bullet-}$ production and increased NOS activity (Figures 4(a) and 4(b)). Neither endothelium-independent relaxation responses induced by SNP nor overall endothelium-dependent relaxation induced by ACh differed significantly in the aortae of control as compared to Epi-treated rats (Figures 4(c) and 4(d)). Acute L-NAME pretreatment, which inhibited NO-dependent relaxation, inhibited relaxation more strongly in Epi-treated rats as compared to controls (Figure 4(e)). Calculation of the AUC revealed that Epi significantly increased endothelial NO-dependent relaxation by approximately 26% versus control and concurrently decreased endothelial NO-independent relaxation in the aorta (Figure 4(f)).

NOS activity was unaffected by Epi in both brain regions investigated (brainstem and cerebellum) (Figures 5(a) and 6(a)). Interestingly, NOS activity in the brainstem and cerebellum correlated positively with total distance traveled (as shown in Figures 5(b) and 6(b)) as well as with central zone distance traveled in the OF ($r = 0.53$, $p < 0.05$, $n = 14$ for brainstem; $r = 0.62$, $p < 0.02$, $n = 14$ for cerebellum).

Gene expression levels of nNOS increased (Figures 5(c) and 6(c)) while iNOS levels remained the same in both brain regions investigated in Epi-treated rats (Figures 5(d) and 6(d)). Interestingly, eNOS and p22phox gene expression were increased in the cerebellum of Epi-treated rats as compared with controls (Figures 6(e) and 6(f)). This effect was not observed in the brainstem.

4. Discussion

This study investigates the effect of subchronic treatment with Epi in young SHR rats, at the peripubertal age, which is a critical developmental period when BP increases rapidly in SHR [39, 40]. We show that continuous Epi treatment during this period significantly prevented BP increase and reduced spontaneous locomotor hyperactivity in SHR. In addition, we show here for the first time that Epi treatment increased erythrocyte deformability in SHR.

In this study, Epi was administered continuously in tap water. It is known that Epi is subject to partial degradation in

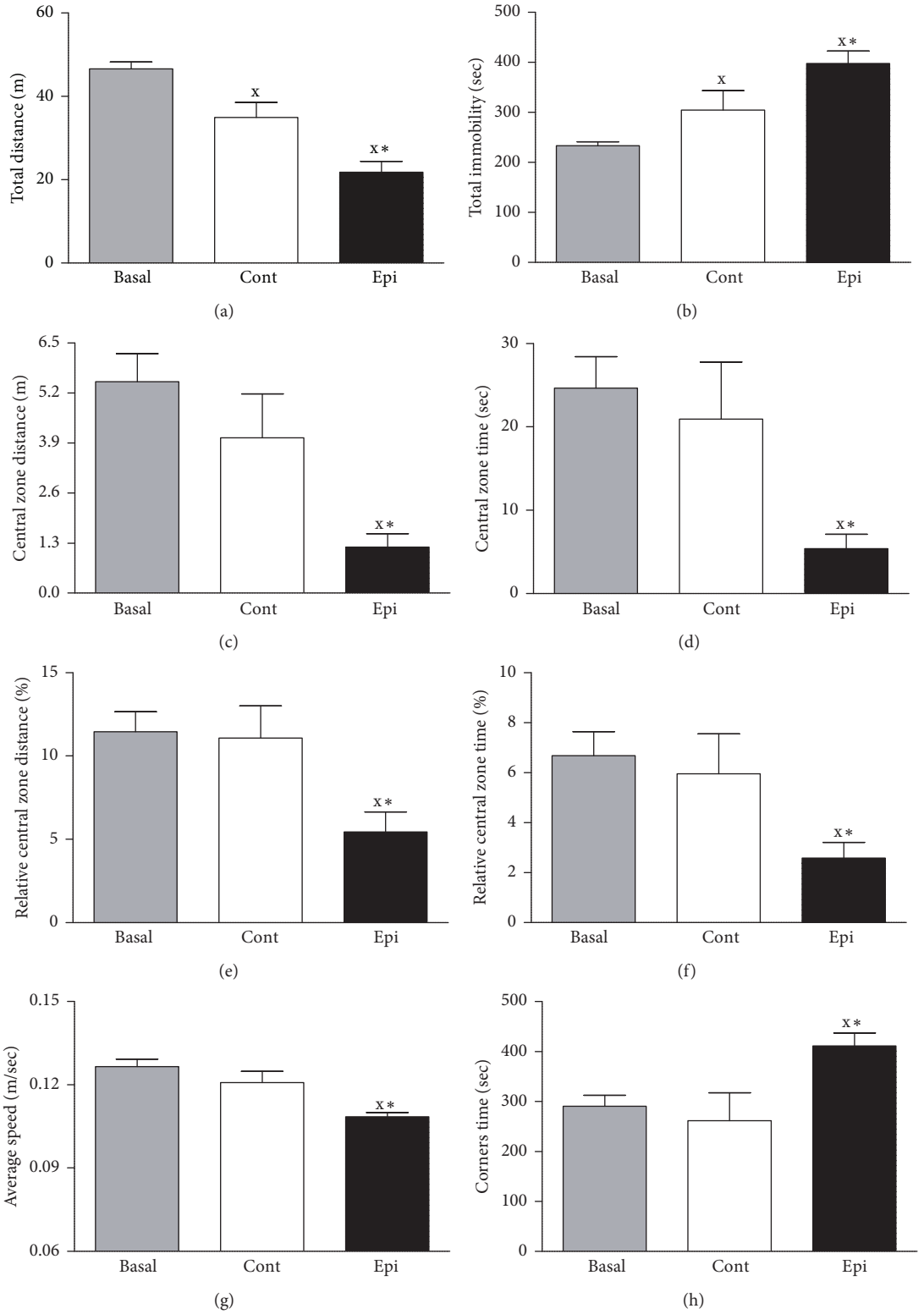

Figure 2: Effect of (−)-epicatechin treatment on open-field behavior of spontaneously hypertensive rats. Total distance traveled (a), total immobility (b), distance traveled in the central zone (c), time spent in the central zone (d), relative central zone distance (e), relative central zone time (f), average speed (g), and time spent in the corners (h). Values represent mean ± SEM; $n = 18$ for Basal, $n = 8$ for Cont, and $n = 10$ for Epi. $^{x}p < 0.05$ versus Basal values; $^{*}p < 0.05$ versus Cont group. Abbreviations: Cont: control group and Epi: (−)-epicatechin-treated group.

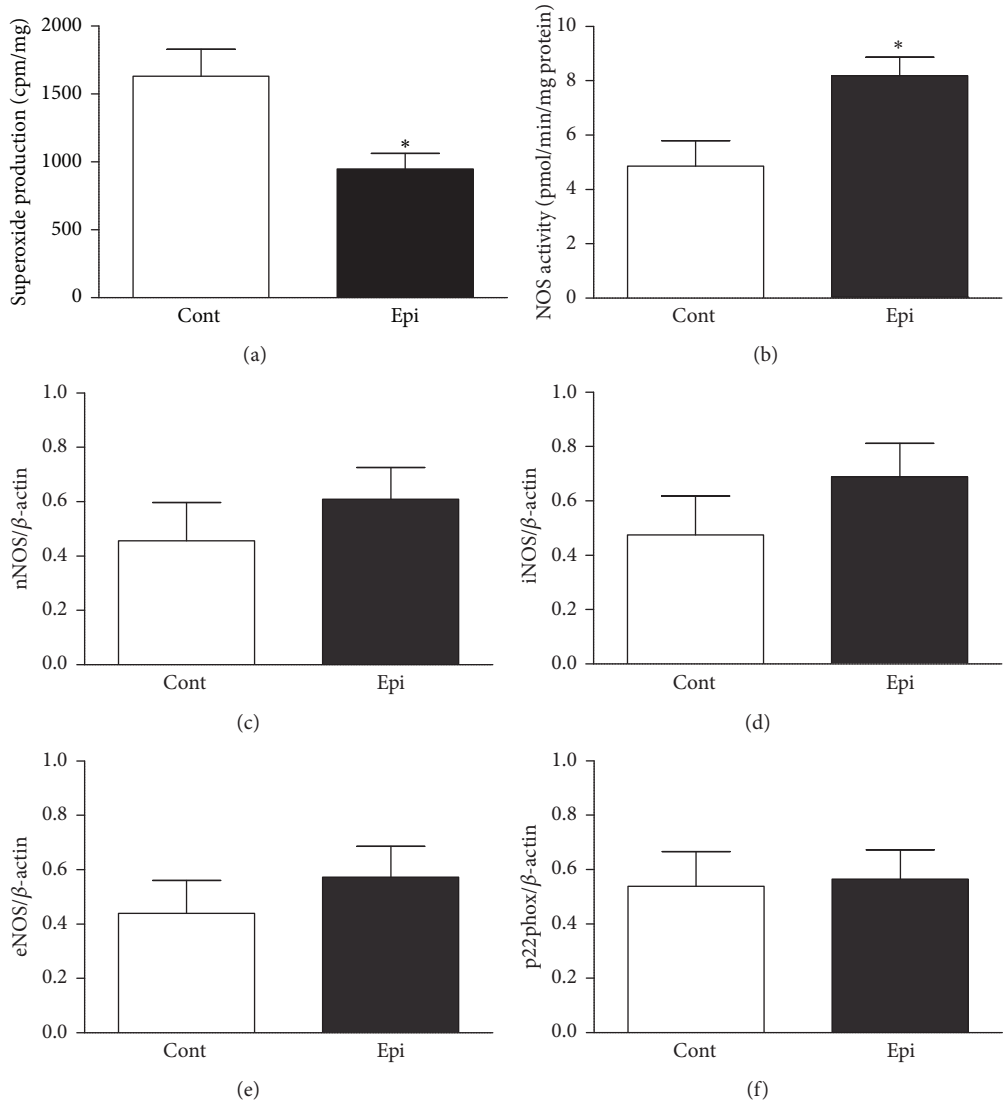

Figure 3: Effect of (−)-epicatechin treatment on superoxide production (a), nitric oxide synthase (NOS) activity (b), gene expression of neuronal NOS (nNOS, c), inducible NOS (iNOS, d), endothelial NOS (eNOS, e), and the p22phox subunit of nicotinamide adenine dinucleotide phosphate oxidase (f) in the left heart ventricle of spontaneously hypertensive rats. Values represent mean ± SEM; $n = 6$–8 for Cont and $n = 8$–10 for Epi. $^{*}p < 0.05$ versus Cont group. Abbreviations: Cont: control group and Epi: (−)-epicatechin-treated group.

water and further metabolism after ingestion. The presence of Epi and/or its metabolites (e.g., 3′-O-methyl epicatechin and 4′-O-methyl epicatechin) in plasma as well as in the brain was detected previously after administration of the same dose of Epi as used in this study [41]. However, despite the fact that biologically active substance(s) may differ from Epi itself, this study demonstrates the significant biological effects of orally administered Epi. We used the given dose of Epi (100 mg/kg/day), as we were interested in possible central effects of Epi, despite the fact that BP-lowering effect can be reached by lower doses. As the relatively high dose of Epi was used in this study, we determined creatinine, uric acid, and urea in plasma at the end of Epi treatment to reveal whether the given dose of Epi is safe or if it produces adverse side effects to kidneys. No signs of renal toxicity of the given dose of Epi were observed in our study (see Supplementary Materials available online at http://dx.doi.org/10.1155/2016/6949020).

The Epi-mediated prevention of hypertension development was associated with elevated plasma TAC and reduced superoxide production in the LHV and aortae of Epi-treated rats. However, these findings were not associated with changes in the gene expression of the p22phox subunit of NADPH oxidase, one of the main sources of $O_2^{\bullet-}$ in the CVS. These findings support other studies in which the antioxidant capacity of Epi was associated either with activation of the enzymes involved in the antioxidant defense system [42] in the heart or with radical-scavenging properties in endothelial cells without affecting NADPH oxidase activity *in vitro* [43, 44]. On the other hand, short-term Epi cotreatment reduced

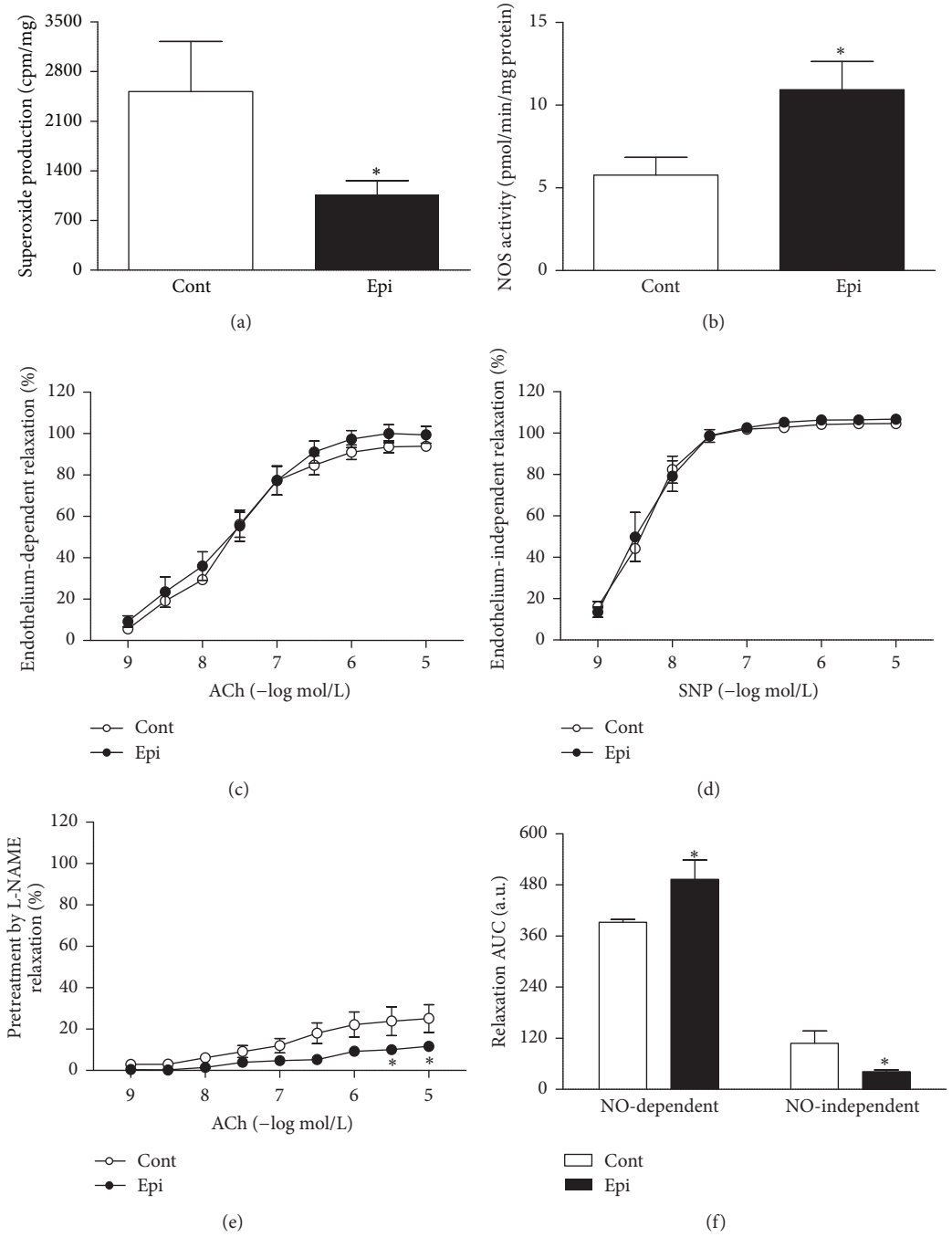

Figure 4: Effect of (−)-epicatechin treatment on superoxide production (a), NOS activity (b), endothelium-dependent relaxation induced by acetylcholine (c), endothelium-independent relaxation induced by SNP (d), inhibitory effect of L-NAME pretreatment (300 μmol/L) on ACh-induced relaxation (e), and NO-dependent and NO-independent components of relaxation (f) in the aorta of spontaneously hypertensive rats. Values represent mean ± SEM; $n = 6$–8 for Cont and $n = 6$–10 for Epi. $^*P < 0.05$ versus Cont group. Abbreviations: ACh: acetylcholine, AUC: area under the curve, a.u.: arbitrary units, Cont: control group, Epi: (−)-epicatechin-treated group, L-NAME: N^G-nitro-L-arginine methyl ester, NO: nitric oxide, NOS: nitric oxide synthase, and SNP: sodium nitroprusside.

protein expression levels of the p47phox subunit of NADPH oxidase in the hearts of rats with L-NAME-induced hypertension [24] and in the renal cortex in fructose-fed rats [45], in contrast to our findings in a genetic model of hypertension. Regarding nitrosative damage, *in vitro* studies revealed that Epi protected cells against peroxynitrite-induced damage [46, 47] similarly as we observed *in vivo* in blood.

In addition to the reduction in superoxide production and increased NOS activity in the aorta and LHV as well as reduced plasma nitrotyrosine concentration, increased aortic

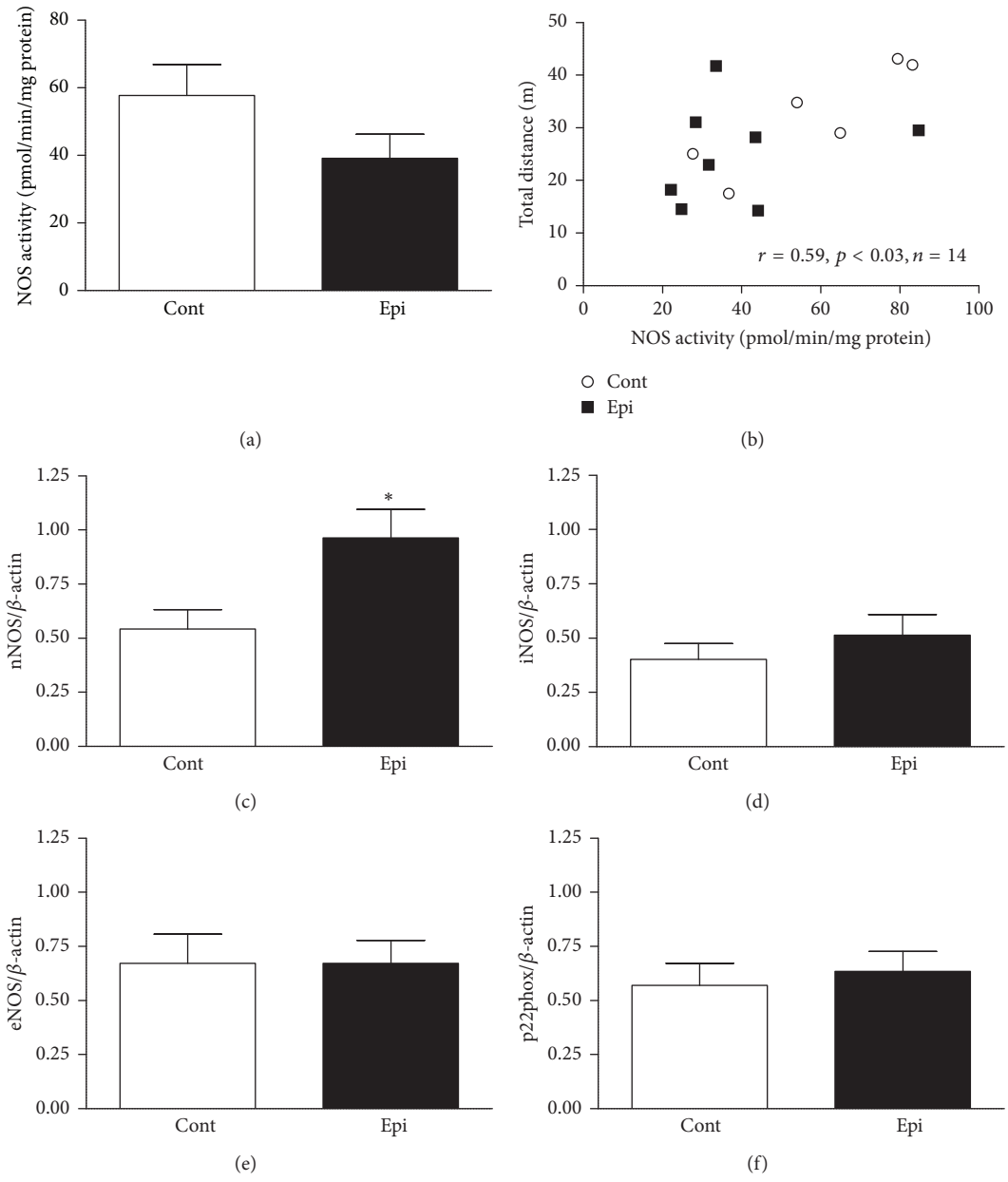

Figure 5: Effect of (−)-epicatechin treatment on nitric oxide synthase activity (a), correlation between total distance traveled in the open-field and nitric oxide synthase activity (b) and gene expression of nNOS (c), iNOS (d), eNOS (e), and the p22phox (f) in the brainstem of spontaneously hypertensive rats. Values represent mean ± SEM; $n = 6$ for Cont and $n = 8$–10 for Epi. $^{*}p < 0.05$ versus Cont group. Abbreviations: Cont: control group, Epi: (−)-epicatechin-treated group, NOS: nitric oxide synthase, eNOS: endothelial NOS, iNOS: inducible NOS, nNOS: neuronal NOS, and p22phox: subunit of nicotinamide adenine dinucleotide phosphate oxidase.

endothelial NO-dependent relaxation also proofs better NO bioavailability in the CVS. Interestingly, no effect of Epi on e/i/nNOS gene expression in the LHV was found in this study, suggesting that Epi influences the catalytic properties of NOS but not its gene expression in the CVS. A similar mechanism was demonstrated previously in cultured endothelial cells [48] as well as in the cardiac tissue of L-NAME-treated rats [24]. Therefore, our study in a genetic model of spontaneous hypertension confirms the ability of Epi to increase the CVS capacity for NO production resulting in elevated NO bioavailability; however, the involvement of individual NOS isoforms remains to be clarified.

Regarding vascular function, acute Epi administration induces both endothelium-dependent and endothelium-independent relaxation in the isolated arteries of normotensive rats and in human arteries [49–51]. The Epi-induced endothelium-dependent relaxation in normotensive rats was primarily mediated by NO [49, 50]. Recent study

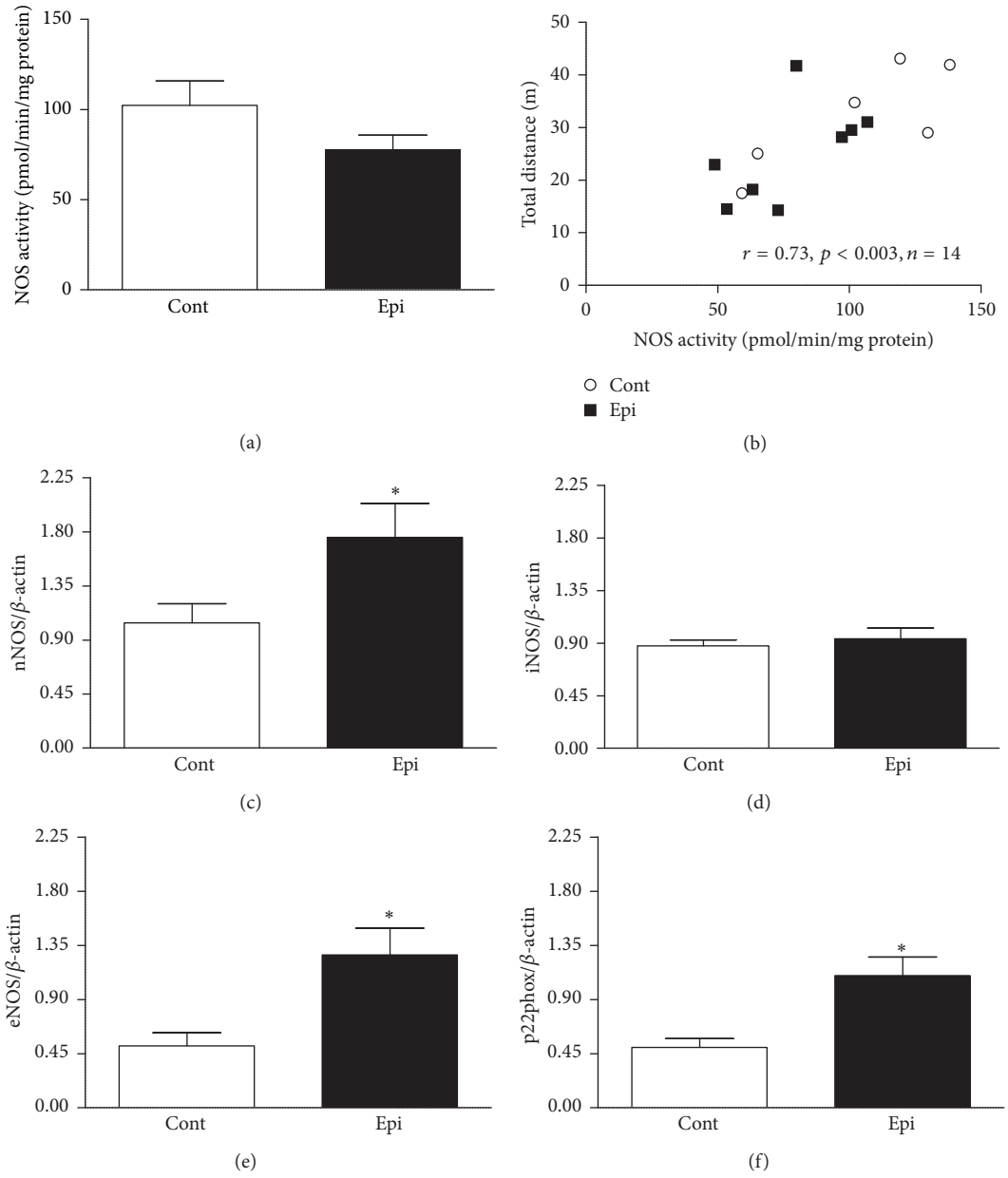

Figure 6: Effect of (−)-epicatechin treatment on nitric oxide synthase (NOS) activity (a), correlation between total distance traveled in the open-field and NOS activity (b) and gene expression of nNOS (c), iNOS (d), eNOS (e), and p22phox (f) in the cerebellum of spontaneously hypertensive rats. Values represent mean ± SEM; $n = 6$ for Cont and $n = 8$–10 for Epi. $^{*}p < 0.05$ versus Cont group. Abbreviations: Cont: control group, Epi: (−)-epicatechin-treated group, eNOS: endothelial NOS, iNOS: inducible NOS, nNOS: neuronal NOS, and p22phox: subunit of nicotinamide adenine dinucleotide phosphate oxidase.

of Moreno-Ulloa et al. has suggested G protein-coupled estrogen receptor (GPER) as a potential mediator of Epi effects in vasculature, which was associated with elevated phosphorylation of eNOS in Wistar rats [52]. We have shown recently that relatively short-term (10-day) dietary administration of Epi reversed endothelial dysfunction in the femoral artery of adult SHR by enhancing the NO-dependent component of relaxation [27]. Similarly, in people with never-treated essential hypertension, administering flavanol-rich dark chocolate (which has a high concentration of Epi) for two weeks normalized the NO-mediated endothelium-dependent relaxation in the brachial artery [53]. In this study, Epi elevated NOS activity in the aorta and enhanced the NO-dependent component of ACh-induced relaxation but failed

to affect overall relaxation in peripubertal SHR. Yet despite the lack of an effect on overall relaxation in the aorta, the improvements in vascular NO bioavailability and the NO-dependent component of relaxation observed in Epi-treated rats in this study may prevent vascular remodeling and reduce vascular wall stiffness [54], both of which are observed in hypertension [55–57].

Furthermore, the increased level of erythrocyte deformability observed in this study suggests improvements in blood flow as well as oxygenation in individual organs, similarly as has been recently observed in humans after two-week cocoa flavanol intake [58]. As erythrocytes contain functional eNOS and NO increases their deformability [59], it is plausible that the positive health effects of Epi are associated with the NO-related modulation of red blood cell properties [60]. Collectively, these observations in various experimental conditions both *in vitro* and *in vivo* suggest multiple mechanisms for the cardioprotective effects of Epi that are associated with improved NO bioavailability in the heart and vasculature as well as with enhancement of mechanical properties of the red blood cells.

In addition to the cardiovascular effects of Epi, our study pointed also to possible central effects. As mentioned above, Epi can cross the BBB [28, 29, 41]. Moreover, BBB was shown to be damaged in hypertension, specifically, in the brainstem and cerebellum [56, 61]. We chose these areas of the brain to focus on because the cerebellum integrates the neural control of movement and plays a role in the pathogenesis of ADHD [62]. The brainstem was selected as it is a part of the brain involved in the control of bodily motor function, in addition to the regulation of cardiac and respiratory functions.

In humans, the consumption of natural polyphenols, including cocoa flavanols, results in an acute improvement in visual and cognitive functions [63, 64], which may be relevant in the treatment of ADHD. Indeed, Pycnogenol®, a polyphenol extract from the bark of the French maritime pine, significantly reduces hyperactivity and improves attention, visual-motor coordination, and concentration in children with ADHD [65]. However, to our knowledge, the effect of Epi on ADHD symptoms has not yet been investigated in humans. Several studies in rodents have demonstrated the variable effects of flavanols on behavior. In Wistar rats, a single dose of cacao mass showed anxiolytic effects, but 2-week consumption did not reduce anxiety-related behavior. Locomotor activity in the OF was unaffected in those rats [66]. Two-week cocoa polyphenolic extract treatment had an antidepressant-like effect in Wistar-Unilever rats subjected to a forced-swim test without accompanying changes in locomotion in the OF [67]. In adult C57BL/6 mice, Epi had an anxiolytic effect as represented by an elevated ratio of distance traveled and time spent in the central zone of the OF compared to periphery [68]. However, it has to be noted that all of these studies were performed in normotensive rodents. We used SHR, which are known to be locomotor hyperactive with high levels of exploratory activity and reduced levels of anxiety compared to normotensive rat strains [5, 69, 70]. In our study, Epi administration attenuated locomotor hyperactivity as determined by decreases in the total distance traveled and the average speed of movement as well as by increases in total immobility. Epi also deceptively elevated anxiety in the OF, as suggested by reductions in total distance traveled and time spent in the central zone (in both absolute and relative values) and increased time spent in the corners. However, considering the innate hyperactivity and low anxiety levels of control SHR, Epi, in fact, corrected their behavioral abnormalities. These alterations were not associated with changes in NOS activity in the selected brain areas. However, in contrast to our findings in the LHV, we observed increased nNOS gene expression in both areas of the brain investigated here; eNOS gene expression increased only in the cerebellum. Interestingly, NOS activity in the brainstem and cerebellum correlated positively with locomotor activity and negatively with anxiety level (determined as a reduction in the central zone distance traveled) in the OF. These correlations were stronger in the cerebellum, suggesting that cerebellar NO-dependent mechanisms are more significantly involved in modulation of locomotor activity in young SHR. Yet, the studies performed to date in rats and in humans have demonstrated the considerable variability of findings on the role of NO in the modulation of behavior as well as the varying effects of NO in different neuroanatomical structures of the brain, which might even be antagonistic on the behavioral level [8, 34].

It is of interest that gene expression levels for the p22phox subunit of NADPH oxidase were increased in the cerebellum following Epi treatment, which is in contrast to the findings reported for the CVS in different animal models of hypertension [24–26]. If the Epi-induced upregulation of NADPH oxidase gene expression was to be followed by translation into functional enzyme, the abovementioned antioxidant effects of Epi could still maintain ROS at physiological levels. Our review of the literature did not reveal any study that has investigated the effect of subchronic Epi treatment on e/i/nNOS or p22phox NADPH oxidase subunit gene expression or activity in the CVS or brain of SHR. Yet our findings suggest that Epi exerts tissue-specific effects on the expression of individual NOS isoforms and NADPH oxidase subunits in SHR. These effects may not correlate with enzyme activity levels in the corresponding tissue [71].

The simultaneous prevention of BP increase and reduced hyperactivity of SHR observed in this study suggest the possibility of a common mechanism(s) underlying both pathologies. One possible mechanism is a reduction in noradrenergic neurotransmission, which is elevated in SHR and associated with high blood pressure and locomotor hyperactivity [62, 72]. In Epi-treated SHR rats, noradrenergic hyperfunction may be diminished by presynaptic α_2-autoreceptor-mediated feedback [62, 73], the improvement of calcium signaling [62, 74], and/or increases in bioavailability of NO [75]. These effects may prevent hypertension development and decrease locomotion in young SHR. Another plausible mechanism is the improvement of regional cerebral blood flow, as its alterations were observed in SHR and children with ADHD [62, 76] and flavanol-rich cocoa consumption improved it in older healthy volunteers [77].

Although our study brought interesting results related to simultaneous prevention of hypertension and reduction

of behavioral hyperactivity in juvenescent rats, there are certain limitations of this study. Firstly, NO production, gene expressions, and vascular function were determined in the aorta. These parameters may differ in smaller arteries, so the effect of Epi, especially in the small resistance arteries, needs to be investigated. Secondly, we did not determine Epi and/or Epi metabolites levels in blood, NOS phosphorylation, and involvement of GPER receptors. Thus, further studies are needed to elucidate the exact bioactive substance(s) and the exact site(s) of action of orally administered Epi in preventing and treating hypertension and behavioral hyperactivity in young subjects.

5. Conclusion

In conclusion, the results presented here showed that oral Epi treatment significantly prevented BP increase and reduced behavioral hyperactivity in young SHR. The mechanism underlying the positive effects of Epi observed in this study was related to improved cardiovascular NO bioavailability, due to elevated NOS activity and reduced $O_2^{\bullet-}$ levels in the CVS concurrently with elevations in plasma antioxidant capacity as well as red blood cell deformability. Altogether, these beneficial alterations could result in reduced sympathetic tone and improved cerebrovascular blood flow and tissue oxygenation, resulting in the prevention of hypertension and the reduction of locomotor hyperactivity. The results of this study may be relevant in pharmacological approaches to the prevention and treatment of hypertension and ADHD comorbidity in young subjects with a significant family history of hypertension. Our data also suggest tissue-specific influences of Epi in SHR that should be taken into account in evaluating the overall effects of Epi-containing foods.

Competing Interests

All authors have no potential financial or ethical competing interests regarding the contents of the submission.

Acknowledgments

The authors thank Mrs. J. Petova and M. Kovacsova for their technical assistance. This study was supported by Grants VEGA 2/0084/14, VEGA 1/0032/14, and APVV-0523-10. The infrastructure used in this study was partially supported by the "ITMS 26240120020–Establishment of the Centre for the Research on Composite Materials for Structural, Engineering and Medical Applications–CEKOMAT II" project.

References

[1] B. Falkner, "Hypertension in children and adolescents: epidemiology and natural history," *Pediatric Nephrology*, vol. 25, no. 7, pp. 1219–1224, 2010.

[2] E. G. Willcutt, "The prevalence of DSM-IV attention-deficit/hyperactivity disorder: a meta-analytic review," *Neurotherapeutics*, vol. 9, no. 3, pp. 490–499, 2012.

[3] H. R. Adams, P. G. Szilagyi, L. Gebhardt, and M. B. Lande, "Learning and attention problems among children with pediatric primary hypertension," *Pediatrics*, vol. 126, no. 6, pp. e1425–e1429, 2010.

[4] G. Cicco and A. Pirrelli, "Red blood cell (RBC) deformability, RBC aggregability and tissue oxygenation in hypertension," *Clinical Hemorheology and Microcirculation*, vol. 21, no. 3-4, pp. 169–177, 1999.

[5] T. Sagvolden, E. D. Hendley, and S. Knardahl, "Behavior of hypertensive and hyperactive rat strains: hyperactivity is not unitarily determined," *Physiology & Behavior*, vol. 52, no. 1, pp. 49–57, 1992.

[6] T. Sagvolden and E. B. Johansen, "Rat models of ADHD," in *Behavioral Neuroscience of Attention Deficit Hyperactivity Disorder and Its Treatment*, pp. 301–315, Springer, Berlin, Germany, 2011.

[7] V. Volke, A. Soosaar, S. Kõks, M. Bourin, P. T. Männistö, and E. Vasar, "7-Nitroindazole, a nitric oxide synthase inhibitor, has anxiolytic-like properties in exploratory models of anxiety," *Psychopharmacology*, vol. 131, no. 4, pp. 399–405, 1997.

[8] F. Freudenberg, A. Alttoa, and A. Reif, "Neuronal nitric oxide synthase (NOS1) and its adaptor, NOS1AP, as a genetic risk factors for psychiatric disorders," *Genes, Brain and Behavior*, vol. 14, no. 1, pp. 46–63, 2015.

[9] M. Majzunova, I. Dovinova, M. Barancik, and J. Y. H. Chan, "Redox signaling in pathophysiology of hypertension," *Journal of Biomedical Science*, vol. 20, no. 1, article 69, 2013.

[10] F. Ng, M. Berk, O. Dean, and A. I. Bush, "Oxidative stress in psychiatric disorders: evidence base and therapeutic implications," *The International Journal of Neuropsychopharmacology*, vol. 11, no. 6, pp. 851–876, 2008.

[11] J. Bouayed, H. Rammal, and R. Soulimani, "Oxidative stress and anxiety: relationship and cellular pathways," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 2, pp. 63–67, 2009.

[12] T. J. Guzik, N. E. J. West, R. Pillai, D. P. Taggart, and K. M. Channon, "Nitric oxide modulates superoxide release and peroxynitrite formation in human blood vessels," *Hypertension*, vol. 39, no. 6, pp. 1088–1094, 2002.

[13] P. Ferdinandy, H. Danial, I. Ambrus, R. A. Rothery, and R. Schulz, "Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure," *Circulation Research*, vol. 87, no. 3, pp. 241–247, 2000.

[14] Z. Szilvássy, T. Csont, T. Páli, M.-T. Droy-Lefaix, and P. Ferdinandy, "Nitric oxide, peroxynitrite and cGMP in atherosclerosis-induced hypertension in rabbits: beneficial effects of cicletanine," *Journal of Vascular Research*, vol. 38, no. 1, pp. 39–46, 2001.

[15] C. G. Fraga and P. Oteiza, "Flavanols and vascular health: molecular mechanisms to build evidence-based recommendations," *Free Radical Biology and Medicine*, vol. 75, supplement 1, p. S12, 2014.

[16] V. Schini-Kerth, "Role of polyphenols in improving endothelial dysfunction in diabetes," *Free Radical Biology and Medicine*, vol. 75, no. 1, pp. S11–S12, 2014.

[17] B. Buijsse, E. J. M. Feskens, F. J. Kok, and D. Kromhout, "Cocoa intake, blood pressure, and cardiovascular mortality: The Zutphen Elderly Study," *Archives of Internal Medicine*, vol. 166, no. 4, pp. 411–417, 2006.

[18] M. L. McCullough, K. Chevaux, L. Jackson et al., "Hypertension, the Kuna, and the epidemiology of flavanols," *Journal of Cardiovascular Pharmacology*, vol. 47, supplement 2, pp. S103–S109, 2006.

[19] S. Desch, J. Schmidt, D. Kobler et al., "Effect of cocoa products on blood pressure: systematic review and meta-analysis," *American Journal of Hypertension*, vol. 23, no. 1, pp. 97–103, 2010.

[20] K. Ried, T. Sullivan, P. Fakler, O. R. Frank, and N. P. Stocks, "Does chocolate reduce blood pressure? A meta-analysis," *BMC Medicine*, vol. 8, article 39, 2010.

[21] S. Ellinger, A. Reusch, P. Stehle, and H.-P. Helfrich, "Epicatechin ingested via cocoa products reduces blood pressure in humans: a nonlinear regression model with a Bayesian approach," *American Journal of Clinical Nutrition*, vol. 95, no. 6, pp. 1365–1377, 2012.

[22] H. Schroeter, C. Heiss, J. Balzer et al., "(−)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 4, pp. 1024–1029, 2006.

[23] M. K. Piskula and J. Terao, "Accumulation of (-)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues," *Journal of Nutrition*, vol. 128, no. 7, pp. 1172–1178, 1998.

[24] B. Piotrkowski, V. Calabró, M. Galleano, and C. G. Fraga, "(-)-Epicatechin prevents alterations in the metabolism of superoxide anion and nitric oxide in the hearts of L-NAME-treated rats," *Food and Function*, vol. 6, no. 1, pp. 155–161, 2015.

[25] M. C. Litterio, M. A. Vazquez Prieto, A. M. Adamo et al., "(−)-Epicatechin reduces blood pressure increase in high-fructose-fed rats: effects on the determinants of nitric oxide bioavailability," *Journal of Nutritional Biochemistry*, vol. 26, no. 7, pp. 745–751, 2015.

[26] M. Gómez-Guzmán, R. Jiménez, M. Sánchez et al., "Epicatechin lowers blood pressure, restores endothelial function, and decreases oxidative stress and endothelin-1 and NADPH oxidase activity in DOCA-salt hypertension," *Free Radical Biology and Medicine*, vol. 52, no. 1, pp. 70–79, 2012.

[27] M. Galleano, I. Bernatova, A. Puzserova et al., "Epicatechin reduces blood pressure and improves vasorelaxation in spontaneously hypertensive rats by NO-mediated mechanism," *IUBMB Life*, vol. 65, no. 8, pp. 710–715, 2013.

[28] L. Wu, Q.-L. Zhang, X.-Y. Zhang et al., "Pharmacokinetics and blood-brain barrier penetration of (+)-Catechin and (−)-Epicatechin in rats by microdialysis sampling coupled to high-performance liquid chromatography with chemiluminescence detection," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 37, pp. 9377–9383, 2012.

[29] A. Faria, D. Pestana, D. Teixeira et al., "Insights into the putative catechin and epicatechin transport across blood-brain barrier," *Food & Function*, vol. 2, no. 1, pp. 39–44, 2011.

[30] D. Mastroiacovo, C. Kwik-Uribe, D. Grassi et al., "Cocoa flavanol consumption improves cognitive function, blood pressure control, and metabolic profile in elderly subjects: the cocoa, cognition, and aging (CoCoA) study—a randomized controlled trial," *American Journal of Clinical Nutrition*, vol. 101, no. 3, pp. 538–548, 2015.

[31] I. Bernatova, M. P. Key, J. B. Lucot, and M. Morris, "Circadian differences in stress-induced pressor reactivity in mice," *Hypertension*, vol. 40, no. 5, pp. 768–773, 2002.

[32] Y. L. Su, L. K. Leung, Y. Huang, and Z.-Y. Chen, "Stability of tea theaflavins and catechins," *Food Chemistry*, vol. 83, no. 2, pp. 189–195, 2003.

[33] A. Puzserova, P. Slezak, P. Balis, and I. Bernatova, "Long-term social stress induces nitric oxide-independent endothelial dysfunction in normotensive rats," *Stress*, vol. 16, no. 3, pp. 331–339, 2013.

[34] N. Sestakova, A. Puzserova, M. Kluknavsky, and I. Bernatova, "Determination of motor activity and anxiety-related behaviour in rodents: methodological aspects and role of nitric oxide," *Interdisciplinary Toxicology*, vol. 6, no. 3, pp. 126–135, 2013.

[35] A. Mataseje, I. Beder, M. Kittova, J. Okkelova, and R. Vazan, "The assessment of erythrocyte deformability by filtration rate," *Bratislavské Lekárske Listy*, vol. 104, no. 4-5, pp. 158–160, 2003.

[36] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, and C. Rice-Evans, "Antioxidant activity applying an improved ABTS radical cation decolorization assay," *Free Radical Biology and Medicine*, vol. 26, no. 9-10, pp. 1231–1237, 1999.

[37] P. Slezak, A. Puzserova, P. Balis et al., "Genotype-related effect of crowding stress on blood pressure and vascular function in young female rats," *BioMed Research International*, vol. 2014, Article ID 413629, 11 pages, 2014.

[38] S. Cacanyiova, F. Kristek, M. Malekova, and K. Ondrias, "Effect of chronic neuronal nitric oxide-synthase inhibition on arterial function and structure in spontaneously hypertensive rats," *Journal of Physiology and Pharmacology*, vol. 63, no. 1, pp. 23–28, 2012.

[39] J. Zicha and J. Kuneš, "Ontogenetic aspects of hypertension development: analysis in the rat," *Physiological Reviews*, vol. 79, no. 4, pp. 1227–1282, 1999.

[40] M. Cebova and F. Kristek, "Age-dependent ultrastructural changes of coronary artery in spontaneously hypertensive rats," *General Physiology and Biophysics*, vol. 30, no. 4, pp. 364–372, 2011.

[41] M. M. Abd El Mohsen, G. Kuhnle, A. R. Rechner et al., "Uptake and metabolism of epicatechin and its access to the brain after oral ingestion," *Free Radical Biology and Medicine*, vol. 33, no. 12, pp. 1693–1702, 2002.

[42] S. D. Quine and P. S. Raghu, "Effects of (−)-epicatechin, a flavonoid on lipid peroxidation and antioxidants in streptozotocin-induced diabetic liver, kidney and heart," *Pharmacological Reports*, vol. 57, no. 5, pp. 610–615, 2005.

[43] Y. Steffen, C. Gruber, T. Schewe, and H. Sies, "Mono-O-methylated flavanols and other flavonoids as inhibitors of endothelial NADPH oxidase," *Archives of Biochemistry and Biophysics*, vol. 469, no. 2, pp. 209–219, 2008.

[44] E. J. B. Ruijters, A. R. Weseler, C. Kicken, G. R. M. M. Haenen, and A. Bast, "The flavanol (−)-epicatechin and its metabolites protect against oxidative stress in primary endothelial cells via a direct antioxidant effect," *European Journal of Pharmacology*, vol. 715, no. 1–3, pp. 147–153, 2013.

[45] P. D. Prince, C. R. Lanzi, J. E. Toblli et al., "Dietary (−)-epicatechin mitigates oxidative stress, NO metabolism alterations, and inflammation in renal cortex from fructose-fed rats," *Free Radical Biology and Medicine*, vol. 90, pp. 35–46, 2016.

[46] P. Schroeder, L.-O. Klotz, and H. Sies, "Amphiphilic properties of (−)-epicatechin and their significance for protection of cells against peroxynitrite," *Biochemical and Biophysical Research Communications*, vol. 307, no. 1, pp. 69–73, 2003.

[47] P. Schroeder, L.-O. Klotz, D. P. Buchczyk, C. D. Sadik, T. Schewe, and H. Sies, "Epicatechin selectively prevents nitration but not oxidation reactions of peroxynitrite," *Biochemical and Biophysical Research Communications*, vol. 285, no. 3, pp. 782–787, 2001.

[48] I. Ramirez-Sanchez, L. Maya, G. Ceballos, and F. Villarreal, "(−)-Epicatechin activation of endothelial cell endothelial nitric oxide synthase, nitric oxide, and related signaling pathways," *Hypertension*, vol. 55, no. 6, pp. 1398–1405, 2010.

[49] Y. Huang, N. W. K. Chan, C. W. Lau, X. Q. Yao, F. L. Chan, and Z. Y. Chen, "Involvement of endothelium/nitric oxide in

vasorelaxation induced by purified green tea (−)epicatechin," *Biochimica et Biophysica Acta—General Subjects*, vol. 1427, no. 2, pp. 322–328, 1999.

[50] A. Aggio, D. Grassi, E. Onori et al., "Endothelium/nitric oxide mechanism mediates vasorelaxation and counteracts vasoconstriction induced by low concentration of flavanols," *European Journal of Nutrition*, vol. 52, no. 1, pp. 263–272, 2013.

[51] A. Novakovic, M. Marinko, A. Vranic et al., "Mechanisms underlying the vasorelaxation of human internal mammary artery induced by (−)-epicatechin," *European Journal of Pharmacology*, vol. 762, pp. 306–312, 2015.

[52] A. Moreno-Ulloa, D. Mendez-Luna, E. Beltran-Partida et al., "The effects of (−)-epicatechin on endothelial cells involve the G protein-coupled estrogen receptor (GPER)," *Pharmacological Research*, vol. 100, pp. 309–320, 2015.

[53] D. Grassi, S. Necozione, C. Lippi et al., "Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives," *Hypertension*, vol. 46, no. 2, pp. 398–405, 2005.

[54] D. Grassi, G. Desideri, S. Necozione et al., "Cocoa consumption dose-dependently improves flow-mediated dilation and arterial stiffness decreasing blood pressure in healthy individuals," *Journal of Hypertension*, vol. 33, no. 2, pp. 294–303, 2015.

[55] M. Safar, P. Chamiot-Clerc, G. Dagher, and J. F. Renaud, "Pulse pressure, endothelium function, and arterial stiffness in spontaneously hypertensive rats," *Hypertension*, vol. 38, no. 6, pp. 1416–1421, 2001.

[56] P. W. Pires, C. M. D. Ramos, N. Matin, and A. M. Dorrance, "The effects of hypertension on the cerebral circulation," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 304, no. 12, pp. H1598–H1614, 2013.

[57] E. N. T. P. Bakker, G. Groma, L. J. A. Spijkers et al., "Heterogeneity in arterial remodeling among sublines of spontaneously hypertensive rats," *PLoS ONE*, vol. 9, no. 9, article e107998, 2014.

[58] C. Heiss, R. Sansone, H. Karimi et al., "Impact of cocoa flavanol intake on age-dependent vascular stiffness in healthy men: a randomized, controlled, double-masked trial," *Age*, vol. 37, no. 3, article 56, 2015.

[59] P. Kleinbongard, R. Schulz, T. Rassaf et al., "Red blood cells express a functional endothelial nitric oxide synthase," *Blood*, vol. 107, no. 7, pp. 2943–2951, 2006.

[60] O. Schnorr, T. Brossette, T. Y. Momma et al., "Cocoa flavanols lower vascular arginase activity in human endothelial cells in vitro and in erythrocytes in vivo," *Archives of Biochemistry and Biophysics*, vol. 476, no. 2, pp. 211–215, 2008.

[61] W. G. Mayhan, F. M. Faraci, and D. D. Heistad, "Disruption of the blood-brain barrier in cerebrum and brain stem during acute hypertension," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 251, no. 6, pp. H1171–H1175, 1986.

[62] V. A. Russell, "Neurobiology of animal models of attention-deficit hyperactivity disorder," *Journal of Neuroscience Methods*, vol. 161, no. 2, pp. 185–198, 2007.

[63] D. T. Field, C. M. Williams, and L. T. Butler, "Consumption of cocoa flavanols results in an acute improvement in visual and cognitive functions," *Physiology & Behavior*, vol. 103, no. 3-4, pp. 255–260, 2011.

[64] F. Jagla and O. Pechanova, "Age-related cognitive impairment as a sign of geriatric neurocardiovascular interactions: may polyphenols play a protective role?" *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 721514, 8 pages, 2015.

[65] J. Trebatická, S. Kopasová, Z. Hradečná et al., "Treatment of ADHD with French maritime pine bark extract, Pycnogenol®," *European Child & Adolescent Psychiatry*, vol. 15, no. 6, pp. 329–335, 2006.

[66] T. Yamada, Y. Yamada, Y. Okano, T. Terashima, and H. Yokogoshi, "Anxiolytic effects of short- and long-term administration of cacao mass on rat elevated T-maze test," *The Journal of Nutritional Biochemistry*, vol. 20, no. 12, pp. 948–955, 2009.

[67] M. Messaoudi, J.-F. Bisson, A. Nejdi, P. Rozan, and H. Javelot, "Antidepressant-like effects of a cocoa polyphenolic extract in Wistar-Unilever rats," *Nutritional Neuroscience*, vol. 11, no. 6, pp. 269–276, 2008.

[68] T. P. Stringer, D. Guerrieri, C. Vivar, and H. van Praag, "Plant-derived flavanol (−)epicatechin mitigates anxiety in association with elevated hippocampal monoamine and BDNF levels, but does not influence pattern separation in mice," *Translational Psychiatry*, vol. 5, article e493, 2015.

[69] A. Ramos, O. Berton, P. Mormède, and F. Chaouloff, "A multiple-test study of anxiety-related behaviours in six inbred rat strains," *Behavioural Brain Research*, vol. 85, no. 1, pp. 57–69, 1997.

[70] C. Gentsch, M. Lichtsteiner, and H. Feer, "Open field and elevated plus-maze: a behavioural comparison between spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats and the effects of chlordiazepoxide," *Behavioural Brain Research*, vol. 25, no. 2, pp. 101–107, 1987.

[71] S. Miyamoto, A. Ochiai, N. Boku et al., "Discrepancies between the gene expression, protein expression, and enzymatic activity of thymidylate synthase and dihydropyrimidine dehydrogenase in human gastrointestinal cancers and adjacent normal mucosa," *International Journal of Oncology*, vol. 18, no. 4, pp. 705–713, 2001.

[72] N. Herring, C. W. Lee, N. Sunderland, K. Wright, and D. J. Paterson, "Pravastatin normalises peripheral cardiac sympathetic hyperactivity in the spontaneously hypertensive rat," *Journal of Molecular and Cellular Cardiology*, vol. 50, no. 1, pp. 99–106, 2011.

[73] B. Langen and R. Dost, "Comparison of SHR, WKY and Wistar rats in different behavioural animal models: effect of dopamine D1 and alpha2 agonists," *ADHD Attention Deficit and Hyperactivity Disorders*, vol. 3, no. 1, pp. 1–12, 2011.

[74] N. Kumar, R. Kant, P. K. Maurya, and S. I. Rizvi, "Concentration dependent effect of (−)-epicatechin on Na^+/K^+-ATPase and Ca^{2+}-ATPase inhibition induced by free radicals in hypertensive patients: comparison with L-ascorbic acid," *Phytotherapy Research*, vol. 26, no. 11, pp. 1644–1647, 2012.

[75] J. Török, "Participation of nitric oxide in different models of experimental hypertension," *Physiological Research*, vol. 57, no. 6, pp. 813–825, 2008.

[76] J. F. Danker and T. Q. Duong, "Quantitative regional cerebral blood flow MRI of animal model of attention-deficit/hyperactivity disorder," *Brain Research*, vol. 1150, no. 1, pp. 217–224, 2007.

[77] F. A. Sorond, L. A. Lipsitz, N. K. Hollenberg, and N. D. L. Fisher, "Cerebral blood flow response to flavanol-rich cocoa in healthy elderly humans," *Neuropsychiatric Disease and Treatment*, vol. 4, no. 2, pp. 433–440, 2008.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 8405957, 12 pages
http://dx.doi.org/10.1155/2016/8405957

Research Article

The Chemical Profile of *Senna velutina* Leaves and Their Antioxidant and Cytotoxic Effects

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Received 4 June 2016; Accepted 14 September 2016

Academic Editor: Jerzy Kruk

Natural products can be a source of biomolecules with antioxidant activity which are able to prevent oxidative stress-induced diseases and show antitumor activity, making them important sources of new anticancer drug prototypes. In this context, this study aimed to analyze the chemical composition of an ethanol extract of *Senna velutina* leaves and to assess its antioxidant and cytotoxic activities in leukemic cells. The antioxidant properties were evaluated using a DPPH free radical scavenging assay and by examining the extract's inhibition of AAPH-induced lipid peroxidation in human erythrocytes. Its cytotoxicity and possible mechanisms of action were assessed in Jurkat and K562 leukemic cell lines. The ethanol extract contained flavonoids, such as epigallocatechin, epicatechin, kaempferol heteroside, rutin, and dimeric and trimeric proanthocyanidin derivatives. The extract exhibited antioxidant activity by scavenging free radicals and antihemolytic action, and it decreased malondialdehyde content in human erythrocytes. Furthermore, the extract also induced leukemic cell death by activating intracellular calcium and caspase-3, decreasing mitochondrial membrane potential, and arresting the cell cycle in S and G2 phases. Hence, *S. velutina* leaf extract contains antioxidant and antileukemic biomolecules with potential applications in diseases associated with oxidative stress and in the inhibition of tumor cell proliferation.

1. Introduction

Several diseases, including cancer, diabetes, atherosclerosis, inflammatory diseases, and premature aging, are related to oxidative stress [1]. Oxidative stress stems from excess of free radicals in the body and low antioxidant activity, resulting in damage to essential biomolecules such as nucleic acids, proteins, and lipids [2].

Cancer is an oxidative stress-related disease that causes high rates of morbidity and mortality in the global population [3]. Leukemias are cancers that affect the cells of the hematopoietic system; depending on their cellular origin and maturity stage, leukemias can be classified as either myeloid or lymphoid and as acute or chronic [4]. Surgery, radiotherapy, and chemotherapy [5] are among the main types of treatment for these cancers.

Biomolecules with anticancer activity at low therapeutic doses and with reduced side effects have been increasingly sought in recent decades [6]. Between 1940 and 2014, 49% of the 174 anticancer drugs that were made available on the market were either natural products or their derivatives [7]. Thus, there is a trend among the general population and the medical community to regard medicinal plants as alternative sources of antitumor drugs, provided that the therapeutic properties of such plants have been scientifically researched and proven [8]. The discoveries of paclitaxel [9], an anticancer drug, and of homoharringtonine [10], which is used in the treatment of acute and chronic myeloid leukemia, are examples of successful cases in the development of medicinal plant-derived drugs.

The search for new molecules with therapeutic properties, including antioxidant and anticarcinogenic activities, is facilitated by the vast biodiversity and bioprospecting potential in Brazil. *Senna* genus has been used in Brazilian folk medicine for its antioxidant, antimicrobial [11], anti-inflammatory [12], antidiabetic [13], and antitumor [14] activities, among other uses.

Taxonomically, some species have been transferred from *Cassia* genus to *Senna* genus [15]. This taxon currently comprises 500–600 species [14, 16], of which many have not been yet characterized with respect to their chemical compositions and biological properties as the arboreal species *Senna velutina* (Vogel) H. S. Irwin & Barneby (Fabaceae, Caesalpinioideae). In this context, the aim of the present study was to determine the chemical composition of an ethanol extract of *S. velutina* leaves and to evaluate its antioxidant and antileukemic activities.

2. Materials and Methods

2.1. Plant Material and Extract Preparation. *S. velutina* leaves were collected following the identification of the plant and authorization of the SISBIO (Sistema de Autorização e Informação em Biodiversidade, permit number 54470-1) in Dourados, Mato Grosso do Sul (S 22°05′545″ and W 055°20′746″), Brazil, oven-dried with the air circulation at a temperature of 45±5°C, and then ground in a Willy-type knife mill. An exsiccated sample was deposited in the Herbarium of the Federal University of Grande Dourados, Mato Grosso do Sul, Brazil, with registration number 4665.

The extract was then prepared by macerating the plant material in an ethanol 95% mixture at room temperature in the dark for 7 days. Then, the extract was filtered, and the residue was further extracted twice using the same process. After 21 days, the filtrate was concentrated in a rotary vacuum evaporator (Gehaka, São Paulo, SP, Brazil) to obtain the ethanol extract of *S. velutina* leaves (ESV). The dry extract yield was 29%, calculated using the following formula: extraction yield (%) = (weight of the freeze-dried extract × 100)/(weight of the original sample). The ESV was stored at −20°C protected from light.

2.2. Chemical Analysis. The extract was analyzed by Ultra Fast Liquid Chromatography (UFLC) (Shimadzu) coupled to Diode Array Detector (DAD) (240–800 nm, Shimadzu) and electrospray ionization time-of-flight (ESI-QTOF-micrOTOF QII) (operating in positive and negative mode, 120–1200 Da, Bruker Daltonics). A C-18 column was used (Kinetex, 2.6 μm, 150 × 2.2 mm, Phenomenex) protected by a guard column of the same material. The mobile phase was as follows: water (solvent A) and acetonitrile (solvent B) both with 0.1% of formic acid in a gradient of 0–2 min 3% B, 2–25 min 3–25% B, and 25–40 min 25–80% B followed by washing and reconditioning of the column (8 minutes). The flow rate was 0.3 mL/min and 1 μL (1 mg/mL) of extract was injected. The other micrOTOF-QII parameters were as follows: temperature, 200°C; N_2 drying gas flow rate, 9 L/min; Nebulizer, 4.0 bar; capillary voltage, −3500 V (negative) and +4500 V (positive); and internal calibration with TFA-NA injected at the end of the chromatographic analysis. The rutin and epicatechin standards were obtained from Sigma-Aldrich with a purity of ≥95%.

2.3. Antioxidant Activity

2.3.1. DPPH Free Radical Scavenging Activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of ESV was evaluated as described in D. Gupta and R. K. Gupta [17], with modifications. In this assay, 0.2 mL of ESV at different concentrations (1–1000 μg/mL) was added to 1.8 mL of DPPH solution (0.11 mM) in 80% ethanol. The mixture was incubated for 30 minutes at room temperature in the dark. Absorbance at 517 nm was then measured spectrophotometrically. Ascorbic acid and butylhydroxytoluene (BHT) were used as reference antioxidants. As a control, 0.2 mL of solvent used to dilute the extract was added to 1.8 mL of DPPH solution (0.11 mM) in 80% ethanol. Two independent experiments were performed in triplicate. The percentage inhibition was calculated relative to the control using the following equation:

$$\text{inhibition of DPPH radical (\%)} = \left(1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100. \quad (1)$$

2.3.2. Inhibition of Lipid Peroxidation in Human Erythrocytes

(1) Preparation of Erythrocyte Suspension. After approval by the Research Ethics Committee (Comitê de Ética em Pesquisa, CEP) of the University Center of Grande Dourados (UNIGRAN, Brazil (CEP process number: 123/12)), peripheral blood from healthy donors was collected into tubes containing sodium citrate which were then centrifuged at 400 ×g for 10 min. The plasma and leukocyte layer were discarded, and the erythrocytes were washed 3 times with 0.9% sodium chloride solution (NaCl) and centrifuged. Finally, 10% erythrocyte suspension was prepared in 0.9% NaCl solution to attain a 2.5% final concentration for further analysis.

(2) Hemolytic Activity and Inhibition of Oxidative Hemolysis. The ability of ESV to protect against lipid peroxidation was

evaluated using an antihemolytic assay in human erythrocytes that were incubated with the oxidant 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH), a thermoinducible initiator of lipid peroxidation, as described by Campos et al. [18]. To evaluate hemolytic activity and inhibition of oxidative hemolysis, erythrocytes were preincubated with either ESV or ascorbic acid (25–125 μg/mL) at 37°C for 30 min before the addition of 0.5 mL of either 0.9% NaCl or 50 mM AAPH (dissolved in 0.9% NaCl). In the assay, 1% ethanol was used as a solvent control, while 0.9% NaCl was used as the baseline hemolysis control. Total hemolysis was induced by incubating erythrocytes with distilled water. Samples were incubated at 37°C with regular stirring. The extent of hemolysis was determined at 60-minute intervals for an incubation period of 5 h. Tubes were centrifuged at 700 ×g for 5 minutes; 0.2 mL of the supernatant was collected and added to 1.8 mL of 0.9% NaCl for spectrophotometric reading at 540 nm. The hemolysis percentage was calculated using the formula $A/B \times 100$, where A is the sample absorbance and B is the absorbance of the total hemolysis sample. Three independent experiments were performed in triplicate.

(3) Malondialdehyde (MDA) Measurements. The inhibition of malondialdehyde (MDA) production, which is a byproduct of lipid peroxidation, was evaluated according to the method described by Campos et al. [18]. Erythrocytes were preincubated at 37°C for 30 min with either ESV or ascorbic acid (25–125 μg/mL) before the addition of 0.5 mL of 50 mM AAPH solution. The mixtures were incubated at 37°C with regular stirring; 1% ethanol was used as a negative control. The MDA concentration was determined at 60-minute intervals for a total of 5 h. To determine the MDA concentration, samples were centrifuged at 700 ×g for 5 min; 0.5 mL of each supernatant was collected and transferred to a tube containing 1 mL of 10 nM thiobarbituric acid (TBA), dissolved in 75 mM monobasic potassium phosphate buffer at pH 2.5. The standard controls used were 500 μL of a 20 mM MDA solution and 1 mL of TBA. Samples were incubated at 96°C for 45 min and allowed to cool before adding 4 mL of n-butyl alcohol and centrifuging at 1600 ×g for 5 min. The resulting supernatant was read at 532 nm in a spectrophotometer. Two independent experiments were performed in triplicate. Sample MDA levels were expressed in nM/mL, according to the following formula:

$$\text{MDA} = \text{Abs}_{\text{sample}} \times \left(\frac{20 \times 220.32}{\text{Abs}_{\text{standard}}} \right). \quad (2)$$

2.4. Cytotoxic Activity

2.4.1. Cell Culture. Leukemia human cell lines Jurkat and K562 were cultivated in RPMI 1640 (Sigma-Aldrich, Germany) culture medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin (Sigma-Aldrich, Germany), and 100 μg/mL streptomycin (Sigma-Aldrich, Germany). Cells were cultured in a humidified incubator containing 5% CO_2 at 37°C.

2.4.2. Cytotoxicity and Cell Death Profile. Cytotoxic activity and the cell death profile were evaluated according to the method described by Paredes-Gamero et al. [19]. Leukemic cells were seeded at 10^5 cells/mL in 96-well microplates and treated with ESV (0–100 μg/mL) for 24 h. Then, the cells were centrifuged 600 ×g for 6 min and resuspended in binding buffer (0.14 M NaCl, 2.5 mM $CaCl_2$, 0.01 M HEPES, and pH 7.4) and incubated at room temperature with 1 μL of Annexin V-FITC (BD Biosciences, San Diego, CA, USA) and 5 μg/mL propidium iodide (PI) Becton Dickinson, USA for 20 min. Sample analysis was performed using Accuri C6 flow cytometer (Becton Dickinson, San Diego, CA, USA), with acquisition of 3,000 events.

2.4.3. Measurement of Mitochondrial Membrane Potential. To evaluate the possible effects of ESV on mitochondrial membrane potential, leukemic cells were incubated with the fluorescent marker JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, Eugene, OR, USA) according to the method described by Moraes et al. [20]. JC-1 probe accumulates in mitochondria in a potential-dependent manner. Viable cells with high mitochondrial membrane potential are stained red. Upon reduction of the mitochondrial membrane potential, cells appear green. In this assay, cells were seeded into 24-well plates (10^5 cells/mL) containing supplemented media and were then incubated with 27.6 μg/mL (Jurkat cells) or 67.5 μg/mL (K562 cells) ESV for 24 h. The cells were then centrifuged and incubated with JC-1 (1 μg/mL) for 15 min at room temperature. Fluorescence readings were performed in a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, San Diego, CA, USA). A total of 10,000 events were collected per sample.

2.4.4. Caspase-3 Activity. Caspase-3 activity was assessed according to the method described by Moraes et al. [20], with minor modifications. Caspase activity was measured by flow cytometer. Leukemic lineages were treated with ESV (27.6 μg/mL) in 24-well microplates (10^5 cells/mL) for 24 h. Then the cells were fixed with 2% paraformaldehyde in PBS for 30 min and permeabilized with 0.01% saponin for 15 min at room temperature. Next, the cells were incubated for 1 h at 37°C with anti-cleaved-caspase 3-FITC antibody (Becton Dickinson, USA). After incubation for 40 min, the fluorescence was analyzed by Accuri C6 flow cytometer (Becton Dickinson, USA). A total of 10,000 events were acquired.

2.4.5. Intracellular Calcium and Pan-Caspase Inhibitors. The roles of intracellular calcium and caspases in ESV-promoted cytotoxicity were evaluated according to Bechara et al. [21], with minor modifications. Jurkat cells were pretreated for 1 h at 37°C under a 5% CO_2 atmosphere with either the intracelullar calcium chelator BAPTA-AM or the pan-caspase inhibitor Z-VAD-FMK; ESV (27.6 μg/mL) was then added to the cells and allowed to incubate for 24 h. Control and treated cells were resuspended in culture medium containing 0.05% trypan blue and were counted in a hemocytometer chamber to determine cell viability (trypan blue exclusion assay).

2.4.6. Cell Cycle Phases. Distribution of cell cycle was determined by PI staining and flow cytometry analysis. Leukemic

lineages (10^5 cells/mL) were treated with ESV (27.6 μg/mL) for 24 h and then were fixed and permeabilized as previously described and treated with 4 mg/mL RNase (Sigma-Aldrich, Germany) for 45 min at 37°C. For DNA labeling, cells were incubated with 5 μg/mL of PI (Sigma Aldrich, Germany). Percentages of cells within cell cycle compartments (G1, S, and G2/M) were determined by Accuri C6 flow cytometer (Becton Dickinson, USA). A total of 10,000 events were acquired.

2.5. Statistical Analyses. The data are shown as the mean ± standard error of the mean (SEM) and were analyzed for statistical significant differences between the groups using Student's *t*-test for comparison between two groups and one-way analysis of variance (ANOVA) followed by Dunnett's test for comparison of more than two groups using Prism 5 GraphPad Software. The results were considered significant when $P < 0.05$.

3. Results

3.1. Chemical Composition of ESV. The metabolites in ESV were identified by interpreting the UV absorption and mass spectra and comparing them with data in the literature. When available, compounds were compared with authentic standards for confirmation.

The identification of compound 2, with *m/z* 305.0660 $[M-H]^-$, was based on the fragmentation pattern of epigallocatechin proposed by Dou et al. [22], namely, *m/z* 261 $[M-CO_2]^-$, *m/z* 219 $[M-C_4H_6O_2]^-$, *m/z* 179 $[M-C_6H_6O_3]^-$, *m/z* 167 $[M-C_7H_6O_3]^-$, and *m/z* 165 $[M-C_7H_8O_3]^-$, and also on the detection of UV absorption at 270 nm.

Compounds 4, 8, and 9 showed UV absorption patterns that were characteristic of flavonols (270 and 340 nm). Their fragmentation patterns were consistent with those of heteroside derivatives of kaempferol, whose main fragment consists of *m/z* 285 $[C_{15}H_9O_6]^-$. Several compounds of this class have been described in *Senna* genus [23, 24].

Dimeric (compounds 6, 7, and 10–14) and trimeric (compounds 15–22) proanthocyanidins, comprising cassiaflavan, afzelechin, epicatechin, epigallocatechin, and naringenin subunits, were also observed. These derivatives, although rare in nature, are often reported in *Senna* genus; some authors consider them to be chemical markers for the genus [25–27]. The UV absorption maxima at 280 nm and the increase in reverse-phase retention time concomitant with decreased hydroxyl group content or increased degree of polymerization are in agreement with the study by Callemien and Collin [28]. Fragmentation patterns obtained by retro-Diels-Alder (RDA) fission, heterocyclic ring fission (HRF), and quinone methide (QM) can be used to characterize the subunits that make up proanthocyanidins [29]. A complete discussion on the elucidation of this class of compounds may be found in the literature [29, 30]. Thus, based on retention times, fragmentation profiles, and comparisons with previously published data, 22 compounds in ESV were characterized (Figure 1 and Table 1).

3.2. DPPH Free Radical Scavenging Activity. ESV was able to scavenge the DPPH free radical, with a 2.5-fold higher IC_{50} and maximum activity values relative to ascorbic acid; however, these values were lower than those obtained with BHT (Table 2).

3.3. Hemolytic Activity and Inhibition of Oxidative Hemolysis. Over the range of tested concentrations, ESV showed no hemolytic activity in human erythrocytes, as no hemolysis was observed after up to 5 h of incubation (Figure 2(a)).

The control antioxidant, ascorbic acid, was able to protect erythrocytes from hemolysis for up to 4 h of incubation when they were exposed to the oxidant AAPH (data not shown). ESV was able to protect erythrocytes for 5 h over the tested 50–125 μg/mL concentration range (Figure 2(b)), demonstrating its powerful antihemolytic activity.

3.4. MDA Measurements. The degree of protection conferred by ESV against AAPH-induced lipid peroxidation in human erythrocytes was evaluated by measuring MDA levels. At ESV concentrations of 100 and 125 μg/mL, the MDA levels were decreased throughout the course of the assay (data not shown) and after 5 h of incubation (Figure 2(c)).

3.5. Cytotoxic Activity and Cell Death Profile. ESV promoted cell death in both tested cell lines. IC_{50} values indicated that ESV was more effective in Jurkat cells than in the erythroleukemic cell line K562 (IC_{50} = 27.6 μg/mL and 67.5 μg/mL, resp.) (Figures 3(a) and 3(b)). ESV treatment promoted double staining in both cell lines. This type of death was evident in 71.9 ± 5.7% of Jurkat cells and 30.1 ± 2.8% of K562 cells after treatment with 40 and 80 μg/mL of extract, respectively (Figures 4(a) and 4(b)).

3.6. Mitochondrial Membrane Potential. The mitochondrial membrane potentials of Jurkat and K562 leukemic cells decreased after 24 h of incubation with ESV, as evidenced by a decrease in red fluorescence and an increase in green fluorescence compared to untreated cells. The mitochondrial membrane potential was reduced by 91.0±4.3% in Jurkat cells and by 74.7 ± 7.3% in K562 cells after treatment with 27.6 and 67.5 μg/mL of extract, respectively (Figures 5(a) and 5(b)).

3.7. Caspase-3 Activity. The Jurkat cell line, which was more sensitive to ESV activity, was used to investigate ESV-promoted cell death mechanisms. The fluorescence histogram (Figure 6(a)) showed a rightward shift (greater fluorescence values), indicating the activation of the apoptosis-inducing enzyme caspase-3. Cleaved caspase-3 levels increased 4.5-fold in ESV-treated cells relative to untreated cells (Figure 6(b)).

3.8. Pan-Caspase Inhibition and Intracellular Calcium Chelation. Jurkat cells were preincubated with the pan-caspase inhibitor Z-VAD-FMK to assess whether ESV cytotoxicity was mainly mediated by caspase activation. Z-VAD-FMK pretreatment did not decrease ESV-induced cell death (Figure 7). However, the intracellular Ca^{2+} chelator BAPTA-AM partially inhibited ESV-induced death in Jurkat cells (Figure 7).

Table 1: Compounds identified in ESV by UFLC-DAD-ESI-QTOF-micrOTOF QII.

ID	Time (min)	UV	$[M\text{-}H]^-$ (m/z)	Molecular formula	Error (ppm)	MS/MS	Compound
1	1.1	—	341.1086	$C_{12}H_{20}O_{11}$	0.6	341: 179	Sugar derivative
2	8.6	270	305.0660	$C_{15}H_{14}O_7$	2.3	305: 261, 219, 179, 167, 165	Epigallocatechin
3	12.5	280	289.0714	$C_{15}H_{14}O_6$	1.3	289: 245, 205, 203	Epicatechin
4	19.1	270/346	593.1524	$C_{27}H_{30}O_{15}$	2.1	593: 447, 285	Kaempferol-*O*-hexoside-deoxyhexoside
5	19.7	270/346	609.1450	$C_{27}H_{30}O_{16}$	1.9	609: 463, 301	Rutin
6	20.5	280	545.1440	$C_{30}H_{26}O_{10}$	2.5	545: 305, 239, 219, 167, 165	Cassiaflavan-epigallocatechin
7	20.8	280	545.1440	$C_{30}H_{26}O_{10}$	2.5	545: 305, 239, 219, 167, 165	Cassiaflavan-epigallocatechin
8	21.2	268/338	593.1522	$C_{27}H_{30}O_{15}$	1.6	593: 447, 285	Kaempferol-*O*-hexoside-deoxyhexoside
9	22.3	270/342	593.1521	$C_{27}H_{30}O_{15}$	1.5	593: 447, 285	Kaempferol-*O*-hexoside-deoxyhexoside
10	22.6	280	529.1489	$C_{30}H_{26}O_9$	2.9	529: 289, 245, 239, 203	Cassiaflavan-epicatechin
11	23.5	280	529.1484	$C_{30}H_{26}O_9$	3.7	529: 289, 245, 239, 203	Cassiaflavan-epicatechin
12	24.9	280	529.1489	$C_{30}H_{26}O_9$	2.9	529: 267, 257, 239, 151	Naringenin-afzelechin
13	28.3	280	513.1551	$C_{30}H_{26}O_8$	0.8	513: 267, 255, 239	Cassiaflavan-afzelechin
14	28.8	280	513.1541	$C_{30}H_{26}O_8$	2.8	513: 267, 255, 239	Cassiaflavan-afzelechin
15	30.2	280	785.2266	$C_{45}H_{38}O_{13}$	3.4	785: 435, 305, 239	Cassiaflavan-cassiaflavan-epigallocatechin
16	30.4	280	785.2285	$C_{45}H_{38}O_{13}$	1.7	785: 435, 305, 239	Cassiaflavan-cassiaflavan-epigallocatechin
17	30.5	280	785.2255	$C_{45}H_{38}O_{13}$	2.0	785: 435, 305, 239	Cassiaflavan-cassiaflavan-epigallocatechin
18	31.1	280	769.2310	$C_{45}H_{38}O_{12}$	2.6	769: 529, 419, 289	Cassiaflavan-cassiaflavan-epicatechin
19	31.3	280	769.2303	$C_{45}H_{38}O_{12}$	1.6	769: 529, 419, 289	Cassiaflavan-cassiaflavan-epicatechin
20	31.5	280	769.2295	$C_{45}H_{38}O_{12}$	0.6	769: 377, 267, 239	Cassiaflavan-naringenin-afzelechin
21	31.7	280	769.2310	$C_{45}H_{38}O_{12}$	2.6	769: 377, 267, 239	Cassiaflavan-naringenin-afzelechin
22	31.8	280	769.2297	$C_{45}H_{38}O_{12}$	0.9	769: 377, 267, 239	Cassiaflavan-naringenin-afzelechin

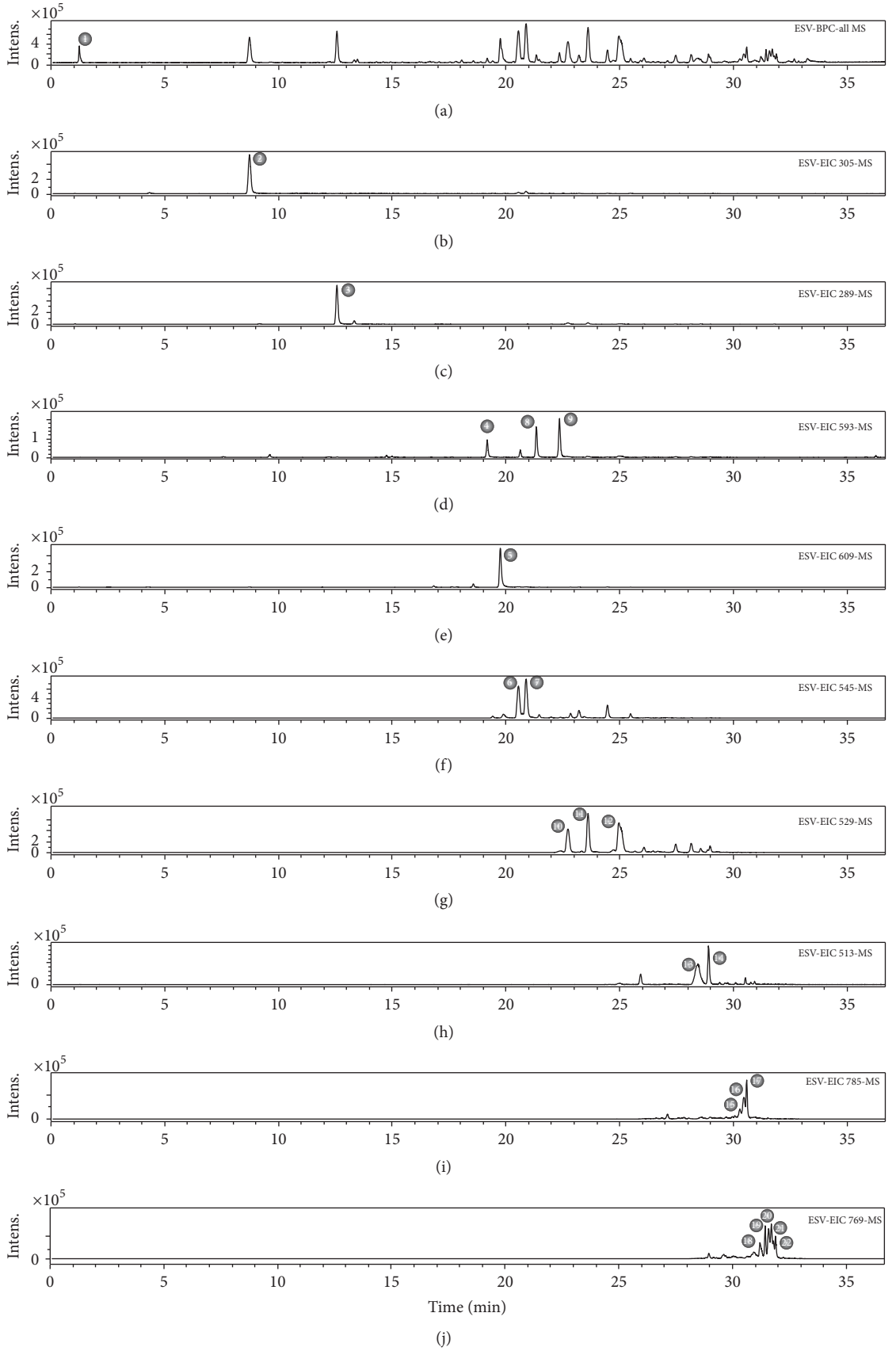

Figure 1: UFLC-DAD-ESI-QTOF-micrOTOF QII chemical profiling (negative mode) of an ethanol extract of *S. velutina* leaves. (a) Base peak chromatograms (BPC). (b) Extract ion chromatogram (EIC) of *m/z* 305. (c) EIC of *m/z* 289. (d) EIC of *m/z* 593. (e) EIC of *m/z* 609. (f) EIC of *m/z* 545. (g) EIC of *m/z* 529. (h) EIC of *m/z* 513. (i) EIC of *m/z* 785. (j) EIC of *m/z* 769.

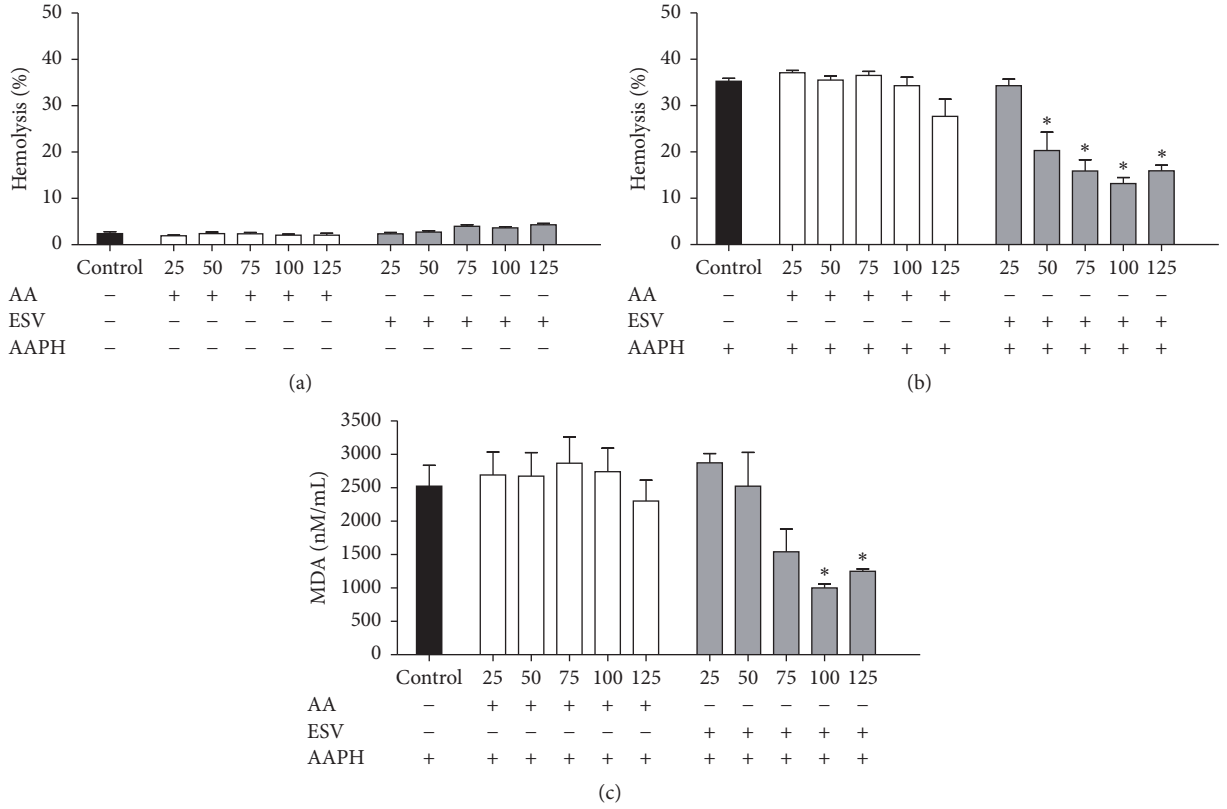

Figure 2: Hemolysis and MDA content in human erythrocytes incubated for 5 hours with ascorbic acid (AA) and ESV (50–125 μg/mL). (a) Hemolytic activity of ESV in the absence of AAPH. (b) Antihemolytic activity after addition of AAPH. (c) Malondialdehyde (MDA) concentration (nM/mL) after addition of the oxidizing agent. $^{*}P < 0.05$ compared to the AAPH-only control (erythrocytes incubated with oxidant only).

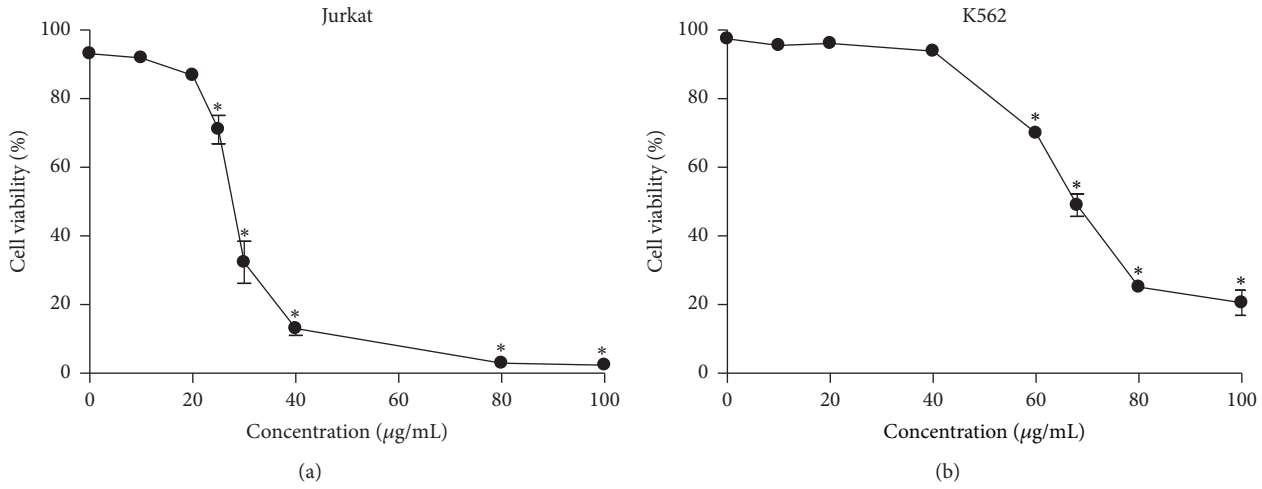

Figure 3: Viability of leukemic Jurkat (a) and K562 (b) cells after treatment with different concentrations of ESV. $^{*}P < 0.05$ compared to the untreated control group.

3.9. Cell Cycle Phases. Histograms were used to show the distributions of cell cycle phases in control and ESV-treated Jurkat cells after 24 h of incubation (Figure 8(a)). The results show a decreased portion of cells in G0/G1 phase (17.4±0.6%) and increased portions of cells in S and G2/M phases (30.7 ± 1.8% and 26.5 ± 1.9%, resp.) (Figure 8(b)). These results indicate that ESV inhibits the progression of cell cycle transitions.

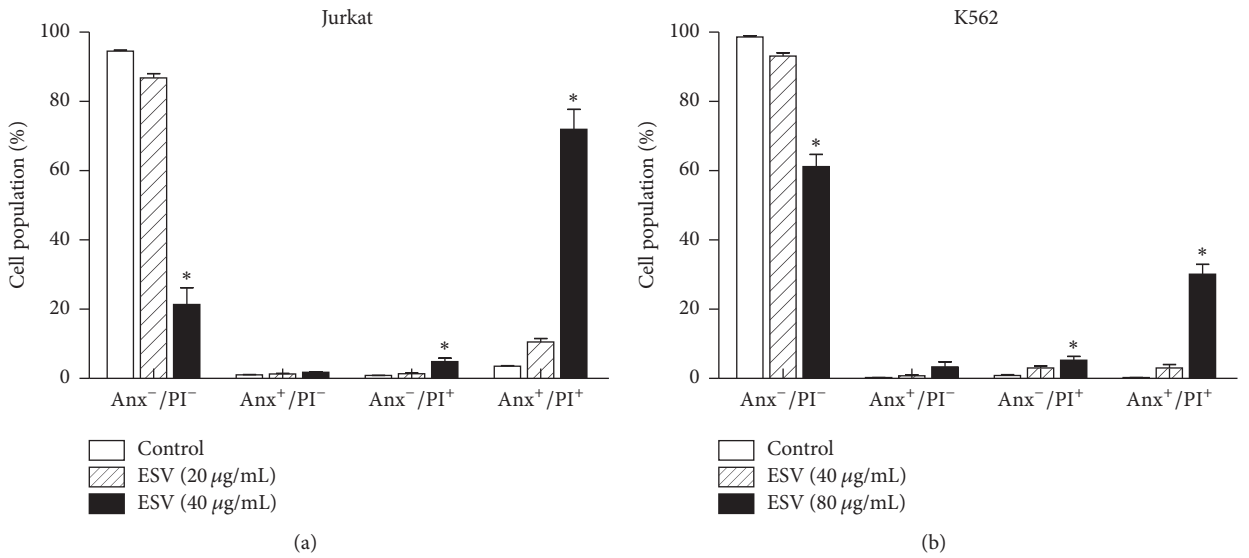

FIGURE 4: Cell death profiles of ESV-treated Jurkat (a) and K562 (b) cells. Anx^-/PI^-, viable cells; Anx^+/PI^-, apoptotic cells; Anx^-/PI^+, necrotic cells; and Anx^+/PI^+, late apoptotic cells. $^*P < 0.05$ compared to the respective control groups.

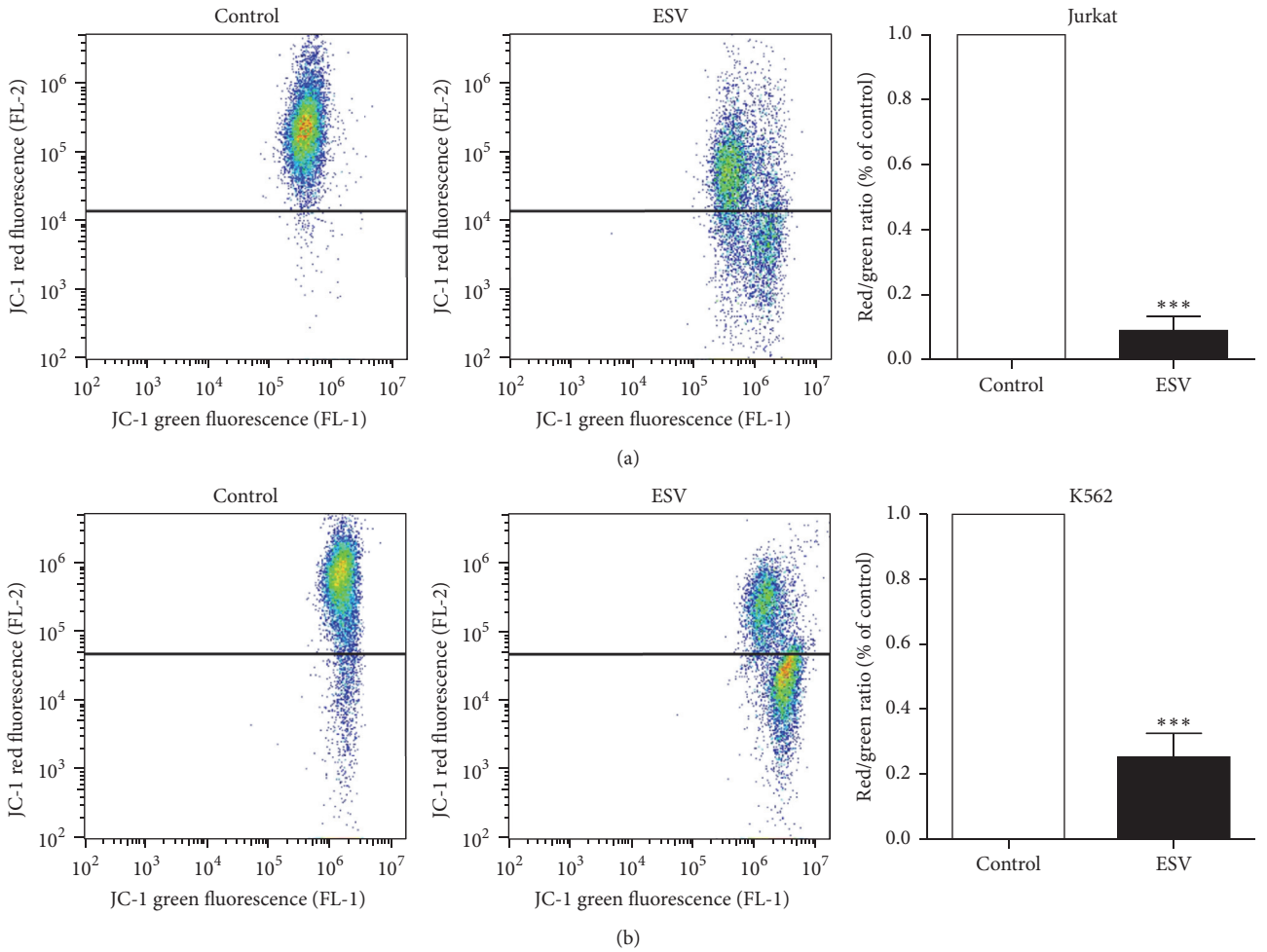

FIGURE 5: Mitochondrial membrane potential of leukemic Jurkat (a) and K562 (b) cells treated with different ESV concentrations. $^{***}P < 0.0001$ compared to the untreated control group.

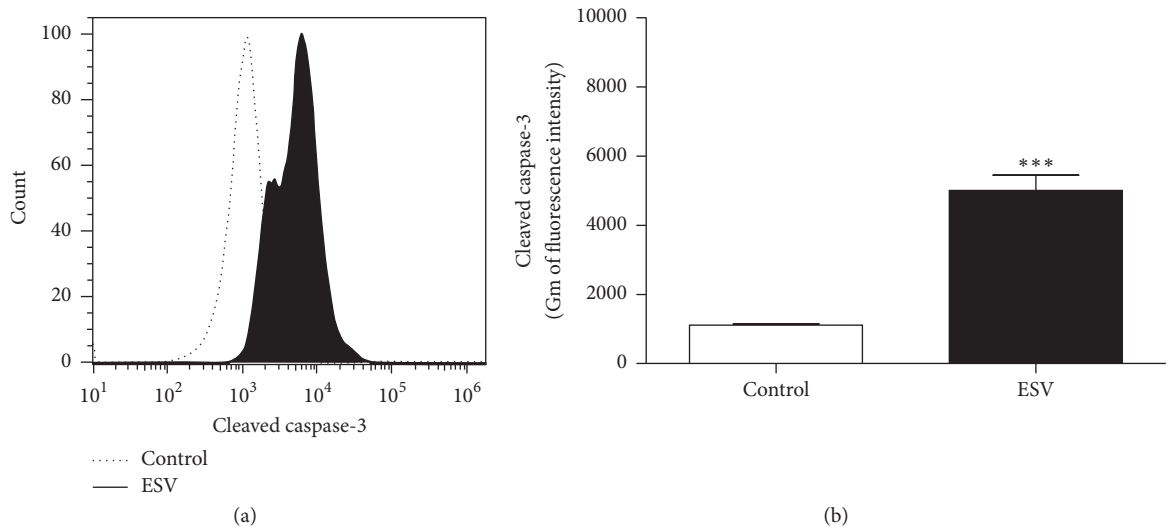

Figure 6: Histogram (a) and representative graph (b) of caspase-3 activation in ESV-treated Jurkat cells. $^{***}P < 0.0001$ compared to the untreated control group.

Table 2: IC_{50} and maximal DPPH radical scavenging activity of standard antioxidants and of ESV.

Sample	IC_{50} (μg/mL)	Maximal inhibition	
		%	μg/mL
Ascorbic acid	2.6 ± 0.8	90.9 ± 1.6	10
BHT	21.3 ± 1.2	92.4 ± 1.2	250
ESV	6.3 ± 1.3	92.4 ± 0.4	25

Values are means ± SEM.

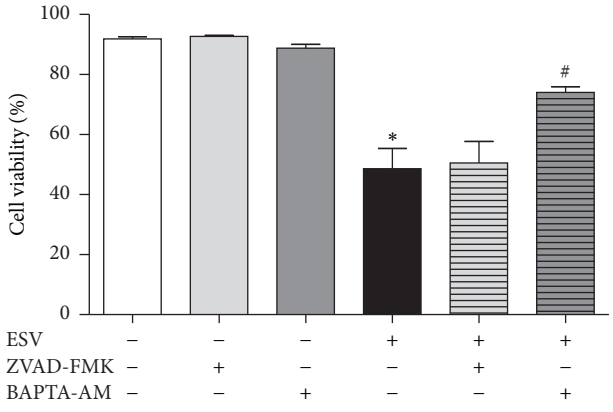

Figure 7: Involvement of caspases (via the pan-caspase inhibitor Z-VAD-FMK) and intracellular calcium (using the chelator BAPTA-AM) in ESV-induced Jurkat cell cytotoxicity. $^{*}P < 0.05$ compared to the untreated control group. $^{\#}P < 0.05$ compared to the ESV group.

4. Discussion

Brazilian biodiversity is rich in active compounds with high potential for development of new therapeutic drugs, particularly antioxidants and anticancer agents. Several plant species found in Brazil have been characterized for their antioxidant and cytotoxic activities in several tumor cell lines [31–33].

In the present study, an ethanol extract of *S. velutina* leaves exhibited antioxidant activity and showed cytotoxic effects against two leukemic cell lines, Jurkat and K562. The antioxidant activity of ESV was demonstrated in AAPH-incubated human erythrocytes; DPPH free radical scavenging and inhibition of lipid peroxidation led to decreases in oxidative hemolysis and malondialdehyde production. This activity of ESV is likely related to the presence of flavone derivatives with antioxidant activity, such as epigallocatechin, epicatechin, rutin, kaempferol glycosides, and dimeric and trimeric proanthocyanidins, in the leaves [34–37].

Flavonoids can donate hydrogen atoms to radicals, protecting against lipid peroxidation, and this ability is associated with the presence of a dihydroxylated B-ring [38]. Similar to other phenolic compounds, the antioxidant activity of flavonoids is ascribed to the presence of free hydroxyl groups in the molecule, and the level of antioxidant activity increases concomitantly with the number of hydroxyl groups [39].

Excess free radicals in the body can promote not only lipid peroxidation but also oxidative DNA damage, leading to the development of early stages of mutagenesis and carcinogenesis [40, 41]. Thus, compounds with antioxidant properties have fundamental roles in preventing diseases such as cancer.

The cytotoxic activity of ESV against leukemic lines was evaluated with an Annexin/PI-stain cell death assay. Furthermore, activation of caspase-3 was observed but was not confirmed to be a main mechanism of cell death when the pan-caspase inhibitor Z-VAD-FMK was used. Different extracts can promote several different death mechanisms simultaneously because of their different compositions. This fact complicates the analysis and identification of specific cell death pathways.

The ESV-induced decrease in mitochondrial membrane potential is consistent with the observed cytotoxic effect of ESV against leukemic cells. The changes in potential are likely due to increased mitochondrial membrane permeability

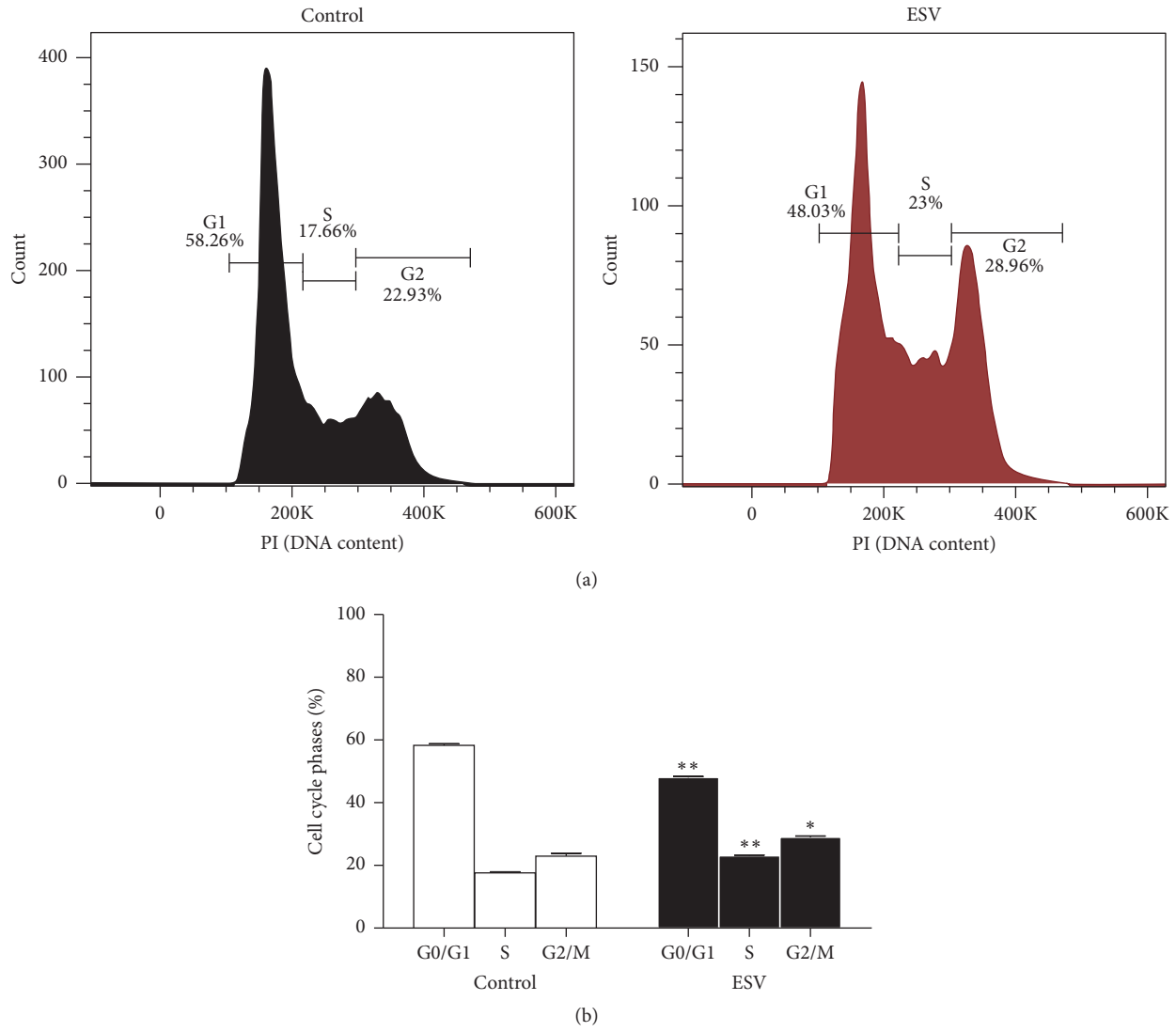

Figure 8: Histogram (a) and representative graph (b) of cell cycle distribution after 24 h of treatment with ESV. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared to the untreated control group.

arising from an increase in intracellular calcium levels, a characteristic of necrotic cell death [42, 43].

The present study demonstrated the involvement of calcium in cell death, as the reduced cell viability of extract-treated cells was reversed by incubation with the calcium chelator BAPTA-AM. High calcium levels promote the opening of mitochondrial permeability transition pores; these pores are nonselective and thus release the contents of the intermembrane space of the mitochondrion [44].

Another mechanism of the ESV-induced cytotoxicity against Jurkat cells consists of cell cycle arrest. ESV promoted a decrease in the number of cells in G0/G1 phase and an increase in the number of cells in S and G2 phases. Established anticancer drugs, such as cisplatin and doxorubicin, exert a similar profile change in the tumor cell cycle [14, 45]. Mueller et al. [46] observed that cisplatin is likely to be active in G2/M phases because cells in these phases are more sensitive to DNA damage, as DNA repair mechanisms are less active than in G1/S phases. Flavonoids are phytochemicals that are known to induce cell cycle arrest by decreasing cellular levels of cyclin B and cyclin-dependent kinase 1, which are responsible for controlling cell cycle progression between S and M stages [47]. Furthermore, one of the major anticancer mechanisms ascribed to flavonoids is their ability to induce cell cycle changes in tumor cell lines [48]. Thus, cyclin-dependent kinase inhibitors (CDKIs) have generated great interest for their ability to arrest the tumor cell cycle and prevent tumor cell proliferation; such cell cycle arrest may be the main mechanism through which ESV operates [49]. However, plant extracts are natural products with complex chemical composition, and biologically active compounds in extracts may act alone or synergistically through different pathways.

In conclusion, an ethanol extract of *S. velutina* leaves exhibited antioxidant activity and showed cytotoxic effects

on leukemic cells by activating intracellular calcium and caspase-3, decreasing mitochondrial membrane potential, and arresting the cell cycle in S and G2 phases.

Abbreviations

AA:	Ascorbic acid
AAPH:	2,2′-Azobis-(2-amidinopropane) dihydrochloride
Abs:	Absorbance
Anx:	Annexin V-FITC
BHT:	Butylhydroxytoluene
Ca^{2+}:	Calcium
$CaCl_2$:	Calcium chloride
DAD:	Diode Array Detector
DPPH:	2,2-Diphenyl-1-picrylhydrazyl
ESI-QTOF-micrOTOF QII:	Electrospray ionization time-of-flight
ESV:	Ethanol extract of *S. velutina* leaves
JC-1:	5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
MDA:	Malondialdehyde
NaCl:	Sodium chloride
PI:	Propidium iodide
SEM:	Standard error of the mean
TBA:	Thiobarbituric acid
UFLC:	Ultra Fast Liquid Chromatography.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by grants of the Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Mato Grosso do Sul (FUNDECT), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Edson Lucas dos Santos, Carlos Alexandre Carollo, and Edgar J. Paredes-Gamero are recipients of fellowships from Brazilian National Research Council (CNPq), Brazil.

References

[1] S. Sultan, "Reviewing the protective role of antioxidants in oxidative stress caused by free radicals," *Asian Pacific Journal of Health Science*, vol. 1, pp. 401–406, 2014.

[2] V. Lobo, A. Patil, A. Phatak, and N. Chandra, "Free radicals, antioxidants and functional foods: impact on human health," *Pharmacognosy Reviews*, vol. 4, no. 8, pp. 118–126, 2010.

[3] R. Siegel, D. Naishadham, and A. Jemal, "Cancer statistics, 2013," *CA: A Cancer Journal for Clinicians*, vol. 63, no. 1, pp. 11–30, 2013.

[4] American Cancer Society, *Leukemia-Chronic Myeloid (Myelogenous) Overview*, 2013.

[5] M. J. S. Asmaa, H. A. N. Al-Jamal, C. Y. Ang, J. M. Asan, A. Seeni, and M. F. Johan, "Apoptosis induction in MV4-11 and K562 human leukemic cells by *Pereskia sacharosa* (Cactaceae) leaf crude extract," *Asian Pacific Journal of Cancer Prevention*, vol. 15, no. 1, pp. 475–481, 2014.

[6] T. Srdic-Rajic, N. Tisma-Miletic, M. Cavic et al., "Sensitization of K562 leukemia cells to doxorubicin by the *Viscum album* extract," *Phytotherapy Research*, vol. 30, no. 3, pp. 485–495, 2016.

[7] D. J. Newman and G. M. Cragg, "Natural products as sources of new drugs from 1981 to 2014," *Journal of Natural Products*, vol. 79, no. 3, pp. 629–661, 2016.

[8] M. J. Balunas and A. D. Kinghorn, "Drug discovery from medicinal plants," *Life Sciences*, vol. 78, no. 5, pp. 431–441, 2005.

[9] A. Nagle, W. Hur, and N. S. Gray, "Antimitotic agents of natural origin," *Current Drug Targets*, vol. 7, no. 3, pp. 305–326, 2006.

[10] O. Prakash, A. Kumar, P. Kumar, and Ajeet, "Anticancer potential of plants and natural products: a review," *American Journal of Pharmacological Sciences*, vol. 1, no. 6, pp. 104–115, 2013.

[11] Y. W. Mak, L. O. Chuah, R. Ahmad, and R. Bhat, "Antioxidant and antibacterial activities of hibiscus (*Hibiscus rosa-sinensis* L.) and Cassia (*Senna bicapsularis* L.) flower extracts," *Journal of King Saud University-Science*, vol. 25, pp. 275–282, 2013.

[12] A. C. Susunaga-Notario, S. Pérez-Gutiérrez, M. Á. Zavala-Sánchez et al., "Bioassay-guided chemical study of the anti-inflammatory effect of *Senna villosa* (Miller) H.S. Irwin & Barneby (Leguminosae) in TPA-induced ear edema," *Molecules*, vol. 19, no. 7, pp. 10261–10278, 2014.

[13] E. Thilagam, B. Parimaladevi, C. Kumarappan, and S. C. Mandal, "α-Glucosidase and α-amylase inhibitory activity of *Senna surattensis*," *Journal of Acupuncture and Meridian Studies*, vol. 6, no. 1, pp. 24–30, 2013.

[14] R. M. Pereira, G. Á. Ferreira-Silva, M. Pivatto et al., "Alkaloids derived from flowers of *Senna spectabilis*, (–)-cassine and (–)-spectaline, have antiproliferative activity on HepG2 cells for inducing cell cycle arrest in G1/S transition through ERK inactivation and downregulation of cyclin D1 expression," *Toxicology in Vitro*, vol. 31, pp. 86–92, 2016.

[15] C. Viegas Junior, A. de Rezende, D. H. S. Silva et al., "Aspectos químicos, biológicos e etnofarmacológicos do gênero *Cassia*," *Química Nova*, vol. 29, no. 6, pp. 1279–1286, 2006.

[16] L. Delumlle and K. Demeyer, *Anthraquinones in Plants—Source, Safety and Applications in Gastrointestinal Health*, Nottingham University Press, 2008.

[17] D. Gupta and R. K. Gupta, "Bioprotective properties of Dragon's blood resin: *in vitro* evaluation of antioxidant activity and antimicrobial activity," *BMC Complementary and Alternative Medicine*, vol. 11, article 13, pp. 1–9, 2011.

[18] J. F. Campos, U. P. Das Santos, P. D. S. Da Rocha et al., "Antimicrobial, antioxidant, anti-inflammatory, and cytotoxic activities of propolis from the stingless bee *Tetragonisca fiebrigi* (Jataí)," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, Article ID 296186, 11 pages, 2015.

[19] E. J. Paredes-Gamero, M. N. C. Martins, F. A. M. Cappabianco, J. S. Ide, and A. Miranda, "Characterization of dual effects induced by antimicrobial peptides: regulated cell death or membrane disruption," *Biochimica et Biophysica Acta—General Subjects*, vol. 1820, no. 7, pp. 1062–1072, 2012.

[20] V. W. R. Moraes, A. C. F. Caires, E. J. Paredes-Gamero, and T. Rodrigues, "Organopalladium compound 7b targets mitochondrial thiols and induces caspase-dependent apoptosis in human

myeloid leukemia cells," *Cell Death & Disease*, vol. 4, article e658, 8 pages, 2013.

[21] A. Bechara, C. M. V. Barbosa, E. J. Paredes-Gamero et al., "Palladacycle (BPC) antitumour activity against resistant and metastatic cell lines: the relationship with cytosolic calcium mobilisation and cathepsin B activity," *European Journal of Medicinal Chemistry*, vol. 79, pp. 24–33, 2014.

[22] J. Dou, V. S. Y. Lee, J. T. C. Tzen, and M.-R. Lee, "Identification and comparison of phenolic compounds in the preparation of oolong tea manufactured by semifermentation and drying processes," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 18, pp. 7462–7468, 2007.

[23] K. V. Rao, A. G. Damu, B. Jayaprakasam, and D. Gunasekar, "Flavonol glycosides from *Cassia hirsuta*," *Journal of Natural Products*, vol. 62, no. 2, pp. 305–306, 1999.

[24] N. H. El-Sayed, A. M. A. Dooh, S. A. M. El-Khrisy, and T. J. Mabry, "Flavonoids of *Cassia italica*," *Phytochemistry*, vol. 31, no. 6, p. 2187, 1992.

[25] S. Morimoto, G. Nonaka, R. Chen, and I. Nishioka, "Tannins and related compounds. LXI. Isolation and structures of novel bi- and triflavanoids from the leaves of *Cassia fistula* L.," *Chemical and Pharmaceutical Bulletin*, vol. 36, no. 1, pp. 39–47, 1988.

[26] T. Hatano, A. Yamashita, T. Hashimoto et al., "Flavan dimers with lipase inhibitory activity from *Cassia nomame*," *Phytochemistry*, vol. 46, no. 5, pp. 893–900, 1997.

[27] J. Coetzee, L. Mciteka, E. Malan, and D. Ferreira, "Structure and synthesis of butiniflavan-epicatechin and -epigallocatechin probutinidins," *Phytochemistry*, vol. 52, no. 4, pp. 737–743, 1999.

[28] D. Callemien and S. Collin, "Use of RP-HPLC-ESI (-)-MS/MS to differentiate various proanthocyanidin isomers in lager beer extracts," *The Journal of the American Society of Brewing Chemists*, vol. 66, pp. 109–115, 2008.

[29] L. Gu, M. A. Kelm, J. F. Hammerstone et al., "Liquid chromatographic/electrospray ionization mass spectrometric studies of proanthocyanidins in foods," *Journal of Mass Spectrometry*, vol. 38, no. 12, pp. 1272–1280, 2003.

[30] S. A. Lazarus, G. E. Adamson, J. F. Hammerstone, and H. H. Schmitz, "High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages," *Journal of Agricultural and Food Chemistry*, vol. 47, no. 9, pp. 3693–3701, 1999.

[31] J. C. Casagrande, L. F. B. Macorini, K. A. Antunes et al., "Antioxidant and cytotoxic activity of hydroethanolic extract from *Jacaranda decurrens* leaves," *PLoS ONE*, vol. 9, no. 11, Article ID e112748, pp. 1–8, 2014.

[32] J. M. Serpeloni, A. F. Leal Specian, D. L. Ribeiro et al., "Antimutagenicity and induction of antioxidant defense by flavonoid rich extract of *Myrcia bella* Cambess. in normal and tumor gastric cells," *Journal of Ethnopharmacology*, vol. 176, pp. 345–355, 2015.

[33] A. S. N. Formagio, D. D. Ramos, M. C. Vieira et al., "Phenolic compounds of *Hibiscus sabdariffa* and influence of organic residues on its antioxidant and antitumoral properties," *Brazilian Journal of Biology*, vol. 75, no. 1, pp. 69–76, 2015.

[34] J. Yang, J. Guo, and J. Yuan, "*In vitro* antioxidant properties of rutin," *LWT—Food Science and Technology*, vol. 41, no. 6, pp. 1060–1066, 2008.

[35] L. S. Chua, "A review on plant-based rutin extraction methods and its pharmacological activities," *Journal of Ethnopharmacology*, vol. 150, no. 3, pp. 805–817, 2013.

[36] E. D. Pauli, G. B. Malta, P. M. Sanchez, I. C. Moreira, and I. S. Scarminio, "Mixture design analysis of solvent extractor effects on epicatechin, epigallocatechin gallate, epigallocatechin and antioxidant activities of the *Camellia sinensis* L. leaves," *Analytical Chemistry Research*, vol. 2, pp. 23–29, 2014.

[37] G. W. Plumb, S. De Pascual-Teresa, C. Santos-Buelga, V. Cheynier, and G. Williamson, "Antioxidant properties of catechins and proanthocyanidins: effect of polymerisation, galloylation and glycosylation," *Free Radical Research*, vol. 29, no. 4, pp. 351–358, 1998.

[38] M. Valko, C. J. Rhodes, J. Moncol, M. Izakovic, and M. Mazur, "Free radicals, metals and antioxidants in oxidative stress-induced cancer," *Chemico-Biological Interactions*, vol. 160, no. 1, pp. 1–40, 2006.

[39] A. Kurek-Górecka, A. Rzepecka-Stojko, M. Górecki, J. Stojko, M. Sosada, and G. Swierczek-Zieba, "Structure and antioxidant activity of polyphenols derived from propolis," *Molecules*, vol. 19, no. 1, pp. 78–101, 2014.

[40] B. Frei, "Reactive oxygen species and antioxidant vitamins: mechanisms of action," *The American Journal of Medicine*, vol. 97, no. 3, pp. 5–13, 1994.

[41] M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *The International Journal of Biochemistry & Cell Biology*, vol. 39, no. 1, pp. 44–84, 2007.

[42] J. J. Lemasters, T. Qian, C. A. Bradham et al., "Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death," *Journal of Bioenergetics and Biomembranes*, vol. 31, no. 4, pp. 305–319, 1999.

[43] N. Festjens, T. Vanden Berghe, and P. Vandenabeele, "Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1757, no. 9-10, pp. 1371–1387, 2006.

[44] K. W. Kinnally, P. M. Peixoto, S.-Y. Ryu, and L. M. Dejean, "Is mPTP the gatekeeper for necrosis, apoptosis, or both?" *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1813, no. 4, pp. 616–622, 2011.

[45] W. Wang, K. Chen, Q. Liu et al., "Suppression of tumor growth by *Pleurotus ferulae* ethanol extract through induction of cell apoptosis, and inhibition of cell proliferation and migration," *PLoS ONE*, vol. 9, no. 7, Article ID e102673, pp. 1–14, 2014.

[46] S. Mueller, M. Schittenhelm, F. Honecker et al., "Cell-cycle progression and response of germ cell tumors to cisplatin *in vitro*," *International Journal of Oncology*, vol. 29, no. 2, pp. 471–479, 2006.

[47] M. Carocho and I. C. F. R. Ferreira, "The role of phenolic compounds in the fight against cancer—a review," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 13, no. 8, pp. 1236–1258, 2013.

[48] R. P. Singh and R. Agarwal, "Natural flavonoids targeting deregulated cell cycle progression in cancer cells," *Current Drug Targets*, vol. 7, no. 3, pp. 345–354, 2006.

[49] G. K. Schwartz and M. A. Shah, "Targeting the cell cycle: a new approach to cancer therapy," *Journal of Clinical Oncology—Biology of Neoplasia*, vol. 23, no. 36, pp. 9408–9421, 2005.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 4320374, 27 pages
http://dx.doi.org/10.1155/2016/4320374

Review Article

Plant-Derived Agents for Counteracting Cisplatin-Induced Nephrotoxicity

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Received 8 June 2016; Accepted 23 August 2016

Academic Editor: Renata Szymanska

Cisplatin (CSP) is a chemotherapeutic agent commonly used to treat a variety of malignancies. The major setback with CSP treatment is that its clinical efficacy is compromised by its induction of organ toxicity, particular to the kidneys and ears. Despite the significant strides that have been made in understanding the mechanisms underlying CSP-induced renal toxicity, advances in developing renoprotective strategies are still lacking. In addition, the renoprotective approaches described in the literature reveal partial amelioration of CSP-induced renal toxicity, stressing the need to develop potent combinatorial/synergistic agents for the mitigation of renal toxicity. However, the ideal renoprotective adjuvant should not interfere with the anticancer efficacy of CSP. In this review, we have discussed the progress made in utilizing plant-derived agents (phytochemicals) to combat CSP-induced nephrotoxicity in preclinical studies. Furthermore, we have also presented strategies to utilize phytochemicals as prototypes for the development of novel renoprotective agents for counteracting chemotherapy-induced renal damage.

1. Introduction

Cisplatin (CSP), chemically known as *cis*-diamminedichloroplatinum-II, is an anticancer agent used in the treatment of testicular, head and neck, ovarian, cervical, and non-small-cell lung cancers [1]. The major issues limiting the clinical use of CSP are its tendency to induce profound nephrotoxicity and ototoxicity [1]. The first occurrence of nephrotoxicity was documented in the clinical trial that evaluated the anticancer effects of CSP. It is estimated that 30% of patients treated with CSP could exhibit elevated serum creatinine levels and reduced glomerular filtration rate, reflecting the development of nephrotoxicity. In addition, these symptoms could occur as early as 10 days after the initiation of CSP chemotherapy. Moreover, nephrotoxicity is considered a determinant side effect of the use of anticancer medications. It is pertinent to note that approximately 50–60% of patients undergoing cancer chemotherapy acquire nosocomial acute kidney injury, which is associated with increased morbidity and mortality rates [1, 2].

The pathophysiological mechanisms purported to underlie CSP-induced nephrotoxicity have been extensively studied, and several hypotheses have been forwarded. To date, oxidative stress, inflammation, and apoptosis pathways have been widely considered as key pathomechanisms involved in the CSP-induced nephrotoxicity [3]. The identified scenario is that the accumulation of CSP in renal tissues results in massive oxidative stress that causes inflammatory damage to the tubular epithelium, which spreads to the renal microvasculature, impedes the blood flow by evoking ischemic injury, and decreases the glomerular filtration rate. These phenotypic events culminate in acute renal failure. To circumvent the CSP-induced nephrotoxicity, several analogs have been developed, which are expected to be less nephrotoxic. In addition, several clinical trials have examined the efficacy of mannitol and furosemide (osmotic and loop diuretics, resp.)

in reducing the renal retention of CSP and, thereby, minimizing the noxious effects on naïve tissue [4]. However, this approach has met with limited clinical success; while the induced nephrotoxicity has been milder, it has not been completely averted. Therefore, there is an urgent need to develop agents that confer renoprotection without compromising the anticancer activity of CSP [1, 5].

2. Phytochemicals as Leads for Attenuating CSP-Induced Nephrotoxicity

Phytochemicals are compounds that are distributed in various plant tissues and are responsible for imparting characteristics such as color and smell but do not possess nutritional value. Importantly, phytochemicals have been used in traditional medicine for several centuries for treating various ailments. There is considerable evidence from *in vitro* preclinical studies that phytochemicals extracted from various plant sources may retard tumor growth and elicit antioxidant and anti-inflammatory effects [6]. Most importantly, the anticancer agent Taxol (paclitaxel) is a phytochemical that was originally identified, extracted, and purified from the bark of the Pacific yew tree (*Taxus brevifolia*) [7]. Currently, most developed drugs are not from plants but are rather chemically synthesized. Recently there is a renewed interest in tapping into the potential of medicinal plants in drug discovery, since phytochemicals are chemically diverse in nature and a considerable receptacle of pharmacophores. This enthusiasm has led to significant research strides in the identification of several potential phytochemicals that are being investigated for their renoprotective actions in preclinical studies.

Extensive investigations over the past decade have provided significant insights into the pathophysiology of CSP-induced nephrotoxicity. A plethora of biochemical pathways and mechanisms have been purported to mediate CSP-elicited nephrotoxicity, including those involved in oxidative/nitrative stress, mitochondrial malfunction, inflammation, and cell death (reviewed in [8–10]). Recently, the involvement of endocannabinoid system has been implicated in the pathogenesis of CSP-induced nephrotoxicity [11, 12].

In this context, we have discussed the developments made with the use of phytochemicals to attenuate the development CSP-induced nephrotoxicity in experimental models. The summary of the effects of phytochemicals in preclinical or *ex vivo* studies or both is provided in Table 1. The chemical structures of phytochemicals that have been tested for potential renoprotective actions against CSP-induced renal toxicity are presented in Table 2. Next, various biochemical pathways recruited by CSP in eliciting renal toxicity and the attenuation of these effects by phytochemicals are illustrated in Figure 1. Furthermore, in the following section, we systematically discussed the effects of various phytochemicals investigated for their potential renoprotection against CSP-induced nephrotoxicity.

2.1. 23-Hydroxytormentic Acid (23-HTA) and Niga-ichigoside F_1 (NIF_1). 23-Hydroxytormentic acid (23-HTA), an aglycone of the triterpenoid glycoside niga-ichigoside F_1 (NIF_1), has been isolated from the unripe fruit of *Rubus coreanus*, a perennial shrub found in southern parts of Korea [13]. Kim et al. [13] and Sohn et al. [14] have demonstrated that 23-HTA and NIF_1 attenuated CSP-induced nephrotoxicity by mitigating oxidative stress and inflammation in renal tissues. However, further mechanistic studies are required to confirm their renoprotective effects against CSP-induced renal toxicity.

2.2. 6-Gingerol. 6-Gingerol is a pungent ingredient of ginger (*Zingiber officinale*), which has demonstrated anti-inflammatory, analgesic, antipyretic, antitumor, and antiproliferative properties [15, 16]. Kuhad et al. [17] reported that gingerol inhibited CSP-induced nephrotoxicity by suppressing oxidative stress. Similarly, another study reported that gingerol elicited renoprotective action by mitigating renal oxidative stress and inflammation [18]. However, further studies are warranted to delineate the precise molecular mechanisms of their renoprotective actions.

2.3. 6-Hydroxy-1-methylindole-3-acetonitrile (6-HMA). 6-HMA is a phytochemical present in *Brassica rapa* roots. In traditional medicine, *B. rapa* has been used to treat a variety of conditions such as hepatitis, jaundice, furuncle, and sore throats [19]. 6-HMA has been demonstrated to improve renal function, augment endogenous antioxidant defenses, and protect kidneys from the noxious effects of CSP. Further, 6-HMA also inhibited CSP-induced death of LLC-PK1 cells (renal proximal tubular epithelial cells derived from porcine kidneys) [19].

2.4. β-Caryophyllene (BCP). β-Caryophyllene (BCP) is a natural sesquiterpene found in several essential oils of spices such as cinnamon, oregano, black pepper, basil, cloves, and other condiments [20]. BCP has been shown to elicit anti-inflammatory [20] and antioxidant effects [21, 22]. Horváth et al. [23] demonstrated that BCP attenuated CSP-induced nephrotoxicity by decreasing oxidative/nitrative stress, inflammation, and cell death pathway activation. Further, mechanistic studies revealed that the renoprotective actions of BCP against CSP-induced renal toxicity were mediated via activation of cannabinoid receptor-2 (CB_2). It is pertinent to note that previous studies have also demonstrated the renoprotective role of CB_2 receptor activation [24]. In addition, several studies have documented the anti-inflammatory phenotype induced by CB_2 receptors activation in preclinical studies [25]. Considering the good safety and tolerability profile of BCP in human subjects, this has excellent prospects for further pharmaceutical development as a renoprotective agent.

2.5. Berberine. Berberine, an isoquinoline alkaloid present in the rhizome, root, and stem bark of several plant species, is especially highly concentrated in berries (*Berberis vulgaris*) [26]. Berberine has been documented to possess antioxidant, anti-inflammatory, and anticancer activities [26]. Berberine

Table 1: Phytochemicals investigated for renoprotective actions against cisplatin- (CSP-) induced nephrotoxicity.

Phytochemical	Dose, duration, and route of administration	Animal model	Cisplatin dose and route of administration	Key findings	Reference
NIF_1 and 23-hydroxytormentic acid	10 mg/kg/day, orally (PO) for 14 days intraperitoneally (i.p.)	Sprague Dawley (SD) rats	7 mg/kg, i.p.	↓ BUN and serum creatinine ↓ MDA production and GSH depletion	[14]
6-Gingerol	12.5, 25, and 50 mg/kg for 5 days (before and after treatment), i.p.	Wistar rats	5 mg/kg, i.p.	↓ oxidative stress	[17]
6-Hydroxy-1-methylindole-3-acetonitrile	5 and 10 mg/kg, single dose, PO	LLC-PK1 cells and SD rats	7 mg/kg, i.p.	↓ BUN, creatinine, and urinary LDH ↑ HO-1 expression, activities of SOD, CAT, GR, MDA, and GSH	[19]
β-Caryophyllene	1–10 mg/kg, i.p. single dose	C57BL/6J mice	25 mg/kg, i.p.	↓ inflammation and dysfunction ↓ NOX-2 and NOX-4 expression, 4-HNE, 3-NT accumulation, and cell death	[23]
Berberine	1–3 mg/kg, single dose, i.p.	BALB/cN mice	13 mg/kg, i.p.	↓ BUN, creatinine, and oxidative/nitrosative stress ↓ NF-κB, TNF-α, COX-2, iNOS, and histopathological changes ↓ p53 and active caspase-3	[27]
Bixin	2.5 and 5 mg/kg for 3 days, i.p.	Wistar rats	5 mg/kg, i.p.	↓ lipid peroxidation and renal glutathione depletion ↓ chromosome aberrations	[29, 30]
C-Phycocyanin	5–50 mg/kg, i.p.	C57BL/6J and CD1 mice	12–18 mg/kg, i.p.	↓ BUN, creatinine, oxidative stress, and apoptosis ↓ p-ERK, p-JNK, and p-p38 expression and Bax, caspase-9, and caspase-3 activation	[31, 32]
Caffeic acid phenethyl ester	10 μM/kg, single dose, i.p.	Wistar Albino rats	7 mg/kg, i.p.	↓ BUN, tubular damage, and oxidative tissue damage ↑ antioxidant enzymes	[33]
Cannabidiol	2.5–10 mg/kg, i.p. (before and after treatment)	C57BL/6J mice	20 mg/kg, i.p.	↓ BUN, creatinine, ROS formation, and 3-NT ↓ PARP, caspase-3/7, and DNA fragmentation ↓ mRNA of TNF-α and IL1 and iNOS and protein expression	[36]
Capsaicin	5 and 10 mg/kg, PO for 6 days, i.p.	SD rats	5 mg/kg, i.p.	↓ BUN, creatinine, MDA, and renal damage	[38]
	2.5, 5, and 10 mg/kg for 5 days, i.p.	C57BL/6 mice	5 mg/kg, i.p.	↑ HO-1 expression	[39]
Cardamonin	10 and 30 mg/kg, PO for 2 weeks	Albino rats	7 mg/kg, i.p.	↑ SOD, GSH ↓ NOX-1, caspase-3 expression, and Bax/Bcl-2 ratio	[41]
Carnosic acid	100 mg/kg, PO for 10 days	Wistar rats	7.5 mg/kg, i.p.	↓ BUN, creatinine, and MDA ↑ GSH levels, catalase, SOD, GST, GPx, and GR activities ↓ caspase-3 activity, apoptosis, and renal damage	[45]
Chrysin	25 or 50 mg/kg 14 days, i.p.	Wistar rats	7.5 mg/kg, i.p.	↓ oxidative stress and apoptosis	[47]

Table 1: Continued.

Phytochemical	Dose, duration, and route of administration	Animal model	Cisplatin dose and route of administration	Key findings	Reference
Cinnamic acid (CA) and cinnamaldehyde (CD)	CA, 50 mg/kg CD, 40 mg/kg, PO for 7 days	SD rats	5 mg/kg, i.p.	↓ urea, creatinine, and MDA content ↑ GSH levels, SOD, CAT, and GPx activities	[49]
Curcumin	100 mg/kg 10 days, i.p.	Wistar rats	7 mg/kg, i.p.	↓ MDA ↑ NAMPT, SIRT1, SIRT3, and SIRT4 levels	[56]
	100 mg/kg, i.p.	C57BL/6J mice	20 mg/kg, i.p.	↓ renal TNF-α, MCP-1, and ICAM-1 mRNA expression	[54]
	8 mg/kg	Wistar rats	5 mg/kg, i.p.	↓ creatinine, TBARS, and MDA	[51]
Cyanidin	10, 20, and 40 μg/mL	HK-2 cells	8 μg/mL	↓ BUN, creatinine, MDA, renal index, and IL-6 ↓ GRP78, p-ERK, caspase-12, and PARP cleavage ↓ apoptosis, DNA damage, ERK activation, and AKT inhibition	[58]
Decursin	20–80 mM	Primary HRCs	20–80 mM	↑ catalase, SOD, and GPx activities ↓ caspases 3 and 9, PARP, DNA fragmentation, and apoptosis	[59]
	10–40 mg/kg 3 days, i.p.	SD rats	5.2 mg/kg, i.p.	↓ BUN and creatinine	[60]
Ellagic acid	10 and 30 mg/kg 9 days, i.p.	SD rats	6 mg/kg, i.p.	↓ creatinine, urea, and kidney injury ↑ total antioxidant status and GSH	[63]
	10 mg/kg 10 days, i.p.	SD rats	7 mg/kg, i.p.	↓ MDA levels and improved antioxidant enzymes ↓ tubular necrosis and tubular dilatation	[65]
Emodin	10 mg/kg for 9 days, i.p.	Wistar rats	6 mg/kg, i.p.	↑ GSH, TAC, GST, GPx, GR, SOD, and CAT ↓ NAG, creatinine, and urea concentration	[69]
Epigallocatechin-3-gallate	l00 mg orally, 2 days	Wistar rats	7 mg/kg, i.p.	↑ SOD, CAT, GPx, and GSH ↓ NF-κB and 4HNE	[72]
	100 mg/kg i.p., single dose	C57BL/6 mice	20 mg/kg, i.p.	↓ p-ERK, GRP78, caspase-12, Fas-L, BAX, and apoptosis	[75, 76]
Genistein	10 mg/kg 3 days 25 μg/L	C57BL/6 mice HK-2 cells	20 mg/kg, i.p. 1 μg/mL	↓ BUN, creatinine, ROS production, tubular damage, and necrosis score ↓ ICAM-1 and MCP-1 expression and NF-κB activation ↓ apoptosis and p53 induction	[77]
Ginsenosides	10–60 μg/mL	LLC-PK1 cells	25 and 500 μM	↓ LDH leakage, renal damage, and apoptosis	[78–80]
Glycyrrhizic acid	75 and 150 mg/kg for 7 days, i.p.	BALB/c and Swiss Albino mice	7 mg/kg, i.p.	↑ GSH, GR, GST, catalase, and GPx activities ↓ BUN and creatinine	[82, 84]
Hesperidin	100 and 200 mg/kg 10 days, i.p.	Wistar rats	7.5 mg/kg, i.p.	↓ BUN, creatinine, and DNA degradation ↑ SOD, GPx, GST, GR, GSH, and catalase activities and vitamin C levels ↓ renal TNF-α levels	[85, 86]

Table 1: Continued.

Phytochemical	Dose, duration, and route of administration	Animal model	Cisplatin dose and route of administration	Key findings	Reference
Isoliquiritigenin	1 mg/kg for 15 days, i.p.	BALB/c mice	5 mg/kg, i.p.	↓ BUN, creatinine, nitrite, and tissue MDA and ROS	[87]
Licochalcone A	1 mg/kg for 15 days, i.p.	BALB/c mice	5 mg/kg, i.p.	↓ BUN, creatinine, nitrite, and MDA	[89]
Ligustrazine	50 and 100 mg/kg, 7 days, i.p.	SD rats	8 mg/kg, i.p.	↓ urinary protein excretion, NAG excretion, creatinine, and BUN ↑ GSH levels, SOD, and GST activities ↓ tubular cell apoptosis	[90]
Luteolin	10 mg/kg 3 days, i.p.	BALB/cN mice	10 and 20 mg/kg, i.p.	↓ renal dysfunction, tubular injury, oxidative stress, BUN, and creatinine ↑ GSH, SOD, and catalase ↓ p53 activation and PUMA-α protein expression	[92]
	50 mg/kg 3 days, i.p.	C57BL/6J mice	20 mg/kg, i.p.	↓ CYP2E1, Bcl-2, 4-HNE, 3-NT, NF-κB, and caspase-3 ↓ MRP4 and MRP2 expression	[93]
Lycopene	6 mg/kg 10 days, i.p.	Wistar rats	7 mg/kg, i.p.	↓ urea and creatinine and MRP2 and MRP4 expression ↑ OAT1, OAT3, OCT1, OCT2, Nrf2, and Bcl-2 expression	[96, 97]
	4 mg/kg 5 days, i.p.	SD and Wistar rats		↑ catalase, GPx, and SOD activities ↓ NF-κB, HSP 60 and HSP 70, and Bax expression	[98]
Naringenin	20 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}, PO for 10 days	Wistar Albino rats	7 mg/kg, intravenous (i.v.)	↓ urea, creatinine, sodium excretion, and renal lipid peroxides ↑ GST activity and renal antioxidant enzymes	[101]
Paeonol	20 mg/kg 3 days, i.p.	BALB/c mice	10–30 mg/kg I.P.	↓ creatinine, BUN, TNF-α, and IL-1β	[102]
Penta-*O*-galloyl-β-D-glucose	20–80 μM	Primary HRC	40 μM	↓ cytotoxicity, apoptosis, PARP cleavage, Bax, and caspase-3 ↓ cytochrome C translocation and ROS production	[106]
Platycodin D	0.1, 1, and 5 mg/kg for 3 days, i.p.	ICR mice	20 mg/kg, i.p.	↓ BUN, creatinine, TBARS, NF-κB activation, ↑ GSH, GPx, and SOD	[108]
Quercetin	100 mg/kg 30 days	Albino rats	12 mg/kg i.p.	↑ GSH, GPX, SOD, CAT, GR, XO, TOS, and TAC ↓ BUN, creatinine, LPO, H_2O_2, and tubular cell necrosis	[110]
	50 mg/kg 3 days	Wistar rats	5 mg/kg, i.p.	↓ Na and K excretion, NAG, LDH, ALP, GGT, and KIM-1 ↓ GSH/GSSG ratio, NFκB, iNOS, ICAM-1, VCAM-1, and renal MPO	[111]
	50 and 100 mg/kg 9 days, i.p.	Fischer-F344 rats	7.5 mg/kg, i.p.	↓ caspase-3/7 activity and DNA fragmentation	[148]

Table 1: Continued.

Phytochemical	Dose, duration, and route of administration	Animal model	Cisplatin dose and route of administration	Key findings	Reference
Resveratrol	25 mg/kg single dose, i.p.	Albino mice	5 mg/kg, i.p.	↓ creatinine, MDA, and LDH leakage	[115]
	10 mg/kg, 7 days	C57BL/6 mice and	20 mg/kg, i.p.	↓ inflammation and necrosis	[116]
	30 μg/mL, i.p.	Fischer rat kidney *in vitro*	7.5/15 μg/mL, i.p.	↓ acetylation of p53 and SIRT1	[117]
Rosmarinic acid	1, 2, and 5 mg/kg 2 days, i.p.	BALB/cN mice	13 mg/kg, i.p.	↓ creatinine and BUN ↓ CYP2E1, HO-1, and 4-HNE expression ↓ NFκB and cleaved caspase-3 expression	[120]
Rutin	75 and 150 mg/kg 21 days	Wistar rats	7 mg/kg, i.p.	↓ BUN, creatinine, H_2O_2, LDH, caspase-3, NFκB, and TNF-α level	[122]
	30 mg/kg 14 days	SD rats	5 mg/kg, i.p.	↑ membrane integrity, GSH, XO, and GGT	[86]
Schizandrin and schizandrin B	10, 25, 50 mg/kg 15 days, i.p.	BALB/c mice	10 mg/kg, i.p.	↓ NFκB activation and p53 activation	[124]
Silibinin	200 mg/kg single dose, i.p.	Wistar rats	5 mg/kg, i.p.	↑ creatinine clearance ↑ glomerular and proximal tubular function	[127, 128]
Sulforaphane	500 μg/kg/day i.v. for 3 days	Wistar rats	7.5 mg/kg, i.p.	↓ p38 MAPK and renal adhesion molecule expressions	[130]
	500 μg/kg/day i.p. for 3 days	Wistar rats	10 mg/kg, i.p.	↓ inflammatory cell infiltration	[131]
Tannic acid	40 and 80 mg/kg 7 days, i.p.	Swiss Albino mice	7 mg/kg, i.p.	↓ BUN, creatinine, p38 MAPK phosphorylation, and PARP cleavage ↓ XOR and LPO; ↑ G6PD, QR, and catalase activities	[136]
Thymoquinone	50 mg/L in drinking water for 5 days	Wistar Albino rats and Swiss Albino mice	5, 7, and 14 mg/kg i.v. in rats i.p. in mice	↓ urea, creatinine, MDA, 8-isoprostane, MRP2, and MRP4 ↑ OAT1, OAT3, OCT1, and OCT2 and survival rate of animals	[139]
Xanthorrhizol	100 and 200 mg/kg for 4 days, i.p.	ICR mice	45 mg/kg, i.p.	↓ BUN, creatinine, and lipid peroxides	[143]

inhibited CSP-induced nephrotoxicity by reducing oxidative stress/nitrative stress, nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) activation, and proinflammatory cytokine expression. In addition, berberine also inhibited apoptosis and diminished the cytochrome P450 (CYP) 2E1 expression in CSP-treated kidneys. CYP2E1 is the primary enzyme involved in the biotransformation of cisplatin, and previous studies have also demonstrated that genetic ablation of CYP2E1 imparted renoprotection against CSP-induce toxicity [27, 28].

2.6. Bixin. Bixin is the main carotenoid found in species of the tropical plant *Annatto* (*Bixa orellana*). Bixin inhibited CSP-induced nephrotoxicity by inhibiting lipid peroxidation and augmenting endogenous antioxidant defenses [29, 30]. However, further mechanistic studies are required to understand its renoprotective properties.

2.7. C-Phycocyanin (C-PC). C-Phycocyanin (C-PC) is a pigment from the blue-green algae, *Spirulina maxima* [31]. C-PC has been shown to mitigate CSP-induced nephrotoxicity via inhibition of oxidative stress, inflammation, and apoptosis. Furthermore, mechanistic studies revealed that C-PC blunted CSP-induced proapoptotic mitogen-activated protein kinase (MAPK) kinase (MEK), B-cell lymphoma 2- (Bcl2-) associated X protein (Bax)/Bcl2 ratio alterations, and caspase-3 activation in renal tissues [31, 32].

2.8. Caffeic Acid Phenethyl Ester (CAPE). Caffeic acid phenethyl ester (CAPE) is an active phenolic compound

Table 2: Structures of phytochemicals investigated for renoprotective action against cisplatin- (CSP-) induced nephrotoxicity.

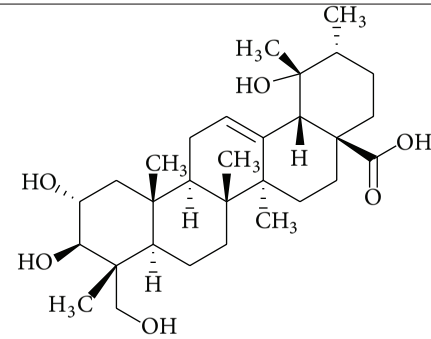
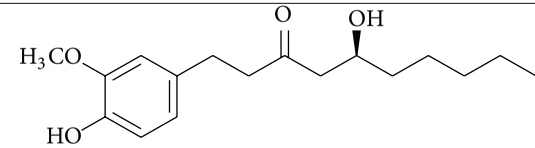
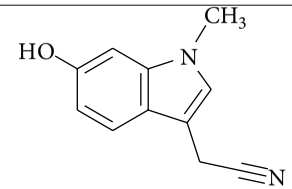
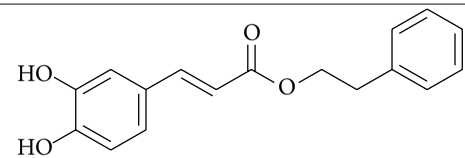
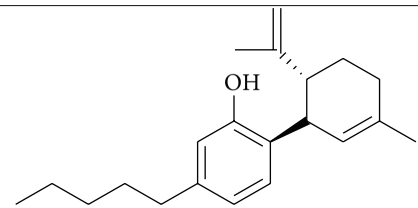
Phytochemical	Structure	Chemical class
23-Hydroxytormentic acid		Carboxylic acid
6-Gingerol		Decanone
6-Hydroxy-1-methylindole-3-acetonitrile		Nitrile
Caffeic acid phenylethyl ester		Ester
Cannabidiol		Monoterpene

Table 2: Continued.

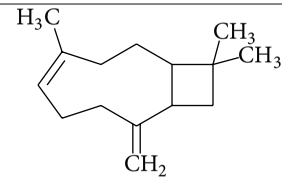
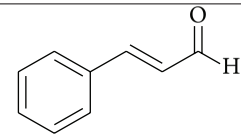
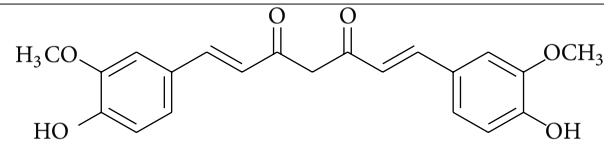
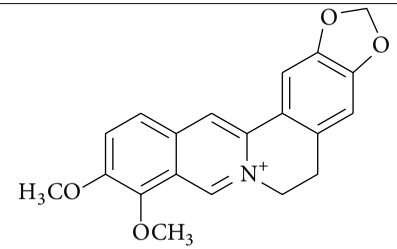
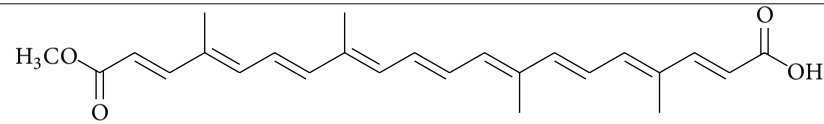
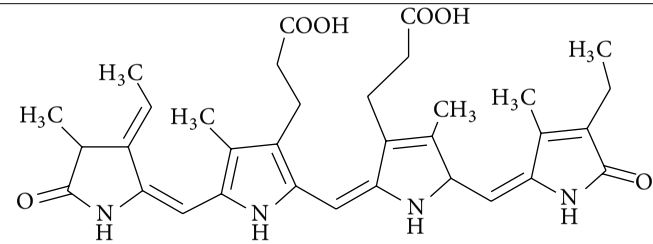
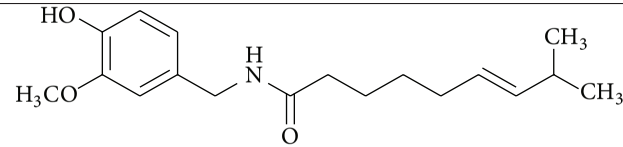
Phytochemical	Structure	Chemical class
β-Caryophyllene		Bicyclic alkene
Cinnamaldehyde		Aldehyde
Curcumin		Diketone
Berberine		Isoquinoline
Bixin		Apocarotenoid
C-Phycocyanin		Phycobiliprotein
Capsaicin		Amide

Table 2: Continued.

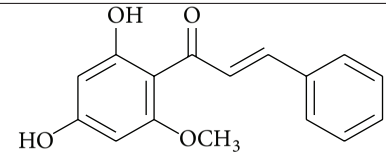
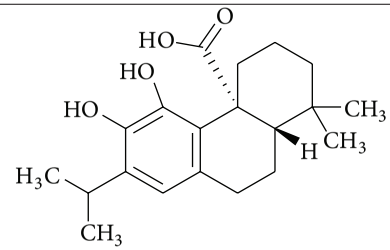
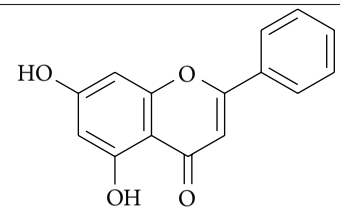
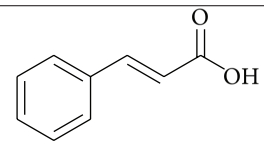
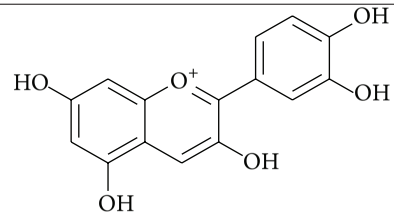
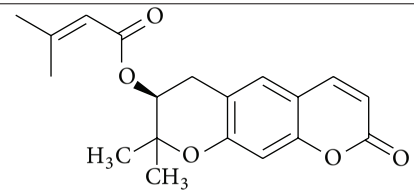
Phytochemical	Structure	Chemical class
Cardamonin		Chalconoid
Carnosic acid		Benzenediol abietane diterpene
Chrysin		Flavonoid
Cinnamic acid		Carboxylic acid
Cyanidin		Anthocyanidin
Decursin		Coumarin

Table 2: Continued.

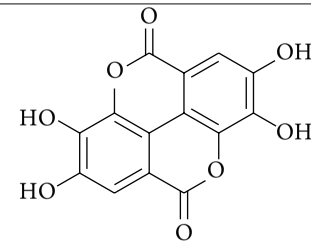
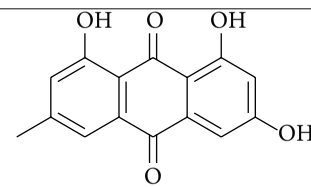
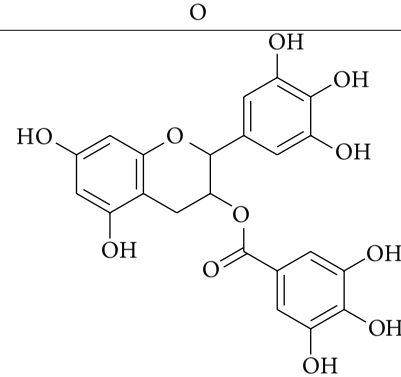
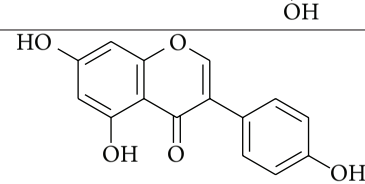
Phytochemical	Structure	Chemical class
Ellagic acid		Chromene-5,10-dione
Emodin		Anthraquinone
Epigallocatechin-3-gallate		Polyphenol
Genistein		Isoflavone

Table 2: Continued.

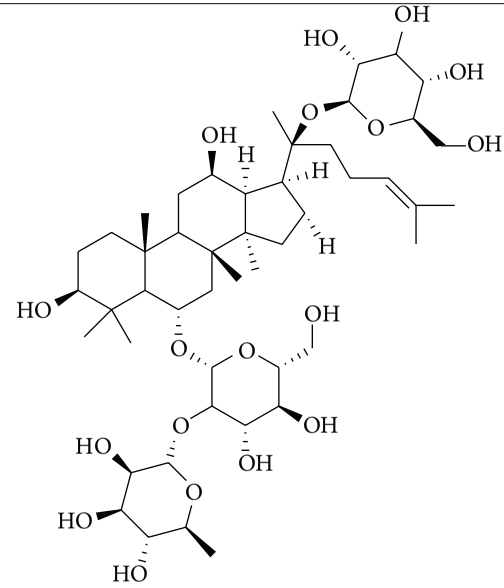
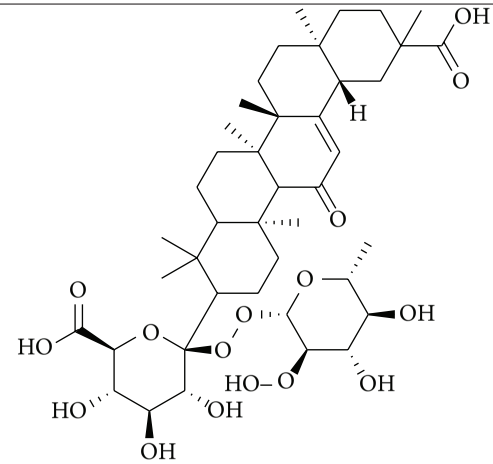
Phytochemical	Structure	Chemical class
Ginsenoside		Triterpene-saponin
Glycyrrhizic acid		Triterpenoid saponin

Table 2: Continued.

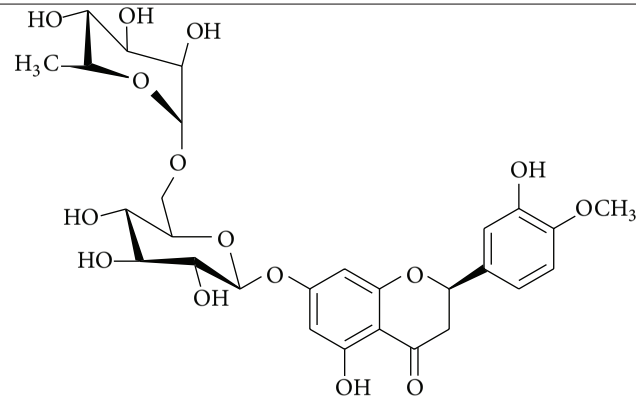
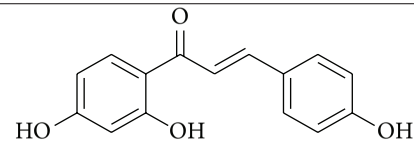
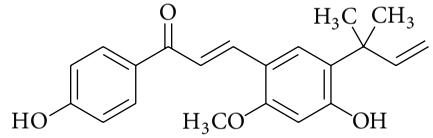
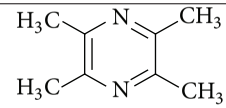
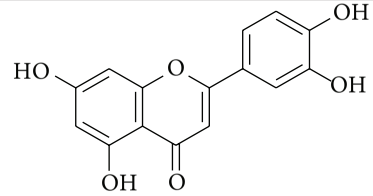
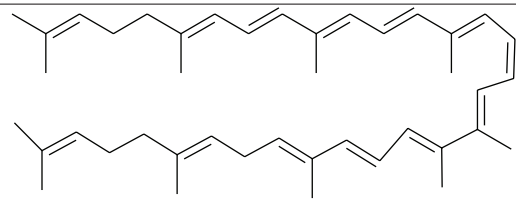
Phytochemical	Structure	Chemical class
Hesperidin		Licorice chalconoid
Isoliquiritigenin		Chalconoid
Licochalcone A		Chalconoid
Ligustrazine		Pyrazine
Luteolin		Flavanone
Lycopene		Carotenoid

Table 2: Continued.

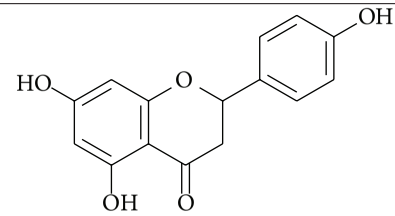
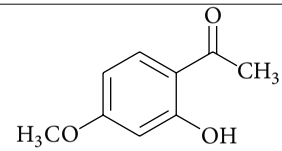
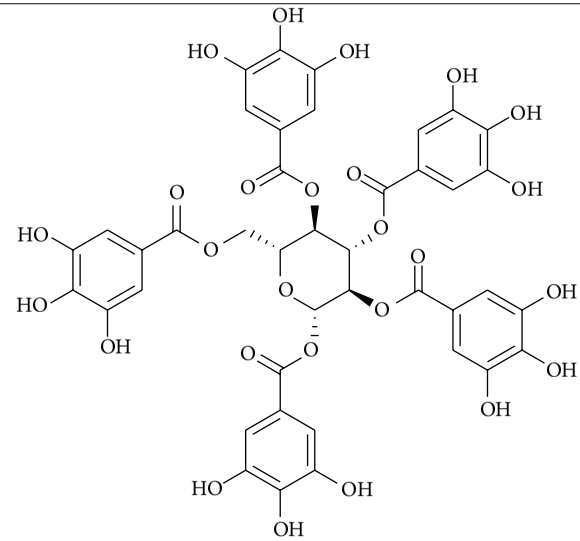
Phytochemical	Structure	Chemical class
Naringenin		Flavanone
Paeonol		Acetophenone
Penta-O-galloyl-B-D-glucose		Glycoside

Table 2: Continued.

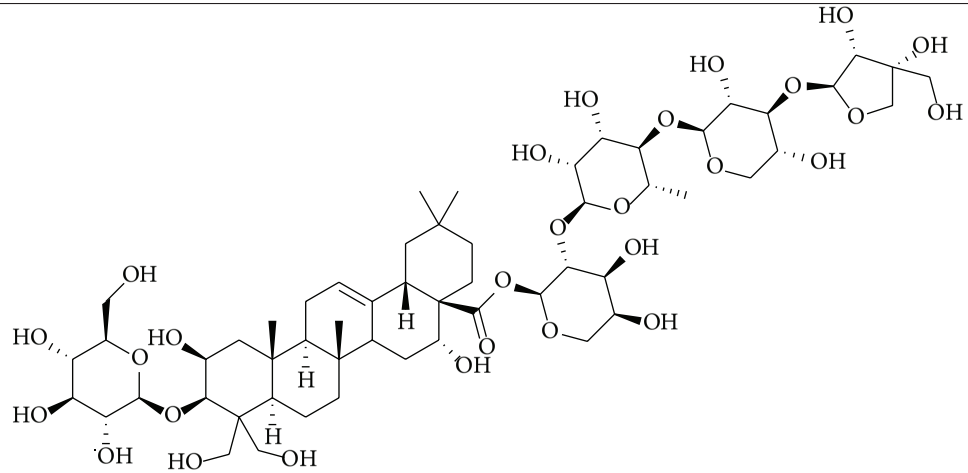
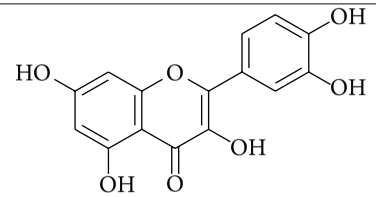
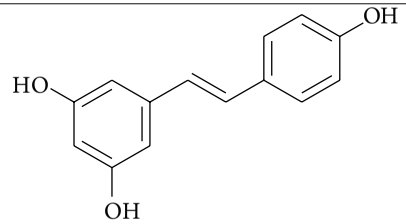
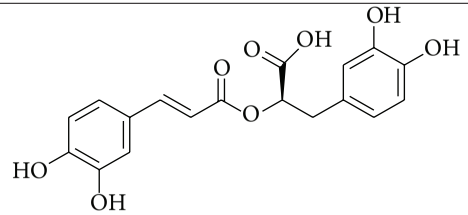
Phytochemical	Structure	Chemical class
Platycodin D		Saponin
Quercetin		Flavonol
Resveratrol		Stilbenoid
Rosmarinic acid		Caffeic acid

Table 2: Continued.

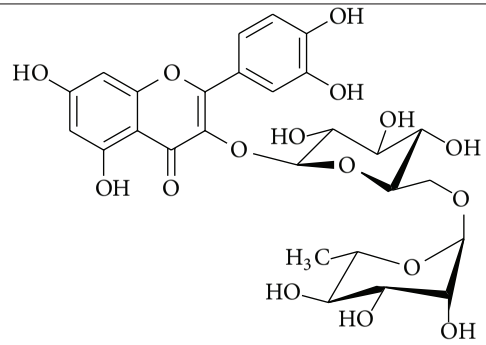
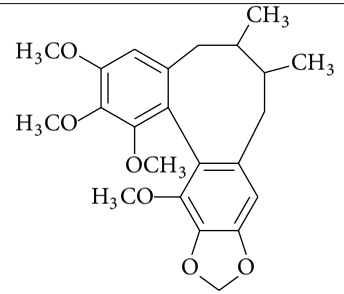
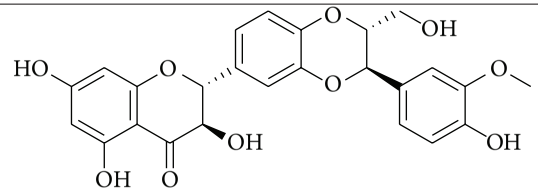
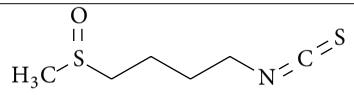
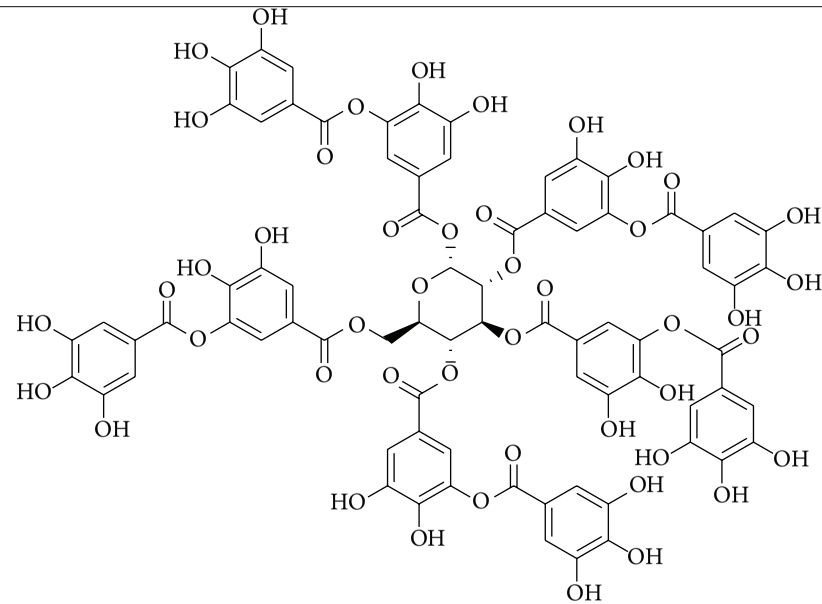
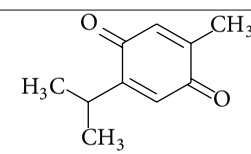
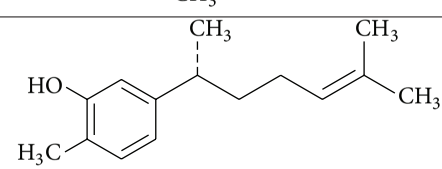
Phytochemical	Structure	Chemical class
Rutin		Chroman-4-one
Schizandrin		Cycloocta[1′,2′:4,5]benzo[1,2-d][1,3]dioxole
Silibinin		Chroman-4-one
Sulforaphane		Isothiocyanate

Table 2: Continued.

Phytochemical	Structure	Chemical class
Tannic acid		Polyphenol
Thymoquinone		1,4-Quinone
Xanthorrhizol		Sesquiterpene

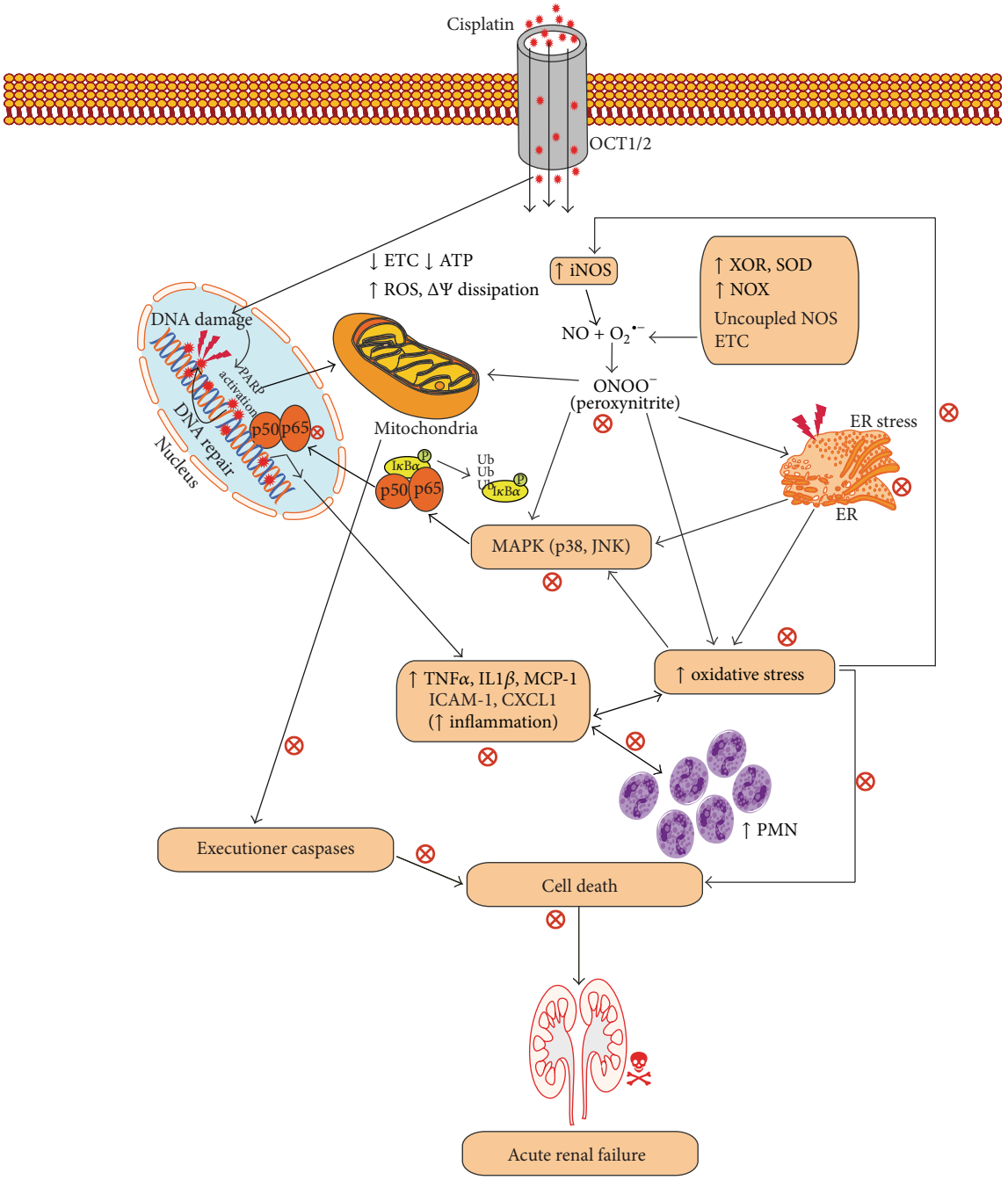


Figure 1: Scheme showing various pathways mediating cisplatin- (CSP-) induced nephrotoxicity and mitigation of this cascade by phytochemicals.

extracted from honeybee propolis [33]. CAPE treatment inhibited CSP-induced renal toxicity by suppressing oxidative stress, inflammation, and apoptosis. Further, CAPE also blunted CYP2E1 activation, thereby inhibiting the biotransformation of CSP [33, 34]. However, further studies are required to investigate whether CAPE provides renoprotection without compromising the anticancer effects of CSP.

2.9. Cannabidiol (CBD). Cannabidiol (CBD) is a phenolic compound and phytocannabinoid extracted from the *Cannabis sativa* (marijuana) plant, and it elicits anti-inflammatory, immunomodulatory, and analgesic effects [35]. CBD attenuated CSP-induced nephrotoxicity by suppressing oxidative stress, inflammation, and apoptosis. It is also pertinent to note that CBD reversed the CSP-induced kidney injury when administered after the onset of renal tissue injury [36]. Furthermore, it is noteworthy that CBD is devoid of psychoactive properties since it does not bind to major cannabinoid receptors and has an excellent safety profile in human subjects. Recently, CBD was approved for the

treatment of childhood epilepsy [25], and it could also be considered as a potent candidate for further development to counteract CSP-induced renal toxicity.

2.10. Capsaicin. Capsaicin is the major pungent ingredient in red peppers and has been used in pain sensation studies based on its stimulation of vanilloid receptor-1, an ion channel protein expressed by nociceptive primary afferent neurons [37]. Capsaicin has been demonstrated to inhibit oxidative stress, inflammation, and apoptosis in the renal tissues of CSP-treated animals. The renoprotective effects were in part due to the activation of heme oxygenase-1 (HO-1) [38, 39].

2.11. Cardamonin. Cardamonin is a flavone found in *Alpinia* plants and has been shown to affect cell-signaling pathways and to possess anticancer and anti-inflammatory properties [40]. Cardamonin increased endogenous antioxidants and decreased oxidative stress and inflammation [41–44].

2.12. Carnosic Acid. Carnosic acid is a naturally occurring polyphenolic diterpenoid molecule present in rosemary (*Rosmarinus officinalis*) [45]. Carnosic acid suppressed CSP-induced nephrotoxicity by mitigating oxidative stress and apoptosis in renal tissues [45]. However, additional studies are required to understand the molecular mechanisms purported to mediate its renoprotective actions.

2.13. Chrysin. Chrysin (5,7-dihydroxyflavone) is a flavonoid extracted from honeybee propolis. Chrysin has been reported to be a potent inhibitor of aromatase and anticancer properties [46]. Sultana et al. demonstrated that treatment of chrysin effectively diminished CSP-induced oxidative stress by improving antioxidant enzyme status and restored membrane integrity of tubular epithelial cells [47]. Furthermore, Khan et al. [48] reported that chrysin attenuated CSP-renal toxicity by inhibiting oxidative stress, p53 expression, DNA damage, and apoptosis.

2.14. Cinnamic Acid and Cinnamaldehyde. The essential oil of cinnamon contains both cinnamic acid (CA) and cinnamaldehyde (CD). These phytochemicals have been documented to possess antioxidant, antibacterial, and anti-inflammatory effects [49]. CA and CD administration to rodents restored kidney function, suppressed oxidative stress, and mitigated the histopathological degeneration induced by CSP [49]. However, additional studies are required to understand the precise molecular mechanism underlying the renoprotective actions of CA and CD.

2.15. Curcumin. Curcumin is a principle curcuminoid (phenolic terpene compound) derived from the Indian curry spice turmeric (*Curcuma longa*) [50]. Curcumin treatment restored CSP-induced depletion of endogenous antioxidants [51–53] and reduced inflammation by suppressing NFκB activation, expression of proinflammatory cytokines, and adhesion molecules [54, 55]. Furthermore, curcumin has been reported to ameliorate CSP-induced renal toxicity by augmenting silent mating type information regulation 2 homolog-1 (SIRT-1) and nuclear factor erythroid-derived 2 (Nrf2), which enhanced endogenous antioxidant defenses and mitochondrial biogenesis [55, 56].

2.16. Cyanidin. Proanthocyanidins are polyphenol derivatives of flavan-3-ol flavonoids derived from grape seed. Proanthocyanidins are reported to possess antioxidant, anti-inflammatory, and antitumor activities [57]. Cyanidin treatment of rodents suppressed CSP-induced renal reactive oxygen species (ROS) generation and enhanced the activation of prosurvival kinases such as extracellular signal-regulated kinase (ERK) and Akt. Furthermore, cyanidin also suppressed CSP-induced renal apoptosis by blunting caspase-3/12 expression, the Bax/Bcl-2 ratio, p53 phosphorylation, and poly adenosine diphosphate (ADP) ribose polymerase (PARP) activation. In addition, cyanidin also suppressed CSP-induced endoplasmic reticulum stress in renal tissues [58]. Collectively these results suggest that cyanidin recruited several prosurvival pathways to counteract CSP-induced renal damage.

2.17. Decursin. Decursin is a natural pyranocoumarin compound isolated from the Korean herb *Angelica gigas* and is reported to possess anticancer activity [59]. Decursin treatment reduced CSP-induced renal toxicity by attenuating oxidative stress, inflammation, and apoptosis pathways in renal cancer cell lines and rodents [59, 60]. Recently, dose escalation studies were conducted to determine the pharmacokinetic profile of decursin in human subjects. From this study, it was inferred that decursin was well tolerated in both sexes and reached a peak plasma concentration in 8–12 h. These observations indicate the efficacy, safety, tissue distribution, and pharmacodynamic properties of decursin in human subjects [61].

2.18. Ellagic Acid. Ellagic acid is a naturally occurring phenolic compound found in fruits such as raspberries, strawberries, and pomegranates [62]. Ellagic acid treatment ameliorated CSP-induced renal toxicity by suppressing the kidney injury molecule (KIM-1) and clusterin protein expression (considered as early indicators of kidney injury) [63]. Furthermore, ellagic acid enhanced the glomerular filtration rate, which corroborated its reduction of inflammatory mediators and apoptotic markers in renal tissues [64]. These findings were correlated with the amelioration of CSP-induced tubular necrosis, degeneration, karyomegaly, and tubular dilatation [65].

2.19. Emodin. Emodin is the most abundant bioactive anthraquinone extracted from the Chinese culinary herb, Rhubarb (*Rheum palmatum*), and it possesses anticancer [66] and antioxidant activities [67]. Emodin treatment increased the cell viability after CSP treatment of normal human renal tubular epithelial cells [68]. In addition, emodin attenuated CSP-induced renal damage by suppressing the activity of *N*-acetyl-beta-D-glucosaminidase (NAG) [69], which is a lysosomal enzyme that is constitutively expressed in the proximal kidney tubule. Owing to its high molecular weight, under physiological conditions, NAG does not void via the kidneys because of its negligible glomerular filtration [70].

However, damage to the renal tubules causes the release of NAG in higher amounts than usual and, hence, it is excreted in the urine, and its serum accumulation is increased [70]. In a separate study, Liu et al. [71] demonstrated that emodin ameliorates CSP-induced apoptosis of rat renal tubular cells *in vitro* by modulating adenosine monophosphate-activated protein kinase (AMPK)/mechanistic target of rapamycin (mTOR) signaling pathways and activating autophagy and in vivo by suppressing caspase-3 activity and apoptosis in renal tissues.

2.20. Epigallocatechin-3-Gallate (EGCG). Epigallocatechin-3-gallate (EGCG) is a phenolic compound present in green tea [72] and is an effective ROS scavenger *in vitro* and *in vivo* [73, 74]. EGCG mitigated CSP-induced nephrotoxicity by inducing the expression of *Nrf-2* and HO-1 and decreasing that of NFκB and proinflammatory cytokines [72]. Furthermore, EGCG also inhibited endoplasmic reticulum (ER) stress-induced apoptosis through the suppression of phosphorylated (p)-ERK, glucose-regulated protein 78 (GRP78), and the caspase-12 pathway [75]. Furthermore, EGCG inhibited the ligand of death receptor Fas (Fas-L); apoptosis regulator, Bax; and the tumor-suppressor protein, p53, while it increased the expression of Bcl-2 and, thereby, inhibited the extrinsic pathways of renal cell apoptosis [76]. All these studies collectively established the renoprotective actions of EGCG.

2.21. Genistein. Genistein is a polyphenol nonsteroidal isoflavonoid phytoestrogen extracted from soybean. Genistein treatment counteracted CSP-induced ROS generation and suppressed NFκB activation, proinflammatory cytokines expression, and apoptosis [77].

2.22. Ginsenosides Rh_4 and Rk_3. Ginseng is the root of *Panax ginseng* and is one of the most widely recommended and intensively studied herbal medicines. Ginsenosides are the secondary metabolites and unique constituents of *Panax* plants. Baek et al. [78] demonstrated that ginsenosides increased cell viability and prevented lactate dehydrogenase (LDH) leakage induced by CSP in normal renal proximal tubular epithelial cells. Furthermore, ginsenosides ameliorated CSP-induced renal damage by mitigating inflammation and apoptosis, which was evidenced by the suppression of DNA damage-induced apoptosis biomarkers such as phosphorylated c-Jun N-terminal kinase (JNK), p53, and cleaved caspase-3 expressions [79, 80].

2.23. Glycyrrhizic Acid. Glycyrrhizin and its aglycone glycyrrhetic acid (GA) are used for various therapeutic purposes in Chinese traditional medicine practice [81]. GA is the hydrophilic part of glycyrrhizin, an active compound found in licorice (*Glycyrrhiza glabra*), which is a conjugate of two molecules of glucuronic acid and GA. It is used as a flavoring agent in candies, pharmaceuticals, and tobacco products [82]. Furthermore, it has been reported to elicit anti-inflammatory, antioxidant, and antitumor activities [83]. GA treatment restored the antioxidant status and improved kidney function, as evidenced by diminished DNA fragmentation [82]. In addition, the renoprotective effects of GA were also associated with the upregulation of Nrf2 and downregulation of NFκB expression, resulting in decreased kidney damage [84].

2.24. Hesperidin. Hesperidin is a pharmacologically active bioflavonoid found in citrus fruits [85]. Hesperidin attenuated CSP-induced renal toxicity by ameliorating oxidative stress, inflammation, and apoptosis [85, 86]. However, additional studies are required to understand the exact molecular mechanism mediating the renoprotection induced by hesperidin.

2.25. Isoliquiritigenin (ISL). Isoliquiritigenin (ISL) is a flavonoid with a chalcone moiety extracted from several *Glycyrrhiza* species [87]. ISL has been shown to exert a variety of biological activities such as antiplatelet aggregation, antioxidant, and anti-inflammatory [88]. ISL exerted a remarkable renoprotective effect against CSP-induced renal toxicity by abrogating oxidative stress and apoptosis [87]. However, the precise molecular mechanisms purported to mediate the renoprotective activity of ISL needs to be explored.

2.26. Licochalcone A (LCA). Licochalcone A (LCA) is a species-specific phenolic constituent of *Glycyrrhiza inflata*. LCA administration to CSP-treated animals restored kidney function markers and decreased oxidative stress [89]. However, the exact mechanism underlying the renoprotection induced by LCA needs to be investigated.

2.27. Ligustrazine. Ligustrazine (tetramethylpyrazine) is an alkaloid compound extracted from the Chinese herb Chuanxiong (*Ligusticum chuanxiong* Hort) [90] and is extensively used in China for the management of myocardial and cerebral infarction [91]. Ligustrazine significantly diminished CSP-induced urinary NAG excretion and renal tubular injury in a dose-dependent manner. Furthermore, ligustrazine also suppressed renal oxidative stress, inflammation, and apoptosis by restoring the Bax/Bcl-2 ratio [90].

2.28. Luteolin. Luteolin is a flavone present in high concentrations in celery, green pepper, and chamomile, and it has been reported to display anti-inflammatory, antioxidant, and anticarcinogenic activities [92, 93]. Luteolin treatment significantly reduced the pathophysiological changes induced by CSP in the kidneys by the suppression of oxidative/nitrative stress, inflammation, and apoptosis [92]. Moreover, luteolin also ameliorated tubular necrosis, which was confirmed using a terminal deoxynucleotidyl transferase (TdT) deoxyuridine 5′-triphosphate (dUTP) nick-end labeling (TUNEL) assay, and it diminished p53 activation and PUMA-α expression, as well as altering the Bax/Bcl-2 ratio [93].

2.29. Lycopene. Lycopene is a carotenoid pigment found in tomato [94]. Lycopene from dietary sources has been shown to reduce the risk of some chronic diseases including cancer and cardiovascular disorders [95]. The administration of lycopene significantly normalized the kidney function

and antioxidant status of CSP-treated animals. Furthermore, lycopene also increased the expression of the organic anion and cation transporters (OAT and OCT, resp.) including OAT1, OAT3, OCT1, and OCT2 in the renal tissues [96–98]. In addition, lycopene also decreased the renal efflux transporters (multidrug resistance-associated protein [MRP]-2 and MRP4) levels and induced Nrf2 activation, which activated the antioxidant defense system [99]. Furthermore, lycopene protected against CSP-induced renal injury by modulating proapoptotic Bax and antiapoptotic Bcl-2 expressions and enhancing heat shock protein (HSP) expression [97].

2.30. Naringenin (NAR). Citrus fruits (such as oranges and grapefruits) are rich in the flavanone naringenin (NAR, aglycone) [100]. NAR diminished the extent of CSP-induced nephrotoxicity by improving renal function and antioxidant enzyme activity and diminishing lipid peroxidation [101]. However, the detailed molecular mechanism of the renoprotective action of NAR against CSP-induced renal tissue injury is still unknown and requires further investigation.

2.31. Paeonol. Paeonol is a major phenolic component of Moutan cortex [102]. In traditional medicine practice, paeonol is used to treat various diseases including atherosclerosis, infections, and other chronic inflammatory disorders [103]. Paeonol improved kidney function and suppressed the levels of proinflammatory cytokines, which attenuated the renal tissue injury induced by CSP [102]. However, additional mechanistic studies are warranted to understand the renoprotective activity of paeonol.

2.32. 1,2,3,4,6-Penta-O-galloyl-β-D-glucose (PGG). 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (PGG) is a polyphenol and water-soluble gallotannin isolated from the Chinese herb *Rhus chinensis* [104]. PGG significantly blocked cytotoxicity and reduced the sub-G1 accumulation of human renal proximal tubular epithelial cells induced by CSP [105]. In addition, PGG suppressed PARP cleavage, caspase-3 activation, cytochrome c release, and upregulation of Bax and p53 expression, which diminished apoptosis in the renal tissues [106].

2.33. Platycodin D (PD). Triterpenoid saponins extracted from the roots of *Platycodon grandiflorum* exhibit a variety of pharmacological activities such as anti-inflammatory, anticancer, and immune-enhancing effects. The saponins in *P. grandiflorum* inhibited inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions by mitigating NFκB activation in CSP-treated kidneys [107]. Furthermore, PD also ameliorated CSP-induced renal injury as revealed by the decreased intraluminal cast formation and diminished epithelial desquamation. These effects were mediated in part by quenching ROS generation and suppressing the apoptosis cascade [108].

2.34. Quercetin. Quercetin is one of the most abundant flavonoids found in several plant species and exerts numerous beneficial effects on health including cardioprotection, anti-inflammatory, anti-proliferative, and anticancer activities [109]. Quercetin ameliorated CSP-induced nephrotoxicity by mitigating oxidative stress, inflammation, and cell death pathways. Specifically, quercetin diminished renal lipid peroxidation, MAPK, and NFκB activation, proinflammatory cytokine expression, and caspase activation, as well as decreasing apoptosis. The improvements in the molecular pathology induced by quercetin corroborated the improved renal function in CSP-treated animals [110–113].

2.35. Resveratrol. Resveratrol is a phenolic compound present in several botanical species such as mulberries, peanuts, red grapes, cranberries, and blueberries [114]. Resveratrol attenuated CSP-induced nephrotoxicity by augmenting the endogenous antioxidant defense system via SIRT1 and Nrf2 activation. Furthermore, it inhibited inflammatory cytokine production by blunting NFκB activation and immune cell infiltration in renal tissues. In addition, resveratrol also inhibited CSP-induced renal apoptosis by downregulating p53 expression and restoring the Bax/Bcl-2 ratio. Furthermore, resveratrol enhanced the chemosensitivity of CSP without compromising its antitumor activity [115–118].

2.36. Rosmarinic Acid. Rosmarinic acid is an ester of caffeic acid that is abundantly present in rosemary (*Rosmarinus officinalis*) [119]. Rosmarinic acid treatment diminished the CSP-induced renal toxicity by attenuating oxidative stress, and this effect was characterized by decreased accumulation of 4-hydroxynonenal (4-HNE) formation with improvement in superoxide dismutase (SOD) activity and glutathione (GSH) levels. The beneficial effects of rosmarinic acid, in part, were mediated by its inhibition of the expression and activity of CYP2E1. In addition, rosmarinic acid inhibited CSP-induced inflammation by blunting NFκB activation and apoptosis by reducing p53 activation and DNA damage [120].

2.37. Rutin. Rutin is a glycone of quercetin, which has been extracted from various citrus fruits [121]. The mechanism of the renoprotection induced by rutin against CSP toxicity is mediated by the suppression of oxidative stress, NFκB activation, inflammatory cytokine expression, and apoptosis [86, 122].

2.38. Schizandrin and Schizandrin B. Schizandrin is a lignan found in the Chinese berry (*Schisandra chinensis*) [123]. Giridharan et al. [124] documented that schizandrin B inhibited CSP-induced oxidative stress, inflammation, and apoptosis by attenuating NFκB, p53 accumulation, and cleaved caspase-3 expression. Furthermore, schizandrin B induced the activation of Nrf2 and its downstream target genes such as HO-1 and gamma-glutamylcysteine synthetase (*GGCS*), which is the rate-limiting enzyme involved in GSH synthesis. Furthermore, schizandrin B also inhibited CSP-induced nicotinamide adenine dinucleotide phosphate (NAD[P]H) dehydrogenase [quinone] 1 (NQO1) enzymatic activity. It is pertinent to note that NQO1 is involved in the one-electron reduction of quinones which produces superoxide and, thereby, propagates oxidative stress [125].

2.39. Silibinin. Silibinin is a flavonoid extracted from *Silybum marianum*, popularly known as the milk thistle [126]. Gaedeke et al. [127] demonstrated that silibinin inhibited CSP-renal damage by preserving the proximal tubular function and ameliorating proteinuria. However, the precise molecular mechanism underlying this action was not investigated. In another study, silibinin protected the kidneys against CSP-induced renal toxicity without compromising the antitumor activity of CSP in rodents [128].

2.40. Sulforaphane. Sulforaphane is an isothiocyanate present in cruciferous vegetables such as broccoli, Brussels sprout, and cabbage [129]. Sulforaphane inhibited CSP-induced renal dysfunction, structural damage, oxidative/nitrative stress, inflammation, and apoptosis. Mechanistically, sulforaphane attenuated MAPK and NFκB activation and stimulated Nrf2 activation [130, 131]. In addition, several synthetic analogs of sulforaphane also exerted renoprotective activity against CSP-induced nephrotoxicity by the aforementioned mechanisms [132].

2.41. Tannic Acid. Tannins belong to the class of polyphenols and have been shown to possess multiple biological activities including anticancer [133], antioxidant, and antimicrobial activities [134]. Yokozawa et al. [135] demonstrated that tannic acid administration restored antioxidant levels, decreased lipid peroxidation, and improve renal function. Tannic acid also decreased CSP-induced DNA fragmentation by diminishing p53 activation [136]. Furthermore, green tea tannin has been reported to restore the kidney function and synergistically enhance the cell death of ovarian cancer cells by CSP [137]. In addition, Tikoo et al. [138] reported that tannic acid decreased PARP cleavage, phosphorylation of p38, and hypoacetylation of histone H4, which diminished kidney injury, indicating the efficacy of tannic acid as a therapeutic drug for CSP-induced nephrotoxicity.

2.42. Thymoquinone. Thymoquinone is a bioactive compound derived from *Nigella sativa* popularly known as black seed oil. Thymoquinone has been shown to exert anti-inflammatory, antioxidant, and antineoplastic effects in both *in vitro* and *in vivo* studies [139]. Thymoquinone was shown to improve kidney function, diminish lipid peroxidation, and augment endogenous antioxidants [139]. In addition, thymoquinone has also been shown to increase the expression of various organic anion and cation transporters such as OAT1, OAT3, OCT1, and OCT2, which are necessary for the renal clearance of xenobiotic agents including toxins and commonly used drugs [140, 141].

2.43. Xanthorrhizol. Xanthorrhizol is one of the major constituents from the rhizomes of *Curcuma xanthorrhiza,* a medicinal plant native to Indonesia [142]. Kim et al. [143] demonstrated the renoprotective action of xanthorrhizol against CSP-induced nephrotoxicity mediated by inhibiting NFκB and activator protein-1 (AP-1) activation, proinflammatory cytokine expression, immune cell infiltration, and apoptosis. Furthermore, mechanistic studies revealed that xanthorrhizol suppressed CSP-induced phosphorylation of c-Jun N-terminal kinase (JNK) and p53, as well as the shutdown of the mitochondria-mediated apoptosis pathway [144].

2.44. Renoprotective Actions of Phytochemicals in Human Studies. The review of the published literature revealed that several preclinical studies reported the renoprotective properties of phytochemicals. Currently, there is no significant evidence from clinical trials indicating that phytochemicals show renoprotective efficacy in human subjects undergoing CSP chemotherapy. However, a recent open-labeled randomized clinical trial undertaken in a small patient population suggested that treatment with cystone (a herbomineral ayurvedic formulation) in combination with CSP chemotherapy improved renal function without compromising the antitumor effects of CSP. However, long-term follow-up data and survival rates were not presented in this study and, therefore, more stringent, well-designed, and controlled clinical trials are warranted to establish the clinical efficacy of cystone in combating CSP-induced nephrotoxicity [145].

3. Conclusion

The analysis of literature suggests that plant-derived agents (phytochemicals) are widely used to prevent the CSP-induced renal toxicity, and it is evident that these compounds exhibited potentially effective renal protection in preclinical studies. However, the major impediment to the clinical translation of these compounds for further pharmaceutical development pertains to the lack of convincing evidence of their bioavailability in human subjects [146, 147]. In addition, the therapeutic indexes for various phytochemicals are presently unknown. Therefore, future studies should investigate the analogs and derivatives of phytochemicals with demonstrable bioavailability in human subjects, and these molecules should be thoroughly investigated in preclinical models for further pharmaceutical development. In addition, most studies reported in the literature demonstrated the prophylactic action of phytochemicals in combating CSP-induced renal tissue injury. However, this approach has major limitations because clinically patients require treatment after and not before the onset of kidney damage. Therefore, future studies should essentially investigate the therapeutic effect of phytochemicals against CSP-induced nephrotoxicity in preclinical models. Specifically, studies must report the effect of phytochemical administration after the establishment of renal tissue injury and present the survival rate of the animal models. Finally, to establish the renoprotective actions of phytochemicals, studies need to be conducted in rodents harboring tumors that are sensitive to CSP. This is to ascertain that the beneficial effects of the phytochemicals do not compromise or interfere with the antitumor activity of CSP.

Abbreviations

CSP: Cisplatin
CB_2: Cannabinoid receptor-2

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B-cells
CYP2E1: Cytochrome P450 2E1
MAPK: Mitogen-activated protein kinase
Bax: B-cell lymphoma 2- (Bcl-2-) associated X protein
Bcl-2: B-cell lymphoma 2
HO-1: Heme oxygenase-1
p53: Tumor-suppressor protein p53
SIRT, Sirtuin: Silent mating type information regulation 2 homolog
Nrf2: Nuclear factor erythroid-derived 2
ROS: Reactive oxygen species
ERK: Extracellular signal-regulated kinases
Akt: Serine/threonine-specific protein kinase (synonym: protein kinase B)
PARP: Poly adenosine diphosphate (ADP) ribose polymerase
KIM-1: Kidney injury molecule-1
NAG: *N*-acetyl-D-glucosamine
AMPK: Adenosine monophosphate-activated protein kinase
mTOR: Mechanistic target of rapamycin
GRP-78: 78 kDa glucose-regulated protein
Fas-L: Fas ligand [synonym: cluster of differentiation antigen 95 (CD95) ligand]
LDH: Lactate dehydrogenase
JNK: c-Jun N-terminal kinases
TUNEL: Terminal deoxynucleotidyl transferase deoxyuridine 5′-triphosphate (dUTP) nick-end labeling
PUMA: p53-upregulated modulator of apoptosis
OAT: Organic anion transporter
OCT: Organic cation transporter
MRP: Multidrug resistance-associated proteins
HSP: Heat shock protein
iNOS: Inducible nitric oxide synthase
COX-2: Cyclooxygenase-2
4-HNE: 4-Hydroxynonenal
SOD: Superoxide dismutase
GSH: Glutathione (reduced)
GCLC: Gamma-glutamyl cysteine synthetase
NQO1: NAD(P)H dehydrogenase [quinone] 1
AP-1: Activator protein-1
BUN: Blood urea nitrogen
MDA: Malondialdehyde
CAT: Catalase
GR: Glutathione reductase
NOX: NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) oxidase
3-NT/3-NY: 3-Nitrotyrosine
TNF-α: Tumor necrosis factor alpha
GPx: Glutathione peroxidase
p38: p38 MAPK
NO: Nitric oxide
IL-1β: Interleukin 1 beta
NAMPT: Nicotinamide phosphoribosyl transferase
MCP-1: Monocyte chemoattractant protein-1
ICAM-1: Intercellular adhesion molecule-1
TBARS: Thiobarbituric acid reactive substances
IL: Interleukin
TAC: Total antioxidant capacity
GST: Glutathione S-transferase
XO: Xanthine oxidase
TOS: Total oxidant status
LPO: Lipid peroxidation
H_2O_2: Hydrogen peroxide
ALP: Alkaline Phosphatase
GSSG: Glutathione disulfide
ICAM: Intercellular adhesion molecule
VCAM: Vascular cell adhesion protein
MPO: Myeloperoxidase
G6PD: Glucose-6-phosphate dehydrogenase
QR: Quinone reductase
I.P.: Intraperitoneal injection
I.V.: Intravenous administration
LLC-PK1: Renal epithelial cells derived from normal pig kidney
HK-2 cells: Proximal tubular epithelial cells from normal human kidney
HRCs: Human primary epithelial cells from cortex and glomeruli
ETC: Electron transport chain
XOR: Xanthine oxidoreductase
CXCL1: Chemokine (C-X-C motif) ligand 1
ER: Endoplasmic reticulum
PMN: Polymorphonuclear (neutrophil).

Competing Interests

There is no conflict of interests to disclose.

Authors' Contributions

Shreesh Ojha and Balaji Venkataraman contributed equally to this article.

Acknowledgments

Mohanraj Rajesh, Shreesh Ojha, and Bassem Sadek were supported by intermural funds from the College of Medicine and Health Sciences and the Office of Graduate Studies and Research, UAE University.

References

[1] H. Fukasawa, R. Furuya, H. Yasuda, T. Yamamoto, A. Hishida, and M. Kitagawa, "Anti-cancer agent-induced nephrotoxicity," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 14, no. 7, pp. 921–927, 2014.

[2] A. Ruggiero, G. Trombatore, S. Triarico et al., "Platinum compounds in children with cancer: toxicity and clinical management," *Anti-Cancer Drugs*, vol. 24, no. 10, pp. 1007–1019, 2013.

[3] T. Karasawa and P. S. Steyger, "An integrated view of cisplatin-induced nephrotoxicity and ototoxicity," *Toxicology Letters*, vol. 237, no. 3, pp. 219–227, 2015.

[4] V. Launay-Vacher, J.-B. Rey, C. Isnard-Bagnis, G. Deray, and M. Daouphars, "Prevention of cisplatin nephrotoxicity: state of the art and recommendations from the European Society of Clinical Pharmacy Special Interest Group on Cancer Care," *Cancer Chemotherapy and Pharmacology*, vol. 61, no. 6, pp. 903–909, 2008.

[5] N. A. G. Dos Santos, M. A. C. Rodrigues, N. M. Martins, and A. C. Dos Santos, "Cisplatin-induced nephrotoxicity and targets of nephroprotection: an update," *Archives of Toxicology*, vol. 86, no. 8, pp. 1233–1250, 2012.

[6] J. S. Bland, "Phytonutrition, phytotherapy, and phytopharmacology," *Alternative Therapies in Health and Medicine*, vol. 2, no. 6, pp. 73–76, 1996.

[7] L. C. Morejohn and D. E. Fosket, "The biochemistry of compounds with anti-microtubule activity in plant cells," *Pharmacology & Therapeutics*, vol. 51, no. 2, pp. 217–230, 1991.

[8] R. P. Miller, R. K. Tadagavadi, G. Ramesh, and W. B. Reeves, "Mechanisms of cisplatin nephrotoxicity," *Toxins*, vol. 2, no. 11, pp. 2490–2518, 2010.

[9] M. Finkel, A. Goldstein, Y. Steinberg, L. Granowetter, and H. Trachtman, "Cisplatinum nephrotoxicity in oncology therapeutics: retrospective review of patients treated between 2005 and 2012," *Pediatric Nephrology*, vol. 29, no. 12, pp. 2421–2424, 2014.

[10] G.-S. Oh, H.-J. Kim, A. Shen et al., "New therapeutic concept of NAD Redox balance for cisplatin nephrotoxicity," *BioMed Research International*, vol. 2016, Article ID 4048390, 12 pages, 2016.

[11] P. Mukhopadhyay, M. Rajesh, H. Pan et al., "Cannabinoid-2 receptor limits inflammation, oxidative/nitrosative stress, and cell death in nephropathy," *Free Radical Biology and Medicine*, vol. 48, no. 3, pp. 457–467, 2010.

[12] P. Mukhopadhyay, H. Pan, M. Rajesh et al., "CB_1 cannabinoid receptors promote oxidative/nitrosative stress, inflammation and cell death in a murine nephropathy model," *British Journal of Pharmacology*, vol. 160, no. 3, pp. 657–668, 2010.

[13] Y.-H. Kim, J.-H. Choi, H.-K. Rim et al., "23-Hydroxytormentic acid and niga-ichgoside F_1 isolated from *Rubus coreanus* attenuate cisplatin-induced cytotoxicity by reducing oxidative stress in renal epithelial LLC-PK_1 cells," *Biological and Pharmaceutical Bulletin*, vol. 34, no. 6, pp. 906–911, 2011.

[14] S.-I. Sohn, H.-K. Rim, Y.-H. Kim et al., "The ameliorative effect of 23-hydroxytormentic acid isolated from Rubus coreanus on cisplatin-induced nephrotoxicity in rats," *Biological and Pharmaceutical Bulletin*, vol. 34, no. 9, pp. 1508–1513, 2011.

[15] 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.

[16] C.-C. Wang, L.-G. Chen, L.-T. Lee, and L.-L. Yang, "Effects of 6-gingerol, an antioxidant from ginger, on inducing apoptosis in human leukemic HL-60 cells," *In Vivo*, vol. 17, no. 6, pp. 641–645, 2003.

[17] A. Kuhad, N. Tirkey, S. Pilkhwal, and K. Chopra, "6-Gingerol prevents cisplatin-induced acute renal failure in rats," *BioFactors*, vol. 26, no. 3, pp. 189–200, 2006.

[18] F. A. P. Rodrigues, M. M. G. Prata, I. C. M. Oliveira et al., "Gingerol fraction from Zingiber officinale protects against gentamicin-induced nephrotoxicity," *Antimicrobial Agents and Chemotherapy*, vol. 58, no. 4, pp. 1872–1878, 2014.

[19] J. H. Moon, J.-S. Shin, J.-B. Kim et al., "Protective effects of 6-hydroxy-1-methylindole-3-acetonitrile on cisplatin-induced oxidative nephrotoxicity via Nrf2 inactivation," *Food and Chemical Toxicology*, vol. 62, pp. 159–166, 2013.

[20] J. Gertsch, M. Leonti, S. Raduner et al., "Beta-caryophyllene is a dietary cannabinoid," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 26, pp. 9099–9104, 2008.

[21] I. Kubo, S. K. Chaudhuri, Y. Kubo et al., "Cytotoxic and antioxidative sesquiterpenoids from Heterotheca inuloides," *Planta Medica*, vol. 62, no. 5, pp. 427–430, 1996.

[22] G. Singh, P. Marimuthu, C. S. de Heluani, and C. A. N. Catalan, "Antioxidant and biocidal activities of *Carum nigrum* (Seed) essential oil, oleoresin, and their selected components," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 1, pp. 174–181, 2006.

[23] B. Horváth, P. Mukhopadhyay, M. Kechrid et al., "β-Caryophyllene ameliorates cisplatin-induced nephrotoxicity in a cannabinoid 2 receptor-dependent manner," *Free Radical Biology & Medicine*, vol. 52, no. 8, pp. 1325–1333, 2012.

[24] P. Mukhopadhyay, M. Rajesh, H. Pan et al., "Cannabinoid-2 receptor limits inflammation, oxidative/nitrosative stress, and cell death in nephropathy," *Free Radical Biology & Medicine*, vol. 48, no. 3, pp. 457–467, 2010.

[25] P. Pacher and G. Kunos, "Modulating the endocannabinoid system in human health and disease—successes and failures," *The FEBS Journal*, vol. 280, no. 9, pp. 1918–1943, 2013.

[26] X.-W. Chen, Y. M. Di, J. Zhang, Z.-W. Zhou, C. G. Li, and S.-F. Zhou, "Interaction of herbal compounds with biological targets: a case study with berberine," *The Scientific World Journal*, vol. 2012, Article ID 708292, 31 pages, 2012.

[27] R. Domitrović, O. Cvijanović, E. Pernjak-Pugel, M. Škoda, L. Mikelić, and Ž. Crnčević-Orlić, "Berberine exerts nephroprotective effect against cisplatin-induced kidney damage through inhibition of oxidative/nitrosative stress, inflammation, autophagy and apoptosis," *Food and Chemical Toxicology*, vol. 62, pp. 397–406, 2013.

[28] H. Liu and R. Baliga, "Cytochrome P450 2E1 null mice provide novel protection against cisplatin-induced nephrotoxicity and apoptosis," *Kidney International*, vol. 63, no. 5, pp. 1687–1696, 2003.

[29] C. R. Silva, L. M. G. Antunes, and M. D. L. P. Bianchi, "Antioxidant action of bixin against cisplatin-induced chromosome aberrations and lipid peroxidation in rats," *Pharmacological Research*, vol. 43, no. 6, pp. 561–566, 2001.

[30] G. C. Dos Santos, L. M. Mendonça, G. A. Antonucci, A. C. dos Santos, L. M. G. Antunes, and M. D. L. P. Bianchi, "Protective effect of bixin on cisplatin-induced genotoxicity in PC12 cells," *Food and Chemical Toxicology*, vol. 50, no. 2, pp. 335–340, 2012.

[31] B. Fernández-Rojas, O. N. Medina-Campos, R. Hernández-Pando, M. Negrette-Guzmán, S. Huerta-Yepez, and J. Pedraza-Chaverri, "C-Phycocyanin prevents cisplatin-induced nephrotoxicity through inhibition of oxidative stress," *Food and Function*, vol. 5, no. 3, pp. 480–490, 2014.

[32] B. J. Lim, J. Y. Jeong, Y.-K. Chang et al., "C-phycocyanin attenuates cisplatin-induced nephrotoxicity in mice," *Renal Failure*, vol. 34, no. 7, pp. 892–900, 2012.

[33] S. Özen, Ö. Akyol, M. Iraz et al., "Role of caffeic acid phenethyl ester, an active component of propolis, against cisplatin-induced nephrotoxicity in rats," *Journal of Applied Toxicology*, vol. 24, no. 1, pp. 27–35, 2004.

[34] A. Kart, Y. Cigremis, M. Karaman, and H. Ozen, "Caffeic acid phenethyl ester (CAPE) ameliorates cisplatin-induced hepatotoxicity in rabbit," *Experimental and Toxicologic Pathology*, vol. 62, no. 1, pp. 45–52, 2010.

[35] R. Mechoulam, M. Peters, E. Murillo-Rodriguez, and L. O. Hanuš, "Cannabidiol—recent advances," *Chemistry & Biodiversity*, vol. 4, no. 8, pp. 1678–1692, 2007.

[36] H. Pan, P. Mukhopadhyay, M. Rajesh et al., "Cannabidiol attenuates cisplatin-Lnduced nephrotoxicity by decreasing oxidative/nitrosative stress, inflammation, and cell death," *Journal of Pharmacology and Experimental Therapeutics*, vol. 328, no. 3, pp. 708–714, 2009.

[37] A. Szallasi and P. M. Blumberg, "Vanilloid (capsaicin) receptors and mechanisms," *Pharmacological Reviews*, vol. 51, no. 2, pp. 159–212, 1999.

[38] Y. Shimeda, Y. Hirotani, Y. Akimoto et al., "Protective effects of capsaicin against cisplatin-induced nephrotoxicity in rats," *Biological & Pharmaceutical Bulletin*, vol. 28, no. 9, pp. 1635–1638, 2005.

[39] S.-H. Jung, H.-J. Kim, G.-S. Oh et al., "Capsaicin ameliorates cisplatin-induced renal injury through induction of heme oxygenase-1," *Molecules and Cells*, vol. 37, no. 3, pp. 234–240, 2014.

[40] M. K. Park, S. H. Jo, H. J. Lee et al., "Novel suppressive effects of cardamonin on the activity and expression of transglutaminase-2 lead to blocking the migration and invasion of cancer cells," *Life Sciences*, vol. 92, no. 2, pp. 154–160, 2013.

[41] R. N. El-Naga, "Pre-treatment with cardamonin protects against cisplatin-induced nephrotoxicity in rats: impact on NOX-1, inflammation and apoptosis," *Toxicology and Applied Pharmacology*, vol. 274, no. 1, pp. 87–95, 2014.

[42] Z. Wei, J. Yang, Y.-F. Xia, W.-Z. Huang, Z.-T. Wang, and Y. Dai, "Cardamonin protects septic mice from acute lung injury by preventing endothelial barrier dysfunction," *Journal of Biochemical and Molecular Toxicology*, vol. 26, no. 7, pp. 282–290, 2012.

[43] Y.-J. Kim, H. Ko, J.-S. Park et al., "Dimethyl cardamonin inhibits lipopolysaccharide-induced inflammatory factors through blocking NF-κB p65 activation," *International Immunopharmacology*, vol. 10, no. 9, pp. 1127–1134, 2010.

[44] J.-H. Lee, S. J. Haeng, M. G. Phan et al., "Blockade of nuclear factor-κB signaling pathway and anti-inflammatory activity of cardamomin, a chalcone analog from *Alpinia conchigera*," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 316, no. 1, pp. 271–278, 2006.

[45] B. D. Sahu, K. K. R. Rentam, U. K. Putcha, M. Kuncha, G. M. N. Vegi, and R. Sistla, "Carnosic acid attenuates renal injury in an experimental model of rat cisplatin-induced nephrotoxicity," *Food and Chemical Toxicology*, vol. 49, no. 12, pp. 3090–3097, 2011.

[46] H. Cho, C.-W. Yun, W.-K. Park et al., "Modulation of the activity of pro-inflammatory enzymes, COX-2 and iNOS, by chrysin derivatives," *Pharmacological Research*, vol. 49, no. 1, pp. 37–43, 2004.

[47] S. Sultana, K. Verma, and R. Khan, "Nephroprotective efficacy of chrysin against cisplatin-induced toxicity via attenuation of oxidative stress," *Journal of Pharmacy and Pharmacology*, vol. 64, no. 6, pp. 872–881, 2012.

[48] R. Khan, A. Q. Khan, W. Qamar et al., "Chrysin abrogates cisplatin-induced oxidative stress, p53 expression, goblet cell disintegration and apoptotic responses in the jejunum of Wistar rats," *British Journal of Nutrition*, vol. 108, no. 9, pp. 1574–1585, 2012.

[49] E.-S. M. El-Sayed, O. M. Abd El-Raouf, H. M. Fawzy, and M. F. Manie, "Comparative study of the possible protective effects of cinnamic acid and cinnamaldehyde on cisplatin-induced nephrotoxicity in rats," *Journal of Biochemical and Molecular Toxicology*, vol. 27, no. 12, pp. 508–514, 2013.

[50] B. H. Ali, H. Marrif, S. A. Noureldayem, A. O. Bakheit, and G. Blundene, "Some biological properties of curcumin: a review," *Natural Product Communications*, vol. 1, no. 6, pp. 509–521, 2006.

[51] L. M. G. Antunes, J. D. C. Darin, and M. D. L. P. Bianchi, "Effects of the antioxidants curcumin or selenium on cisplatin-induced nephrotoxicity and lipid peroxidation in rats," *Pharmacological Research*, vol. 43, no. 2, pp. 145–150, 2001.

[52] A. Kuhad, S. Pilkhwal, S. Sharma, N. Tirkey, and K. Chopra, "Effect of curcumin on inflammation and oxidative stress in cisplatin-induced experimental nephrotoxicity," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 25, pp. 10150–10155, 2007.

[53] M. I. Waly, M. S. Al Moundhri, and B. H. Ali, "Effect of curcumin on cisplatin- and oxaliplatin-induced oxidative stress in human embryonic kidney (HEK) 293 cells," *Renal Failure*, vol. 33, no. 5, pp. 518–523, 2011.

[54] M. Ueki, M. Ueno, J. Morishita, and N. Maekawa, "Curcumin ameliorates cisplatin-induced nephrotoxicity by inhibiting renal inflammation in mice," *Journal of Bioscience and Bioengineering*, vol. 115, no. 5, pp. 547–551, 2013.

[55] J. Trujillo, Y. I. Chirino, E. Molina-Jijón, A. C. Andérica-Romero, E. Tapia, and J. Pedraza-Chaverrí, "Renoprotective effect of the antioxidant curcumin: recent findings," *Redox Biology*, vol. 1, no. 1, pp. 448–456, 2013.

[56] S. Ugur, R. Ulu, A. Dogukan et al., "The renoprotective effect of curcumin in cisplatin-induced nephrotoxicity," *Renal Failure*, vol. 37, no. 2, pp. 332–336, 2015.

[57] D. Bagchi, A. Garg, and R. L. Krohn, "Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice," *General Pharmacology*, vol. 30, no. 5, pp. 771–776, 1998.

[58] S. Gao, T. Chen, M.-Y. Choi, Y. Liang, J. Xue, and Y.-S. Wong, "Cyanidin reverses cisplatin-induced apoptosis in HK-2 proximal tubular cells through inhibition of ROS-mediated DNA damage and modulation of the ERK and AKT pathways," *Cancer Letters*, vol. 333, no. 1, pp. 36–46, 2013.

[59] J. H. Kim, S.-J. Jeong, H.-Y. Kwon et al., "Decursin prevents cisplatin-induced apoptosis via the enhancement of antioxidant enzymes in human renal epithelial cells," *Biological and Pharmaceutical Bulletin*, vol. 33, no. 8, pp. 1279–1284, 2010.

[60] H. I. Jiang Cheng-Zhe and C. Seyoung, "Seyoung: Decursin mediated protection on cisplatin-induced nephrotoxicity in SD Rats and BDF1 mice," *Journal of Northeast Agricultural University*, vol. 19, no. 1, pp. 50–56, 2012.

[61] J. Zhang, L. Li, T. W. Hale et al., "Single oral dose pharmacokinetics of decursin and decursinol angelate in healthy adult men and women," *PLoS ONE*, vol. 10, no. 2, Article ID e0114992, 2015.

[62] R.-F. Wang, W.-D. Xie, Z. Zhang et al., "Bioactive compounds from the seeds of *Punica granatum* (pomegranate)," *Journal of Natural Products*, vol. 67, no. 12, pp. 2096–2098, 2004.

[63] N. Al-Kharusi, H. A. Babiker, S. Al-Salam et al., "Ellagic acid protects against cisplatin-induced nephrotoxicity in rats: a dose-dependent study," *European Review for Medical and Pharmacological Sciences*, vol. 17, no. 3, pp. 299–310, 2013.

[64] A. M. El-Garhy, O. M. Abd El-Raouf, B. M. El-Sayeh, H. M. Fawzy, and D. M. Abdallah, "Ellagic acid antiinflammatory

and antiapoptotic potential mediate renoprotection in cisplatin nephrotoxic rats," *Journal of Biochemical and Molecular Toxicology*, vol. 28, no. 10, pp. 472–479, 2014.

[65] A. Ateşşahín, A. O. Çeríbaşi, A. Yuce, Ö. Bulmus, and G. Çikim, "Role of ellagic acid against cisplatin-induced nephrotoxicity and oxidative stress in rats," *Basic & Clinical Pharmacology & Toxicology*, vol. 100, no. 2, pp. 121–126, 2007.

[66] Y.-C. Chang, T.-Y. Lai, C.-S. Yu et al., "Emodin induces apoptotic death in murine myelomonocytic leukemia WEHI-3 cells in vitro and enhances phagocytosis in leukemia mice in vivo," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 523596, 13 pages, 2011.

[67] J.-M. Yon, I.-J. Baek, B. J. Lee, Y. W. Yun, and S.-Y. Nam, "Emodin and [6]-gingerol lessen hypoxia-induced embryotoxicities in cultured mouse whole embryos via upregulation of hypoxia-inducible factor 1α and intracellular superoxide dismutases," *Reproductive Toxicology*, vol. 31, no. 4, pp. 513–518, 2011.

[68] M. I. Waly, B. H. Ali, I. Al-Lawati, and A. Nemmar, "Protective effects of emodin against cisplatin-induced oxidative stress in cultured human kidney (HEK 293) cells," *Journal of Applied Toxicology*, vol. 33, no. 7, pp. 626–630, 2013.

[69] B. H. Ali, S. Al-Salam, I. S. Al Husseini et al., "Abrogation of cisplatin-induced nephrotoxicity by emodin in rats," *Fundamental & Clinical Pharmacology*, vol. 27, no. 2, pp. 192–200, 2013.

[70] C. Bazzi, C. Petrini, V. Rizza et al., "Urinary *N*-acetyl-β-glucosaminidase excretion is a marker of tubular cell dysfunction and a predictor of outcome in primary glomerulonephritis," *Nephrology Dialysis Transplantation*, vol. 17, no. 11, pp. 1890–1896, 2002.

[71] H. Liu, L.-B. Gu, Y. Tu, H. Hu, Y.-R. Huang, and W. Sun, "Emodin ameliorates cisplatin-induced apoptosis of rat renal tubular cells in vitro by activating autophagy," *Acta Pharmacologica Sinica*, vol. 37, no. 2, pp. 235–245, 2016.

[72] K. Sahin, M. Tuzcu, H. Gencoglu et al., "Epigallocatechin-3-gallate activates Nrf2/HO-1 signaling pathway in cisplatin-induced nephrotoxicity in rats," *Life Sciences*, vol. 87, no. 7-8, pp. 240–245, 2010.

[73] H. Pan, J. Chen, K. Shen et al., "Mitochondrial modulation by epigallocatechin 3-gallate ameliorates cisplatin induced renal injury through decreasing oxidative/nitrative stress, inflammation and NF-kB in mice," *PLoS ONE*, vol. 10, no. 4, Article ID e0124775, 2015.

[74] S. Fatima, N. Al-Mohaimeed, S. Arjumand, N. Banu, N. Al-Jameil, and Y. Al-Shaikh, "Effect of pre- and post-combined multidoses of epigallocatechin gallate and coenzyme Q10 on cisplatin-induced oxidative stress in rat kidney," *Journal of Biochemical and Molecular Toxicology*, vol. 29, no. 2, pp. 91–97, 2015.

[75] B. Chen, G. Liu, P. Zou et al., "Epigallocatechin-3-gallate protects against cisplatin-induced nephrotoxicity by inhibiting endoplasmic reticulum stress-induced apoptosis," *Experimental Biology and Medicine*, vol. 240, no. 11, pp. 1513–1519, 2015.

[76] P. Zou, J. Song, B. Jiang et al., "Epigallocatechin-3-gallate protects against cisplatin nephrotoxicity by inhibiting the apoptosis in mouse," *International Journal of Clinical and Experimental Pathology*, vol. 7, no. 8, pp. 4607–4616, 2014.

[77] M. J. Sung, D. H. Kim, Y. J. Jung et al., "Genistein protects the kidney from cisplatin-induced injury," *Kidney International*, vol. 74, no. 12, pp. 1538–1547, 2008.

[78] S. H. Baek, X. L. Piao, U. J. Lee, H. Y. Kim, and J. H. Park, "Reduction of cisplatin-induced nephrotoxicity by ginsenosides isolated from processed ginseng in cultured renal tubular cells," *Biological & Pharmaceutical Bulletin*, vol. 29, no. 10, pp. 2051–2055, 2006.

[79] M.-S. Han, I.-H. Han, D. Lee et al., "Beneficial effects of fermented black ginseng and its ginsenoside 20(S)-Rg3 against cisplatin-induced nephrotoxicity in LLC-PK1 cells," *Journal of Ginseng Research*, vol. 40, no. 2, pp. 135–140, 2016.

[80] J. Y. Park, P. Choi, T. Kim et al., "Protective effects of processed ginseng and its active ginsenosides on cisplatin-induced nephrotoxicity: in vitro and in vivo studies," *Journal of Agricultural and Food Chemistry*, vol. 63, no. 25, pp. 5964–5969, 2015.

[81] S. Rahman and S. Sultana, "Glycyrrhizin exhibits potential chemopreventive activity on 12-*O*-tetradecanoyl phorbol-13-acetate-induced cutaneous oxidative stress and tumor promotion in Swiss albino mice," *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 22, no. 3, pp. 363–369, 2007.

[82] W. Arjumand and S. Sultana, "Glycyrrhizic acid: a phytochemical with a protective role against cisplatin-induced genotoxicity and nephrotoxicity," *Life Sciences*, vol. 89, no. 13-14, pp. 422–429, 2011.

[83] L. A. Baltina, "Chemical modification of glycyrrhizic acid as a route to new bioactive compounds for medicine," *Current Medicinal Chemistry*, vol. 10, no. 2, pp. 155–171, 2003.

[84] C.-H. Wu, A.-Z. Chen, and G.-C. Yen, "Protective effects of glycyrrhizic acid and 18β-glycyrrhetinic acid against cisplatin-induced nephrotoxicity in BALB/c mice," *Journal of Agricultural and Food Chemistry*, vol. 63, no. 4, pp. 1200–1209, 2015.

[85] B. D. Sahu, M. Kuncha, G. J. Sindhura, and R. Sistla, "Hesperidin attenuates cisplatin-induced acute renal injury by decreasing oxidative stress, inflammation and DNA damage," *Phytomedicine*, vol. 20, no. 5, pp. 453–460, 2013.

[86] K. M. Kamel, O. M. Abd El-Raouf, S. A. Metwally, H. A. Abd El-Latif, and M. E. El-sayed, "Hesperidin and rutin, antioxidant citrus flavonoids, attenuate cisplatin-induced nephrotoxicity in rats," *Journal of Biochemical and Molecular Toxicology*, vol. 28, no. 7, pp. 312–319, 2014.

[87] C. K. Lee, S. H. Son, K. K. Park, J. H. Y. Park, S. S. Lim, and W. Y. Chung, "Isoliquiritigenin inhibits tumor growth and protects the kidney and liver against chemotherapy-induced toxicity in a mouse xenograft model of colon carcinoma," *Journal of Pharmacological Sciences*, vol. 106, no. 3, pp. 444–451, 2008.

[88] 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.

[89] C. K. Lee, S. H. Son, K. K. Park et al., "Licochalcone a inhibits the growth of colon carcinoma and attenuates cisplatin-induced toxicity without a loss of chemotherapeutic efficacy in mice," *Basic and Clinical Pharmacology and Toxicology*, vol. 103, no. 1, pp. 48–54, 2008.

[90] X.-H. Liu, J. Li, Q.-X. Li, Y.-X. Ai, and L. Zhang, "Protective effects of ligustrazine on cisplatin-induced oxidative stress, apoptosis and nephrotoxicity in rats," *Environmental Toxicology and Pharmacology*, vol. 26, no. 1, pp. 49–55, 2008.

[91] L. L. Li, Z. R. Zhang, T. Gong, L. L. He, and L. Deng, "Simultaneous determination of Gastrodin and Ligustrazine hydrochloride in dog plasma by gradient high-performance liquid chromatography," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 41, no. 4, pp. 1083–1087, 2006.

[92] R. Domitrović, O. Cvijanović, E. P. Pugel, G. B. Zagorac, H. Mahmutefendić, and M. Škoda, "Luteolin ameliorates cisplatin-induced nephrotoxicity in mice through inhibition of platinum accumulation, inflammation and apoptosis in the kidney," *Toxicology*, vol. 310, pp. 115–123, 2013.

[93] K. P. Kang, S. K. Park, D. H. Kim et al., "Luteolin ameliorates cisplatin-induced acute kidney injury in mice by regulation of p53-dependent renal tubular apoptosis," *Nephrology Dialysis Transplantation*, vol. 26, no. 3, pp. 814–822, 2011.

[94] K. Sahin, N. Sahin, and O. Kucuk, "Lycopene and chemotherapy toxicity," *Nutrition and Cancer*, vol. 62, no. 7, pp. 988–995, 2010.

[95] O. Kucuk, F. H. Sarkar, W. Sakr et al., "Phase II randomized clinical trial of lycopene supplementation before radical prostatectomy," *Cancer Epidemiology Biomarkers and Prevention*, vol. 10, no. 8, pp. 861–868, 2001.

[96] K. Sahin, M. Tuzcu, N. Sahin, S. Ali, and O. Kucuk, "Nrf2/HO-1 signaling pathway may be the prime target for chemoprevention of cisplatin-induced nephrotoxicity by lycopene," *Food and Chemical Toxicology*, vol. 48, no. 10, pp. 2670–2674, 2010.

[97] A. Dogukan, M. Tuzcu, C. A. Agca et al., "A tomato lycopene complex protects the kidney from cisplatin-induced injury via affecting oxidative stress as well as Bax, Bcl-2, and HSPs expression," *Nutrition and Cancer*, vol. 63, no. 3, pp. 427–434, 2011.

[98] A. Atessahin, S. Yilmaz, I. Karahan, A. O. Ceribasi, and A. Karaoglu, "Effects of lycopene against cisplatin-induced nephrotoxicity and oxidative stress in rats," *Toxicology*, vol. 212, no. 2-3, pp. 116–123, 2005.

[99] F. Erman, M. Tuzcu, C. Orhan, N. Sahin, and K. Sahin, "Effect of lycopene against cisplatin-induced acute renal injury in rats: organic anion and cation transporters evaluation," *Biological Trace Element Research*, vol. 158, no. 1, pp. 90–95, 2014.

[100] S. Kawaii, Y. Tomono, E. Katase et al., "Quantitative study of flavonoids in leaves of *Citrus* plants," *Journal of Agricultural and Food Chemistry*, vol. 48, no. 9, pp. 3865–3871, 2000.

[101] O. A. Badary, S. Abdel-Maksoud, W. A. Ahmed, and G. H. Owieda, "Naringenin attenuates cisplatin nephrotoxicity in rats," *Life Sciences*, vol. 76, no. 18, pp. 2125–2135, 2005.

[102] H. Lee, G. Lee, H. Kim, and H. Bae, "Paeonol, a major compound of moutan cortex, attenuates Cisplatin-induced nephrotoxicity in mice," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 310989, 7 pages, 2013.

[103] H.-S. Chung, M. Kang, C. Cho et al., "Inhibition of nitric oxide and tumor necrosis factor-alpha by moutan cortex in activated mouse peritoneal macrophages," *Biological and Pharmaceutical Bulletin*, vol. 30, no. 5, pp. 912–916, 2007.

[104] A. S. Hofmann and G. G. Gross, "Biosynthesis of gallotannins: formation of polygalloylglucoses by enzymatic acylation of 1,2,3,4,6-penta-O-galloylglucose," *Archives of Biochemistry and Biophysics*, vol. 283, no. 2, pp. 530–532, 1990.

[105] A. Dörrenhaus, J. I. F. Müller, K. Golka, P. Jedrusik, H. Schulze, and W. Föllmann, "Cultures of exfoliated epithelial cells from different locations of the human urinary tract and the renal tubular system," *Archives of Toxicology*, vol. 74, no. 10, pp. 618–626, 2000.

[106] H.-G. Ryu, S.-J. Jeong, H.-Y. Kwon et al., "Penta-O-galloyl-β-D-glucose attenuates cisplatin-induced nephrotoxicity via reactive oxygen species reduction in renal epithelial cells and enhances antitumor activity in Caki-2 renal cancer cells," *Toxicology in Vitro*, vol. 26, no. 2, pp. 206–214, 2012.

[107] K. S. Ahn, E. J. Noh, H. L. Zhao, S. H. Jung, S. S. Kang, and Y. S. Kim, "Inhibition of inducible nitric oxide synthase and cyclooxygenase II by *Platycodon grandiflorum* saponins via suppression of nuclear factor-κB activation in RAW 264.7 cells," *Life Sciences*, vol. 76, no. 20, pp. 2315–2328, 2005.

[108] T.-W. Kim, I.-B. Song, H.-K. Lee et al., "Platycodin D, a triterpenoid sapoinin from *Platycodon grandiflorum*, ameliorates cisplatin-induced nephrotoxicity in mice," *Food and Chemical Toxicology*, vol. 50, no. 12, pp. 4254–4259, 2012.

[109] A. W. Boots, G. R. M. M. Haenen, and A. Bast, "Health effects of quercetin: from antioxidant to nutraceutical," *European Journal of Pharmacology*, vol. 585, no. 2-3, pp. 325–337, 2008.

[110] O. A. Almaghrabi, "Molecular and biochemical investigations on the effect of quercetin on oxidative stress induced by cisplatin in rat kidney," *Saudi Journal of Biological Sciences*, vol. 22, no. 2, pp. 227–231, 2015.

[111] H. D. Coletta Francescato, T. M. Coimbra, R. S. Costa, and M. D. L. P. Bianchi, "Protective effect of quercetin on the evolution of cisplatin-induced acute tubular necrosis," *Kidney and Blood Pressure Research*, vol. 27, no. 3, pp. 148–158, 2004.

[112] E. B. Behling, M. C. Sendão, H. D. C. Francescato, L. M. G. Antunes, R. S. Costa, and M. D. L. P. Bianchi, "Comparative study of multiple dosage of quercetin against cisplatin-induced nephrotoxicity and oxidative stress in rat kidneys," *Pharmacological Reports*, vol. 58, no. 4, pp. 526–532, 2006.

[113] M. Aldemir, E. Okulu, K. Kösemehmetoğlu et al., "Evaluation of the protective effect of quercetin against cisplatin-induced renal and testis tissue damage and sperm parameters in rats," *Andrologia*, vol. 46, no. 10, pp. 1089–1097, 2014.

[114] A. M. Rimando, W. Kalt, J. B. Magee, J. Dewey, and J. R. Ballington, "Resveratrol, pterostilbene, and piceatannol in *Vaccinium* berries," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 15, pp. 4713–4719, 2004.

[115] A.-M. M. Osman, S. A. Telity, Z. A. Damanhouri et al., "Chemosensitizing and nephroprotective effect of resveratrol in cisplatin-treated animals," *Cancer Cell International*, vol. 15, article 6, 2015.

[116] D. H. Kim, Y. J. Jung, J. E. Lee et al., "Sirt1 activation by resveratrol ameliorates cisplatin-induced renal injury through deacetylation of p53," *American Journal of Physiology—Renal Physiology*, vol. 301, no. 2, pp. F427–F435, 2011.

[117] M. A. Valentovic, J. G. Ball, J. Mike Brown et al., "Resveratrol attenuates cisplatin renal cortical cytotoxicity by modifying oxidative stress," *Toxicology in Vitro*, vol. 28, no. 2, pp. 248–257, 2014.

[118] C. L. Do Amaral, H. D. C. Francescato, T. M. Coimbra et al., "Resveratrol attenuates cisplatin-induced nephrotoxicity in rats," *Archives of Toxicology*, vol. 82, no. 6, pp. 363–370, 2008.

[119] M. Petersen and M. S. J. Simmonds, "Rosmarinic acid," *Phytochemistry*, vol. 62, no. 2, pp. 121–125, 2003.

[120] R. Domitrović, I. Potočnjak, Ž. Crnčević-Orlić, and M. Škoda, "Nephroprotective activities of rosmarinic acid against cisplatin-induced kidney injury in mice," *Food and Chemical Toxicology*, vol. 66, pp. 321–328, 2014.

[121] S. Habtemariam and G. Lentini, "The therapeutic potential of rutin for diabetes: an update," *Mini-Reviews in Medicinal Chemistry*, vol. 15, no. 7, pp. 524–528, 2015.

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

[123] A. Panossian and G. Wikman, "Pharmacology of *Schisandra chinensis* Bail.: an overview of Russian research and uses in

medicine," *Journal of Ethnopharmacology*, vol. 118, no. 2, pp. 183–212, 2008.

[124] V. V. Giridharan, R. A. Thandavarayan, H. N. Bhilwade, K. M. Ko, K. Watanabe, and T. Konishi, "Schisandrin B, attenuates cisplatin-induced oxidative stress, genotoxicity and neurotoxicity through modulating NF-κB pathway in mice," *Free Radical Research*, vol. 46, no. 1, pp. 50–60, 2012.

[125] M. Li, J. Jin, J. Li et al., "Schisandrin B protects against nephrotoxicity induced by cisplatin in HK-2 cells via Nrf2-ARE activation," *Acta Pharmaceutica Sinica*, vol. 47, no. 11, pp. 1434–1439, 2012.

[126] P. Surai, "Silymarin as a natural antioxidant: an overview of the current evidence and perspectives," *Antioxidants*, vol. 4, no. 1, pp. 204–247, 2015.

[127] J. Gaedeke, L. M. Fels, C. Bokemeyer, U. Mengs, H. Stolte, and H. Lentzen, "Cisplatin nephrotoxicity and protection by silibinin," *Nephrology Dialysis Transplantation*, vol. 11, no. 1, pp. 55–62, 1996.

[128] C. Bokemeyer, L. M. Fels, T. Dunn et al., "Silibinin protects against cisplatin-induced nephrotoxicity without compromising cisplatin or ifosfamide anti-tumour activity," *British Journal of Cancer*, vol. 74, no. 12, pp. 2036–2041, 1996.

[129] J. W. Fahey and P. Talalay, "Antioxidant functions of sulforaphane: a potent inducer of phase II detoxication enzymes," *Food and Chemical Toxicology*, vol. 37, no. 9-10, pp. 973–979, 1999.

[130] C. E. Guerrero-Beltrán, M. Calderón-Oliver, E. Tapia et al., "Sulforaphane protects against cisplatin-induced nephrotoxicity," *Toxicology Letters*, vol. 192, no. 3, pp. 278–285, 2010.

[131] L. Gaona-Gaona, E. Molina-Jijón, E. Tapia et al., "Protective effect of sulforaphane pretreatment against cisplatin-induced liver and mitochondrial oxidant damage in rats," *Toxicology*, vol. 286, no. 1–3, pp. 20–27, 2011.

[132] T. Kim, Y.-J. Kim, I.-H. Han et al., "The synthesis of sulforaphane analogues and their protection effect against cisplatin induced cytotoxicity in kidney cells," *Bioorganic & Medicinal Chemistry Letters*, vol. 25, no. 1, pp. 62–66, 2015.

[133] T. Okuda, T. Yoshida, and T. Hatano, "Hydrolyzable tannins and related polyphenols," *Progress in the Chemistry of Organic Natural Products*, vol. 66, pp. 1–117, 1995.

[134] M. M. Cowan, "Plant products as antimicrobial agents," *Clinical Microbiology Reviews*, vol. 12, no. 4, pp. 564–582, 1999.

[135] T. Yokozawa, T. Nakagawa, K. I. Lee, E. J. Cho, K. Terasawa, and S. Takeuchi, "Effects of green tea tannin on cisplatin-induced nephropathy in LLC-PK1 cells and rats," *The Journal of Pharmacy and Pharmacology*, vol. 51, no. 11, pp. 1325–1331, 1999.

[136] S. T. Ahmad and S. Sultana, "Tannic acid mitigates cisplatin-induced nephrotoxicity in mice," *Human & Experimental Toxicology*, vol. 31, no. 2, pp. 145–156, 2012.

[137] Y. Sun, T. Zhang, B. Wang, H. Li, and P. Li, "Tannic acid, an inhibitor of poly(ADP-ribose) glycohydrolase, sensitizes ovarian carcinoma cells to cisplatin," *Anti-Cancer Drugs*, vol. 23, no. 9, pp. 979–990, 2012.

[138] K. Tikoo, D. K. Bhatt, A. B. Gaikwad, V. Sharma, and D. G. Kabra, "Differential effects of tannic acid on cisplatin induced nephrotoxicity in rats," *FEBS Letters*, vol. 581, no. 10, pp. 2027–2035, 2007.

[139] O. A. Badary, M. N. Nagi, O. A. Al-Shabanah, H. A. Al-Sawaf, M. O. Al-Sohaibani, and A. M. Al-Bekairi, "Thymoquinone ameliorates the nephrotoxicity induced by cisplatin in rodents and potentiates its antitumor activity," *Canadian Journal of Physiology and Pharmacology*, vol. 75, no. 12, pp. 1356–1361, 1997.

[140] Q. Huang, R. T. Dunn II, S. Jayadev et al., "Assessment of cisplatin-induced nephrotoxicity by microarray technology," *Toxicological Sciences*, vol. 63, no. 2, pp. 196–207, 2001.

[141] R. Ulu, A. Dogukan, M. Tuzcu et al., "Regulation of renal organic anion and cation transporters by thymoquinone in cisplatin induced kidney injury," *Food and Chemical Toxicology*, vol. 50, no. 5, pp. 1675–1679, 2012.

[142] H. Itokawa, F. Hirayama, K. Funakoshi, and K. Takeya, "Studies on the antitumor bisabolane sesquiterpenoids isolated from *Curcuma xanthorrhiza*," *Chemical & Pharmaceutical Bulletin*, vol. 33, no. 8, pp. 3488–3492, 1985.

[143] S. H. Kim, K. O. Hong, J. K. Hwang, and K.-K. Park, "Xanthorrhizol has a potential to attenuate the high dose cisplatin-induced nephrotoxicity in mice," *Food and Chemical Toxicology*, vol. 43, no. 1, pp. 117–122, 2005.

[144] O. H. Kyoung, K. H. Jae, K.-K. Park, and S. H. Kim, "Phosphorylation of c-Jun N-terminal Kinases (JNKs) is involved in the preventive effect of xanthorrhizol on cisplatin-induced hepatotoxicity," *Archives of Toxicology*, vol. 79, no. 4, pp. 231–236, 2005.

[145] M. A. El-Ghiaty, O. M. H. Ibrahim, S. M. Abdou, and F. Z. Hussein, "Evaluation of the protective effect of Cystone® against cisplatin-induced nephrotoxicity in cancer patients, and its influence on cisplatin antitumor activity," *International Urology and Nephrology*, vol. 46, no. 7, pp. 1367–1373, 2014.

[146] P. Anand, A. B. Kunnumakkara, R. A. Newman, and B. B. Aggarwal, "Bioavailability of curcumin: problems and promises," *Molecular Pharmaceutics*, vol. 4, no. 6, pp. 807–818, 2007.

[147] M. G. Novelle, D. Wahl, C. Diéguez, M. Bernier, and R. De Cabo, "Resveratrol supplementation: where are we now and where should we go?" *Ageing Research Reviews*, vol. 21, pp. 1–15, 2015.

[148] P. D. Sanchez-Gonzalez, F. J. Lopez-Hernandez, F. Perez-Barriocanal, A. I. Morales, and J. M. Lopez-Novoa, "Quercetin reduces cisplatin nephrotoxicity in rats without compromising its anti-tumour activity," *Nephrology Dialysis Transplantation*, vol. 26, no. 11, pp. 3484–3495, 2011.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5381540, 8 pages
http://dx.doi.org/10.1155/2016/5381540

Review Article

The Multiple Facets of Lutein: A Call for Further Investigation in the Perinatal Period

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Received 29 June 2016; Accepted 11 August 2016

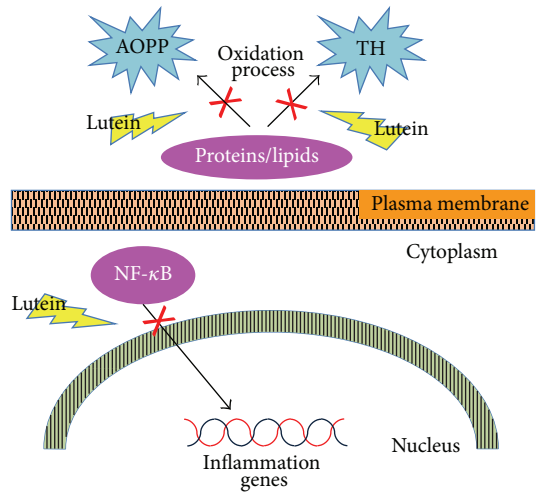
Academic Editor: Renata Szymanska

Lutein may have important antioxidant actions in free-radical-mediated diseases, in addition to its well-known antioxidant and cytoprotective effects on macula and photoreceptors. The peculiar perinatal susceptibility to oxidative stress indicates that prophylactic use of antioxidants as lutein could help to prevent or at least to reduce oxidative stress related diseases in newborns. Since lutein is not synthesized by humans, the intake primarily depends on diet or supplementation. Newborns receive lutein exclusively from breast milk. Lutein supplementation in term newborns has been reported to reduce oxidative stress and increase antioxidant capacities in the first days of life. Innovative frontiers concerning lutein supplementation are orientated toward cardiometabolic health improvement and cognitive benefits. The safety of lutein as an antioxidant agent has been confirmed in experimental and clinical studies, but its routine use is not recommended in perinatal period. This review summarizes what is known about the role of lutein as an antioxidant and anti-inflammatory agent in animal model and humans.

1. Structure and Location of Lutein

Lutein is a fat-soluble pigment, belonging to the family of carotenoids, which encompasses about 700 members in nature. Carotenoids are divided into two classes according to their chemical structure: the carotenes (hydrocarbons, such as β-carotene and lycopene) and the xanthophylls (polar compounds including oxygen atoms in their structure, such as lutein and its structural isomer zeaxanthin) [1]. Since xanthophyll biosynthesis occurs exclusively in plants, algae, bacteria, and certain fungi [2], the primary intake of lutein depends on diet or supplementation. Lutein and zeaxanthin can be found in yellow-orange food, such as egg yolk and corn [3], but especially in dark green vegetables such as turnip greens, kale, parsley, spinach, and broccoli [4]. Lutein intake from dietary sources is strongly associated with plasma concentrations [5]. Indeed, it has been shown that in humans every 10% increase in dietary lutein corresponds to a 2,4% increase in serum lutein concentration [6]. In human body lutein is stored in the eye (retina, rod outer segments, and lens) [7, 8] and other places in human body including skin [9], cervix, brain, and breasts.

The chemical structure of lutein ($C_{40}H_{56}O_2$) consists of 40-carbon, hence known as tetraterpenoids, with alternating single and double carbon-carbon bonds with attached methyl side groups. The presence of a hydroxyl group at both ends of the molecule distinguishes lutein and zeaxanthin from other carotenoids and it is responsible for the high chemical reactivity with singlet oxygen [10–12]. The presence of electrons localized over the entire length of the hydrocarbon chain molecules allows the neutralization of free radicals (FR) [13]. Due to its modest aqueous solubility, lutein is usually localized in the inner core of the cell membranes or bound to proteins [14]. Since cell membranes are the first structures attacked by FR, the anchor of lutein guarantees protection. Lutein also crosses the blood-brain barrier and the placenta; its presence is three times higher in breast milk and colostrum, compared to those of other carotenoids, as a result of an active secretion from the bloodstream. The plasma levels of lutein in the mother correlate with carotenoid status in



AOPP: advanced oxidative protein products

TH: total hydroperoxides

Figure 1: Schematic representation of anti-inflammatory and antioxidant effects of lutein.

the newborn [15]. In the neonatal period, fresh human milk is the main source of lutein [16]. Mature human milk can be stored safely in a freezer and heated in a microwave oven without loss of carotenoids [17]. Lutein-enriched infant formulas are now available. Oral supplementation represents an alternative source that has been demonstrated to decrease oxidative stress (OS) biomarkers and increase biological antioxidant potential in the first days of human life [18, 19].

2. Antioxidant, Anti-Inflammatory, and Neuroprotective Properties

Several antioxidant activities have been ascribed to lutein: inhibition of membrane lipids peroxidation, particularly in photoreceptors, which have plenty of polyunsaturated fatty acids; direct antioxidant action; and anti-inflammatory and immunomodulatory properties.

In a rat model of endotoxin-induced uveitis lutein blocks the degradation of inhibitory kB-a from the cytosolic fraction and prevents NF-kappa-B (NF-κB) translocation, decreasing inducible gene transcription and synthesis of inflammatory mediators (Figure 1) [20, 21].

Paraquat and hydrogen peroxide-induced apoptosis are neutralized by lutein in cultured retina photoreceptors promoting survival and differentiation [22]. Lutein also avoids the photooxidation of phosphatidyl-pyridinium bisretinoid (A2-PE), which may activate a cascade of events leading to the formation of reactive species in retinal pigment epithelial cells [23]. Moreover, lutein supplementation in retinal pigment epithelial cells prevents the proteasome inactivation in response to photooxidation and modulates inflammation-related genes [24].

In lipopolysaccharide- (LPS-) stimulated macrophages line, lutein has been found to decrease intracellular hydrogen peroxide (H_2O_2) accumulation by scavenging superoxide anion and H_2O_2 [25]. In the same study, lutein has been found to inhibit the expression of proinflammatory genes by suppressing nuclear factor NF-κB translocation and reducing LPS-induced secretion of tumor necrosis factor- (TNF-) α and interleukin-1β. Similar anti-inflammatory mechanisms have been observed *in vitro* in both models of gastric epithelial cells [26] and microglia [27]. Lutein also significantly reduces skin inflammatory responses in ultraviolet-irradiated keratinocytes [28].

Moreover, lutein acts as a competitive inhibitor of cytosolic calcium-dependent phospholipase A_2 inhibiting arachidonic acid release from a macrophage cell line [29]. In vascular smooth muscle cells, platelet-derived growth factor and extracellular H_2O_2 stimulation induce FR production, which is attenuated by lutein [30].

The protective effects of lutein against protein oxidation, lipid peroxidation, and DNA damage induced by OS have been reported also in human lens epithelial cells where lutein supplementation increased reduced glutathione (GSH) levels and reduced/oxidized GSH ratio [31].

Supplementation with lutein has anti-inflammatory, neuroprotective, and antiangiogenic properties. In mice receiving three-month lutein supplementation, the outer nuclear layer thickness histopathologically examined was significantly greater than in the nonsupplemented group. In the same cohort, retinal expression of proinflammatory mediators such as inducible nitric oxide synthase, TNF-α, cyclooxygenase-2, IL-1β, and vascular endothelial growth factor was significantly lower in supplemented mice [32].

The administration of lutein affords neuroprotective effect against transient cerebral ischemic injury in mice since it is able to significantly increase reduced/oxidized GSH ratio as well as activities of antioxidant enzymes (superoxide dismutase, GSH peroxidase, and catalase) [33].

Lutein suppresses STAT3 activation by inflammatory cytokines and extracellular signal-regulated kinase activation, slowing DNA damage and preserving a-wave electroretinogram amplitude in mouse models [34]. Lutein plays a neuroprotective role in retinal ganglion cells against N-methyl-D-aspartate-induced retinal damage in rats [35].

Lastly, lutein treatment significantly decreased OS in rat model of skeletal ischemia/reperfusion injury by down-regulating oxidative stress and inflammatory mechanisms [36].

3. Lutein and Cognitive Function

Recent papers report how lutein, predominantly accumulating in the brain, is positively associated with improved cognitive function in the elderly [37]. Macular pigment optical density, which is a stable measure of lutein and zeaxanthin in the retina, is consistent with better global cognition, verbal learning and fluency, and processing and perceptual speed in old people [38–40]. Moreover, lutein improves cognitive scores after 4-month supplementation in old women [41] and ameliorates visual processing speed and visual motor behavior in young subjects [42].

Due to the encouraging findings of a positive impact of lutein on brain function, growing interest focuses on identifying possible lutein functions in neurodegenerative diseases such as Parkinson disease (PD) and Alzheimer disease (AD). It has been suggested that lutein offers benefits against neuronal damage occurring in AD by virtue of its mitochondrial protective, antioxidant, and antiapoptotic properties. In a randomized, double-blind clinical trial, AD patients were daily supplemented for six months with macular carotenoids (10 mg meso-zeaxanthin, 10 mg lutein, and 2 mg zeaxanthin) [43]. The authors found significant improvements in visual function and increase of macular pigment density in patients with AD after lutein supplementation while cognitive function was not influenced. In PD-mice model, lutein has been found to protect nigral dopaminergic neurons by enhancing antioxidant defense mechanisms and diminishing mitochondrial dysfunction and apoptotic death [44]. Lutein reversed the loss of nigral dopaminergic neurons by inhibiting the activation of proapoptotic markers (Bax and caspases 3, 8, and 9) and enhancing antiapoptotic marker (Bcl-2) expressions, with significant reduction in motor abnormalities. These findings pave the way to a beneficial employment of lutein for neurodegenerative therapy even if its potential protective function against these diseases remains to be explored.

4. Lutein and the Eye

In human eye macular pigment is composed of three carotenoids including lutein in equal concentrations to zeaxanthin and meso-zeaxanthin [45–47]. The *macula lutea* is a yellow, circular area 5-6 mm in diameter, located in the central and posterior portion of the primate retina. The macula includes the majority of photoreceptors and it is responsible for central vision and high-resolution visual acuity. Neuronal lipid bilayer membranes in the retina are especially vulnerable to oxidative damage because of exposure to high oxygen concentration. Since lutein is soluble in polyunsaturated phospholipid membrane domains, it plays a pivotal role against OS in retinal tissues. Retinal vulnerability to hypoxia-ischemia is evident especially as a result of photochemical damage, primarily located in the outer layers of the central region of the retina, regarding both photoreceptors and retinal pigment epithelium [48]. Laboratory studies have suggested that photochemical damage is triggered by oxidative events leading to retinal cells apoptosis [49]. In particular, ocular exposure to sunlight, UV, and short blue light-emitting lamps may lead to cataract and retinal degeneration through a photooxidation reaction. In photooxidation reactions, phototoxic chromophores in the eye are able to absorb light but they subsequently turn to an unstable state (singlet and then a triplet state) producing FR [49]. Antioxidant quenchers as lutein can prevent the phototoxic reactions damage. In fact, due to its chemical structures with extensive conjugated bonds, lutein is able to absorb light of the blue range wavelength (400–500 nm) preventing light-induced retinal damage [50, 51]. Moreover, lutein acts as an effective quencher of singlet molecular oxygen ($^1O_2$) in the retina during OS conditions, preventing lipid peroxidation and the accumulation of FR responsible for photoreceptor apoptosis [11, 12, 52]. OS also occurs in the inner part of the retina, particularly within axons of retinal ganglion cells which are rich in mitochondria and consequently sensitive to FR harmful effects compared to neuron soma [53].

OS is the main consequence of retinal ischemia which was found to underlie diabetic retinopathy (DR) and retinopathy of prematurity (ROP) [54]. In both DR and ROP early ischemia due to abnormal retinal blood supply leads to abnormal neovascularization and subsequent hemorrhages and blindness. In preterm babies the hypoxic injury is caused by an imbalance between an increased metabolic demand and delayed retinal vascular development due to the suppression of growth factor in a hyperoxic environment [55]. DR hyperglycemia and decrease in blood flow produce retinal ischemia [56]. Hyperglycemia induces several changes including leukostasis, vasoconstriction, and a proinflammatory state that also cause hypoxia in the retina. The early proinflammatory changes can directly provoke hypoxia in the retina.

Furthermore, lutein is well known to be protective against senile cataract by influencing changes in glutathione oxidation, which is responsible for the increased susceptibility of the nucleus to oxidative damage in older lenses [57]. Protective effects of lutein have been also demonstrated in age-related macular degeneration (AMD). AMD is a major cause of visual impairment and blindness among people 65 years or older. It is due to the decrease in naturally protective antioxidant systems and the increase in UV and visible light-absorbing endogenous phototoxic chromophores that produce reactive oxygen species [58]. Lutein counteracts stress-induced changes in the retinal pigment epithelium promoting tight junction repair and suppresses inflammation both by direct scavenging and by induction of endogenous antioxidant enzymes.

Sustained supplementation of lutein, zeaxanthin, and meso-zeaxanthin was demonstrated to be effective in increasing macular pigment, contrast sensitivity, and visual function in early AMD [59]. These three carotenoids showed also beneficial effects on visual performance in various retinal diseases [60].

In a large multicenter double-masked clinical trial called Age-Related Eye Disease Study 2 (AREDS2), participants were randomly assigned to receive four different treatments: (1) 10 mg lutein + 2 mg zeaxanthin; (2) fish oil containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); (3) lutein + zeaxanthin + DHA + EPA; and (4) placebo. Lutein + zeaxanthin formulation significantly decreased the progression to advanced AMD [61].

Interestingly, lutein, meso-zeaxanthin, and zeaxanthin supplementation has been reported to be effective in ameliorating contrast sensitivity in healthy population (free of retinal disease) by increasing retinal concentrations of these carotenoids [62]. A recent meta-analysis by Ma et al. reports that lutein, zeaxanthin, and meso-zeaxanthin supplementation improves macular pigment optical density in both AMD and healthy subjects with a dose-response relationship [63].

5. Lutein and Cardiometabolic Health

Due to its antioxidant and anti-inflammatory capacity, lutein has been shown to exert a positive influence in promoting cardiovascular health and decreasing the risk of Coronary Artery Disease (CAD). Animal studies show that lutein contributes to prevention of atherosclerosis development by decreasing malondialdehyde and oxidized low-density lipoprotein levels and reducing inflammatory cytokines such as interleukin- (IL-) 10 [64]. Furthermore, in ApoE-deficient mice supplemented with lutein for 24 weeks NADPH oxidase was inhibited and peroxisome proliferator-activated receptor expression was increased by lutein, protecting against high fat diet-induced atherosclerosis [65].

The possible beneficial cardiovascular effect of a lutein-rich diet in humans, particularly in preventing arterial plaque formation, has been reported in the Atherosclerosis Risk in Communities (ARIC) and the Carotid Ultrasound Disease Assessment (CUDAS) studies [66, 67]. An inverse association between plasmatic lutein and atherosclerosis is also shown in the Los Angeles Atherosclerosis Study [68]. While the benefits regarding hypertension are uncertain [69], lutein has been reported to counteract OS produced after myocardial ischemia/reperfusion damage [70, 71]. Upon reperfusion, neutrophils accumulate and produce an inflammatory response with increased generation of highly reactive oxygen species, which are responsible for myocytes apoptosis [70]. Consequently, limiting myocardial injury may prevent contractile dysfunction, reducing morbidity and mortality associated with CAD [72].

A recent meta-analysis also showed a lower risk of coronary heart disease, stroke, and metabolic syndrome in high-lutein blood concentration subjects or lutein-supplemented subjects [73].

6. Lutein and Oxidative Stress in Perinatal Period

Oxidative stress is defined as an imbalance between free radicals, such as nitric oxide ($NO^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), and H_2O_2, and antioxidants, promoting overabundance of FR. The newborn is particularly susceptible to OS due to the sudden transition from uterine life, relatively hypoxic, to extrauterine environment, with significantly higher oxygen concentrations. Other predisposing factors are the rapid tissue growth and perinatal conditions characterized by increased concentrations of FR and free iron, such as chorioamnionitis, placental hypoperfusion, neonatal hypoxic-ischemic events, inflammation, or fetal-placental transfusion.

Several preterm newborn's diseases, such as retinopathy of prematurity (ROP), bronchopulmonary dysplasia, intraventricular hemorrhage, periventricular leukomalacia, necrotizing enterocolitis, oxidative hemolysis, and renal failure [74–78], recognize in OS a pathogenetic role. These pathological conditions were grouped into a larger entity defined as "FR disease of the newborn" [79].

Therefore one of the goals of modern neonatology is to protect the infant from oxidative damage by reducing the production of FR or promoting the development of antioxidant systems. Vitamins, FR inhibitors, and scavengers have been used as antioxidant drugs in clinical and experimental studies with uncertain results. Among them, lutein represents one of the antioxidant strategies with clinical application in the perinatal period [80]. Newborns receive lutein from breast milk: lutein is the predominant carotenoid in mature breast milk [81, 82]. Breast-feeding infants intake of lutein depends on multiple factors such as maternal lutein intake, alcohol consuming, smoking [83], and maternal body mass index; for example, breast milk of obese mothers was found to have lower lutein content [84]. A recent paper by Vishwanathan et al. shows that lutein is the prevalent carotenoid in the developing infant brain and its concentration is lower in preterms compared to term neonates perhaps for lack of supplementation [85].

Few data are currently available about the effects of lutein supplementation in newborns. Lutein may play a role in visual development, being involved in cell maturation in the developing macula [86]. Moreover, a recent clinical trial showed that lutein supplementation may improve neuroretinal health (assessed through electroretinography recording the voltage change across the retina after light stimulus) in preterm newborn infants [87]. Although oral lutein is well absorbed by preterm babies [88], it has not yet been verified whether dietary lutein enhances visual development in infants [89] and the mechanisms remain largely not understood.

Since ROP is OS-related disease, a striking interest has been focused on the possible role of lutein in preventing it [90, 91]. ROP is a two-phase disease affecting preterm infants. At first, the hyperoxic stimulus during oxygen supplementation downregulates the vascular endothelial growth factor with subsequent interruption of retinal vessel growth. Afterwards, the condition of relative hypoxia of the retina, occurring when the babies stop oxygen therapy, leads to the abnormal proliferation of vessels (neovascularization) and consequently OS [92].

Data regarding the possible benefits of lutein supplementation in preventing ROP are scarce and no consensus has been achieved yet. In a multicenter, randomized-controlled trial, the incidence of ROP in very low birth weight infants was found not to decrease after lutein supplementation. Similar findings were described also in another randomized-controlled trial by Dani et al. [93]. Romagnoli et al. showed a strong antioxidant capacity of lutein, which significantly increased the biological antioxidant potential but not efficacy to reduce the occurrence/severity of ROP [94]. Although a significant linear correlation was reported between plasma lutein concentration and total antioxidant status, supplementation with lutein orally was ineffective in enhancing biological antioxidant capacity in preterm babies [95]. Conversely, clinical trials in term healthy newborns indicated that orally supplemented lutein was effective in enhancing biological antioxidant potential and reducing lipid peroxidation [18, 19]. As demonstrated so far, lutein has a well-ascertained antioxidant and anti-inflammatory role, while the capacity

preventing OS-related newborn diseases remains uncertain, probably due to the multifactorial nature of the pathological processes or the need for higher daily doses.

Further clinical trials are needed to evaluate therapeutic effects of lutein on preterm and term infant morbidity, particularly the free-radical-mediated diseases of the newborn.

7. Conclusions

Due to its antioxidant anti-inflammatory properties and safety, lutein has been considered as a promising molecule in several fields of application. Neonatal age is a vulnerable period regarding the threatening effects of OS on the developing tissues. Neonates, especially if preterm, are defenseless against the oxidative cellular injury because of both several prooxidant events, such as the exposition to a relatively hyperoxic environment with enhanced generation of FR, and deficient antioxidant systems. Additional neonatal conditions (inflammation, hypoxia, ischemia, and free iron release) may also worsen OS damage. As a consequence, a great deal of interest has been focused on antioxidant treatments. The efficacy of lutein in counteracting oxidative damage has been tested in human adult diseases, such as atherosclerosis, AMD, and senile cataract. This evidence calls for a further investigation in infants. Since humans do not synthesize lutein, lutein supplementation should be undertaken in maternal diet and in all non-lutein-enriched formula fed newborns, lacking an adequate dietary intake.

Competing Interests

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

Acknowledgments

The authors thank EURAIBI (Europe Against Infant Brain Injury) Foundation for its grant.

References

[1] A.-A. Arteni, M. Fradot, D. Galzerano et al., "Structure and conformation of the carotenoids in human retinal macular pigment," *PLoS ONE*, vol. 10, no. 8, Article ID e0135779, 2015.

[2] S. Takaichi, "Carotenoids in algae: distributions, biosyntheses and functions," *Marine Drugs*, vol. 9, no. 6, pp. 1101–1118, 2011.

[3] A. Perry, H. Rasmussen, and E. J. Johnson, "Xanthophyll (lutein, zeaxanthin) content in fruits, vegetables and corn and egg products," *Journal of Food Composition and Analysis*, vol. 22, no. 1, pp. 9–15, 2009.

[4] O. Sommerburg, J. E. E. Keunen, A. C. Bird, and F. J. G. M. van Kuijk, "Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eyes," *British Journal of Ophthalmology*, vol. 82, no. 8, pp. 907–910, 1998.

[5] L. Natarajan, C. L. Rock, J. M. Major et al., "On the importance of using multiple methods of dietary assessment," *Epidemiology*, vol. 15, no. 6, pp. 738–745, 2004.

[6] C. L. Rock, M. D. Thornquist, M. L. Neuhouser et al., "Diet and lifestyle correlates of lutein in the blood and diet," *Journal of Nutrition*, vol. 132, no. 3, 2002.

[7] O. G. Sommerburg, W. G. Siems, J. S. Hurst, J. W. Lewis, D. S. Kliger, and F. J. G. M. van Kuijk, "Lutein and zeaxanthin are associated with photoreceptors in the human retina," *Current Eye Research*, vol. 19, no. 6, pp. 491–495, 1999.

[8] S. S. Ahmed, M. N. Lott, and D. M. Marcus, "The macular xanthophylls," *Survey of Ophthalmology*, vol. 50, no. 2, pp. 183–193, 2005.

[9] K. Pezdirc, M. J. Hutchesson, R. L. Williams et al., "Consuming high-carotenoid fruit and vegetables influences skin yellowness and plasma carotenoids in young women: a single-blind randomized crossover trial," *Journal of the Academy of Nutrition and Dietetics*, vol. 116, no. 8, pp. 1257–1265, 2016.

[10] H. D. Martin, C. Ruck, M. Schmidt et al., "Chemistry of carotenoid oxidation and free radical reactions," *Pure and Applied Chemistry*, vol. 71, no. 12, pp. 2253–2262, 1999.

[11] F. Böhm, R. Edge, and T. G. Truscott, "Interactions of dietary carotenoids with singlet oxygen ($^1O_2$) and free radicals: potential effects for human health," *Acta Biochimica Polonica*, vol. 59, no. 1, pp. 27–30, 2012.

[12] R. Edge, D. J. McGarvey, and T. G. Truscott, "The carotenoids as anti-oxidants—a review," *Journal of Photochemistry and Photobiology B: Biology*, vol. 41, no. 3, pp. 189–200, 1997.

[13] A. Alves-Rodrigues and A. Shao, "The science behind lutein," *Toxicology Letters*, vol. 150, no. 1, pp. 57–83, 2004.

[14] P. S. Bernstein, B. Li, P. P. Vachali et al., "Lutein, zeaxanthin, and meso-zeaxanthin: the basic and clinical science underlying carotenoid-based nutritional interventions against ocular disease," *Progress in Retinal and Eye Research*, vol. 50, pp. 34–66, 2016.

[15] B. S. Henriksen, G. Chan, R. O. Hoffman et al., "Interrelationships between maternal carotenoid status and newborn infant macular pigment optical density and carotenoid status," *Investigative Ophthalmology and Visual Science*, vol. 54, no. 8, pp. 5568–5578, 2013.

[16] J. Bettler, J. P. Zimmer, M. Neuringer, and P. A. DeRusso, "Serum lutein concentrations in healthy term infants fed human milk or infant formula with lutein," *European Journal of Nutrition*, vol. 49, no. 1, pp. 45–51, 2010.

[17] K. J. M. Tacken, A. Vogelsang, R. A. van Lingen, J. Slootstra, B. D. Dikkeschei, and D. van Zoeren-Grobben, "Loss of triglycerides and carotenoids in human milk after processing," *Archives of Disease in Childhood: Fetal & Neonatal Edition*, vol. 94, no. 6, pp. F447–F450, 2009.

[18] S. Perrone, M. Longini, B. Marzocchi et al., "Effects of lutein on oxidative stress in the term newborn: a pilot study," *Neonatology*, vol. 97, no. 1, pp. 36–40, 2009.

[19] S. Perrone, M. Tei, M. Longini et al., "Lipid and protein oxidation in newborn infants after lutein administration," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 781454, 7 pages, 2014.

[20] X.-H. Jin, K. Ohgami, K. Shiratori et al., "Inhibitory effects of lutein on endotoxin-induced uveitis in Lewis rats," *Investigative Ophthalmology and Visual Science*, vol. 47, no. 6, pp. 2562–2568, 2006.

[21] T. Ashino, R. Yamanaka, M. Yamamoto et al., "Negative feedback regulation of lipopolysaccharide-induced inducible nitric oxide synthase gene expression by heme oxygenase-1 induction in macrophages," *Molecular Immunology*, vol. 45, no. 7, pp. 2106–2115, 2008.

[22] A. J. Chucair, N. P. Rotstein, J. P. SanGiovanni, A. During, E. Y. Chew, and L. E. Politi, "Lutein and zeaxanthin protect photoreceptors from apoptosis induced by oxidative stress: relation with docosahexaenoic acid," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 11, pp. 5168–5177, 2007.

[23] S. R. Kim, K. Nakanishi, Y. Itagaki, and J. R. Sparrow, "Photooxidation of A2-PE, a photoreceptor outer segment fluorophore, and protection by lutein and zeaxanthin," *Experimental Eye Research*, vol. 82, no. 5, pp. 828–839, 2006.

[24] Q. Bian, S. Gao, J. Zhou et al., "Lutein and zeaxanthin supplementation reduces photooxidative damage and modulates the expression of inflammation-related genes in retinal pigment epithelial cells," *Free Radical Biology and Medicine*, vol. 53, no. 6, pp. 1298–1307, 2012.

[25] J.-H. Kim, H.-J. Na, C.-K. Kim et al., "The non-provitamin A carotenoid, lutein, inhibits NF-κB-dependent gene expression through redox-based regulation of the phosphatidylinositol 3-kinase/PTEN/Akt and NF-κB-inducing kinase pathways: role of H_2O_2 in NF-κB activation," *Free Radical Biology and Medicine*, vol. 45, no. 6, pp. 885–896, 2008.

[26] Y. Kim, J. H. Seo, and H. Kim, "β-Carotene and lutein inhibit hydrogen peroxide-induced activation of NF-κB and IL-8 expression in gastric epithelial AGS cells," *Journal of Nutritional Science and Vitaminology*, vol. 57, no. 3, pp. 216–223, 2011.

[27] W. Wu, Y. Li, Y. Wu, Y. Zhang, Z. Wang, and X. Liu, "Lutein suppresses inflammatory responses through Nrf2 activation and NF-κB inactivation in lipopolysaccharide-stimulated BV-2 microglia," *Molecular Nutrition and Food Research*, vol. 59, no. 9, pp. 1663–1673, 2015.

[28] J. Oh, J. H. Kim, J. G. Park et al., "Radical scavenging activity-based and AP-1-targeted anti-inflammatory effects of lutein in macrophage-like and skin keratinocytic cells," *Mediators of Inflammation*, vol. 2013, Article ID 787042, 8 pages, 2013.

[29] H. S. Song, H. R. Kim, M. C. Kim, Y. H. Hwang, and S. S. Sim, "Lutein is a competitive inhibitor of cytosolic Ca^{2+}-dependent phospholipase A_2," *Journal of Pharmacy and Pharmacology*, vol. 62, no. 12, pp. 1711–1716, 2010.

[30] H.-M. Lo, Y.-J. Tsai, W.-Y. Du, C.-J. Tsou, and W.-B. Wu, "A naturally occurring carotenoid, lutein, reduces PDGF and H_2O_2 signaling and compromises migration in cultured vascular smooth muscle cells," *Journal of Biomedical Science*, vol. 19, article 18, 2012.

[31] S. Gao, T. Qin, Z. Liu et al., "Lutein and zeaxanthin supplementation reduces H_2O_2-induced oxidative damage in human lens epithelial cells," *Molecular Vision*, vol. 17, pp. 3180–3190, 2011.

[32] H. L. Ramkumar, J. Tuo, D. F. Shen et al., "Nutrient supplementation with n3 polyunsaturated fatty acids, lutein, and zeaxanthin decrease A2E accumulation and *VEGF* expression in the retinas of *Ccl2/Cx3cr1*-deficient mice on *Crb1*^{rd8} background," *The Journal of Nutrition*, vol. 143, no. 7, pp. 1129–1135, 2013.

[33] Y.-X. Sun, T. Liu, X.-L. Dai, Q.-S. Zheng, B.-D. Hui, and Z.-F. Jiang, "Treatment with lutein provides neuroprotection in mice subjected to transient cerebral ischemia," *Journal of Asian Natural Products Research*, vol. 16, no. 11, pp. 1084–1093, 2014.

[34] Y. Ozawa, M. Sasaki, N. Takahashi, M. Kamoshita, S. Miyake, and K. Tsubota, "Neuroprotective effects of lutein in the retina," *Current Pharmaceutical Design*, vol. 18, no. 1, pp. 51–56, 2012.

[35] C. Zhang, Z. Wang, J. Zhao et al., "Neuroprotective effect of Lutein on NMDA-induced retinal ganglion cell injury in rat retina," *Cellular and Molecular Neurobiology*, vol. 36, no. 4, pp. 531–540, 2016.

[36] F. Cheng, Q. Zhang, F.-F. Yan, J.-F. Wan, and C.-S. Lin, "Lutein protects against ischemia/reperfusion injury in rat skeletal muscle by modulating oxidative stress and inflammation," *Immunopharmacology and Immunotoxicology*, vol. 37, no. 4, pp. 329–334, 2015.

[37] E. J. Johnson, R. Vishwanathan, M. A. Johnson et al., "Relationship between serum and brain carotenoids, α-tocopherol, and retinol concentrations and cognitive performance in the oldest old from the georgia centenarian study," *Journal of Aging Research*, vol. 2013, Article ID 951786, 13 pages, 2013.

[38] R. Vishwanathan, A. Iannaccone, T. M. Scott et al., "Macular pigment optical density is related to cognitive function in older people," *Age and Ageing*, vol. 43, no. 2, Article ID aft210, pp. 271–275, 2014.

[39] L. M. Renzi, M. J. Dengler, A. Puente, L. S. Miller, and B. R. Hammond Jr., "Relationships between macular pigment optical density and cognitive function in unimpaired and mildly cognitively impaired older adults," *Neurobiology of Aging*, vol. 35, no. 7, pp. 1695–1699, 2014.

[40] D. Kelly, R. F. Coen, K. O. Akuffo et al., "Cognitive function and its relationship with macular pigment optical density and serum concentrations of its constituent carotenoids," *Journal of Alzheimer's Disease*, vol. 48, no. 1, pp. 261–277, 2015.

[41] E. J. Johnson, "A possible role for lutein and zeaxanthin in cognitive function in the elderly," *The American Journal of Clinical Nutrition*, vol. 96, no. 5, pp. 1161S–1165S, 2012.

[42] E. R. Bovier, L. M. Renzi, and B. R. Hammond, "A double-blind, placebo-controlled study on the effects of lutein and zeaxanthin on neural processing speed and efficiency," *PLoS ONE*, vol. 9, no. 9, Article ID e108178, 2014.

[43] J. M. Nolan, E. Loskutova, A. Howard et al., "The impact of supplemental macular carotenoids in Alzheimer's disease: a randomized clinical trial," *Journal of Alzheimer's Disease*, vol. 44, no. 4, pp. 1157–1169, 2015.

[44] J. Nataraj, T. Manivasagam, A. J. Thenmozhi, and M. M. Essa, "Lutein protects dopaminergic neurons against MPTP-induced apoptotic death and motor dysfunction by ameliorating mitochondrial disruption and oxidative stress," *Nutritional Neuroscience*, vol. 19, no. 6, pp. 237–246, 2016.

[45] L. M. Renzi, B. R. Hammond Jr., M. Dengler, and R. Roberts, "The relation between serum lipids and lutein and zeaxanthin in the serum and retina: results from cross-sectional, case-control and case study designs," *Lipids in Health and Disease*, vol. 11, article 33, 2012.

[46] R. A. Bone, J. T. Landrum, G. W. Hime, A. Cains, and J. Zamor, "Stereochemistry of the human macular carotenoids," *Investigative Ophthalmology & Visual Science*, vol. 34, no. 6, pp. 2033–2040, 1993.

[47] J. M. Nolan, K. Meagher, S. Kashani, and S. Beatty, "What is meso-zeaxanthin, and where does it come from?" *Eye*, vol. 27, no. 8, pp. 899–905, 2013.

[48] J. Wu, S. Seregard, and P. V. Algvere, "Photochemical damage of the retina," *Survey of Ophthalmology*, vol. 51, no. 5, pp. 461–481, 2006.

[49] R. Straight and J. D. Spikes, "Photosensitized oxidation of biomolecules," in *Singlet Oxygen*, A. A. Frimer, Ed., pp. 91–143, CRC Press, Boca Raton, Fla, USA, 1985.

[50] A. Junghans, H. Sies, and W. Stahl, "Macular pigments lutein and zeaxanthin as blue light filters studied in liposomes," *Archives of Biochemistry and Biophysics*, vol. 391, no. 2, pp. 160–164, 2001.

[51] B. R. Wooten and B. R. Hammond, "Macular pigment: influences on visual acuity and visibility," *Progress in Retinal and Eye Research*, vol. 21, no. 2, pp. 225–240, 2002.

[52] K. Koushan, R. Rusovici, W. Li, L. R. Ferguson, and K. V. Chalam, "The role of lutein in eye-related disease," *Nutrients*, vol. 5, no. 5, pp. 1823–1839, 2013.

[53] M. Almasieh, A. M. Wilson, B. Morquette, J. L. Cueva Vargas, and A. Di Polo, "The molecular basis of retinal ganglion cell death in glaucoma," *Progress in Retinal and Eye Research*, vol. 31, no. 2, pp. 152–181, 2012.

[54] S.-Y. Li, Z. J. Fu, and A. C. Y. Lo, "Hypoxia-induced oxidative stress in ischemic retinopathy," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 426769, 10 pages, 2012.

[55] M. E. Hartnett, "Studies on the pathogenesis of avascular retina and neovascularization into the vitreous in peripheral severe retinopathy of prematurity (an American Ophthalmological Society thesis)," *Transactions of the American Ophthalmological Society*, vol. 108, pp. 96–119, 2010.

[56] G. B. Arden and S. Sivaprasad, "Hypoxia and oxidative stress in the causation of diabetic retinopathy," *Current Diabetes Reviews*, vol. 7, no. 5, pp. 291–304, 2011.

[57] D. C. Beebe, N. M. Holekamp, and Y.-B. Shui, "Oxidative damage and the prevention of age-related cataracts," *Ophthalmic Research*, vol. 44, no. 3, pp. 155–165, 2010.

[58] S. Beatty, H.-H. Koh, M. Phil, D. Henson, and M. Boulton, "The role of oxidative stress in the pathogenesis of age-related macular degeneration," *Survey of Ophthalmology*, vol. 45, no. 2, pp. 115–134, 2000.

[59] K. O. Akuffo, J. M. Nolan, A. N. Howard et al., "Sustained supplementation and monitored response with differing carotenoid formulations in early age-related macular degeneration," *Eye*, vol. 29, no. 7, pp. 902–912, 2015.

[60] R. Crosby-Nwaobi, P. Hykin, T. Peto, and S. Sivaprasad, "An exploratory study evaluating the effects of macular carotenoid supplementation in various retinal diseases," *Clinical Ophthalmology*, vol. 10, pp. 835–844, 2016.

[61] E. Y. Chew, T. E. Clemons, J. P. SanGiovanni et al., "Secondary analyses of the effects of lutein/zeaxanthin on age-related macular degeneration progression: AREDS2 report no. 3," *JAMA Ophthalmology*, vol. 132, no. 2, pp. 142–149, 2014.

[62] J. M. Nolan, R. Power, J. Stringham et al., "Enrichment of macular pigment enhances contrast sensitivity in subjects free of retinal disease: central retinal enrichment supplementation trials—report 1," *Investigative Ophthalmology & Visual Science*, vol. 57, no. 7, pp. 3429–3439, 2016.

[63] L. Ma, R. Liu, J. Du, T. Liu, S. Wu, and X. Liu, "Lutein, zeaxanthin and meso-zeaxanthin supplementation associated with macular pigment optical density," *Nutrients*, vol. 8, no. 7, article 426, 2016.

[64] J. E. Kim, J. O. Leite, R. DeOgburn, J. A. Smyth, R. M. Clark, and M. L. Fernandez, "A lutein-enriched diet prevents cholesterol accumulation and decreases oxidized LDL and inflammatory cytokines in the aorta of guinea pigs," *The Journal of Nutrition*, vol. 141, no. 8, pp. 1458–1463, 2011.

[65] H. Han, W. Cui, L. Wang et al., "Lutein prevents high fat diet-induced atherosclerosis in ApoE-deficient mice by inhibiting NADPH oxidase and increasing PPAR expression," *Lipids*, vol. 50, no. 3, pp. 261–273, 2015.

[66] S. B. Kritchevsky, G. S. Tell, T. Shimakawa et al., "Provitamin A carotenoid intake and carotid artery plaques: the Atherosclerosis Risk in Communities Study," *The American Journal of Clinical Nutrition*, vol. 68, no. 3, pp. 726–733, 1998.

[67] B. M. McQuillan, J. Hung, J. P. Beilby, M. Nidorf, and P. L. Thompson, "Antioxidant vitamins and the risk of carotid atherosclerosis: the perth carotid ultrasound disease assessment study (CUDAS)," *Journal of the American College of Cardiology*, vol. 38, no. 7, pp. 1788–1794, 2001.

[68] J. H. Dwyer, M. J. Paul-Labrador, J. Fan, A. M. Shircore, C. N. Bairey Merz, and K. M. Dwyer, "Progression of carotid intima-media thickness and plasma antioxidants: the Los Angeles Atherosclerosis study," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 2, pp. 313–319, 2004.

[69] A. Hozawa, D. R. Jacobs, M. W. Steffes, M. D. Gross, L. M. Steffen, and D.-H. Lee, "Circulating carotenoid concentrations and incident hypertension: the Coronary Artery Risk Development in Young Adults (CARDIA) study," *Journal of Hypertension*, vol. 27, no. 2, pp. 237–242, 2009.

[70] S. A. Saeed, M. A. Waqar, A. J. Zubairi et al., "Myocardial ischaemia and reperfusion injury: reactive oxygen species and the role of neutrophil," *Journal of the College of Physicians and Surgeons Pakistan*, vol. 15, no. 8, pp. 507–514, 2005.

[71] J. L. Zweier and M. A. H. Talukder, "The role of oxidants and free radicals in reperfusion injury," *Cardiovascular Research*, vol. 70, no. 2, pp. 181–190, 2006.

[72] R. S. Adluri, M. Thirunavukkarasu, L. Zhan et al., "Cardioprotective efficacy of a novel antioxidant mix VitaePro against ex vivo myocardial ischemia-reperfusion injury," *Cell Biochemistry and Biophysics*, vol. 67, no. 2, pp. 281–286, 2013.

[73] E. T. M. Leermakers, S. K. L. Darweesh, C. P. Baena et al., "The effects of lutein on cardiometabolic health across the life course: a systematic review and meta-analysis," *American Journal of Clinical Nutrition*, vol. 103, no. 2, pp. 481–494, 2016.

[74] S. Perrone, P. Vezzosi, M. Longini et al., "Biomarkers of oxidative stress in babies at high risk for retinopathy of prematurity," *Frontiers in Bioscience: Elite Edition*, vol. 1, no. 2, pp. 547–552, 2009.

[75] S. Perrone, M. Mussap, M. Longini et al., "Oxidative kidney damage in preterm newborns during perinatal period," *Clinical Biochemistry*, vol. 40, no. 9-10, pp. 656–660, 2007.

[76] S. Perrone, M. L. Tataranno, A. Santacroce, S. Negro, and G. Buonocore, "The role of oxidative stress on necrotizing enterocolitis in very low birth weight infants," *Current Pediatric Reviews*, vol. 10, no. 3, pp. 202–207, 2014.

[77] S. Perrone, M. Tataranno, and G. Buonocore, "Oxidative stress and bronchopulmonary dysplasia," *Journal of Clinical Neonatology*, vol. 1, no. 3, pp. 109–114, 2012.

[78] S. Perrone, M. L. Tataranno, G. Stazzoni, A. Del Vecchio, and G. Buonocore, "Oxidative injury in neonatal erythrocytes," *Journal of Maternal-Fetal and Neonatal Medicine*, vol. 25, no. 5, pp. 104–108, 2012.

[79] S. Perrone, M. L. Tataranno, S. Negro et al., "Early identification of the risk for free radical-related diseases in preterm newborns," *Early Human Development*, vol. 86, no. 4, pp. 241–244, 2010.

[80] B. R. Hammond, "The dietary carotenoids lutein and zeaxanthin in pre-and- postnatal development," *Funtional Food Reviews*, vol. 4, pp. 130–137, 2012.

[81] F. J. Schweigert, K. Bathe, F. Chen, U. Büscher, and J. W. Dudenhausen, "Effect of the stage of lactation in humans on carotenoid levels in milk, blood plasma and plasma lipoprotein fractions," *European Journal of Nutrition*, vol. 43, no. 1, pp. 39–44, 2004.

[82] K. J. M. Tacken, A. Vogelsang, R. A. van Lingen, J. Slootstra, B. D. Dikkeschei, and D. van Zoeren-Grobben, "Loss of

triglycerides and carotenoids in human milk after processing," *Archives of Disease in Childhood: Fetal and Neonatal Edition*, vol. 94, no. 6, pp. F447–F450, 2009.

[83] P. Galan, F. E. Viteri, S. Bertrais et al., "Serum concentrations of β-carotene, vitamins C and E, zinc and selenium are influenced by sex, age, diet, smoking status, alcohol consumption and corpulence in a general French adult population," *European Journal of Clinical Nutrition*, vol. 59, no. 10, pp. 1181–1190, 2005.

[84] P. G. Panagos, R. Vishwanathan, A. Penfield-Cyr et al., "Breast-milk from obese mothers has pro-inflammatory properties and decreased neuroprotective factors," *Journal of Perinatology*, vol. 36, no. 4, pp. 284–290, 2016.

[85] R. Vishwanathan, M. J. Kuchan, S. Sen, and E. J. Johnson, "Lutein and preterm infants with decreased concentrations of brain carotenoids," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 59, no. 5, pp. 659–665, 2014.

[86] B. R. Hammond Jr., "Possible role for dietary lutein and zeaxanthin in visual development," *Nutrition Reviews*, vol. 66, no. 12, pp. 695–702, 2008.

[87] L. P. Rubin, G. M. Chan, B. M. Barrett-Reis et al., "Effect of carotenoid supplementation on plasma carotenoids, inflammation and visual development in preterm infants," *Journal of Perinatology*, vol. 32, no. 6, pp. 418–424, 2012.

[88] C. Romagnoli, C. Tirone, S. Persichilli et al., "Lutein absorption in premature infants," *European Journal of Clinical Nutrition*, vol. 64, no. 7, pp. 760–761, 2010.

[89] J. P. Zimmer and B. R. Hammond Jr., "Possible influences of lutein and zeaxanthin on the developing retina," *Clinical Ophthalmology*, vol. 1, pp. 25–35, 2007.

[90] J. C. Rivera, P. Sapieha, J.-S. Joyal et al., "Understanding retinopathy of prematurity: update on pathogenesis," *Neonatology*, vol. 100, no. 4, pp. 343–353, 2011.

[91] O. Ates, H. H. Alp, I. Caner et al., "Oxidative DNA damage in retinopathy of prematurity," *European Journal of Ophthalmology*, vol. 19, no. 1, pp. 80–85, 2009.

[92] M. E. Hartnett, "The effects of oxygen stresses on the development of features of severe retinopathy of prematurity: knowledge from the 50/10 OIR model," *Documenta Ophthalmologica*, vol. 120, no. 1, pp. 25–39, 2010.

[93] C. Dani, I. Lori, F. Favelli et al., "Lutein and zeaxanthin supplementation in preterm infants to prevent retinopathy of prematurity: a randomized controlled study," *The Journal of Maternal-Fetal & Neonatal Medicine*, vol. 25, no. 5, pp. 523–527, 2012.

[94] C. Romagnoli, C. Giannantonio, F. Cota et al., "A prospective, randomized, double blind study comparing lutein to placebo for reducing occurrence and severity of retinopathy of prematurity," *Journal of Maternal-Fetal and Neonatal Medicine*, vol. 24, no. 1, pp. 147–150, 2011.

[95] S. Costa, C. Giannantonio, C. Romagnoli et al., "Effects of lutein supplementation on biological antioxidant status in preterm infants: a randomized clinical trial," *Journal of Maternal-Fetal and Neonatal Medicine*, vol. 26, no. 13, pp. 1311–1315, 2013.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 2124638, 9 pages
http://dx.doi.org/10.1155/2016/2124638

Review Article

Mechanisms and Clinical Application of Tetramethylpyrazine (an Interesting Natural Compound Isolated from Ligusticum Wallichii): Current Status and Perspective

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Received 17 June 2016; Accepted 9 August 2016

Academic Editor: Renata Szymanska

Tetramethylpyrazine, a natural compound from *Ligusticum wallichii* (*Chuan Xiong*), has been extensively used in China for cardiovascular and cerebrovascular diseases for about 40 years. Because of its effectiveness in multisystems, especially in cardiovascular, its pharmacological action, clinical application, and the structural modification have attracted broad attention. In this paper its mechanisms of action, the clinical status, and synthetic derivatives will be reviewed briefly.

1. Introduction

Tetramethylpyrazine (ligustrazine, TMP) is a natural compound isolated from Chinese herbal medicine *Ligusticum wallichii* (*Chuan Xiong*), which has been extensively used for medicinal purpose for more than 2000 years. TMP was firstly isolated in 1957 and has been increasingly studied for its action on myocardial and cerebral infarction since 1970s [1]. In the past decades, researchers explored other pharmacological capabilities of TMP in various diseases, such as coronary heart disease, diabetes, cancers, and liver injury. Accordingly, laboratory study verified the regulation ability of this agent in multiple molecular targets, such as anti-inflammation, antioxidant, antiplatelet, and antiapoptosis. The pharmacology of TMP has been well reviewed in the past [2–4]. This paper will briefly summarize the pharmacological mechanisms and its clinical application status; moreover research about TMP derivatives, also a highly popular topic due to its inherent low bioavailability, will then be discussed [5] (see Figure 1).

2. Medicinal Use of TMP

2.1. Cardiovascular System. The cardiovascular pharmacological effects of TMP aroused widespread interest among researchers in recent years [2, 4]. There are considerable documents supporting the view that this monomer can be a promising botanical remedy for cardiovascular diseases. The possible mechanism of its action might include modulating ion channels, stimulating the release of NO production, inhibiting vascular smooth muscle cell proliferation and migration, scavenging ROS, regulating inflammation and apoptosis, and preventing platelet aggregation (see Figure 2).

2.1.1. Regulation of Cardiac Inotropic and Vascular Functions

(1) Ion Channels. TMP was described as "calcium antagonist" [6] and produced a vasodilation effect via inhibiting Ca^{2+} influx and the release of intracellular Ca^{2+} at first [7]. Tsai et al. [8] introduced the cultured vascular smooth muscle (A7r5) to prove that TMP can affect the calcium influx, at least partly, by mediating the opening of potassium channel. Moreover, Kim et al. [9] verified that TMP-induced

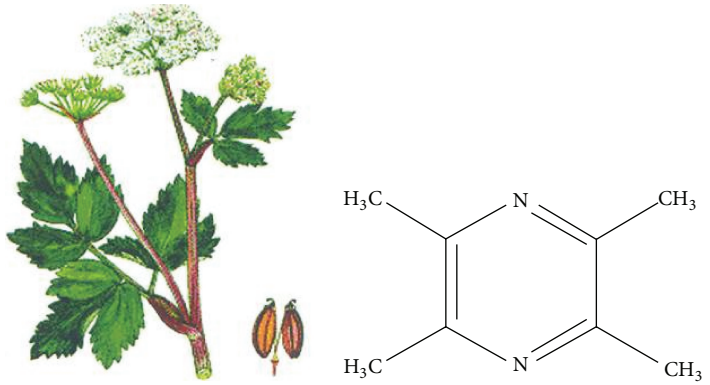

Figure 1: Illustration of *Ligusticum wallichii* (*Chuan Xiong*) plant (a) and chemical structure of ligustrazine (b).

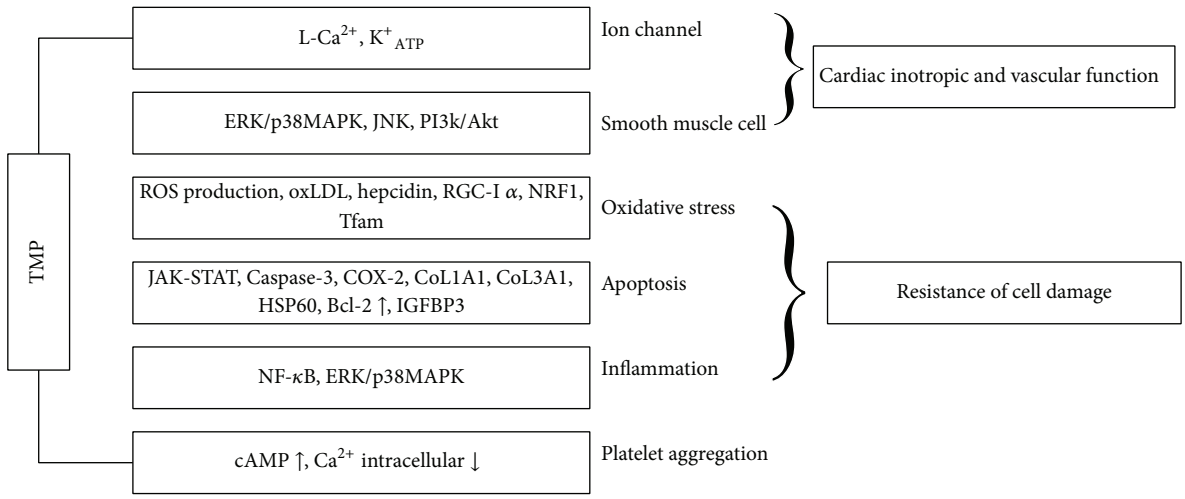

Figure 2: Putative mechanisms underlying cardiovascular protective effects of TMP.

vasorelaxation in isolated rat aortic rings was determined by ATP-dependent potassium channels. TMP was also reported to have a direct effect on L-type calcium current ($I_{Ca\text{-}L}$), since it can reduce calcium transient in a dose-dependent manner when applied to rabbit ventricular myocyte [10]. The combination of tetramethylpyrazine phosphate (TMPP) and Ginsenoside-Rb1 (Rb1) in $cTnT^{R141W}$ mouse model also obtained some benefit; the downregulated level of calmodulin 1 and calcium/calmodulin-dependent protein kinase IIβ (Camk2b) indicated that TMP might regulate Ca^{2+}/CaM/CaMKII [11]. However, current evidence is quite preliminary; the specific link between TMP and ion channels still needs to be thoroughly investigated. Despite the potentially involved ions which had been listed in the publications, the corresponding pathways, genes, or cytokines that might be responsible for the activation of ion channels are barely understood.

(2) Nitric Oxide Pathway. TMP can stimulate NO production in pulmonary arteries of rat [12]; Lv et al. [13] demonstrated that Akt and the endothelial isoform of nitric oxide synthase (eNOS) phosphorylation were significantly upregulated after TMP pretreatment *in vivo*; this effect could be blocked by NO synthase (NOS) inhibitor consequently. PI3K/Akt pathway might play a pivotal role in activating eNOS and increasing NO production. Numerous researches verified the result of TMP on NO production; they believed that there are certain kinds of relationship between TMP and Akt, while the result remains controversial. Some reported TMP exerts an inhibition role in phosphorylation of Akt in N9 microglial cells [14], although others claimed that TMP can activate Akt in vascular endothelial cells [15]. Despite different cell phenotypes involved in these experiments, the relationship between TMP and Akt pathway requires further exploration. Besides, there is no clear and standard therapeutic dosages of TMP, which makes the antioxidative effect of TMP not as evident as proposed.

(3) Smooth Muscle Cell Proliferation and Migration. TMP can suppress the proliferation of VSMC in rabbit aortic vascular [16]. Additionally, there is a study which intended to investigate the effect of TMP on the airway smooth muscle; their

data indicate that TMP might suppress the airway smooth cells proliferation via ERK1/2 signaling pathway, as the level of PDGF and p-ERK 1/2 proteins has decreased significantly in the TMP group [17]. Recently, another researcher [18] pointed out that TMP can inhibit PDGF-BB induced proliferation of VSMC, and results expressed that differentiated VSMC can be reversed by TMP and ERK and p38 MAPK might be involved in this process. Interestingly, most studies emphasized inhibitory effect on proliferation when TMP was administered before proliferation occurs, although few related mechanism or targets were identified.

2.1.2. Resistance of Cell Damage

(1) Oxidative Stress. Clinical and laboratory studies on herbal medicine draw special attention to ROS-pathway-mediated injury in CVDs [19]. The scavenging ROS function of TMP on hypoxia induced pulmonary vascular leakage had been explored [20], and H_2O_2-induced human umbilical vein endothelial cells (HUVECs) were also employed to evaluate protective effect of TMP on oxidative stress, as well as its antiapoptotic properties [21]. By testing its effect on C2C12 myotube, Gao and his coworkers [22] reported that TMP could restrain mitochondrial ROS generation and upregulate the expression of PGC1, NRF1, and Tfam, which reflects mitochondrial biogenesis. TMP also exerts an endothelium protective property via downregulating the expression of ICAM-1 and HSP60 [23]. Considering the precise mechanism is still not clearly clarified, further studies focusing on the specific roles of TMP should be emphasized.

(2) Apoptosis. TMP can decrease the ANP mRNA expression in cardiomyocyte hypertrophy rat model and suppress the level of pJAK2, pJAK1, or pSTAT3, demonstrating that TMP can inhibit JAK-STAT signal transduction [24]. Researchers evaluated the effect of tetramethylpyrazine phosphate (TMPP) on the dilated cardiomyopathy (DCM) and reported that TMPP can prevent the progressive LV dilation and systolic dysfunction, as well as the collagen deposition and gene expression reduction of procollagens COL1A1 and COL3A1 [25]. Moreover, the upregulated level of Bcl-2 and the reduction of Caspase-3 also had been observed in apoptosis myocyte after TMP intervention [26]. TMP can exert antiapoptosis ability by inhibiting macrophage COX-2 [27]. Recently, a piece of work [28] successfully tested the protective effect of TMP in H9c2 cardiomyoblasts; the result is consistent with previous report from Zheng et al. [29].

(3) Inflammation. TMP can restrain LPS-induced IL-8 overexpression in HUVECs at both the protein and mRNA levels, which is possibly due to blocking the activation of the NF-kB-dependent pathway; the involvement of ERK and p38 MAPK signaling pathway has also been observed [30]. Hepcidin has emerged as a positive regulator of atherosclerotic plaque destabilization since 2007 by Sullivan [31]; its expression might be regulated by TMP [32]. SD rats with high-fat diet for 8 weeks were employed in the study; TMP group was injected with TMP at 40 mg/(kg·d), while hepcidin group was injected with heparin at 5 mg/(kg·d). After treatment, relevant markers such as blood lipid, hepcidin, ET-1, ROS, MDA, and SOD were detected. The results supported that the protective effect of TMP on endothelium might be related to inhibiting overexpressed level of hepcidin. However, direct pathways involved in regulating hepcidin need to be investigated in the future.

2.1.3. Antiplatelet. Platelet aggregation plays a key role in the pathogenesis of atherothrombosis, and a variety of Chinese herbals have been examined for their antiplatelet property [33]. TMP has been commonly reported on its effect of antiplatelet since the 1980s [34]; stimulating cAMP production and inhibiting intracellular calcium mobilization were assumed to be the potential mechanism [35]. There is plenty of evidence for the suppression of platelet aggregation, although few publications were related to the platelet release reaction. A piece of research was conducted on patients who were diagnosed with acute coronary syndrome and received percutaneous coronary intervention. After TMP treatment, the level of CD63, an indicator of platelet activation, decreased significantly [36]. TMP might have an effect on inhibition of platelet release, although no experimental study is conducted to verify the role of TMP in platelet release reaction.

2.2. Protection on Cerebra and Spinal Cord Injury. The application of TMP in the treatment of ischemic stroke has been well documented for ages [37]. Tsai and Liang [38] directly evaluated its ability to penetrate blood brain barrier by using microdialysis technique that provides evidence for the following studies on central effect of TMP. Its neuroprotective property is partly due to modulating thioredoxin transcription [39] and downregulating the expression of neuronal isoform of NO synthase (nNOS) [40]. TMP also could attenuate the inflammation associated with ischemia by regulating the expression of NF-E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), which plays a role against ischemic reperfusion brain injury [40, 41]. Researchers also claimed that TMP can protect mitochondrial function and enzymatic antioxidants [42]; nevertheless sufficient evidence is needed. Recently, there is a piece of evidence for TMP on functional recovery and dendritic plasticity after ischemia [43]. The neuroprotective effects of TMP have also been tested on spinal cord injury [44, 45]. Moreover, other researchers [46] explored anti-inflammatory properties of TMP in Alzheimer's disease. Considering there is limited publications currently in this field, rigorous experiments can be warranted.

2.3. Cancer. Liu et al. [47] firstly investigated TMP for lymphocytes proliferation response. Later on, it has been tested on various cancers, such as leukemia [48, 49], lung cancer [50, 51], ovarian carcinoma [52], liver cancer [53], glioma [54], osteosarcoma [55], chemotherapy-resistant breast cancer [56], and prostate cancer [57]; the probable mechanism includes anti-inflammatory and promoting apoptosis. Studies intended to examine the effect of TMP derivatives like tetramethylpyrazine hydrochloride (TMPH) had yielded a similar conclusion. Large quantities of agents are currently

reported as an anticarcinogen via numerous pathways, as determined in experimental environments, while few display the same effects in clinic. How to apply the laboratory finding in the clinical practice is a critical issue that remains to be settled.

2.4. Diabetes. As there is abundant evidence for the vascular protective action of TMP, Lee and his colleagues hypothesized to investigate this characteristic on the diabetic model at the outset. Streptozotocin-induced diabetic mice model was adopted to test lipid peroxidation level, which is one of marked pathological changes in diabetes, and the result indicated that TMP can effectively alleviate glucose and blood urea nitrogen concentration (BUN) [58]. Later on, researchers also attempt to conduct similar experiments on streptozotocin-induced diabetic nephropathy rat model; their result was consistent with the previous one on the mice model. Moreover, they demonstrated that the level of insulin, angiotensin II, and P-selectin also decreased [58, 59]. Additionally, there is a piece of study reporting protective role of TMP regarding this disorder [60].

2.5. Liver Injury. Liu et al. [61] detected hepatoprotective effect of TMP on acute econazole-induced liver injury. The probable explanation for this action includes inhibition of membrane lipid peroxidation [62] and oxidative stress [63]. Likewise, recent evidence indicated that TMP exerts a protective property on sepsis-induced acute liver injury mainly by ameliorating the aquaporin 8 expression [64]. Besides, there are also publications related to its inhibitory effect on hepatic fibrosis, PI3/AKT, and ERK pathways, and NLRP3 inflammasome pathway might be engaged [65, 66].

2.6. Renal Injury. TMP can attenuate the Cisplatin-induced nephrotoxicity in rats; antioxidative stress might be one of the proposed mechanisms [67, 68]. Similar effect had also been tested in rat renal tubular cells [69]. Its therapeutic effect on hepatic/renal ischemia-reperfusion injury in rats [70], as well as the fibrosis of renal interstitial, had been verified [71, 72]. Moreover, TMP can protect rat renal tubular cells from adriamycin-induced apoptosis [73, 74]; this action, to some extent, is due to the inhibition of p38 MAPK and FoxO1 pathways [75].

2.7. Others. Since TMP was reported to possess a broad spectrum of pharmacological effects, such as antioxidant, anti-inflammatory, antifibrosis effects, diseases, such as asthma and colitis, suffered from such pathological changes and had been further investigated [76–80]. However, the precise mechanism still needs to be further explored.

3. Current Status of Therapeutic Uses of TMP

The injection solution of TMP has been broadly used especially in China to treat ischemic stroke [81], coronary heart disease [36], diabetic nephropathy [82], and knee osteoarthritis [83]. Large amount of research is about the efficacy of TMP injection. For example, in a systematic review, they evaluated the efficacy of 22 Chinese patent medicines for stroke; there are 11 randomized controlled trials with 1652 patients related to TMP injection [84]. Although, it seems that lots of evidence have stated the efficacy, the reliability of these trials is doubtful. Few of the studies report the adverse events [82, 85, 86]; the poor methodology makes the problem even worse. Even for the treatment of the same disease, the dosage of TMP injection is quite different. Similar problem was confronted when talking about the treatment course. What is more, there are lots of combination uses of other herbal injections during the treatment, which might be difficult to figure out the interactions. Due to the low quality of clinical trials in this field, the safety concerns about the herbal injection have long been a major dispute [87].

4. Approaches to Improve the Bioavailability of TMP

4.1. Problems Associated with Drug Delivery. Early pharmacokinetic research has determined the metabolism rate of TMP and verified its *in-vivo* short half-life of $T_{1/2} = 2.89$ h. Besides, the accumulated toxicity is another potential threat to the patients for keeping an effective concentration via frequently administration [88, 89]. Considering the drug delivery deficiency of TMP [90], enormous experiments were conducted to improve its pharmacological activity since the 1990s. There are two primary approaches which can improve the biological activity.

4.2. Improvement of Dosage Form. The conventional TMP dosage forms include injection, oral tablet, and capsule. Conventionally, the oral administration, is the most preferable route for chronic diseases. However, this form might not be suitable, considering that hepatic first-pass metabolism can probably result in a lower bioavailability [5]. However, the intravenous injection does not help; drug concentration of injection form was peaked within 20 min but was undetected after 120 min [38]. A great deal of formulations had been proposed to solve the deficiency during drug delivery, such as porosity osmotic pump, microemulsions, ethosomes, and transdermal patch. The administration route has changed accordingly; transdermal, intranasal, intraperitoneal injection, and ocular delivery were introduced [5, 91, 92]. It is of importance to quantify the drug concentration in different sites (such as brain, liver, and skin). Research related to *in vitro* drug release, *in vivo* distribution, and the optimum dosage is urgently needed. Another unsatisfactory situation is that most of the newly designed forms were conducted at laboratory level, and extensive clinical evidence needs to be layed out to validate the clinical effect accordingly.

4.3. Structural Modification of TMP. The structural formula of TMP shows that pyrazine largely determined its pharmacodynamics, while the side chain might be mainly responsible for its pharmacokinetics. Given its inherent characteristics, the structure modification to improve the bioavailability has been broadly investigated, which opened new perspective for drug discovery. During the

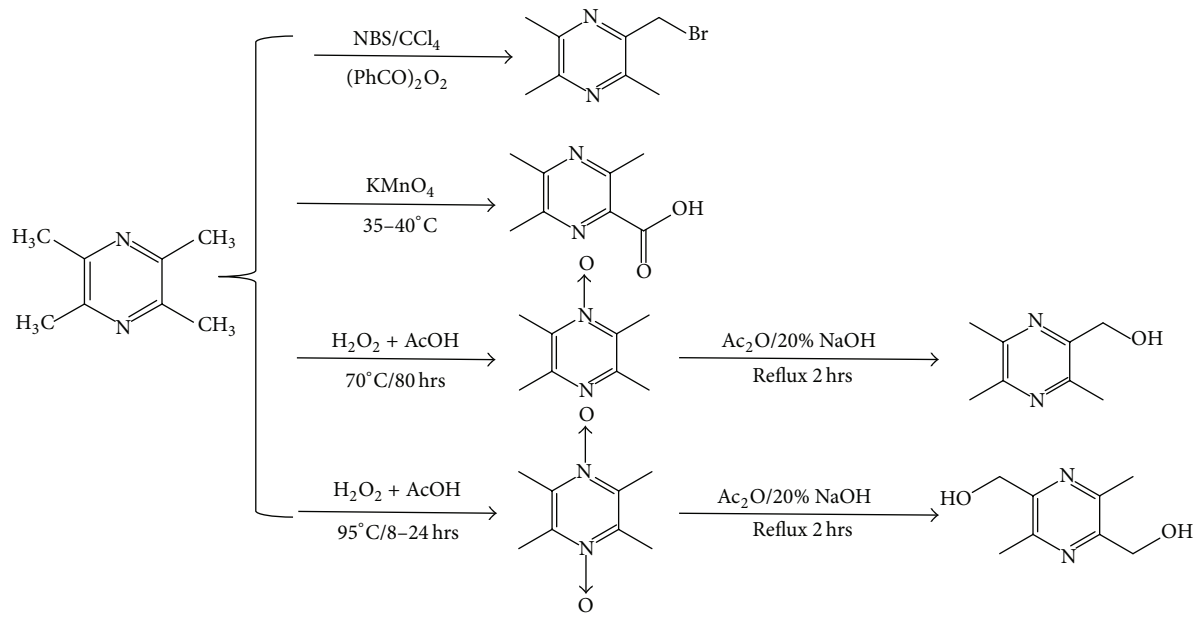

Figure 3: Primary intermediates of TMP.

past decades, over 300 novel TMP derivatives had been designed and synthesized [93]. In general, most of the modifications were derived from 4 primary intermediates of TMP. They are 2-bromomethyl-3,5,6-trimethylpyrazine (TMP-Br), 3,5,6-trimethylpyrazine-2-yl (TMP-OH), 3,5,6-trimethylpyrazine-2-carboxylic acid (TMP-COOH), and 2,5-dimethylol-3,6-dimethylpyrazine (OH-TMP-OH) (Figure 3). The first step of the structural modification is the synthesis of these four fundamental compounds. With the obtained intermediates, we can easily link other reactive groups with TMP. Not only can small chemical groups be used for structural modification, but considerable herbal compounds were also introduced into synthesis process, for example, TMP-ferulic acid derivatives that combined TMP and ferulic acid, another active ingredient of *Ligusticum wallichii*, as precursors. Both TMP and ferulic acid had been extensively reported because of their antiplatelet aggregation effect [94]. Given the structural properties, calcium antagonists also could be combined with pyrazine to form new derivatives. Further study should be conducted, enabling a better understanding of the pharmacokinetics of these derivatives. After modification, further experiment is needed to demonstrate the biological activities and pharmacological characteristics of original derivatives. After 21 new derivatives had been developed, Li and his coworkers conducted experiment to evaluate the protective effect of newly designed derivatives on the HUVECs. The data proved that some of the new compounds had better protective effect compared with the reference drug [95]. Most of the results seem preferable, which reported a better effect over TMP. Although the interaction and toxicity of new derivatives have not been studied comprehensively, the active parts of the new drugs, as well as their metabolism parameters, and signal transmission might be different from TMP, which also needs to be further confirmed.

5. Conclusion

As an effective and multitarget product, TMP is promising and worthy of further investigation. Large amount of trials had been conducted to assess its clinical efficacy; their results seem too good to be true. So far, there is no clear evidence that gives us the standard dosage, treatment course, as well as the reporting of adverse events which is necessary for clinical trials. It would be more helpful if standardized usage for TMP was established. In that case, the results of clinical trial would be more persuasive, providing reliable evidence for decision making in practice. Despite enormous interests in the clinical uses, there is still a great deal of challenge facing the academics, such as the effectiveness, pharmacological effect, and toxicity. Nowadays, the action and mechanism of TMP have been investigated in multisystems and multi-diseases, and the involved pathways and targets seem quite complex. With advances in the technique of laboratory, such as genomic, proteomic data, as well as spectrometric analysis, our understanding of herbal compounds like TMP will be more systematic. As our knowledge about its mechanism as well as disease pathophysiology has been expanded, TMP can be used more extensively and more targeted. In the future, more preclinical studies to determine its mechanisms and potential toxic effects and to answer questions related to absorption and metabolism are needed. After this preclinical work has been completed, well-designed clinical studies can be conducted. In summary, multifunction and the structural advantage make TMP a promising candidate for further study to achieve maximum therapeutic efficacy and minimum toxicity.

Competing Interests

The authors declare that there is no conflict of interests.

Authors' Contributions

Yue Liu conceived the topic and helped to draft the paper. Yingke Zhao collected references and wrote the paper. Keji Chen helped to draft the manuscript.

Acknowledgments

This work was supported by National Natural Science Foundation of China (no. 81403266) and Innovative Team Project of China Academy of Chinese Medical Sciences.

References

[1] S. K. Guo, K. J. Chen, Z. H. Qian, W. L. Weng, and M. Y. Qian, "Tetramethylpyrazine in the treatment of cardiovascular and cerebrovascular diseases," *Planta Medica*, vol. 47, no. 2, article 89, 1983.

[2] M. Guo, Y. Liu, and D. Shi, "Cardiovascular actions and therapeutic potential of tetramethylpyrazine (active component isolated from *Rhizoma Chuanxiong*): roles and mechanisms," *BioMed Research International*, vol. 2016, Article ID 2430329, 9 pages, 2016.

[3] H.-J. Gao, P.-F. Liu, P.-W. Li et al., "Ligustrazine monomer against cerebral ischemia/ reperfusion injury," *Neural Regeneration Research*, vol. 10, no. 5, pp. 832–840, 2015.

[4] W. Qian, X. Xiong, Z. Fang, H. Lu, and Z. Wang, "Protective effect of tetramethylpyrazine on myocardial ischemia-reperfusion injury," *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 107501, 9 pages, 2014.

[5] D. Mei, S. Mao, W. Sun, Y. Wang, and T. Kissel, "Effect of chitosan structure properties and molecular weight on the intranasal absorption of tetramethylpyrazine phosphate in rats," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 70, no. 3, pp. 874–881, 2008.

[6] X. B. Zhou, L. Salganicoff, and R. Sevy, "The pharmacological effect of ligustrazine on human platelets," *Acta Pharmaceutica Sinica*, vol. 20, no. 5, pp. 334–339, 1985.

[7] C. Y. Kwan, E. E. Daniel, and M. C. Chen, "Inhibition of vasoconstriction by tetramethylpyrazine: does it act by blocking the voltage-dependent Ca channel?" *Journal of Cardiovascular Pharmacology*, vol. 15, no. 1, pp. 157–162, 1990.

[8] C.-C. Tsai, T.-Y. Lai, W.-C. Huang et al., "Tetramethylpyrazine as potassium channel opener to lower calcium influx into cultured aortic smooth muscle cells," *Planta Medica*, vol. 69, no. 6, pp. 557–558, 2003.

[9] E.-Y. Kim, J.-H. Kim, and M.-R. Rhyu, "Endothelium-independent vasorelaxation by ligusticum wallichii in isolated rat aorta: comparison of a butanolic fraction and tetramethylpyrazine, the main active component of ligusticum wallichii," *Biological and Pharmaceutical Bulletin*, vol. 33, no. 8, pp. 1360–1363, 2010.

[10] Z. Ren, J. Ma, P. Zhang et al., "The effect of ligustrazine on L-type calcium current, calcium transient and contractility in rabbit ventricular myocytes," *Journal of Ethnopharmacology*, vol. 144, no. 3, pp. 555–561, 2012.

[11] D. Lu, H.-T. Shao, W.-P. Ge et al., "Ginsenoside-RB1 and tetramethylpyrazine phosphate act synergistically to prevent dilated cardiomyopathy in cTnTR141W transgenic mice," *Journal of Cardiovascular Pharmacology*, vol. 59, no. 5, pp. 426–433, 2012.

[12] W. Peng, D. Hucks, R. M. Priest, Y. M. Kan, and J. P. T. Ward, "Ligustrazine-induced endothelium-dependent relaxation in pulmonary arteries via an NO-mediated and exogenous L-arginine-dependent mechanism," *British Journal of Pharmacology*, vol. 119, no. 5, pp. 1063–1071, 1996.

[13] L. Lv, S.-S. Jiang, J. Xu, J.-B. Gong, and Y. Cheng, "Protective effect of ligustrazine against myocardial ischaemia reperfusion in rats: the role of endothelial nitric oxide synthase," *Clinical and Experimental Pharmacology and Physiology*, vol. 39, no. 1, pp. 20–27, 2012.

[14] H.-T. Liu, Y.-G. Du, J.-L. He et al., "Tetramethylpyrazine inhibits production of nitric oxide and inducible nitric oxide synthase in lipopolysaccharide-induced N9 microglial cells through blockade of MAPK and PI3K/Akt signaling pathways, and suppression of intracellular reactive oxygen species," *Journal of Ethnopharmacology*, vol. 129, no. 3, pp. 335–343, 2010.

[15] Y. Kang, M. Hu, Y. Zhu, X. Gao, and M.-W. Wang, "Antioxidative effect of the herbal remedy Qin Huo Yi Hao and its active component tetramethylpyrazine on high glucose-treated endothelial cells," *Life Sciences*, vol. 84, no. 13-14, pp. 428–436, 2009.

[16] S. Li, J.-H. Wang, and S.-L. Chen, "Inhibitory effect of ligustrazine on proliferation of rabbit vascular smooth muscle cells after arterial injury," *Acta Pharmacologica Sinica*, vol. 20, no. 10, pp. 917–922, 1999.

[17] Y.-J. Qu, H.-B. Bai, C.-Z. Wang, J.-D. Xu, T.-T. Zhang, and Z.-Y. Han, "Inhibition of tetramethylpyrazine on the proliferation of rat airway smooth muscle cells," *Chinese Pharmacological Bulletin*, vol. 26, no. 6, pp. 814–818, 2010.

[18] L. Yu, X. Huang, K. Huang, C. Gui, Q. Huang, and B. Wei, "Ligustrazine attenuates the platelet-derived growth factor-BB-induced proliferation and migration of vascular smooth muscle cells by interrupting extracellular signal-regulated kinase and P38 mitogen-activated protein kinase pathways," *Molecular Medicine Reports*, vol. 12, no. 1, pp. 705–711, 2015.

[19] L. Li, X. Zhou, N. Li, M. Sun, J. Lv, and Z. Xu, "Herbal drugs against cardiovascular disease: traditional medicine and modern development," *Drug Discovery Today*, vol. 20, no. 9, pp. 1074–1086, 2015.

[20] L. Zhang, M. Deng, and S. Zhou, "Tetramethylpyrazine inhibits hypoxia-induced pulmonary vascular leakage in rats via the ROS-HIF-VEGF pathway," *Pharmacology*, vol. 87, no. 5-6, pp. 265–273, 2011.

[21] W.-M. Li, H.-T. Liu, X.-Y. Li et al., "The effect of tetramethylpyrazine on hydrogen peroxide-induced oxidative damage in human umbilical vein endothelial cells," *Basic and Clinical Pharmacology and Toxicology*, vol. 106, no. 1, pp. 45–52, 2010.

[22] X. Gao, X.-L. Zhao, Y.-H. Zhu et al., "Tetramethylpyrazine protects palmitate-induced oxidative damage and mitochondrial dysfunction in C2C12 myotubes," *Life Sciences*, vol. 88, no. 17-18, pp. 803–809, 2011.

[23] H.-J. Wu, J. Hao, S.-Q. Wang, B.-L. Jin, and X.-B. Chen, "Protective effects of ligustrazine on TNF-α-induced endothelial dysfunction," *European Journal of Pharmacology*, vol. 674, no. 2-3, pp. 365–369, 2012.

[24] M.-H. Gao, L. Zhang, B. Li, S.-R. Ren, and B. Zhang, "Effect of tetramethylpyrazine on JAK-STAT signal transduction in cardiomyocyte hypertrophy," *Chinese Journal of Cellular and Molecular Immunology*, vol. 27, no. 5, pp. 519–524, 2011.

[25] H.-P. Zhao, D. Lü, W. Zhang et al., "Protective action of tetramethylpyrazine phosphate against dilated cardiomyopathy

in cTnTR141W transgenic mice," *Acta Pharmacologica Sinica*, vol. 31, no. 3, pp. 281–288, 2010.

[26] Y. Yang, Z.-H. Li, H. Liu, W.-D. Shi, and J. Zhang, "Inhibitory effect of tetramethylpyrazine preconditioning on overload training-induced myocardial apoptosis in rats," *Chinese Journal of Integrative Medicine*, vol. 21, no. 6, pp. 423–430, 2014.

[27] J. Y. Wan, D. Y. Ye, P. Wu, L. Zhang, X. Gong, and Y. Huang, "Effect of tetramethylpyrazine on lipopolysaccharides induced macrophage cyclo-oxidase-2 expression and apoptosis of cardiac myocytes," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 24, no. 10, pp. 906–911, 2004.

[28] K.-H. Lin, W.-W. Kuo, A.-Z. Jiang et al., "Tetramethylpyrazine ameliorated hypoxia-induced myocardial cell apoptosis via HIF-1α/JNK/p38 and IGFBP3/BNIP3 inhibition to upregulate PI3K/Akt survival signaling," *Cellular Physiology and Biochemistry*, vol. 36, no. 1, pp. 334–344, 2015.

[29] H. Zheng, S. Wang, P. Zhou, W. Liu, and F. Ni, "Effects of Ligustrazine on DNA damage and apoptosis induced by irradiation," *Environmental Toxicology and Pharmacology*, vol. 36, no. 3, pp. 1197–1206, 2013.

[30] X.-Y. Li, J.-L. He, H.-T. Liu, W.-M. Li, and C. Yu, "Tetramethylpyrazine suppresses interleukin-8 expression in LPS-stimulated human umbilical vein endothelial cell by blocking ERK, p38 and nulear factor-κB signaling pathways," *Journal of Ethnopharmacology*, vol. 125, no. 1, pp. 83–89, 2009.

[31] J. L. Sullivan, "Macrophage iron, hepcidin, and atherosclerotic plaque stability," *Experimental Biology and Medicine*, vol. 232, no. 8, pp. 1014–1020, 2007.

[32] M.-Y. Sun, C.-Y. Guo, J.-S. Wang et al., "Correlation between high expression of hepcidin and vascular endothelial damage as well as intervention of tetramethylpyrazine," *Chinese Traditional and Herbal Drugs*, vol. 46, no. 15, pp. 2265–2269, 2015.

[33] Y. Liu, H.-J. Yin, D.-Z. Shi, and K.-J. Chen, "Chinese herb and formulas for promoting blood circulation and removing blood stasis and antiplatelet therapies," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 184503, 8 pages, 2012.

[34] G. Y. Zeng, Y. P. Zhou, L. Y. Zhang, and Y. Zhang, "Effects of tetramethylpyrazine on cardiac haemodynamics in dogs," *Acta Pharmaceutica Sinica*, vol. 17, no. 3, pp. 182–186, 1982.

[35] S.-Y. Liu and D. M. Sylvester, "Antithrombotic/antiplatelet activity of tetramethylpyrazine," *Thrombosis Research*, vol. 58, no. 2, pp. 129–140, 1990.

[36] Z.-Q. Chen, L. Hong, and H. Wang, "Effect of tetramethylpyrazine on platelet activation and vascular endothelial function in patients with acute coronary syndrome undergoing percutaneous coronary intervention," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 27, no. 12, pp. 1078–1081, 2007.

[37] K. J. Chen and K. Chen, "Ischemic stroke treated with *Ligusticum chuanxiong*," *Chinese Medical Journal*, vol. 105, no. 10, pp. 870–873, 1992.

[38] T.-H. Tsai and C.-C. Liang, "Pharmacokinetics of tetramethylpyrazine in rat blood and brain using microdialysis," *International Journal of Pharmaceutics*, vol. 216, no. 1-2, pp. 61–66, 2001.

[39] J. Jia, X. Zhang, Y.-S. Hu et al., "Protective effect of tetraethyl pyrazine against focal cerebral ischemia/reperfusion injury in rats: therapeutic time window and its mechanism," *Thrombosis Research*, vol. 123, no. 5, pp. 727–730, 2009.

[40] X. Xiao, Y. Liu, C. Qi et al., "Neuroprotection and enhanced neurogenesis by tetramethylpyrazine in adult rat brain after focal ischemia," *Neurological Research*, vol. 32, no. 5, pp. 547–555, 2010.

[41] M. Li, X. Zhang, L. Cui et al., "The neuroprotection of oxymatrine in cerebral ischemia/reperfusion is related to nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated antioxidant response: role of Nrf2 and hemeoxygenase-1 expression," *Biological and Pharmaceutical Bulletin*, vol. 34, no. 5, pp. 595–601, 2011.

[42] S.-Y. Li, Y.-H. Jia, W.-G. Sun et al., "Stabilization of mitochondrial function by tetramethylpyrazine protects against kainate-induced oxidative lesions in the rat hippocampus," *Free Radical Biology and Medicine*, vol. 48, no. 4, pp. 597–608, 2010.

[43] J.-B. Lin, C.-J. Zheng, X. Zhang, J. Chen, W.-J. Liao, and Q. Wan, "Effects of tetramethylpyrazine on functional recovery and neuronal dendritic plasticity after experimental stroke," *Evidence-based Complementary and Alternative Medicine*, vol. 2015, Article ID 394926, 10 pages, 2015.

[44] Z. Xiao, J. Hu, H. Lu et al., "Effect of tetramethylpyrazine on the expression of macrophage migration inhibitory factor in acute spinal cord injury in rats," *Journal of Central South University (Medical Sciences)*, vol. 37, no. 10, pp. 1031–1036, 2012.

[45] J.-W. Shin, J.-Y. Moon, J.-W. Seong et al., "Effects of tetramethylpyrazine on microglia activation in spinal cord compression injury of mice," *The American Journal of Chinese Medicine*, vol. 41, no. 6, pp. 1361–1376, 2013.

[46] M. Kim, S.-O. Kim, M. Lee et al., "Tetramethylpyrazine, a natural alkaloid, attenuates pro-inflammatory mediators induced by amyloid β and interferon-γ in rat brain microglia," *European Journal of Pharmacology*, vol. 740, pp. 504–511, 2014.

[47] J. Liu, W. Qiang, and S. Ye, "Effect of tetramethylpyrazine on lymphocytes proliferation response of murine splenocytes," *Journal of West China University of Medical Sciences*, vol. 26, no. 2, pp. 177–179, 1995.

[48] N. Li, X.-H. Jia, and J.-Y. Wang, "Effects of tetramethylpyrazine on apoptosis of human leukemia cells and the expressions of apoptotic-relevant proteins," *Tumor*, vol. 34, no. 10, pp. 919–923, 2014.

[49] X.-J. Wang, Y.-H. Xu, G.-C. Yang, H.-X. Chen, and P. Zhang, "Tetramethylpyrazine inhibits the proliferation of acute lymphocytic leukemia cell lines via decrease in GSK-3β," *Oncology Reports*, vol. 33, no. 5, pp. 2368–2374, 2015.

[50] X.-Y. Xu, P.-K. Yan, G. Chen, and D.-F. Liao, "Inhibition of tetramethylpyrazine on Lewis lung carcinomas, microvessel growth and VEGF expression in mice," *Chinese Pharmacological Bulletin*, vol. 20, no. 2, pp. 151–154, 2004.

[51] C.-Y. Zheng, W. Xiao, M.-X. Zhu, X.-J. Pan, Z.-H. Yang, and S.-Y. Zhou, "Inhibition of cyclooxygenase-2 by tetramethylpyrazine and its effects on A549 cell invasion and metastasis," *International Journal of Oncology*, vol. 40, no. 6, pp. 2029–2037, 2012.

[52] J. Yin, C. Yu, Z. Yang et al., "Tetramethylpyrazine inhibits migration of SKOV3 human ovarian carcinoma cells and decreases the expression of interleukin-8 via the ERK1/2, p38 and AP-1 signaling pathways," *Oncology Reports*, vol. 26, no. 3, pp. 671–679, 2011.

[53] J. Cao, Q. Miao, S. Miao et al., "Tetramethylpyrazine (TMP) exerts antitumor effects by inducing apoptosis and autophagy in hepatocellular carcinoma," *International Immunopharmacology*, vol. 26, no. 1, pp. 212–220, 2015.

[54] Z. Chen, X. Pan, A. G. Georgakilas et al., "Tetramethylpyrazine (TMP) protects cerebral neurocytes and inhibits glioma by

down regulating chemokine receptor CXCR4 expression," *Cancer Letters*, vol. 336, no. 2, pp. 281–289, 2013.

[55] Y. Wang, Q. Fu, and W. Zhao, "Tetramethylpyrazine inhibits osteosarcoma cell proliferation via downregulation of NF-κB in vitro and in vivo," *Molecular Medicine Reports*, vol. 8, no. 4, pp. 984–988, 2013.

[56] Y. Zhang, X. Liu, T. Zuo, Y. Liu, and J. H. Zhang, "Tetramethylpyrazine reverses multidrug resistance in breast cancer cells through regulating the expression and function of P-glycoprotein," *Medical Oncology*, vol. 29, no. 2, pp. 534–538, 2012.

[57] J. Han, J. Song, X. Li et al., "Ligustrazine suppresses the growth of HRPC Cells through the inhibition of cap-dependent translation via both the mTOR and the MEK/ERK pathways," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 15, no. 6, pp. 764–772, 2015.

[58] L.-M. Lee, C.-F. Liu, and P.-P. Yang, "Effect of tetramethylpyrazine on lipid peroxidation in streptozotocin-induced diabetic mice," *The American Journal of Chinese Medicine*, vol. 30, no. 4, pp. 601–608, 2002.

[59] Y.-J. Fu, Y. Zhou, J.-Q. Pan, X.-M. Zhang, and J.-H. Lü, "The rapeutic effects and mechanisms of tetramethylpyrazine on streptozocin-induced-nephropathy in type 2 diabetic rats," *Chinese Pharmaceutical Journal*, vol. 47, no. 22, pp. 1807–1812, 2012.

[60] Q.-H. Yang, Y. Liang, Q. Xu, Y. Zhang, L. Xiao, and L.-Y. Si, "Protective effect of tetramethylpyrazine isolated from Ligusticum chuanxiong on nephropathy in rats with streptozotocin-induced diabetes," *Phytomedicine*, vol. 18, no. 13, pp. 1148–1152, 2011.

[61] C.-F. Liu, C.-C. Lin, L.-T. Ng, and S.-C. Lin, "Hepatoprotective and therapeutic effects of tetramethylpyrazine on acute econazole-induced liver injury," *Planta Medica*, vol. 68, no. 6, pp. 510–514, 2002.

[62] E. C. So, K.-L. Wong, T.-C. Huang, S.-C. C. Tasi, and C.-F. Liu, "Tetramethylpyrazine protects mice against thioacetamide-induced acute hepatotoxicity," *Journal of Biomedical Science*, vol. 9, no. 5, pp. 410–414, 2002.

[63] C. Lu, Y. Jiang, F. Zhang et al., "Tetramethylpyrazine prevents ethanol-induced hepatocyte injury via activation of nuclear factor erythroid 2-related factor 2," *Life Sciences*, vol. 141, pp. 119–127, 2015.

[64] J.-Q. Wang, L. Zhang, X.-G. Tao et al., "Tetramethylpyrazine upregulates the aquaporin 8 expression of hepatocellular mitochondria in septic rats," *Journal of Surgical Research*, vol. 185, no. 1, pp. 286–293, 2013.

[65] F. Zhang, Z. Zhang, D. Kong et al., "Tetramethylpyrazine reduces glucose and insulin-induced activation of hepatic stellate cells by inhibiting insulin receptor-mediated PI3K/AKT and ERK pathways," *Molecular and Cellular Endocrinology*, vol. 382, no. 1, pp. 197–204, 2014.

[66] X. Wu, F. Zhang, X. Xiong et al., "Tetramethylpyrazine reduces inflammation in liver fibrosis and inhibits inflammatory cytokine expression in hepatic stellate cells by modulating NLRP3 inflammasome pathway," *IUBMB Life*, vol. 67, no. 4, pp. 312–321, 2015.

[67] B. H. Ali, M. Al-Moundhri, M. T. Eldin, A. Nemmar, S. Al-Siyabi, and K. Annamalai, "Amelioration of cisplatin-induced nephrotoxicity in rats by tetramethylpyrazine, a major constituent of the chinese herb ligusticum wallichi," *Experimental Biology and Medicine*, vol. 233, no. 7, pp. 891–896, 2008.

[68] Z. Lan, K. S. Bi, and X. H. Chen, "Ligustrazine attenuates elevated levels of indoxyl sulfate, kidney injury molecule-1 and clusterin in rats exposed to cadmium," *Food and Chemical Toxicology*, vol. 63, pp. 62–68, 2014.

[69] Y.-M. Sue, C.-F. Cheng, C.-C. Chang, Y. Chou, C.-H. Chen, and S.-H. Juan, "Antioxidation and anti-inflammation by haem oxygenase-1 contribute to protection by tetramethylpyrazine against gentamicin-induced apoptosis in murine renal tubular cells," *Nephrology Dialysis Transplantation*, vol. 24, no. 3, pp. 769–777, 2009.

[70] J.-L. Chen, T. Zhou, W.-X. Chen et al., "Effect of tetramethylpyrazine on P-selectin and hepatic/renal ischemia and reperfusion injury in rats," *World Journal of Gastroenterology*, vol. 9, no. 7, pp. 1563–1566, 2003.

[71] X.-P. Yuan, L.-S. Liu, Q. Fu, and C.-X. Wang, "Effects of ligustrazine on ureteral obstruction-induced renal tubulointerstitial fibrosis," *Phytotherapy Research*, vol. 26, no. 5, pp. 697–703, 2012.

[72] J. Li, J. Yu, Y. Liu et al., "Expression of the matrix metalloproteinases and the tissue inhibitor of metalloproteinase factors are affected by tetramethylpyrazine treatment in a renal interstitial fibrosis rat model," *Journal of Hard Tissue Biology*, vol. 23, no. 3, pp. 309–316, 2014.

[73] C.-Y. Cheng, Y.-M. Sue, C.-H. Chen et al., "Tetramethylpyrazine attenuates adriamycin-induced apoptotic injury in rat renal tubular cells NRK-52E," *Planta Medica*, vol. 72, no. 10, pp. 888–893, 2006.

[74] S.-H. Juan, C.-H. Chen, Y.-H. Hsu et al., "Tetramethylpyrazine protects rat renal tubular cell apoptosis induced by gentamicin," *Nephrology Dialysis Transplantation*, vol. 22, no. 3, pp. 732–739, 2007.

[75] X. Gong, Q. Wang, X. Tang et al., "Tetramethylpyrazine prevents contrast-induced nephropathy by inhibiting p38 MAPK and FoxO1 signaling pathways," *American Journal of Nephrology*, vol. 37, no. 3, pp. 199–207, 2013.

[76] X.-W. Che, Y. Zhang, H. Wang, and W. Wang, "Effect of ligustrazine injection on levels of interleukin-4 and interferon-γ in patients with bronchial asthma," *Chinese Journal of Integrative Medicine*, vol. 14, no. 3, pp. 217–220, 2008.

[77] N.-F. Ji, Y.-C. Xie, M.-S. Zhang et al., "Ligustrazine corrects Th1/Th2 and Treg/Th17 imbalance in a mouse asthma model," *International Immunopharmacology*, vol. 21, no. 1, pp. 76–81, 2014.

[78] X. Y. Xu, L. Ye, G. Chen, J. D. Feng, W. H. Chen, and Y. Y. Hu, "Effect of tetramethylpyrazine on expression of vascular cell adhesion molecule-1 in mice with ulcerative colitis," *China Journal of Chinese Materia Medica*, vol. 31, no. 19, pp. 1608–1611, 2006.

[79] X. He, Z. Zheng, X. Yang, Y. Lu, N. Chen, and W. Chen, "Tetramethylpyrazine attenuates PPAR-γ antagonist-deteriorated oxazolone-induced colitis in mice," *Molecular Medicine Reports*, vol. 5, no. 3, pp. 645–650, 2012.

[80] Y. Lu, M. Zhu, W. Chen et al., "Tetramethylpyrazine improves oxazolone-induced colitis by Inhibiting the NF-κB pathway," *Clinical & Investigative Medicine*, vol. 37, no. 1, pp. E1–E9, 2014.

[81] X. Ni, S. Liu, and X. Guo, "Medium- and long-term efficacy of ligustrazine plus conventional medication on ischemic stroke: a systematic review and meta-analysis," *Journal of Traditional Chinese Medicine*, vol. 33, no. 6, pp. 715–720, 2013.

[82] B. Wang, Q. Ni, X. Wang, and L. Lin, "Meta-analysis of the clinical effect of ligustrazine on diabetic nephropathy," *The American Journal of Chinese Medicine*, vol. 40, no. 1, pp. 25–37, 2012.

[83] J.-Z. Hu, C.-Y. Luo, M. Kang, H.-B. Lü, G.-H. Lei, and Z. Dai, "Therapeutic effects of intraarticular injection of ligustrazine on knee osteoarthritis," *Journal of Central South University*, vol. 31, no. 4, pp. 591–594, 2006.

[84] B. Wu, M. Liu, H. Liu et al., "Meta-analysis of traditional Chinese patent medicine for ischemic stroke," *Stroke*, vol. 38, no. 6, pp. 1973–1979, 2007.

[85] H. Shao, L. Zhao, F. Chen, S. Zeng, S. Liu, and J. Li, "Efficacy of ligustrazine injection as adjunctive therapy for angina pectoris: a systematic review and meta-analysis," *Medical Science Monitor*, vol. 21, pp. 3704–3715, 2015.

[86] J.-S. Li, H.-F. Wang, Y.-P. Bai, S.-Y. Li, X.-Q. Yu, and Y. Li, "Ligustrazine injection for chronic pulmonary heart disease: a systematic review and meta-analysis," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 792726, 8 pages, 2012.

[87] S. A. Jordan, D. G. Cunningham, and R. J. Marles, "Assessment of herbal medicinal products: challenges, and opportunities to increase the knowledge base for safety assessment," *Toxicology and Applied Pharmacology*, vol. 243, no. 2, pp. 198–216, 2010.

[88] R. Xu, Y. Li, and X. Huang, "Pharmacokinetic developments in ligustrazine," *Journal of Anhui Traditional Chinese Medicine College*, vol. 21, pp. 58–61, 2002.

[89] C. Ligong, Y. Yili, and N. Shanqiu, "Research on the mechanism of the action of tetramethylpyrazine on mesenteric capillary of rabbits," *Journal of Chinese Microcirculation*, vol. 1, article 5, 1998.

[90] W. Cai, S. N. Dong, and Y. Q. Lou, "HPLC determination of tetramethylpyrazine in human serum and its pharmacokinetic parameters," *Acta Pharmaceutica Sinica*, vol. 24, no. 12, pp. 881–886, 1989.

[91] L. Wei, N. Marasini, G. Li, C. S. Yong, J. O. Kim, and Q. Quan, "Development of ligustrazine-loaded lipid emulsion: formulation optimization, characterization and biodistribution," *International Journal of Pharmaceutics*, vol. 437, no. 1-2, pp. 203–212, 2012.

[92] Z. Tang, Q. Wang, H. Xu, and W. Zhang, "Microdialysis sampling for investigations of tetramethylpyrazine following transdermal and intraperitoneal administration," *European Journal of Pharmaceutical Sciences*, vol. 50, no. 3-4, pp. 454–458, 2013.

[93] K. Xu, P. Wang, X. Xu et al., "An overview on structural modifications of ligustrazine and biological evaluation of its synthetic derivatives," *Research on Chemical Intermediates*, vol. 41, no. 3, pp. 1385–1411, 2015.

[94] X. Ran, L. Ma, C. Peng, H. Zhang, and L.-P. Qin, "Ligusticum chuanxiong Hort: a review of chemistry and pharmacology," *Pharmaceutical Biology*, vol. 49, no. 11, pp. 1180–1189, 2011.

[95] Z. Li, F. Yu, L. Cui et al., "Ligustrazine derivatives. Part 8: design, synthesis, and preliminary biological evaluation of novel ligustrazinyl amides as cardiovascular agents," *Medicinal Chemistry*, vol. 10, no. 1, pp. 81–89, 2014.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9156510, 13 pages
http://dx.doi.org/10.1155/2016/9156510

Research Article

Properties of Flavonoids Isolated from the Bark of *Eysenhardtia polystachya* and Their Effect on Oxidative Stress in Streptozotocin-Induced Diabetes Mellitus in Mice

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Received 10 April 2016; Accepted 8 June 2016

Academic Editor: Renata Szymanska

Six new flavonoids 2′,4′-dihydroxychalcone-6′-O-β-D-glucopyranoside (**1**), α,3,2′,4′-tetrahydroxy-4-methoxy-dihydrochalcone-3′-C-β-glucopyranosy-6′-O-β-D-glucopyranoside (**2**), 7-hydroxy-5,8′-dimethoxy-6′α-L-rhamnopyranosyl-8-(3-phenyl-trans-acryloyl)-1-benzopyran-2-one (**3**), 6′7-dihydroxy-5,8-dimethoxy-8(3-phenyl-trans-acryloyl)-1-benzopyran-2-one (**4**), 9-hydroxy-3,8-dimethoxy-4-prenylpterocarpan (**5**), and α,4,4′-trihydroxydihydrochalcone-2′-O-β-D-glucopyranoside (**6**) were isolated from bark of *Eysenhardtia polystachya*. Antidiabetic activity of compounds **1**–**5** in terms of their cellular antioxidant and free radical scavenging and also in streptozotocin- (STZ-) induced diabetic mice was evaluated on liver transaminases, lipid peroxidation, total bilirubin, total protein, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (CSH-Px), and glutathione reductase (GSH). Results indicated that **1**–**5** scavenged 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl ($^{\bullet}OH$), nitric oxide radicals ($NO^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), radical cation ($ABTS^{\bullet+}$), and hydrogen peroxide (H_2O_2) radical, and protection against H_2O_2 induced BSA damage was also observed. Furthermore, **1**–**5** showed ability to decrease the oxidative stress in H9c2 cell. Diabetic mice present high levels of lipid peroxide, total protein, SGPT, SGOT, ALP, and TB. However, treatment of STZ-induced diabetes in mice with **1**–**5** reduced levels of these enzymes leading to protector effect of liver. In addition, with treatment with **1**–**5**, increases in radical scavenging enzymes of CSH-Px, SOD, GSH, and CAT have also been observed in diabetic mice. The antioxidant properties of compounds **1**–**5** are a promising strategy for ameliorating therapeutic effects by avoiding disorders in the normal redox reactions in healthy cells which consequently could alleviate complications of diabetes.

1. Introduction

Oxidative stress is a consequence of the increased reactive oxygen species (ROS) production and/or a decrease in their elimination. ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (OH), nitrogen oxide (NO), and lipid peroxides are formed in aerobic metabolism as normal products but also are produced under pathophysiological conditions in elevated rates. ROS can be responsible for the attack to biological macromolecules such as nucleic acids, proteins, membrane lipids, and carbohydrates, causing damage in the cell, which has been implicated in cardiovascular diseases, cancer, neurodegenerative disorders, and diabetes [1]. Natural antioxidants present in the majority of plants help in preventing mutagenesis, aging, and carcinogenesis and reduce oxidative damage due to their radical scavenging effect [2].

The tree *Eysenhardtia polystachya* (Ortega) Sarg, belonging to the Leguminosae family, is known as "palo azul" and has widely been used as antirheumatic, for the treatment of

nephrolithiasis and bladder disorders developed in diabetes [3]. Phytochemical studies indicate that *E. polystachya* contains polyphenols [4]. In another study, isoflavones displayed moderate cytotoxic activity against KB cell lines [5]. In previous studies, methanol-water extract from the bark of *E. polystachya* was evaluated in *in vitro* assays, showing antioxidant potential, hypoglycemic, and AGEs inhibition capacity [6]. In this study, we investigated the antioxidant properties and protective activity of 5 flavonoids isolates from the bark of *E. polystachya* in *in vitro* and *in vivo* assays.

2. Materials and Methods

2.1. General Experimental Procedures. IR spectra were determined on a Perkin-Elmer 1720 FTIR. ^{1}H-^{1}H, ^{13}C, DEPT, ID, DQF-COSY, TOCSY, and HMBC experiments were recorded on a Bruker DRX-300 NMR spectrometer, operating at 599.19 MHz for ^{1}H and 150.86 MHz for ^{13}C, using the UXNMR software package; chemical shifts are expressed in δ (ppm) using TMS as an internal standard [7]. HREIMS were recorded on a JEOL HX 110 mass spectrometer (JEOL, Tokyo, Japan). TLC and column chromatography were carried out using precoated TLC silica gel 60 F254 aluminium sheets from Sigma-Aldrich (St. Louis, USA) and silica gel 60 (230–400 mesh, Merck Co., New Jersey, USA); solvents used as eluents were purchased from Fermont (California, USA). DCFH-DA were purchased from Degussa (Cincinnati, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, USA).

2.2. Plant Material. Bark of *E. polystachya* was collected in October 2014 near Tula, State of Mexico. A voucher specimen (number 49584) is deposited at the Herbarium of Universidad Autonoma Metropolitana-Xochimilco, Mexico.

2.3. Extraction and Isolation. The dried bark *E. polystachya* (5 kg) was milled and macerated with distilled water and methanol (1 : 1) for 15 days. The extract was evaporated under reduced pressure at 40°C, affording 400 g which was partitioned sequentially with n-hexane, $CHCl_3$, and MeOH. The methanol extract was adsorbed on 50 g of silica gel 60 (70−230-mesh ASTM), loaded onto a column of silica gel of 50 cm length and 7 cm diameter, 300 g silica gel, and then eluted with CH_2Cl_2/EtOAc 9 : 2 to yield 9 subfractions (PA-1 to PA-9). The elutions were examined by TLC and each one of them was determined as antioxidant activity. The fractions having antioxidant activity were PA-5 and PA-7. PA-5 was subjected to silica gel column chromatography eluted with EtOAc/hexane 7 : 2 to yield 7 subfractions (PA5-1 to PA5-7). Subfractions PA5-2 and PA5-7 were subjected to gel filtration over Sephadex LH-20 eluted with $CHCl_3$/MeOH (6 : 1), and this led to the isolation of compounds **7**–**13** which were combined and further purified by repeated preparative thin layer chromatography (PTLC) eluting with ethyl ether/acetone/methanol (1.5/3/0.5) gradient system. This led to the isolation of compounds **7** (65.0 mg), **8** (13.5 mg), **9** (76.4 mg), **10** (15.8 mg), **11** (49.2 mg), **12** (29.37 mg), and **13** (48.5 mg). PA-7 was subjected to silica gel column chromatography eluted with EtOAc/hexane 9 : 3 to yield 6 subfractions (PA7-1 to PA7-6). Fractions PA7-4 and PA7-5 were combined and further crude mixture was subjected to preparative thin layer chromatography (PTLC) eluting with ethyl ether/acetone/methanol (4/2/1.0) gradient system. Subfractions 1–3 were purified by gel filtration over Sephadex LH-20 eluted with CH_2Cl_2-CH_3OH 1 : 1 increasing concentration of MeOH yielding compounds **1** (67.1 mg), **2** (85.4 mg), **3** (72.8 mg), **4** (96.7 mg), **5** (90.2 mg), and **6** (42.9 mg).

2.4. Antioxidant Activity In Vitro

2.4.1. Scavenging Effects on DPPH Radicals. 1 mL solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH, 0.2 mmol/L) in methanol was added to 4 mL of sample. The mixture was shaken vigorously and was allowed to stand at room temperature for 30 min. Absorbance of the resulting solution was measured at 517 nm using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) [8].

2.5. Chelating Activity on Metal Ions. 40 μmol/L $FeCl_2$ was reacted with flavonoids for 5 min. Then ferrozine (200 μmol/L) was added and the mixture was left to stand for another 10 min. The absorbance at 562 nm was determined spectrophotometrically (UV-1800, Shimadzu, Kyoto, Japan) [8].

2.6. Trolox Equivalent Antioxidant Capacity (TEAC) Assay. 2 mmol/L H_2O_2 alone in 30 mmol/L acetate buffer with pH 3.6 was incubated for 30 min at room temperature producing 10 mmol/L of ABTS radical cation ($ABTS^{\bullet+}$). 80 μL of this solution was added to 0.4 mol/L acetate buffer (pH 5.8, 800 μL) and to 20 μL of the sample solutions; after exactly 5 min the absorbance was read at 734 nm. A dose-response curve was plotted for Trolox and antioxidant ability was expressed as TEAC value [9].

2.7. Scavenging Effects on Nitrite Oxide (NO). In the test tubes to 0.1 mL of sodium nitroprusside solution (25 mmol/L) was added 150 μg/mL of the sample; then the tubes were incubated for 150 min at room temperature. Griess reagent (0.3 mL of 1 g/L naphthylethyl-enediamine dihydrochloride and 0.3 mL of 10 g/L sulfanilamide in 5% H_3PO_4) was added to the incubation solution. The absorbance was immediately read at 570 nm and referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent [10].

2.8. Effects on the Oxidation of BSA Induced by H_2O_2. 0.2 mL of samples was incubated with 1 mL BSA (40 mg/mL), 0.4 mL H_2O_2 (20 mmol/L), and 0.4 mL of 20 mmol/L phosphate buffer (pH 7.4), for 2 h at 37°C. 5,5-Dithio-bis(2-nitrobenzoic acid) (DTNB, 1 mL, 2 mmol/L) was then added and the mixture was left to stand for another 30 min. The absorbance was measured at 410 nm. The free thiol concentration of samples was calculated based on the standard curve prepared by using various concentrations of L-cysteine.

2.9. Superoxide Radical Scavenging Activity ($O_2^{\bullet-}$). The reaction mixture is carried out in final volume of 3 mL containing 6 mM EDTA, 20 μM riboflavin, 58 mM phosphate buffer at pH 7.6, and 50 μM of nitroblue tetrazolium chloride (NBT).

The reaction mixture is exposed for 15 min to 40 V under fluorescence lamp to initiate the reaction. The absorbance was determined at 560 nm [11].

2.10. Hydroxyl Radical Scavenging Activity. The reaction mixture contained phosphate buffer (0.1 mM, pH 7.4), 2-deoxy-2-ribose (2.8 mM), ferric chloride (20 μM), hydrogen peroxide (500 μM), ascorbic acid (100 μM), and EDTA (100 μM) and test sample (10–1000 $\mu g/mL^{-1}$) in a final volume of 1 mL. The mixture was incubated at 37°C for 1 h. Then 0.8 mL of the mixture was added to 2.8% of trichloroacetic acid (TCA) solution (1.5 mL), followed by sodium dodecyl sulphate (0.2 mL) and a thiobarbituric acid (TBA) solution (1 mL of 50 mM at 1% in sodium hydroxide). The mixture was heated (90°C for 20 min) to develop the colour. After cooling, the absorbance was determined at 532 nm [12].

2.11. Hydrogen Peroxide Radical Scavenging Activity. Compounds at concentrations of 1 mg/mL were dissolved in 3.4 mL of a 0.1 M phosphate buffer (pH 7.4) solution and then were mixed with 600 μL of a 43 mM solution of H_2O_2 prepared in the same buffer and their concentration was measured by reading absorbance values at 230 nm of the reaction mixtures [13].

2.12. Cellular Oxidative Stress Inhibition. The intracellular accumulation of ROS in the H9c2 rat cardiomyoblast cell line was determined with carboxy-2′,7′-dichloro-dihydrofluorescein diacetate (DCFHDA) method [14]. Cells H9c2 rat heart-derived embryonic myocytes are obtained from American Type Culture Collection, Manassas, VA, USA (CRL-1446), which was cultured with DMEM/F12 supplemented with 100 U/mL penicillin G, 10% (v/v) foetal bovine serum, 2 mM/L-glutamine, and 100 mg/mL streptomycin. Cells were incubated using 5% CO_2 and 95% air at 37°C, after the cells were plated in 35-mm culture dishes at 5.0×10^{-4} cells/cm^2. Then, the medium is changed with a new one. Cells seeded on 96-well plates were incubated with DCFHDA probe for 40 min. At the end of this period, medium was removed and cells were exposed to the flavonoids under investigation at a concentration of 10, 50, and 100 μg/mL. After incubating exposed cells at 37°C for 24 h, fluorescence was measured at 488 nm (excitation) and 535 nm (emission) wavelengths on a microplate reader (Molecular Devices Spectra MAX Gemini X).

2.13. Evaluation of Oxidative Stress Markers In Vivo

2.13.1. Animals Care Conditions. The study was conducted in male CD1 mice, weighing about 25–30 g. Before and during the experiment, animals were fed a standard laboratory diet (Mouse Chow 5015, Purina) with free access to water. Mice were procured from the bioterium of ENCB and were housed in microloan boxes in a controlled environment (temperature 25 ± 2°C). Animals were acclimatized for a period of three days in their new environment before the initiation of experiment. Litter in cages was renewed three times a week to ensure hygiene and maximum comfort for animals. The experiments reported in this study were following the guidelines stated in Principles of Laboratory Animal Care (NIH publication 85-23, revised 1985) and the Mexican Official Normativity (NOM-062-Z00-1999). All animals procedures were performed in accordance with the recommendations for the care and use of laboratory animals (756/lab/ENCB).

2.14. Induction of Mild Diabetes (MD). Mild diabetes type 2 was induced in overnight fasted mice by a single intraperitoneal injection of 45 mg/kg streptozotocin (STZ) dissolved in 0.1 mol/L cold citrate buffer (pH 4.5), 15 min after the intraperitoneal administration of 120 mg/kg nicotinamide. The STZ treated animals were allowed to drink 5% glucose solution overnight to overcome drug induced hypoglycemia. After 7 days of development of diabetes, mice with moderate diabetes having persistent hyperglycaemia with more than 200 mg/dL were used for further experimentation [15].

2.15. Experimental Design. Eight groups ($n = 10$) of diabetic mice were used to determine the chronic effect of **1**–**5**. Each group was submitted to a specific treatment, as follows. Normal control and mild diabetic rats groups were fed with normal diet and drinking water *ad libitum* and were given saline by gastric gavage Mice with mild diabetes daily treated with the compounds isolated by an oral gavage at doses of 20 mg/kg of weight. The flavonoids were dissolved in distilled water and administered orally as a daily dose for four weeks. Mice were fasted overnight and sacrificed by cervical dislocation. Blood samples were collected in EDTA coated tubes. Livers, kidney, and pancreas were removed, washed with cold saline, and stored at −80°C.

2.16. Antioxidant Parameters Levels in Serum, Liver, Pancreas, and Kidney. Activities of superoxide dismutase (SOD), serum catalase (CAT), glutathione peroxidase (CSH-Px), and glutathione reductase (GSH) were measured by assay kits purchased from Cayman Chemical (Michigan, USA), and the procedures were according to the kits instructions. The protein concentration was determined by the Bradford method [16] as described in the Bio-Rad protein assay kit. Lipid peroxidation (LPO) is used as an indicator of the oxidative stress in tissues and was estimated by the method of Fraga et al. [17], expressed as μM/g of liver and kidney tissue. Malondialdehyde (MDA) as thiobarbituric acid reactive substance was measured at 532 nm spectrophotometrically [18]. Serum glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (SALP), total bilirubin (TB), and total protein were determined by using a commercial Diagnostic Kit Biocompare, BioVision, Biocompare, and Thermo Scientific, respectively.

3. Results and Discussion

3.1. Characterization of the Isolated Compounds. The MeOH extract was subjected to multiple chromatographic purifications to afford dihydrochalcones **1**–**13** (Figures 1 and 3). The spectral data of the six new compounds isolated (**1**–**6**) are presented as follows.

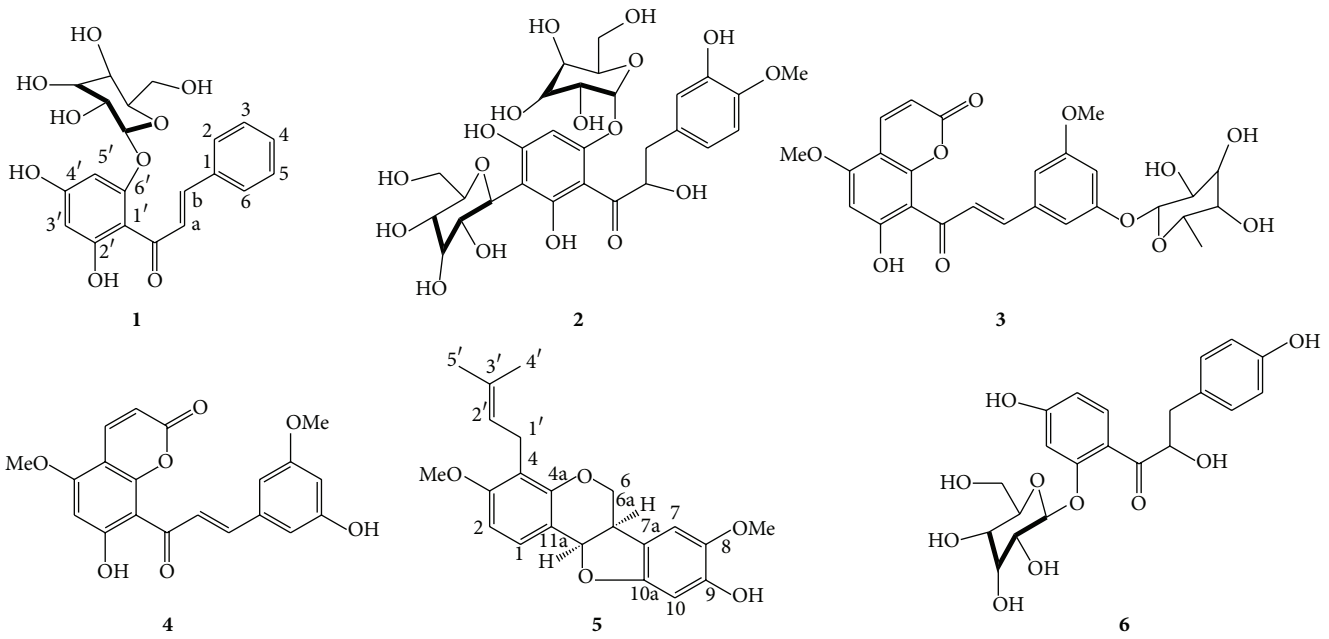

Figure 1: New compounds isolated from *Eysenhardtia polystachya*.

2′,4′-Dihydroxychalcone-6′-O-*β*-D-glucopyranoside (**1**) was isolated as dark yellow amorphous powder, mp 138–140°C; UV λ_{max} 362, 301, and 238 nm; IR (KBr) cm^{-1}: 3341, 2957, 2761, 1611, 1597, 1506, and 1463; and HRESIMS *m/z* 418.1270 calc. For $C_{21}H_{22}O_9$, 418.1264, and ^1H and ^{13}C NMR data see Table 1.

Compound **1** was isolated from ethanol-water 1 : 1 extract of bark of *Eysenhardtia polystachya* after purification through silica gel column chromatography. Compound **1** showed a molecular ion at *m/z* 418.1270 in its HREIMS and the presence of 21 carbons on the ^{13}C NMR spectrum suggested a molecular formula $C_{21}H_{22}O_9$. DEPT experiments classified the protonated carbon signals into one methylene, fourteen methines, and six quaternary carbons. The IR spectrum of **1** showed absorption bands at ν_{max} 3341, 2957, 2761, 1597, 1506, 1463, and 1611 cm^{-1} showing the presence of hydroxy, aromatic, and carbonyl groups. The ^1H NMR spectrum showed the presence of unsubstituted ring B (δ_H 7.12, 2H, m, H-2, 6, 7.46, 3H, m, H-3, 4, 5), and it showed a pair of doublets of δ_H 8.24 (H-*β*) and δ_H 7.50 (H-*α*) with the vicinal coupling constant ^3J = 15.8 Hz due to *trans*-*α*,*β*-unsaturated ketone protons. Its ketone carbonyl was confirmed with the signal at δ_C 202.69, C-*α* (δ_C 141.31), and C-*β* (δ_C 130.60) in the ^{13}C NMR being indicative of chalcone skeleton. NMR spectrum showed signals for one chelated hydroxyl group δ_H 13.2 (1H, s, OH-2′) and one singlet at δ_H 6.81 assigned to OH-4′. In addition ^1H NMR spectrum showed the presence of two aromatic protons in ring A (Table 1). These were placed at C-3′ and C-5′ based on correlation of NOESY and HMBC spectrum (Figure 2). In the HMBC spectrum the long range correlation of H-3′ with C-1′ (δ_C 103.79) and C-4′ (δ_C 148.36), H-5′ with C-1′ (δ_C 103.79), C-3′ (δ_C 114.82), C-4′ (δ_C 148.36), and C-6′ (δ_C 161.21), and H-1″ with C-6′ (δ_C 130.60), C-2″ (δ_C 78.36), and C-5″ (δ_C 71.17) was observed. Thus, the structure of compound **1** was established as 2′,4′-dihydroxychalcone-6′-O-*β*-D-glucopyranoside (Figure 1).

α,3,2′,4′-Tetrahydroxy-4-methoxy-dihydrochalcone-3′-C-*β*-glucopyranosy-6′-O-*β*-D-glucopyranoside (**2**) was isolated as dark yellow amorphous powder, mp 150–152°C; UV λ_{max} 235, 264, 302, and 312 nm; IR (KBr) cm^{-1}: 3339, 1621, 1598, 1510, and 1460; and HRESIMS *m/z* 644.1936 calc. For $C_{28}H_{36}O_{17}$, 644.1952, and ^1H and ^{13}C NMR data see Table 1.

Compound **2** was also isolated as dark yellow amorphous powder. The aliphatic hydroxyl group of compound **3** has *α* position similarly to **2**. The singlet at δ_H 3.85 was assigned to an aromatic methoxyl group which showed long range correlation with C-4 (δ_C 145.05) in the HMBC spectrum indicated to be linked at position C-4, whereas the doublets at δ_H 4.85 and δ_H 5.01 were attributed to two *β*-glucopyranose moieties on the basis of the coupling constant (7.5 and 7.3 Hz). In the HMBC spectrum of **2**, the long range correlations of anomeric proton H-1″ (δ_H 4.85) with C-2′ (δ_c 164.13), C-3′ (δ_c 107.88), C-4′ (δ_c 165.34), C-2″ (δ_c 74.73), C-3″ (δ_c 78.85), and C-5″ (δ_c 77.32) were observed. These signals suggested that glucose unit should be directly linked at position C-3′. Also the anomeric proton H-1‴ (δ_H 5.01) correlation with C-6′ (δ_c 164.04), C-2‴ (δ_{δ_C} 78.65), and C-5‴ (δ_c 78.01) indicated that the other glucose unit should be linked to C-6′ through a glycosidic bond (Table 1). ^1H NMR spectrum showed the typical pattern of a 2′,3′,4′,6′-tetrasubstituted and 3,4-disubstituted chalcone with a singlet for one proton at δ_H 6.68 could be assigned to the C-5′ indicating that this position is free. Analysis of the HMBC and COSY spectra of **3** allowed for the complete assignment of all protons and carbons (Figure 2). Consequently, the structure of compound **3** was elucidated as *α*,3,2′,4′-tetrahydroxy-4-methoxy-dihydrochalcone-3′-C-*β*-glucopyranosy-6′-O-*β*-D-glucopyranoside (Figure 1).

Table 1: ^{1}H NMR and ^{13}C NMR spectral data for compounds **1–4** (δ in ppm, J in Hz).

	1		**2**		**3**		**4**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
α	7.50 (1H, d, 15.8)	141.31	4.70, d, (1H, 7.5)	78.65	4.72, d, (1H, 7.5)	78.85	—	—
β	8.24 (1H, d, 15.8)	130.60	2.99, dd, (1H, 16.7, 3.0)	41.00	3.02, dd, (1H, 16.7, 3.0)	41.00	—	—
β	—	—	2.81, dd, (1H, 16.7, 13.2)	—	2.82, dd, (1H, 16.7, 13.2)	—	—	—
C=O	—	202.69	—	204.20	—	204.20	—	—
1	—	142.55	—	128.49	—	128.82	—	—
2	7.12 (2H, m, H-2, 6)	128.43	7.22 (1H, dt, 8.5, 2.0)	131.38	7.26 (1H, d, 2.0)	114.81	—	198.21
3		126.57	6.71 (1H, dt, 8.5, 2.0)	116.27	—	144.61	7.97, d (1H, 10.1)	143.48
4	7.46 (3H, m, H-3, 4, 5)	126.46	—	164.03	—	145.05	7.70, d (1H, 10.1)	126.71
4a	—	—	—	—	—	—	—	105.97
5		128.57	6.51 (1H, dd, 8.5, 1.9)	114.86	7.0 (1H, d, 8.0)	114.59	—	143.48
6		130.60	7.26 (1H, dd, 8.5, 2.0)	130.16	7.67 (1H, dd, 8.0, 2.0)	146.64	6.47, s	106.82
7							—	163.17
8								105.07
8a								159.82
1′	—	103.79	—	120.52	—	104.76	—	204.21
2′	6.92 (1H, s, OH-2′)	157.57	—	164.03	—	164.13	7.32, d (1H, 15.4)	142.53
3′	6.52 (1H, d, J = 2.4)	114.82	6.68, s	111.30	—	107.88	7.47, d (1H, 15.4)	132.26
4′	6.81 (1H, s, OH-4′)	148.36	—	144.59	—	165.34	—	132.68
5′	6.42 (1H, dd, 8.6, 2.4)	107.52	6.44 (1H, d, 8.9)	108.67	6.68, s	162.45	6.27, 1H, m	107.14
6′	—	161.21	7.71 (1H, d, 8.9)	131.78	—	164.04	—	163.56
7′							6.32 (1H, dd, 1.7, 1.7)	106.29
8′							—	166.12
9′							6.40, 1H, m	101.23
1″	Glc-6: 5.02 (1H, d, 7.4)	106.19	Glc-2: 5.02 (1H, d, 7.3)	106.12	Glc-3: 4.85 (1H, d, 7.5)	106.07	Rham-1: 5.05, brs	100.8
2″	3.51 (1H, m, H-2″, 3″)	78.36	3.53 (1H, m, H-2″, 3″)	78.84	3.79 (1H, t, 11.0)	74.73	3.89, m	71.50
3″		75.59		74.02	3.55 (1H, m)	78.85	3.12, m	71.62
4″	3.43 (1H, m, H-4″, 5″)	71.82	3.44 (1H, m, H-4″, 5″)	71.81	3.5 (1H, m)	70.27	3.12, m	72.76
5″		71.17		71.23	3.43 (1H, m)	77.32	3.89, m	68.32
6″	3.88 (2H, dd, 2.0, 12 H-6″)	66.11	3.78 (2H, dd, 2.0, 12 H-6″)	61.33	4.30 (2H, dd, 2.0, 12 H-6″)	61.33	1.15 (3H, d, 7.0)	17.98
1‴					Glc-6: 5.01 (1H, d, 7.3)	104.75		
2‴					3.52 (1H, m, H-2‴, 3‴)	78.65		

Table 1: Continued.

	1		2		3		4	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
3‴						74.03		
4‴					3.44 (1H, m, H-4‴, 5‴)	71.25		
5‴						78.01		
6‴					3.88 (2H, dd, 2.0, 12 H-6‴)	62.16		
OMe					3.85, s	56.78	3.39, s, 3.46, s	60.69, 55.79
OH-4	9.10, s							
OH-2′	13.2, s							
OH-4′	6.81, s							
OH-7′							13.59, s	
OH-7							14.15, s	

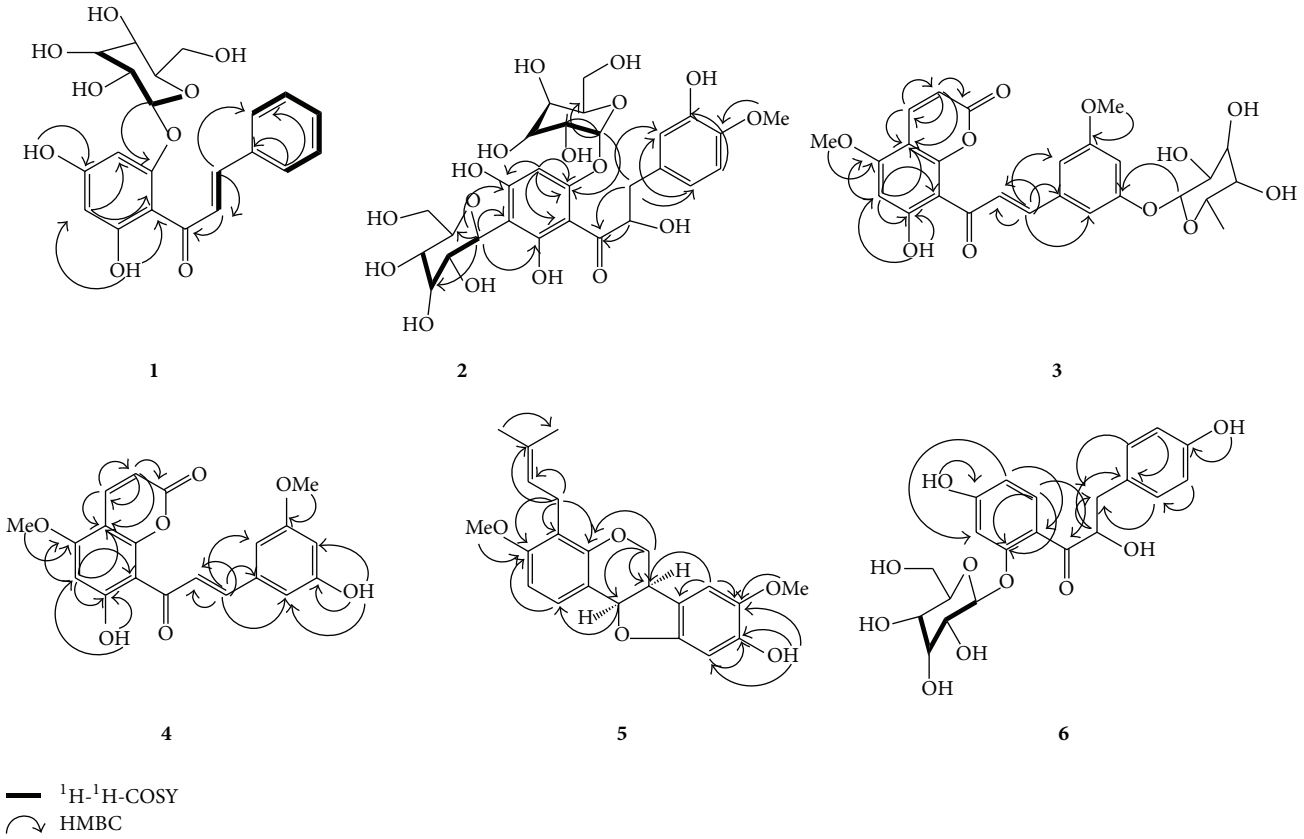

Figure 2: Correlation of ^{1}H-^{1}H-COSY and HMBC compounds **1**–**6**.

7-Hydroxy-5,8′-dimethoxy-6′α-L-rhamnopyranosyl-8-(3-phenyl-trans-acryloyl)-1-benzopyran-2-one (**3**) was isolated as dark yellow amorphous powder, mp 142–144°C; UV λ_{max} 249 and 381 nm; IR (KBr) cm^{-1}: 3439, 1659, 1619, 1521, and 1443; HRESIMS *m/z* 514.1483 calc. For $C_{26}H_{26}O_{11}$, 514.1475, and ^{1}H and ^{13}C NMR data see Table 1.

Compound **3** was isolated as dark yellow amorphous powder. Its molecular formula was assigned as $C_{26}H_{26}O_{11}$ on the basis of HRESIMS and ^{13}C NMR data. The IR spectrum showed characteristic signals for OH (3439 cm^{-1}), aromatic ring (1521 and 1443 cm^{-1}), and α,β-unsaturated ketone (1659 and 1619 cm^{-1}). The UV spectrum at 249 and 381 nm confirmed the existence of unsaturated functional groups. The ^{1}H NMR spectrum showed one single proton at δ_H 14.15 (1H, s, 7-OH) and two methoxyl groups at δ_H 3.39 (3H, s) and δ_H 3.46 (3H, s), while the resonances of two trans-coupled olefinic protons were recorded at δ_H 7.32 (1H, d, J = 15.4 Hz, H-2′) and 7.32 (1H, d, J = 15.4 Hz, H-3′)

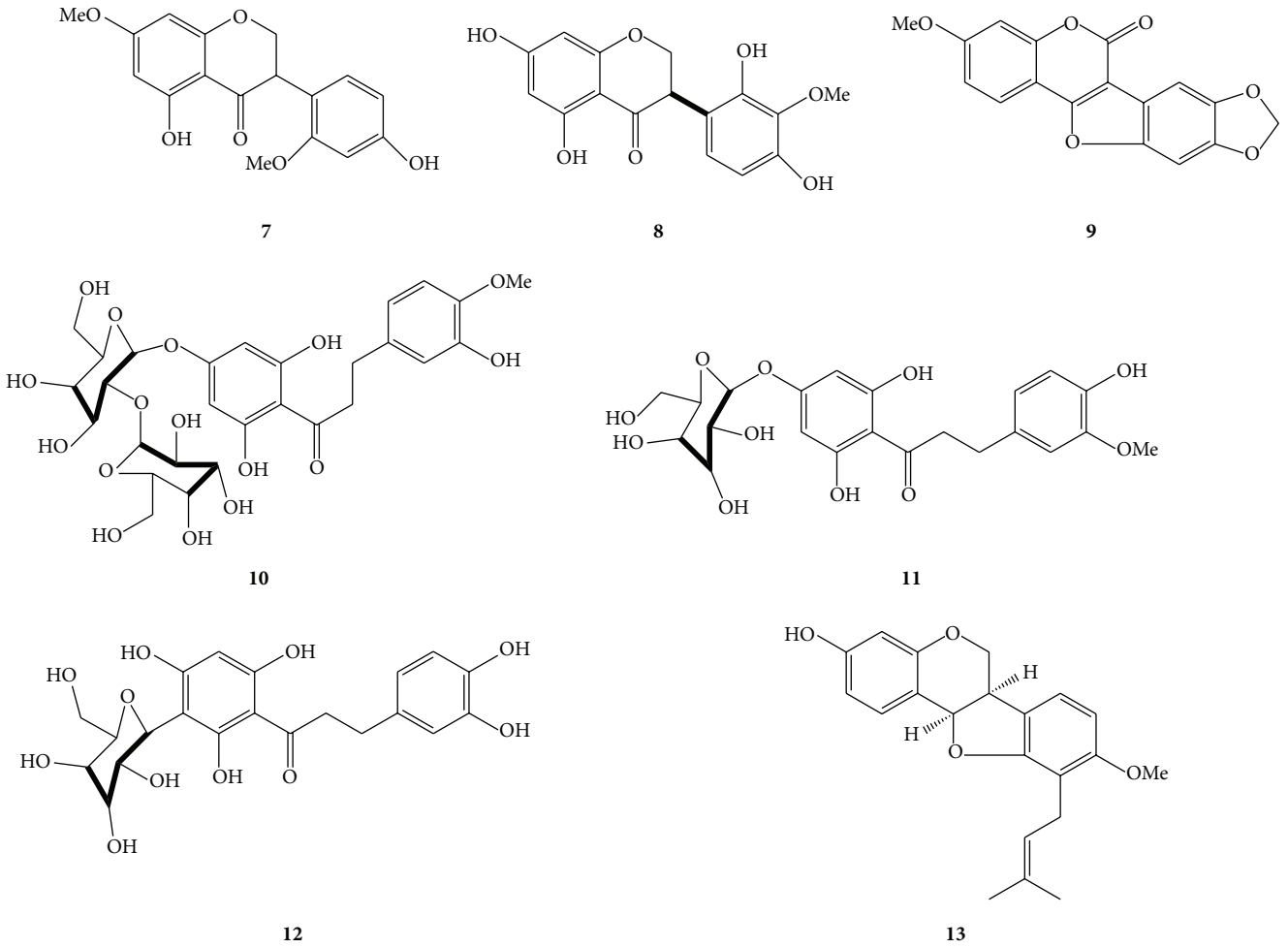

Figure 3: Known compounds isolated from *Eysenhardtia polystachya*.

as well as two olefinic protons δ_H 6.58 (1H, d, J = 10.1 Hz, H-3) and δ_H 7.50 (1H, d, J = 10.1 Hz, H-4). In addition ^{1}H NMR spectrum showed aromatic signals of 4H at 6.32–6.47 and also exhibited signals due to α-L-rhamnopyranosyl at δ_H 5.05 (1H, brs, H-1$''$) and δ_H 1.15 (3H, d, 7.0 Hz, H-6$''$). ^{13}C NMR and DEPT spectra (Table 1) indicated signals corresponding to 26 carbons which were classified into three CH_3 groups, thirteen groups, and ten quaternary carbons. The attachment of the rhamnopyranosyl moiety was deduced to be at C-6$'$, by the HMBC spectrum in which the anomeric proton of the rhamnopyranosyl moiety at δ_H 5.05 (1H, brs, H-1$''$) showed long range correlation with C-6$'$ at δ_C 163.56. ^{13}C NMR data, with the corresponding literature, suggested that **3** exhibited the skeletons of a neoflavone and a chalcone [19, 20]. The HBMC correlation of H-3 at δ_H 6.58 with C-2 (δ_C 168.21) and C-4a (δ_C 105.97) and H-4 with C-3 (δ_C 143.48) revealed the existence of the neoflavone skeleton. The HMBC relationship of the double bond group (δ_H 7.97 and δ_H 7.70) indicated a fragment of O@CAC@CAC, showing the skeleton of a chalcone. Compound **3** possesses a methoxyl group at C-5 suggested by a ^{13}C-^{1}H long range coupling from OCH_3-5 (δ_H 3.39) to C-5 (δ_C 143.48) and another methoxyl group in C-8$'$ indicated by correlations observed in OCH_3-8$'$ (δ_H 3.46) to C-8$'$ (δ_C 166.12) in the HMBC spectrum (Figure 2). This was confirmed by NOE correlation between OMe-5 to H-6 and OMe-8$'$ with H-9$'$. On the basis of these data the structure is proposed as 7-hydroxy-5,8$'$-dimethoxy-6$'\alpha$-L-rhamnopyranosyl-8-(3-phenyl-trans-acryloyl)-1-benzopyran-2-one (Figure 1).

6$'$7-Dihydroxy-5,8-dimethoxy-8(3-phenyl-trans-acryloyl)-1-benzopyran-2-one (**4**) was isolated as yellow amorphous powder, mp 156–158°C; UV λ_{max} 228 and 349 nm; and HRESIMS m/z 396.0863 (calcd $C_{20}H_{16}O_7$). For ^{1}H and ^{13}C NMR data, see Table 2.

The NMR spectra of compound **4** showed similarities to those of **3** indicating a close structure. The C-6$'$ rhamnopyranosyl moiety of **3** is replaced with a hydroxyl group at δ_C 168.38 as revealed by the spectra of HMBC and COSY showing the corresponding correlations (Figure 2). On the basis of these data the structure is proposed as 6$'$7-dihydroxy-5,8-dimethoxy-8(3-phenyl-trans-acryloyl)-1-benzopyran-2-one (Figure 1).

9-Hydroxy-3,8-dimethoxy-4-prenylpterocarpan (**5**) was isolated as dark yellow amorphous powder, mp 142–134°C;

Table 2: ^{1}H NMR and ^{13}C NMR spectral data for compounds **5-6** (δ in ppm, J in Hz).

	5		**6**	
	δ_H	δ_C	δ_H	δ_C
1	—		7.15, d (1H, 8.5)	128.72
2	—	198.16	6.52, d (1H, 8.5)	110.83
3	7.96, d (1H, 10.1)	143.48	—	147.76
4	7.71, d (1H, 10.1)	126.69	—	115.84
4a	—	105.95	—	154.59
5		143.41	—	—
6α	6.41, s	105.82	3.59, t (1H, 10.2)	69.37
6β	—	—	4.31, dd (1H, 4.9, 10.2)	69.55
6a	—	—	3.50, (1H, m, 10.2, 7.0, 5.0)	40.48
6b	—	—	—	116.56
7		168.38	6.73, s	108.07
8		105.08	—	152.51
8a		159.81	—	—
9			—	163.17
10			6.49, s	99.01
10a			—	154.59
11a			5.20, d (1H, 7.0)	77.75
11b			—	112.23
1′		204.19	3.43, d (2H, 8)	24.61
2′	7.31, d (1H, 15.4)	142.54	5.29, d (1H, 8)	121.62
3′	7.47, d (1H, 15.4)	132.26	—	137.49
4′	—	132.68	1.81, 3H, s	18.75
5′	6.26, 1H, m	108.31	1.70, 3H, s	24.61
6′	—	166.38	—	—
7′	6.32 (1H, dd, 1.7, 1.7)	105.79		
8′	—	166.12		
9′	6.40, 1H, m	101.24		
OMe-3	—	—	3.11, s	55.57
OMe-8	—	—	3.35, s	60.64
OH-9	—	—	8.12, s	—

UV λ_{max} 212, 292, and 344 nm; IR (KBr) cm^{-1}: 3389, 1619, 1547, 1483, and 1429; and HRESIMS *m/z* 368.1641 calc. For $C_{22}H_{24}O_5$, 368.1624, and ^{1}H and ^{13}C NMR data see Table 2.

Compound **5** was isolated as yellow amorphous powder. Its molecular formula was assigned as $C_{22}H_{24}O_5$ on the basis of HRESIMS and ^{13}C NMR data. In IR spectrum were observed absorption bands for hydroxyl groups at 3389 cm^{-1} and aromatic groups at 1619, 1547, 1483, and 1429 cm^{-1}. Its molecular formula was established by HRESIMS and ^{13}C NMR data as $C_{22}H_{24}O_5$. ^{13}C NMR and DEPT spectrum indicated that it contains 22 carbons, including four methyls, two methylenes, seven methines, and nine quaternary carbons. A pterocarpan structure was indicated to ^{1}H MR spectrum due to the splitting pattern of the protons at δ_H 4.31 (dd, J = 4.9, 10.2 Hz, H-6β), δ_H 3.59, (t, J = 10.2, H-6α), δ_H 3.50 (m, H-6a), and δ_H 5.20 (d, J = 7.2, 11a) related to the protons of the heterocyclic ring B. The HMQC spectrum suggested the presence of two OCH_3 groups at δ_H 3.11 (3H, s, OMe-3) and δ_H 3.35 (3H, s, OMe-8), confirming its presence in the ^{13}C NMR with signals to δ_C 55.57 (OMe-3) and δ_C 60.64 (OMe-8). HMBC spectrum shows the relations between 3.11 (3H, s, OMe-3) and δ_C 147.76 (s, C-3) and between 3.77 (3H, s, OMe-8) and δ_C 152.51 (s, C-8) show two -OMe that joined with C-3 and C-8 and the relations between OH δ_H 8.12 (br, s, HO-) and δ_C 99.01 (C-10) and between δ_C 163.17 (s, C-9) and δ_C 152.51 (s, C-8) indicate that hydroxyl group is at C-9. The proton doublets at δ_H 7.15 and δ_H 6.52 suggested *ortho*-coupled position. The correlation of H-1′ (δ_H 3.43) with C-4 (δ_C 115.84), C-4a (δ_C 154.59), and C-3 (δ_C 147.76) in HMBC spectrum indicated that prenyl group was located at C-4 of A ring. The HMBC spectra (Figure 2) show correlation of H-11a (δ_H 5.20) with C-1 (δ_C 77.75), C-6a (δ_C 40.48), and C-11b (δ_C 77.75) and H-6a (δ_H 3.50) with C-6a (δ_C 69.37), C-11a (δ_C 77.75), and C-6b (δ_C 116.56) (Figure 2). NOESY correlation of the 11a proton (δ_H 5.20) with H-1 (δ_H 7.15) suggested that H-11a is equatorially oriented *cis*-ring fusion between rings B and C. The cisconfiguration of 6a and 11a was confirmed based on the evidence from the chemical shift and J value (7.0 Hz) compared with values reported in the literature for

Table 3: Scavenging effects of **1–5** on different *in vitro* assays.

Assay	Scavenging effects (IC_{50} μg/mL)					
	1	**2**	**3**	**4**	**5**	Reference compounds
DPPH radical	39.39 ± 2.57	83.43 ± 5.11	12.49 ± 3.98	10.21 ± 4.23	15.45 ± 7.51	Ascorbic acid 54.14 ± 2.87 Gallic acid 3.76 ± 0.82
NO radical	48.69 ± 6.27	85.04 ± 4.83	30.29 ± 3.48	25.47 ± 4.70	31.52 ± 2.93	Ascorbic acid 31.27 ± 5.21 Quercetin 17.30 ± 1.98
$O_2^{\bullet-}$	8.87 ± 1.05	18.86 ± 2.19	6.03 ± 0.39	5.76 ± 0.73	7.21 ± 3.22	Ascorbic acid 5.11 ± 0.48
Hydroxyl radical	44.13 ± 4.28	62.34 ± 3.78	37.45 ± 2.58	33.17 ± 6.83	36.52 ± 5.40	Ascorbic acid 30.29 ± 6.17
Hydrogen peroxide	91.81 ± 4.57	143.13 ± 7.55	80.73 ± 6.76	87.68 ± 9.43	101.03 ± 10.88	Ascorbic acid 443.76 ± 7.66
	Scavenging effects (%)					
Chelating activity 50 μg/mL	76	43	89	84	85	Ascorbic acid 91
Peroxidationof BSA						Ascorbic acid 94
100 μg/mL	30	25	36	38	32	
200 μg/mL	55	48	60	64	59	
300 μg/mL	83	65	90	97	92	
	TEAC (nM)					
TEAC: ABTS	28.11 ± 2.48	49.51 ± 4.15	19.38 ± 3.29	16.23 ± 2.15	20.34 ± 2.17	Curcumin 12.04 ± 4.19

The data represent the means ± SD of three determinations. IC_{50}: concentration required to inhibit 50% of the activity; DPPH: 2,2-diphenyl-1-picrylhydrazyl; $O_2^{\bullet-}$: superoxide radical; TEAC: ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging capacity in Trolox equivalence (nanomoles); BSA: bovine serum albumin; Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

cis (6.6 Hz) and trans (13.4) [21]. Key correlations in **5** are shown in Figure 2. Therefore, the structure of compound **6** is 9-hydroxy-3,8-dimethoxy-4-prenylpterocarpan (Figure 1).

α,4,4′-Trihydroxydihydrochalcone-2′-O-β-D-glucopyranoside (**6**) was isolated as dark yellow amorphous powder, mp 146-147°C; UV λ_{max} 263, 293, and 328 nm; IR (KBr) cm^{-1}: 3341, 2957, 2761, 1611, 1597, 1506, and 1463; and HRESIMS *m/z* 418.1270 calc. For $C_{21}H_{22}O_9$, 418.1264, and ^1H and ^{13}C NMR data see Table 1.

Compound **6** was isolated as dark yellow amorphous powder. The positive HREIMS analysis showed an ion peak at *m/z* 436.1374 which corresponded to the molecular formula $C_{21}H_{24}O_{10}$. The IR showed absorption of hydroxyl (3341 cm^{-1}) and aromatic rings (1647 and 1489 cm^{-1}) functions. Aliphatic proton was assigned to ABX system at δ_H 4.70 (1H, d, 7.5 Hz, H-α), δ_H 2.99 (1H, 16.7, 3.0 Hz, H-β), and δ_H 2.81 (1H, 16.7, 3.2 Hz, H-β) suggesting the presence of -CO-CH (OH)-CH2– moiety. Compound **6** showed the presence of a glucopyranosyl moiety which was linked to C-3′ according to the HSQC correlation of anomeric proton at δ_H 5.02 (br, d, J = 7.3 Hz, H-1″) with C-3′ (δ_C 164.03). The anomeric configuration was assigned as β for the glucopyranosyl group from their coupling constants. The downfield shift of C-4′ at δ_C 144.59 was indicative that it has a hydroxy group. Aromatic protons H-3′ (δ_H 6.68), H-5′ (δ_H 6.44), and H-6′ (δ_H 7.71) suggested a 2′,4′-dioxygenation in a trisubstituted ring, which is corroborated by the NOESY and the HMBC spectrum (Figure 2). The AA′XX′ spin system (δ_H 6.71, 7.22) of A ring indicates 1,4-disubstitution, whereas the chemical shift of C-4 (δ_C 164.03) reveals hydroxyl group at this position. Therefore, the structure of compound **6** was identified as α,4,4′-trihydroxydihydrochalcone-2′-O-β-D-glucopyranoside (Figure 1).

In addition, seven known compounds were also isolated (Figure 3) by comparing the spectroscopic data with literature and were identified as 5,4′-dihydroxy-7,2′-dimethoxyl-isoflavone (**7**) [22], (3R)-5,7-2′,4′-tetrahydroxyl-3′-methoxyl-isoflavanone (**8**) [23], flemichapparin C (**9**) [24], neohesperidin dihydrochalcone (**10**) [25], hesperetin dihydrochalcone glucoside (**11**) [26], aspalathin (**12**) [27], and *sandwicensis* (**13**) [28].

3.2. Antioxidant Activity. Radical scavenging capacities of compounds **1–5** isolated from *E. polystachya* are given in Table 3. However, compound **6** displayed lower scavenging value and the data are not presented here. Chalcones **4**, **3**, **5,** and **1** showed significant scavenging activity in DPPH radical with IC_{50} values of 10.21 to 19.39 μg/mL. These results suggested that these compounds showed the highest hydrogen-donating capacity towards the DPPH radical.

Hydroxyl radicals are found in living systems that react with almost all biomolecules as they are high oxidant. The orders of reactivity of the flavonoids were similar to that of DPPH. Compound **4** (33.17 μg/mL) was found to exhibit the highest hydroxyl radical scavenging effect followed by **5** (36.52 μg/mL) and **3** (37.45 μg/mL). Scavenging of hydrogen peroxide by the isolated compound was found to be better compared to the ascorbic acid (IC_{50} 443.76 μg/mL). Among the tested flavonoids, the best scavenging of hydrogen peroxide activity was presented by **3** (IC_{50} 80.73 μg/mL), **4** (IC_{50} 87.68 μg/mL), and **1** (IC_{50} 91.13 μg/mL). The presence in the –OH group in ring A and -OCH3 in ring B showed good antioxidant activity on scavenging of hydrogen peroxide activity compared to electron-withdrawing substituents at paraposition.

Superoxide radical is a free radical harmful to cellular components, precursor for many reactive oxygen species. Superoxide radical scavenging capacity followed an activity order of chalcones **4**, **3,** and **5**.

As evident from Table 3, the isolated compound exerted nitric oxide scavenging activities with values of IC_{50} of 25.47 to 75.04 μg/mL. Compound **4** showed the highest scavenging effects on nitric oxide, followed by **3**, **5,** and **1**. Reactive nitrogen species (N_2O_4, NO_3, N_3O_4, NO, and NO_2) are formed during the reaction with superoxides or oxygen which are responsible for altering the functional and structural cellular components.

The ability to chelate and deactivate transition metals is an important mechanism of antioxidant activity, which catalyzes Fenton-type reactions and hydroperoxide decomposition. Therefore, it was considered of importance to study chelating ability of the isolated compound with the metal. The chelating activity of flavonoids to suppress the formation of ferrozine-Fe^{2+} complex decreased in the order of **4** > **5** > **3** > **1** > **2**.

Chalcones **4** and **3** and pterocarpan **5** also showed good significant scavenging activities of Trolox (16.23 to 20.34 nM) indicating their good antioxidant effect.

The inhibition of the isolated compound on the oxidation of BSA induced by H_2O_2 was also determined. Exposure of H_2O_2 to BSA produced a decrease of free thiol groups level. Results are shown in Table 3; the isolated compound significantly inhibited the oxidation of BSA induced by H_2O_2 in a dose-dependent manner. Among the five compounds tested, **4** showed the strongest antioxidant activity at a concentration of 300 mg/mL preventing the 97% of the depletion of protein thiol groups. These results indicated that hydrophilic antioxidant moiety in the compounds has the ability to scavenge H_2O_2 reducing thiol groups oxidation in BSA.

The antioxidant activity in all tested assays indicates that the compound with chalcone skeleton showed the best inhibitory activity when the dihydrochalcone as in compounds **4**, **3,** and **1** indicated that chalcones having electron donor group in the phenyl ring A in orthoposition to carbonyl moiety showed better antioxidant activity. Moreover, the activity decreasing obviously with glycosidation such as only **1** showed a moderate inhibitory activity, and the other glycoside **2** showed a low inhibitory effect. However, pterocarpan showed a good antioxidant activity in various oxidation systems.

Table 4: Effect of **1–5** on cellular oxidation stress reduction activity.

Group	DCF fluorescence (%)
Blank	0.7 ± 0.004
Control	89.2 ± 3.32
1	
10 μg/mL	79.4 ± 5.32
50 μg/mL	64.3 ± 1.83
100 μg/mL	44.6 ± 4.39
2	
10 μg/mL	86.5 ± 6.17
50 μg/mL	68.4 ± 5.38
100 μg/mL	55.2 ± 7.35
3	
10 μg/mL	72.3 ± 7.09
50 μg/mL	60.8 ± 4.21
100 μg/mL	39.6 ± 3.19
4	
10 μg/mL	69.8 ± 3.26
50 μg/ml	52.1 ± 2.99
100 μg/mL	35.1 ± 2.67
5	
10 μg/mL	73.4 ± 5.16
50 μg/mL	58.5 ± 6.43
100 μg/mL	38.3 ± 4.29
Ascorbic acid 25 μg/mL	19.1 ± 1.46

Fluorescence (488 nm (excitation) and 535 nm (emission)) was finally quantified after 24 h incubation. Data is shown as mean ± SD of the experiments (3 replicates each).

Antioxidants compounds have been shown to delay, inhibit, and prevent the oxidation, possibly through the mechanisms interacting with biological systems as scavenging free radicals, absorbing oxygen radicals, and chelating of the metal ions [29].

3.3. Flavonoids Prevent Palmitate-Induced ROS Production and Oxidative Stress in H9c2 Cells. The ability of **1–5** to decrease the oxidative stress in cells was determined. It is well known that concentrations of palmitate at 500 μm increase oxidative stress and stimulate ROS production in H9c2 cell. H_2O_2 model has been used to determine the oxidative stress because it can generate superoxide anion radical. Pretreatment with flavonoids for 1 hr at 20 μM significantly decreased ROS production preventing oxidative stress in H9c2 cell and palmitate-induced ROS production using DCFH-HA (for $H_2O_2^-$) and DHE (for O_2^-) test. The oxidative stress reduction potential of **1–5** is given in Table 4. The capacity of 10, 50, and 100 μg/mL flavonoids to reduce the oxidative stress was compared with that of 25 μg/mL of ascorbic acid. The values of the mean fluorescent intensity indicated that the isolated compound significantly reduces the increase of ROS in palmitate-induced cells. The antioxidant effectiveness *in vitro* of the isolated compound can be possibly due to their ability to act as free radical scavengers, quenchers of singlet

Table 5: Antioxidative status of mice and biochemical parameters at the end of the experimental period.

Organ	Group	SOD (U/min^{-1})	CAT (U/s^{-1})	CSH-Px (U/mL^{-1})	GSH (U/mL^{-1})	MDA ($nmol/mL^{-1}$)
Kidney	N	2.2 ± 0.34^{a}	0.74 ± 0.005^{b}	68.65 ± 2.43^{a}	25.27 ± 4.31^{a}	29.12 ± 3.57^{a}
	MD	2.8 ± 0.26^{b}	0.59 ± 0.002^{c}	56.41 ± 5.19^{b}	5.29 ± 1.84^{c}	25.60 ± 5.16^{b}
	MD + **1**	3.6 ± 0.34^{c}	0.79 ± 0.005^{b}	71.65 ± 6.73^{c}	16.21 ± 1.53^{b}	27.93 ± 4.52^{a}
	MD + **2**	3.1 ± 0.39^{b}	0.76 ± 0.006^{a}	67.62 ± 4.10^{a}	13.07 ± 3.76^{a}	26.62 ± 1.47^{a}
	MD + **3**	3.7 ± 0.39^{c}	0.82 ± 0.006^{a}	73.80 ± 6.14^{b}	17.23 ± 1.95^{b}	28.18 ± 3.52^{c}
	MD + **4**	3.8 ± 0.42^{c}	0.84 ± 0.009^{a}	79.37 ± 4.42^{a}	19.87 ± 2.25^{c}	29.14 ± 5.32^{c}
	MD + **5**	3.7 ± 0.39^{c}	0.82 ± 0.004^{a}	74.26 ± 5.28^{a}	19.01 ± 4.78^{c}	28.01 ± 4.11^{c}
Liver	N	6.5 ± 0.18^{a}	0.82 ± 0.011^{a}	99.41 ± 6.16^{a}	48.76 ± 4.84^{a}	26.35 ± 4.25^{a}
	MD	5.8 ± 0.13^{b}	0.58 ± 0.005^{c}	39.34 ± 5.63^{c}	26.48 ± 2.60^{b}	23.78 ± 2.17^{b}
	MD + **1**	6.2 ± 0.72^{a}	0.71 ± 0.006^{b}	79.59 ± 3.54^{a}	40.63 ± 3.62^{c}	25.77 ± 2.83^{a}
	MD + **2**	6.0 ± 0.28^{a}	0.65 ± 0.004^{a}	51.24 ± 6.10^{a}	35.89 ± 4.29^{c}	24.21 ± 4.76^{a}
	MD + **3**	6.3 ± 0.51^{a}	0.73 ± 0.003^{b}	90.21 ± 3.29^{b}	44.21 ± 7.36^{a}	26.53 ± 2.80^{a}
	MD + **4**	6.4 ± 0.73^{a}	0.79 ± 0.005^{a}	93.26 ± 5.17^{b}	47.02 ± 5.42^{c}	27.91 ± 5.52^{a}
	MD + **5**	6.1 ± 0.45^{a}	0.76 ± 0.002^{a}	89.38 ± 6.35^{b}	44.32 ± 6.15^{a}	27.22 ± 5.16^{a}
Pancreas	N	4.03 ± 0.37^{a}	0.74 ± 0.007^{a}	359.56 ± 4.67^{a}	51.26 ± 3.35^{a}	28.56 ± 5.42^{a}
	MD	2.90 ± 0.67^{c}	0.65 ± 0.003^{c}	325.39 ± 6.23^{b}	19.48 ± 1.84^{b}	24.21 ± 3.43^{b}
	MD + **1**	3.70 ± 0.49^{b}	0.72 ± 0.004^{b}	342.29 ± 4.67^{c}	43.17 ± 3.27^{c}	26.55 ± 2.67^{ac}
	MD + **2**	3.06 ± 0.30^{a}	0.69 ± 0.005^{a}	330.56 ± 7.52^{b}	30.84 ± 5.63^{d}	25.19 ± 6.54^{a}
	MD + **3**	3.80 ± 0.59^{b}	0.73 ± 0.005^{b}	346.99 ± 6.43^{c}	45.80 ± 3.52^{c}	26.56 ± 2.90^{ac}
	MD + **4**	3.89 ± 0.47^{b}	0.75 ± 0.008^{a}	350.48 ± 5.39^{a}	47.22 ± 5.42^{d}	27.84 ± 4.53^{a}
	MD + **5**	3.12 ± 0.62^{c}	0.76 ± 0.009^{a}	349.14 ± 5.84^{d}	45.74 ± 6.21^{d}	27.18 ± 5.84^{a}

Each value represents the mean ± SEM from 6 rats. Values within columns bearing the same lower case letters (a, b, c, and d) are not different at $p < 0.05$ and are not in any particular order. Normal control (N) and diabetic control (MD).

Table 6: Effects of **1–5** on serum enzyme levels in hyperglycaemic and normal mice.

Group	Glucose (mg/dL)	SGOT (IU/L)	SGPT (IU/L)	ALP (KA units)	TB (mg/dL)	Total protein (g/dL)
Normal control	99.4 ± 5.67	140.18 ± 8.15	99.31 ± 4.59	30.17 ± 3.52	0.65 ± 0.07	8.1 ± 2.14
Control diabetic MD	256.7 ± 8.43^{a}	234.23 ± 5.71^{a}	200.06 ± 7.34^{a}	71.86 ± 5.48^{a}	0.46 ± 0.06^{a}	5.1 ± 1.6^{a}
MD + **1**	—	168.87 ± 5.15^{b}	135.61 ± 6.82^{b}	54.78 ± 1.98^{b}	0.51 ± 0.07^{b}	6.3 ± 1.84^{b}
MD + **2**	—	182.04 ± 4.17^{b}	145.71 ± 6.04^{b}	63.62 ± 4.26^{b}	0.49 ± 0.04^{b}	5.9 ± 0.98^{b}
MD + **3**	—	163.61 ± 7.48^{b}	129.72 ± 4.48^{b}	50.09 ± 3.56^{b}	0.55 ± 0.05^{b}	6.8 ± 2.74^{b}
MD + **4**	—	150.29 ± 6.41^{b}	122.27 ± 5.67^{b}	45.30 ± 2.92^{b}	0.58 ± 0.03^{b}	7.1 ± 2.31^{b}
MD + **5**	—	156.52 ± 5.42^{b}	128.30 ± 7.03^{b}	48.41 ± 2.68^{b}	0.56 ± 0.08^{b}	6.9 ± 1.95^{b}

Each value represents the mean ± SEM ($n = 6$ rats). $^{a}p < 0.001$ compared with normal control group and $^{b}p < 0.001$ compared with MD control group. ALP: alkaline phosphatase; SGOT: serum glutamate oxaloacetate transaminase; SGPT: serum glutamate; TB: total bilirubin.

O_2 formation, to complex with prooxidant metal ions and reducing agents [30].

3.4. Effects of Flavonoids on Oxidative Stress Markers and Aminotransferases Activities. There were no changes of blood glucose level after 30 days of administration of **1–5**. There was a significant increase of lipid peroxide in the liver, kidney, and pancreas in diabetes mice. However, the administration of **1–5** improved these levels in the treated groups with respect to the diabetic control group as shown in Table 5. The production of ROS including hydroxyl, superoxide anions and hydrogen peroxide, nitric oxide, DPPH, ABTS radicals, protein oxidation, and lipid peroxidation can be enhanced by treatment with isolated **1–5**, and increases in radical scavenging enzymes of CSH-Px, SOD, GSH, and CAT have also been observed in liver, pancreas, and kidney in the group of diabetic animals treated.

Elevation of serum biomarker enzymes such as total protein, SGPT, SGOT, ALP, and TB was observed in diabetic mice indicating deterioration in liver function (Table 6) which may be because STZ produced liver damage causing leakage of these enzymes into the blood [31]. However, treatment of STZ-induced diabetes in mice with **1–5** reduced levels of these enzymes leading to hepatoprotective effect.

3.5. Effects of Flavonoids on TBARS Levels. In diabetic mice, elevated levels of oxidative stress are due to protein glycation, autoxidation of glucose, low activities of antioxidant

Table 7: Effect of **1–5** on malondialdehyde concentration in liver and kidney of normal and diabetic mice.

Groups (mg/kg)	TBARS (μM/g)	
	Liver	Kidney
Normal control	0.99 ± 0.08	1.9 ± 0.04
MD control	1.60 ± 0.09[a]	2.7 ± 0.06[a]
MD + **1**	1.07 ± 0.06[b]	2.0 ± 0.09[b]
MD + **2**	1.21 ± 0.02[b]	2.3 ± 0.07[b]
MD + **3**	0.99 ± 0.07[b]	1.9 ± 0.08[b]
MD + **4**	0.97 ± 0.03[b]	1.8 ± 0.04[b]
MD + **5**	0.95 ± 0.05[b]	1.6 ± 0.06[b]

All values are expressed as mean ± SEM, $n = 10$. [a] $p < 0.05$ when compared to normal control group. [b] $p < 0.01$ when compared to diabetic control group.

enzymes, and lipid peroxidation [32]. Consistent with this finding, decreased activities of antioxidant enzymes, such as GSH, SOD, GST, and GPx, and a marked increase in the concentration of TBARS indicate increase lipid peroxidation leading to decrease of the antioxidant defense mechanisms to avoid overproduction of ROS which leads to tissue injury [33]. Supplementation to diabetic mice with the isolated compound resulted in a significant ($p < 0.05$) diminution in lipid peroxidation levels, in liver and kidney compared with diabetic control, and the observed reduction of TBARS towards normal levels (Table 7). These results suggest a marked inhibition of the isolated compound on ROS generation in STZ-induced diabetic mice. TBARS levels are an index of oxidative stress and endogenous lipid peroxidation which intensifies with increasing free radicals production. Therefore, in diabetic patients measurement of TBA-reactive substance levels is used to determine the level of oxidative stress. In addition, increased lipid peroxidation in diabetic mice might be due to stimulation of hepatic triglyceride synthesis [34].

4. Conclusion

In this study were isolated and characterized five biologically active compounds from bark of *Eysenhardtia polystachya*. The flavonoids were found to be 2′,4′-dihydroxychalcone-6′-O-β-D-glucopyranoside (**1**), α,3,2′,4′-tetrahydroxy-4-methoxy-dihydrochalcone-3′-C-β-glucopyranosy-6′-O-β-D-glucopyranoside (**2**), 7-hydroxy-5,8′-dimethoxy-6′α-L-rhamnopyranosyl-8-(3-phenyl-trans-acryloyl)-1-benzopyran-2-one (**3**), 6′7-dihydroxy-5,8-dimethoxy-8(3-phenyl-trans-acryloyl)-1-benzopyran-2-one (**4**), and 9-hydroxy-3,8-dimethoxy-4-prenylpterocarpan (**5**). The isolated compounds were evaluated for their antioxidant potentials in *in vitro* and *in vivo* assay finding that compound **4** is the most potent antioxidant. Our data indicates that isolated **1–5** from the bark of *Eysenhardtia polystachya* have an ability to reduce oxidative stress under diabetic conditions, prevent and/or delay the onset renal, pancreatic, and hepatic damage through decreasing of lipid peroxidation, antioxidant properties, and increasing radical scavenging enzymes activity, also reduce intracellular reactive oxygen species, and they consequently could alleviate complications of diabetes. In addition, the antioxidant properties of compounds **1–5** are a promising strategy for ameliorating therapeutic effects by avoiding disorders in the normal redox reactions in healthy cells.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

References

[1] K. Jomova and M. Valko, "Advances in metal-induced oxidative stress and human disease," *Toxicology*, vol. 283, no. 2-3, pp. 65–87, 2011.

[2] Z. E. Suntres, "Role of antioxidants in paraquat toxicity," *Toxicology*, vol. 180, no. 1, pp. 65–77, 2002.

[3] R. M. G. Perez, R. Vargas, G. S. Perez, and S. Zavala, "Anti-urolithiatic activity of *Eysenhardtia polystachya* aqueous extract on rats," *Phytotherapy Research*, vol. 12, no. 2, pp. 144–145, 1998.

[4] D. T. Burns, B. G. Dalgarno, P. Gargan, and J. Grimshaw, "An isoflavone and a coumestan from eysenhardtia polystachya-Robert Boyle's fluorescent acid-base indicator," *Phytochemistry*, vol. 23, no. 1, pp. 167–169, 1984.

[5] L. Alvarez, M. Y. Rios, C. Esquivel et al., "Cytotoxic isoflavans from *Eysenhardtia polystachya*," *Journal of Natural Products*, vol. 61, no. 6, pp. 767–770, 1998.

[6] R. M. Gutierrez and E. G. Baez, "Evaluation of antidiabetic, antioxidant and antiglycating activities of the *Eysenhardtia polystachya*," *Pharmacognosy Magazine*, vol. 10, no. 38, pp. S404–S418, 2014.

[7] P. Nolis and T. Parella, "Spin-edited 2D HSQC–TOCSY experiments for the measurement of homonuclear and heteronuclear coupling constants: application to carbohydrates and peptides," *Journal of Magnetic Resonance*, vol. 176, no. 1, pp. 15–26, 2005.

[8] H.-Y. Chen and G.-C. Yen, "Antioxidant activity and free radical-scavenging capacity of extracts from guava (*Psidium guajava* L.) leaves," *Food Chemistry*, vol. 101, no. 2, pp. 686–694, 2007.

[9] O. Erel, "A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation," *Clinical Biochemistry*, vol. 37, no. 4, pp. 277–285, 2004.

[10] L. Marcocci, J. J. Maguire, M. T. Droylefaix, and L. Packer, "The nitric oxide-scavenging properties of *Ginkgo biloba* extract EGb 761," *Biochemical and Biophysical Research Communications*, vol. 201, no. 2, pp. 748–755, 1994.

[11] A. Kumaran and R. J. Karunakaran, "In vitro antioxidant activities of methanol extracts of five *Phyllanthus* species from India," *LWT-Food Science and Technology*, vol. 40, no. 2, pp. 344–352, 2007.

[12] B. Halliwell and J. M. C. Gutteridge, "Role of free radicals and catalytic metal ions in human disease: an overview," *Methods in Enzymology*, vol. 186, no. 3, pp. 1–85, 1990.

[13] R. J. Ruch, S. J. Cheng, and J. E. Klauning, "Prevention of cito-toxicity and inhibition of intercelular communication by anti-oxidant catechins from Reactive oxygen Chinese Green tea," *Carcinogenesis*, vol. 10, no. 6, pp. 1003–1008, 1989.

[14] A. Aranda, L. Sequedo, L. Tolosa et al., "Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay: A quantitative method for oxidative stress assessment of nanoparticle-treated cells," *Toxicology in Vitro*, vol. 27, no. 2, pp. 954–963, 2013.

[15] A. Tahara, A. Matsuyama-Yokono, R. Nakano, Y. Someya, and M. Shibasaki, "Hypoglycaemic effects of antidiabetic drugs in streptozotocin-nicotinamide-induced mildly diabetic and streptozotocin-induced severely diabetic rats ," *Basic and Clinical Pharmacology and Toxicology*, vol. 103, no. 6, pp. 560–568, 2008.

[16] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[17] C. G. Fraga, B. E. Leibovitz, and A. L. Tappel, "Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes," *Free Radical Biology and Medicine*, vol. 4, no. 3, pp. 155–161, 1988.

[18] M. Uchiyama and M. Mihara, "Determination of malonaldehyde precursor in tissues by thiobarbituric acid test," *Analytical Biochemistry*, vol. 86, no. 1, pp. 271–278, 1978.

[19] T. Quadri-Spinelli, J. Heilmann, T. Rali, and O. Sticher, "Bioactive coumarin derivatives from the fern *Cyclosorus interruptus*," *Planta Medica*, vol. 66, no. 8, pp. 728–733, 2000.

[20] H. Wei, X. Zhang, G. Wu et al., "Chalcone derivatives from the fern *Cyclosorus parasiticus* and their anti-proliferative activity," *Food and Chemical Toxicology*, vol. 60, pp. 147–152, 2013.

[21] L. Jiménez-González, M. Álvarez-Corral, M. Muñoz-Dorado, and I. Rodríguez-García, "A concise and diastereoselective total synthesis of *cis* and *trans*-pterocarpans," *Chemical Communications*, vol. 7, no. 21, pp. 2689–2691, 2005.

[22] M. Maver, E. F. Queiroz, J.-L. Wolfender, and K. Hostettmann, "Flavonoids from the stem of *Eriophorum scheuchzeri*," *Journal of Natural Products*, vol. 68, no. 7, pp. 1094–1098, 2005.

[23] X.-Z. Huang, X.-S. Bai, H. Liang et al., "Cytotoxic isoflavanones from *Uraria clarkei*," *Bulletin of the Korean Chemical Society*, vol. 34, no. 5, pp. 1421–1424, 2013.

[24] D. T. Burns, B. G. Dalgarno, P. E. Gargan, and J. Grimshaw, "An isoflavone and a coumestan from *Eysenhardtia polystachya*-Robert Boyle's fluorescent acid-base indicator," *Phytochemistry*, vol. 23, no. 1, pp. 167–169, 1984.

[25] D. H. Waalkens-Berendsen, M. E. M. Kuilman-Wahls, and A. Bär, "Embryotoxicity and teratogenicity study with neohesperidin dihydrochalcone in rats," *Regulatory Toxicology and Pharmacology*, vol. 40, no. 1, pp. 74–79, 2004.

[26] S. Roowi and A. Crozier, "Flavonoids in tropical *Citrus* species," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 22, pp. 12217–12225, 2011.

[27] M. J. Simpson, D. Hjelmqvist, C. López-Alarcón et al., "Anti-peroxyl radical quality and antibacterial properties of rooibos infusions and their pure glycosylated polyphenolic constituents," *Molecules*, vol. 18, no. 9, pp. 11264–11280, 2013.

[28] P. Innok, T. Rukachaisirikul, S. Phongpaichit, and A. Suksamrarn, "Fuscacarpans A-C, new pterocarpans from the stems of *Erythrina fusca*," *Fitoterapia*, vol. 81, no. 6, pp. 518–523, 2010.

[29] 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.

[30] G. Ruberto and M. T. Baratta, "Antioxidant activity of selected essential oil components in two lipid model systems," *Food Chemistry*, vol. 69, no. 2, pp. 167–174, 2000.

[31] J. J. Holst, "The physiology of glucagon-like peptide 1," *Physiological Reviews*, vol. 87, no. 4, pp. 1409–1439, 2007.

[32] T. O'Brien, T. T. Nguyen, and B. R. Zimmerman, "Hyperlipidemia and diabetes mellitus," *Mayo Clinic Proceedings*, vol. 73, no. 10, pp. 969–976, 1998.

[33] B. M. Matkovics, M. Kotorman, I. S. Varga, D. Q. Hai, and C. Varga, "Oxidative stress in experimental diabetes induced by streptozotocin," *Acta Physiologica Hungarica*, vol. 85, no. 1, pp. 29–38, 1998.

[34] F. Pociot, J. L. Reimers, and H. U. Andersen, "Nicotinamide—biological actions and therapeutic potential in diabetes prevention," *Diabetologia*, vol. 36, no. 6, pp. 574–576, 1993, Proceedings of the IDIG Workshop, Copenhagen, Denmark, December 1992.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3403586, 6 pages
http://dx.doi.org/10.1155/2016/3403586

Research Article

In Vitro Evaluation of the Antioxidant Activity and Wound Healing Properties of Jaboticaba (*Plinia peruviana*) Fruit Peel Hydroalcoholic Extract

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Received 17 June 2016; Accepted 25 July 2016

Academic Editor: Pavel Pospisil

Jaboticaba is a fruit from a native tree to Brazil, *Plinia peruviana*. Jaboticaba peels are an important source of antioxidant molecules such as phenolic compounds. This study aimed to evaluate *in vitro* the activity of a hydroalcoholic extract of jaboticaba fruit peels (HEJFP) in wound healing processes and antioxidant activity in murine fibroblasts (L929 cell line). HEJFP concentrations (0.5, 1, 5, 10, 25, 50, 100, and 200 μg/mL) were tested in MTT assay and cell proliferation was verified at 100 μg/mL after 24 h and at 25, 50, and 100 μg/mL after 48 h of extract exposure. Evaluation of antioxidant activity was performed at 0.5, 5, 25, 50, and 100 μg/mL HEJFP concentrations. Cell treatment with HEJFP at 25, 50, and 100 μg/mL for 24 h followed by H_2O_2 exposure for 3 h showed a strong cytoprotective effect. *In vitro* scratch wound healing assay indicated that none of tested HEJFP concentrations (0.5, 5, 25, 50, and 100 μg/mL) were capable of increasing migration rate after 12 h of incubation. These results demonstrate a positive effect of HEJFP on the wound healing process on L929 fibroblasts cell line, probably due to the antioxidant activity exhibited by phytochemicals in the extract.

1. Introduction

Wound healing is a process divided into three interactive and overlapping phases classified as inflammation, tissue formation, and tissue remodeling [1]. During inflammation, neutrophils and monocytes invade the injury tissue and start to secrete proteolytic enzymes, proinflammatory cytokines, and growth factors. Besides, these cells also secrete reactive oxygen species (ROS), important molecules that defend the body against bacteria and microorganism invasion [2].

The next phase known as tissue formation is characterized by proliferation and migration of fibroblasts and keratinocytes from the wound edge to the wound bed [3]. Angiogenesis is triggered and leads to the formation of granulation tissue, which is important to support the nutrients and oxygen supply in injured tissue [4–6]. In this tissue, fibroblasts become myofibroblasts which synthesize and deposit extracellular matrix (ECM) compounds, especially collagen. Besides, these cells are responsible for wound contraction and maturation of the granulation tissue [7].

At remodeling phase, there is a reduction on cellularity due to the apoptosis of myofibroblasts, endothelial cells, and inflammatory cells. The synthesis of ECM is reduced and ECM's components are modified as the matrix is remodeled [8].

Impaired wound healing is a problem that may be caused by uncontrolled inflammatory and immune responses, microbial infection, and excessive ROS production [9]. Excessive amounts of ROS may modify and/or degrade ECM proteins and damage dermal fibroblasts and keratinocytes functions. Besides, ROS-mediated transcription causes the maintained proinflammatory cytokines secretion and induction of matrix metalloproteases [10].

Jaboticaba is a fruit from a native tree to Brazil, that is, *Plinia peruviana*. Studies have shown important biological properties of anthocyanins, mainly those related to anti-inflammatory activity and antioxidative stress [11]. Jaboticaba fruit peels are the main source of anthocyanins in the fruit and it has been used in traditional medicine to treat diarrhea, skin irritation, hemoptysis, and asthma [12–14]. This study aimed to evaluate the antioxidant activity of a HEJFP and its role in wound healing processes as migration and proliferation of murine fibroblasts (L929 cell line).

2. Materials and Methods

2.1. Plant Material Collection and Extraction. Fruits of *P. peruviana* were collected from a backyard format planting system during the harvest season (spring, 2014) in Guaxupé, Minas Gerais, Brazil. The plant was authenticated by Dr. Marcos Sobral and a voucher specimen (FLOR 55902) was preserved at FLOR herbarium (Department of Botany, Federal University of Santa Catarina, Florianópolis, southern Brazil).

The fruit peels of jaboticaba were lyophilized and powdered by an electric grinder. The dried and powdered biomass was added to 50% ethanol solution (v/v), pH 3.6 (1 : 10 w/v). The mixture was microwaved (three pulses of five seconds with 60 seconds of interval between each of the pulses) to extract the compounds of interest. The HEJFP was recovered by filtration on cellulose membranes under vacuum.

2.2. Determination of Total Phenolic Content. The total phenolic content of HEJFP was measured spectrophotometrically [15]. For that, HEJFP was diluted in 50% ethanol solution, pH 3.6 (1 : 10 v/v). Subsequently, 1 mL of HEJFP previously diluted was added to 5 mL 95% methanol solution. After this second dilution, sample (1 mL) was added to 1 mL 95% ethanol solution, 5 mL distilled water, and 0.5 mL Folin-Ciocalteu's reagent and incubated for 7 min.

After incubation, 1 mL 5% sodium carbonate solution was added and kept in the darkness at room temperature for 1 h. A blank solution was prepared as described above replacing the sample by 50% of ethanol solution, pH 3.6. The absorbance was measured at 725 nm, using a UV-Vis spectrophotometer (BEL LGS 53, BEL Engineering, Monza, Italy).

The total phenolic compounds were quantified using a standard curve of gallic acid. The results were expressed as mg gallic acid equivalents/g dry weight of jaboticaba biomass.

2.3. Determination of the Total Flavonoid Content. The determination of total flavonoids was based on aluminum chloride colorimetric method [16].

Previously, HEJFP was diluted in 50% ethanol solution, pH 3.6 (1 : 10 v/v). 0.5 mL of diluted HEJFP was added to 2.5 mL ethanol and 0.5 mL 2% aluminum chloride diluted in methanol and incubated for 1 h. A blank solution was prepared as described above replacing the sample by 50% of ethanol solution, pH 3.6. The absorbance was measured at 420 nm in a UV-Vis spectrophotometer (BEL LGS 53, BEL Engineering, Monza, Italy). The quantification of total flavonoids was carried out using a quercetin standard curve. The results were expressed as mg quercetin equivalents/g dry weight of jaboticaba biomass.

2.4. Antioxidant Activity (DPPH Assay). The 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay is a chemical method that measures the capacity of a compound to scavenge free radicals based on the decrease in absorbance during the reaction [17]. A stock solution of 0,0079 g of DPPH was diluted in 2.5 mL methanol. This solution was further diluted in a concentration of 1 : 100 (v/v) in 80% methanol (v/v). The absorbance of this DPPH solution should be around 0.5 and 0.6. The HEJFP, previously diluted in 50% ethanol, pH 3.6, at 1 : 100 (v/v), was added to DPPH/80% methanol solution (1 : 30 v/v). The capacity of the HEJFP to inhibit DPPH radicals was measured spectrophotometrically at 515 nm, after incubation for 5, 10, 20, 30, 40, and 50 min in the dark, at room temperature. The same procedure described above was used to test the 50% ethanol, pH 3.6, solution to ensure that the solvent was not reacting with DPPH/80% methanol solution. The percentage of inhibition of DPPH radicals was calculated by the following formula (Abs. = absorbance):

$$\text{inhibition DPPH (\%)} = \frac{\text{Abs. DPPH/80\% methanol solution} - \text{Abs. HEJFP}}{\text{Abs. DPPH/80\% methanol solution}} \times 100. \quad (1)$$

2.5. Cell Proliferation and Viability Assay Using L929 Fibroblast. L929 mouse fibroblast cells were seeded at a density of 5×10^3 cells/well into a 96-well plate in DMEM culture medium supplemented with 10% FBS and incubated at 37°C, in a humidified 5% CO_2 atmosphere overnight. After incubation, DMEM was replaced by DMEM 10% FBS containing 0.5, 1, 5, 10, 25, 50, 100, and 200 μg/mL (dry weight) of HEJFP, except in control, where the culture medium was replaced by fresh DMEM. Cells were incubated for 24 h and 48 h, at 37°C, in a humidified 5% CO_2 atmosphere. Afterwards, the culture medium was replaced by 100 μL of fresh DMEM along with 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) per well and incubated in the dark, for 3 h, at 37°C, in a humidified 5% CO_2 atmosphere. A negative control without cells with 100 μL of DMEM and 10 μL of MTT solution was required. Subsequently, 85 μL of culture medium was removed and 50 μL of DMSO was added onto each well and incubated for more 10 min, at 37°C, in a humidified 5% CO_2 atmosphere. After homogenizing formazan crystals, the absorbance at 540 nm was determined by an ELISA plate reader. The percentage of cell proliferation/viability was calculated and compared to control (100% of viability).

2.6. Hydrogen Peroxide-Induced Oxidative Stress in L929 Fibroblast Cells and Evaluation of Cell Survival. Hydrogen peroxide was used for induction of oxidative stress as described by Balekar et al. [18] and Ponnusamy et al. [19]. The L929 fibroblast cells were seeded at a density of 5×10^3 cells/well into a 96-well plate in DMEM supplemented with 10% FBS and incubated at 37°C, in a humidified 5% CO_2

atmosphere overnight. A curve with H_2O_2 concentrations (0.0625, 0.125, 0.25, 0.5, and 1.0 mM) was built to determine H_2O_2 dose which decreases cell viability by 80% after 24 h of exposure using MTT assay. The chosen concentration was 1.0 mM of H_2O_2. Subsequently, L929 fibroblast cells were seeded at a density of 5×10^3 cells/well into a 96-well plate containing DMEM culture medium supplemented with 10% FBS and incubated overnight at 37°C, in a humidified 5% CO_2 atmosphere. After incubation, DMEM with 10% FBS containing 0.5, 5, 25, 50, and 100 μg/mL (dry weight) of HEJFP was used to treat cells in different times as follows: (1) cells were treated for 24 h followed by 1.0 mM of H_2O_2 exposure for 3 h, (2) cells were exposed concomitantly to HEJFP and 1.0 mM of H_2O_2 for 24 h, and (3) cells were exposed to 1.0 mM of H_2O_2 for 3 h followed by cells treatment with HEJFP for 24 h. Evaluation of cell survival was performed using MTT assay as described above.

2.7. Scratch Assay. The stimulatory effect of HEJFP on migration of L929 cells was determined as described by Balekar et al. [18]. The L929 fibroblast cells were seeded at a density of 5×10^5 cells/well into a 24-well plate containing DMEM culture medium supplemented with 10% FBS and incubated overnight at 37°C, in a humidified 5% CO_2 atmosphere. After incubation, DMEM was completely removed and the adherent cell layer was scratched with a sterile yellow pipette tip. Cellular debris was removed by washing off with phosphate buffer saline (PBS). The cells were treated with DMEM with 10% FBS containing 0.5, 5, 25, 50, and 100 μg/mL (dry weight) of HEJFP. Controls received only fresh DMEM. To avoid proliferation of cells, mitomycin C (10 μg/mL) was added in each well along with control and HEJFP-treated cells; this way only migration was evaluated. The cells were incubated (at 37°C in humidified 5% CO_2 atmosphere for 12 h) and then the recording of images of the scratch area was carried out in two different points, using a built-in camera in the microscope (40x magnification) at 0 h (just after scratching cells) and at 12 h after incubation with HEJFP and control. Data were analyzed with ImageJ 1.42q imaging software (National Institutes for Health, US) in order to determine the width of the scratch and thus to calculate the rate of migration of cells by the following formula:

$$\text{migration rate } (\%) = \frac{\text{distance within scratch } (0\,\text{h}) - \text{distance within scratch } (12\,\text{h})}{\text{distance within scratch } (0\,\text{h})} \times 100. \quad (2)$$

2.8. Statistical Analysis. Data were collected and summarized, followed by statistical analysis using one-way ANOVA and Tukey's test. P values lower than 0.05 were considered to be statistically significant. The values were expressed as mean ± SD or median as indicated in figures' captions.

3. Results

3.1. Total Phenolic and Flavonoid Contents of the Hydroalcoholic Extract. The total phenol and flavonoid contents of HEJFP were 92,2 ± 9,75 mg gallic acid equivalent/g and 6,43 ± 0,49 mg quercetin equivalent/g, respectively.

3.2. Antioxidant Activity. DPPH radical scavenging of HEJFP was measured in different times to determine the peak of antioxidant capacity. After 5 min of incubation, HEJFP inhibited 83.6% of DPPH radicals, showing an excellent antioxidant activity in few minutes of reaction. At 30 min of incubation, 91% scavenging activity was achieved and it remained until 50 minutes of reaction (Table 1).

3.3. Cell Proliferation and Viability. The effect of HEJFP on both cell proliferation and viability was evaluated in L929 murine fibroblasts cell line in different concentrations after 24 h and 48 h, using MTT assay.

HEJFP was able to promote cell proliferation at 100 μg/mL after 24 h and at 25, 50, and 100 μg/mL after 48 h. The concentration of 200 μg/mL was shown to be cytotoxic in both times of exposure, decreasing significantly cell viability. For the other concentrations assayed, the cell viability was higher than 80% (Figure 1).

3.4. Hydrogen Peroxide-Induced Oxidative Stress and Cell Survival. L929 fibroblast cells were treated with 1.0 mM H_2O_2 as a model study of oxidative stress and resulted in decrease of cell viability by 90% after 24 h of exposure. The antioxidant potential of the HEJFP was tested before and after H_2O_2 exposure for 3 h and concomitantly with H_2O_2 for 24 h. The HEJFP was not effective in protecting cells against oxidative stress before or concomitantly with exposure to H_2O_2, resulting in low rate of cell survival. However, when cells were first treated with HEJFP for 24 h, followed by H_2O_2 exposure for 3 h, the tested concentrations of 25, 50, and 100 μg/mL protected the cells against adverse effects caused by H_2O_2-induced oxidative stress and maintained the cell viability (Figure 2).

3.5. Scratch Assay. L929 murine fibroblasts cell line was tested through the scratch assay to determine the capacity of these cells to migrate under HEJFP stimulus (Figure 3). L929 cells have a fast migration rate; then the time to evaluate the cell migration was determined as 12 h upon exposure to HEJFP. At 24 h, scratch is almost closed, making it difficult to analyze images.

Although concentrations of 0.5 and 100 μg/mL increased the cell migration rate after 12 h, the effect was not significant when compared to control (Table 2).

4. Discussion

Plant extracts can be efficient in helping the wound healing process if they contain phytochemicals with antimicrobial and antioxidant activities and free radical scavengers and

Table 1: Antioxidant activity of HEJFP determined by the DPPH assay.

Incubation time (min)	% inhibition of DPPH radical
5	83.6 ± 1.83
10	88.09 ± 1.52
20	90.18 ± 1.02
30	91.01 ± 0.42
40	91.36 ± 1.01
50	91.88 ± 1.28

Values are mean ± SD ($n = 3$).

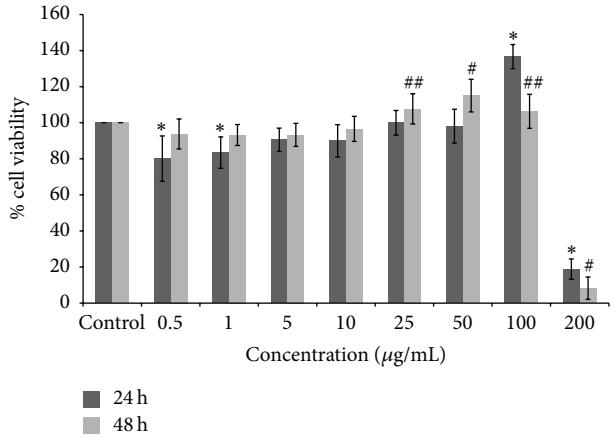

Figure 1: Percentage of survival of L929 fibroblast cells treated with HEJFP after 24 h and 48 h. Data are expressed as a mean ± SD ($n = 18$). ∗ indicates $P < 0.01$ against control for 24 h; # indicates $P < 0.01$ and ## indicates $P < 0.05$ against control for 48 h.

Table 2: Scratch length (μm) and cell migration rate (%) of L929 murine fibroblasts treated with HEJFP determined by the scratch assay.

	Length within the scratch (μm)	% migration rate
Time 0	859.97 ± 113.9	0
Control	246.73 ± 62.84	71.31 ± 7.3
0.5 μg/mL	157.56 ± 53.58	81.68 ± 6.23
5 μg/mL	261.91 ± 99.32	69.54 ± 11.55
25 μg/mL	263.36 ± 73.4	71.42 ± 8.53
50 μg/mL	245.79 ± 138.65	69.37 ± 16.12
100 μg/mL	183.31 ± 91.15	78.57 ± 10.6

Values are mean ± SD ($n = 8$).

active compounds that enhance mitogenic activity, angiogenesis, collagen production, and DNA synthesis [20].

Jaboticaba fruit peel has a promising potential as a wound healing enhancer duo to its biomass rich in phenolic compounds. Indeed, those secondary metabolites have a well-known antioxidant activity that prevents tissue damage and stimulates wound healing [11, 21].

The evaluation of the effectiveness of HEJFP in wound healing process was performed *in vitro* using L929 murine fibroblasts cell line. Nowadays, cell culture is a popular and effective method to test the sensitivity of cells to selected groups present in the microenvironment. Fibroblasts cell cultures have been proposed as a method for testing wound healing activity *in vitro* [22].

Hydrogen peroxide-induced oxidative stress is an alternative to evaluate extract's antioxidant activity in cells. H_2O_2 is an important molecule in wound healing process, the effect of which shall be under control of a molecular antioxidant apparatus such as SOD, GPx, and phospholipid hydroperoxide glutathione peroxidase [23]. A cytoprotective effect of HEJFP was detected when oxidative stress was induced to cells after the treatment with the extract. In this sense, a plausible assumption takes into account the fact that the protective effect of the extract could be related to the antioxidant activity thereof, corroborated by the results obtained through DPPH assay.

Xu et al. [11] also found a protective effect against hydrogen peroxide-induced oxidative stress in keratinocytes and fibroblasts of black soybean seed coat extract. The authors assigned this effect to the antioxidant activity of anthocyanins, metabolites that belong to phenolic group and are found in abundance in jaboticaba fruit peels.

Cell proliferation and migration are two extremely important features during the tissue formation phase in the wound healing. Scratch assay is a form to mimic a wound *in vitro* and evaluate the cell migration rate. Once the cell monolayer is disrupted, the loss of cell-cell interaction results in increasing concentration of growth factors and cytokines at the wound edge, initiating migration and proliferation of cells [24]. Interestingly, although the HEJFP was not able to increase cell migration rate, the extracts at 25, 50, and 100 μg/mL promoted fibroblasts proliferation. This mitogenic effect is a positive event for wound healing process because fibroblasts are important cells involved in wound contraction and ECM production [18].

5. Conclusion

HEJFP has been shown to be *in vitro* a potential plant extract, enhancing the wound healing process. The cytoprotective effect of HEJFP in fibroblasts against hydrogen peroxide-induced oxidative stress can be assigned to its phenolic compounds, which have been proven to be strong antioxidants. Besides, HEJFP induced mitogenic activity of fibroblasts, an important feature in the wound healing process. Further investigations are necessary to isolate and identify the compounds responsible for these activities, as previous findings refer to the ellagic acid as a major compound in the HEJFP (unpublished data). Besides, *in vitro* studies measuring antioxidant enzymes will help understand the mechanisms underlying the effects described herein for the wound healing process.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

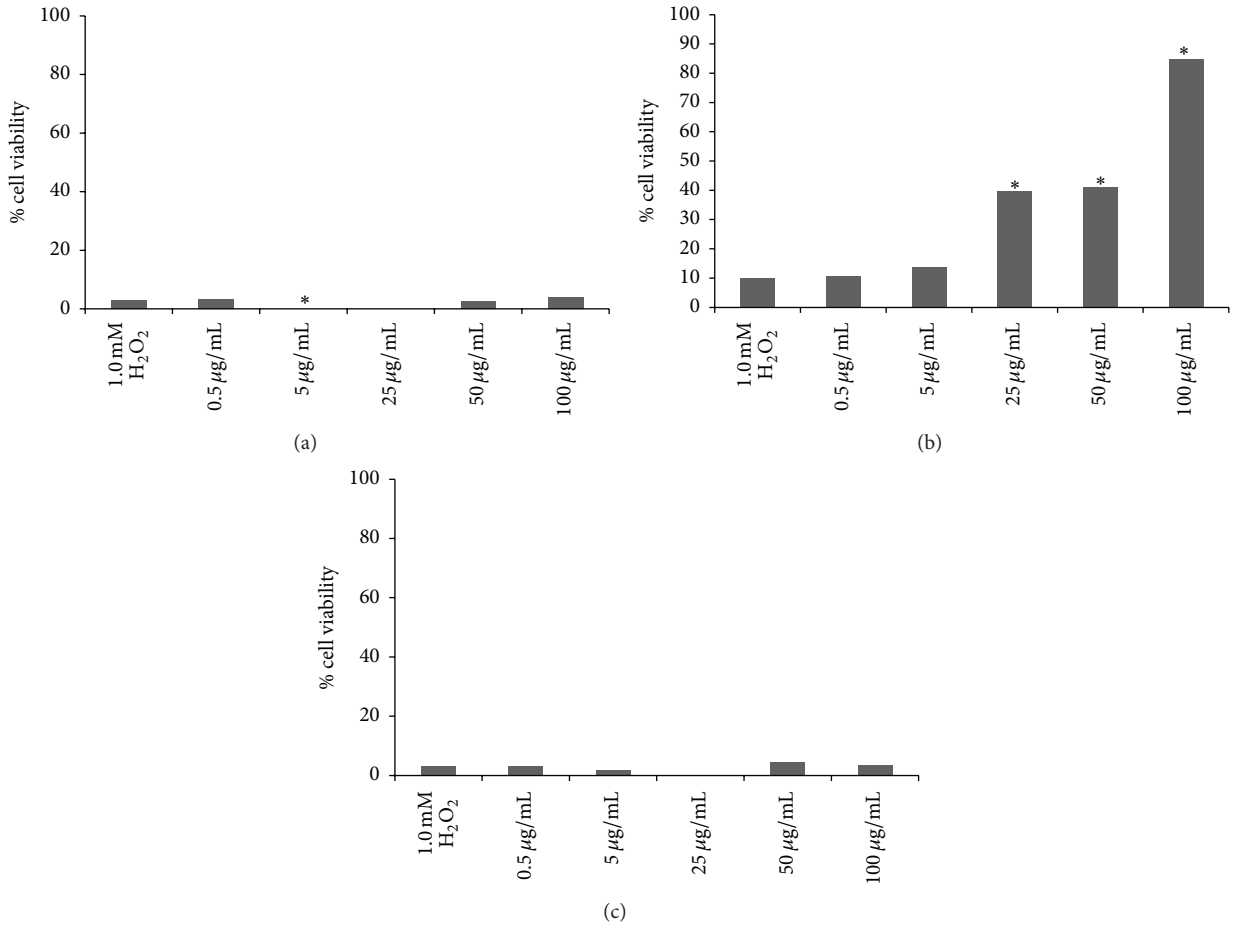

Figure 2: Viability (%) of cells treated with HEJFP after H_2O_2 exposure (a) and before H_2O_2 exposure (b) and concomitantly with H_2O_2 (c). Data are expressed as a median ($n = 16$). $*$ indicates $P < 0.01$ against 1.0 mM H_2O_2 control.

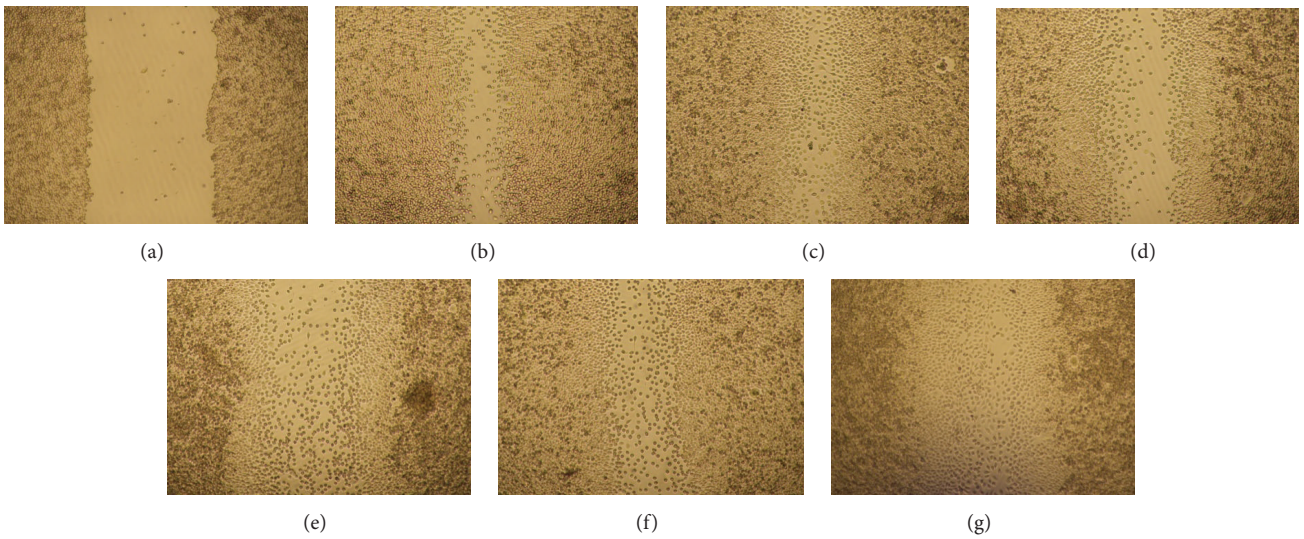

Figure 3: Microscopy images of L929 fibroblast cells migration after scratch ((a)—time 0) and after 12 h of HEJFP treatment. (b) Control, (c) 0.5 μg/mL, (d) 5 μg/mL, (e) 25 μg/mL, (f) 50 μg/mL, and (g) 100 μg/mL.

References

[1] A. J. Singer and R. A. F. Clark, "Cutaneous wound healing," *New England Journal of Medicine*, vol. 341, no. 10, pp. 738–746, 1999.

[2] R. A. F. Clark, "Wound repair: overview and general considerations," in *The Molecular and Cellular Biology of Wound Repair*, pp. 3–50, Plenum Press, New York, NY, USA, 1996.

[3] D. Neagos, V. Mitran, G. Chiracu et al., "Skin wound healing in a free floating fibroblast populated collagen lattice model," *Romanian Journal of Biophysics*, vol. 16, no. 3, pp. 157–168, 2006.

[4] C. Dunnill, T. Patton, J. Brennan et al., "Reactive oxygen species (ROS) and wound healing: the functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process," *International Wound Journal*, vol. 12, no. 6, pp. 1–8, 2015.

[5] P. Martin, "Wound healing—aiming for perfect skin regeneration," *Science*, vol. 276, no. 5309, pp. 75–81, 1997.

[6] G. C. Gurtner, S. Werner, Y. Barrandon, and M. T. Longaker, "Wound repair and regeneration," *Nature*, vol. 453, no. 7193, pp. 314–321, 2008.

[7] B. Hinz and G. Gabbiani, "Cell-matrix and cell-cell contacts of myofibroblasts: role in connective tissue remodeling," *Thrombosis and Haemostasis*, vol. 90, no. 6, pp. 993–1002, 2003.

[8] A. Desmoulière, M. Redard, I. Darby, and G. Gabbiani, "Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar," *The American Journal of Pathology*, vol. 146, no. 1, pp. 56–66, 1995.

[9] M. Gangwar, M. K. Gautam, S. Ghildiyal, G. Nath, and R. K. Goel, "*Mallotus philippinensis* Muell. Arg fruit glandular hairs extract promotes wound healing on different wound model in rats," *BMC Complementary and Alternative Medicine*, vol. 15, article 123, 2015.

[10] R. Moseley, J. E. Stewart, P. Stephens, R. J. Waddington, and D. W. Thomas, "Extracellular matrix metabolites as potential biomarkers of disease activity in wound fluid: lessons learned from other inflammatory diseases?" *British Journal of Dermatology*, vol. 150, no. 3, pp. 401–413, 2004.

[11] L. Xu, T. H. Choi, S. Kim et al., "Anthocyanins from black soybean seed coat enhance wound healing," *Annals of Plastic Surgery*, vol. 71, no. 4, pp. 415–420, 2013.

[12] J. Morton, *Fruits of Warm Climates*, Julia Morton, Miami, Fla, USA, 1987.

[13] H. Lorenzi, *Árvores Brasileiras: Manual de Identificação e Cultivo de Plantas Arbóreas Nativas do Brasil*, Instituto Plantarum, Nova Odessa, Brazil, 2000.

[14] A. M. V. Cruz and M. A. C. Kaplan, "Uso medicinal de espécies das famílias Myrtaceae e Melastomataceae no Brasil," *Floresta Ambiente*, vol. 11, no. 1, pp. 47–52, 2004.

[15] R. Randhir, P. Shetty, and K. Shetty, "L-DOPA and total phenolic stimulation in dark germinated fava bean in response to peptide and phytochemical elicitors," *Process Biochemistry*, vol. 37, no. 11, pp. 1247–1256, 2002.

[16] R. D. Petry, G. G. González Ortega, and W. B. Silva, "Flavonoid content assay: influence of the reagent concentration and reaction time on the spectrophotometric behavior of the aluminium chloride—flavonoid complex," *Pharmazie*, vol. 56, no. 6, pp. 465–470, 2001.

[17] Y. K. Kim, Q. Guo, and L. Packer, "Free radical scavenging activity of red ginseng aqueous extracts," *Toxicology*, vol. 172, no. 2, pp. 149–156, 2002.

[18] N. Balekar, N. G. Katkam, T. Nakpheng, K. Jehtae, and T. Srichana, "Evaluation of the wound healing potential of *Wedelia trilobata* (L.) leaves," *Journal of Ethnopharmacology*, vol. 141, no. 3, pp. 817–824, 2012.

[19] Y. Ponnusamy, N. J.-Y. Chear, S. Ramanathan, and C.-S. Lai, "Polyphenols rich fraction of *Dicranopteris linearis* promotes fibroblast cell migration and proliferation *in vitro*," *Journal of Ethnopharmacology*, vol. 168, pp. 305–314, 2015.

[20] P. K. Ghosh and A. Gaba, "Phyto-extracts in wound healing," *Journal of Pharmacy and Pharmaceutical Sciences*, vol. 16, no. 5, pp. 760–820, 2013.

[21] A. Scalbert, C. Manach, C. Morand, C. Rémésy, and L. Jiménez, "Dietary polyphenols and the prevention of diseases," *Critical Reviews in Food Science and Nutrition*, vol. 45, no. 4, pp. 287–306, 2005.

[22] Y. Abe, K. Inagaki, A. Fujiwara, and K. Kuriyama, "Wound healing acceleration of a novel transforming growth factor-β inducer, SEK-1005," *European Journal of Pharmacology*, vol. 408, no. 2, pp. 213–218, 2000.

[23] H. Steiling, B. Munz, S. Werner, and M. Brauchle, "Different types of ROS-scavenging enzymes are expressed during cutaneous wound repair," *Experimental Cell Research*, vol. 247, no. 2, pp. 484–494, 1999.

[24] C.-C. Liang, A. Y. Park, and J.-L. Guan, "*In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*," *Nature Protocols*, vol. 2, no. 2, pp. 329–333, 2007.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 7910340, 8 pages
http://dx.doi.org/10.1155/2016/7910340

Research Article

Antioxidant and Antihyperlipidemic Effects of *Campomanesia adamantium* O. Berg Root

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Received 4 February 2016; Revised 21 April 2016; Accepted 30 May 2016

Academic Editor: Jerzy Kruk

Campomanesia adamantium O. Berg, popularly known as guavira, has been used in Brazilian traditional medicine for reduction of serum lipid. The present study was carried out to investigate the antioxidant and antihyperlipidemic effects of *Campomanesia adamantium* root aqueous extract (ExCA). Phenolic compounds were quantified in the ExCA and gallic and ellagic acids were identified by HPLC. ExCA showed efficiency in 2,2-diphenyl-1-picrylhydrazyl free radical scavenging, with IC_{50} similar to butylhydroxytoluene control, and protected the erythrocytes against lipid peroxidation induced by 2,2′-azobis(2-methylpropionamidine) dihydrochloride, reducing generated malondialdehyde. Hyperlipidemic Wistar rats treated daily by gavage during eight weeks with ExCA (200 mg/kg of body weight) showed reduced serum level of total cholesterol and triglycerides, similar to normolipidemic rats and hyperlipidemic rats treated with simvastatin (30 mg/kg of body weight) and ciprofibrate (2 mg/kg of body weight). Moreover, the treatment with ExCA also decreased malondialdehyde serum level in the hyperlipidemic rats. The body weight and organ mass were unmodified by ExCA in hyperlipidemic rats, except an increase of liver mass; however, the hepatic enzymes, alanine aminotransferase and aspartate aminotransferase, were unchanged. Together, these results confirm the potential value of *Campomanesia adamantium* root for lowering lipid peroxidation and lipid serum level, improving risk factors for cardiometabolic diseases development.

1. Introduction

Dyslipidemia is characterized by higher serum level of total cholesterol and triglycerides, accompanied by reduction of high-density lipoprotein (HDL). In 2008, more than 17.3 million people died from cardiovascular diseases [1], such as atherosclerosis, which can lead to stroke and myocardial infarction [2].

Concomitant with increased serum lipid level, decreasing of antioxidant capacity of the organism has been observed, which contributes to endothelial dysfunction present in atherosclerosis and connects this alteration with modified metabolic state [3]. Reactive species formation occurs continuously in the body especially in consequence of oxidative metabolic process for energy generation. In lower concentration, these molecules have physiological function in cellular signalization and proliferation. However, in higher concentration it reactive species lead oxidative damage to protein, lipid, and nucleic acid, affecting key cellular structures [4].

The body has endogenous mechanisms, enzymatic and nonenzymatic, for neutralizing the excess of reactive species and decreasing possible cell damages [5]. The excess of oxidants agents which are not neutralized defines the oxidative stress present in dyslipidemia, obesity, and atherosclerosis [6].

Medical plants, rich in vitamins and secondary metabolites, have been source of inhibitors of the endogenous synthesis of cholesterol, as well as natural antioxidants [7, 8]. In Brazil, the fruit of *Campomanesia adamantium* O. Berg

(Myrtaceae) is used for nutrition and, in the traditional medicine, the leaves and root are used for treatment of diabetes and dyslipidemia. Similar effects have been described for other species of the genus such as hypolipidemic and antiplatelet effects [7, 9], antiulcerogenic effects [10], reducing body weight [11], and antidiabetic effects [12]. Additionally, phytochemical studies have showed phenol and flavonoids in the leaves of *Campomanesia adamantium*, which has been described as molecules with high potential antioxidant [13].

In this context, this study was carried out to investigate the antioxidant and antihyperlipidemic effects of the root aqueous extract of *Campomanesia adamantium* in rats with high fructose diet-induced hyperlipidemia (HFD).

2. Materials and Methods

2.1. Botanical Material and Obtaining Extract. *Campomanesia adamantium* O. Berg roots were collected in Dourados, MS, under coordinates S 22°02′47.9″ W 055°08′14.3″. They were sanitized, dried in an oven with air circulation at 45°C, and ground in a Willy-type knife mill. A voucher specimen was deposited in the herbarium DDMS/UFGD number 4108.

The extract was prepared by repeat extractions of the pulverized material using accelerated solvent extractor (ASE® 150-Dionex). The samples were placed in a cell of 100 mL and extracted with distilled water at a temperature of 125°C in static two cycles of 5 min each time, with 80% of the volume of washing and 60-second purge. The extracts were combined in an aqueous medium and then lyophilized to obtain the dry extract, yield 6%.

2.2. High Performance Liquid Chromatography Coupled with Diode Array Detector (HPLC-DAD). ExCA chemical profile was determined by HPLC-DAD (Shimadzu SPD-M20A, Japan) using a reversed phase column C-18 (250 mm × 4.6 mm, 5.0 μm, Shimadzu, Japan) mobile phase water (A) and acetonitrile (B), both with 1% acetic acid. The elution gradient was 5% of eluent B over 3 min and 3–60% of eluent B over 3–27 min and another 6 min to return the initial condition and restabilize the column. The flow rate was 1.0 mL/min and the injected volume was 20 μL. Compounds were quantified at 270 nm in triplicate analysis. The quantification was performed using a regression curve of five points, 0.5–50 μg/mL for gallic acid (Sigma-Aldrich, USA) and 2.5–100 μg/mL for ellagic acid (Sigma-Aldrich, USA). Analyzed tracks were linear (detector response/concentration). Peak areas were correlated with the concentration according to the calibration curve and the correlation coefficient (r) > 0.9998 for both compounds.

2.3. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Free Radical Scavenging. The activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging was available according to D. Gupta and R. K. Gupta [14] method. Briefly, 200 μL of extract (0.1–2000 μL) was mixed with 1.8 mL of DPPH 0.11 mM in 80% ethanol solution. The mixture was homogenized and incubated at room temperature in the dark for 30 min. The reading was performed in a spectrophotometer at 517 nm. Ascorbic acid and butylhydroxytoluene (BHT) were used as reference antioxidants. The tests were performed in triplicate in three independent experiments. The percentage of inhibition was calculated relative to the control (ascorbic acid and BHT) using the following equation:

$$\text{inhibition activity (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100. \quad (1)$$

2.4. Protection against 2,2′-Azobis(2-Methylpropionamidine) dihydrochloride (AAPH) Induced Hemolysis. The antioxidant activity of the extract was determined by erythrocytes protection capacity against hemolysis induced by peroxyl radicals generated by thermal decomposition of the hydrochloride 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), following the methodology described by Campos et al. [15]. For this, peripheral blood of healthy donors was collected into tubes containing anticoagulant (approved by the ethics committee of the Federal University of Grande Dourados under protocol number 123/12). Then, the blood sample was centrifuged at 1500 rpm for 10 min. After centrifugation, the plasma cell and the upper layer containing leukocytes were removed, preserving only the lower layer comprising red cells. The erythrocytes were washed three times with the addition of approximately 4 mL of saline and centrifuged at 2000 rpm for 10 min. After washing, a suspension of 10% erythrocytes was prepared. The *Campomanesia adamantium* extract was solubilized in 0.9% NaCl at concentrations of 50, 75, 100, and 125 μg/mL. The same procedure was carried out with ascorbic acid (an antioxidant standard).

In 250 μL of 10% erythrocyte sample, 250 μL aliquots were added at their respective concentrations analysis (50, 75, 100, and 125 μg/mL); this mixture was incubated at 37°C for 30 min, and then 500 μL of AAPH (0.406 g of AAPH diluted with 15 mL of 0.9% NaCl) was added. The material remained incubated at 37°C under gentle mixing for up to 180 min. Aliquots of 200 μL samples were removed and diluted in 1800 μL of 0.9% NaCl for analysis in a spectrophotometer at 540 nm at 60 min intervals. The percentage hemolysis was determined by measuring the absorbance of the supernatant (A) and compared to the total hemolysis (B), using the following equation:

$$\text{hemolysis (\%)} = \frac{A}{B} \times 100. \quad (2)$$

2.5. Lipid Peroxidation of Erythrocytes Induced by 2,2′-Azobis(2-Methylpropionamidine) (AAPH) dihydrochloride. We collected 5 mL of healthy blood donor for the preparation of the erythrocytes solution (H) 20%. The malondialdehyde (MDA), a product of lipid peroxidation of the erythrocyte, was performed as described by Campos et al. [15]. Samples of H (2.5%), H+AAPH, H+AAPH+ascorbic acid, and H+AAPH+ExCA were incubated for 180 min at 37°C. Concentrations available were 50, 75, 100, 125, 250, and 500 μg/mL of ascorbic acid and ExCA was added to an aliquot of 0.5 mL of each sample and 0.5 mL of 20%

trichloroacetic acid. An aliquot of 0.5 mL was removed from this mixture and added to tubes containing 1 mL of the reagent thiobarbituric acid (TBA) 10 nM incubated at 94°C for 45 min. After this period, the samples were kept at room temperature for 15 min, followed by addition of 3 mL of butanol, followed by agitation and centrifugation at 3000 rpm. The supernatant absorbance was read in a spectrophotometer at 532 nm, and three independent assays were performed in triplicate.

2.6. High-Fructose Diet-Induced Hyperlipidemia. High-fructose diet was prepared by mixing 330 g of commercial rodent chow (Labina) and 660 g of fructose and presented caloric value of 377 ± 4 kcal/100 g of chow. Commercial rodent chow (Labina) with caloric value of 332 ± 1 kcal/100 g of chow was used as control.

2.7. Experimental Design

2.7.1. Animals. All procedures with animals were performed in accordance with the ethical principles of animal experimentation adopted by the National Council of Animal Experimentation Control (CONCEA) and were approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Grande Dourados (UFGD) under the protocol number 025/2012.

Wistar rats with about 60 days weighing between 140 and 150 g of body weight (BW) were pretreated with ad libitum diet rich in fructose for 16 weeks to induce hyperlipidemia, which was confirmed by the serum levels of total cholesterol, 79.0 ± 2.9 to 128.0 ± 14.5 mg/dL, and triglyceride levels of 137.3 ± 23.0 to 243.3 ± 32.4 mg/dL. After this assessment, the animals continue receiving the diet rich in fructose and were randomly divided into four experimental groups (n = 8 per group) as follows: HFD (high-fructose diet + 300 μL of water), HFD-S (HFD + 30 mg of simvastatin by kg of BW), HFD-C (HFD + 2 mg ciprofibrate by kg of BW), and HFD-ExCA (HFD + 200 mg of *Campomanesia adamantium* root aqueous extract by kg of BW). For in vivo treatment, the lyophilized extract, ciprofibrate, and simvastatin were dissolved in distilled water daily before use and administered by gavage.

Animals fed with standard rodent commercial chow and 300 μL of water by gavage (n = 8 per group) formed the CT group and they were considered normolipidemic.

2.7.2. Organs and Tissues Available and Biochemical Analysis. After euthanasia the liver, heart, lung, kidney, spleen, soleus, and extensor digitorum longus (EDL) muscle were isolated and weighed. The collected blood was centrifuged at 3000 rpm for 10 min and the serum was used to measure total cholesterol, HDL cholesterol, triglycerides, and aminotransferases (AST and ALT) with support of equipment Integra 400 Plus (Roche™).

2.7.3. Dosage of Malondialdehyde (MDA). The concentration of MDA as index of lipid peroxidation was determined by incubating 200 μL of serum from the animal of the groups HFD and HFD-ExCA for 45 min at 93°C with 1 mL of 10 nmol TBA. After this period, the samples were kept at room temperature for 15 min and added to 3 mL of butanol with subsequent agitation and centrifugation for 5 min at 3000 rpm. The supernatant absorbance was performed at 532 nm [15].

Table 1: Antioxidant activity: IC_{50} and percentage of maximum activity of DPPH free radical scavenging of *Campomanesia adamantium* root aqueous extract (ExCA) compared to standard antioxidant, ascorbic acid, and BHT.

Sample	IC_{50} (μg/mL)	N	Maximum activity	
			%	μg/mL
Ascorbic acid	2.5 ± 0.6	(4)	98	25
BHT	36.1 ± 9.1	(4)	93	500
ExCA	37.3 ± 4.1	(4)	96	250

Values are expressed as mean ± SEM.

2.7.4. Liver Lipids. Quantification of total lipids in the liver was performed in lyophilized sample subjected to extraction with chloroform and methanol (2 : 1) according to Association of Official Analytical Chemists, 1970.

2.8. Statistical Analysis. Data are shown as the mean ± standard error of the mean and were submitted to one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls posttest. The results were considered significant when $P < 0.05$.

3. Results

3.1. Chemical Profile. The chemical profile of the extract showed the presence of gallic acid in 6.5 min and ellagic acid in 18.38 min and other metabolites containing the same chromophore of the ellagic acid (Figure 1). The concentration of gallic acid and ellagic acid was 2.83 ± 0.01 and 15.48 ± 0.50 μg/mg of ExCA, respectively.

3.2. Antioxidant Activity. Considering the presence of antioxidants, gallic acid, and ellagic acid in the extract, we investigated the DPPH free radical scavenging activity in order to determine the in vitro antioxidant activity. From the various concentrations tested, the half-maximal inhibitory concentration (IC_{50}) of DPPH free radical scavenging and the maximum activity of ExCA were calculated, which are shown in Table 1. IC_{50} of ExCA was similar to BHT but was about 15 times higher than that of ascorbic acid.

After checking the DPPH free radical capture capability, we investigated the antioxidant activity of ExCA in in vitro cellular model. The ExCA protected the human erythrocyte of the hemolysis induced by AAPH until 180 min at all concentrations evaluated similarly to ascorbic acid, except in the concentration of 50 mg/mL (Figure 2).

The ability of the ExCA to protect against AAPH-induced lipid peroxidation of human erythrocytes was evaluated through the dosage of MDA. ExCA provided concentration-dependent reduction in the generation of MDA similar to ascorbic acid (Figure 3).

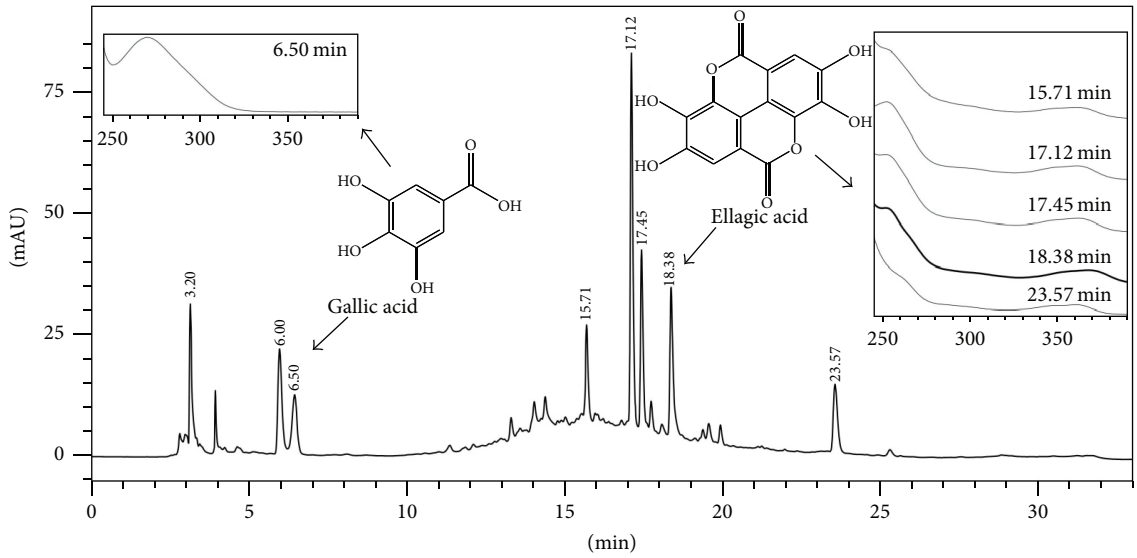

Figure 1: Identification of compounds in the aqueous extract of *Campomanesia adamantium* roots by HPLC-DAD (270 nm).

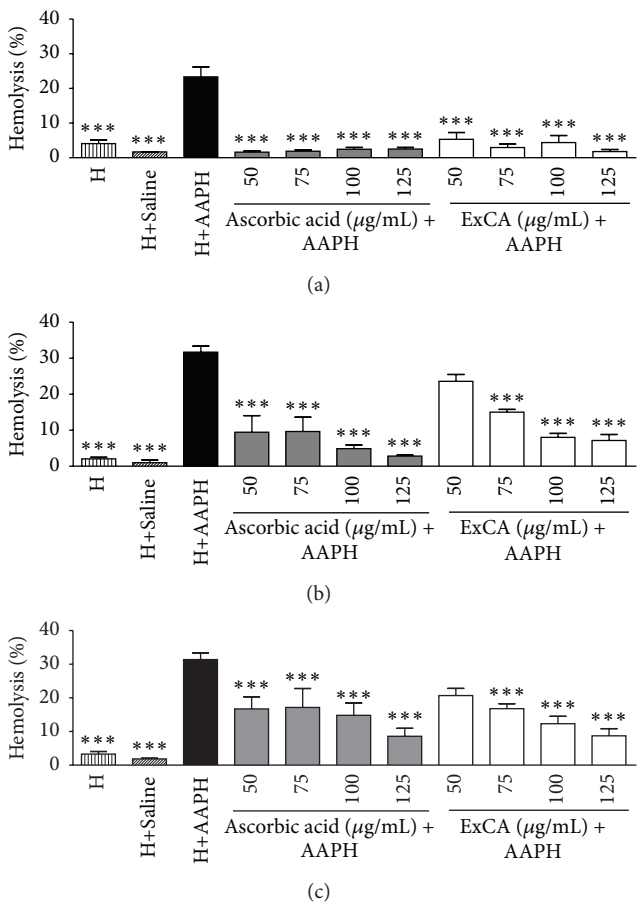

Figure 2: Hemolysis assessment at (a) 60, (b) 120, and (c) 180 min after addition of AAPH in 2.5% erythrocytes (H) incubated with different concentrations (50–125 μg/mL) of ascorbic acid and *Campomanesia adamantium* root aqueous extract (ExCA). $^{***}P < 0.001$ versus H+AAPH.

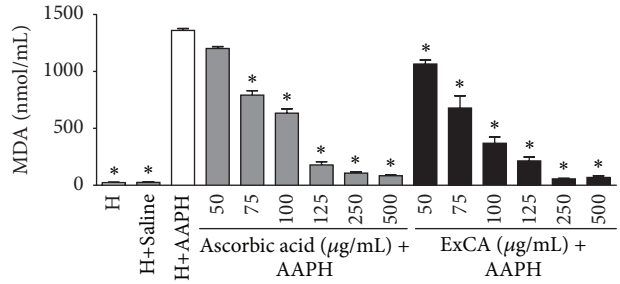

Figure 3: Malondialdehyde (MDA) content at 180 min after addition of the AAPH hemolysis inducer in 2.5% erythrocytes (H) incubated with different concentrations (50–500 μg/mL) of ascorbic acid and *Campomanesia adamantium* root aqueous extract (ExCA). $^{*}P < 0.001$ versus H+AAPH.

3.3. Hypolipidemic Activity. During the eight weeks of treatment, the evolution of body weight was similar between all the animals fed with HFD (Figure 4(a)) and higher than CT group. However, a reduction in caloric intake of animals in HFD groups was observed, compared to the CT group (Figure 4(b)).

The assessment of organ weights reveals that HFD-ExCA and HFD-C groups showed recovery of the liver mass compared to CT group and higher than HFD group (Table 2). Additionally, no differences were observed in serum levels of liver injury markers (AST and ALT) and other organs investigated (Table 2).

Confirming the antioxidant activity of ExCA, we investigated the lipid lowering in vivo activity of the extract. Animals treated with ExCA show decreased serum levels of total cholesterol and triglycerides, when compared with hyperlipidemic animals of the HFD group, similar to those presented by the animals treated with simvastatin (HFD-S), ciprofibrate (HFD-C), and normolipidemic animals (CT)

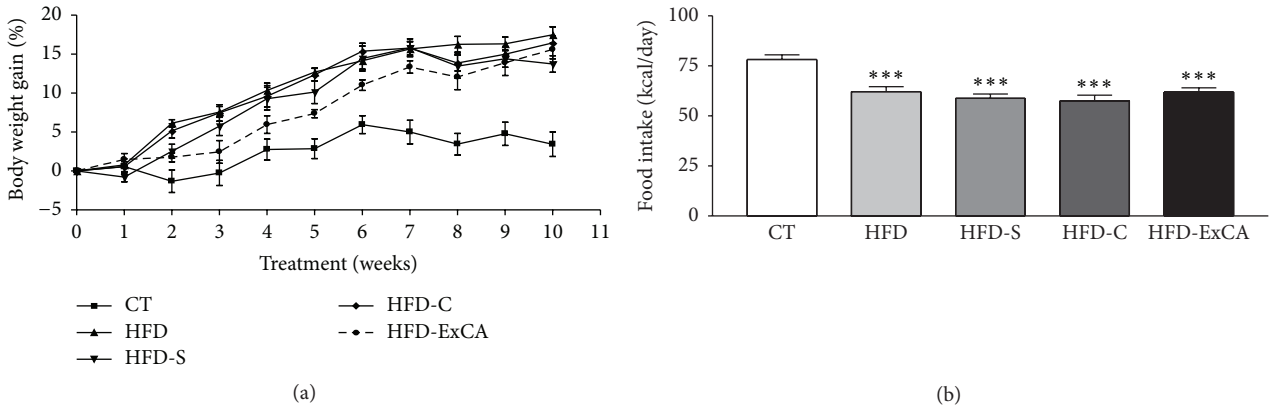

Figure 4: (a) Body weight gain and (b) daily caloric intake of normolipidemic (CT) and hyperlipidemic rats treated with water (HFD), simvastatin (30 mg/kg of BW, HFD-S), ciprofibrate (2 mg/kg of BW, HFD-C), and *Campomanesia adamantium* root aqueous extracts (200 mg/kg of BW, HFD-ExCA) during eight weeks by gavage. $n = 8$ in each group. $^{***}P < 0.001$ versus CT group.

Table 2: Tissues, organs, and serum levels of AST and ALT of normolipidemic (CT) and hyperlipidemic Wistar rats treated with water (HFD), simvastatin (HFD-S), ciprofibrate (HFD-C), and *Campomanesia adamantium* root aqueous extract (HFD-ExCA).

Parameters	CT	HFD	HFD-S	HFD-C	HFD-ExCA
Soleus (g/100 g of BW)	0.03 ± 0.001	0.03 ± 0.003	0.03 ± 0.001	0.03 ± 0.003	0.02 ± 0.004
EDL (g/100 g of BW)	0.01 ± 0.002	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.002
Heart (g/100 g of BW)	0.32 ± 0.01	0.34 ± 0.01	0.33 ± 0.004	0.30 ± 0.04	0.31 ± 0.009
Kidney (g/100 g of BW)	0.60 ± 0.02	0.54 ± 0.01	0.61 ± 0.01	0.56 ± 0.08	0.59 ± 0.01
Liver (g/100 g of BW)	2.89 ± 0.03	2.71 ± 0.05	2.85 ± 0.06	3.08 ± 0.06***	3.09 ± 0.07***
AST (U/L)	174.0 ± 6.5	178.0 ± 14.5	219.5 ± 19.8	195.0 ± 15.9	154.1 ± 15.2
ALT (U/L)	53.2 ± 3.3	65.7 ± 7.0	64.8 ± 5.8	65.0 ± 4.1	71.9 ± 7.4

BW, body weight; EDL, extensor digitorum longus muscle; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Mean ± SEM. $^{***}P < 0.001$ versus HFD group.

(Figures 5(a) and 5(b)). There was no change in HDL serum levels among the groups investigated (Figure 5(c)).

Considering the chemical constituents detected in ExCA and its antioxidant activity as well as reduction of serum lipids, we investigated the serum levels of malondialdehyde (MDA) in vivo. The hyperlipidemic animals treated with ExCA show decreased MDA serum levels compared to controls of the HFD group (Figure 6).

4. Discussion

Our results show by first time that the *Campomanesia adamantium* root aqueous extract reduces serum levels of total cholesterol and triglycerides similar to the reference drugs used for treatment of dyslipidemia and possesses antioxidant properties. These activities probably are due to its chemical constituents, partially the ellagic and gallic acids derivatives present in the ExCA, and other unidentified phenolic compounds including flavonoids.

Phenolic compounds are among the major classes of secondary metabolites of plants and several studies have demonstrated their capacity for radical free scavenging [16, 17].

The ellagic and gallic acids derivatives, which present in ExCA, have a direct action in the capture of free radicals [18] and, in addition, are capable of stimulating the activity of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [19, 20], contributing to the antioxidant actions observed. The action of the ExCA in the capture of free radicals was demonstrated in this study and its possible action on antioxidant enzymes may be evaluated in future studies.

The activity of free radical scavenging contributes to lower generation of malondialdehyde (MDA). This is a toxic product resulting from the degradation of the cell membrane by lipid peroxidation which leads to release of unsaturated fatty acids and phospholipids disintegration, causing cell ruptures and mutations [21]. The ExCA reduced the generation of MDA in erythrocytes induced with AAPH, which probably contributes to the conservation of the cell structure. This effect demonstrated in vitro was confirmed in vivo, since reduction of MDA in the serum of hyperlipidemic animals treated with ExCA was observed. Amin and Nagy [22] correlated phenolic compounds present in the extracts of various plants with reduced peroxidation and production of MDA in rats treated with high-fat diet and protection against LDL oxidation.

The oxidation of the LDL decreases their interaction with specific membrane receptors and increases its permanence in the bloodstream, making oxidized LDL a proinflammatory marker [23, 24]. These structural and functional changes of LDL favor the development of atherosclerotic

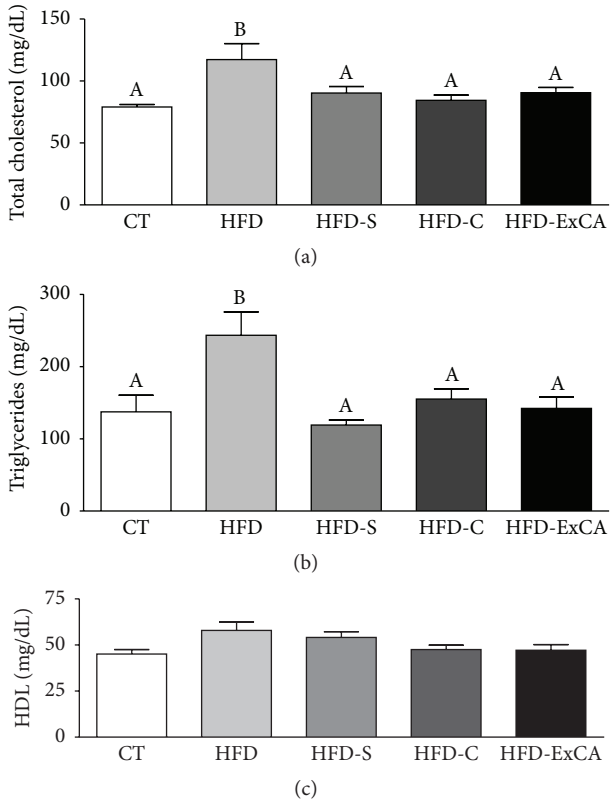

Figure 5: (a) Total cholesterol, (b) triglycerides, and (c) HDL cholesterol serum levels of normolipidemic (CT) and hyperlipidemic rats treated with water (HFD), simvastatin (30 mg/kg of BW, HFD-S), ciprofibrate (2 mg/kg of BW, HFD-C), and *Campomanesia adamantium* root aqueous extracts (200 mg/kg of BW, HFD-ExCA) during eight weeks by gavage. $n = 8$ in each group. Different superscript letters indicate significant differences at $P < 0.05$ between groups.

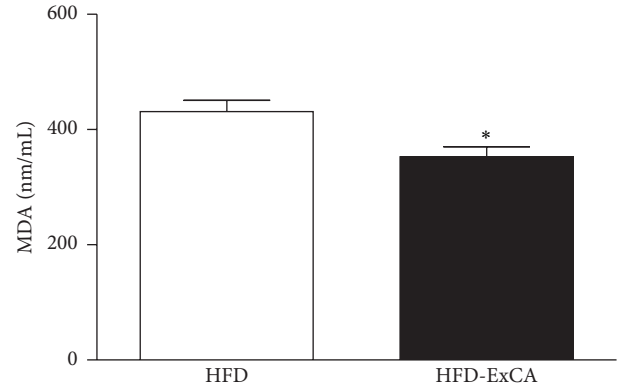

Figure 6: Malondialdehyde (MDA) serum level in hyperlipidemic rats treated with water (300 μL, HFD) and *Campomanesia adamantium* root aqueous extract (200 mg/kg of BW, HFD-ExCA) during eight weeks by gavage. $n = 8$ in each group. $^{*}P < 0.05$ versus HFD group.

plaque in blood vessels, concomitant with the increase in lipid peroxidation products, such as malondialdehyde [25]. Antioxidants, as ExCA, can reduce oxidation of LDL and attenuate the atherosclerotic vascular lesion, lowering stroke occurrence under this condition [26]. Kannan and Quine [27] found that ellagic acid administered to rats in a model of hypertrophy and arrhythmias induced experimentally reduced hyperlipidemia and lipid peroxidation in these animals because of its ability to inhibit the activity of 3-hydroxy-3-methylglutaryl-CoA reductase. In addition, ellagic acid has anti-inflammatory activity [28, 29] and renal protective effect attributed to its antioxidant potential [19].

Medicinal plants, beyond antioxidant activity, have been widely used as lipid-lowering agents [22, 30] and several studies have confirmed this ability [9, 31, 32], as observed for ExCA, which was capable of reducing total serum cholesterol and triglycerides similar to drugs currently used for the treatment of hypercholesterolemia and hypertriglyceridemia, ciprofibrate and simvastatin, respectively. The concomitant reduction in cholesterol and triglycerides induced by ExCA is an advantage, since the hypertriglyceridemia rarely occurs as a single factor and is almost always accompanied by high levels of total cholesterol [33]. Thus, the search of new drugs that have broad effects and are less toxic and capable of reducing serum lipid and controlling oxidative stress associated with dyslipidemia is growing [20]. These effects were described by Usta et al. [34] who describe many benefits of ellagic acid that is present in many fruits and nuts with antioxidant power and lowering lipid. Although the liver mass of the animals treated with the ExCA has increased, the marker enzymes of liver damage (AST and ALT) were unchanged.

The mechanism involved in cholesterol reduction may be inhibition of the enzyme HMG-CoA reductase, as observed by Klafke et al., [7] for *Campomanesia xanthocarpa*. However, the reduction of triglyceride possibly involves increased lipolysis either by raising the catabolism of VLDL and chylomicrons or by the activity of lipoprotein lipase; mechanisms have not been described yet for *Campomanesia* gender.

Together, the results reveal the antioxidant effect *Campomanesia adamantium* root aqueous extract and its ability to lower lipid, making it promising to prevent or treat dyslipidemia and furthermore expand our knowledge of plants used in traditional medicine.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The authors would like to thank the Foundation to Support to Development of Education, Science and Technology of Mato Grosso do Sul State (FUNDECT), Brazilian National Research Council (CNPq), and Coordination of Improvement of Higher Level (CAPES). Edson Lucas dos Santos, Maria do Carmo Vieira, and Carlos Alexandre Carollo are recipients of fellowship from Brazilian National Research Council (CNPq), Brazil.

References

[1] S. Mendis, P. Puska, and B. Norrving, *Global Atlas on Cardiovascular Disease Prevention and Control*, World Health Organization, Geneva, Switzerland, 2011.

[2] Y.-J. Geng and L. Jonasson, "Linking immunity to atherosclerosis: Implications for vascular pharmacology-a tribute to Göran K. Hansson," *Vascular Pharmacology*, vol. 56, no. 1-2, pp. 29–33, 2012.

[3] S. Lacroix, C. Des Rosiers, J.-C. Tardif, and A. Nigam, "The role of oxidative stress in postprandial endothelial dysfunction," *Nutrition Research Reviews*, vol. 25, no. 2, pp. 288–301, 2012.

[4] M. T. Elnakish, H. H. Hassanain, P. M. Janssen, M. G. Angelos, and M. Khan, "Emerging role of oxidative stress in metabolic syndrome and cardiovascular diseases: important role of Rac/NADPH oxidase," *The Journal of Pathology*, vol. 231, no. 3, pp. 290–300, 2013.

[5] A. M. Pisoschi and A. Pop, "The role of antioxidants in the chemistry of oxidative stress: a review," *European Journal of Medicinal Chemistry*, vol. 97, article 7855, pp. 55–74, 2015.

[6] D. Pastori, R. Carnevale, and P. Pignatelli, "Is there a clinical role for oxidative stress biomarkers in atherosclerotic diseases?" *Internal and Emergency Medicine*, vol. 9, no. 2, pp. 123–131, 2014.

[7] J. Z. Klafke, M. A. da Silva, T. F. Panigas et al., "Effects of *Campomanesia xanthocarpa* on biochemical, hematological and oxidative stress parameters in hypercholesterolemic patients," *Journal of Ethnopharmacology*, vol. 127, no. 2, pp. 299–305, 2010.

[8] P. Palozza, A. Catalano, R. E. Simone, M. C. Mele, and A. Cittadini, "Effect of lycopene and tomato products on cholesterol metabolism," *Annals of Nutrition and Metabolism*, vol. 61, no. 2, pp. 126–134, 2012.

[9] J. Z. Klafke, M. Arnoldi Da Silva, M. Fortes Rossato et al., "Antiplatelet, antithrombotic, and fibrinolytic activities of *Campomanesia xanthocarpa*," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 954748, 8 pages, 2012.

[10] B. E. O. Markman, E. M. Bacchi, and E. T. M. Kato, "Antiulcerogenic effects of Campomanesia xanthocarpa," *Journal of Ethnopharmacology*, vol. 94, no. 1, pp. 55–57, 2004.

[11] M. W. Biavatti, C. Farias, F. Curtius et al., "Preliminary studies on *Campomanesia xanthocarpa* (Berg.) and *Cuphea carthagenensis* (Jacq.) J.F. Macbr. aqueous extract: weight control and biochemical parameters," *Journal of Ethnopharmacology*, vol. 93, no. 2-3, pp. 385–389, 2004.

[12] A. S. Vinagre, Â. D. R. O. Rönnau, S. F. Pereira, L. U. Da Silveira, E. D. F. Wiilland, and E. S. Suyenaga, "Anti-diabetic effects of *Campomanesia xanthocarpa* (Berg) leaf decoction," *Brazilian Journal of Pharmaceutical Sciences*, vol. 46, no. 2, pp. 167–177, 2010.

[13] A. C. R. F. Pascoal, C. A. Ehrenfried, M. N. Eberlin, M. É. A. Stefanello, and M. J. Salvador, "Free radical scavenging activity, determination of phenolic compounds and HPLC-DAD/ESI-MS profile of *Campomanesia adamantium* leaves," *Natural Product Communications*, vol. 6, no. 7, pp. 969–972, 2011.

[14] D. Gupta and R. K. Gupta, "Bioprotective properties of Dragon's blood resin: *in vitro* evaluation of antioxidant activity and antimicrobial activity," *BMC Complementary and Alternative Medicine*, vol. 11, article 13, 2011.

[15] J. F. Campos, U. P. dos Santos, L. F. B. Macorini et al., "Antimicrobial, antioxidant and cytotoxic activities of propolis from *Melipona orbignyi* (Hymenoptera, Apidae)," *Food and Chemical Toxicology*, vol. 65, pp. 374–380, 2014.

[16] 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.

[17] D. A. Vattem, R. Ghaedian, and K. Shetty, "Enhancing health benefits of berries through phenolic antioxidant enrichment: focus on cranberry," *Asia Pacific Journal of Clinical Nutrition*, vol. 14, no. 2, pp. 120–130, 2005.

[18] P. P. Singh and S. M. Chauhan, "Activity-guided isolation of antioxidants from the leaves of Terminalia arjuna," *Natural Product Research*, vol. 28, no. 10, pp. 760–763, 2014.

[19] V. Vijaya Padma, P. Kalai Selvi, and S. Sravani, "Protective effect of ellagic acid against TCDD-induced renal oxidative stress: modulation of CYP1A1 activity and antioxidant defense mechanisms," *Molecular Biology Reports*, vol. 41, no. 7, pp. 4223–4232, 2014.

[20] P.-C. Chao, C.-C. Hsu, and M.-C. Yin, "Anti-inflammatory and anti-coagulatory activities of caffeic acid and ellagic acid in cardiac tissue of diabetic mice," *Nutrition and Metabolism*, vol. 6, article 33, 2009.

[21] L.-F. Wang, "Physiological and molecular responses to variation of light intensity in rubber tree (*Hevea brasiliensis* Muell. Arg.)," *PLoS ONE*, vol. 9, no. 2, Article ID e89514, 2014.

[22] K. A. Amin and M. A. Nagy, "Effect of Carnitine and herbal mixture extract on obesity induced by high fat diet in rats," *Diabetology and Metabolic Syndrome*, vol. 1, article 17, 2009.

[23] A. Fan, X. Wu, H. Wu et al., "Atheroprotective effect of Oleoylethanolamide (OEA) targeting oxidized LDL," *PLoS ONE*, vol. 9, no. 1, Article ID e85337, 2014.

[24] M. Estruch, J. L. Sánchez-Quesada, J. Ordóñez Llanos, and S. Benítez, "Electronegative LDL: a circulating modified LDL with a role in inflammation," *Mediators of Inflammation*, vol. 2013, Article ID 181324, 13 pages, 2013.

[25] H. Heinle, U. Brehme, O. Kelber, W. Schneider, and D. Weiser, "Oxidative stress in atherogenesis: basic mechanisms and problems of therapy with antioxidants," *Experimental and Clinical Cardiology*, vol. 6, no. 2, pp. 77–80, 2001.

[26] Z. Bahadoran, P. Mirmiran, and F. Azizi, "Dietary polyphenols as potential nutraceuticals in management of diabetes: a review," *Journal of Diabetes and Metabolic Disorders*, vol. 12, article 43, 2013.

[27] M. M. Kannan and S. D. Quine, "Ellagic acid inhibits cardiac arrhythmias, hypertrophy and hyperlipidaemia during myocardial infarction in rats," *Metabolism: Clinical and Experimental*, vol. 62, no. 1, pp. 52–61, 2013.

[28] D. C. Favarin, J. R. de Oliveira, C. J. F. de Oliveira, and A. D. P. Rogerio, "Potential effects of medicinal plants and secondary metabolites on acute lung injury," *BioMed Research International*, vol. 2013, Article ID 576479, 12 pages, 2013.

[29] C. D. F. Alves, G. N. Angeli, D. C. Favarin et al., "The effects of proresolution of ellagic acid in an experimental model of allergic airway inflammation," *Mediators of Inflammation*, vol. 2013, Article ID 863198, 9 pages, 2013.

[30] H. Shimoda, J. Tanaka, M. Kikuchi et al., "Effect of polyphenol-rich extract from walnut on diet-induced hypertriglyceridemia in mice via enhancement of fatty acid oxidation in the liver," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 5, pp. 1786–1792, 2009.

[31] J. Lachman, M. Šulc, K. Faitová, and V. Pivec, "Major factors influencing antioxidant contents and antioxidant activity in grapes and wines," *International Journal of Wine Research*, vol. 1, no. 1, pp. 101–121, 2009.

[32] A. Basu, M. Wilkinson, K. Penugonda, B. Simmons, N. M. Betts, and T. J. Lyons, "Freeze-dried strawberry powder improves lipid profile and lipid peroxidation in women with metabolic syndrome: baseline and post intervention effects," *Nutrition Journal*, vol. 8, no. 1, article 43, 2009.

[33] E. A. Brinton, "Management of hypertriglyceridemia for prevention of atherosclerotic cardiovascular disease," *Cardiology Clinics*, vol. 33, no. 2, pp. 309–323, 2015.

[34] C. Usta, S. Ozdemir, M. Schiariti, and P. E. Puddu, "The pharmacological use of ellagic acid-rich pomegranate fruit," *International Journal of Food Sciences and Nutrition*, vol. 64, no. 7, pp. 907–913, 2013.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5846865, 13 pages
http://dx.doi.org/10.1155/2016/5846865

Research Article

Protective Effects of Soy Oligopeptides in Ultraviolet B-Induced Acute Photodamage of Human Skin

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Received 5 February 2016; Revised 26 May 2016; Accepted 1 June 2016

Academic Editor: Renata Szymanska

Aim. We explored the effects of soy oligopeptides (SOP) in ultraviolet B- (UVB-) induced acute photodamage of human skin *in vivo* and foreskin *ex vivo*. *Methods*. We irradiated the forearm with 1.5 minimal erythemal dose (MED) of UVB for 3 consecutive days, establishing acute photodamage of skin, and topically applied SOP. Erythema index (EI), melanin index, stratum corneum hydration, and transepidermal water loss were measured by using Multiprobe Adapter 9 device. We irradiated foreskin *ex vivo* with the same dose of UVB (180 mJ/cm^2) for 3 consecutive days and topically applied SOP. Sunburn cells were detected by using hematoxylin and eosin staining. Apoptotic cells were detected by using terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Cyclobutane pyrimidine dimers (CPDs), p53 protein, Bax protein, and Bcl-2 protein were detected by using immunohistochemical staining. *Results*. Compared with UVB group, UVB-irradiated skin with topically applied SOP showed significantly decreased EI. Compared with UVB group, topical SOP significantly increased Bcl-2 protein expression and decreased CPDs-positive cells, sunburn cells, apoptotic cells, p53 protein expression, and Bax protein expressions in the epidermis of UVB-irradiated foreskin. *Conclusion*. Our study demonstrated that topical SOP can protect human skin against UVB-induced photodamage.

1. Introduction

When the skin receives more than the maximum tolerated dose of ultraviolet (UV) irradiation, an acute phototoxic reaction can occur. Ultraviolet B (UVB) can induce 100–800 times greater biological effects in the skin compared with ultraviolet A (UVA) of the same dose, and the most visual indicator of acute injury to skin caused by UVB is erythema. During erythema formation, there is infiltration of inflammatory cells in the dermis and sunburn cells in the epidermis. Sunburn cells are apoptotic keratinocytes, which show nuclear shrinkage and uniform cytoplasm and are eosinophilic and deeply stained. Sunburn cell formation is a defense mechanism for preventing malignant changes of epithelial cells [1].

Pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs) are two major types of DNA lesions induced by UVB [2]. CPDs are formed more abundantly and repaired less efficiently than the 6-4PPs and are therefore considered as the predominant UVB-induced DNA photolesions [2, 3]. If DNA lesions cannot be repaired in time, UVB-damaged cells will be removed by apoptosis, and p53 tumor suppressor protein is the key regulatory factor of apoptosis [4–6].

Soy oligopeptides (SOP) are hydrolysates obtained from proteolytic hydrolysis of soybean proteins, consisting of 3–6 amino acids with <1000 low molecular weight peptides, mainly 300–700 kDa [7]. Recently, various biological functions of soybean oligopeptides have been found such as antioxidant, blood pressure lowering, and blood lipid lowering effects [8, 9]. Our previous study (written in Chinese) has confirmed that soybean oligopeptides could effectively relieve photodamage induced by UVB irradiation in mice [10].

In this study, we conducted *in vivo* and *ex vivo* studies using human skin. In a clinical study, we topically applied SOP on UVB-irradiated forearm skin. We evaluated the erythema index (EI), melanin index (MI), transepidermal water loss (TEWL), and stratum corneum (SC) hydration of the irradiated skin at certain time points. In an *ex vivo* study, we applied SOP on UVB-irradiated foreskin. We detected sunburn cells, apoptotic cells, p53 protein, Bax protein, Bcl-2

protein, and CPDs. We aimed to evaluate the effects of SOP in UVB-irradiated human skin and the underlying mechanisms.

2. Materials and Methods

2.1. Ethics Statement. This study was approved by the institutional review board of Nanjing Medical University, Nanjing, China (approval number 2013-SRFA-025). Written informed consent was obtained from all participants before taking part in this research.

2.2. SOP Preparation. SOP was prepared from soybean protein isolates (SPI) obtained from Jilin Fuji Protein Co. Ltd. (Jilin, China), as described previously [11]. Alcalase (obtained from Novozymes Biological Co., Tianjin, China) at a ratio of 600,000 U/kg (enzyme/protein substrate) was added to the solution and the hydrolysis was kept at pH 8.5 by continuous addition of 20% NaOH. The degree of hydrolysis (DH) of soybean protein was calculated by using the pH stat method. After the DH reached around 10–15%, the suspension was cooled down to 50°C and added with Protex 13 FL (purchased from Genencor, Division of Danisco, Wuxi, China) at a ratio of 200,000 U/kg (enzyme/protein substrate). Then, the mixture was incubated at 50°C until the DH reached 20–25%. The reaction was stopped by heating the mixture to 90°C for 15 min to inactivate the enzyme, and the resulting hydrolysate was centrifuged at 15,000 ×g for 10 min (SYGQ105 tube centrifuge, Shanghai Shiyuan Bioengineering Equipment Co., Shanghai, China). The supernatant was filtered with UF-5000 ultrafiltration equipment (molecular weight cut-off 5,000 Da, Xinda Membrane Tech. Co., Hefei, China) and then evaporated with a double-effect falling film evaporator (OE2, OECH Machinery Equipment Co., Ltd., Hefei, China) at 0.10 ± 0.02 MPa and 60 ± 5°C, until the solid content of the concentrated liquid reached 30–40%. The concentrated solution containing peptides was dried with a spray drier (YG30, Wuxi City Sunlight Drier Factory, Wuxi, China) at a 15 kg/h flow rate with inlet temperature of 160–180°C and outlet temperature of 80–90°C. The peptides present in SOP extract were analyzed and quantified using HPLC. The peptide and free amino acid contents of SOP were 82.5 ± 1.13% and 3.7 ± 0.28%, respectively. The molecular weight distribution of SOP was mainly below 1,000 Da (85.4%), 56.7% of which were 140–500 Da. SOP creams were custom-order produced by Infinitus Ltd., China, and were used for *in vivo* and *ex vivo* experiments.

2.3. In Vivo Study Protocol

2.3.1. Volunteer Recruitment. Nine healthy male volunteers who were in the range from 23 to 26 years old with Fitzpatrick skin types III to IV were enrolled in the study. All volunteers had no light-related skin and systemic diseases. All volunteers denied any drug use in the past month prior to and throughout the experiment. Sunlight exposure on the experimental site was avoided throughout the experiment.

2.3.2. Group Division and Treatments. The flexor side of the left forearm was selected as the experimental site. The selected UVB dose was 180 mJ/cm^2. There were eight areas of 1.5 cm × 1.5 cm designated as the following 8 groups: (1) negative control group; (2) vehicle control group; (3) SOP group; (4) UVB group; (5) UVB + vehicle group; (6) UVB + 2.5 IU/mL SOP group; (7) UVB + 5.0 IU/mL SOP group; (8) UVB + 10.0 IU/mL SOP group. Hence, UVB dose = UVB irradiation intensity × irradiation time (s). The UVB irradiation apparatus was from Sigma High-Tech Co., Ltd. (Shanghai, China). UVB irradiation was delivered by using a Philips TL 20W/12 (Eindhoven, Netherlands) at an intensity of 1.5 mW/cm^2, a fluorescent bulb emitting 280–320 nm wavelength with a peak at 313 nm. Irradiation output was monitored using a UV-meter (Waldmann, Villingen-Schwenningen, Germany).

Five minutes after irradiation with 180 mJ/cm^2 UVB, SOP cream (provided by Infinitus Ltd., China) at 3 different concentrations (2.5, 5.0, and 10.0 IU/mL) was topically applied on the selected areas. This procedure was done for 3 consecutive days. MI, EI, TEWL, and SC hydration were detected 1, 3, and 10 days after the last treatment.

2.3.3. Detection of Skin Indexes Using Multifunctional Skin Test. The experimental site was cleansed with warm water, free from skin care products or drugs, and the volunteers were requested to have a seat and rest for 2 hours. The experimental site was then examined using Multiprobe Adapter (MPA) 9 device (CK Electronic, Germany) in a room with no direct sunlight and ventilation at a room temperature of 25-26°C and relative humidity of 50–60%. MI and EI were detected using Mexameter MX 18 probe, TEWL was detected using Tewameter TM 300 probe, and SC hydration was detected using Corneometer CM 825 probe. The instrument used in the present study has been sent to the manufacturer for calibration biannually.

2.4. Ex Vivo Study Protocol

2.4.1. Foreskin Preparation. Fresh and healthy foreskins from 20 cases (ages ranged from 20 to 30 years) were obtained by circumcisions as approved by the Ethics Committee in the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). The subcutaneous tissue was removed by scraping with forceps. The foreskin was washed 3 times with saline, each time for 3 minutes. The foreskin was cut into 4 parts and the size of each part was 0.5 cm × 1.0 cm. Roswell Park Memorial Institute (RPMI) medium (containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20 μM β-mercaptoethanol) was added to a Petri dish. The foreskin was placed into the Petri dish with the dermis facing downwards and the bottom immersed in the culture medium, whilst the epidermis was above the culture medium surface [12].

2.4.2. Group Division. The foreskins were randomly divided into 4 groups: (1) control group; (2) UVB group; (3) UVB + vehicle group; (4) UVB + 5.0 IU/mL SOP group. Each group was detected at 4 different time points (8, 24, 48, and 72 h after UVB irradiation).

2.4.3. UVB Irradiation and SOP Application Ex Vivo. UVB groups, UVB + vehicle groups, and UVB + SOP groups were placed into 24-well culture dish. The epidermal side of the foreskin was irradiated once by UVB at a dose of 180 mJ/cm^2 and an intensity of 1.5 mW/cm^2. 5 min after UVB irradiation, SOP cream was applied evenly on the epidermis of the foreskin by using a sterile cotton swab. Each foreskin was divided into 4 parts, immersed in the culture medium, and placed in the 37°C incubator for different times (8, 24, 48, or 72 h).

2.4.4. Histopathology Using Hematoxylin and Eosin (H&E) Staining. Tissues were fixed in 10% formalin and embedded in paraffin. Vertical sections (6 μm) were cut, mounted on glass slides, and stained with H&E. Microscopic evaluation (Olympus, Japan) and digital photography of the slides were taken. The mean number of sunburn cells (with a pyknotic nucleus and densely stained eosinophilic cytoplasm) was quantified in five random fields at 400 times magnification.

2.4.5. TUNEL Assay. To evaluate apoptosis, Terminal dUTP Nicked Labelling was used as a marker for apoptosis. TUNEL *in situ* detection kit was purchased from KeyGen Biotech, China. In brief, tissue sections (5 μm) after deparaffinization and re-hydration were permeabilized with proteinase K (30 mg/mL) for 1 h at 37°C. Thereafter, the sections were quenched of endogenous peroxidase activity using 3% hydrogen peroxide for 10 min. After thorough washing with 1x PBS, sections were incubated with equilibration buffer for 10 min, and then the TdT reaction mixture was added to the sections, except for the negative control, and incubated at 37°C for 1 h. The reaction was stopped by immersing the sections in 2x saline– sodium citrate buffer for 15 min. Sections were then added with streptavidin– HRP (1 : 500) for 30 min at room temperature, and after repeated washings, sections were incubated with substrate DAB until color development. The micrographs were obtained using a light microscope (Olympus, Japan) and the mean level of apoptotic cells (with brown granules in the nucleus) was quantified in five random fields at ×400 magnification.

2.4.6. Immunohistochemical Staining. Briefly, skin samples were processed conventionally before paraffin embedding, and serial 4 μm sections were cut and were deparaffinized and rehydrated with water. Endogenous peroxidase activity was blocked by 10 min incubation with 3% hydrogen peroxide, and slides were then incubated with 0.125% trypsin for 10 min at 37°C and then with 1 N HCl for 30 min at room temperature. Slides were washed with PBS and then incubated for 30 min with 10% goat serum in PBS. Sections were then incubated with CPDs-specific monoclonal antibody (Sigma Co. Ltd., St. Louis, MO, USA), p53-specific monoclonal antibody (Sigma Co. Ltd., St. Louis, MO, USA), Bax-specific monoclonal antibody (Santa Cruz Co., USA), and Bcl-2-specific monoclonal antibody (Santa Cruz Co., USA). Bound anti-CPDs antibody was detected by incubation with biotinylated goat anti-mouse IgG1 followed by peroxidase-labeled streptavidin. Slides were developed with 3,30-diaminobenzidine (DAB, Fuzhou Maixin Biotech. Co., Ltd.) as a substrate for 3–5 min. The sections were then rinsed with distilled water and counterstained with H&E, cleared, and mounted. The DAB-peroxidase reaction gave a brown reaction product. The mean level of CPDs and p53-positive cells was quantified under a light microscope (Olympus, Japan) in five random fields at ×400 magnification. Bax was mainly expressed as brownish materials in the cytoplasm. Bcl-2 was localized in the cytoplasm and cell membrane.

2.4.7. Immunohistochemical Analysis. Immunohistochemical evaluation was done as described by Bayramgürler et al. [13]. The proportion of the immunoreactive cells was assessed as follows: 0 meant there are no immunoreactive cells; 1 meant 1–25% of the cells are immunoreactive; 2 meant 26–50% of the cells are immunoreactive; 3 meant 51–75% of the cells are immunoreactive; 4 meant more than 75% of the cells are immunoreactive. The staining intensity of the cells was graded as 0 (no staining), 1 (light staining), 2 (moderate staining), and 3 (intense staining). An immunoreactivity intensity distribution index (IRIDI) was calculated as follows: the score of the staining intensity multiplied by the score of the proportion of the immunoreactive cells.

2.5. Statistical Analysis. Statistical analysis was performed by using SPSS for Windows version 16.0 (SPSS, Chicago, IL, USA). The statistical analysis for the *in vivo* study was carried out with the paired t-test. The statistical analysis for the *ex vivo* study was carried out with the Kruskal-Wallis H test and Mann-Whitney U test, and all were two-sided tests. Data were expressed as the mean ± SD for each group. A P value of less than 0.05 ($P < 0.05$) was considered statistically significant.

3. Results

3.1. In Vivo Study

3.1.1. The Effect of Topical SOP on the EI of UVB-Irradiated Human Skin. One, three, and ten days after UVB irradiation, the skin EI of all UVB-irradiated groups was significantly increased ($P < 0.05$) compared with negative control group. Compared with UVB group, the skin EI in UVB + 5.0 IU/mL SOP and UVB + 10.0 IU/mL SOP groups was significantly decreased ($P < 0.05$), whilst that of UVB + 2.5 IU/mL SOP group showed no significant difference ($P > 0.05$). The lowest skin EI was achieved by UVB + 5.0 IU/mL SOP group ($P < 0.05$). There was no significant difference in the skin EI among nonirradiated groups ($P > 0.05$) (Figure 1(a)).

3.1.2. The Effect of Topical SOP on the MI of UVB-Irradiated Human Skin. One day after UVB irradiation, there was no significant difference in the skin MI of UVB-irradiated groups compared with negative control group ($P > 0.05$), but it was significantly increased on days 3 and 10 ($P < 0.05$ and $P < 0.001$, resp.). Compared with UVB group, UVB + SOP groups showed no significant difference in the skin MI ($P > 0.05$). There was no significant difference in the skin MI among nonirradiated groups ($P > 0.05$) (Figure 1(b)).

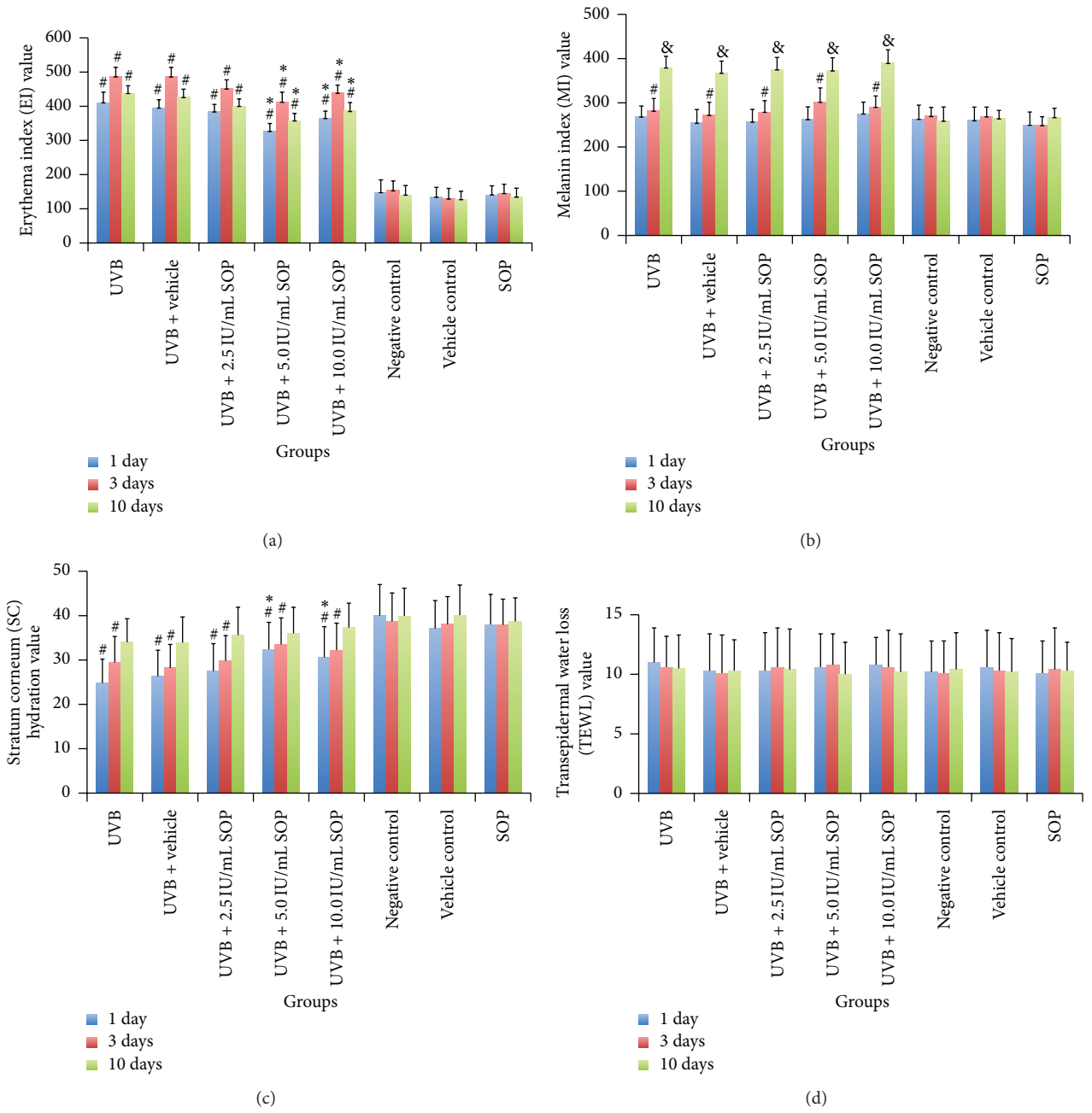

FIGURE 1: (a) Erythema index (EI) value in each group as detected by Mexameter MX 18 probe of MPA 9 device. (b) Melanin index (MI) value in each group as detected by Mexameter MX 18 probe of MPA 9 device. (c) Stratum corneum (SC) hydration value in each group as detected by Corneometer CM 825 probe of MPA 9 device. (d) Transepidermal water loss (TEWL) value in each group as detected by Tewameter TM 300 probe of MPA 9 device. Compared with ultraviolet B (UVB) group, the skin EI in UVB + 5.0 IU/mL soy oligopeptides (SOP) and UVB + 10.0 IU/mL SOP groups was significantly decreased ($P < 0.05$) and UVB + 2.5 IU/mL SOP group showed no significant difference ($P > 0.05$); the skin MI in UVB + SOP groups showed no significant difference ($P > 0.05$); the SC hydration on day 1 in UVB + 5.0 IU/mL SOP and UVB + 10 IU/mL SOP groups was significantly increased ($P < 0.05$), whilst that of days 3 and 10 showed no significant difference ($P > 0.05$), and the skin TEWL in UVB + SOP groups showed no significant difference ($P > 0.05$). The statistical analysis was carried out with the paired t-test; data were expressed as the mean ± SD for each group; $^{\&}P < 0.001$ when compared with negative control group of the same period; $^{\#}P < 0.05$ when compared with negative control group of the same period; $^{*}P < 0.05$ when compared with UVB group of the same period.

3.1.3. The Effect of Topical SOP on the SC Hydration of UVB-Irradiated Human Skin. One and three days after UVB irradiation, the SC hydration in each irradiated group was significantly decreased ($P < 0.05$) compared with negative control group, but there was no significant difference on day 10 ($P > 0.05$). Compared to UVB group, the SC hydration on day 1 in UVB + 5.0 IU/mL SOP and UVB + 10.0 IU/mL SOP groups was significantly increased ($P < 0.05$), whilst that of days 3

and 10 showed no significant difference ($P > 0.05$). There was no significant difference in the SC hydration among nonirradiated groups ($P > 0.05$) (Figure 1(c)).

3.1.4. The Effect of Topical SOP on TEWL of UVB-Irradiated Human Skin. One, three, and ten days after UVB irradiation, the skin TEWL in all UVB-irradiated groups showed no significant difference ($P > 0.05$) compared with negative control group. Compared with UVB group, the skin TEWL in UVB + SOP groups on days 1, 3, and 10 also showed no significant difference ($P > 0.05$). There was no significant difference in the skin TEWL among nonirradiated groups ($P > 0.05$) (Figure 1(d)).

3.2. Ex Vivo Study

3.2.1. The Effect of Topical SOP on the Proportion of CPDs-Positive Cells in the Epidermis after UVB Irradiation. After UVB irradiation, the epidermis CPDs-positive cells were stained brownish by immunohistochemical staining. There were no CPDs-positive cells in the epidermis of the control group. UVB-irradiated groups showed a large number of CPDs-positive cells in the epidermis, and the highest number was observed at 8 h. Then, 8, 24, 48, and 72 h after UVB irradiation, the proportion of CPDs-positive cells in UVB + SOP groups was significantly decreased compared with UVB group ($P < 0.05$). The difference in the proportion of CPDs-positive cells between UVB + vehicle and UVB groups was not significant ($P > 0.05$) (Figures 2(a) and 2(b)).

3.2.2. The Effect of Topical SOP on the Proportion of Sunburn Cells in the Epidermis after UVB Irradiation. Here, 8, 24, 48, and 72 h after UVB irradiation, there were a large number of sunburn cells in the epidermis of UVB-irradiated groups, and the highest number was observed at 24 h. The proportion of sunburn cells in the epidermis of UVB + SOP groups at 8, 24, 48, and 72 h after UVB irradiation was significantly decreased compared with UVB group ($P < 0.05$). The difference between UVB group and UVB + vehicle groups at different time points was not significant ($P > 0.05$) (Figures 3(a) and 3(b)).

3.2.3. The Effect of Topical SOP on the Proportion of Apoptotic Cells in the Epidermis after UVB Irradiation. The apoptotic cells were stained brown by TUNEL staining. There was no apoptotic cell in the epidermis of the control group. There were a large number of apoptotic cells in the epidermis of UVB-irradiated groups, and the highest number was at 24 h. Compared with UVB group, the proportion of apoptotic cells in UVB + SOP groups at 8, 24, 48, and 72 h was significantly decreased ($P < 0.05$). There was no significant difference between UVB + vehicle and UVB groups ($P > 0.05$) (Figures 4(a) and 4(b)).

3.2.4. The Effect of Topical SOP on the p53 Protein in the Epidermis after UVB Irradiation. The p53 protein expression in the epidermis of control group at 8, 24, 48, and 72 h was relatively weak. The p53 protein expression in the epidermis of UVB-irradiated groups at each time point was increased, and the highest p53 protein expression was achieved at 24 h. Compared with UVB group, the p53 protein expression in UVB + SOP groups at 8, 24, and 48 h was significantly decreased ($P < 0.01$), whilst there was no significant difference in the IRIDI of p53 protein at 72 h ($P > 0.05$). There was no significant difference in the IRIDI of p53 protein between UVB + vehicle and UVB groups ($P > 0.05$) (Table 1, Figure 5).

3.2.5. The Effect of Topical SOP on the Epidermis Bax Proteins after UVB Irradiation. The Bax protein expression in the epidermis of control group at 8, 24, 48, and 72 h was weak. The Bax protein expression in the epidermis of all UVB-irradiated groups at all time points was increased, and the highest Bax protein expression was at 24 h. Compared with UVB group, UVB + SOP group showed significantly decreased expression of Bax protein in the epidermis at 8, 24, and 48 h ($P < 0.05$), whilst there was no significant difference in the IRIDI of Bax protein at 72 h ($P > 0.05$). There was no significant difference in the IRIDI of Bax protein between UVB + vehicle and UVB groups ($P > 0.05$) (Table 2, Figure 6).

3.2.6. The Effect of Topical SOP on the Epidermis Bcl-2 Proteins after UVB Irradiation. The Bcl-2 protein expression in the epidermis of all UVB-irradiated groups at 8, 24, 48, and 72 h was significantly decreased ($P < 0.05$) compared with control group. Compared with UVB group, UVB + SOP group showed significantly increased expression of Bcl-2 protein in the epidermis at 48 and 72 h ($P < 0.05$). There was no expression of Bcl-2 protein detected in the UVB and UVB + vehicle groups at 8 and 24 h and the expression of Bcl-2 protein in UVB + SOP groups was also very weak. There was no significant difference in the Bcl-2 protein expression between UVB + vehicle and UVB groups ($P > 0.05$) (Table 3, Figure 7).

4. Discussion

Erythema reactions produced by skin exposure to simulated UV can be divided into immediate and delayed erythema. UVB irradiation above erythemal dose can cause skin inflammatory erythema and delayed hyperpigmentation, requiring several days to several weeks to subside. Minimal erythema dose (MED) is the amount of UV radiation that will produce minimal erythema (sunburn or redness caused by engorgement of capillaries) of an individual's skin within 24 hours following exposure. Lim et al. demonstrated that 2.5 MED of UVB irradiation increased skin EI and reached the highest value on day 2 after irradiation, whilst the skin MI was obviously increased on day 3 [14]. In accordance with this, we found that 180 mJ/cm^2 of UVB irradiation for 3 consecutive days significantly increased skin EI on day 1 after irradiation, whilst skin MI was increased later on day 3. Topical 5.0 IU/mL and 10.0 IU/mL SOP significantly decreased skin EI in UVB-irradiated skin, indicating that a certain amount of SOP could accelerate erythema to subside. However, topical SOP of different doses made no significant difference in the skin MI of UVB-irradiated skin, indicating that topical SOP could not

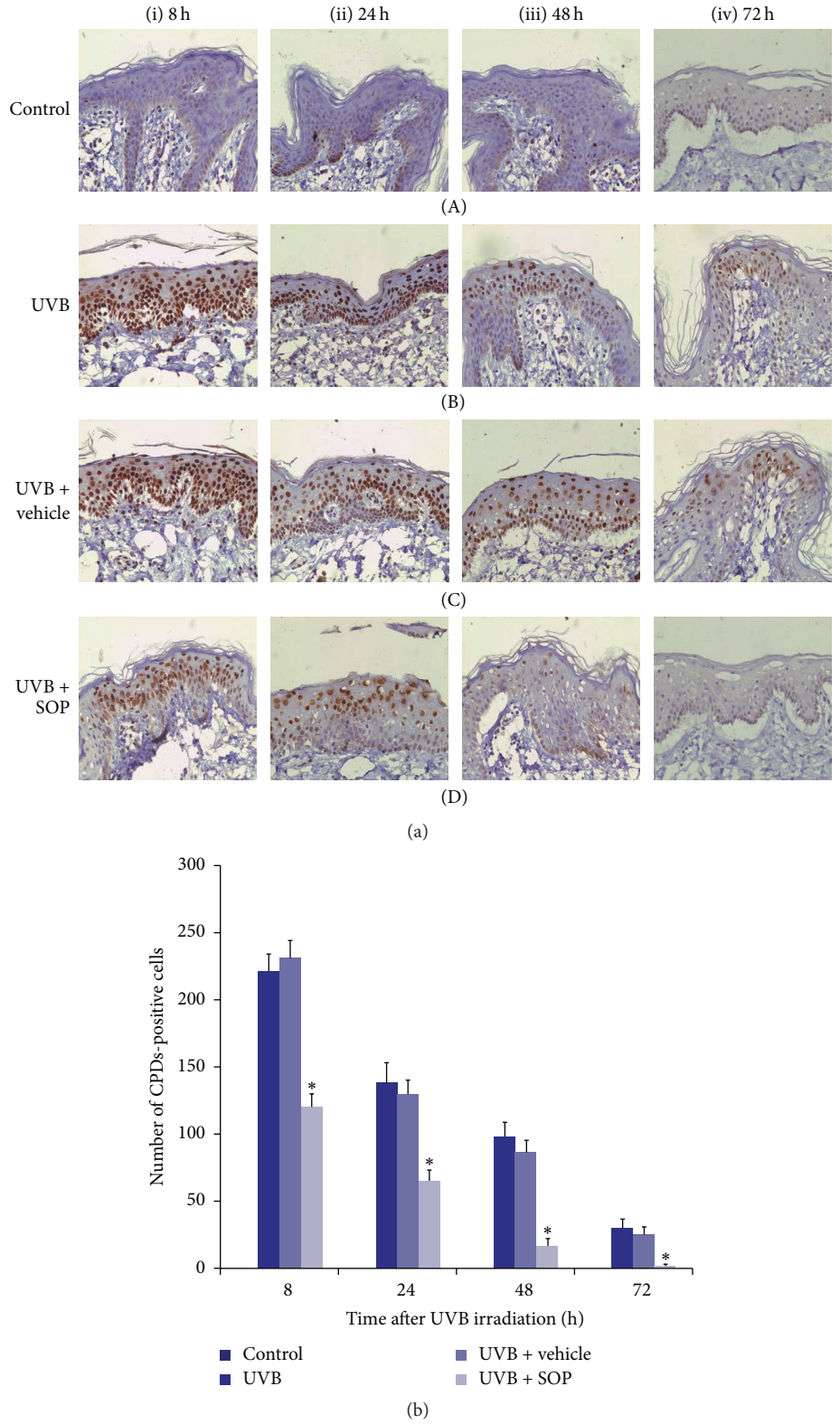

Figure 2: (a) Cyclobutane pyrimidine dimers- (CPDs-) positive cells in the epidermis of each group, stained by immunohistochemical staining and detected by light microscopy with ×400 magnifications. (A) Control; (B) UVB group; (C) UVB + vehicle group; (D) UVB + 5.0 IU/mL SOP group. (i) 8 h; (ii) 24 h; (iii) 48 h; (iv) 72 h. (b) The number of CPDs-positive cells in the epidermis of each group. Then, 8, 24, 48, and 72 h after ultraviolet B (UVB) irradiation, the number of CPDs-positive cells in UVB + 5.0 IU/mL SOP groups was significantly decreased compared with UVB group ($P < 0.05$); stained by immunohistochemical staining and detected by light microscopy with ×400 magnifications; $^{*}P < 0.05$ when compared with the UVB group.

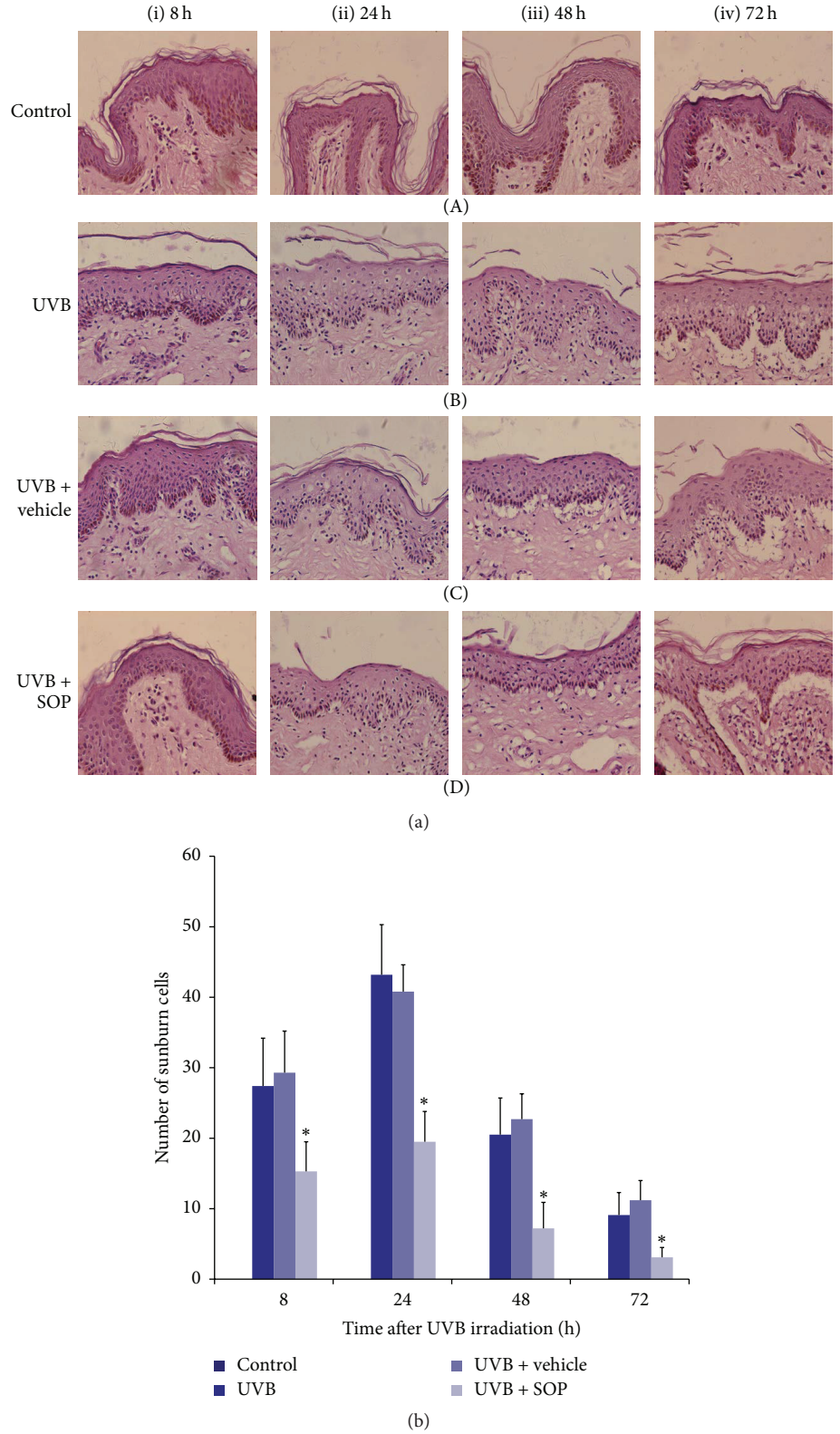

Figure 3: (a) Sunburn cells in the epidermis of each group, stained by hematoxylin and eosin (H&E) staining and detected by light microscopy with ×400 magnifications. (A) Control; (B) UVB group; (C) UVB + vehicle group; (D) UVB + 5.0 IU/mL SOP group. (i) 8 h; (ii) 24 h; (iii) 48 h; (iv) 72 h. (b) The number of sunburn cells in the epidermis of each group. The number of sunburn cells in the epidermis of UVB + 5.0 IU/mL SOP groups at 8, 24, 48, and 72 h after UVB irradiation was significantly decreased compared with UVB group ($P < 0.05$). Stained by H&E staining and detected by light microscopy with ×400 magnifications. $^{*}P < 0.05$ when compared with the UVB group.

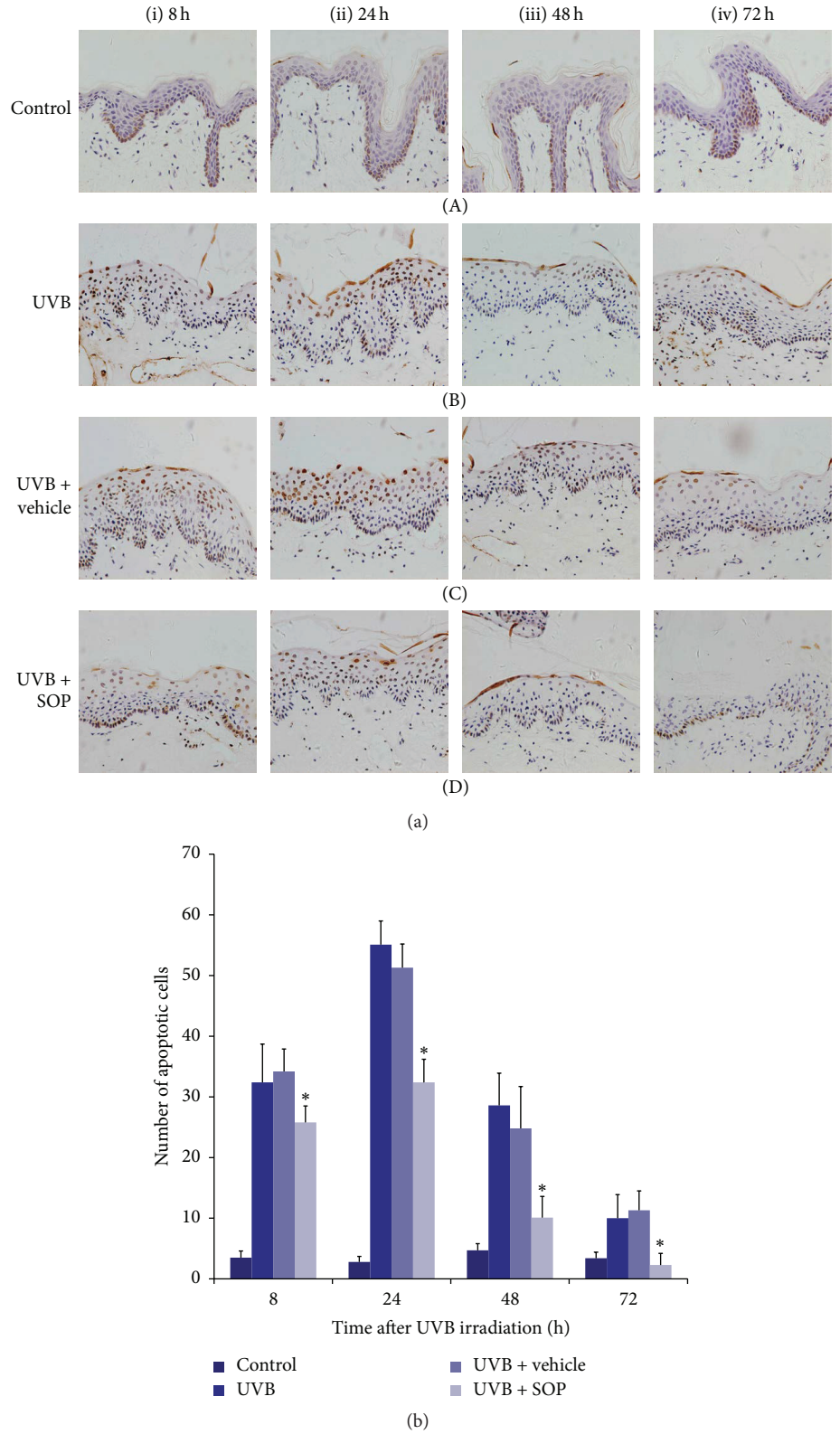

Figure 4: (a) Apoptotic cells in the epidermis of each group, stained by TUNEL staining and detected by light microscopy with ×400 magnifications. (A) Control; (B) UVB group; (C) UVB + vehicle group; (D) UVB + 5.0 IU/mL SOP group. (i) 8 h; (ii) 24 h; (iii) 48 h; (iv) 72 h. (b) The number of apoptotic cells in the epidermis of each group. Compared with UVB group, the number of apoptotic cells in UVB + 5.0 IU/mL SOP groups at 8, 24, 48, and 72 h was significantly decreased ($P < 0.05$). Stained by TUNEL staining and detected by light microscopy with ×400 magnifications. $^{*}P < 0.05$ when compared with the UVB group.

Table 1: p53 protein expression in UVB-irradiated epidermis after topical application with SOP (IRIDI).

Group division	8 h		24 h		48 h		72 h	
	Mean score	SE	Mean score	SE	Mean score	SE	Mean score	SE
Control	0.2	0-1	0.4	0-1	0.2	0-1	0.2	0-1
UVB	2.2	2-3	4.0	3–6	2.4	2-3	1.2	0–2
UVB + vehicle	2.4	2-3	4.2	3–6	2.4	2-3	1.4	1-2
UVB + 5.0 IU/mL SOP	1.0*	0–2	1.4*	1-2	0.8*	0-1	0.6	0-1

UVB: ultraviolet B; SOP: soy oligopeptides; IRIDI: immunoreactivity intensity distribution index. *$P < 0.01$ when compared with UVB group of the same period.

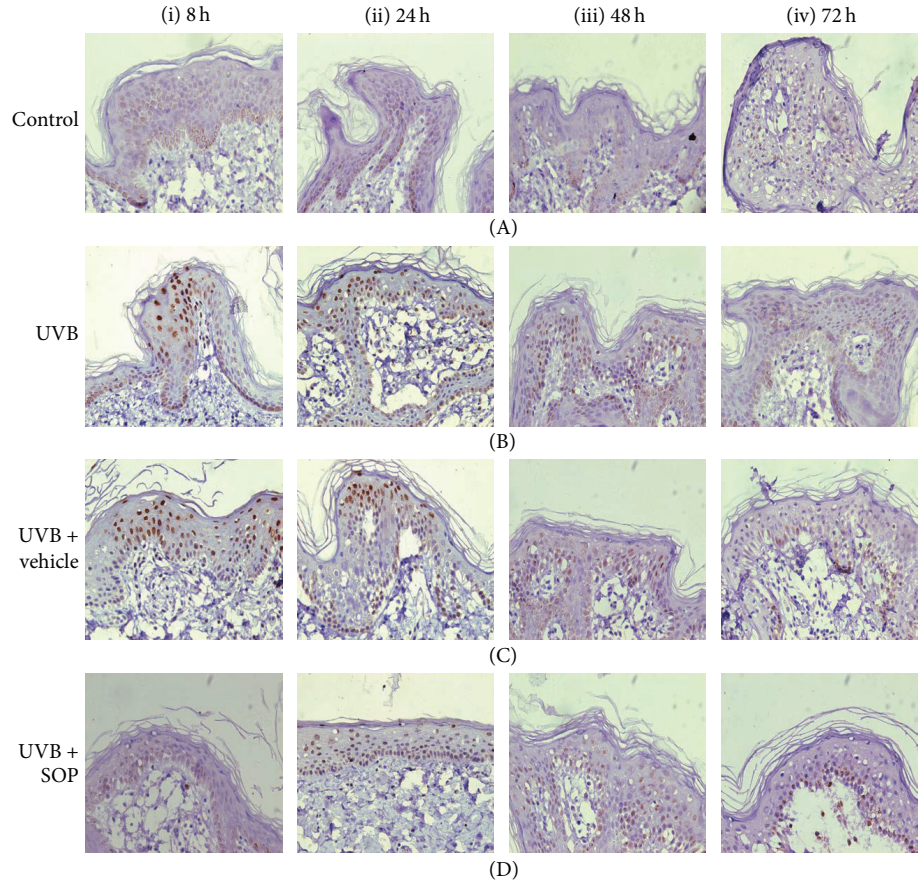

Figure 5: p53 protein expression in the epidermis of each group, stained by immunohistochemical staining and detected by light microscopy with ×400 magnifications. (A) Control; (B) UVB group; (C) UVB + vehicle group; (D) UVB + 5.0 IU/mL SOP group. (i) 8 h; (ii) 24 h; (iii) 48 h; (iv) 72 h.

improve delayed hyperpigmentation induced by 180 mJ/cm^2 of UVB irradiation for 3 consecutive days.

In this experiment, two noninvasive main indicators of skin barrier function, namely, SC hydration and TEWL, were measured. Studies found that high-dose or prolonged UV irradiation could impair skin barrier function [15, 16]. Our results showed that there was no significant change in the TEWL of irradiated groups, indicating that 180 mJ/cm^2 of UVB irradiation for 3 consecutive days made no significant impacts on TEWL. This might be related to the UVB dose and/or irradiation method. Meanwhile, topical SOP made no significant changes in the TEWL of both irradiated and nonirradiated skin. Nevertheless, topical SOP significantly increased the SC hydration in UVB-irradiated skin treated with SOP on day 1 after UVB irradiation, however, not on days 3 and 10. Moreover, topical SOP did not increase SC hydration in nonirradiated skin. This suggests that topical SOP could not improve SC hydration and TEWL values of both normal and UVB-irradiated skin. There are many internal and external factors that could affect the SC hydration, such as water channel protein, epidermal lipids, and the quality and quantity of moisturizing factor [17]. In this experiment, topical SOP could not increase SC hydration maybe due to failure to make impacts on those possible factors.

Based on the results of our clinical study, we found that topical SOP could reduce UVB-induced erythema reaction.

Table 2: Bax protein expression in UVB-irradiated epidermis after topical application with SOP (IRIDI).

Group division	8 h		24 h		48 h		72 h	
	Mean score	SE	Mean score	SE	Mean score	SE	Mean score	SE
Control	0.4	0-1	0.2	0-1	0.2	0-1	0.2	0-1
UVB	2.8	2–4	4.4	4–6	1.8	1-2	1.2	1-2
UVB + vehicle	3.0	2–4	4.0	4–6	1.6	1-2	1.2	1-2
UVB + 5.0 IU/mL SOP	1.8*	1-2	2.2*	2-3	0.8*	0-1	0.8	0-1

UVB: ultraviolet B; SOP: soy oligopeptides; IRIDI: immunoreactivity intensity distribution index. *$P < 0.05$ when compared with UVB group of the same period.

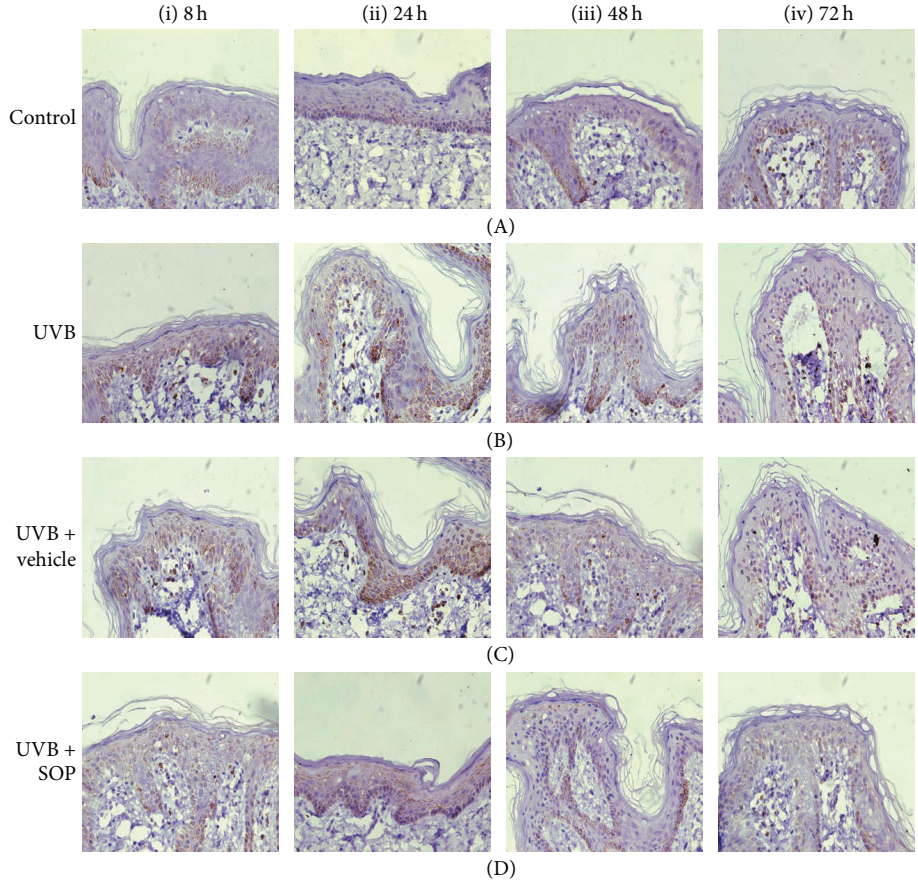

Figure 6: Bax protein expression in the epidermis of each group, stained by immunohistochemical staining and detected by light microscopy with ×400 magnifications. (A) Control; (B) UVB group; (C) UVB + vehicle group; (D) UVB + 5.0 IU/mL SOP group. (i) 8 h; (ii) 24 h; (iii) 48 h; (iv) 72 h.

UV-induced erythema is related to UVB-induced DNA damage and apoptosis. When the skin receives UVB, cellular DNA directly absorbs UVB and forms light products, including a large number of CPDs [18]. Intracellular accumulation of CPDs would hinder DNA replication and transcription [19]. If CPDs cannot be repaired in time, cells with damaged DNA would be cleared through activation of p53 apoptotic pathway mechanism. In UV-induced erythema, there is infiltration of sunburn cells in the epidermis, which are apoptotic keratinocytes [1]. Therefore, we further carried out *ex vivo* experiments to explore the possible mechanisms by which topical SOP reduced UVB-induced erythema.

Acute skin photodamage caused by short-term high-dose UV irradiation can increase the amount of reactive oxygen species in the skin, resulting in DNA damage, inducing keratinocytes to undergo apoptosis and manifest themselves as sunburn cells. We detected sunburn cells using H&E and at the same time we detected apoptotic cells using TUNEL assay. We found that there were a large number of sunburn and apoptotic cells in the epidermis after UVB irradiation, which reached the highest number at 24 h and gradually reduced with time. The number of apoptotic cells detected by TUNEL assay was slightly higher than the number of sunburn cells detected by H&E staining. This might be because TUNEL

Table 3: Bcl-2 protein expression in UVB-irradiated epidermis after topical application with SOP (IRIDI).

Group division	8 h		24 h		48 h		72 h	
	Mean score	SE	Mean score	SE	Mean score	SE	Mean score	SE
Control	2.2	1–3	2.4	2-3	2.2	1–3	2.6	2-3
UVB	$0^{\#}$	0	$0^{\#}$	0	$0.2^{\#}$	0-1	$0.8^{\#}$	0-1
UVB + vehicle	$0^{\#}$	0	$0^{\#}$	0	$0.4^{\#}$	0-1	$0.8^{\#}$	0-1
UVB + 5.0 IU/mL SOP	$0.8^{\#}$	0-1	$0.2^{\#}$	0-1	$1.2^{\#*}$	1-2	$1.6^{\#*}$	1-2

UVB: ultraviolet B; SOP: soy oligopeptides; IRIDI: immunoreactivity intensity distribution index. $^{\#}P < 0.05$ when compared with negative control group of the same period; $^{*}P < 0.05$ when compared with UVB group of the same period.

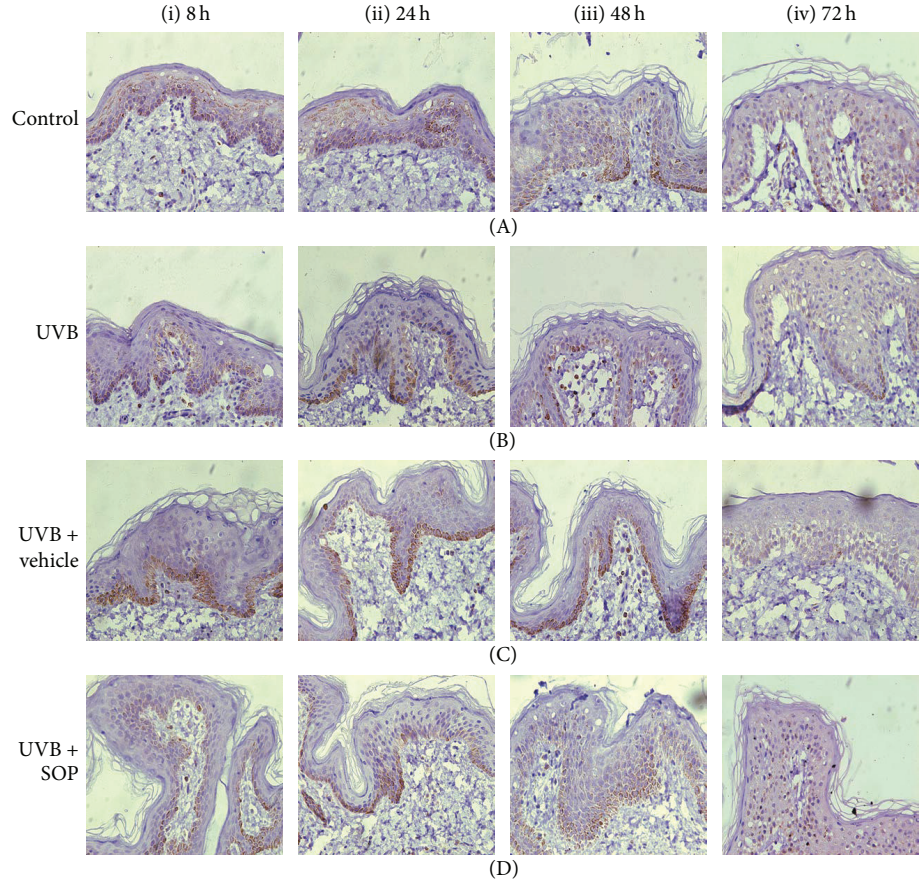

Figure 7: Bcl-2 protein expression in the epidermis of each group, stained by immunohistochemical staining and detected by light microscopy with ×400 magnifications. (A) Control; (B) UVB group; (C) UVB + vehicle group; (D) UVB + 5.0 IU/mL SOP group. (i) 8 h; (ii) 24 h; (iii) 48 h; (iv) 72 h.

assay detects the DNA breaks which occur very early in apoptosis, prior to changes in cell morphology, thus capable of detecting the apoptotic cells which cannot yet be recognized based on changes in morphology [20]. Our results demonstrate that topical SOP significantly reduced the number of sunburn and apoptotic cells in UVB-irradiated epidermis. This indicates that topical SOP accelerated removal of apoptotic cells.

CPDs are the main light product produced in UV-irradiated cells, accounting for 70–80%, and have a low rate of repair. Their absorption peak is within UVB wavelength range. Therefore, detection of UVB-induced CPDs formation and removal is important for the evaluation of DNA damage severity and repair. CPDs can occur after the skin is irradiated by UV and reached a peak after 4 h. Because CPDs removal in the skin is relatively slow, there is still a high level of CPDs in the skin 24 h after UV irradiation, even after several days [21]. In accordance with this, our experimental result showed that there were a large number of CPDs-positive cells after UVB irradiation, and the highest number was observed at 8 h, and it gradually decreased with time. Several studies found that some antioxidants, such as green tea polyphenols and retinyl esters, have a positive contribution to the removal of CPDs [22, 23]. We found that topical SOP application on UVB-irradiated skin significantly decreased the number of CPDs-positive cells compared with that of UVB group, indicating

that topical SOP application accelerated the removal of CPDs. Our previous study on topical SOP effects in UVB-induced skin photodamage of mice confirmed that SOP had a strong antioxidant capacity, increased skin SOD and GSH-px activities, and accelerated removal of free radicals [10]. The positive effect of SOP may be mainly through the absorption of UV and clearance of oxygen free radicals produced from UVB irradiation, thus reducing damage caused by oxygen free radicals in CPDs nucleotide excision repair pathway. Therefore, the removal of CPDs could be accelerated through the antioxidant properties of SOP.

p53 is a tumor suppressor gene and has an important role in UVB-induced skin lesions. After p53-gene-knockout mice were irradiated by UV, the number of sunburn cells in their skin was obviously decreased compared with that of normal mice [24]. This suggests that p53 expression plays an important role in the formation of UV-induced sunburn cells. As a transcription factor, p53 protein regulates a series of downstream target gene expressions and arrests the cell cycle at G1 phase, promoting DNA repair [4]. If the DNA cannot be repaired, p53 can induce Bax activation and downregulation of Bcl-2, causing apoptosis [5, 6]. Our study showed that, after UVB irradiation, there were increased p53 and Bax protein expressions and decreased Bcl-2 protein expression compared to control group, promoting apoptosis in the dermis. In accordance with this, we found increased apoptotic cells in the epidermis after UVB irradiation. However, topical SOP application significantly decreased UVB-induced p53 and Bax protein expressions and also significantly increased Bcl-2 protein expression at 48 and 72 h after UVB irradiation.

In conclusion, our study demonstrated that topical SOP could reduce UVB-induced erythema but have no impacts on hyperpigmentation and SC hydration. Topical SOP could accelerate removal of UVB-induced CPDs and apoptotic cells through decreasing UVB-induced p53 and Bax protein expressions and increasing Bcl-2 protein expression, thereby protecting against UVB-induced photodamage.

Competing Interests

The SOP cream and vehicle control used in the present study were kindly provided by Infinitus Ltd., China. However, Infinitus Ltd., China, had no influences on the design and conduct of the study. The authors have declared no other competing interests.

Authors' Contributions

Bing-rong Zhou and Li-wen Ma contributed equally to the paper.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation, China (81573072 and 81301384), and the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

References

[1] E. Guzman, J. L. Langowski, and L. Owen-Schaub, "Mad dogs, Englishmen and apoptosis: the role of cell death in UV-induced skin cancer," *Apoptosis*, vol. 8, no. 4, pp. 315–325, 2003.

[2] J.-L. Ravanat, T. Douki, and J. Cadet, "Direct and indirect effects of UV radiation on DNA and its components," *Journal of Photochemistry and Photobiology B: Biology*, vol. 63, no. 1–3, pp. 88–102, 2001.

[3] N. Kobayashi, S. Katsumi, K. Imoto et al., "Quantitation and visualization of ultraviolet-induced dna damage using specific antibodies: application to pigment cell biology," *Pigment Cell Research*, vol. 14, no. 2, pp. 94–102, 2001.

[4] M. Nakanishi, H. Niida, H. Murakami, and M. Shimada, "DNA damage responses in skin biology-Implications in tumor prevention and aging acceleration," *Journal of Dermatological Science*, vol. 56, no. 2, pp. 76–81, 2009.

[5] E. C. Thornborrow and J. J. Manfredi, "The tumor suppressor protein p53 requires a cofactor to activate transcriptionally the human BAX promoter," *Journal of Biological Chemistry*, vol. 276, no. 19, pp. 15598–15608, 2001.

[6] B. L. Arasada, A. Bommareddy, X. Zhang, K. Bremmon, and C. Dwivedi, "Effects of α-santalol on proapoptotic caspases and p53 expression in UVB irradiated mouse skin," *Anticancer Research*, vol. 28, no. 1, pp. 129–132, 2008.

[7] D. Elia, K. Stadler, V. Horváth, and J. Jakus, "Effect of soy- and whey protein-isolate supplemented diet on the redox parameters of trained mice," *European Journal of Nutrition*, vol. 45, no. 5, pp. 259–266, 2006.

[8] S. Nagaoka, K. Miwa, M. Eto, Y. Kuzuya, G. Hori, and K. Yamamoto, "Soy protein peptic hydrolysate with bound phospholipids decreases micellar solubility and cholesterol absorption in rats and Caco-2 cells," *Journal of Nutrition*, vol. 129, no. 9, pp. 1725–1730, 1999.

[9] Z.-I. Shin, R. Yu, S.-A. Park et al., "His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 6, pp. 3004–3009, 2001.

[10] L. W. Ma, H. W. Zhao, B. R. Zhou et al., "Protective effects of soy oligopeptides on UVB-induced skin photoaging in mice," *Chinese Journal of Dermatology and Venereology*, vol. 28, no. 2, pp. 119–122, 2014.

[11] M.-Y. Cai, R.-Z. Gu, C.-Y. Li et al., "Pilot-scale production of soybean oligopeptides and antioxidant and antihypertensive effects in vitro and in vivo," *Journal of Food Science and Technology*, vol. 51, no. 9, pp. 1866–1874, 2014.

[12] F. M. Arellano, C. E. Wentworth, A. Arana, C. Fernández, and C. F. Paul, "Risk of lymphoma following exposure to calcineurin inhibitors and topical steroids in patients with atopic dermatitis," *Journal of Investigative Dermatology*, vol. 127, no. 4, pp. 808–816, 2007.

[13] D. Bayramgürler, S. K. Özkara, R. Apaydin, C. Erçin, and N. Bilen, "Heat shock proteins 60 and 70 expression of cutaneous lichen planus: comparison with normal skin and psoriasis vulgaris," *Journal of Cutaneous Pathology*, vol. 31, no. 9, pp. 586–594, 2004.

[14] S. H. Lim, S. M. Kim, Y. W. Lee, K. J. Ahn, and Y. B. Choe, "Change of biophysical properties of the skin caused by ultraviolet radiation-induced photodamage in Koreans," *Skin Research and Technology*, vol. 14, no. 1, pp. 93–102, 2008.

[15] S. J. Jiang, A. W. Chu, Z. F. Lu, M. H. Pan, D. F. Che, and X. J. Zhou, "Ultraviolet B-induced alterations of the skin barrier and epidermal calcium gradient," *Experimental Dermatology*, vol. 16, no. 12, pp. 985–992, 2007.

[16] Y. Takagi, H. Nakagawa, H. Kondo, Y. Takema, and G. Imokawa, "Decreased levels of covalently bound ceramide are associated with ultraviolet B-induced perturbation of the skin barrier," *Journal of Investigative Dermatology*, vol. 123, no. 6, pp. 1102–1109, 2004.

[17] A. V. Rawlings and C. R. Harding, "Moisturization and skin barrier function," *Dermatologic Therapy*, vol. 17, supplement 1, pp. 43–48, 2004.

[18] G. Mathonnet, C. Léger, J. Desnoyers, R. Drouin, J.-P. Therrien, and E. A. Drobetsky, "UV wavelength-dependent regulation of transcription-coupled nucleotide excision repair in p53-deficient human cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 12, pp. 7219–7224, 2003.

[19] Y.-H. You, D.-H. Lee, J.-H. Yoon, S. Nakajima, A. Yasui, and G. P. Pfeifer, "Cyclobutane pyrimidine dimers are responsible for the vast majority of mutations induced by UVB irradiation in mammalian cells," *The Journal of Biological Chemistry*, vol. 276, no. 48, pp. 44688–44694, 2001.

[20] M. Archana, Bastian,, T. L. Yogesh, and K. L. Kumaraswamy, "Various methods available for detection of apoptotic cells—a review," *Indian Journal of Cancer*, vol. 50, no. 3, pp. 274–283, 2013.

[21] J. M. Sheehan, N. Cragg, C. A. Chadwick, C. S. Potten, and A. R. Young, "Repeated ultraviolet exposure affords the same protection against DNA photodamage and erythema in human skin types II and IV but is associated with faster DNA repair in skin type IV," *Journal of Investigative Dermatology*, vol. 118, no. 5, pp. 825–829, 2002.

[22] S. K. Katiyar, "Skin photoprotection by green tea: antioxidant and immunomodulatory effects," *Current Drug Targets. Immune, Endocrine & Metabolic Disorders*, vol. 3, no. 3, pp. 234–242, 2003.

[23] C. Antille, C. Tran, O. Sorg, P. Carraux, L. Didierjean, and J.-H. Saurat, "Vitamin A exerts a photoprotective action in skin by absorbing ultraviolet B radiation," *Journal of Investigative Dermatology*, vol. 121, no. 5, pp. 1163–1167, 2003.

[24] F. R. de Gruijl and P. Voskamp, "Photocarcinogenesis—DNA damage and gene mutations," *Cancer Treatment and Research*, vol. 146, pp. 101–108, 2009.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 7453849, 7 pages
http://dx.doi.org/10.1155/2016/7453849

Research Article

Resveratrol Reduces the Incidence of Portal Vein System Thrombosis after Splenectomy in a Rat Fibrosis Model

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Received 1 April 2016; Accepted 7 June 2016

Academic Editor: Jerzy Kruk

Purpose. To investigate the preventive effect of resveratrol (RES) on the formation of portal vein system thrombosis (PVST) in a rat fibrosis model. *Methods*. A total of 64 male SD rats, weighing 200–300 g, were divided into five groups: Sham operation, Splenectomy I, Splenectomy II, RES, and low molecular weight heparin (LMWH), with the former two groups as nonfibrosis controls. Blood samples were subjected to biochemical assays. Platelet apoptosis was measured by flow cytometry. All rats were euthanized for PVST detection one week after operation. *Results*. No PVST occurred in nonfibrosis controls. Compared to Splenectomy II, the incidences of PVST in RES and LMWH groups were significantly decreased (both $p < 0.05$). Two rats in LMWH group died before euthanasia due to intra-abdominal hemorrhage. In RES group, significant decreases in platelet aggregation, platelet radical oxygen species (ROS) production, and increase in platelet nitric oxide (NO) synthesis and platelet apoptosis were observed when compared with Splenectomy II (all $p < 0.001$), while in LMWH group only significant decrease in platelet aggregation was observed. *Conclusion*. Prophylactic application of RES could safely reduce the incidence of PVST after splenectomy in cirrhotic rat. Regulation of platelet function and induction of platelet apoptosis might be the underlying mechanisms.

1. Introduction

Hepatic cirrhosis was most commonly caused by viral hepatitis and alcohol abuse [1] with hepatic insufficiency and portal hypertension being the most serious consequences [2]. If untreated, the 5-year survival of decompensated cirrhosis was only 15% [3]. The ideal treatment option for cirrhotic patients was liver transplantation. However, due to severe shortage of donor livers, splenectomy and pericardial devascularization were still the primary method for the patients with cirrhosis and portal hypertension to alleviate thrombocytopenia and reduce the risk of gastrointestinal hemorrhage in some countries such as China.

Portal vein system thrombosis (PVST) referred to thrombosis in the portal vein, splenic, and superior mesenteric veins or intrahepatic portal vein branches [4]. It was widely recognized as a potential fatal complication after splenectomy, leading to bowel infarction, upper gastrointestinal bleeding, and even hepatic coma [5, 6]. With the development of advanced imaging devices, it has been found that PVST after splenectomy is not a rare complication with an incidence of 5%–25% [7]. Previous studies of PVST mainly focused on the incidence, diagnosis, and treatment of PVST after splenectomy in patients with myelodysplastic syndromes [8], hemolytic anemia [9], and splenic tumors [10]. In recent years, it has been found that PVST after splenectomy also occurred in cirrhotic patients despite the presence of endogenous coagulopathy [11]. However, the pathogenesis and characteristics of PVST in these patients remain unclear. Recently, some studies showed that both pro- and anticoagulation elements were concomitantly reduced in liver cirrhosis, thus maintaining an intricate balance of coagulation [12, 13]. Under such circumstance, a series of local and systemic changes subject to splenectomy, including hemodynamic changes of the portal venous system [13], local vascular pathological changes [6], and blood hypercoagulability [6], as well as irrational use of coagulants [14], might be breaking the balance and finally contribute to the formation of PVST.

Resveratrol (trans-3,5,4′-trihydroxystilbene, RES), a natural polyphenol, was first isolated from the roots of white hellebore in 1940 [15] and is now found to be present in various plant species such as berries, peanuts, and particularly grape skins [16]. Numerous studies have demonstrated its diverse biologic effects, such as antioxidative effect [17], anti-inflammatory activity [18], antiviral activity [19], and antiplatelet aggregation activity [20]. Previous study in animal model had demonstrated that RES exerted protective effect on rats subjected to PVST via its antioxidant and antiaggregant properties [21]. However, the effect of RES on the formation of PVST after splenectomy remains unclear.

In light of these, the present study aimed to investigate the preventive effect of RES on the formation of PVST in rats after splenectomy in the context of liver cirrhosis. The findings will provide some clues that RES can be a potential antithrombotic agent for reducing PVST formation after splenectomy for cirrhotic patients.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley (SD) rats weighing between 200 and 300 g 9 to 10 weeks old were purchased from the Laboratory Animal Center of Xi'an Jiaotong University Health Science Center (Xi'an, China). Liver fibrosis was induced by intraperitoneal injection of carbon tetrachloride (CCl_4, Sinopharm Chemical Reagent Co. Ltd. Shanghai, China; 0.6 mL/kg of body weight) in olive oil, twice a week for six weeks, and was confirmed by liver biopsy when animals were euthanized. All animals were allowed free access to water and standard laboratory chow, except for an overnight fast before undergoing surgery. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication number 85-23, revised in 1996). All procedures were reviewed and approved by the Ethics Committee, Xi'an Jiaotong University Health Science Center.

2.2. Reagents. Resveratrol was purchased from Xi'an Sino-Herb Bio-technology Company (Xi'an, China). Dimethyl sulfoxide (DMSO) and RPMI-1640 were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). The RES was dissolved and sterilized in DMSO and then diluted in RPMI-1640 to 5 mg/mL.

2.3. Experimental Design. We estimated that a total of 64 rats would be needed to detect a difference between groups, with a one-tailed chi-square test ($\alpha = 0.05$ and $\beta = 0.20$), when the forecasting incidences of PVST in nonfibrosis controls, fibrotic rats without anticoagulation, and fibrotic rats with anticoagulation were 1% [22], 40% [23], and around 10% [24], respectively. Then the numbers in each group were determined as follows with an experimental-to-control animal ratio of 2 : 1 as previously reported [25]: two groups of age-matched normal rats served as nonfibrosis controls: Sham operation group and Splenectomy group I with 8 rats in each group. Sham operation group (Sham): a 5 cm midline abdominal incision was made to expose the spleen and laparotomy was performed with no splenectomy; Splenectomy group I (Splenectomy I): splenectomy was carried out. The other 48 rats with liver fibrosis were randomized into Splenectomy group II, RES group, and LMWH group, with 16 rats in each group, as follows: Splenectomy group II (Splenectomy II): animals underwent the same surgical procedure as Splenectomy I; RES preconditioning group (RES): animals underwent the same surgical procedure as Splenectomy group I/II and received RES (50 mg/d per nasogastric tube) for 10 consecutive days before operation; and LMWH treatment group (LMWH): animals underwent the same surgical procedure as Splenectomy group I/II and received Enoxaparin (1.5 mg/kg, subcutaneously) on postoperative days (POD) 1, 2, and 3. Rats were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg; Fujian Gutian Pharmaceutical Co., Ltd, Fujian, China). All animals were euthanized with an overdose of ketamine (150 mg/kg IP) followed by exsanguinations 1 week after operation. Blood was collected from the inferior vena cava in plastic tubes containing sodium heparin (1000 units/mL) as anticoagulant at a ratio of 9 : 1 v/v. Blood samples were centrifuged at 150 ×g at room temperature for 10 min and platelet-rich plasma (PRP) was taken and immediately processed. Portal vein, superior mesenteric vein, and splenic vein were dissected and opened for the detection of PVST.

2.4. Platelet Aggregation Evaluation. 5 mL of PRP was added with equal volume of the washing buffer (140 mM NaCl, 0.5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM saccharose, and pH 6) and centrifuged at 800 ×g for 15 min. The pellet was resuspended in washing buffer and washed twice. Then the platelets were suspended in Krebs solution (118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.7 mM $MgSO_4$, 5.6 mM glucose, and pH 7.4) at a count of 1×10^8/mL.

Platelet aggregation was initiated by adding 10 μg/mL of collagen (Wuhan Boster Biological Technology, Ltd. Wuhan, China) as agonist to 0.5 mL platelet suspension and was determined using a computerized dual channel Chronolog Aggregometer (Chrono-Log Corporation, Havertown, PA) at 37°C as previously described [26]. At the end of the experiment, platelet suspensions were immediately centrifuged at 12000 ×g for 30 s and the cell-free supernatant was snap frozen in liquid nitrogen and stored at −80°C for further analysis.

2.5. Platelet ROS Evaluation. For the measurement of the generation of ROS by activated platelets, washed platelets in Krebs solution (1×10^8/mL) were loaded with 5 μM of 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma, Saint-Louis, Missouri, USA) for 15 min at 37°C. Then the platelet samples were centrifuged at 800 ×g for 10 min and resuspended in 500 μL of Krebs solution. The ROS-generated fluorescence was read using a Becton Dickinson flow cytometer (FACSCalibur; USA) equipped with a 488 nm wavelength argon laser, 510–540 nm band pass filter, as described [27].

2.6. Platelet NO Evaluation. NO release by platelets during platelet aggregation was estimated by measuring NO degradation products, nitrite plus nitrate, in platelet supernatants. Briefly, frozen samples of platelets supernatant, as described earlier, were deproteinized with 3 M ZnSO4, 30% v/v, for 15 min and then centrifuged at 12,000 g for 5 min. After discarding the pellet, the clean supernatant was added with activated cadmium beads for overnight incubation at room temperature. Then, the culture was centrifuged and the supernatant was used for NO degradation products concentration determination by using a colorimetric nonenzymatic assay kit (Oxford Biomedical Research, Oxford, MI, USA) as reported previously [28].

2.7. Flow Cytometry Analysis. For measuring platelet apoptosis, an Annexin-V Apoptosis Detection Kit FITC (eBioscience, San Diego, CA, USA) was used according to manufacturer's instructions.

2.8. Statistical Analysis. Continuous data were presented as mean ± SD and categorical data were presented as frequencies. Statistical differences were calculated by Student's t-test or chi-square test using SPSS 11.5 statistical software (SPSS Inc., Chicago, IL, United States). A p value <0.05 was considered statistically significant.

3. Results

3.1. Incidence and Distribution of PVST after Splenectomy. No PVST occurred in Sham group and Splenectomy group I. For the other three groups of rats with liver fibrosis, the incidence of PVST 1 week after operation was 43.75% (7/16) in Splenectomy group II, 6.25% (1/16) in RES group, and 7.14% (1/14) in LMWH group (two rats in this group died at POD 4 and 5, resp., due to intra-abdominal hemorrhage). Both pretreatment with RES (50 mg/d per nasogastric tube for 10 days) and a short term postoperative administration of Enoxaparin (1.5 mg/kg subcutaneously on POD 1, 2, and 3) showed a significant decrease in the incidence of PVST than in Splenectomy group II ($p = 0.037$ and $p = 0.039$, resp.).

Of the 7 cases of PVST in Splenectomy group II, there were 5 in the splenic vein and 2 in the portal and splenic veins. For the one case in RES group and one case in LMWH group, it was located in the portal vein and splenic vein, respectively. It is notable that splenic vein thrombosis occurred in 8 out of 9 cases (88.9%).

3.2. Inhibitory Effect of RES and Enoxaparin on Rat Platelet Aggregation. Data on the effects of RES and Enoxaparin on platelet aggregation induced by collagen are presented in Figure 1. Platelet aggregation in rats with liver fibrosis 1 week after splenectomy (Splenectomy group II) was significantly higher than that in rats without liver fibrosis (Sham and Splenectomy group I) (all $p < 0.001$). Both pretreatment with RES (50 mg/d per nasogastric tube for 10 days) and a short term postoperative administration of Enoxaparin (1.5 mg/kg subcutaneously on POD 1, 2, and 3) caused a significant reduction in collagen-induced platelet aggregation 1 week after splenectomy, compared with Splenectomy group II (all $p < 0.001$).

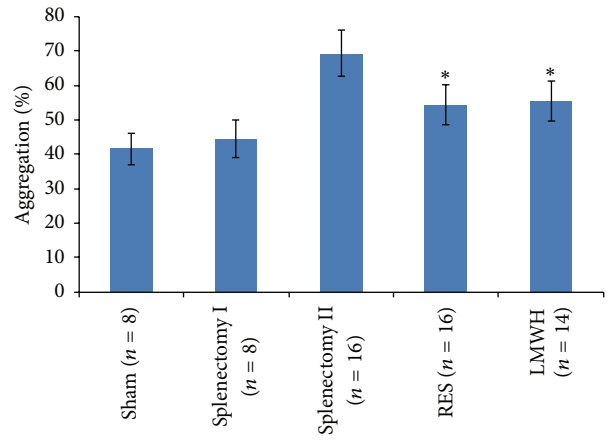

Figure 1: Inhibitory effects of RES and Enoxaparin on rat platelet aggregation. Rats received RES (50 mg/d per nasogastric tube) for 10 days before operation or a short term postoperative administration of Enoxaparin (1.5 mg/kg subcutaneously on POD 1, 2, and 3) and the blood was collected 1 week after operation. Washed platelets aggregation (1×10^8 platelets/mL) stimulated with 10 μg/mL of collagen was determined. Results are shown as mean ± SD. $^*p < 0.001$ compared with Splenectomy group II.

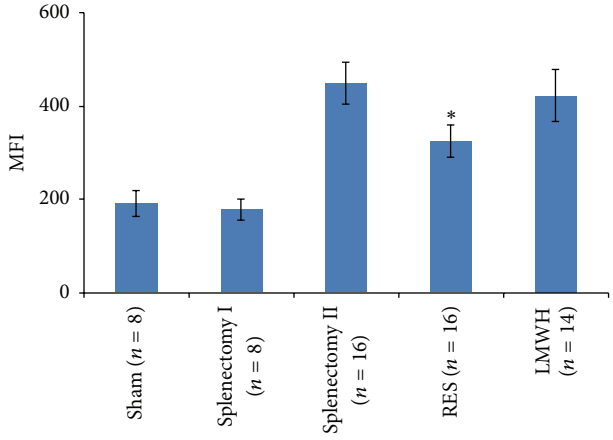

Figure 2: Inhibitory effect of RES on ROS generation in rat platelets. Rats received RES (50 mg/d per nasogastric tube) for 10 days before operation and ROS generation was quantified in collagen (10 μg/mL)-activated platelets. Results are shown as mean ± SD. $^*p < 0.001$ compared with Splenectomy group II. MFI: Mean Fluorescence Index.

3.3. Inhibitory Effect of RES on ROS Generation in Rat Platelets. Compared to Sham and Splenectomy group I, ROS formation by collagen-stimulated platelets increased obviously in fibrosis rat 1 week after splenectomy (all $p < 0.001$). However, this increase in ROS formation was significantly alleviated by in vivo pretreatment with RES ($p < 0.001$), but not by postoperative administration of Enoxaparin (Figure 2).

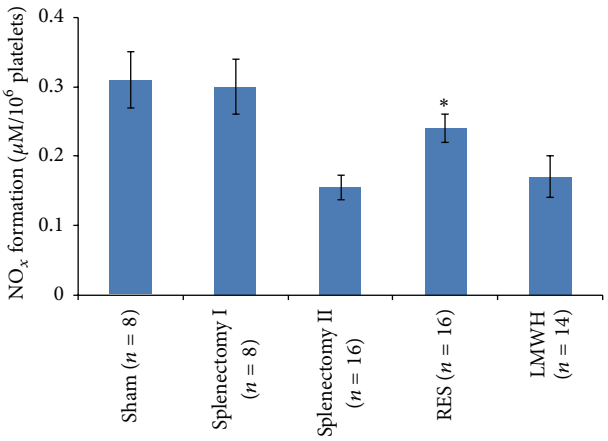

Figure 3: Stimulating effect of RES on NO release in rat platelets. Rats received RES (50 mg/d per nasogastric tube) for 10 days before operation and NO release by platelets during platelet aggregation was estimated in platelet supernatants. Results are shown as mean ± SD. $^*p < 0.001$ compared with Splenectomy group II.

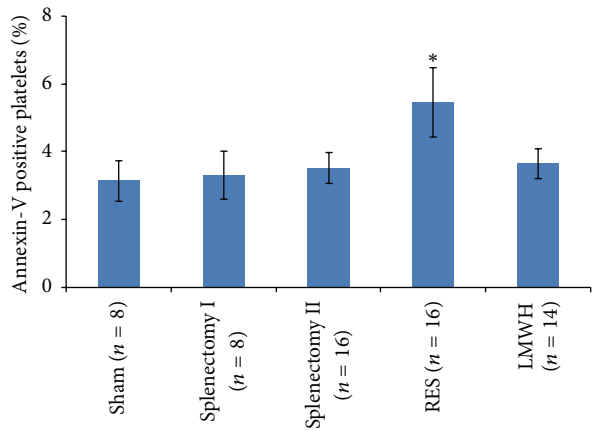

Figure 4: Induction of rat platelets apoptosis by RES. Rats received RES (50 mg/d per nasogastric tube) for 10 days before operation and apoptosis of rat platelets was measured by flow cytometry with an Annexin-V Apoptosis Detection Kit. Results are shown as mean ± SD. $^*p < 0.001$ compared with the other four groups.

3.4. Stimulating Effect of RES on NO Release in Rat Platelets. Compared to Sham and Splenectomy group I, NO release during platelet aggregation decreased obviously in fibrosis rat 1 week after splenectomy (all $p < 0.001$). However, this decrease in NO release was significantly alleviated by in vivo pretreatment with RES ($p < 0.001$) but not by postoperative administration of Enoxaparin (Figure 3).

3.5. Induction of Rat Platelets Apoptosis by RES. As seen in Figure 4, significantly increased Annexin-V positive platelets representing phosphatidylserine (PS) exposure were observed in RES group (5.46 ± 1.02%) compared to that in Sham group (3.15±0.6%), Splenectomy group I (3.31±0.7%), Splenectomy group II (3.52 ± 0.45%), and LMWH group (3.66 ± 0.44%) (all $p < 0.001$), suggesting more platelet apoptosis occurring in rats pretreated with RES.

4. Discussion

Although the pathogenesis of PVST following splenectomy is still controversial, it is generally agreed that it is related to blood hypercoagulability [6], hemodynamic changes of the portal venous system [13], local vascular pathological changes [6], and irrational use of coagulants [14], and so forth. Lower preoperative platelet counts, postoperative thrombocytosis, wider preoperative portal/splenic vein diameter, prolonged prothrombin time (PT), periesophagogastric devascularization, and mutation of prothrombin genes and deficiency in protein C and protein S have been considered as risk factors of PVST after splenectomy [23, 29].

For cirrhotic patients, preoperative platelet count was generally lower than normal due to concurrent hypersplenism. Under such condition, removal of spleen would lead to a platelet rebound phenomenon and the soaring count and augmented aggregation competence of platelet after operation would contribute to the hypercoagulable state, which, in combination with other underlying causes, may synergistically result in the formation of PVST.

Currently, the prophylaxis of PVST after splenectomy in liver cirrhosis remains controversial. The main concern is that prophylactic anticoagulation might induce relevant complications, mainly related to bleeding. So far, the management of PVST after splenectomy is mainly based on individual experience [29]. Several recent pilot studies had demonstrated the feasibility, safety, and efficacy of prophylactic anticoagulation with decreased incidence of PVST and low rate of bleeding complications comparable to controls [30–34]. However, due to the small number of cases in individual studies, more results with adequate power are required to confirm these observations.

The activation of thrombin is a crucial step in the formation of thrombosis. LMWH could suppress the activation of thrombin via suppressing coagulation active factor X. Moreover, LMWH could reduce platelet aggregation induced by thrombin and inhibit platelet aggregation in response to adenosine diphosphate (ADP) [35]. In the present study, compared to Splenectomy group II, both the incidence of PVST and platelet aggregation were significantly decreased in LMWH group ($p < 0.05$ and $p < 0.001$, resp.). However, two rats in LMWH group died before euthanasia due to intra-abdominal hemorrhage, suggesting that the anticoagulants under such conditions should be used more carefully. We speculated that a possible reason might be the increased release of tissue plasminogen activator and consequent fibrinolysis induced by LMWH.

One of the most studied actions of RES was its antiplatelet aggregation property. RES exerted this function through multiple mechanisms. First, RES could inhibit both type I collagen mRNA expression and platelet adhesion to the collagen, which was the initiation of platelet activation [36]; second, RES could interfere with platelet aggregation through inhibiting Ca^{2+} influx into thrombin-stimulated platelets [37]; third,

RES could reduce tissue factor (TF) activity by inhibiting nuclear factor-κB/Rel-dependent transcription in both endothelial cells and monocytes [38].

Besides, RES was shown to reduce oxidative stress by different mechanisms including activation of antioxidant enzymes, inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and chelation of metal catalysts [39], thus reducing the formation of ROS. Moreover, RES could enhance NO production by stimulated platelets via promoting the phosphorylation of protein kinase B and vasodilator stimulated phosphoprotein (VASP) [39]. RES also increased NO production by increasing NO synthase activity (NOS) [40]. All these activities also contributed to the antiplatelet aggregation activity of RES.

Of late, there were reports of plant-derived molecules, such as RES, causing platelet apoptosis [41, 42]. Further studies showed that RES triggered platelet apoptosis by multiple mechanisms including caspase-9, caspase-3, and caspase-8, gelsolin and actin cleavage, truncated Bid (tBid) formation, Bcl-2-associated X protein (Bax) translocation, cytochrome c release, phosphatidylserine (PS) exposure, and dissipation of the mitochondrial membrane potential, suggesting that RES-induced platelet apoptosis may be mediated by both intrinsic and extrinsic apoptotic pathway [43].

A previous study had reported that RES exerted protective effect on rats subjected to PVST via its antioxidant and antiaggregant properties [21]. The authors found that, compared with the rats subjected to PVST alone, significant increases in both tissue and plasma levels of reduced glutathione (GSH), as well as tissue c-AMP level, and decreased tissue malondialdehyde (MDA) level were observed among rats receiving RES (60 mg/d per nasogastric tube) for 10 days before being subjected to PVST. However, the effect of RES on the formation of PVST after splenectomy remains to be elucidated.

In the present study, we investigate the preventive effect of RES on the formation of PVST after splenectomy in a rat fibrosis model. The reported interval between splenectomy and PVST development varied and in our previous study the interval was 4 days [23]. Taking this into consideration, we choose 1 week as the end point of observation. Our results showed that the incidence of PVST in RES group, similar to LMWH group, was significantly decreased compared to Splenectomy group II, and no animal in RES group died due to postoperative complications such as bleeding. Moreover, we further studied the effect of RES on platelet aggregation, platelet ROS production, platelet NO production, and platelet apoptosis. As expected, platelet aggregation and platelet ROS production were significantly higher in Splenectomy II group rats than those in rats without liver cirrhosis 1 week after operation, while pretreatment with RES (50 mg/d per nasogastric tube for 10 days) showed a significant decrease in platelet aggregation and platelet ROS production than in Splenectomy group II. Besides, our results demonstrated that platelet NO production was significantly decreased in Splenectomy group II rats than that in rats without liver cirrhosis 1 week after operation, while pretreatment with RES showed a significant increase in platelet NO production than in Splenectomy group II. These findings affirmed the results of previous studies showing that ROS act as second messengers to activate platelets via NO inactivation [44]. PS exposure on cellular surface represents the most universal and best characterized target recognition signal, leading to phagocytes-mediated clearance of the apoptotic cells [45]. Our results also demonstrated that, in RES group, statistically significant increase in Annexin-V positive platelets representing PS exposure was observed when compared with the other four groups. More importantly, we provided the first evidence supporting the preventive effect of RES on the formation of PVST after splenectomy in the setting of cirrhotic, which are consistent with previous findings, suggesting the antiplatelet aggregation, antioxidative, vasorelaxing, and apoptosis-promoting effects of RES [39, 41–43]. However, additional studies with longer follow-up time are needed to affirm the results.

Taken together, our present study has provided evidence for the first time that RES exerts its preventive effect on PVST formation after splenectomy, through simultaneously inhibiting aggregation, decreasing ROS, and increasing NO production, as well as stimulating apoptosis effects on platelets, in a rat fibrosis model. RES, as a natural substance found in multiple plants, could be a promising candidate in the development of an effective antithrombotic therapy for the prevention of PVST after splenectomy for cirrhotic patients.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Meng Xu and Wanli Xue contributed equally.

References

[1] S. P. Starr and D. Raines, "Cirrhosis: diagnosis, management, and prevention," *American Family Physician*, vol. 84, no. 12, pp. 1353–1359, 2011.

[2] D. Ganem and A. M. Prince, "Hepatitis B virus infection—natural history and clinical consequences," *The New England Journal of Medicine*, vol. 350, no. 11, pp. 1118–1129, 2004.

[3] C. Haché and J.-P. Villeneuve, "Lamivudine treatment in patients with chronic hepatitis B and cirrhosis," *Expert Opinion on Pharmacotherapy*, vol. 7, no. 13, pp. 1835–1843, 2006.

[4] S. Parikh, R. Shah, and P. Kapoor, "Portal vein thrombosis," *American Journal of Medicine*, vol. 123, no. 2, pp. 111–119, 2010.

[5] A. Pietrabissa, C. Moretto, G. Antonelli, L. Morelli, E. Marciano, and F. Mosca, "Thrombosis in the portal venous system after elective laparoscopic splenectomy," *Surgical Endoscopy and Other Interventional Techniques*, vol. 18, no. 7, pp. 1140–1143, 2004.

[6] K. M. Stamou, K. G. Toutouzas, P. B. Kekis et al., "Prospective study of the incidence and risk factors of postsplenectomy thrombosis of the portal, mesenteric, and splenic veins," *Archives of Surgery*, vol. 141, no. 7, pp. 663–669, 2006.

[7] R. Charco, J. Fuster, C. Fondevila, J. Ferrer, E. Mans, and J. C. García-Valdecasas, "Portal vein thrombosis in liver transplantation," *Transplantation Proceedings*, vol. 37, no. 9, pp. 3904–3905, 2005.

[8] R. A. Mesa, D. S. Nagorney, S. Schwager, J. Allred, and A. Tefferi, "Palliative goals, patient selection, and perioperative platelet management: outcomes and lessons from 3 decades of splenectomy for myelofibrosis with myeloid metaplasia at the Mayo Clinic," *Cancer*, vol. 107, no. 2, pp. 361–370, 2006.

[9] E. R. Winslow, L. M. Brunt, J. A. Drebin, N. J. Soper, M. E. Klingensmith, and E. Dunn, "Portal vein thrombosis after splenectomy," *American Journal of Surgery*, vol. 184, no. 6, pp. 631–636, 2002.

[10] M. Ikeda, M. Sekimoto, S. Takiguchi et al., "High incidence of thrombosis of the portal venous system after laparoscopic splenectomy: a prospective study with contrast-enhanced CT scan," *Annals of Surgery*, vol. 241, no. 2, pp. 208–216, 2005.

[11] N. Kinjo, H. Kawanaka, T. Akahoshi et al., "Risk factors for portal venous thrombosis after splenectomy in patients with cirrhosis and portal hypertension," *British Journal of Surgery*, vol. 97, no. 6, pp. 910–916, 2010.

[12] A. Tripodi, M. Primignani, V. Chantarangkul et al., "An imbalance of pro- vs anti-coagulation factors in plasma from patients with cirrhosis," *Gastroenterology*, vol. 137, no. 6, pp. 2105–2111, 2009.

[13] A. Tripodi and P. M. Mannucci, "The coagulopathy of chronic liver disease," *The New England Journal of Medicine*, vol. 365, no. 2, pp. 147–156, 2011.

[14] T. Soyer, A. O. Ciftci, F. C. Tanyel, M. E. Şenocak, and N. Büyükpamukçu, "Portal vein thrombosis after splenectomy in pediatric hematologic disease: risk factors, clinical features, and outcome," *Journal of Pediatric Surgery*, vol. 41, no. 11, pp. 1899–1902, 2006.

[15] N. Namasivayam, "Chemoprevention in experimental animals," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 60–71, 2011.

[16] J. A. Baur and D. A. Sinclair, "Therapeutic potential of resveratrol: the in vivo evidence," *Nature Reviews Drug Discovery*, vol. 5, no. 6, pp. 493–506, 2006.

[17] E. L. Robb, M. M. Page, B. E. Wiens, and J. A. Stuart, "Molecular mechanisms of oxidative stress resistance induced by resveratrol: specific and progressive induction of MnSOD," *Biochemical and Biophysical Research Communications*, vol. 367, no. 2, pp. 406–412, 2008.

[18] G. Lanzilli, A. Cottarelli, G. Nicotera, S. Guida, G. Ravagnan, and M. P. Fuggetta, "Anti-inflammatory effect of resveratrol and polydatin by in vitro IL-17 modulation," *Inflammation*, vol. 35, no. 1, pp. 240–248, 2012.

[19] Y. Abba, H. Hassim, H. Hamzah, and M. M. Noordin, "Antiviral activity of resveratrol against human and animal viruses," *Advances in Virology*, vol. 2015, Article ID 184241, 7 pages, 2015.

[20] I. K. Toliopoulos, Y. V. Simos, S. Oikonomidis, and S. C. Karkabounas, "Resveratrol diminishes platelet aggregation and increases susceptibility of K562 tumor cells to natural killer cells," *Indian Journal of Biochemistry and Biophysics*, vol. 50, no. 1, pp. 14–18, 2013.

[21] V. Kirimlioglu, H. Sozen, S. Turkoglu, and M. Haberal, "Protective effect of resveratrol, a red wine constituent polyphenol, on rats subjected to portal vein thrombosis," *Transplantation Proceedings*, vol. 40, no. 1, pp. 290–292, 2008.

[22] R. Licinio, M. Principi, G. Losurdo, N. M. Castellaneta, E. Ierardi, and A. Di Leo, "Low molecular weight heparin in portal vein thrombosis of cirrhotic patients: only therapeutic purposes?" *The Scientific World Journal*, vol. 2014, Article ID 895839, 6 pages, 2014.

[23] S. Wu, Z. Wu, X. Zhang, R. Wang, and J. Bai, "The incidence and risk factors of portal vein system thrombosis after splenectomy and pericardial devascularization," *Turkish Journal of Gastroenterology*, vol. 26, no. 5, pp. 423–428, 2015.

[24] M. Primignani, G. Tosetti, and V. La Mura, "Therapeutic and clinical aspects of portal vein thrombosis in patients with cirrhosis," *World Journal of Hepatology*, vol. 7, no. 29, pp. 2906–2912, 2015.

[25] K. H. Song, C. S. Youn, C. L. Lee et al., "Increased expression of neuregulin 1 and erbB2 tyrosine kinase in the bladder of rats with cyclophosphamide-induced interstitial cystitis," *International Neurourology Journal*, vol. 19, no. 3, pp. 158–163, 2015.

[26] R. H. Raghavendra and K. A. Naidu, "Spice active principles as the inhibitors of human platelet aggregation and thromboxane biosynthesis," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 81, no. 1, pp. 73–78, 2009.

[27] P. Zhang, J. Du, L. Zhao et al., "The role of intraplatelet reactive oxygen species in the regulation of platelet glycoprotein Ibα ectodomain shedding," *Thrombosis Research*, vol. 132, no. 6, pp. 696–701, 2013.

[28] S. Momi, R. Caracchini, E. Falcinelli, S. Evangelista, and P. Gresele, "Stimulation of platelet nitric oxide production by nebivolol prevents thrombosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 34, no. 4, pp. 820–829, 2014.

[29] M.-X. Li, X.-F. Zhang, Z.-W. Liu, and Y. Lv, "Risk factors and clinical characteristics of portal vein thrombosis after splenectomy in patients with liver cirrhosis," *Hepatobiliary and Pancreatic Diseases International*, vol. 12, no. 5, pp. 512–519, 2013.

[30] H. Kawanaka, T. Akahoshi, N. Kinjo et al., "Impact of antithrombin III concentrates on portal vein thrombosis after splenectomy in patients with liver cirrhosis and hypersplenism," *Annals of Surgery*, vol. 251, no. 1, pp. 76–83, 2010.

[31] H. Wang, D. Kopac, R. Brisebois, C. Sample, and A. M. J. Shapiro, "Randomized controlled trial to investigate the impact of anticoagulation on the incidence of splenic or portal vein thrombosis after laparoscopic splenectomy," *Canadian Journal of Surgery*, vol. 54, no. 4, pp. 227–231, 2011.

[32] W. Lai, S.-C. Lu, G.-Y. Li et al., "Anticoagulation therapy prevents portal-splenic vein thrombosis after splenectomy with gastroesophageal devascularization," *World Journal of Gastroenterology*, vol. 18, no. 26, pp. 3443–3450, 2012.

[33] H. Kawanaka, T. Akahoshi, S. Itoh et al., "Optimizing risk stratification in portal vein thrombosis after splenectomy and its primary prophylaxis with antithrombin III concentrates and danaparoid sodium in liver cirrhosis with portal hypertension," *Journal of the American College of Surgeons*, vol. 219, no. 5, pp. 865–874, 2014.

[34] C. Hongwei, L. Zhang, L. Maoping, Z. Yong, D. Chengyou, and L. Dewei, "Era of liver transplantation: combined anatomic splenectomy and anticoagulant therapy in prevention of portal vein thrombosis after splenectomy," *Hepato-Gastroenterology*, vol. 62, no. 138, pp. 405–409, 2015.

[35] T. T. Horlocker and J. A. Heit, "Low molecular weight heparin: biochemistry, pharmacology, perioperative prophylaxis regimens, and guidelines for regional anesthetic management," *Anesthesia and Analgesia*, vol. 85, no. 4, pp. 874–885, 1997.

[36] S. Godihaud, S. Krisa, B. Couronné et al., "Deactivation of cultured human liver myofibroblasts by *trans*-resveratrol, a grapevine-derived polyphenol," *Hepatology*, vol. 31, no. 4, pp. 922–931, 2000.

[37] Z. Wang, J. Zou, Y. Huang, K. Cao, Y. Xu, and J. M. Wu, "Effect of resveratrol on platelet aggregation in vivo and in vitro," *Chinese Medical Journal*, vol. 115, no. 3, pp. 378–380, 2002.

[38] A. Di Santo, A. Mezzetti, E. Napoleone et al., "Resveratrol and quercetin down-regulate tissue factor expression by human stimulated vascular cells," *Journal of Thrombosis and Haemostasis*, vol. 1, no. 5, pp. 1089–1095, 2003.

[39] F. Messina, G. Guglielmini, M. Curini, S. Orsini, P. Gresele, and M. C. Marcotullio, "Effect of substituted stilbenes on platelet function," *Fitoterapia*, vol. 105, pp. 228–233, 2015.

[40] P. Gresele, C. Cerletti, G. Guglielmini, P. Pignatelli, G. de Gaetano, and F. Violi, "Effects of resveratrol and other wine polyphenols on vascular function: an update," *Journal of Nutritional Biochemistry*, vol. 22, no. 3, pp. 201–211, 2011.

[41] S. T. Towhid, E.-M. Schmidt, E. Schmid et al., "Thymoquinone-induced platelet apoptosis," *Journal of Cellular Biochemistry*, vol. 112, no. 11, pp. 3112–3121, 2011.

[42] L.-M. Lien, C.-C. Su, W.-H. Hsu et al., "Mechanisms of andrographolide-induced platelet apoptosis in human platelets: regulatory roles of the extrinsic apoptotic pathway," *Phytotherapy Research*, vol. 27, no. 11, pp. 1671–1677, 2013.

[43] K. H. Lin, G. Hsiao, C. M. Shih, D. S. Chou, and J. R. Sheu, "Mechanisms of resveratrol-induced platelet apoptosis," *Cardiovascular Research*, vol. 83, no. 3, pp. 575–585, 2009.

[44] F. Violi and P. Pignatelli, "Platelet NOX, a novel target for antithrombotic treatment," *Thrombosis and Haemostasis*, vol. 111, no. 5, pp. 817–823, 2014.

[45] L.-P. Erwig and P. M. Henson, "Clearance of apoptotic cells by phagocytes," *Cell Death and Differentiation*, vol. 15, no. 2, pp. 243–250, 2008.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 2853543, 9 pages
http://dx.doi.org/10.1155/2016/2853543

Research Article

Biofunctional Activities of *Equisetum ramosissimum* Extract: Protective Effects against Oxidation, Melanoma, and Melanogenesis

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Received 26 February 2016; Revised 28 April 2016; Accepted 8 May 2016

Academic Editor: Pavel Pospisil

Equisetum ramosissimum, a genus of Equisetaceae, is a medicinal plant that can be separated into ethyl acetate (EA), dichloromethane (DM), *n*-hexane (Hex), methanol (MeOH), and water extracts. EA extract was known to have potent antioxidative properties, reducing power, DPPH scavenging activity, and metal ion chelating activity. This study compared these five extracts in terms of their inhibiting effects on three human malignant melanomas: A375, A375.S2, and A2058. MTT assay presented the notion that both EA and DM extracts inhibited melanoma growth but did not affect the viabilities of normal dermal keratinocytes (HaCaT) or fibroblasts. Western blot analyses showed that both EA and DM extracts induced overexpression of caspase proteins in all three melanomas. To determine their roles in melanogenesis, this study analyzed their *in vitro* suppressive effects on mushroom tyrosinase. All extracts except for water revealed moderate suppressive effects. None of the extracts affected B16-F10 cells proliferation. EA extract inhibited cellular melanin production whereas DM extract unexpectedly enhanced cellular pigmentation in B16-F10 cells. Data for modulations of microphthalmia-associated transcription factor, tyrosinase, tyrosinase-related protein 1, and tyrosinase-related protein 2 showed that EA extract inhibited protein expression mentioned above whereas DM extract had the opposite effect. Overall, the experiments indicated that the biofunctional activities of EA extract contained in food and cosmetics protect against oxidation, melanoma, and melanin production.

1. Introduction

Mitochondria, chloroplasts, and peroxisomes produce reactive oxygen species (ROS) through respiration and photosynthesis [1, 2]. Studies suggested that the changes in cellular homeostasis caused by high ROS levels can result in oxidative damage [3]. To avoid ROS oxidative injuries, the defense radical scavenging systems used by the human being were separated into enzymatic and nonenzymatic mechanisms. Antioxidant enzymes and substances could reduce oxidative damage by decreasing production of ROS and radicals [2]. These agents include glutathione and catalase, glutathione reductase, superoxide dismutase, and glutathione peroxidase. Others include α-lipoic acid, carotenoids, coenzyme Q10, flavonoids, antioxidative minerals (copper, zinc, manganese, and selenium), and cofactors (folic acid and vitamins A, B1, B2, B6, B12, C, and E). Generally, the above antioxidative materials are applied in synergic ways with each other against various free radical types [1–3].

Malignant melanoma is among the most incursive and life-threatening malignant tumors [4]. Skin cancer can result from exposure to ultraviolet (UV) radiation emitted by the sun and by halogen lamps. Experimental studies of metastatic melanoma are very challenging because systemic treatments are often ineffective and the rapid spread of melanoma cells to retain an intensive property of the cellular spreading which happens later is pathologically confusing [5]. Although melanoma is not a major cause of tumorous symptoms, melanoma is a major cause of death in patients with skin cancer. Treating melanoma is difficult due to its resistance to conventional chemoradiotherapy [6]. No effective therapies for metastatic melanoma are currently available, and effective drugs are urgently needed.

Melanocytes are located in the basal epidermal layer and in the hair follicles. Generally, UV radiation produces pigment by diametric stimulation of melanocytes [7]. Melanin pigmentation has been recognized by many factors; the permeation of sunlight is a well recognized source of melanin pigmentation; specifically, UV radiation causes darkening of the skin and/or sunburn. UV is the most common reason of changes in the visible countenance of human skin. Unusual melanogenesis is a characteristic of many human skin disorders, including abnormal pigmentation, nevi, and melanoma [8].

Many recent studies have investigated the biological functions of natural extracts and their potential applications as health foods, as active ingredients in cosmetics, and as leading compounds in new medicines [9, 10]. *Equisetum ramosissimum,* a genus of Equisetaceae, is a medicinal plant administered to treat hemorrhage, urethritis, jaundice, and hepatitis [11]. Although the antioxidant activities of *E. ramosissimum* were identified [12], its biological activities have not been examined. Therefore, this study elucidated the potential protective effects of *E. ramosissimum* extract against oxidation, melanoma, and melanogenesis.

2. Materials and Methods

2.1. Chemicals and Reagents. Ascorbic acid (vitamin C), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-3,4-dihydroxyphenylalanine (L-DOPA), dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethanol, ethylenediaminetetraacetic acid (EDTA), ferrous chloride ($FeCl_2{\cdot}4H_2O$), ferric chloride ($FeCl_3$), kojic acid, methanol, potassium ferricyanide ($K_3Fe(CN)_6$), 3-tert-butyl-4-hydroxyanisole (BHA), and L-tyrosine were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD, USA). Other chemical buffers and reagents were purchased at the highest available purity and quality.

2.2. Plant Material Extraction and Isolation. Two authors of this study (Dr. Chieh-Chih Shih and Professor Zhi-Hong Wen) prepared the extracts as follows. First, methanol (2.2 L) was requested in two extraction procedures of *E. ramosissimum* from a powder consisting of ground *E. ramosissimum* plant, and the extract was then refluxed for 30 minutes. After a filtering procedure, the extract was concentrated to obtain methanol (MeOH) crude extract, 9.52 g. The crude extract was added to distilled water (200 mL) and then partitioned with *n*-hexane (Hex, 1.95 g), dichloromethane (DM, 0.67 g), and ethyl acetate (EA, 0.26 g), which left an aqueous layer (H_2O, 6.44 g). All fractions were concentrated, freeze-dried, dissolved in DMSO to obtain a stock solution (500 mg/mL), and then diluted with DMEM to the required concentrations.

2.3. Assays of Antioxidant Effects

2.3.1. Reducing Power Assay. Assays of the reducing power of the crude extracts were performed as described in the literature [13]. Briefly, dissimilar concentrations of each extract were blended with 85 μL of 67 mM sodium phosphate buffer (pH 6.8) and 2.5 μL of 20% $K_3Fe(CN)_6$. The admixture was kept at 50°C for 20 min. After addition of 160 μL 10% TCA, the admixture was centrifuged for 10 min at 3,000 g. The supernatant (75 μL) was mixed with 2% $FeCl_3$ (25 μL), and absorbance was read with a spectrophotometer (BioTek Co., Winooski, VT, USA) at 700 nm with a BHA solution as a positive control. High absorbance was interpreted as a high capacity for metal ion reduction.

2.3.2. DPPH$^\bullet$ Radical Scavenging Ability Assay. DPPH is a stable free radical with a violet color. Reaction of DPPH$^\bullet$ with an antioxidant provides hydrogen, which results in decreased absorbance at 517 nm. DPPH assay was performed as described previously, with some minor modification [13]. Various concentrations of *E. ramosissimum* extracts were added to 100 μL of aqueous stable DPPH$^\bullet$ (60 μM) solution and allowed to stand at room temperature for 60 min. Vitamin C was used as a positive control. Low absorbance was interpreted as a high DPPH scavenging ability. The calculation for free radical scavenging activity (%) is done as follows:

$$\text{Scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%. \quad (1)$$

2.3.3. Metal Chelating Activity. The chelating properties of ferrous ions (Fe^{2+}) were explored using a method described

previously [14]. Briefly, various concentrations of extract were dissolved in DMSO and added to a 10 μL solution of $FeCl_2 \cdot 4H_2O$ (2 mM). Next, 20 μL ferrozine (5 mM) was added, and the admixture was shaken vigorously for 10 minutes. The absorbance was 562 nm. EDTA was used as a positive control, and the formula employed to calculate metal chelating activity is as follows:

$$\text{Metal chelating activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%. \quad (2)$$

2.4. Cell Line Cultures. Human and rat melanoma cell lines were obtained from Bioresource Collection and Research Center (Taiwan): A375 (BCRC number 60039), A375.S2 (BCRC number 60263), A2058 (BCRC number 60240), and B16-F10 (BCRC number 60031). Human fibroblasts were separated from the foreskin primary culture (Institutional Review Board, KMUH-IRB-990269). Cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics. Human skin keratinocytes, HaCaT cells, were cultured in Keratinocyte-SFM (Gibco, USA) supplemented with bovine pituitary extract and human recombinant epidermal growth factor. All cell lines were cultured in 5% CO_2 at 37°C.

2.5. MTT Assay of Cell Viability. The influences of extracts on cell development were estimated with MTT assay [15]. Cells were seeded at 8×10^3 cells/well in 96-well plates and incubated for 24 h before addition of extracts. After 24 h, MTT solution was dispensed into each well. After 2 h, the culture medium was discarded, and DMSO was added to each well. The absorbance of the formazan salt was 595 nm, and the cell viability was computed as follows:

$$\text{Cell viability (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%. \quad (3)$$

2.6. Western Blot Analysis. This analysis was performed as described previously with some minor modifications [16]. 1×10^6 cells were treated with extracts or with the vehicle control for 24 h, and the cells were then harvested and lysed with RIPA lysis buffer. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and next transferred into a polyvinylidene fluoride membrane. The membranes were incubated with corresponding primary antibodies and afterwards incubated with secondary antibodies corresponding with the primary antibodies. The signals were visualized with a chemiluminescence detection kit (Amersham, Piscataway, NJ, USA).

2.7. Mushroom Tyrosinase Measurement. Mushroom tyrosinase activity was evaluated as described previously with some minor modifications [17]. Samples were incubated with mushroom tyrosinase (25 U/mL), and L-tyrosine (2 mM) in phosphate buffer (pH 6.8) was added. The mixtures were then kept at 37°C for 30 minutes. Kojic acid was used as a positive control. Tyrosinase inhibitory activity was determined by the following equation:

$$\text{Mushroom tyrosinase inhibition (\%)} = \frac{[(A - B) - (C - D)]}{(A - B)} \times 100\%, \quad (4)$$

where A is the optical density (OD_{490}) without testing extract; B is OD_{490} with tyrosinase and without testing extract; C is OD_{490} with testing extract; and D is OD_{490} with tyrosinase and without testing extract.

2.8. Melanin Quantification Assessment. This analysis was demonstrated as described in the literature with some minor modifications [17]. Cell pellets were liquefied with 1.0 N NaOH, warmed to 80°C for 1 hour, and centrifuged at 10,000 g for 10 minutes. The quantity of melanin was determined by extracting the supernatant and running it through the spectrophotometer, which gave a result of 475 nm.

2.9. Statistical Analysis. Biofunctional assays of the *E. ramosissimum* extracts in each platform were performed in triplicate. Results were expressed as means ± SD. Analysis of variance was used for data analysis. A p value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Antioxidant Activity of E. ramosissimum. Free radicals have roles in signal travel and in physiological, metabolic, and immune reactions. Even though free radicals are needed for normal healthy biochemical processes in the body, they have severe negative health effects [18, 19]. One of the study aims was to test antioxidative properties, which were examined by ferric reducing powers, DPPH radical scavenging capacities, and metal chelating power activities.

First, a simple, rapid, and reliable test was used to measure Fe(III)-ferricyanide complex synthesis. In this test, the extracts reducing properties of *E. ramosissimum* were indicated by changes in the color of the solution (from light yellow to different shades of green and blue). Table 1 presented the notion that the reducing power of EA extract resulted in stronger dose-dependent suppressive effects compared to the other four extracts and showed the highest scavenging of 0.87 ± 0.23 at 200 μg/mL.

The second oxidation inhibitory assay was DPPH radical scavenging test. As antioxidants stabilize DPPH radicals, the color of DPPH solution changes from violet to yellow as diphenylpicrylhydrazine is formed. Table 1 illustrated the results for the five extracts, and the comparisons presented the notion that the EA extract had the strongest radical scavenging effects in a dose-dependent manner.

Within the oxidative conditions, ferrozine can develop complexes with Fe^{2+} to be measured quantitatively. In the presence of chelating materials, complex constructions are dislocated, which lightens the red color of the complex. Table 1 demonstrated the data for the last evaluation

Table 1: Antioxidant activities of *E. ramosissimum* extracts, including reducing power, DPPH free radical scavenging activity, and ferrous ion chelating power.

Extracts (μg/mL)	Reducing power (OD_{700})			DPPH scavenging capacity (%)			Chelating activity (%)		
	50	100	200	50	100	200	50	100	200
EA	0.60 ± 0.06	0.79 ± 0.06	0.87 ± 0.23	<10.0	15.23 ± 2.76	43.41 ± 7.68	20.47 ± 3.57	34.69 ± 5.03	44.56 ± 1.32
DM	0.59 ± 0.03	0.65 ± 0.07	0.68 ± 0.04	<10.0	<10.0	<10.0	15.57 ± 1.39	17.49 ± 0.13	20.19 ± 1.72
Hex	0.56 ± 0.11	0.65 ± 0.10	0.68 ± 0.03	<10.0	<10.0	<10.0	17.98 ± 4.50	20.71 ± 0.33	22.53 ± 6.07
MeOH	0.58 ± 0.01	0.67 ± 0.13	0.73 ± 0.02	<10.0	10.69 ± 1.66	15.60 ± 1.28	10.63 ± 1.65	18.89 ± 1.19	20.43 ± 0.07
Water	0.62 ± 0.03	0.65 ± 0.10	0.70 ± 0.01	<10.0	<10.0	14.87 ± 0.73	10.77 ± 0.79	13.33 ± 4.55	13.52 ± 0.59
Vitamin C[a]	—	—	—	—	85.55 ± 0.48	—	—	—	—
BHA[b]	—	1.64 ± 0.29	—	—	—	—	—	—	—
EDTA[c]	—	—	—	—	—	—	—	95.49 ± 0.05	—

All statistics are presented as average values ± SD; $n = 3$. [a]Vitamin C (100 mM) was utilized as a positive control for DPPH assay; [b]EDTA (100 mM) was used as a positive control for analysis of metal chelating ability; [c]BHA (100 mM) was applied as a positive control for analysis of reducing power. Assays not performed in this study were indicated by dashes.

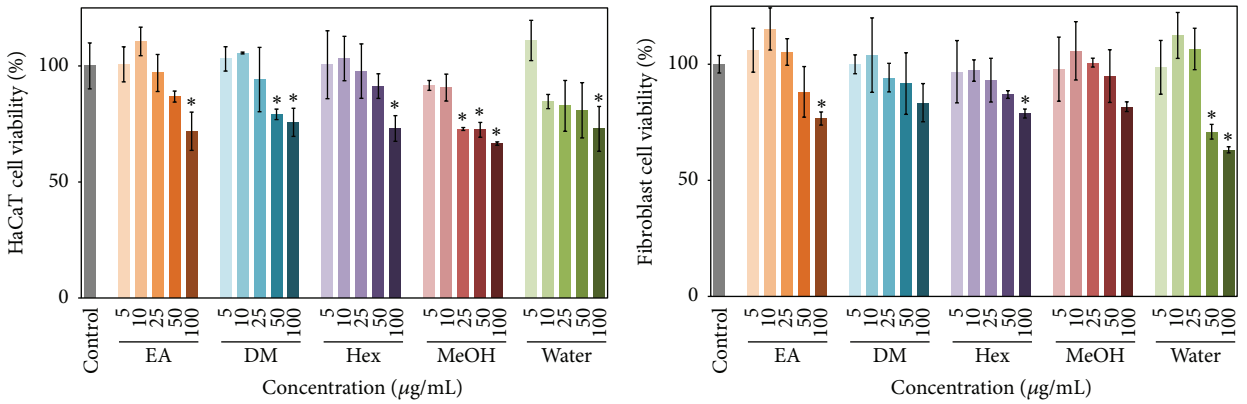

Figure 1: Effects of *E. ramosissimum* extracts on viability of normal human cells according to MTT assay. Suppression of cell viability was measured in (a) HaCaT and (b) fibroblast cells cultured with 5, 10, 25, 50, and 100 μg/mL EA, DM, Hex, MeOH, and water extracts. The results for the control group cultured without extracts were shown on the left (gray line). All experimental data were presented as average values ± SD; $n = 3$; $^*p < 0.05$.

of antioxidative properties. The extracts showed low-to-moderate Fe^{2+} scavenging activities at concentrations of 50–200 μg/mL, and EA extract possessed the highest value of 44.56 ± 1.32 at 200 μg/mL.

3.2. Cytotoxicity of E. ramosissimum on Human Melanoma Cells and Normal Cells. Melanoma is a malignant tumor that starts in a certain type of skin cell and is activated when the abnormal cells in the affected part of the body begin to proliferate in an uncontrolled manner [20]. Metastatic malignant melanomas are highly resistant to existing therapies and have a very poor prognosis, and thus new treatment strategies are urgently needed. In early stages of the development of new chemoprotective substances, the main considerations are normal cell allergic responses, sensitivity and potential reactions, and toxic side effects [9]. MTT method was applied to evaluate the cytotoxic effectivenesses of *E. ramosissimum* extracts on normal human skin cells, including epidermal keratinocytes (HaCaT) and dermal fibroblasts in Figure 1. These two cells were treated with various concentrations (0 to 100 μg/mL) to compare dose-dependent impacts. In both cells, high doses (100 μg/mL) of the five *E. ramosissimum* extracts had minor effects, and all cellular viabilities exceeded 65% after a 24-hour treatment. That is, the *E. ramosissimum* extracts had no severe discernible toxic effects on human normal cells.

After establishing the fact that the *E. ramosissimum* extracts did not cause major injuries to normal cells, the melanoma platforms were used to investigate their antiproliferative effects. Cell line A375 was from a malignant melanoma of a 54-year-old female; and cell line A375.S2 comprised cells which were differentiated from A375 cells; and cell line A2058 comprised highly invasive melanoma cells [21]. In Figure 2, we demonstrated the cytotoxicities of the three cell lines, notably, and high doses (50 and 100 μg/mL) of the EA, DM, and Hex extracts from *E. ramosissimum* had much larger influences on A375 and A375.S2 cells compared to the A2058 cells. For example, after 24 h treatment with 50 and 100 μg/mL EA extract, the viabilities of the A375 and A375.S2 cells decreased to 30% whereas the viability of the A2058 cells only

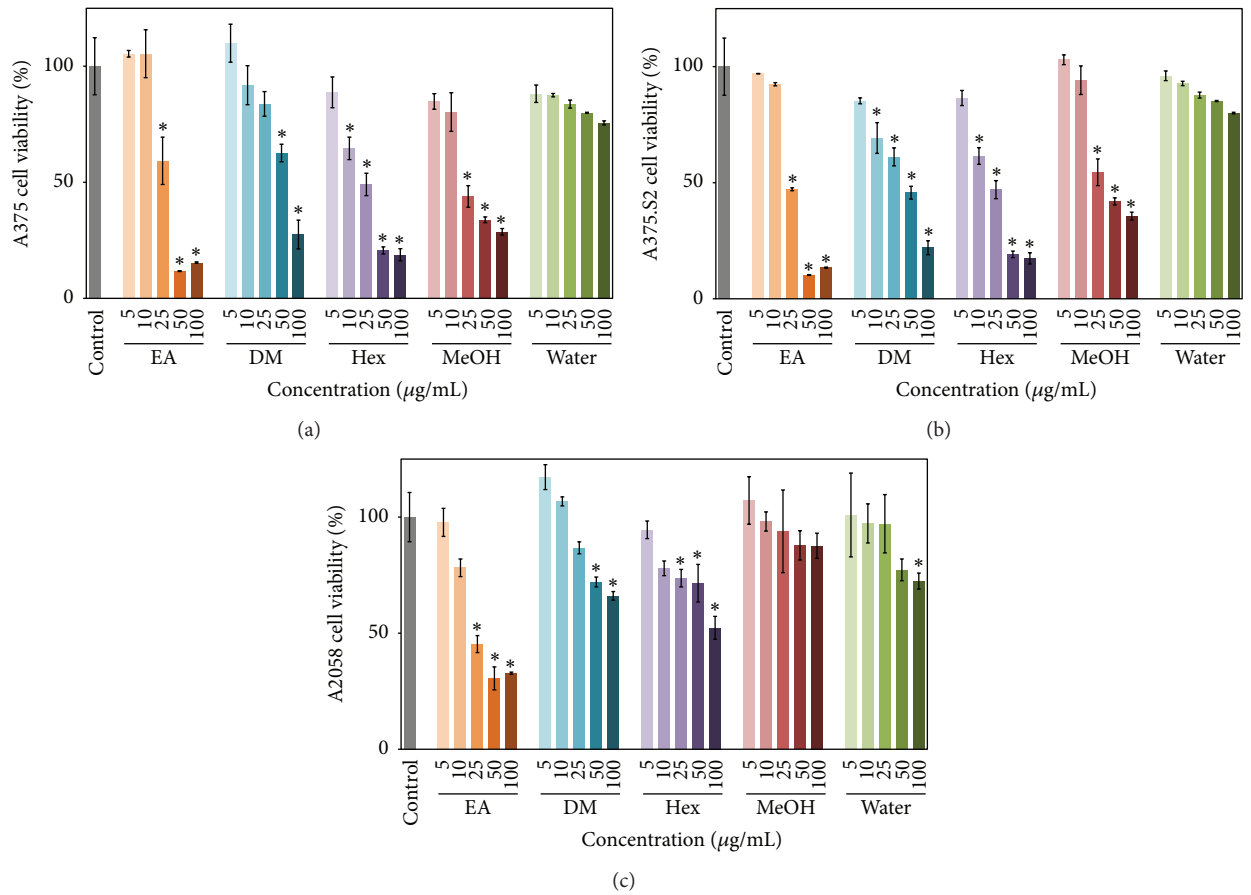

FIGURE 2: Effects of *E. ramosissimum* extracts on viability of human melanoma cells according to MTT assay. Suppression of cell viability was measured in (a) A375, (b) A375.S2, and (c) A2058 cells cultured with 5, 10, 25, 50, and 100 μg/mL EA, DM, Hex, MeOH, and water. The results for the control group cultured without extract were demonstrated on the left (gray line). All experimental data were presented as average values ± SD; $n = 3$; $^{*}p < 0.05$.

decreased to 50%. MeOH extract had no effect on the A2058 cells, and the water extract had no apparent cytotoxic effect on the three melanoma cell types. According to our statistical data, the *E. ramosissimum* extracts have little harmful effects on normal skin cells, and these extracts, particularly EA, DM, and Hex extracts, actually inhibit melanoma cellular proliferation.

3.3. Effects of EA and DM Extracts on Caspase Proteins in Melanoma Cells. Caspase is a family of cysteine-aspartic proteases that mediates type I programmed cell death (apoptosis), and more than 10 family members have been identified so far [22]. The activation of caspase-associated proteins is essential for apoptosis induced by various apoptotic stimuli. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation; however, the failure of cancer cell apoptosis is a major contributor to tumor development and autoimmune disease [23].

Caspase-9 initiates an apoptotic cascade by cleaving and activates caspase-3 [24]. However, the maturation of caspase-9 requires autocatalytic cleavage by apoptosomes released by damaged mitochondria [25]. The cleavage of caspase-3 activates caspase-6 and caspase-7; the protein itself is processed and activated by caspase-8, caspase-9, and caspase-10. The activation of caspase-3 induces cellular apoptosis and proteolysis in specific substrates [26]. Figure 3 showed how caspase affected the apoptotic process induced by EA and DM extracts of *E. ramosissimum* in the three human melanoma cells. Remarkable alterations caused by molecular proteins associated with apoptosis included increased proteolysis induced by caspase-3 and caspase-9. A low concentration (10 μg/mL) of EA extract induced stimulated enzymes in A375, A375.S2, and A2058 cells. Although DM extract also triggered caspase-3 and caspase-9, its effect was smaller compared to a similar dose of EA extract. Notably, 50 μg/mL DM extract was needed to trigger caspase-3 and caspase-9 in A2058 cells. The cellular proteins caspase-3 and caspase-9 have important roles in the regulation of nuclear DNA damage caused by apoptosis and in the decomposition of organelles. Our experiments demonstrated that EA induced caspase protein changes, and similar results were also shown from DM extract, but lower.

3.4. Mushroom Tyrosinase Inhibition. In mammals, the rate-limiting enzyme tyrosinase is the most important enzyme of

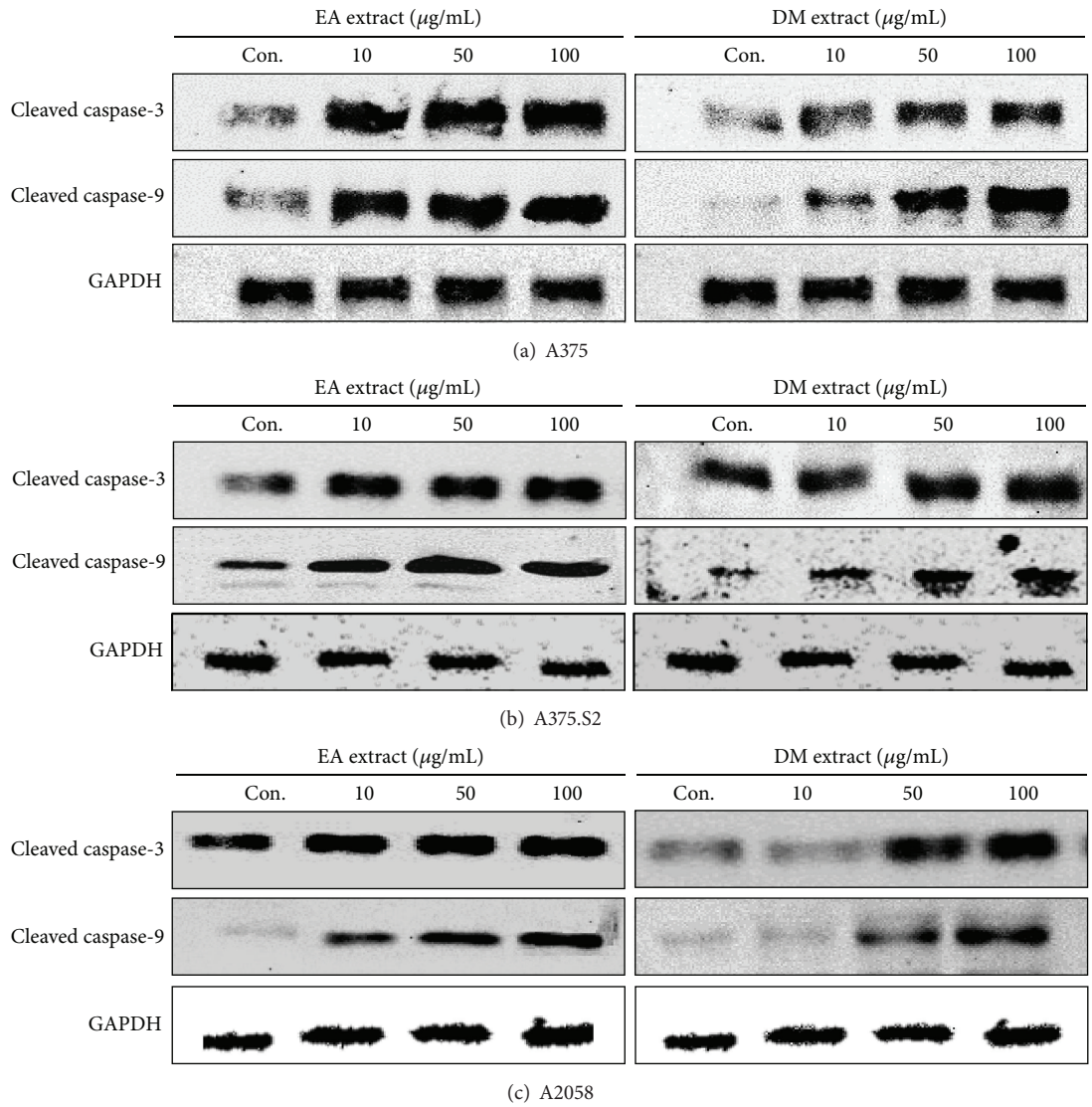

Figure 3: Expressions of caspase proteins in (a) A375, (b) A375.S2, and (c) A2058 cells after treatment with 10, 50, and 100 μg/mL EA and DM extracts for 24 h. Band darkness indicated relative protein expressions in comparison with GAPDH.

pigment biosynthesis reactions. Other enzymes only adjust to differences in synthesis of eumelanin and pheomelanin [10, 27, 28]. To determine whether the extracts inhibited melanin synthesis by suppressing tyrosinase, *in vitro* tyrosinase activity was tested in mushroom type. In Table 2, it was shown that all extracts except for water had dose-dependent (5–100 μg/mL) inhibiting effects on the mushroom tyrosinase system. At a concentration of 100 μg/mL, the repressive effect of EA extract was slightly lower than that of kojic acid. At this concentration, EA had the strongest inhibiting effect (approximately 40%).

3.5. Melanin Content of B16-F10 Cells. Melanin is the source of skin color and can protect the skin from UV radiation damage which induces DNA mutations. Despite its protective functions, superabundance of melanin negatively affects the skin, which then causes social problems [29, 30]. To demonstrate the skin whitening effects of *E. ramosissimum* and its inhibiting effects on melanogenesis, melanin alterations were measured after treatments with *E. ramosissimum* extracts. In Figure 4(a), it was demonstrated that the five fraction extracts

Table 2: Mushroom tyrosinase inhibition by different concentrations of *E. ramosissimum* extracts.

Concentrations (μg/mL)	Mushroom tyrosinase inhibition (%)		
	5	50	100
EA	22.42 ± 0.25	24.40 ± 4.90	38.93 ± 3.09
DM	20.69 ± 4.53	21.30 ± 1.10	23.79 ± 3.84
Hex	20.54 ± 0.64	22.33 ± 0.69	23.82 ± 4.25
MeOH	18.54 ± 2.70	20.45 ± 0.36	23.27 ± 1.67
Water	17.88 ± 3.46	19.37 ± 0.12	23.82 ± 1.24
Kojic acid[a]	—	—	30.23 ± 5.68

All statistics are presented as average values ± SD; $n = 3$. [a]Kojic acid (100 μg/mL) was applied as a positive control. Assays not performed in this study were indicated by dashes.

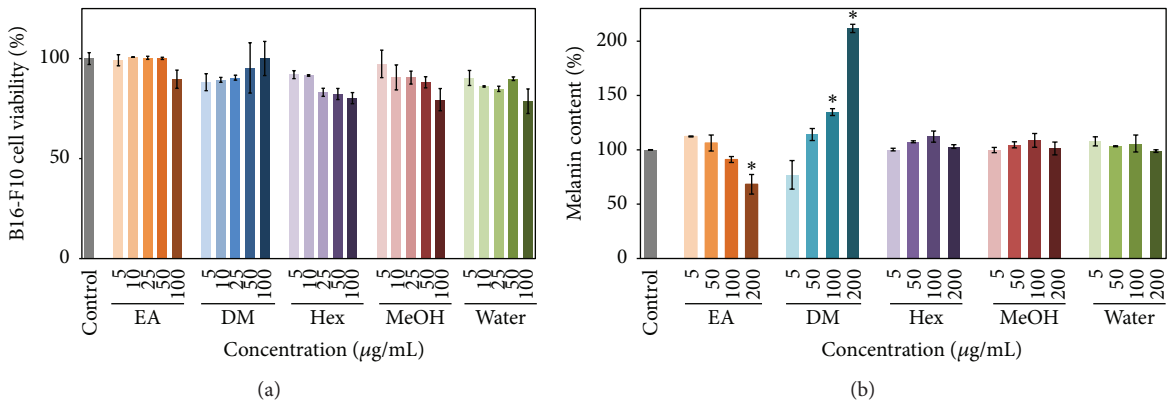

Figure 4: (a) Effects of *E. ramosissimum* extracts on viability of B16-F10 cells according to MTT assay and (b) effects of *E. ramosissimum* extracts on melanin content quantification of all extracts were processed with 5, 50, 100, and 200 μg/mL, respectively. The results for the control group cultured without extracts were shown on the left (gray line). All experimental results were presented as average values ± SD; $n = 3$; $^{*}p < 0.05$.

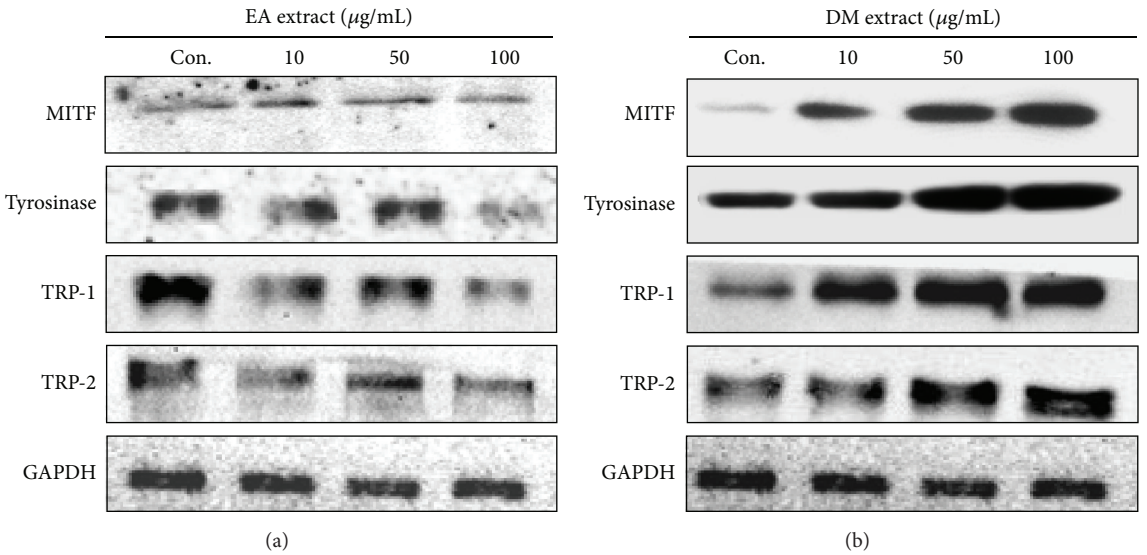

Figure 5: Expressions of tyrosinase, TRP-1, TRP-2, and MITF after treatment with *E. ramosissimum* extracts. B16-F10 cells were treated with EA and DM extracts at concentrations of 10, 50, and 100 μg/mL for 24 h. Protein expressions were shown in comparison with GAPDH.

did not substantially harm B16 cell viability. In Figure 4(b), we presented the notion that the EA extract had the ability to decrease melanin production by about 32% at 200 μg/mL in a dose-dependent trend from concentrations of 5 to 200 μg/mL. In contrast, 200 μg/mL DM extract substantially augmented melanin production (112%), and the effects of DM extract were dose-dependent. Other extracts, including Hex, MeOH, and water, did not substantially change melanin production, even at the maximum experimental dose of 200 μg/mL. These experimental results suggested that the EA extract had potential applications as a whitening agent in cosmetic products whereas the DM extract could be used as a skin darkening agent.

3.6. Expression of Melanogenesis-Related Proteins in B16-F10 Cells. A well known role of microphthalmia-associated transcription factor (MITF) in melanogenesis is normal synthesis of melanin in melanocytes. Expression of MITF increases melanin production by facilitating biosynthesis of tyrosinase, tyrosinase-related protein 1 (TRP-1), and TRP-2 [31]. Expression of MITF is also significant for stabilizing tyrosinase protein and modulating its catalytic activity [8, 32]. To elucidate the mechanisms of its activity, expressions of melanogenesis-related proteins were analyzed by western blot. Figure 5 presented the notion that EA extract concentrations ranging from 10 to 100 μg/mL reduced expressions of MITF, tyrosinase, TRP-1, and TRP-2 in a dose-dependent manner. Conversely, DM extract promoted expressions of MITF, tyrosinase, TRP-1, and TRP-2 in B16-F10 cells in a dose-dependent manner. Both of these phenomena were consistent with the data for melanin content shown in Figure 4(b).

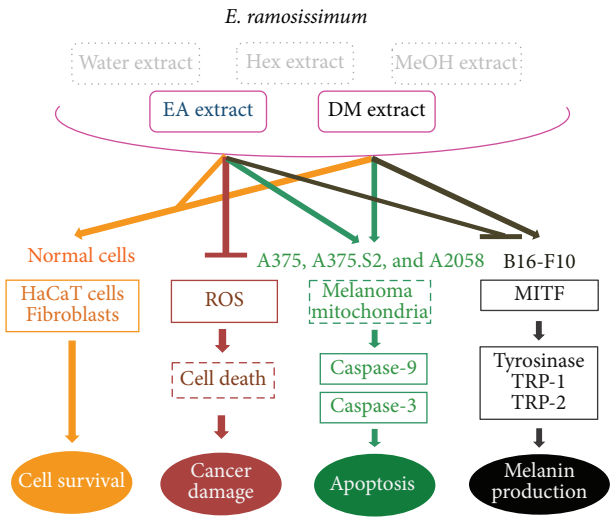

Figure 6: Schematic diagram of biofunctions of *E. ramosissimum* extracts in human skin cells, including normal cell survival, apoptotic pathways of melanoma, and melanogenesis.

4. Conclusion

In summary, the experiments in this study demonstrated that the most beneficial of the five fraction extracts of *E. ramosissimum* was EA because of its multiple biofunctional properties (Figure 6). The experimental outcomes showed that, by acting as an antioxidant ingredient and electron donor, EA extract discontinued or terminated free radical chain reactions. In human melanoma, EA and DM extracts affected the viabilities of melanoma cells and showed low toxicity in both normal human cells, HaCaT cells and fibroblasts. To understand the mechanisms of cell death, we performed western blot analyses of protein expressions in melanoma cells, which pointed out that both extracts induced caspase-3 and caspase-9, both of which have vital roles in apoptosis. Research evaluations of the potential use of EA extract as a whitening agent illustrated that it inhibited mushroom tyrosinase activity and the synthesis of melanin; in contrast, DM extract increased the quantity of melanin. Western blot analyses showed that EA and DM extracts decreased and increased melanin content, respectively, by regulating MITF, tyrosinase, Trp-1, and Trp-2. Whereas this study established the biological functions of *E. ramosissimum*, our future studies will further investigate the components and mechanisms of these compounds.

Competing Interests

The authors have no competing interests regarding the publication of this study.

Authors' Contributions

Pin-Hui Li and Yu-Pin Chiu equally contributed to this study.

Acknowledgments

This work was supported by grants from the Ministry of Science and Technology, Taiwan (MOST104-2622-E-037-001; MOST104-2622-E-037-003-CC2; MOST104-2221-E-037-005-MY2; and MOST104-2628-E-037-001-MY3), from the Center for Stem Cell Research, Kaohsiung Medical University, Kaohsiung, Taiwan (KMU-TP104G00 and KMUTP104G02-05), from Kaohsiung Medical University, Taiwan (KMU-DK105005), from Kaohsiung Medical University, NSYSU-KMU Joint Research Project, Taiwan (NSYSUKMU105-P 007), and from the Center for Infectious Disease and Cancer Research, Kaohsiung Medical University, Taiwan (KMU-TP104E18).

References

[1] K. Apel and H. Hirt, "Reactive oxygen species: metabolism, oxidative stress, and signal transduction," *Annual Review of Plant Biology*, vol. 55, pp. 373–399, 2004.

[2] Á. Gutiérrez-Uzquiza, M. Arechederra, P. Bragado, J. A. Aguirre-Ghiso, and A. Porras, "p38α mediates cell survival in response to oxidative stress via induction of antioxidant genes: effect on the p70S6K pathway," *Journal of Biological Chemistry*, vol. 287, no. 4, pp. 2632–2642, 2012.

[3] C. Richardson, S. Yan, and C. G. Vestal, "Oxidative stress, bone marrow failure, and genome instability in hematopoietic stem cells," *International Journal of Molecular Sciences*, vol. 16, no. 2, pp. 2366–2385, 2015.

[4] K.-N. Kim, G. Ahn, S.-J. Heo et al., "Inhibition of tumor growth in vitro and in vivo by fucoxanthin against melanoma B16F10 cells," *Environmental Toxicology and Pharmacology*, vol. 35, no. 1, pp. 39–46, 2013.

[5] A. Ascenso, T. Pedrosa, S. Pinho et al., "The effect of lycopene preexposure on UV-B-irradiated human keratinocytes," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 8214631, 15 pages, 2016.

[6] C.-C. Lee, L.-Y. Chiou, J.-Y. Wang et al., "Functional ginger extracts from supercritical fluid carbon dioxide extraction via in vitro and in vivo assays: antioxidation, antimicroorganism, and mice xenografts models," *The Scientific World Journal*, vol. 2013, Article ID 210845, 8 pages, 2013.

[7] R. Ghosh, D. Guha, S. Bhowmik, and S. Karmakar, "Antioxidant enzymes and the mechanism of the bystander effect induced by ultraviolet C irradiation of A375 human melanoma cells," *Mutation Research*, vol. 757, no. 1, pp. 83–90, 2013.

[8] W.-S. Liu, Y.-D. Kuan, K.-H. Chiu et al., "The extract of *Rhodobacter sphaeroides* inhibits melanogenesis through the MEK/ERK signaling pathway," *Marine Drugs*, vol. 11, no. 6, pp. 1899–1908, 2013.

[9] C.-C. Lee, Y.-T. Chen, C.-C. Chiu, W.-T. Liao, Y.-C. Liu, and H.-M. David Wang, "Polygonum cuspidatum extracts as bioactive antioxidaion, anti-tyrosinase, immune stimulation and anticancer agents," *Journal of Bioscience and Bioengineering*, vol. 119, no. 4, pp. 464–469, 2015.

[10] M.-G. Lee, S.-Y. Kuo, S.-Y. Yen et al., "Evaluation of *Cinnamomum osmophloeum* Kanehira extracts on tyrosinase suppressor, wound repair promoter, and antioxidant," *The Scientific World Journal*, vol. 2015, Article ID 303415, 7 pages, 2015.

[11] 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.

[12] S. Paulsamy, D. Moorthy, K. Nandakumar et al., "Evaluation of *in vitro* antioxidant potential of methanolic extracts of the ferns, *Actiniopteris radiata* (Sw) Link and *Equisetum ramosissimum* Desf," *International Journal of Research and Development in Pharmacy & Life Sciences*, vol. 2, no. 3, pp. 451–455, 2013.

[13] H.-M. Wang, Y.-T. Chou, Z.-L. Hong et al., "Bioconstituents from stems of *Synsepalum dulcificum* Daniell (Sapotaceae) inhibit human melanoma proliferation, reduce mushroom tyrosinase activity and have antioxidant properties," *Journal of the Taiwan Institute of Chemical Engineers*, vol. 42, no. 2, pp. 204–211, 2011.

[14] H.-M. Wang, J.-L. Pan, C.-Y. Chen et al., "Identification of anti-lung cancer extract from *Chlorella vulgaris* C-C by antioxidant property using supercritical carbon dioxide extraction," *Process Biochemistry*, vol. 45, no. 12, pp. 1865–1872, 2010.

[15] H.-M. Wang, C.-Y. Chen, and Z.-H. Wen, "Identifying melanogenesis inhibitors from *Cinnamomum subavenium* with in vitro and in vivo screening systems by targeting the human tyrosinase," *Experimental Dermatology*, vol. 20, no. 3, pp. 242–248, 2011.

[16] C.-L. Lin, R.-F. Chen, J. Y.-F. Chen et al., "Protective effect of caffeic acid on paclitaxel induced anti-proliferation and apoptosis of lung cancer cells involves NF-κb pathway," *International Journal of Molecular Sciences*, vol. 13, no. 5, pp. 6236–6245, 2012.

[17] X. Chen, B. Zhang, X. Yuan et al., "Isoliquiritigenin-induced differentiation in mouse melanoma B16F0 cell line," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 534934, 11 pages, 2012.

[18] X. H. Pan, X. L. Zhang, H. L. Sun, J. Zhang, M. Yan, and H. Zhang, "Autophagy inhibition promotes 5-fluorouraci-induced apoptosis by stimulating ROS formation in human non-small cell lung cancer A549 cells," *PLoS ONE*, vol. 8, no. 2, Article ID e56679, 2013.

[19] A. Godic, B. Poljšak, M. Adamic, and R. Dahmane, "The role of antioxidants in skin cancer prevention and treatment," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 860479, 6 pages, 2014.

[20] D.-L. Ma, L.-J. Liu, K.-H. Leung et al., "Antagonizing STAT3 dimerization with a rhodium(III) complex," *Angewandte Chemie—International Edition*, vol. 53, no. 35, pp. 9178–9182, 2014.

[21] M. Liu and S. Douthwaite, "Resistance to the macrolide antibiotic tylosin is conferred by single methylations at 23S rRNA nucleotides G748 and A2058 acting in synergy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 23, pp. 14658–14663, 2002.

[22] T. Nakagawa, H. Zhu, N. Morishima et al., "Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β," *Nature*, vol. 403, no. 6765, pp. 98–103, 2000.

[23] H. A. Harrington, K. L. Ho, S. Ghosh, and K. C. Tung, "Construction and analysis of a modular model of caspase activation in apoptosis," *Theoretical Biology and Medical Modelling*, vol. 5, article 26, 2008.

[24] P.-F. Wu, C.-C. Chiu, C.-Y. Chen, and H.-M. D. Wang, "7-Hydroxydehydronuciferine induces human melanoma death via triggering autophagy and apoptosis," *Experimental Dermatology*, vol. 24, no. 12, pp. 930–935, 2015.

[25] Y. Pang, X. C. Bai, C. Yan et al., "Structure of the apoptosome: mechanistic insights into activation of an initiator caspase from *Drosophila*," *Genes & Development*, vol. 29, no. 3, pp. 277–287, 2015.

[26] S.-C. Lo, Y. Wang, M. Weber, J. L. Larson, K. Scearce-Levie, and M. Sheng, "Caspase-3 deficiency results in disrupted synaptic homeostasis and impaired attention control," *The Journal of Neuroscience*, vol. 35, no. 5, pp. 2118–2132, 2015.

[27] J. V. Gruber and R. Holtz, "Examining the impact of skin lighteners in vitro," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 702120, 7 pages, 2013.

[28] E. Neagu, G. Paun, C. Albu, and G.-L. Radu, "Assessment of acetylcholinesterase and tyrosinase inhibitory and antioxidant activity of *Alchemilla vulgaris* and *Filipendula ulmaria* extracts," *Journal of the Taiwan Institute of Chemical Engineers*, vol. 52, pp. 1–6, 2015.

[29] M. Brenner and V. J. Hearing, "The protective role of melanin against UV damage in human skin," *Photochemistry and Photobiology*, vol. 84, no. 3, pp. 539–549, 2008.

[30] C.-Y. Chen, L.-C. Lin, W.-F. Yang, J. Bordon, and H.-M. D. Wang, "An updated organic classification of tyrosinase inhibitors on melanin biosynthesis," *Current Organic Chemistry*, vol. 19, no. 1, pp. 4–18, 2015.

[31] K. S. Hoek, N. C. Schlegel, O. M. Eichhoff et al., "Novel MITF targets identified using a two-step DNA microarray strategy," *Pigment Cell and Melanoma Research*, vol. 21, no. 6, pp. 665–676, 2008.

[32] J.-Y. Lee, "Down-regulation of MITF, TRP-1, TRP-2, and tyrosinase expressions by compounds isolated from Pruni persicae Flos in murine B16F10 melanoma," *Journal of the Korean Society for Applied Biological Chemistry*, vol. 57, no. 6, pp. 827–834, 2014.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9681425, 6 pages
http://dx.doi.org/10.1155/2016/9681425

Research Article

Antioxidant and Hypolipidemic Activity of the Hydroethanolic Extract of *Curatella americana* L. Leaves

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Received 16 February 2016; Accepted 12 April 2016

Academic Editor: Renata Szymanska

High levels of reactive oxygen species in the body and hyperlipidemia are key factors for the development of cardiovascular diseases such as atherosclerosis. The present study investigated the antioxidant and hypolipidemic activity of hydroethanolic extract of *Curatella americana* L. leaves (ExC). The antioxidant activity of ExC was assessed by 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) scavenging capacity and protection against hemolysis induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), followed by quantification of malondialdehyde (MDA). Wistar rats with hyperlipidemia induced by high-fructose diet (60%) were treated for 60 days with water, simvastatin (30 mg·Kg^{-1}), ciprofibrate (2 mg·Kg^{-1}), and ExC (200 mg·Kg^{-1}). ExC revealed IC_{50} of 6.0 ± 0.5 μg·mL^{-1}, an intermediary value among positive controls used in the assay of DPPH scavenging capacity. At all concentrations (50 to 125 μg·mL^{-1}) and times (60 to 240 min) evaluated, ExC protected erythrocytes against AAPH-induced hemolysis, which was confirmed by lower MDA levels. *In vivo* tests showed a reduction of 34 and 45%, respectively, in serum concentration of cholesterol and triglycerides in hyperlipidemic rats treated with ExC, a similar effect compared to the reference drugs, simvastatin and ciprofibrate, respectively. Together, the results showed the antioxidant activity of ExC and its ability to improve the serum lipid profile in hyperlipidemic rats.

1. Introduction

Hyperlipidemia causes about 17 million deaths worldwide each year [1]; in addition, it is also a key factor for the development of heart and coronary diseases and atherosclerosis. Atherosclerosis is a chronic inflammatory disease triggered by multiple factors, with strong contribution of endothelial damage related to lipid peroxidation. This endothelial dysfunction increases the permeation of low-density lipoproteins (LDL) through the intima layer, resulting in oxidation and formation of atherosclerotic damage [2, 3]. In order to control this imbalance, the body has enzymatic and nonenzymatic antioxidant defense mechanisms [4] capable of preventing the deleterious effects of oxidation, inhibiting lipid peroxidation, free radicals scavenging, and maintaining redox balance in cells.

In addition to endogenous antioxidants, there are antioxidants from exogenous sources. The beneficial effects of foods have been linked to the presence of bioactive compounds and other nutrients. Examples of biomolecules that have antioxidant potential are phenolic compounds such as isoflavones, phenolic acids, catechins, chlorogenic acids, anthocyanins, and terpenes [5]. Thus, plants have been described as an alternative to the development of new drugs [6] applied to treatment of many diseases such as hypercholesterolemia, ulcers, depurative blood, and cancer [7–9].

Curatella americana L. is a member of the Dilleniaceae family, popularly known in Brazil as "lixa or lixeira" [10].

The beneficial effects of *C. americana* have been described in scientific research and indicated by its popular use. The anti-inflammatory, analgesic, antihypertensive, and vasodilator effects of the hydroethanolic extract of *Curatella americana* L. leaves have been evaluated in [11–13]. In folk medicine, leaf decoction is used as an antiseptic and astringent; bark infusion is used for the treatment of cold and healing wounds, ulcers, diabetes, and hypertension [14].

In this context, the aim of this study was to evaluate the antioxidant and hypolipidemic activity of the hydroethanolic extract of *Curatella americana* L. leaves (ExC) on rats with hyperlipidemia induced by high-fructose diet.

2. Material and Methods

2.1. Plant Material and Extract Preparation. *C. americana* L. leaves were collected in Mato Grosso do Sul, Brazil. The plant material was dried (45–50°C), crushed, and macerated in ethanol : water (80 : 20, v/v) at room temperature for seven days. After this period, the extract was filtered, concentrated in a rotary vacuum evaporator (FISATOM), and lyophilized. The lyophilized ExC was stored at 4°C and protected from light.

2.2. Dosage of Phenolic Compounds, Total Flavonoids, and Saponins. The concentration of phenolic compounds in samples was determined by the spectrophotometric method described by [15] using the Folin-Ciocalteu method. Three ExC measurements were performed, the average being presented in mg of gallic acid equivalents (GAE) per 100 g of sample.

The content of total flavonoids was determined according to methodology described by [16], with some adaptations, using 2% aluminum chloride solution in methanol as reagent. Extract solutions were prepared in methanol : water (1 : 1) at concentration of 10 $mg \cdot mL^{-1}$. About 0.5 mL ExC was added to 4.5 mL methanolic solution of 2% hydrate aluminum chloride. After 30 min at rest, the absorbance of solutions was read at 415 nm.

The presence of saponins was evaluated by preparing 10 mg of ExC dissolved in 2 mL of ethanol. Then, 5 mL of boiling water was added; the sample was vigorously shaken and allowed to stand for 20 min. According to [17], foaming indicates the presence of saponins.

2.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Activity. The antioxidant activity of the hydroethanolic extract of *C. americana* L. leaves was evaluated using technique described and adapted by [18] of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging. DPPH solutions were prepared (0.11 mM) using ascorbic acid and butylhydroxytoluene (BHT) as positive controls and ExC at concentration of 20 $mg \cdot mL^{-1}$ in 80% ethanol solution. Serial dilutions were prepared based on these solutions in the concentrations investigated. To establish the half-maximal inhibitory concentration (IC_{50}) of DPPH free radical scavenging, the samples were tested in serial dilutions (0.1, 1, 5, 10, 25, 50, 100, 500, and 1000 μg/mL) and analyzed by means of nonlinear regression using the Prism 5 GraphPad Software. Samples were assessed by spectrophotometer at 517 nm. The absorbance of each sample was divided by the absorbance of DPPH and multiplied by one hundred to represent the antioxidant activity in percentage. All independent experiments were performed in triplicate.

2.4. Protection against Hemolysis Induced by 2,2′-Azobis(2-amidinopropane) Dihydrochloride (AAPH). Protection against lipid peroxidation of the extract was evaluated by hemolysis technique induced by 2,2-azobis 2-amidinopropane dihydrochloride (AAPH), described in [19]. About 5 mL of peripheral blood was collected from healthy donors, stored in tubes with sodium citrate (protocol approved by Ethics Research Committee: protocol number 123/12), and subsequently centrifuged at 2000 rpm for 5 min. The buffy coat was removed from plasma. The remaining erythrocytes underwent three washes with saline (0.9% NaCl) at 1500 rpm to remove possible interferences, with the supernatant discarded after each washing cycle. Subsequently, a 10% erythrocyte solution was prepared in saline.

The erythrocyte solution was incubated with distilled water (total hemolysis) and hemolysis induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH; Sigma-Aldrich®) (50 mM) alone or concomitant with the standard antioxidant, ascorbic acid (AA), and ExC at concentrations of 50, 75, 100, and 125 $\mu g \cdot mL^{-1}$, reaching a final concentration of red blood cells of 2.5%. Aliquots were taken every 60 min after the start of incubation for 240 min, which were read in spectrophotometer at 540 nm. Three independent experiments were performed in duplicate.

2.5. Dosage of Malondialdehyde (MDA). A 20% suspension was used to assess the protective effects of ExC against lipid peroxidation. The dosage of MDA was evaluated after 240 min of incubation at 37°C with and without addition of 500 μL of peroxyl radicals generated by thermal decomposition of 50 mM AAPH diluted in saline (0.9% NaCl). For this, after the incubation period, an aliquot of 0.5 mL of the reaction mixture was collected and was added to 0.5 mL of 20% trichloroacetic acid with subsequent homogenization. An aliquot of 0.5 mL was removed from this mixture and added into tubes previously pipetted with 1 mL of thiobarbituric acid reagent (TBA) 10 nM and incubated in water bath at 94°C for 45 min. After this period, samples were kept at room temperature for 15 min, followed by addition of 3 mL of butanol with subsequent agitation and centrifugation as described by [20]. Reading of the supernatant absorbance was carried out in spectrophotometer at 532 nm. Three independent experiments were performed in duplicate.

2.6. Hyperlipidemia Induced by High-Fructose Diet. The experimental procedures were approved by the Ethics Research Committee on Animal Experiments of UFGD under protocol number 022/2012. Wistar rats weighting approximately 156 ± 9 g were pretreated for 90 days with high-fructose diet (66%), prepared with 330 g of commercial chow (Labina) mixed with 660 g of fructose to induce

Table 1: IC_{50} and maximum activity of DPPH free radical scavenging of standard antioxidants and the hydroethanolic extract of *C. americana* L. leaves (ExC).

Treatment	IC_{50} ($\mu g \cdot mL^{-1}$)	n^*	Maximum activity	
			%	$\mu g \cdot mL^{-1}$
Ascorbic acid	1.8 ± 0.4	2	92.3 ± 0.8	10
BHT	18.3 ± 4.5	2	93.7 ± 1.3	500
ExC	6.0 ± 0.5	3	96.5 ± 1.2	25

$^*n$ = number of independent experiments in triplicate. BHT = butylhydroxytoluene.

hyperlipidemia. Concomitantly, normoglycemic rats were kept in commercial rodent chow (Labina) during all experimental period constituting the control group (control diet group, CD). All rats were kept in controlled light cycle and temperature with feed and water being offered *ad libitum*.

2.7. Experimental Design. Normoglycemic ($n = 5$) and hyperlipidemic Wistar rats (28) were assessed in this study. Hyperlipidemic rats were divided into four groups ($n = 7$ each) and daily provided by gavage for 60 days of water (control), simvastatin (30 $mg \cdot Kg^{-1}$ of body weight, simvastatin group), ciprofibrate (2 $mg \cdot Kg^{-1}$ of body weight, ciprofibrate group), and ExC (200 $mg \cdot Kg^{-1}$ of body weight, ExC group). At the end of treatment and after euthanasia, organs, tissue, and blood were collected for analysis.

2.8. Biochemical Analysis. The blood collected was centrifuged at 3000 rpm for 10 min and serum was used to measure total cholesterol, HDL-cholesterol, triglycerides, aminotransferases (AST and ALT), urea, and creatinine with support of Integra 400 Plus equipment (Roche™).

2.9. Statistical Analysis. Data are shown as mean ± standard error of the mean and were submitted to one-way analysis of variance (ANOVA) followed by Tukey posttest. The results were considered significant when $P < 0.05$.

3. Results

3.1. Chemical Profile. The concentration of total phenolic compounds and flavonoids was 391 ± 5.0 mg EAG·100 g^{-1} of ExC and 59 ± 3.6 mg EQ·100 g^{-1} of ExC, and analyses were positive for saponins.

3.2. Antioxidant Activity. Considering the presence of potentially antioxidant substances in ExC, an *in vitro* evaluation of DPPH free radical scavenging at different concentrations was performed. The 50% inhibitory concentration (IC_{50}) and the maximum activity in assay of DPPH free radical scavenging of ExC were approximately one-third that of BHT and three times higher than that of ascorbic acid as shown in Table 1.

The antihemolytic potential of ExC was evaluated in erythrocytes submitted to lipid peroxidation and consequent hemolysis induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) for 240 min at different concentrations. ExC decreased the hemolysis of erythrocytes induced by AAPH in a time dependent manner, but independent of evaluated dose as shown in Figure 1.

Thus, the release of malonaldehyde (MDA) that occurs during the lipid peroxidation process was assessed. Erythrocytes incubated with ExC for 240 min at 125 $\mu g \cdot mL^{-1}$ presented reduced MDA concentrations compared with control sample ($P < 0.001$) and similar to samples incubated with ascorbic acid (Figure 2).

3.3. Hypolipidemic Effect. Rats treated with ExC showed decreased serum levels of total cholesterol and triglycerides, 34% and 45%, respectively, compared to control hyperlipidemic rats. Similar results were observed for the standard drugs, ciprofibrate, used to control cholesterol, and simvastatin, used to control triglycerides. Other biochemical parameters evaluated regarding the hepatic and renal functions were similar among groups investigated (Table 2). However, the analysis between hyperlipidemic and normolipidemic rats (*t*-test analyses) has shown an increase in the serum levels of AST in both groups treated with simvastatin and ciprofibrate, which did not occur in ExC group.

4. Discussion

The aim of this study was to evaluate the antioxidant and hypolipidemic activities of the hydroethanolic extract of *C. americana* L. leaves related to both human health conditions and interest in the development of new drugs.

The oxidative balance in the body is regulated by endogenous and exogenous mechanisms, in which the excess of free radicals is related to many diseases [21]. The control of the excess of oxidative molecules includes especially exogenous intake of antioxidant molecules, which are largely found in plants [22]. The chemical composition of these plants has shown that same classes of polyphenols can exert such function such as flavonoids [23, 24]. The capacity of ExC of DPPH free radicals scavenging was intermediary among standard antioxidants and approximately three times higher than that of BHT. It is noteworthy that both controls used are isolated molecules, which stimulates new studies for the isolation of compounds from ExC, in which the presence of phenolic compounds and flavonoids was identified. In [25], investigating *Dillenia suffruticosa*, a plant of the Dilleniaceae family, identified the presence of polyphenols, although in lower amounts than those observed for *C. americana* L., and these compounds were correlated with antioxidant activity. In addition, another compound common to these two plants is saponin, which has shown hypocholesterolemic and anti-inflammatory activities; however, it has also been described to be able to promote destabilization of the cell membrane and induce hemolysis due to its emulsifying action [26–31], which was not observed for ExC at *in vivo* and *in vivo* studies.

ExC decreased lipid peroxidation induced by AAPH, protecting erythrocytes from cell death similarly to ascorbic acid, which is a vitamin with antioxidant capacity as demonstrated by decreased lipid peroxidation and malonaldehyde (MDA) production [32]. The lower levels of MDA produced during

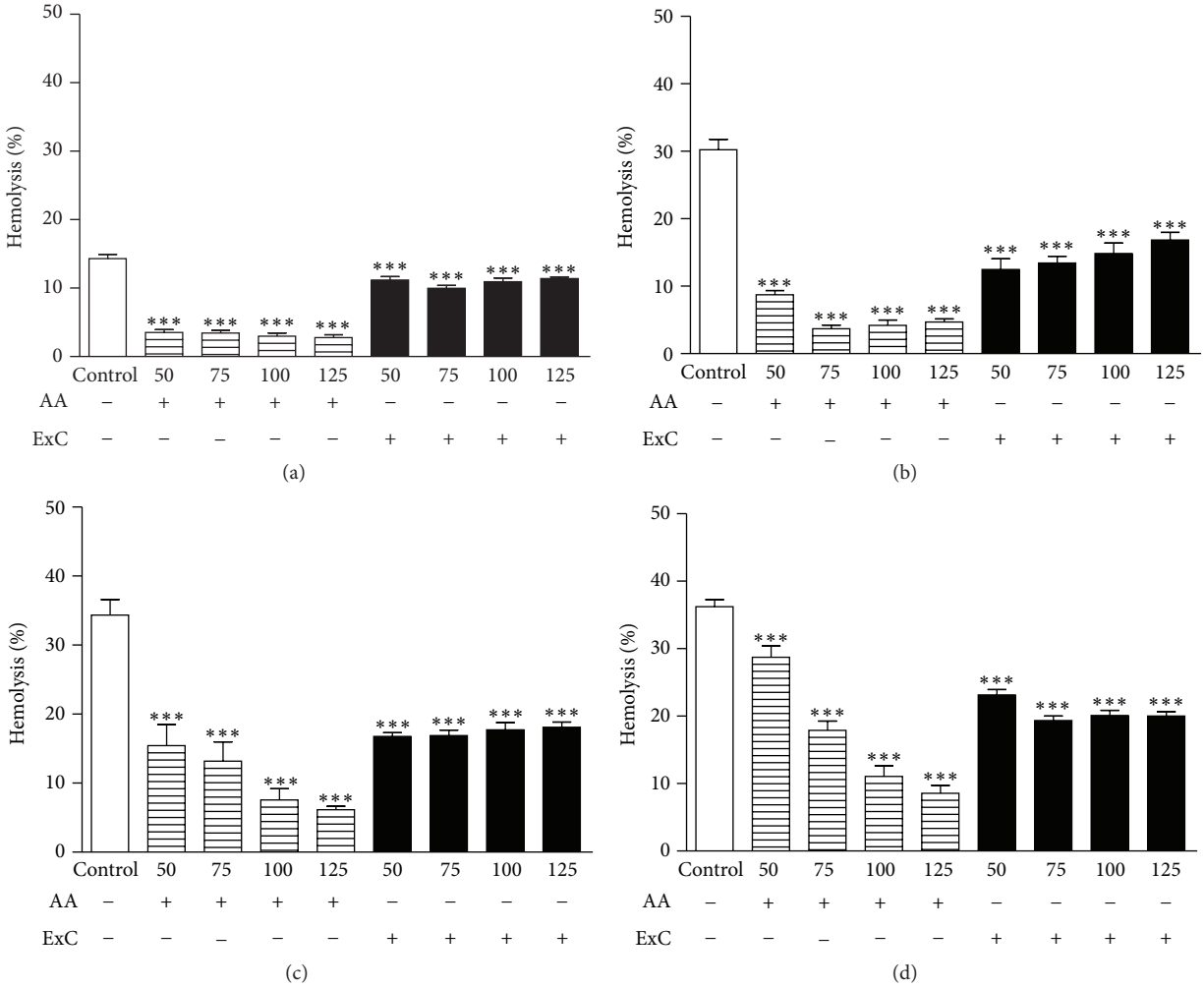

Figure 1: Hemolysis assessment at (a) 60, (b) 120, (c) 180, and (d) 240 min after addition of AAPH in erythrocytes at 2.5% (control) incubated with different concentrations (50–125 $\mu g \cdot mL^{-1}$) of ascorbic acid (AA) and hydroethanolic extract of *C. americana* L. leaves (ExC). $^{***}P < 0.0001$ versus control samples.

hemolysis induced by AAPH in samples incubated with ExC confirm the antioxidant action, also corroborated by [33] in the study of antioxidant activity of *Toona sinensis* leaves. The damage to the cell membrane resulting from lipid peroxidation induced by reactive species occurs in many diseases such as atherosclerosis, obesity, diabetes, hypertension, and cancer [21].

The importance of new products in the treatment and prevention of dyslipidemias becomes essential to reduce the mortality and morbidity due to cardiovascular complications. In addition, the search for less toxic drugs has increased the interest of the scientific community for natural products. The ExC showed to able to manage hyperlipidemia induced by high-fructose diet, reducing serum levels of total cholesterol and triglycerides, without signs of change in hepatic and renal function, suggesting that ExC is safe in the evaluated conditions. Ciprofibrate is a drug widely used to control cholesterol; however, it is contraindicated in patients with renal and hepatic disorders [34]. In this study, the ciprofibrate group showed an increase in liver and kidney weight (data not show), although the serum levels of ALT, creatinine, and urea remained unchanged. When compared to normolipidemic rats, the hyperlipidemic group treated with ciprofibrate presented an increase in serum level of AST.

The hypolipidemic activity of natural products can be correlated to the presence of flavonoids due to their properties of inhibiting cholesterol biosynthesis and absorption and modifying the activity of lipogenic and lipolytic enzymes, leading to reduced lipid metabolism [35–37], as observed in hyperlipidemic rats treated with ExC, which showed significant reduction in the levels of total cholesterol and triglycerides. Other molecules able to decrease the serum level of cholesterol are saponins [38], also present in ExC. It is very interesting that ExC was able to decrease both serum level of cholesterol and total triglycerides.

In conclusion, our results showed that *Curatella americana* L. leaves reduce oxidative stress by free radical scavenging and protect against lipid peroxidation and is also able to manage hyperlipidemia by decreasing serum level of cholesterol and triglycerides, similarly to standard drugs.

Table 2: Serum lipid profile and hepatic and renal parameters of normolipidemic and hyperlipidemic Wistar rats induced by high-fructose diet (66%) treated with water (control), simvastatin (20 mg·kg^{-1} of body weight, simvastatin), ciprofibrate (2 mg·kg^{-1} of body weight, ciprofibrate), and hydroethanolic extract of *C. americana* L. leaves (ExC) (200 mg·kg^{-1} of body weight, ExC).

Parameters	Normolipidemic	Hyperlipidemic			
		Control	Simvastatin	Ciprofibrate	ExC
Total cholesterol (mg·dL^{-1})	80.0 ± 2.0^{a}	118.3 ± 13.0^{b}	91.3 ± 5.2^{ab}	86.4 ± 4.3^{a}	77.9 ± 4.1^{a}
HDL-cholesterol (mg·dL^{-1})	45.0 ± 2.4^{a}	58.0 ± 4.4^{b}	54.0 ± 3.1^{a}	47.0 ± 2.4^{a}	46.0 ± 2.3^{a}
Triglycerides (mg·dL^{-1})	138.3 ± 23.0^{a}	225.0 ± 33.0^{b}	119.0 ± 7.0^{a}	155.0 ± 14.0^{ab}	136.9 ± 7.5^{a}
AST (U·L^{-1})	174.0 ± 6.5	178.0 ± 14.5	220.0 ± 20.1	209.0 ± 9.4	191.8 ± 12.3
ALT (U·L^{-1})	53.2 ± 3.3	65.4 ± 7.0	64.9 ± 5.6	65.0 ± 4.0	64.3 ± 9.3
Urea (U·L^{-1})	23.0 ± 2.0	30.0 ± 3.1	21.0 ± 1.4	26.0 ± 3.7	24.0 ± 3.8
Creatinine (U·L^{-1})	0.28 ± 0.02	0.34 ± 0.03	0.32 ± 0.02	0.30 ± 0.02	0.27 ± 0.02

Mean values followed by different superscript letters indicate significant difference ($P < 0.05$).

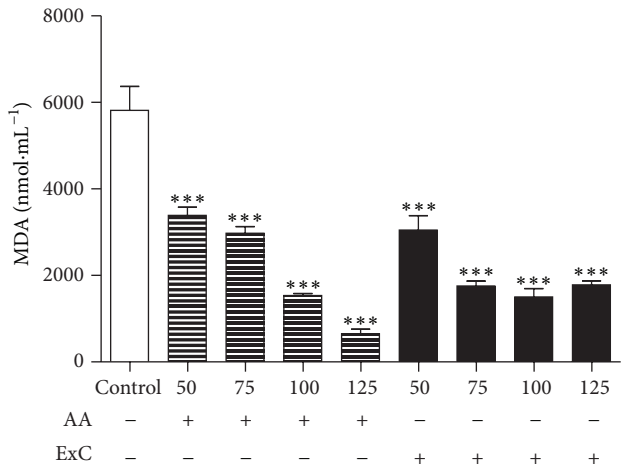

Figure 2: Malondialdehyde (MDA) concentration at 240 min after the addition of AAPH hemolysis inducer in 2.5% erythrocytes incubated with different concentrations (50–125 μg·mL^{-1}) of ascorbic acid (AA) and hydroethanolic extract of *C. americana* L. leaves (ExC) compared with control samples. $^{***}P < 0.001$ versus control samples.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The authors would like to thank the Foundation to Support to Development of Education, Science and Technology of Mato Grosso do Sul State (FUNDECT), Brazilian National Research Council (CNPq), and Coordination of Improvement of Higher Level (CAPES). Edson Lucas dos Santos is recipient of fellowship from Brazilian National Research Council (CNPq), Brazil.

References

[1] WHO, *Global Atlas on Cardiovascular Disease Prevention and Control*, vol. 3, World Health Organization, Geneva, Switzerland, 2011.

[2] G. Vogiatzi, D. Tousoulis, and C. Stefanadis, "The role of oxidative stress in atherosclerosis," *Hellenic Journal of Cardiology*, vol. 50, no. 5, pp. 402–409, 2009.

[3] H. Li, S. Horke, and U. Förstermann, "Vascular oxidative stress, nitric oxide and atherosclerosis," *Atherosclerosis*, vol. 237, no. 1, pp. 208–219, 2014.

[4] F. Bonomini, S. Tengattini, A. Fabiano, R. Bianchi, and R. Rezzani, "Atherosclerosis and oxidative stress," *Histology and Histopathology*, vol. 23, pp. 381–390, 2008.

[5] I. Zanotti, M. Dall'Asta, P. Mena et al., "Atheroprotective effects of (poly) phenols: a focus on cell cholesterol metabolism," *Food and Function*, vol. 6, no. 1, pp. 13–31, 2015.

[6] J. B. Calixto, "Twenty-five years of research on medicinal plants in Latin America: a personal view," *Journal of Ethnopharmacology*, vol. 100, no. 1-2, pp. 131–134, 2005.

[7] P. M. de Souza, P. M. de Sales, L. A. Simeoni, E. C. Silva, D. Silveira, and P. de Oliveira Magalhães, "Inhibitory activity of α-amylase and α-glucosidase by plant extracts from the Brazilian cerrado," *Planta Medica*, vol. 78, no. 4, pp. 393–399, 2012.

[8] C. E. M. De Toledo, E. A. Britta, L. F. Ceole et al., "Antimicrobial and cytotoxic activities of medicinal plants of the Brazilian cerrado, using Brazilian cachaa as extractor liquid," *Journal of Ethnopharmacology*, vol. 133, no. 2, pp. 420–425, 2011.

[9] F. Melo e Silva, J. E. de Paula, and L. S. Espindola, "Evaluation of the antifungal potential of Brazilian Cerrado medicinal plants," *Mycoses*, vol. 52, no. 6, pp. 511–517, 2009.

[10] C. R. Silva, P. M. Vieira, S. C. Santos, and L. Chen-Chen, "Assessment of genotoxicity and cytotoxicity of 'lixeira' (*Curatella americana* L.) using the prophage λ induction test (SOS inductest)," *Anais da Academia Brasileira de Ciências*, vol. 84, no. 1, pp. 149–156, 2012.

[11] M. S. Alexandre-Moreira, M. R. Piuvezam, C. C. Araújo, and G. Thomas, "Studies on the anti-inflammatory and analgesic activity of *Curatella americana* L.," *Journal of Ethnopharmacology*, vol. 67, no. 2, pp. 171–177, 1999.

[12] M. F. Guerrero, P. Puebla, R. Carrón, M. L. Martín, L. Arteaga, and L. S. Román, "Assessment of the antihypertensive and vasodilator effects of ethanolic extracts of some Colombian medicinal plants," *Journal of Ethnopharmacology*, vol. 80, no. 1, pp. 37–42, 2002.

[13] C. A. Hiruma-Lima, C. M. Rodrigues, H. Kushima et al., "The anti-ulcerogenic effects of *Curatella americana* L.," *Journal of Ethnopharmacology*, vol. 121, no. 3, pp. 425–432, 2009.

[14] P. M. de Medeiros, A. H. Ladio, and U. P. Albuquerque, "Patterns of medicinal plant use by inhabitants of Brazilian urban and rural areas: a macroscale investigation based on available literature," *Journal of Ethnopharmacology*, vol. 150, no. 2, pp. 729–746, 2013.

[15] A. Meda, C. E. Lamien, M. Romito, J. Millogo, and O. G. Nacoulma, "Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity," *Food Chemistry*, vol. 91, no. 3, pp. 571–577, 2005.

[16] S. A. Liberio, A. L. A. Pereira, R. P. Dutra et al., "Antimicrobial activity against oral pathogens and immunomodulatory effects and toxicity of geopropolis produced by the stingless bee *Melipona fasciculata* Smith," *BMC Complementary and Alternative Medicine*, vol. 11, article 108, pp. 1–10, 2011.

[17] M. E. P. Martucci, R. C. H. De Vos, C. A. Carollo, and L. Gobbo-Neto, "Metabolomics as a potential chemotaxonomical tool: application in the genus Vernonia Schreb," *PLoS ONE*, vol. 9, no. 4, Article ID e93149, 2014.

[18] J. C. Casagrande, L. F. Macorini, K. A. Antunes et al., "Antioxidant and cytotoxic activity of hydroethanolic extract from Jacaranda decurrens leaves," *PloS ONE*, vol. 9, no. 11, Article ID e112748, 2014.

[19] M. J. Valente, A. F. Baltazar, R. Henrique, L. Estevinho, and M. Carvalho, "Biological activities of Portuguese propolis: protection against free radical-induced erythrocyte damage and inhibition of human renal cancer cell growth *in vitro*," *Food and Chemical Toxicology*, vol. 49, no. 1, pp. 86–92, 2011.

[20] J. F. Campos, U. P. dos Santos, L. F. B. Macorini et al., "Antimicrobial, antioxidant and cytotoxic activities of propolis from Melipona orbignyi (Hymenoptera, Apidae)," *Food and Chemical Toxicology*, vol. 65, pp. 374–380, 2014.

[21] Y. Taniyama and K. K. Griendling, "Reactive oxygen species in the vasculate: molecular and cellular mechanisms," *Hypertension*, vol. 42, pp. 1075–1081, 2003.

[22] R. H. Liu, "Health-promoting components of fruits and vegetables in the diet," *Advances in Nutrition*, vol. 4, no. 3, pp. 384S–392S, 2013.

[23] E. J. Middleton, "Effect of plant flavonoids on immune and inflammatory cell function," in *Flavonoids in the Living System*, vol. 439 of *Advances in Experimental Medicine and Biology*, pp. 175–182, Springer, Berlin, Germany, 1998.

[24] G. W. Dryden, M. Song, and C. McClain, "Polyphenols and gastrointestinal diseases," *Current Opinion in Gastroenterology*, vol. 22, no. 2, pp. 165–170, 2006.

[25] N. Armania, L. S. Yazan, S. N. Musa et al., "*Dillenia suffruticosa* exhibited antioxidant and cytotoxic activity through induction of apoptosis and G_2/M cell cycle arrest," *Journal of Ethnopharmacology*, vol. 146, no. 2, pp. 525–535, 2013.

[26] G. Francis, Z. Kerem, H. P. S. Makkar, and K. Becker, "The biological action of saponins in animal systems: a review," *British Journal of Nutrition*, vol. 88, no. 6, pp. 587–605, 2002.

[27] A. D. Bangham, R. W. Horne, A. M. Glauert, J. T. Dingle, and J. A. Lucy, "Action of saponin on biological cell membranes," *Nature*, vol. 196, pp. 952–955, 1962.

[28] S. Nasri and H. Ben Salem, "Effect of oral administration of *Agave americana* or *Quillaja saponaria* extracts on digestion and growth of Barbarine female lamb," *Livestock Science*, vol. 147, no. 1–3, pp. 59–65, 2012.

[29] N. Monterrosas-Brisson, M. L. Arenas Ocampo, E. Jiménez-Ferrer et al., "Anti-inflammatory activity of different agave plants and the compound Cantalasaponin-1," *Molecules*, vol. 18, no. 7, pp. 8136–8146, 2013.

[30] R. Gilabert-Oriol, K. Mergel, M. Thakur et al., "Real-time analysis of membrane permeabilizing effects of oleanane saponins," *Bioorganic and Medicinal Chemistry*, vol. 21, no. 8, pp. 2387–2395, 2013.

[31] L. L. C. Voutquenne, C. Lavaud, G. Massiot, and L. Le Men-Olivier, "Structure-activity relationships of haemolytic saponins," *Pharmaceutical Biology*, vol. 40, no. 4, pp. 253–262, 2002.

[32] H. Abdollahzad, S. Eghtesadi, I. Nourmohammadi, M. Khadem-Ansari, H. Nejad-Gashti, and A. Esmaillzadeh, "Effect of vitamin C supplementation on oxidative stress and lipid profiles in hemodialysis patients," *International Journal for Vitamin and Nutrition Research*, vol. 79, no. 5-6, pp. 281–287, 2009.

[33] Y.-C. Hseu, W.-H. Chang, C.-S. Chen et al., "Antioxidant activities of *Toona sinensis* leaves extracts using different antioxidant models," *Food and Chemical Toxicology*, vol. 46, no. 1, pp. 105–114, 2008.

[34] N. Broeders, C. Knoop, M. Antoine, C. Tielemans, and D. Abramowicz, "Fibrate-induced increase in blood urea and creatinine: is gemfibrozil the only innocuous agent?" *Nephrology Dialysis Transplantation*, vol. 15, no. 12, pp. 1993–1999, 2000.

[35] N. M. Borradaile, L. E. de Dreu, P. H. R. Barrett, C. D. Behrsin, and M. W. Huff, "Hepatocyte ApoB-containing lipoprotein secretion is decreased by the grapefruit flavonoid, naringenin, via inhibition of MTP-mediated microsomal triglyceride accumulation," *Biochemistry*, vol. 42, no. 5, pp. 1283–1291, 2003.

[36] S. C. Whitman, E. M. Kurowska, J. A. Manthey, and A. Daugherty, "Nobiletin, a citrus flavonoid isolated from tangerines, selectively inhibits class A scavenger receptor-mediated metabolism of acetylated LDL by mouse macrophages," *Atherosclerosis*, vol. 178, no. 1, pp. 25–32, 2005.

[37] J.-M. Brusq, N. Ancellin, P. Grondin et al., "Inhibition of lipid synthesis through activation of AMP kinase: an additional mechanism for the hypolipidemic effects of berberine," *Journal of Lipid Research*, vol. 47, no. 6, pp. 1281–1288, 2006.

[38] S. Patel, D. Santani, M. Shah, and V. Patel, "Anti-hyperglycemic and anti-hyperlipidemic effects of bryonia laciniosa seed extract and its saponin fraction in streptozotocin-induced diabetes in rats," *Journal of Young Pharmacists*, vol. 4, no. 3, pp. 171–176, 2012.