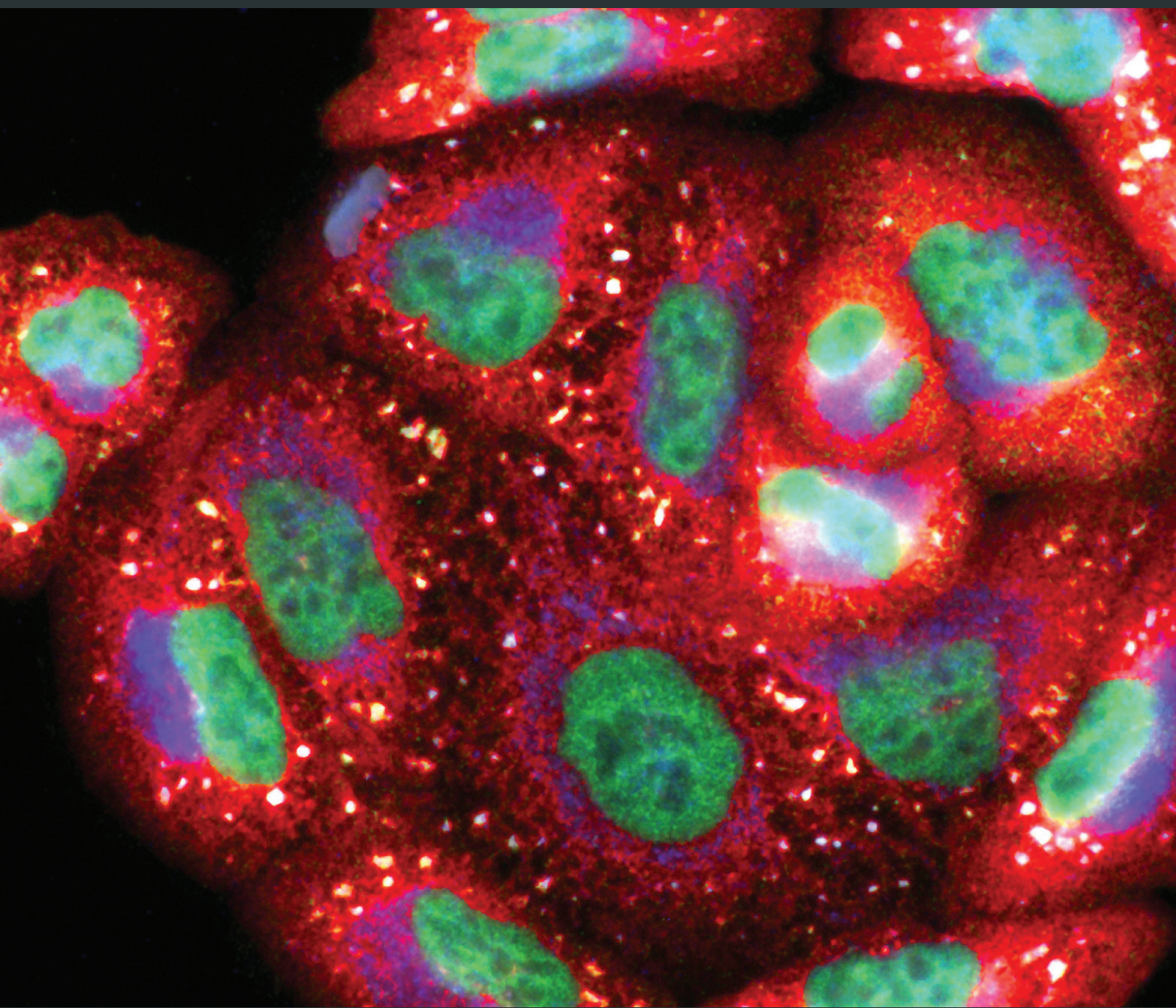
Oxidative Medicine and Cellular Longevity

Antioxidant Phytochemicals at the Pharma-Nutrition Interface

Lead Guest Editor: Elena Azzini
Guest Editors: Gian L. Russo and Jasminka Giacometti



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https://doi.org/10.1155/2017/6986143

Editorial

Antioxidant Phytochemicals at the Pharma-Nutrition Interface

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Received 25 October 2017; Accepted 25 October 2017; Published 8 November 2017

Noncommunicable diseases (NCDs), including obesity, diabetes, cardiovascular diseases, and cancer, represent an emerging global health issue. Nutrition, as known, represents one of the most important aspects of health; several researchers have shown that nutrition plays a crucial role in the prevention of food deficiencies, behavioural disorders, and chronic diseases. The last decades saw the proliferation of studies on the healthy benefits of specific classes of nutrients. Among these, phytochemicals took the lead for their capacity to act as antioxidants, a function which inspired the so-called "antioxidant hypothesis" or "free radical theory" for degenerative diseases originated back in the late 1980s–middle 1990s. After more than twenty years of intense researches, phytochemicals remain an *éminence grise* in the area of the natural remedies against degenerative diseases. In fact, if, from one side, their functional pleiotropy guarantees multiple therapeutic and preventive effects, their low bioavailability and high metabolic transformation represent an unsolved issue on the way to demonstrate a clear structure-function relationship in regulating cellular physiology.

The aim of the present special issue was not to propose a solution for all the questions surrounding the mechanisms of action of phytochemicals but to bring the attention of the readers on specific themes with a selection of high quality research articles and reviews, hoping to stimulate their thinking. This special issue comprising nine papers is focused on various aspects of phytochemicals effects when administered as extracts in animal and cellular models. The articles have been selected on the basis of fundamental ideas/concepts rather than the thoroughness of techniques employed. The papers are organised as follows.

D. de Almeida Bauer Guimarães et al. suggest that pitaya extract (PE) may have a protective effect against breast cancer. PE showed high antioxidant activity, and high values of anthocyanins induced a selective decrease in cell proliferation caused by PE in MCF-7 (ER^+) cell line and an increase in G_0/G_1 phase followed by a decrease in G_2/M phase. Also, PE induced apoptosis in MCF-7 (ER^+) cell line and suppressed $BRCA_1$, $BRCA_2$, PRAB, and Erα gene expression.

D. Załuski et al. highlighted the fruits of *Eleutherococcus* species rich in polyphenols as a new income source of agriculture and industry in natural products and foods. These fruits act as antioxidants, induce apoptosis in Jurkat 45 leukemic cell line, and inhibit the activity of MMP-1, MMP-2, MMP-3, and MMP-9.

L. Song et al. reported a preclinical systematic review about G-Rg1 as a promising potential neuroprotective against PD model through different mechanisms including antineuroinflammatory, antioxidative, and antiapoptotic effects. On the other hand, a letter by Yi-bo et al. expressed comment on "A Preclinical Systematic Review of Ginsenoside-Rg1 in Experimental Parkinson's Disease" by inviting the authors to a more conservative conclusion. Thus, further controlled studies in animals should be attempted to establish the

G-Rg1 as a drug candidate, rather than confirmed by clinical trials immediately.

V. R. Pasupuleti et al. have given a review of several health benefits of honeybee products used as food, such as honey, propolis, and royal jelly, on metabolic diseases, cancers, and others diseases.

T. Bonamigo et al. described how the chemical constituents of propolis, such as phytosterols, terpenes, aromatic acids, and tocopherol, are important in the control of diseases. In the study of antioxidant, cytotoxic, and toxic activities of ethanol extracts of propolis (EEPs) obtained from the Brazilian stingless bees *Scaptotrigona depilis* (EEP-S) and *Melipona quadrifasciata anthidioides* (EEP-M), they indicated the cytotoxic activity of EEPs against erythroleukemic cells and necrosis as the main mechanism of death observed. In addition, cytotoxic doses of EEPs were not toxic against *Caenorhabditis elegans*. Bioactive mixture found in EEP-S and EEP-M can be used in the control of diseases associated with oxidative stress and tumour cell proliferation.

Z. Tuzcu et al. showed that cinnamon reduces the hyperlipidemia, inflammation, and oxidative stress through activating transcription factors and antioxidative defense signaling pathway in high-fat diet- (HFD-) fed rats. Cinnamon polyphenol also suppressed the expression of hepatic sterol regulatory element-binding protein-1c (SREBP-1c), liver X receptor (LXR-α), ATP-citrate lyase, fatty acid synthase (FAS), and nuclear factor kappa B p65 (NF-κB p65) and enhanced the peroxisome proliferator-activated receptor alpha (PPAR-α), insulin receptor substrate 1 (IRS-1), nuclear factor-E2-related factor-2 (Nrf2), and heme oxygenase 1 (HO-1) expressions in the HFD rat livers.

J.-T. Liu et al. investigated the molecular mechanism supporting the protective effect of red yeast rice (RYR; *Monascus purpureus*-fermented rice) in limiting the vascular complications of diabetes. Using an ex vivo cellular model represented by human bone marrow-derived proangiogenic cells (PACs) isolated from healthy donors and treated with nontoxic concentrations of RYR extract (<50 μg/ml), the authors demonstrated its capacity to induce time-dependently Nrf2 nuclear translocation in PAC cells which resulted in a dose-dependent induction of HO-1 mRNA expression and, in parallel, a dose- and time-dependent increase in HO-1 protein expression. Using multiple and complementary approaches, the authors confirmed the beneficial effects of RYR extract showing its ability to inhibit senescence and oxidative stress (production of ROS) following treatment of PAC cells with high glucose (30 mM). Although the direct target of RYR, which triggers the cascade of reactions, leading to the activation of HO-1, has not been identified, Chen's group optimistically proposes that RYR extract may serve as alternative and complementary medicine in decreasing the vascular complications of diabetes.

A. Molfino et al. analyze the active, anti-inflammatory role of the specialized proresolving mediators (SPMs), defined as a family of lipid mediators derived from omega-6 and omega-3 polyunsaturated fatty acids (PUFA) and including well-known bioactive compounds, such as lipoxins (omega-6 PUFA derived) and D-/E-series resolvins, protectins, and maresins (omega-3 PUFA derived). The authors focused on the anti-inflammatory and proresolving role of omega-3-derived SPM in critical illnesses. They firstly reviewed the importance of genetic signature in determining the level of expression of SPMs, their biosynthetic isomers, and pathways in trauma patients. Although still circumstantial, literature data suggest that circulating levels of PUFAs and PUFA metabolites may influence the inflammatory responses via the NF-κB pathway. It remains to be determined the cause-effect relationship between severe inflammatory pathological conditions and inactivation of specific SPM pathways. The anti-inflammatory or proresolving effects of PUFAs in clinics are encouraging since the latest clinical meta-analysis suggests a reduced ICU length of stay in patients following cardiac surgery and supplemented with omega-3 PUFA-enriched parenteral emulsions. A. Molfino et al. conclude that evidence are increasing in favor of a protective role of omega-3 PUFA-derived SPMs as anti-inflammatory and proresolving nutrients, although future work must be devoted to assess the optimal lipid emulsions and their specific uses.

E. Azzini et al. highlighted the following key points: the healthy relationship between anthocyanin supplementation and their antiobesity effects suffers of the same contradictions and doubtable interpretations, which emerge when the beneficial responses of other phytochemicals towards different degenerative diseases are considered; the different dosage applied in animal versus clinical studies; the complex metabolism and biotransformation to which anthocyanins and phytochemicals are subjected in the intestine and tissues; the possibility that different components present in the supplemented mixtures can interact generating antagonistic, synergistic, or additive effects difficult to predict; and the difference between prevention and therapy. The authors concluded that the evolution of the field must seriously consider the need to establish new and adequate cellular and animal models, which may, in turn, allow the design of more efficient and prevention-targeted clinical studies.

Long duration and slow progression of NCDs could be counteracted by modulating the intake of macro- and micronutrients. The use of food components (including polyphenols, glucosinolates, PUFAs, fibers, and friendly bacteria) among patients on conventional pharmacological therapy should be carefully assessed due to the possibility of food-drug interactions. Even if several food compounds may exert a prophylactic function within the human body, their bioavailability and bioactivity have high interindividual variability and the mechanisms of biological action of food extracts and bioactive compounds remain to be elucidated. Consequently, well-designed randomized clinical trials are needed to clarify the precise role of food components and/or new food in human health.

Acknowledgments

The Editors wish to thank all authors who submitted their research to this special issue sharing our interest for this special issue.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 7623954, 2 pages
https://doi.org/10.1155/2017/7623954

Letter to the Editor

Comment on "A Preclinical Systematic Review of Ginsenoside-Rg1 in Experimental Parkinson's Disease"

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Received 27 April 2017; Accepted 2 October 2017; Published 1 November 2017

Academic Editor: Elena Azzini

We read the recently published systematic review of ginsenoside-Rg1 in experimental Parkinson's disease with a great deal of interest [1]. The authors concluded that G-Rg1 exerted potential neuroprotective functions against PD. However, the conclusion should be more conservative because the selection criteria in the meta-analysis are flawed and most preclinical studies of G-Rg1 in experimental Parkinson's disease have bias, which would decrease the reliability of these results. First, the authors chose TH-positive dopamine neurons and levels of TH protein in the SNpc as outcomes. However, loss of TH expression is not necessarily related to cells dying [2, 3], following MPTP and 6-OHDA. A temporal association of tyrosine nitration or cysteine oxidation with inactivation of TH activity in vitro suggests that this covalent posttranslational modification is responsible for the in vivo loss of TH function [4, 5]. So use of TH alone is insufficient to judge dopamine neurons loss; more outcomes should be added in this meta-analysis, such as numbers of Nissl stain-positive cells. Second, in Table 1, the authors did not state the timing of G-Rg1 treatment. Treatment with G-Rg1 before or after MPTP injection is totally different. We also reviewed included papers in this meta-analysis and found almost all studies pretreated with G-Rg1 before MPTP injection. It seemed that these studies did not strictly follow the protocol of Jackson-Lewis and Przedborski (2007) for the MPTP mouse model of Parkinson's disease [6]. All of them did not prove whether G-Rg1 would interfere with MPTP toxicokinetic or pretreatment, or whether coadministration with G-Rg1 may invalidate the interpretation of the data. It is uncertain whether G-Rg1 could prevent the uptake by blocking data, prevent the conversion of MPTP to MPP, detoxify MPTP, and many other possibilities. So, the method of pretreatment with G-Rg1 may not be scientific. Third, all studies in this meta-analysis count cell numbers immediately after the last injection of MPTP. This may lead to higher results as it takes time for cells to die and for the debris to be removed [2, 3], which could be an experimental flaw.

In conclusion, the authors have set out to prove the benefits of the G-Rg1 without critically reviewing the studies. The conclusion should be more conservative. Further, carefully controlled studies in animals should be attempted to see if G-Rg1 is a drug candidate rather than be confirmed by clinical trials immediately.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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[1] L. Song, M. B. Xu, X. L. Zhou, D. P. Zhang, S. L. Zhang, and G. Q. Zheng, "A preclinical systematic review of ginsenoside-Rg1 in experimental Parkinson's disease," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 2163053, 14 pages, 2017.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 1259510, 21 pages
https://doi.org/10.1155/2017/1259510

Review Article

Honey, Propolis, and Royal Jelly: A Comprehensive Review of Their Biological Actions and Health Benefits

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Received 17 February 2017; Accepted 9 April 2017; Published 26 July 2017

Academic Editor: Jasminka Giacometti

Background. There are several health benefits that honeybee products such as honey, propolis, and royal jelly claim toward various types of diseases in addition to being food. *Scope and Approach*. In this paper, the effects of honey, propolis, and royal jelly on different metabolic diseases, cancers, and other diseases have been reviewed. The modes of actions of these products have also been illustrated for purposes of better understanding. *Key Findings and Conclusions*. An overview of honey, propolis, and royal jelly and their biological potentials was highlighted. The potential health benefits of honey, such as microbial inhibition, wound healing, and its effects on other diseases, are described. Propolis has been reported to have various health benefits related to gastrointestinal disorders, allergies, and gynecological, oral, and dermatological problems. Royal jelly is well known for its protective effects on reproductive health, neurodegenerative disorders, wound healing, and aging. Nevertheless, the exact mechanisms of action of honey, propolis, and royal jelly on the abovementioned diseases and activities have not been not fully elucidated, and further research is warranted to explain their exact contributions.

1. Introduction

Apiculture is the science and art of prolonging, sustaining, and retaining health by using products obtained from honeybee hives, such as honey, bee bread, bee venom, bee pollen, propolis, and royal jelly. Recent years have seen the fast application of bee products in both traditional and modern medicine. Currently, many studies are targeted toward investigating directed health benefits and pharmacological properties of bee products due to their efficacies, leading to the increasing development of nutraceuticals and functional food from these products. The concept of functional food refers to food that has the ability to promote better physiological or psychological health compared to traditional remediated and nutritional food. These effects positively contribute toward excellent health maintenance, well-being, and reduced chronic illness [1]. The present review focuses on the potential health benefits of bee products, including honey, propolis, and royal jelly.

Honey is a sweet liquid processed by the honey bee. Honey is recognized worldwide due to its high nutritive components that are beneficial for human well-being. It has been traditionally used by Egyptians, Greeks, Romans, and Chinese to heal wounds and diseases of the gut, including gastric ulcers. It has also been used as a remedy for cough, sore throat, and earaches [2]. In India, Lotus honey has been traditionally used to treat eye infections and other diseases. In addition to being used externally, honey is also used internally [3] as a functional food to provide energy and nourishment to enhance vital organs in the body [4]. This has been in practice since ancient times. The active components of honey, such as glucose, fructose, flavonoid, polyphenols, and organic acids, play an important role in its quality [5]. Honey is being produced in many countries all over the

world and is recognized as an important medicine as well as energy-providing food due to its functional properties and nutritional values. Additionally, honey is well known for its biological, physiological, and pharmacological activities.

Propolis is generally known as the "bee glue", which is a generic name that refers to the resinous substance accumulated by the bees from different types of plants. The word "propolis" is derived from Greek to mean defense for "pro" and city or community for "polis", or the beehive, in other words [6]. Propolis functions in sealing holes and cracks and for the reconstruction of the beehive. It is also used for smoothing the inner surface of the beehive, retaining the hive's internal temperature (35°C), preventing weathering and invasion by predators. Furthermore, propolis hardens the cell wall and contributes to an aseptic internal environment. Propolis generally becomes soft and sticky upon heating [7]. It also possesses a pleasant smell. Propolis and its extracts have numerous applications in treating various diseases due to its antiseptic, anti-inflammatory, antioxidant, antibacterial, antimycotic, antifungal, antiulcer, anticancer, and immunomodulatory properties.

Royal jelly, a white and viscous jelly-like substance, is a form of hypopharyngeal and mandibular gland secretion from the worker bees. It is also known as a "superfood" that is solely consumed by the queen bee. Royal jelly is also fed to the honeybee larvae upon hatching and helps to nurture the brood [8]. It is the exclusive nutriment offered to the immature young larvae in their first 2-3 days of maturation besides being used as a food specifically for the queen bee throughout her entire life cycle. Royalactin is the main compound in royal jelly that allows the morphological change of a larva into the queen bee [9]. This superfood is the main reason for the longevity of the queen bee compared to the other bees. Royal jelly is widely used as a dietary nutritional complex to help combat various chronic health conditions. Furthermore, it is one of the profitable remedies for human beings in both traditional and modern medicine. Many pharmacological activities such as antibacterial, antitumor, antiallergy, anti-inflammatory, and immunomodulatory effects have also been attributed to it.

2. Chemical Composition of Honey, Propolis, and Royal Jelly

Honey is also known as a supersaturated sugar solution. Natural honey is composed of 82.4% carbohydrates, 38.5% fructose, 31% glucose, 12.9% other sugars, 17.1% water, 0.5% protein, organic acids, multiminerals, amino acids, vitamins, phenols, and a myriad of other minor compounds. In addition, honey consists of minor amounts of bioactive components, including phenolic acid, flavonoid, and α-tocopherol [10]. Honey constituents with health benefits include phenolic acids, flavonoids, ascorbic acid, proteins, carotenoids, and certain enzymes, such as glucose oxidase and catalase [11].

Propolis is the third most important component of bee products. It is composed mainly of resin (50%), wax (30%), essential oils (10%), pollen (5%), and other organic compounds (5%) [12]. Phenolic compounds, esters, flavonoids, terpenes, beta-steroids, aromatic aldehydes, and alcohols are the important organic compounds present in propolis [13]. Twelve different flavonoids, namely, pinocembrin, acacetin, chrysin, rutin, luteolin, kaempferol, apigenin, myricetin, catechin, naringenin, galangin, and quercetin; two phenolic acids, caffeic acid and cinnamic acid; and one stilbene derivative called resveratrol have been detected in propolis extracts by capillary zone electrophoresis [14]. Propolis also contains important vitamins, such as vitamins B1, B2, B6, C, and E and useful minerals such as magnesium (Mg), calcium (Ca), potassium (K), sodium (Na), copper (Cu), zinc (Zn), manganese (Mn), and iron (Fe). A few enzymes, such as succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase, and acid phosphatase, are also present in propolis [15].

Royal jelly consists of water (50%–60%), proteins (18%), carbohydrates (15%), lipids (3%–6%), mineral salts (1.5%), and vitamins [16]. Based on modern spectrometric analysis, approximately 185 organic compounds have been detected in royal jelly. Royalactin is the most important protein present in royal jelly. In addition, royal jelly is composed of a significant number of bioactive compounds, including 10-hydroxy-2-decenoic acid (HAD), which has some immunomodulatory properties [17]. Fatty acid, proteins, adenosine monophosphate (AMP) N1 oxide, adenosine, acetylcholine, polyphenols, and hormones such as testosterone, progesterone, prolactin, and estradiol are other useful bioactive components reported to be present in royal jelly [18].

3. Bioactive Compounds in Honey, Propolis, and Royal Jelly

Honey, propolis, and royal jelly are highly rich in bioactive compounds (Table 1). Essential and nonessential compounds, such as polyphenols and vitamins occurring naturally as part of food chains, are considered bioactive. These compounds are naturally present in food and confer useful health benefits. Phenolic compounds are bioactive compounds. Phenols are defined as organic compounds with an aromatic ring that is chemically bonded to one or additional hydrogenated substituents in the presence of corresponding functional derivatives [19].

In honey, propolis, and royal jelly, phenolic compounds are commonly present as flavonoids [20]. Various phenolic compounds contribute to the functional properties of bee products, including their antioxidant, antimicrobial, antiviral, anti-inflammatory, antifungal, wound healing, and cardioprotective activities [21]. Figure 1 summarizes the important biological efficacies of bee products.

4. Health Benefits of Honey

4.1. Wound Management. Honey has traditionally been used to treat wounds, insect bites, burns, skin disorders, sores, and boils. Scientific documentation of the wound-healing capabilities of honey validates its efficacy as a promoter of wound repair and an antimicrobial agent [37]. Honey promotes the activation of dormant plasminogen in the wound matrix, which results in the dynamic expression of the proteolytic

Table 1: Important bioactive compounds in honey, propolis, and royal jelly.

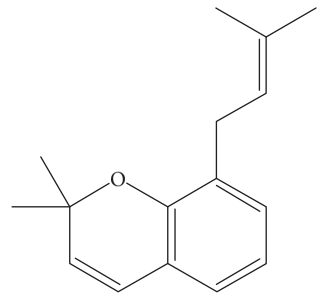
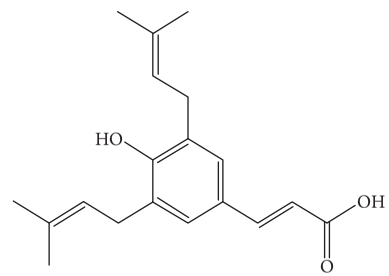
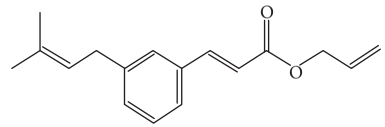
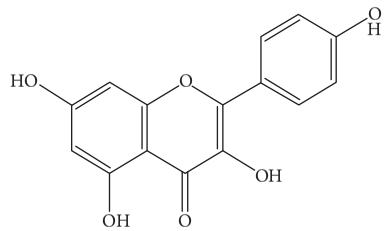
Type of bee product	Bioactive compound	Chemical structure	Biological activity	References
Propolis	Phenolic compound: 2, 2-dimethyl-8-prenylchromene		Antimicrobial	Viuda-Martos et al. [22]
Propolis	Phenolic compound: 4-hydroxy-3, 5-diprenyl cinnamic acid (artepillin C)		Antimicrobial, anti-inflammatory, anticancer	Viuda-Martos et al. [22]
Propolis	Phenolic compound: 3-prenyl cinnamic acid allyl ester		Antimicrobial	Viuda-Martos et al. [22]
Propolis	Phenolic compound: kaempferide		Antitumor, anticancer	Viuda-Martos et al. [22], [23]

Table 1: Continued.

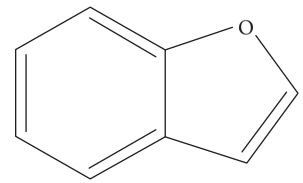
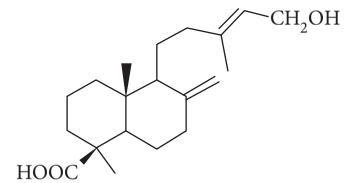
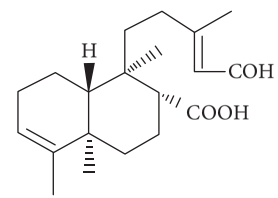
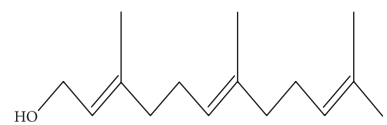
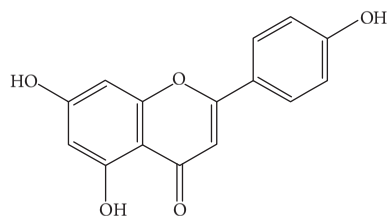
Type of bee product	Bioactive compound	Chemical structure	Biological activity	References
Propolis	Phenolic compound: propolis benzofuran		Antifungal	Viuda-Martos et al. [22], [23]
Propolis	Terpenoid: isocupressic acid, a labdane diterpenoid		Antifungal	Viuda-Martos et al. [22] (Khalil & Sulaiman [23])
Propolis	Terpenoid:13C-symphyoreticulic acid, a clerodane diterpenoid		Antitumor	Viuda-Martos et al. [22], [24]
Propolis	Terpenoid: esters of long-chain fatty acids, (3-hydroxystearic acid ($n = 11$) procrim a; 3-hydroxystearic acid ($n = 13$), procrim b and a pentacyclic triterpenoid (lupeol))		Antioxidant, antimicrobial, antitumor	(Salatino et al. [25]), Viuda-Martos et al. [22], (Huang et al. [13])
Propolis	Terpenoid: farnesol, a sesquiterpenoid		Antifungal	Viuda-Martos et al. [22], (Cotoras et al. [26])
Propolis, honey	Flavonoid: apigenin		Antibacterial, anti-inflammatory	Viuda-Martos et al. [22], (Khalil & Sulaiman [23])

Table 1: Continued.

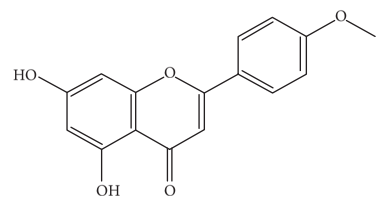
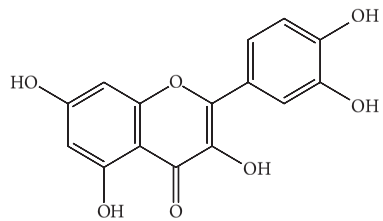
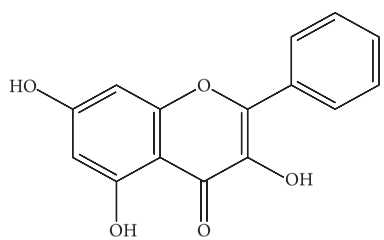
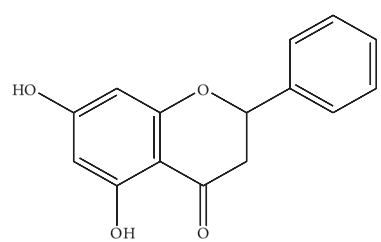
Type of bee product	Bioactive compound	Chemical structure	Biological activity	References
Honey, propolis	Flavonoid: acacetin		Antiallergy, anticancer	Viuda-Martos et al. [22], (Khalil & Sulaiman [23])
Honey, propolis	Flavonoid: quercetin		Anticancer, antiallergy, antibacterial, anti-inflammatory	Viuda-Martos et al. [22], (Khalil & Sulaiman [23])
Honey, propolis	Flavonoid: galangin		Anticancer, antioxidant	Viuda-Martos et al. [22], (Khalil & Sulaiman [23])
Honey, propolis	Flavonoid: pinocembrin		Antimicrobial, anticancer	Viuda-Martos et al. [22], (Khalil & Sulaiman [23])

Table 1: Continued.

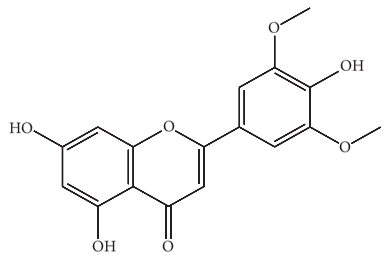
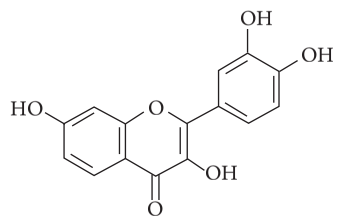
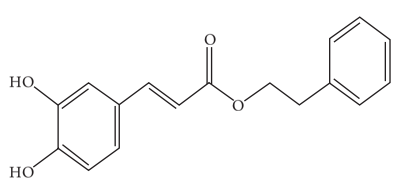
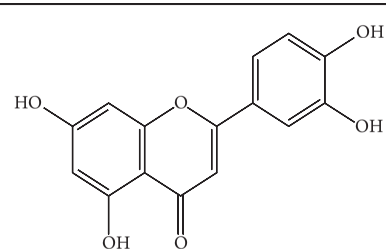
Type of bee product	Bioactive compound	Chemical structure	Biological activity	References
Honey, propolis	Flavonoid: chrysin		Antibacterial, anti-inflammatory, anticancer	Viuda-Martos et al. [22], (Khalil & Sulaiman [23])
Honey, propolis	Flavonoid: fisetin		Antibacterial, antiallergy, anticancer	Viuda-Martos et al. [22], (Abubakar et al. [27])
Honey, propolis	Flavonoid: caffeic acid phenethyl ester		Antitumor, anticancer	Viuda-Martos et al. [22], (Khalil & Sulaiman [23])
Propolis, royal jelly	10-hydroxyl-2-decenoic acid		Antibiotic, antitumor	Izuta et al. [28]
Honey	Flavonoid: luteolin		Antioxidant, anti-inflammatory, antitumor	Lin et al. [29], Mijanur et al. [30]

Table 1: Continued.

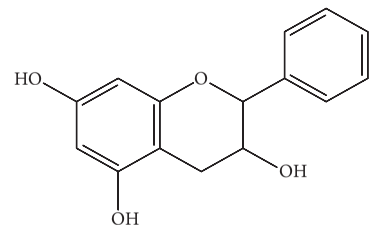
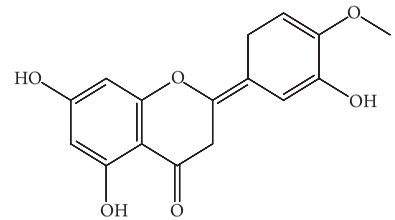
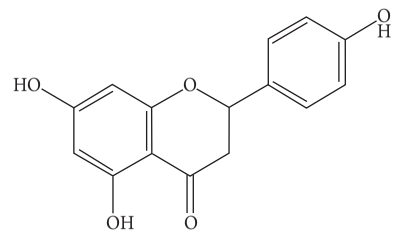
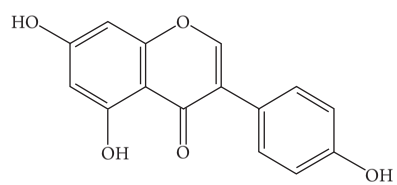
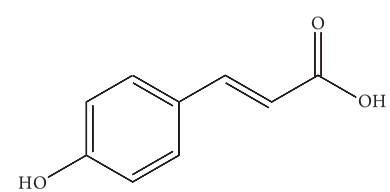
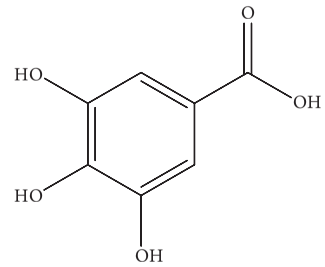
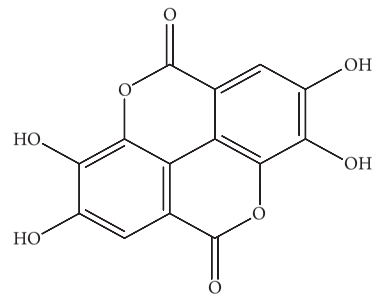
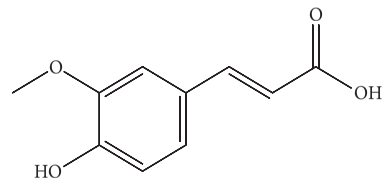
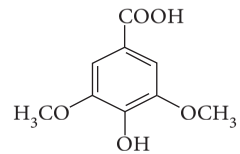
Type of bee product	Bioactive compound	Chemical structure	Biological activity	References
Honey	Flavonoid: pinobanksin		Antioxidant	Ajao et al. [31]
Honey	Flavonoid: hesperetin		Antioxidant, anti-inflammatory	Kassim et al. [32], Mijanur et al. [30]
Honey	Flavonoid: naringenin		Neuroprotective, antioxidant	Badruzzaman Khan et al. [33], Mijanur et al. [30]
Honey	Flavonoid: genistein		Anticancer	Tahir et al. [34], Mijanur et al. [30]
Honey	Phenolic acid: p-coumaric acid		Antigenotoxic, neuroprotective	Vauzour et al. [35], Mijanur et al. [30])

Table 1: Continued.

Type of bee product	Bioactive compound	Chemical structure	Biological activity	References
Honey	Phenolic acid: gallic acid		Antianxiolytic	Mansouri et al. [36], Mijanur et al. [30]
Honey	Phenolic acid: ellagic acid		Antioxidant, chemopreventive, antiproliferative	Mijanur et al. [30]
Honey	Phenolic acid: ferulic acid		Antioxidant, anti-inflammatory, neuroprotective	Mijanur et al. [30]
Honey	Phenolic acid: syringic acid		Antioxidant, anticancer	Mijanur et al. [30], Tahir et al. [34]

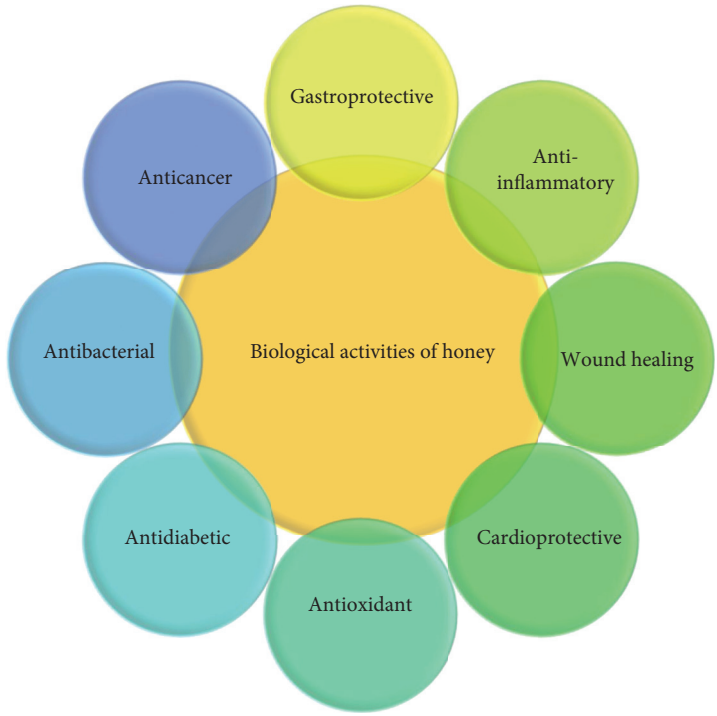


Figure 1: Various types of biological activities of honey products.

enzyme. Plasmin causes blood clot retraction and fibrin destructions. It is an enzyme that breaks down fibrin clots with attached dead tissues in the wound bed [38].

Clinical evidence supporting the effectiveness, specificity, and sensitivity of honey in wound care indicates that the performance of conventional and modern wound care dressing is inferior to that using honey [39]. Certain cases have shown that honey stimulates wound-healing properties even in infected wounds that do not respond to antiseptics or antibiotics and wounds that have been infected with antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Natarajan et al. 2001). Honey also aids autolytic debridement and accelerates the growth of healthy granulated wound bed [40].

Malodor is a general attribute of severe wounds caused by anaerobic bacterial species belonging to *Bacteroides* spp. and *Peptostreptococcus* spp. [41]. Malodourous compounds, such as ammonia, amines, and sulfur, are produced by bacteria during the metabolism of amino acids from putrefied serum and tissue proteins. These compounds are replaced by lactic acids as honey dispenses a substantial amount of glucose, a substrate metabolized by bacteria in preference to amino acids [42]. The therapeutic effects observed after honey application include fast healing, wound cleansing, clearance of infection, tissue regeneration, minimized inflammation, and increased comfort during dressing due to lower extent of tissue adhesion [43].

4.2. Pediatric Care. Honey also controls skin damage near stomas, such as ileostomy and colostomy, by enhancing epithelialization of the afflicted skin surface [44]. Honey has a beneficial effect on pediatric dermatitis caused by excessive use of napkins and diapers, eczema, and psoriasis. The effect of honey mixed with beeswax and olive oil was investigated on patients with psoriasis or atopic dermatitis condition. A clinical trial showed that a mixture containing honey was extremely well tolerated and caused significant improvements. Honey consists of various nitric oxide metabolites, which reduce the incidence of skin infection in psoriasis [45].

4.3. Diabetic Foot Ulcer (DFU). Consumption of honey is a low-cost and effective therapy for the treatment of DFU. DFU is often complicated by microbial infections and slows the healing process. Apart from the infection, symptoms such as pain, swelling, and redness might not be present for diabetic peripheral neuropathy patients due to their reduced immune response, which further complicates the diagnosis [46]. A review indicated that using honey for the treatment of venous ulcers yielded positive outcomes with good acceptance rates from the patients [47]. Honey is used in wound management and is effective among patients with locally infected wounds, DFU, Charcot foot ulcerations, and complex comorbid conditions that have failed hospital management [48]. In addition, there is excellent tolerability and minimal trauma to the wound bed in the presence of honey.

4.4. Gastrointestinal (GI) Disorder. Natural honey is composed of enzymes that facilitate the absorption of molecules, such as sugars and starch. The sugar molecules in honey are in a form that can be easily absorbed by the body. Honey also provides some nutrients, such as minerals, phytochemicals, and flavonoids, that aid digestive processes in the body [49]. Pure honey has bactericidal properties against pathogenic bacteria and enteropathogens, including *Salmonella* spp., *Escherichia coli*, *Shigella* spp., and many other Gram-negative species [50].

The gastrointestinal tract (GIT) contains many important beneficial microbes. For example, *Bifidobacteria* is one of the microorganisms present primarily for the sustenance of a healthy GI system. It has been suggested that consuming foods rich in probiotics can increase the population of *Bifidobacteria* in the GIT. The biological activities and development of this bacteria are further enhanced in the presence of prebiotics. Studies have shown that natural honey contains high amount of prebiotics [51]. Some in vitro and in vivo experimental trials on honey have reported it as a prominent dietary supplement that hastens the growth of *Lactobacillus* and *Bifidobacteria* and catalyzes their probiotic potency in the GIT [52, 53]. Under in vitro conditions, prebiotic ingredients in honey such as inulin, oligofructose, and oligosaccharides promoted the increase in the numbers of *Lactobacillus acidophilus* and *L. plantarum* by 10–100 folds, which was beneficial for the intestinal microbiota [54].

4.5. Oral Health. Honey is useful for the treatment of many oral diseases, including periodontal disease, stomatitis, and halitosis. In addition, it has also been applied for the prevention of dental plaque, gingivitis, mouth ulcers, and periodontitis. The antibacterial and anti-inflammatory properties of honey can stimulate the growth of granulation tissue, leading to the repair of damaged cells [55]. *Porphyromonas gingivalis* is a Gram-negative bacteria that causes periodontitis. Honey exerts antimicrobial activity against this anaerobic bacteria and prevents periodontal disease [56]. Inflammation of mucous membranes in the mouth (stomatitis) may induce redness and swelling of oral tissues and cause distinct and painful ulcers. Honey penetrates into the tissues very quickly and is effective against stomatitis [57, 58]. Halitosis is an oral health condition that causes malodorous breath. Most of the odor in the oral cavity is caused by the activity of degrading microbes [59]. A recent study has reported that honey consumption ameliorates halitosis due to its strong antibacterial activity resulting from its methylglyoxal component [60].

4.6. Pharyngitis and Coughs. Pharyngitis, commonly known as sore throat, is an acute infection induced by *Streptococcus* spp. in the oropharynx and nasopharynx [61]. In addition to streptococci, viruses, nonstreptococcal bacteria, fungi, and irritants such as chemical pollutants may also cause sore throat. Manuka honey is effective for treating sore throat with its anti-inflammatory, antiviral, and antifungal properties. Honey coats the inner lining of the throat and destroys the harmful microbes while simultaneously soothing the throat [62, 63].

A survey has demonstrated that honey is superior to other treatments for cough induced by upper respiratory infections, including dextromethorphan and diphenhydramine [64]. The antioxidant and antimicrobial properties of honey aided in minimizing persistent cough and ameliorated sleep for both children and adults following honey intake (2.5 ml). A comparative study on children with different natural products reported that honey was found to be the widely used remedy for pneumonia 82.4% [65].

4.7. Gastroesophageal Reflux Disease. Gastroesophageal reflux disease (GERD) is a mucosal infection caused by contents of abnormal gastric reflux into the esophagus and even the lungs. Symptoms of GERD include heartburn, inflammation, and acid regurgitation. Consumption of honey helps this condition by coating the esophagus and stomach lining, thus preventing the upward flow of food and gastric juice. Honey can further stimulate the tissues on the sphincter to assist in their regrowth and finally reduce the chances of acid reflux [66].

4.8. Dyspepsia, Gastritis, and Peptic Ulcer. Dyspepsia is a chronic disease in which the GI organs, mainly the stomach and first part of the small intestine, function abnormally. It is a disease that causes epigastric pain, heartburn, bloating, and nausea as symptoms. Dyspepsia is the preliminary symptom of peptic ulcer which could eventually cause cancer. Gastritis refers to the irritation and inflammation of the lining of the stomach wall. Peptic ulcer denotes erosions or open painful ulcers on the lining of the stomach or duodenum. Honey have been identified as a potent inhibitor for gastritis and the peptic ulcer causing agent, *Helicobacter pylori* (*H. pylori*) [67]. Clinical surveys have shown that honey decreased the secretion of gastric acid and increased the healing effect. Thus, honey is taken as a dietary supplement for its antibacterial properties and protective effect [68]. The high sugar content and low pH in honey are the results of glucose oxidative conversion to gluconic acid by glucose oxidase. This mechanism releases hydrogen peroxide, which functions as an antibacterial agent. Glucose oxidase also acts on fibroblasts and epithelial cell activators required for the healing of ulcers caused by *H. pylori* [51].

4.9. Gastroenteritis. Gastroenteritis, known as stomach or gastric flu, causes inflammation of the digestive tract. This condition may be due to foodborne, waterborne, and person-to-person spread of infectious agents. The symptoms of gastroenteritis include dehydration, watery diarrhea, bloating, abdominal cramps, and nausea. There are many infectious agents, such as *Salmonella*, *Shigella*, and *Clostridium*, that can cause this condition [69]. A clinical study by Abdulrahman, 2010, has reported the treatment of infantile gastroenteritis using honey. The study found that replacing the glucose in standard electrolyte oral rehydration solution (ORS) with honey reduced the recovery time of patients with gastroenteritis because the high sugar content in honey boosts electrolyte and water reabsorption in the gut [70].

4.10. Constipation and Diarrhea. Chronic constipation is a common and multifarious illness characterized by intolerable defecation (irregular stools and difficult stool passage). Difficult stool passage includes symptoms such as straining, hard to expel stool, a sense of incomplete evacuation, hard or lumpy stools, and prolonged time to pass stool [71]. Diarrhea is defined as a high frequency of bowel movements with watery stool. Honey has minimized the pathogenesis and duration of viral diarrhea compared to conventional antiviral therapy [72]. In another case, people diagnosed with inflammatory bowel syndrome (IBS) experiencing severe

diarrhea or constipation, bloating, and stomach discomfort was successfully treated with raw Manuka honey on an empty stomach [73].

4.11. Liver and Pancreatic Diseases. Honey helps to soothe pain, balance liver systems, and neutralize toxins. Complications in the liver system can be attributed to oxidative damage. Honey exhibits antioxidant activities that have a potential protective effect on the damaged liver. A study on paracetamol-induced liver damage rats showed that the antioxidant and hepatoprotective activity of honey minimized liver damage [74]. Honey, which has a 1 : 1 ratio of fructose to glucose, may help to promote better blood sugar level, which is useful for those suffering from fatty liver disease since it provides adequate glycogen storage in liver cells. Insufficient glycogen storage in the liver releases stress hormones that impair glucose metabolism over time. Impaired glucose metabolism leads to insulin resistance and is the main factor of fatty liver disease. Another study reported significant reduction in blood glucose levels after treatment with Tualang honey [75, 76].

4.12. Metabolic and Cardiovascular Health. Natural wild honey exerts cardioprotective and therapeutic impacts against epinephrine-induced cardiac disorders and vasomotor dysfunctions. A harmonized relationship between radical scavenging activity and the total phenolic content of honey has been observed [77]. Honey intake showed a significant reduction in risk factors of metabolic and cardiovascular diseases. Honey exhibits cardioprotective effects such as vasodilation, balancing vascular homeostasis, and improvements in lipid profile [78]. Flavonoids in honey improves coronary vasodilation, decreases the ability of platelets to form clots, prevents oxidation of low-density lipoproteins (LDL), increases high-density lipoproteins (HDL), and improves endothelial functions [79].

A study conducted to compare the metabolic response of honey has indicated its ameliorative effects against metabolic syndromes (MetS) [80]. MetS is denoted by hyperglycemia, hypertension, abdominal obesity, dyslipidemia, and intensified adaptability towards diabetes, kidney, and heart diseases. Polyphenols in honey reduce atherosclerotic lesions through the downregulation of inflammatory and angiogenic mechanisms [81]. A clinical study conducted on patients with hyperlipidemia showed that honey decreased total cholesterol (TC) and noticeably prevented the rise in plasma glucose levels. Nitric oxide (NO) is a metabolite present in honey that also has cardioprotective functions [82].

4.13. Cancer and Oncogenesis

4.13.1. Breast Cancer. Imbalance in estrogen signaling pathways and propagating levels of estrogens have important roles in breast cancer growth and propagation [83]. Treatments for breast cancer are associated with targeting the estrogen receptor (ER) signaling pathway. Phytoestrogens are a subclass of phytochemicals with a common structure to the mammalian estrogen that enables them to bind to estrogen receptors. Several experimental studies have investigated the efficiency of honey in modulating the ER signaling pathway [84]. Another study has shown that honey has biphasic activity in MCF-7 cells. This biphasic activity of honey is represented by an antiestrogenic effect at lower concentrations and an estrogenic effect at higher concentrations, which is caused when phytoestrogens bind to estrogen receptors [85]. Moreover, quercetin has been reported to induce apoptotic effects through ER α- and ER β-dependent mechanisms. On the other hand, cytotoxic activities of Tualang honey in human breast cancer cells were demonstrated by elevated secretion of lactate dehydrogenase (LDH) and further illustrated the cytotoxic properties of honey. The study also showed that honey only exerts cytotoxic effects on breast cancer line and not on nonmalignant breast cells. Therefore, this indicates that Tualang honey shows highly specific and selective cytotoxic effects towards breast cancer cell lines and has a good potential as a chemotherapeutic agent [86].

4.13.2. Liver Cancer. The most common type of liver cancer is hepatocellular carcinoma (HCC). The antitumor effects of honey on liver cancer cells have been investigated in various experimental studies. Treatment of HepG2 cells with honey minimized the amount of nitric oxide (NO) levels in the cells and decreased the HepG2 cell number greatly. This increased the overall antioxidant profile of the cells. The survival of HepG2 cells is promoted by reactive oxygen species (ROS), and adequate levels of ROS trigger cell proliferation and differentiation. Decreasing the amount of NO resulting from honey treatment supported this study. Thus, reduced ROS and enhanced antioxidant efficacy inhibit cancerous cell proliferation and lowered the number of HepG2 cells [84]. Another study done by Abdel Aziz et al. investigated the effects of honey on HepG2 cell lines. The report showed that honey exerted cytotoxic, antimetastatic, and antiangiogenic effects on HepG2 cells based on different concentrations [87].

4.13.3. Colorectal Cancer. Most colorectal cancers begin as a polyp, which generally starts on the inner lining of the colon or rectum and grows towards the center. Some polyps are not dangerous but some will eventually grow into adenomas and can eventually result in cancer. A study [88] that investigated the chemopreventive effects of Gelam and Nenas monofloral honeys against colon cancer cell lines found that the honey inhibited proliferation of colon cancer cells. Hydrogen peroxide-induced inflammation in the colon cancer cells was used to examine the effect of honey. The results showed that honey curbed inflammation in the cancerous cells [88]. Another study was done to investigate the apoptotic effects of crude honey on colon cancer cell lines. The study confirmed the antiproliferative effect of honey in these cells. In addition, at high phenolic concentrations (such as those of quercetin and flavonoids), significant antiproliferative action against colon cancer cells was observed [89].

The molecular mechanisms resulting in the antiproliferative and anticancer effects of honey include cell cycle arrest, activation of mitochondrial pathway, induction of mitochondrial outer membrane permeabilization, induction of apoptosis, modulation of oxidative stress, reduction of inflammation, modulation of insulin signaling, and inhibition of angiogenesis in cancer cells (Figure 2). In addition,

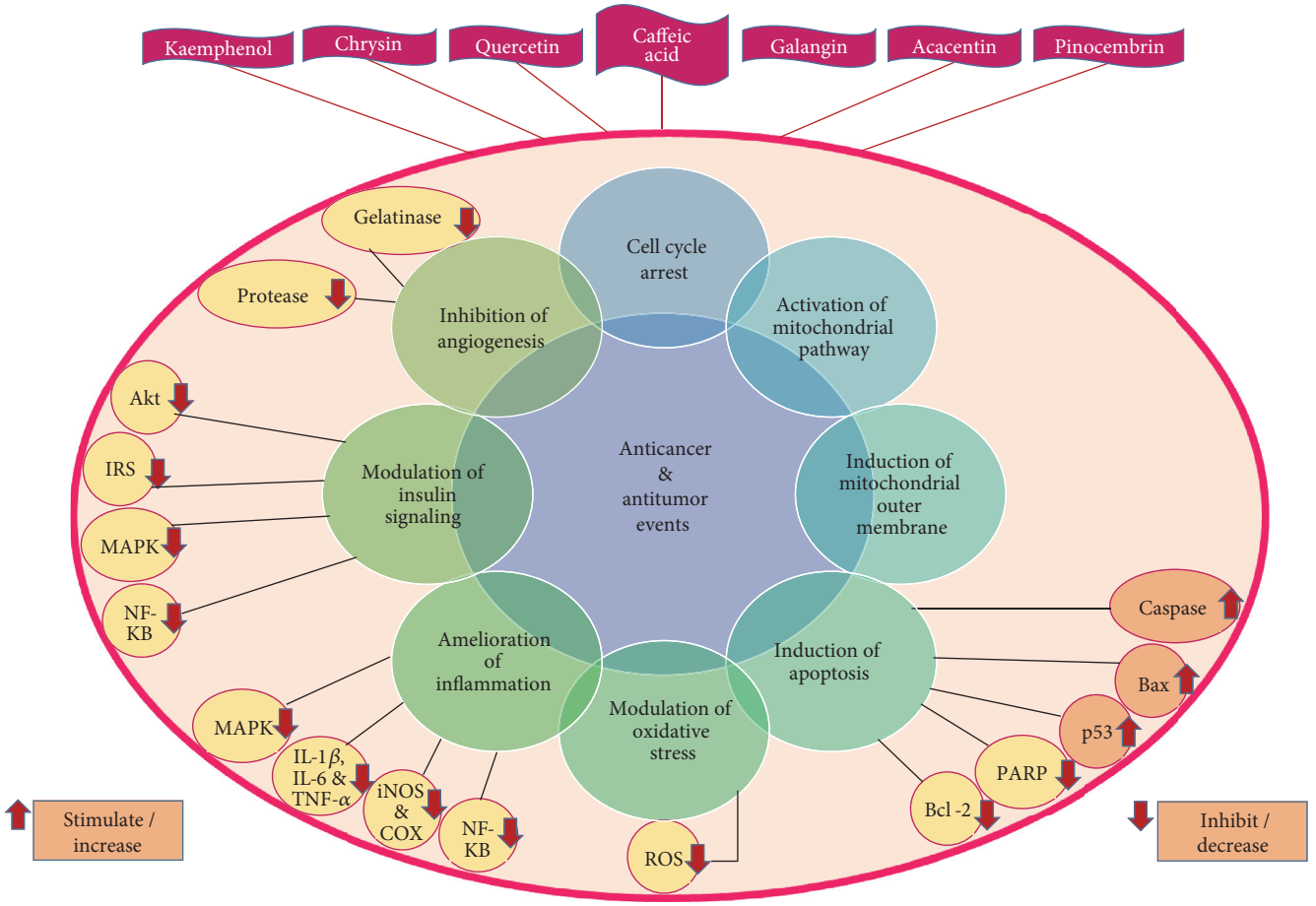


Figure 2: Molecular mechanisms responsible for anticancer and antitumor activities of honey products. IRS—insulin receptor substrate, MAPK—mitogen-activated protein kinase, NF-κB—nuclear factor kappa B, IL-1β—interleukin-1 beta, IL-6—interleukin-6, TNF-α—tumor necrosis factor alpha, iNOS—inducible nitric oxide synthase, COX—cyclooxygenase, ROS—reactive oxygen species, Bcl-2—B-cell lymphoma-2, and PARP—poly (ADP-ribose) polymerases.

honey shows potential effects on cancer cell by modulating proteins, genes, and cytokines that promote cancer.

Several components of honey such as chrysin, quercetin, and kaempferol have been shown to arrest cell cycle at various phases such as G0/G1, G1, and G2/M in human melanoma, renal, cervical, hepatoma, colon, and esophageal adenocarcinoma cell lines. The mitochondrial pathway entails a chain of interactions between stimuli such as nutrients, physical stress, oxidative stress, and damage during major cancer treatments including chemotherapy and radiotherapy. These stimuli cause several proteins located within the intermembrane space (IMS) of the mitochondria, such as cytochrome c, to be released, which eventually culminates in the death of the cell. Flavonoids in honey are effective in activating the mitochondrial pathway and discharging proteins with potential cytotoxicity. Induction of mitochondrial outer membrane permeabilization (MOMP) is the most prevalent anticancer mechanism, which causes the leakage of proteins from the IMS and inevitably results in cell death. Honey induces MOMP in cancer cell lines by decreasing the mitochondrial membrane potential. Honey has also been documented for amplifying the apoptotic effect of tamoxifen by intensified depolarization of the mitochondrial membrane. Flavonoid constituents of honey, such as quercetin, have been shown to trigger MOMP and lead to cancer cell death [84].

Apoptosis is a programmed cell death functioning to control cell growth and remove damaged cells from the system. This process also involves MOMP and results in the discharge of IMS proapoptotic proteins such as cytochrome c to activate caspase cascades which results in further disruption of mitochondria and finally results in cancer cell death. Influence of honey on enzymes, genes, and transcription factors corresponding to apoptosis has been investigated. Poly (ADP-ribose) polymerases (PARP) are crucial enzymes involved in apoptosis and DNA repair. Inhibition of PARP activity renders the cells unable to repair damaged DNA and pass through the G2 and M phases of the cell cycle. Thus, cell cycle is arrested. Because DNA repair is impaired due to nonfunctioning PARP, the cells are being classified as damaged, and consequently, apoptosis activity may be augmented.

Inhibition of PARP activity by flavonoids in honey is a potential strategy for targeting cancers with defective DNA-damage repair. Bcl-2 and Bax are antiapoptotic and proapoptotic proteins, respectively. Bcl-2 is generally overexpressed

in cancer. Tumor suppressor p53 is a transcription factor commonly inactivated in various types of tumors. It modulates transcription of genes involved in apoptosis [84, 90]. Honey enhances the upregulation of Bax and downregulation of Bcl-2. In addition, it activates caspases 3 and 9 and induces p53, thereby inhibiting cancer.

Low levels of ROS intensify cell proliferation while high levels lead to oxidative damage that contributes to various types of cancer. Regulation of redox homeostasis is vital for normal cell growth and proliferation. In this regard, honey is an influential antioxidant and free radical scavenger. The inhibitory effect of honey on cancer growth and proliferation is due to its ability to modulate oxidative stress. Honey exhibits anticancer properties via antioxidant or pro-oxidant mechanisms that are selectively dependent on the state of oxidative stress in the cancer cells. If cancer growth is rapid under high levels of ROS, honey acts as an antioxidant to prevent cancer cell growth by minimizing oxidative stress and scavenging the ROS. On the other hand, under low levels of ROS, it may also act as a pro-oxidant and promotes cancer cell growth by further generation of ROS and maximizing oxidative stress. Thus, the effects of honey on cancer cell death are different under different conditions [84].

Inflammation is a contributing factor for the dysregulation of physiological processes, which leads to various malignancies and cancers. Mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) are the two main pathways responsible for inflammatory response in cells. Activation of MAPK and NF-κB activates proinflammatory genes and generates inflammatory proteins or cytokines. These include cyclooxygenase-2 (COX-2), C-reactive protein (CRP), lipoxygenase-2 (LOX-2), interleukins (IL-1β, IL-6), and TNF-α. These components play crucial roles in both angiogenesis and inflammatory responses corresponding to cancer. IL-1β, IL-6, and TNF-α are cytokines that trigger cancer cell proliferation by maintaining the inflammatory phenotype in the tumor microenvironment. On the other hand, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) yield essential endogenous factors responsible for the tumor progression. The actions of iNOS can be either inductive or inhibitory depending on the tumor types.

Biological responses which facilitate inflammation can promote tumorigenesis as severe inflammation is the major factor for the development of cancer cells. Treating and soothing of inflammation aid to suppress the configuration of malignant and benign tumors. Honey helps to reduce the promotion and tumorigenesis and progression of cancer by reducing the expression of MAPK and NF-κB in cancerous cells. MAPK cascades are the main signaling pathways in the regulation of cell proliferation, survival, and differentiation. NF-κB is a transcription factor which is vital in the regulation of immune responses, inflammation, and oncogenesis. NF-κB translocation to the nucleus and reduced IκBα degradation help to regulate the expression of genes involved in apoptosis and proliferation that are responsible for the development of cancer. Flavonoids found in honey have been shown to induce apoptosis and prevent the release of IL-1β, IL-6, TNF-α, iNOS, and COX-2 [84].

Tumors, malignancies, and cancers are usually enhanced by obesity and insulin-resistant type 2 diabetes mellitus. PI3K/Akt is an important pathway in insulin signaling. The PI3K/Akt pathway is also recognized in modulating substrates that are related to cellular growth, survival, and progression. Elevated levels of MAPK, NF-κB, and insulin receptor substrate 1 (IRS-1) along with reduced levels of Akt expression have been actively linked to the development of insulin resistance. Honey components such as quercetin revive insulin resistance by increasing the expression of Akt while reducing the expression of IRS, MAPK, and NF-κB. Modulation of insulin signaling by honey leads to anticancer activities [84].

Honey has debridement effects by boosting epithelialization and stimulates the development of granulation tissue through its angiogenic effect on the vasculature. Honey selectively stimulates angiogenesis in noncancer tissues through the production of hydrogen peroxide while inhibiting angiogenesis in cancer tissues. Honey has antiangiogenic effects that prevents the wound-healing response, reduces the viability of cancer cells, and lowers the incidence of metastasis by inhibiting the activities of gelatinase and protease. Honey prevents the development of cancer by blocking the three main stages of cancer formation known as initiation, proliferation, and progression [84].

5. Health Benefits of Propolis

5.1. Gastrointestinal Disorder. Infection with parasites usually occurs upon contact with an infected surface. The symptoms of parasitic infection of the GI tract include abdominal pain, diarrhea, bloating, and nausea. Propolis has been reported to have several biological efficacies including anticancer, antioxidant, and anti-inflammatory activities (Figure 3). There are a few studies that reported the clinical use of propolis in the treatment of viral infections. In one study, the in vitro effect of propolis ethanolic extract on the growth and adherence of *Giardia duodenalis trophozoites* was evaluated [91]. Propolis was shown to inhibit growth and adherence of the trophozoites. It also promoted the detachment of these parasitic organisms. Its efficacy against giardiasis has also been reported in a clinical study whereby children and adults with giardiasis-given propolis showed a cure rate between 52% and 60%, whereas those given the conventional drug showed a 40% cure rate. Another experimental study showed that propolis has antihistaminergic, anti-inflammatory, antiacid, and anti-*H. pylori* activities that can be used to treat gastric ulceration [92].

5.2. Gynecological Care. Widespread causes of indicative vaginitis are bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC). The depletion of *Lactobacillus* spp. in the vagina is a distinguished feature of vaginal infections. The infection is accompanied by an overgrowth of vaginal pathogens such as yeast-like fungi and an elevated vaginal pH. Diabetes patients are more prone to having vaginal infections caused by *Candida albicans*. A study conducted on the application of 5% aqueous propolis solution resulted in an improvement in vaginal well-being [93]. In addition

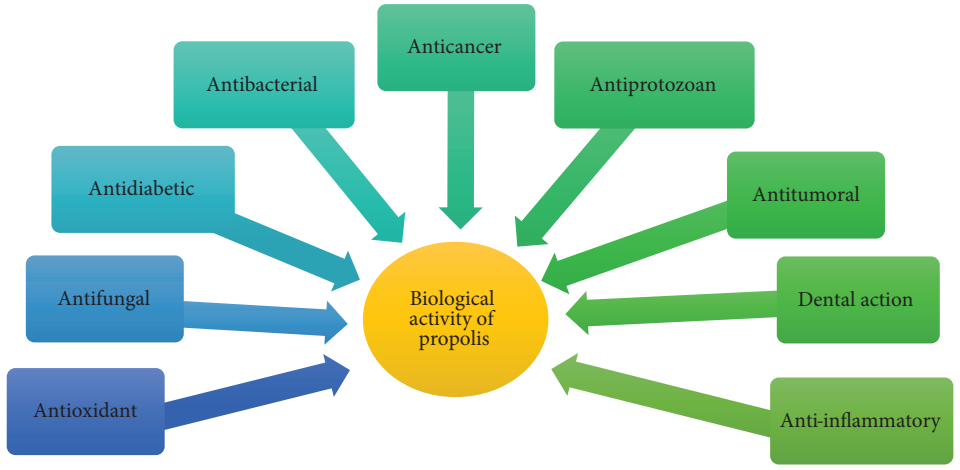


Figure 3: The biological activities of propolis.

to providing antibiotic and antimycotic actions, propolis provides early symptomatic relief due to its anesthetic properties. Thus, propolis may be used for Recurrent Vulvovaginal Candidiasis (RVVC) and can be an alternative option for patients who are unable to take antibiotics due to a concurrent pharmacological treatment. The effectiveness of propolis against conventional antifungal nystatin has shown satisfactory results. Propolis extract solution (PES) also show low toxicity in human cells and can be an alternative treatment for chronic vaginitis. In addition, PES has antifungal properties and it can be used as antibiofilm material for RVVC to counteract biofilm growth of *C. albicans* and resistance in antifungal drug [94].

5.3. Oral Health. The oral cavity has an abundant bacterial microflora and excessive bacterial growth may lead to several conditions such as oral diseases. Studies have shown that propolis may restrict bacterial-plaque development and periodontitis-causing pathogens because of its antibacterial properties [95]. Propolis solutions exert a selectively lower cytotoxic action on human gum fibroblasts compared to chlorhexidine. In addition to that, mouthwash containing propolis have shown effectiveness in healing surgical wounds. This encourages the use of propolis in solutions used as mouthwash [96]. Propolis solution can also be used to disinfect toothbrushes [97]. A 3% ethanolic extract of propolis toothpaste gel showed greater potency against gingivitis caused by dental plague in a group of patients [98]. Propolis extracts have also helped cure halitosis, a condition where an individual experiences unpleasant breath predominantly due to poor oral hygiene. Propolis toothpaste or mouthwash is used for their ability to reduce growth of bacterial plaque and pathogenic microflora that causes gingivitis and periodontitis. Thus, propolis also plays a role as a therapeutic agent [95].

5.4. Oncological Treatment. A study reported that propolis has potential towards human breast cancer treatment due to its antitumor activity by inducing apoptosis on human breast cancer cells. It also exhibits low or no toxicity towards normal cells due to its selectively toxic properties against tumor cells and is believed that propolis may become a prominent agent to treat breast cancer [99]. Another study investigating the effect of ethanolic extract of Algerian propolis on melanoma tumor growth has shown that galangin, a common flavonoid in propolis remarkably induced apoptosis and inhibited melanoma cells in vitro [100]. Turkish propolis has also been shown to exert a selective cytotoxic action on human lung cancer cells by inducing endoplasmic reticulum stress, apoptosis, and caspase activity and by reducing the mitochondrial membrane potential. This indicates that propolis is able to minimize the cancer cell proliferation [101].

5.5. Dermatological Care. Propolis is widely used in dermatological products such as creams and ointments. Its use in skin care products is based on its antiallergy, anti-inflammation, antimicrobial properties, and promotive action on collagen synthesis. A recent study comparing the effect of propolis and the conventional drug silver sulfadiazine showed that propolis notably decreased free radical activity in healing the wound beds which supported the repair process. A clinical study on acne patients using ethanolic extract propolis showed its high efficacy in the treatment of acne vulgaris [102]. Propolis also shows positive collagen metabolism in the wound during the healing process by increasing the collagen content of tissues [103]. A study demonstrated the use of propolis as an alternative therapy for wound healing to promote wound closure, especially under conditions such as human diabetic foot ulcer (DFU) [104].

The molecular mechanisms responsible for the wound-healing activity of propolis is shown in Figure 4. Fibronectin (FN) is a multifunctional glycoprotein of high molecular weight, which influences the structural stability and functional properties of various organs and tissues (Stoffels, 2013). The fibronectin matrix and its accumulation are essential for cell migration, cell proliferation, cell differentiation, cell adhesion, apoptosis, cellular signaling, angiogenesis, collagen biosynthesis, re-epithelialization, clot formation,

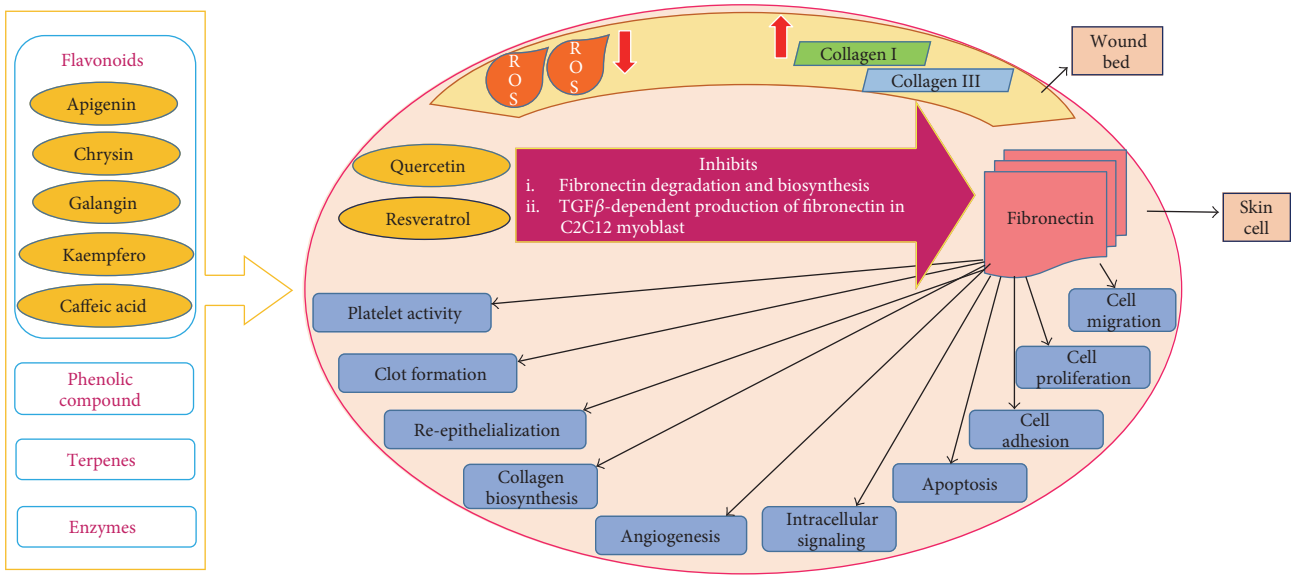


Figure 4: Molecular mechanism targeting wound-healing activity of propolis.

and platelet activity. Fibronectins are also important in the repair mechanisms for conditions such as glycoprotein intensified degradation, which leads to a defective cellular microenvironment and affliction in the structure of granulation tissues. This condition may prevent the wound from healing or inhibit the repair process. The accumulation of fibronectin in the extracellular space also modulates the secretion of other repairing components such as collagen type I and type III, tenascin, laminin, and fibrillin.

Propolis has demonstrated favorable effects in the wound-healing process such as antifungal and antibacterial activities due to its components such as flavonoids, phenolic compounds, terpenes, and enzymes. It also reduces the activity of free radicals (ROS) in the wound bed favoring the repair process. Propolis has also demonstrated great effects on collagen metabolism by increasing the amount of both type I and type III collagens in tissues. The reduction of ROS and accumulation of collagen aid in balancing the extracellular matrix and generating granulation tissues. Propolis is a potential apitherapeutic agent that is able to modify the metabolism of fibronectin by developing a fibrous network of extracellular matrix and inhibiting fibronectin disintegration. The active components in propolis such as quercetin and resveratrol inhibited fibronectin biosynthesis and TGF?-dependent production of fibronectin, respectively, in C2C12 myoblasts. Both the components play important roles in regulating the expression of fibronectins. Studies have also shown that mobility and migration of epithelial cells are dependent on reduced fibronectin content in the extracellular matrix. Reduced amounts of this glycoprotein in propolis effectively treated wounds and produced granulation tissues. Therefore, the influence of propolis on fibronectin metabolism may alter the mechanism of wound healing [103]. Several health benefits of propolis related to gastrointestinal, gynecological care, oral health, skin care, and oncological treatments are tabulated in Table 2.

6. Health Benefits of Royal Jelly

Royal jelly is one of the honey bee products which have potential towards various human disease treatments. Figure 5 depicts the biological activities of royal jelly as an antioxidant, antitumor, antiaging, neurotropic, and anti-inflammatory agent.

6.1. Reproductive Health. A randomized clinical study has reported that royal jelly is effective in reducing premenstrual syndrome [105]. A randomized clinical trial study reported the effectiveness of royal jelly in treatment of urinary problems and promotion of life quality in postmenopausal women [106]. Royal jelly has protective effects against Oxymetholone-induced reproductive toxin (OXM), which is an active steroid derived from testosterone as a defense mechanism. Recent studies have reported that royal jelly protects against the oxidative injuries in the mouse testes and that it contains spermatogenesis-stimulating compounds, which inhibit the production of proinflammatory cytokines [107]. Another study on male rabbits has indicated its positive effects on fertility, semen quality and output, and concentration of testosterone, total proteins, and glucose in the blood. The number of dead and abnormal sperm decreased with the reduction of biomarkers of oxidative stress [108]. Royal jelly has been traditionally used to treat menopause symptoms by rebalancing the hormonal concentration in the blood, decreasing follicle-stimulating hormones (FSH) and increasing the estrogen concentration in aged mice. A study showed that the changes in hormone levels resulting from royal jelly increased the amount of ovulated oocytes and their quality in aged rats [109].

The molecular mechanisms responsible for the antiaging activity of royal jelly are shown in Figure 6. The quality of oocytes decreases with age and the depleted follicle pool hastens hormonal dysregulation. This hormonal dysfunction is responsible for the reduction in ovarian follicle size and

Table 2: Selected propolis activities according to the health benefits.

Health benefits	Propolis activity	Type of studies	Authors
GI disorder	Antiparasitic	Humans	Freitas et al. 2006 [91]
	Antiulceration	Animals	Paulino et al. 2015 [92]
Gynecological care	Antifungal	Human	Imhof et al. 2005 [93]
	Antifungal and antibiofilm	Human	Capoci et al. 2015 [94]
Oral health	Antibacterial	Laboratory	Pereira et al. 2011 [95]
	Daily mouthwash	Human	Jain et al. 2014 [96]
	Toothpaste disinfection	Laboratory	Bertolini et al. 2012 [97]
	Toothpaste against gingivitis	Human	Skaba et al. 2013 [98]
	Oral therapeutic agent	Human	Pereira et al. 2011 [95]
Oncology treatment	Anti-breast cancer	Human	Xuan et al. 2014 [99]
	Antimelanoma cancer	Animals	Benguedouar et al. 2015 [100]
	Anti-lung cancer	Human	Demir et al. 2016 [101]
Dermatology care	Acne vulgaris	Human	Ali et al. 2015 [102]
	Collagen metabolism	Animals	Olczyk et al. 2014 [103]
	Diabetic foot ulcer	Human	Henshaw et al. 2014 [104]

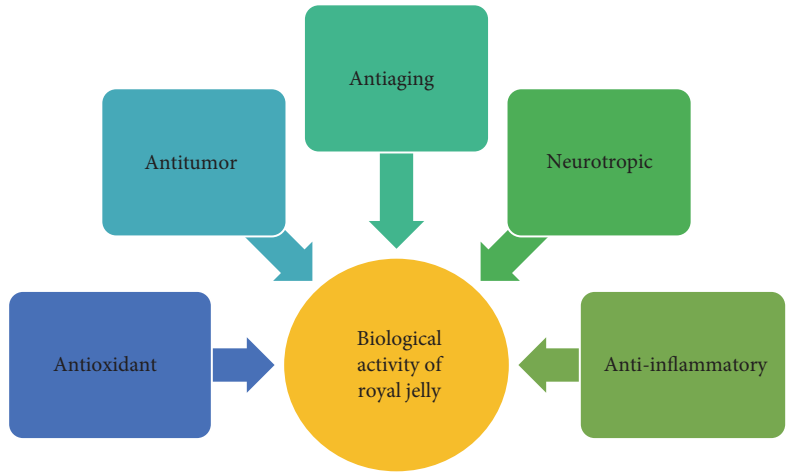


Figure 5: Different types of biological activities of royal Jelly.

oocyte quality. Oxidative stress is the main cause of aging. Increased oxidative stress and continuous ovulation causes loss of antioxidants such as SOD, catalase, and glutathione S-transferase (GST). It also minimizes the size of the follicle pool and oocyte quality. Oxidative stress is controlled by glutathione (GSH), glutathione S-transferase (GST), Glutathione S-Transferase Theta 1 (GSTT1), Bax, and Bcl-2. GSH, GST, and GSTT1 are direct ROS scavengers, which play a vital role in removing oxidative stress from the cell. Higher expression of Bax and lower expression of Bcl-2 also promote aging and reduces oocyte quality.

FSH and luteinizing hormone (LH) are the hormones involved in the aging process. The amount of FSH and LH is controlled by estrogen (E2) and inhibin from the ovarian cells. Reduction of the follicle pool size results in an inadequate release of estrogen and inhibin, which results in a rise in FSH levels. This process then aids in the reduction of the follicle pool size and affects oocyte quality. This process promotes aging in the ovaries. In young ovarian cells, higher amount of estrogen (E2) and inhibins are needed to decrease the level of FSH and LH. This adaptation can be overcome by antiaging therapies such as supplemental consumption of royal jelly. The major active component present in royal jelly is 10-hydroxyl-2-decenoic acid. This compound enhances the synthesis of ovulation hormones, maintaining a lower expression of FSH and LH in young ovarian cells. It is also efficient in preventing the depletion of follicle pool and in enhancing hormonal regulation. Thus, royal jelly helps in preventing the aging process and is an influential antiaging product [109].

6.2. Neurodegenerative and Aging Diseases. Poor mental state and performance such as in the case of Alzheimer's disease (AD) are mostly experienced by elderly individuals due to aging. Royal jelly stimulates physical and mental functions for the elderly and increases their appetite and weight. A

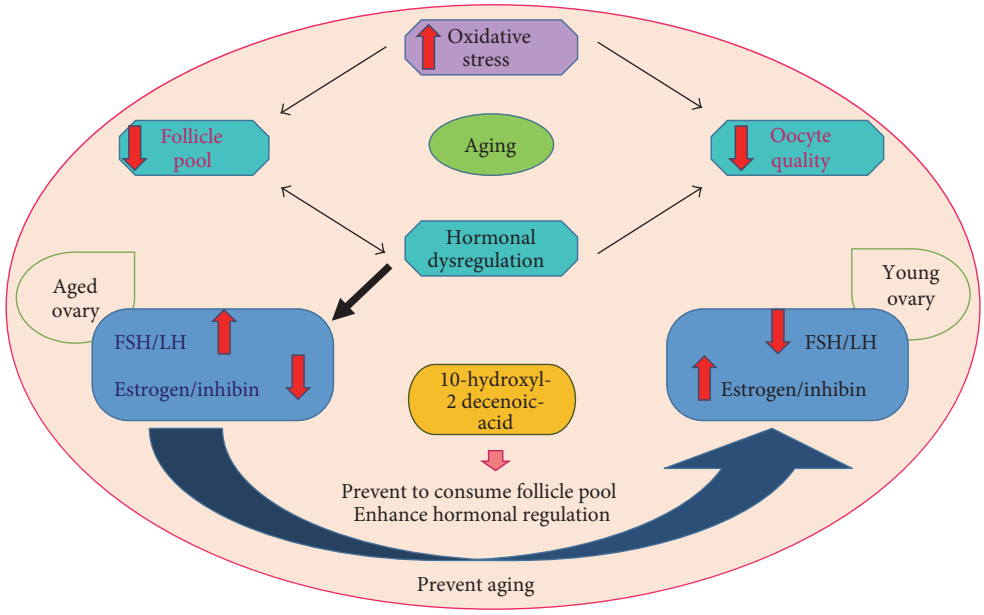


Figure 6: Molecular mechanism responsible for the antiaging activity of royal jelly.

Table 3: Reports on health benefits of royal jelly.

Health benefits	Propolis activity	Type of studies	Authors
Reproductive care	Antioxidant	Animals	El-Hanoun 2012 [108]
	Hormone balance	Animals	Imai et al. 2012 [109]
	Antioxidative agent	Animals	Najafi et al. 2014 [107]
	Reduce premenstrual syndrome	Humans	Taavoni et al. 2014 [105]
	Postmenopausal treatment	Humans	Seyyedi et al. 2016 [106]
Neurodegenerative and aging disease	Longevity promoting	Animals	Honda et al. 2011 [111]
	Alzheimer's disease	Animals	Zamani et al. 2012 [110]
	Mental health	Human	Morita et al. 2012 [112]
Wound healing	Fibroblast migration	Animals	Kim et al. 2010 [113]
	Collagen production	Human	Park et al. 2011 [114]
	Vasodilatation	Human	Siavash et al. 2015 [115]

study showed that royal jelly exerted neuroprotective effects in AD [110]. The behavioral and neurochemical effect of royal jelly was chemically examined in aged rats. The study confirmed a better cognitive performance and increased the life span in the older animals that had been given royal jelly. Another study reported that royal jelly contains longevity-promoting factors and extends the lifespan in the nematode *Caenorhabditis elegans* [111]. Another study have also reported the improved mental health in human upon ingestion of royal jelly for six months [112]. A few studies on the health benefits of royal jelly are given in Table 3.

6.3. Wound Healing. Royal jelly enhances wound-healing activity. In both in vivo and in vitro wound-healing models, under the effect of royal jelly, human fibroblasts were able to migrate and increase levels of sphingolipids by decreasing the secretion and formation of collagen. Thus, royal jelly shortened the curing period of desquamated skin lesions [113]. Another study on the use of royal jelly have also exhibited protective action on human skin against ultraviolet B-induced photoaging by promoting collagen production [114]. Royal jelly dressing is also an effective way of treating diabetic foot ulcers besides standard treatments. This is due to its vasodilation effects around the affected wound, which can help to dilate the blood vessels to enhance blood flow. It also helps to prevents infections due to its antimicrobial activities [115].

7. Conclusion and Future Prospects

The present review focused on the potential health benefits of bee products such as honey, propolis, and royal jelly. These products are highly rich in active components such as flavonoids, phenolic acid, phenolic compounds, terpenes, and enzymes, which have biological functions in preventing some diseases and promoting good health. Honey, propolis, and

royal jelly have distinct efficacies with significant nutritional properties and functional values. Thus, these bee products can be developed into potent apitherapeutic agents. However, some precautions need to be taken in case of allergens associated with bee products and in finding the right intake dosage. Hence, it is necessary to conduct further studies to determine the critical mechanisms related to the pharmacological activities of these bee products and the appropriate amounts that can be taken in order to obtain promising health benefits.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

The authors acknowledge the financial supports from the Research Acculturation Collaborative Effort (RACE) (R/RACE/A07.00/01147A/ 001/2015/000237) and a research university team grant (RUT) (1001/PPSP/853005).

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 2740364, 11 pages
https://doi.org/10.1155/2017/2740364

Review Article

Antiobesity Effects of Anthocyanins in Preclinical and Clinical Studies

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Received 31 March 2017; Revised 23 May 2017; Accepted 13 June 2017; Published 13 July 2017

Academic Editor: Silvana Hrelia

The natural phytochemicals present in foods, including anthocyanins, might play a role in attenuating obesity by producing a decrease in weight and adipose tissue. This review focused on current knowledge about anthocyanins' role in obesity and its related comorbidities reported in animal models and humans. We summarized their target identification and mechanism of action through several pathways and their final effects on health and well-being. Into consideration of ongoing researches, we highlighted the following key points: a healthy relationship between anthocyanin supplementation and antiobesity effects suffers of the same pros and cons evidenced when the beneficial responses to other phytochemical treatments towards different degenerative diseases have been considered; the different dosage applied in animal versus clinical studies; the complex metabolism and biotransformation to which anthocyanins and phytochemicals are subjected in the intestine and tissues; the possibility that different components present in the supplemented mixtures can interact generating antagonistic, synergistic, or additive effects difficult to predict, and the difference between prevention and therapy. The evolution of the field must seriously consider the need to establish new and adequate cellular and animal models which may, in turn, allow the design of more efficient and prevention-targeted clinical studies.

1. Introduction

The obesity and its related comorbidities represent an emerging global health issue. In 2014, WHO estimated overall that about 13% of the world's adult population (11% of men and 15% of women) were obese. Additionally, the worldwide prevalence of obesity is more than doubled between 1980 and 2014 [1]. Obesity is an abnormal excess accumulation of fat in adipose tissue due to an imbalance between energy intake and energy expenditure. The daily energy expenditure consists of basal metabolism, thermogenesis, which includes all processes to produce heat and energy expenditure for physical activity level. The key element to counteract obesity is moderate weight loss (5–10%) over time through an integrated treatment [2]. This is expressed by

(i) Energy intake: a low-calorie diet providing about 800–1500 kcal/day exerts a slow and progressive weight loss and allows to achieve weight reduction;

(ii) Physical activity: the association between diet with physical activity leads to a greater weight loss but also to an easier weight control compared to only low calorie diet;

(iii) Behavior and lifestyle modification;

(iv) Drug therapy: all drugs act by modulating the neurotransmitter release at neuronal level, regulating, in this way, hunger and appetite.

More recently, natural phytochemicals present in foods, including anthocyanins, due to their chemical structure, seem to be able to exert several pharmacologic activities, mainly antioxidant and anti-inflammatory actions [3]. It has been reported that the anthocyanins might play a role in attenuating obesity by producing a decrease in weight and adipose tissue [4]. Controversial results were obtained in animals and human studies in obesity condition [5–11] by type of supplementation (fresh and/or commercial product or pure compound), dose, length of the study, and by different sizes and clinical characteristics of the enrolled sample. Furthermore, the pharmacological actions of anthocyanins could not be fully recognized without knowing the effects of treatment of anthocyanins alone, the effects of nonanthocyanin molecules, and the possible synergistic action between anthocyanins and the complex mixture of phytochemicals present in whole foods [12]. Thus, this review is focused on the current knowledge about anthocyanins' role in obesity and its related comorbidities reported in animal models and humans.

2. Anthocyanins

2.1. Anthocyanin Chemistry. Anthocyanins are water-soluble glycosides of polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium or flavylium salts as displayed in Figure 1.

A lot of molecules have been identified [13] by spectrometric measurements, but plant foods are rich in mainly six anthocyanidins (free of sugar usually known as aglycone), including pelargonidin (Pg), cyaniding (Cy), petunidin (Pt), peonidin (Pn), delphinidin (Dp), and malvidin (Mv). Their distribution in fruits and vegetables is Cy 50%, Dp 12%, Pg 12%, Pn 12%, Pt 7%, and Mv 7%. In fact, 90% of anthocyanins are based on cyanidin, delphinidin, and pelargonidin and their methylated derivatives [14]. These compounds are different from each other by the number of hydroxyl substituent groups on the B ring and their methylation degree and by nature, number, and location of sugars attached to the molecule (anthocyanins) (Figure 2) [15].

Generally, the conjugated sugar bonded to carbon 2, 3, and 5 are glucose, arabinose, rhamnose, and galactose. It is possible to distinguish between mono-, di-, and triglucosides. Kong et al. [16] have reported the presence of the 3-glucoside derivatives 2.5 more frequent than the 3,5-diglucosides. Glycosylation increases water solubility and stability due to hydrogen bonding; therefore, anthocyanidins rarely occur in their free form due to their high reactivity.

Esterification of sugars represents a key factor for chemical structure of these pigments, and it is based on carboxylic acids, including caffeic, ferulic, sinapic, and the p-coumaric acid and on aliphatic acids as well as acetic acid, the malonic, oxalic, and succinic acid. On the same anthocyanin, it could be present up to three esterifying agents. All these variables contribute to define the unique composition in anthocyanins of foodstuff.

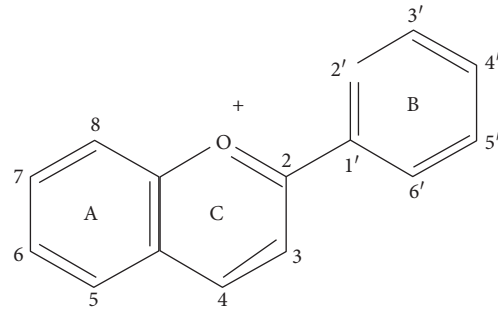

Figure 1: Chemical structures of flavylium cation.

The anthocyanins are very stable and highly colored at low pH (pH 1–3). On increasing pH, these molecules change their chemical structure, as well as color. At pH 1–3, the flavylium cation is the predominant species and contributes to purple and red colours. pH 4-5 generates the colorless carbinol. At pH 6-7, quinoidal blue-violet species are dominant, at pH 7-8 the chalcone species, also practically colorless. In the aqueous phase, these four chemical forms are in equilibrium with each other (Figure 3); the prevalent form depends on the pH of the solution. More particularly, the bioavailability of anthocyanins is low due to their sensitivity to changes in pH [17].

2.2. Dietary Anthocyanins Source. Plant phenolic fraction is composed by a heterogeneous mixture of molecules belonging to different families with varying chemical structures which content represents a peculiar characteristic of plant tissues. Anthocyanins, belonging to flavonoids family, are water-soluble pigments responsible for most of the red, blue, and purple colors of fruits and vegetables and other plant tissues or products. Anthocyanins, as well as plant phytochemicals, play several and varied functions, but their main activities are to protect plants from oxidative risk posed by various environmental stressors (sunlight and other environmental agents) and to defense plants from fungal, bacterial, or viral infections. Recently, their dietary consumption has been associated to several health benefits by free radical scavenging, antioxidant capacities and antibacterial activity [18, 19]. Nowadays, it does not exist a recommended daily allowance for these molecules although a consumption between 250–400 mg/d, respecting the seasonality of food sources, has been proposed for total flavonoids [20]. European Prospective Investigation into Cancer and Nutrition (EPIC) [21] estimated a total anthocyanidin mean intake of 64·88 (SE 1·86) mg/d and 44·08 (SE 2·45) mg/d for men and women, respectively (Turin, Italy). In plant-derived anthocyanin-rich foods, the relative abundance and composition of anthocyanins differs due to intrinsic and extrinsic factors, such as genetic and agronomic variation, light intensity and type, temperature, harvest time, storage, and processing condition. Additionally, data on food anthocyanins composition and content are limited and debated [22]. Zamora-Ros et al. [20] have reported a dietary data set in combination with anthocyanidins content data from two databases USDA database for the flavonoid content of selected foods [23] and Phenol-Explorer [24]. This database includes 1877 food

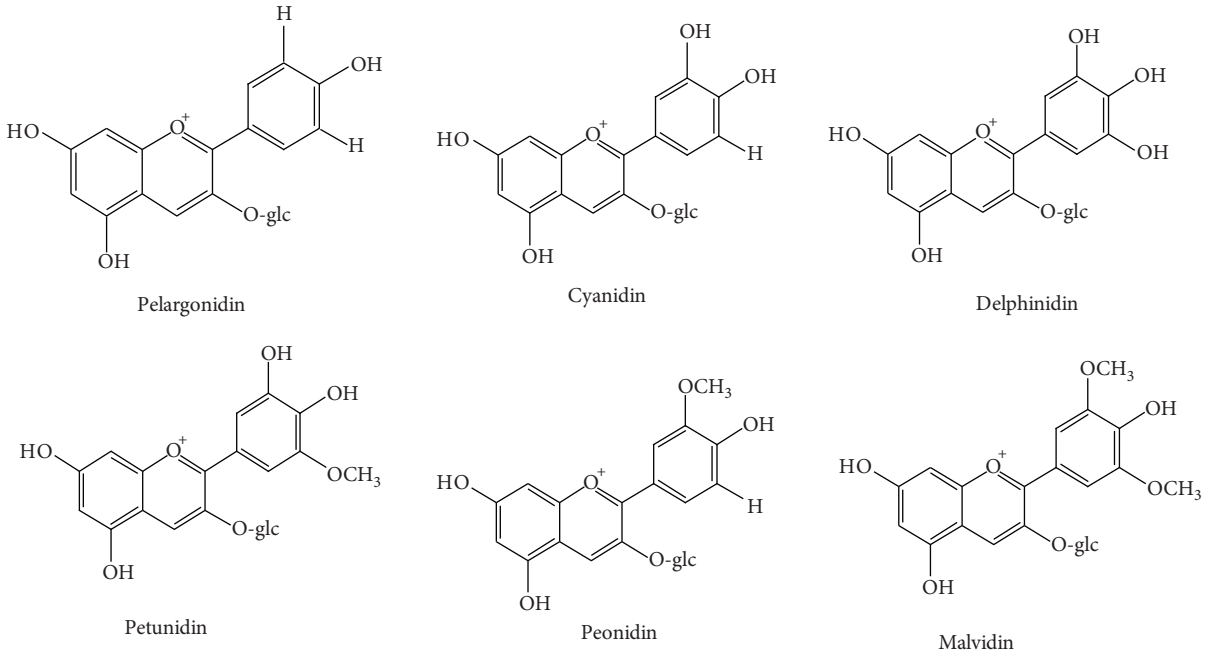

Figure 2: Main six anthocyanidins present in plant foods.

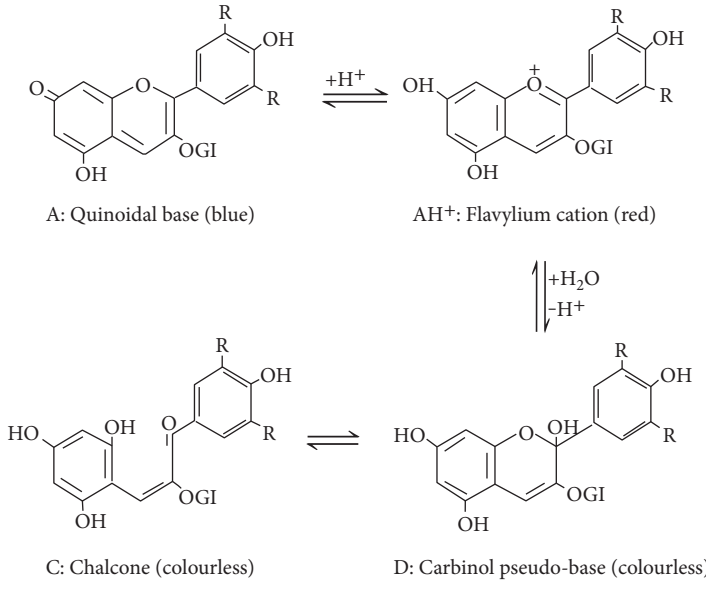

Figure 3: The four chemical forms in equilibrium with each other: the case of Malvidina 3-O-glucoside structures.

items; the anthocyanidins are calculated as the sum of the available forms (glycosides and aglycones) in the literature and expressed as anthocyanidin aglycones per 100 mg fresh weight. To exert their bioactivities and so to enhance health benefits, the anthocyanins should be absorbed at useful dose and properly metabolized to reach specific organ target. More particularly, the bioavailability of anthocyanins is low due to their sensitivity to changes in pH.

2.3. Anthocyanin Bioavailability. Anthocyanins are generally stable at pH values of 3.5 or below, therefore stable under stomach conditions (pH 2). However, these molecules are degraded quickly at higher pH values, such as the intestinal tract (pH 7), so the beneficial absorption and nutritional value could be significantly reduced. Different effects have been observed after acute and chronic consumption of anthocyanin-rich foods or their extracts demonstrating the high interindividual bioavailability and bioactivity [25, 26]. Several metabolic pathways, tissue distribution, and effects have been reviewed [27, 28]. The absorption, metabolism, and excretion of anthocyanins depend on food matrix, including other antioxidants and macronutrients present in

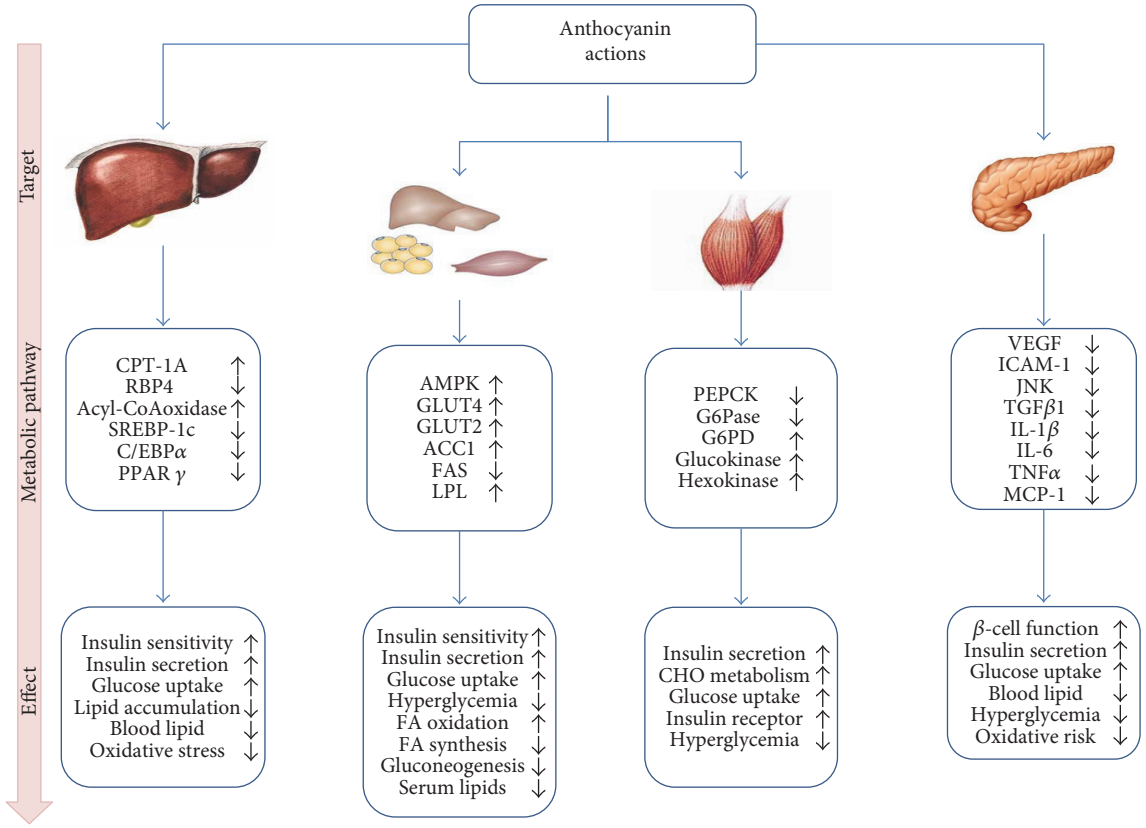

Figure 4: Proposed anthocyanins target identification and mechanism-of-action through several pathways and their final effects on health and well-being. CPT1A: carnitine palmitoyltransferase-1A; RBP4: retinol binding protein-4; SREBP1c: sterol regulatory element binding protein-1c; C/EBPα: CCAAT enhancer binding protein-α; PPAR γ: peroxisomal proliferator-activated receptor gamma; AMPK: 5′ adenosine monophosphate-activated protein kinase; GLUT4: glucose transporter 4; GLUT2: glucose transporter 2; ACC1: acetyl-CoA carboxylase; FAS: fatty acid synthase; LPL: lipoprotein lipase; PEPCK: phosphoenolpyruvate carboxykinase; G6pase: glucose-6-phosphatase; G6PDH: glucose-6-phosphate dehydrogenase; VEGF: vascular endothelial growth factor; ICAM-1: intercellular adhesion molecule; JNK: c-Jun N-terminal kinase; TGFβ1: transforming growth factor-beta; IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha; MCP-1: monocyte-chemo-attractant protein-1.

the usual diet, which consequently affects anthocyanin bioavailability. The pharmacokinetics of anthocyanins and metabolites from several foods suggest that the gastrointestinal tract is their primary target [29–31]. Fang [32] summarized how some anthocyanins could be efficiently absorbed from the gastrointestinal lumen, undergo extensive first-pass metabolism, and enter the systemic circulation as metabolites. Nevertheless, the production of several metabolites poorly absorbed and rapidly removed from plasma does not impair to ameliorate obesity and related comorbidities [33, 34]. Furthermore, Cardona et al. [35] reviewed that, like drugs and/or xenobiotics, these molecules are processed in the body by enzymatic activities of the gut microbial community. On releasing, their metabolites contribute to the maintenance of gut health by the modulation of the gut microbial balance through the stimulation of the growth of beneficial bacteria and the inhibition of pathogen bacteria, exerting prebiotic-like effects. The interindividual differences in the composition of the microbiota could contribute to differences in bioavailability and bioefficacy of anthocyanins and their metabolites. Moreover, Smeriglio et al. [36] highlighted the influence of human enzymes polymorphism involved in biotrasformation mechanisms on anthocyanins bioavailability. Finally, studies on the gut microbiota modulation by anthocyanins and their mechanisms are incomplete. In Figure 4, we summarized anthocyanin's target identification and mechanism of action through several pathways and their final effects on health and well-being. Briefly, they act on the skeletal muscle, liver, pancreas, and adipose tissue for stimulating different inflammatory cytokines, metabolic enzymes, and signaling pathways, which exert anti-inflammatory, antioxidative, and metabolic-stabilizing activity. These mechanisms are associated with stabilization of obesity and diabetes, improvements in blood pressure and lipid profiles, decreased atherosclerotic development, and improved vascular function.

3. The Role of Anthocyanins in Obesity: Animal Studies

In the last several years, many research groups carried out studies to identify the link between anthocyanin compounds and hyperlipidemia, hyperglycemia, hypertension,

inflammation, and immunity that cause diabetes, cardiovascular diseases, and other inflammation-related diseases. Obesity is one of public health problems because it leads to the development of metabolic disorders. Anthocyanins are known as compounds that can modulate mechanisms of the homeostasis of glucose, lipids, and amino acids and suppress inflammation. Increasing attention has been given to the development of alternative strategies and possible therapies targeting differentiation of adipogenesis, glucose transport and intake, attenuation of inflammation, and changes in the immune response. During long-term and low-level inflammation, usually present in obese subjects, alterations presented in the metabolism could lead to changed immunity [37].

Understanding the effects of anthocyanins on human health and disease prevention could promote interest in drug discovery and the potential of diets in the prevention of obesity and several diseases.

Prior et al. [38] reported that supplemented diet with a blueberry extract (BBE) significantly decreased body weight and body fat accumulation in obese C57BL/6 mice fed with high-fat (HF) diet, while intake of wild blueberry powder (WBP) did not induce body fat accumulation. The same authors, in the following study [26], reported that consumption of blueberry juice (BBJ) did not reduce body weight and the weight of white adipose tissue (epididymal and retroperitoneal fat) in obese mice [26, 39]. A fermented blueberry-blackberry beverage also mitigates the development of obesity and reduces fasting blood glucose in C57BL/6J mice [40]. In addition, the consumption of both BBJ and mulberry juice (MJ) resulted in a decrease of body weight, decrease of the serum cholesterol, and reduced resistance to insulin (IR) as well as reduced lipid accumulation and decreased leptin secretion [41].

Beneficial effects of BB anthocyanins reflect the ability of BB anthocyanins to change mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) stress signaling pathways, which might suggest their cytoprotective and anti-inflammatory role in the pathology of obesity [39].

Seymour et al. [42] described that supplementation with 2% (wt/wt) blueberry powder (BBP) reduced the weight of intraperitoneal fat and enhanced the activity of the peroxisome proliferator-activated receptor (PPAR) in white adipose tissue (WAT) and skeletal muscle in Zucker-fatty rats [42]. Following the above mentioned studies, Vendrame et al. [43] reported that supplementation with 8% (wt/wt) wild blueberry powder (WBP) after 8 weeks of feeding significantly increased blood adiponectin levels as well as reduced the levels of inflammatory markers in WAT [43] and improved dyslipidemia [6]. However, WBP did not obtain reduced fasting blood glucose (FBG) and insulin levels in obese Zucker rats in this study [44].

Bilberries can also reduce inflammation, prevent chronic hypertension, and thus mitigate the development of obesity as reported by Mykkänen et al. [45]. Black elderberry (BE) is one of the richest sources of anthocyanins, which improved some metabolic disturbances present in diet-induced obese C57BL/6J mouse model by lowering serum triglycerides (TAG), inflammatory markers, and IR [46].

Consequences caused by the intake of HF diet, such as hepatic steatosis, adipose hypertrophy, and IR, were attenuated by the intake of mulberry ethanol extract (MEE). Treatment with MEE altered the expression profile of genes involved in the lipid metabolism and acted as a protective mechanism on induced fatty acid oxidation, as well as it decreased fatty acid and cholesterol biosynthesis in vivo [47]. Wu et al. [48] reported that anthocyanins purified from Chinese mulberry (*Morus australis Poir*), such as cyanidin-3-glucoside, cyanidin-3-rutinoside, and pelargonidin-3-glucoside, after 12 weeks significantly inhibited body weight gain, reduced the IR, lowered the size of adipocytes, attenuated lipid accumulation, and decreased leptin secretion.

Goka fruit (GF), rich in anthocyanin, improved glucose tolerance and insulin sensitivity and reduced plasma insulin and hepatic accumulation of lipid in HF diet-induced obese mice after GF treatment for 12 weeks. In addition, GF administration to HF mice arose obesity-associated IR and hepatic lipid accumulation through the modulation of AMPK activity and lipid metabolism-associated gene expression [49]. Similar results were obtained by other authors with Cornelian cherries (*Cornus mas*) [50–52] and sweet orange (*Citrus sinensis* L. Osbeck) [53].

Hypolipidemic, antioxidant, and anti-inflammatory properties of blueberry (BB), blackberry (BK), and blackcurrant (BC) in a diet-induced obesity (DIO) mouse model were reported by Kim et al. [54]. Since BB, BK, and BC vary in the anthocyanin composition, their effect on plasma lipids and adipose macrophage infiltration in DIO mice was different. However, no differences were found in their antioxidant capacity and anti-inflammatory potential after BB, BK, and BC administration in DIO mice [55].

Boušová et al. [55] demonstrated that a cranberry extract- (CBE-) enriched diet differently modulated antioxidant enzymes and redox status in obese and nonobese mice. This study was designed to test the consumption of a CBE-enriched diet (2%) for 28 days and to compare the antioxidant status of nonobese and obese mice in a model with monosodium glutamate-induced obesity. CBE treatment increased the thiol content in the plasma and the glutathione S-transferase activity in the erythrocytes in both obese and nonobese mice. However, in the obese animals, the malondialdehyde (MDA) level in the erythrocytes was reduced, while hepatic NAD(P)H:quinone oxidoreductase and catalase activities in erythrocytes and small intestine were increased.

Anthocyanins can induce changes in adipose tissue, such as those in the expression levels of adipocytokines. The supplementation with black chokeberry (*Aronia melanocarpa*) juice concentrate (AJC) decreases epididymal fat (for −30%) and positively influences on adiponectin in male C57BL/6J mice. Therefore, this supplementation prevented weight gain and modulated markers of obesity [56]. Takahashi et al. [57] found that anthocyanin-rich Aronia fruits could suppress visceral fat accumulation and hyperglycemia through the inhibition of pancreatic lipase activity and thus lead to a reduction of intestinal lipid absorption in rats after feeding for 4 weeks.

Polyphenol-rich blackcurrant extract (BCE) prevented inflammation in male C57BL/6J mice fed with modified AIN-93M diet for 12 weeks [58]. The percentage of adipocyte size of the epididymal fat was lower than that in the control. The mRNA expression of genes involved in mitochondrial biogenesis, such as PPARα, PPARδ, UCP-2, UCP-3, and mitochondrial transcription factor A, were increased in the skeletal muscle in the group BCE-fed mice. Tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) mRNA were lower in splenocytes from BCE-fed mice than in the control in model-stimulated inflammation with lipopolysaccharides [58].

Gut microbes are important due to their possible involvement in the development of obesity process and chronic inflammation as well as IR [59]. In addition to being designed to reduce adiposity and hepatic lipogenesis in vivo, table grape consumption may alter gut microbiota. Sulfidogenic bacteria intestinal abundance was decreased, and the abundance of beneficial bacteria was increased in C57BL/6J mice with 3% grapes for 11 weeks. Obese mice fed with the grape polyphenol-rich diet had lower percentages of body fat and amounts of WAT and improved glucose tolerance compared to the HF-fed controls. In addition, these treatments with grape altered gut community structure and WAT inflammation [60].

4. The Role of Anthocyanins in Obesity: Human Studies

Despite the encouraging results obtained by preclinical studies in animal models, the role of anthocyanins in obesity deriving from clinical trials remains controversial. Few interesting reviews have been published in recent years on this topic, and all of them concluded that further interventional studies are needed to assess the preventive effects of anthocyanin-containing foods in obesity, diabetes, and metabolic syndrome due to the difficulty to establish the optimal dose and to identify the ideal food matrix for the best anthocyanin supplementation [27, 61]. Observational studies are more prone to suggest an antiobesity role of this class of polyphenols. In a recent work which analyzed food frequency questionnaires from about 124,000 participants from three different cohorts (Health Professionals Follow-up Study, Nurses' Health Study, and Nurses' Health Study II), the authors observed that an increased consumption of several flavonoid subclasses, including anthocyanins, was associated with weight loss in both men and women, aged 27–65, in a follow-up of 24 years. For example, in four years, increasing the daily consumption of BB by one half cup resulted in a weight loss of about 1.03 kg, less than 0.5 kg/year, a small but significant decrease potentially associated with health improvement [62]. Two interventional studies ended up with positive outcomes of blueberries supplementation in obese men and women at risk of cardiovascular disease and insulin resistance. In one case, 48 subjects, largely women, with metabolic syndrome and an average BMI of $37.8 \pm 2.3\,kg/m^2$, received for 8 weeks a blueberry beverage containing 50 g of freeze-dried blueberries (corresponding to 742 mg of total anthocyanins and 1624 mg total polyphenols). In the blueberry-supplemented group, systolic and diastolic blood pressures, plasma oxidized LDL, and serum malondialdehyde and hydroxynonenal concentrations decreased greater and significantly than in the control group, suggesting that blueberry beverage could contribute to ameliorate improve metabolic syndrome and related cardiovascular risk factors [63]. In the second study, 32 obese men and women with a BMI between 32 and $45\,kg/m^2$ and insulin resistant received twice daily a smoothie with blueberry (45 g of powder contained 1462 mg of total polyphenolics of which 668 mg represented anthocyanins) for 6 weeks. The most interesting and significant change regraded insulin sensitivity which improved in the blueberry group (1.7 ± 0.5 mg·kg $FFM^{-1}\cdot min^{-1}$) compared to the placebo group (0.4 ± 0.4 mg·kg $FFM^{-1}\cdot min^{-1}$) [64].

In a different experimental approach, the administration of a so-called "gastrointestinal microbiome modulator" (GIMM) containing inulin, oat β-glucan, blueberry anthocyanins, and blueberry polyphenols in a small number overweight or obese individuals (30 subjects, age 18–70) reduced in four weeks the desire to eat in the GIMM group compared to placebo [65]. However, it is difficult to extrapolate from the data obtained which component(s) can be more directly responsible for the increased sense of satiety compared to the other measured outcomes, such as improved glucose tolerance. Supplementation with red orange juice rich in anthocyanins in 30 overweight healthy human volunteers for 12 weeks also resulted in a significant antiobesity effect measured as reduction in body mass index (BMI; $1.11 \pm 0.09\,kg/m^2$) compared to the placebo group ($0.15 \pm 0.09\,kg/m^2$) [66].

Less encouraging results have been obtained in the QUENC trial, where 16 healthy volunteers aged 18–65 years with a BMI in the range of 25–35 kg/m^2 consumed 3 sachets (8.3 g/sachet) per day of purple carrots for 4 weeks, providing a total daily intake of anthocyanin of 118.5 mg (259.2 mg total polyphenols/die). At the end of the trial, these obese subjects did not show any evidence of reduced body mass, and sense of appetite as well as changes in other markers of inflammation and lipid metabolism [67].

In the studies where other metabolic dysfunctions associated with obesity have been considered, such as metabolic syndrome, the supplementation with anthocyanin-enriched foods, for example, berry, in general, did not change weight or body composition [61] with references and tables therein. An exception is represented by a study where bilberry supplementation induced a decrease in weight and waist circumference in obese women [68]. Fluctuating results on the beneficial effects of anthocyanin supplementation in overweight and obese subjects and in individuals affected by metabolic syndrome have also been observed when other clinical parameters have been measured, such as diastolic and systolic blood pressure, dyslipidemia, blood glucose, and insulin resistance [27, 61, 69, 70]. To explain the alternate results observed in these studies, based on the supplementation of different berries (strawberry, cranberry, bilberry, sea buckthorn, blueberry, chokeberry, etc.), variations among

Table 1: Clinical studies retrieved from the http://ClinicalTrials.gov database on anthocyanins and obesity.

Trial number status	Conditions and dosage	Objectives	Primary outcome	Number of subjects (age/sex)
NCT02613715 (completed)	Overweight and obesity (250 ml of blackberry juice)	To evaluate the bioavailability of blackberry juice anthocyanins in normal weight and overweight/obese adults	Plasma concentrations of anthocyanins and anthocyanin metabolites	18 (18–40/M-F)
NCT01180712 (recruiting)	Type 2 diabetes (1.4 g of concentrated blackberry extract mirtoselect provided by Indena S.p.A.)	To determine the effects of anthocyanin supplementation in the form of a concentrated blackberry extract on insulin resistance and inflammation particularly in the adipose tissue	Oral glucose tolerance test	60 (40–70/M)
NCT01883401 (completed)	Dyslipidemia; obesity (25–50 g freeze-dried strawberries/day)	To determine the effects of low and high doses of freeze-dried strawberries in cardiovascular risk factors in subjects with abdominal adiposity and dyslipidemia	Change in lipids and lipoproteins	60 (19–72/M-F)
NCT01005420 (completed)	Insulin sensitivity (45 g of blueberry powder)	To evaluate the effect of blueberry powder on insulin sensitivity in obese, nondiabetic, and insulin-resistant subjects.	Insulin sensitivity	37 (>20/M-F)
NCT02689765 (completed)	Insulin resistance; glucose and lipid metabolism disorders; type 2 diabetes (80 g of a mixture of fresh blueberries and blackcurrants)	To characterize the potential effects of anthocyanins, purified from bilberries and blackcurrants, on metabolic zabnormalities commonly associated with type 2 prediabetes	Change in fasting glucose and HbA1C	160 (40–75/M-F)
NCT01705093 (unknown)	Childhood obesity; cardiovascular disease (50 g of flavonoid-rich freeze-dried strawberry powder)	To verify if strawberry intake can lead to improvements in select measures of cardiovascular function in overweight and obese adolescent males	Vascular function measured by peripheral arterial tonometry	25 (14–18/M)
NCT02035592 (active, but not recruiting)	Insulin resistance; metabolic syndrome X (13–26 g of freeze-dried blueberry powder per day)	To determine the dose-dependent impact of blueberry powder intake on insulin sensitivity and resistance, cardiovascular disease risk factors, and lung and cognitive function in overweight and obese participants with metabolic syndrome	Insulin resistance	144 (50–74/M-F)
NCT02291250 (recruiting)	Type 2 diabetes (200 g of blackcurrants, which contain anthocyanins, or 200 g greencurrants, which naturally contain no anthocyanins)	To investigate the acute effect blackcurrants on glucose metabolism in overweight/obese volunteers	Plasma glucose area under the curve	16 (21–70/M-F)
NCT02800967 (active, but not recruiting)	Hypertension; overweight (pure Aronia juice with appx. polyphenol content of 1000 mg gallic acid equivalents/100 ml)	To investigate the effects of Aronia juice polyphenols on platelet function and other CVD risk factors in subjects with moderate CVD risk	Changes in the percentage of P-selectin and glycoprotein IIbIIIa (GPIIbIIIa) positive platelets, percentage of platelet-monocyte, and platelet-neutrophil aggregates	84 (30–50/M-F)
NCT01564498 (recruiting)	Hypertension; hypercholesterolemia; type II diabetes; obesity; inflammation (300–500 g of cooked white/purple potatoes per day, or 300–500 g of raw orange/purple carrots per day)	To verify if purple vegetables, rich in polyphenolic compounds including anthocyanins, can have higher antioxidant and other biological activities than more lightly coloured versions of these foods	Blood cholesterol	60 (18–65/M-F)

the levels of anthocyanins in these berries can be easily hypothesized. It is not difficult to imagine that anthocyanin content changes quantitatively and qualitatively in distinct food sources. However, cause-effect relationships between chemical composition of anthocyanin supplements and their clinical outcomes is not easy to demonstrate and to approach experimentally. This goal may represent one of the challenges of this field in the next years.

The uncertainty of the clinical studies proving antiobesity effects of anthocyanins is confirmed by data summarized in Table 1. Here, we interrogated the http://ClinicalTrials.gov database, a service of the U.S. National Institutes of Health, for "anthocyanins" and "obesity" and retrieved a total of 16 studies (last update March 28, 2017). Those more closely related to the scope of this review have been reported in Table 1: all represent interventional studies and none of their results have been posted. It is easily to predict that, when and if results will be available, these trials will probably provide only limited breakthroughs in the field for the following reasons: (i) small number of enrolled subjects; (ii) wide age range in the enrolled subjects (young, adults, and elderly) which may generate confounding results due to the age-related differences in the physiopathology of obesity and related diseases; and (iii) wide differences in the type, composition, origin, doses, and duration of anthocyanins supplemented. The absence of standardized preparations of anthocyanins represents, in our opinion, an important confounding factor largely responsible for the variability and controversial results of clinical trials commented above. In fact, different typologies of anthocyanin formulations are generally employed in these studies (Table 1). In addition, another important aspect to be considered is the doses of anthocyanins necessary to reach the desired biological effects, which depend upon the complex pharmacokinetics of these compounds, as recently and exhaustively reviewed [36].

5. Conclusion

From the data reported above, it is easy to conclude that the potential functional relationship existing between anthocyanin supplementation and antiobesity effects suffers of the same pros and cons evidenced when the beneficial responses to other phytochemical treatments towards different degenerative diseases have been considered. In general, the positive and encouraging outcomes deriving from of preclinical studies (cell lines and animal models) are contradicted or, at least, diminished moving to case-control trials. The reasons behind this partial failure are complex and not easy to address. They certainly involve the different dosages applied in animal versus clinical studies, the complex metabolism and biotransformation to which anthocyanins and phytochemicals are subjected in the intestine and tissues, the possibility that different components present in the supplemented mixtures (e.g., berry extracts) can interact generating antagonistic, synergistic, or additive effects difficult to predict, and so on. However, we would like to highlight another issue which may contribute to generate confusion in the field, that is, the difference between prevention and therapy: prevention implies low doses, but long duration of the treatments (years), while therapy is associated with higher doses (and potentially side effects), but shorter time of administration. As an example, it is risky to hypothesize that anthocyanins can prevent obesity based on studies on already obese mice that received pharmacological doses of these phytochemicals. We believe that the evolution of the field must seriously consider the need to establish new and adequate cellular and animal models which may, in turn, allow the design of more efficient and prevention-targeted clinical studies.

Abbreviations

Pg: Pelargonidin
Cy: Cyaniding
Pt: Petunidin
Pn: Peonidin
Dp: Delphinidin
MV: Malvidin
BMI: Body mass index
LDL: Low density lipoprotein
FFM: Fat mass and fat-free mass
GIMM: Gastrointestinal microbiome modulator
CPT1A: Carnitine palmitoyltransferase-1A
RBP4: Retinol binding protein-4
SREBP1c: Sterol regulatory element binding protein-1c
C/EBPα: CCAAT enhancer binding protein-α
PPAR γ: Peroxisomal proliferator-activated receptor gamma
AMPK: 5′ Adenosine monophosphate-activated protein kinase
GLUT4: Glucose transporter 4
GLUT2: Glucose transporter 2
ACC1: Acetyl-CoA carboxylase
FAS: Fatty acid synthase
LPL: lipoprotein lipase
PEPCK: Phosphoenolpyruvate carboxykinase
G6pase: Glucose-6-phosphatase
G6PDH: Glucose-6-phosphate dehydrogenase
VEGF: Vascular endothelial growth factor
ICAM-1: Intercellular adhesion molecule
JNK: c-Jun N-terminal kinase
TGFβ1: Transforming growth factor-beta
IL-6: Interleukin-6
TNF-α: Tumor necrosis factor alpha
MCP-1: Monocyte-chemo-attractant protein-1.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 7865073, 13 pages
https://doi.org/10.1155/2017/7865073

Research Article

Pitaya Extracts Induce Growth Inhibition and Proapoptotic Effects on Human Cell Lines of Breast Cancer via Downregulation of Estrogen Receptor Gene Expression

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Received 13 January 2017; Revised 11 April 2017; Accepted 7 May 2017; Published 6 July 2017

Academic Editor: Elena Azzini

Breast cancer is one of the most prevalent cancers in the world and is also the leading cause of cancer death in women. The use of bioactive compounds of functional foods contributes to reduce the risk of chronic diseases, such as cancer and vascular disorders. In this study, we evaluated the antioxidant potential and the influence of pitaya extract (PE) on cell viability, colony formation, cell cycle, apoptosis, and expression of $BRCA_1$, $BRCA_2$, PRAB, and Erα in breast cancer cell lines (MCF-7 and MDA-MB-435). PE showed high antioxidant activity and high values of anthocyanins (74.65 ± 2.18). We observed a selective decrease in cell proliferation caused by PE in MCF-7 (ER^+) cell line. Cell cycle analysis revealed that PE induced an increase in G_0/G_1 phase followed by a decrease in G_2/M phase. Also, PE induced apoptosis in MCF-7 (ER^+) cell line and suppressed $BRCA_1$, $BRCA_2$, PRAB, and Erα gene expression. Finally, we also demonstrate that no effect was observed with MDA-MB-435 cells (ER^-) after PE treatment. Taken together, the present study suggests that pitaya may have a protective effect against breast cancer.

1. Introduction

Breast cancer is the most frequently diagnosed type of cancer around the world [1], and it is a complex disease caused by progressive genetic mutations, associated with other factors [2]. Various complications, including deaths from the disease associated with breast cancer, are due to metastasis. The rates of metastasis and mortality in breast cancer patients have decreased because of early diagnosis by mammographic screening and the implementation of adjuvant therapy. Currently, breast cancer control primarily involves surgical procedures and radiotherapy and is often supported by adjuvant chemotherapy or hormone therapies. This disease is highly resistant to chemotherapy, and there is still no effective cure for patients with advanced stages of the disease, especially in cases of hormone-independent cancer [3].

Several evidences, supported by epidemiological studies, indicate that prolonged exposure to sex hormones is one of the well-defined risk factors for breast cancer [4, 5]. Despite the fact that the majority of breast cancers are ER^+, and hormonal intervention is used to prevent disease recurrence and/or progression, the mechanisms through which estrogen contributes to malignant transformation of mammary epithelium are poorly understood. ER^- tumors are associated with a worse short-term prognosis [6] and have weaker associations with reproductive risk factors [7] than ER^+ tumors.

Mutations in $BRCA_1$ are associated with predisposition to ER^- breast tumors, whereas most known common susceptibility loci for breast cancer show stronger associations with ER^+ than with ER^- tumors [8].

Carcinogenesis process results in the dysfunction of several regulatory features that keep the cells in check [9]. The balanced diet, with the diversified consumption of fruits and vegetables, exposes the body to several phenolic compounds. Over the last decade, these compounds have been widely studied and associated with benefits to human health. However, as there is a wide range of vegetables, species varieties, and differences in the compositions of these foods as well as the different localities of cultivation around the world, much research has yet to be done to elucidate the compounds present in these natural foods and their effective effects on the good health [10, 11].

Some reports support that the belief that components of food can affect the development of cancer in both beneficial and detrimental ways [12, 13]. Healthy lifestyle changes, including a better diet and regular exercise, can prevent up to 40% of breast cancers [14]. The role of fresh fruits and vegetables is to help prevent or lessen the action of free radicals [15].

The pitaya is also known as the "dragon fruit," since it has a bright red peel with overlapping green fins that cover the fruit, a fact that has gained popularity in different countries of the world [16]. *Hylocereus polyrhizus*, which has red-skinned fruits with red meat, *Hylocereus undatus* (red pitaya), which has red-skinned fruits with white flesh, and *Hylocereus megalanthus* (yellow pitaya), which has yellow skin, are the most commercialized and consumed [17]. Red dragon fruit (*Hylocereus polyrhizus*) or sometimes called red pitaya has been comprehensively researched for its bioactive compounds.

Many compounds present in pitaya are responsible for many pharmacological activities such as antitumor, antioxidant, and anti-inflammatory actions. Bioactive compounds have been reported to modify specific carcinogenic processes, including cancer metabolism, hormonal balance, transcription factors, cell cycle control, apoptosis, inflammation, angiogenesis, and metastasis [18]. Potential mechanisms for cancer prevention of bioactive compounds in fruits include prevention of DNA adduct formation, enhanced carcinogen elimination, decrease inflammatory processes, and a direct cytotoxic effect on tumor cells [19, 20].

Recent reports have indicated that pitaya extract may have a role in the prevention and treatment of breast cancer [3, 21]. However, further studies on their role in the chemoprevention of breast cancer are warranted. In this context, the aim of the study was to evaluate the antiproliferative and proapoptotic effects of pitaya extract in MCF7 (ER^+) and MDA-MB-435 (ER^-) cell lines.

2. Methods

2.1. Sample and Extraction. The red pitaya (*Hylocereus polyrhizus*) were obtained from Petropólis (Rio de Janeiro State, Brazil). Hydroalcoholic extract was obtained from the pulp of the fruits. Fruits were washed in tap water, and the pulp was separated from the skins and seeds. Approximately 50 g of pulp of pitaya was extracted with 50 mL of ethanol and 50 mL of distilled water and then shaken for 2 h. After the pulp maceration period, the hydrohalic extract of pitaya was filtered on Whatman number 1 filter paper and the residual ethanol was evaporated under low pressure at 55°C. The extracts were then lyophilized and frozen at −20°C for use in the other experiments. Usually, 50 g of pulp yields 3 g of lyophilized extract.

2.2. Anthocyanin. Anthocyanins were extracted according to the method described by Abdel-Aal et al. [22] with slight modifications. Initially, 1 g of pitaya was extracted twice by mixing with 30 mL of methanol acidified with 1.0 N HCl (85 : 15, *v*/*v*) and shaking on a shaker at 4°C for 24 hr. The crude extracts were filtered with Whatman number 1 paper. The filtrate absorbance readings were taken at 535 nm, in Turner Model 340 spectrophotometer. To determine the anthocyanin values, we considered the dilution coefficients and the extinction coefficient of cyaniding 3-galactoside (98.2).

2.3. Antioxidant Activity Analyses

2.3.1. Oxygen-Radical Absorbance Capacity Assay (ORAC). The ORAC procedure used an automated plate reader (SpectraMax i3x, Molecular Devices, USA) with 96-well plates [23, 24]. Experiments were conducted in phosphate buffer pH 7.4 at 37°C. Peroxyl radical was generated using 2,2′-azobis (2-amidino-propane) dihydrochloride which was prepared fresh for each run. Fluorescein was used as the substrate. Fluorescence conditions were as follows: excitation at 485 nm and emission at 520 nm. The standard curve was linear between 0 and 50 mM Trolox. Results are expressed as μmol TE/g.

2.3.2. Ferric Reducing Ability (FRAP). The extracts were measured for antioxidant activity by FRAP according to Rufino et al. [25]. Aliquots of 2.7 mL of TPTZ reagent (ferric 2,4,6-tripyridyl-s-triazine) were mixed with 0.5 mL of sample extract. After 30 min at 37°C temperature, the absorbance was read at 595 nm. The antioxidant capacity (FRAP) was expressed as Fe^{3+} equivalents (μmol Fe^{3+}/g dry basis).

2.3.3. DPPH Assay. Aliquots of 0.5 mL of the extracts were mixed with 2.5 mL DPPH methanolic solution (0.06 mM) and allowed to react for 1 hour, in the dark. Measurements were performed at 515 nm applying a Turner 340 spectrophotometer. Analysis was performed in triplicates, and the decline in the DPPH radical absorbance concentration caused by the extracts was compared to a Trolox standard. The results were expressed as μmol Trolox equivalents/g dry basis [19].

2.4. Cell Culture and Treatment Protocol. Cell lines were obtained from the Rio de Janeiro Cell Bank that certified their identity and quality (INMETRO—Rio de Janeiro, RJ, Brazil). Human breast adenocarcinoma cell lines (MCF-7 and MDA-MB-435) were plated in 25 cm^2 tissue culture flasks (5.0×10^6 cells/flask) and maintained routinely in the Dulbecco's modified Eagle's medium—high glucose (DMEM) supplemented

with 10% fetal bovine serum (FBS) and 1% penicillin (PS), pH 7.4, under 5% CO2 atmosphere. Stock flasks were grown to 70% confluence and subcultured routinely. Medium renewal was done 3 times weekly. For each experiment, cells were seeded at 3.5×10^5 cells/cm^2 density in 6 and 2×10^4 cells/cm^2 densities in 96-well plates for cell cycle and cell proliferation analyses, respectively. After 24 h, medium was removed and cells were treated with increasing concentrations of PE (500 and 1000 μg/mL) dissolved in DMEM. The controls, DMEM and DMEM+ 2% DMSO, were included on each plate. The cells were then incubated for 24 and 48 hours.

2.5. Cell Viability Assay

2.5.1. MTT Assay. The status of cancer cell line viability was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay (Sigma, New York, USA) wherein the substance is a pale yellow substrate that is reduced by living cells to yield a dark blue formazan product. This requires active mitochondria, and even recently, dead cells do not reduce significant amounts of MTT. Exponentially growing cells were adjusted to 2.0×10^4/cm^2 with DMEM, plated in 96-well plates (Corning, Tewksbury, MA) at 200 μL/well, and incubated for 24 h according to the routine procedure. The cells were then incubated with PE (500 and 1000 μg/mL) for 24 and 48 h. Each well was also incubated with MTT (10 μL/well; 5 g/mL) for 4 h. At 85 μL/well, the liquid was removed, and at 50 μL/well, sodium dodecyl sulfate was added to dissolve the solid residue. Finally, the absorbance was measured using a microplate reader (POLARIS—CELER®) at 570 nm. The cell proliferation inhibition rate (CPIR) was calculated using the following formula: CPIR = (1 − average value of experimental group/average value of control group) × 100%.

2.5.2. Test of Colony Formation (CFU). Breast cancer cell lines were adjusted at a density of 10^3 cells/per well in a 6-well plate in DMEM culture medium containing 10% FBS for 48 h. After this step, the cells were treated with PE at 500 and 1000 μg/mL with medium replace every 5 days. After 18 days, colonies were fixed with 4% paraformaldehyde (Sigma, St. Louis, USA) in PBS containing 4% sucrose (Vetec, Rio de Janeiro, Brazil) for 20 min and then stained with 0.005% crystal violet (Vetec, Rio de Janeiro, Brazil) overnight at room temperature. For colonic analyses, they were washed five times with PBS for 5 min and 50 cells were counted using an Axiovert inverted microscope (Carl Zeiss, Oberkochen, Germany).

2.5.3. Trypan Blue Exclusion Test of Cell Viability. Cells were grown to about 80% confluence in 6-well plates and treated for 24 h and 48 h with red PE at 500 μg/mL and 1000 μg/mL. Adherent and nonadherent cells were collected, and viability was assessed by mixing aliquots of cell suspensions with equal volumes of 0.4% trypan blue (GibcoBRL). Cells that accumulated the dye were considered dead.

2.6. Cell Cycle. Cells were rinsed briefly with calcium and magnesium-free phosphate-buffered saline and detached with trypsin at room temperature. After centrifugation, the cells were washed twice with phosphate-buffered saline and were resuspended in 500 μL of ice-cold Vindelov solution [20] containing 0.1% Triton X-100, 0.1% citrate buffer and 0.1 mg/mL RNase, and 50 mg/mL propidium iodide (Sigma Chemical Co., St. Louis, MO). After 15 min of incubation, cell suspension was analyzed for DNA content by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). The relative proportions of cells with DNA content haploid subG_1 (<2n), diploid G_0/G_1 (2n), S phase (>2n but <4n), and G_2/M phase (4n) were acquired and analyzed using CellQuest and WinMDI 2.9, respectively. The percentage of cell population at a particular phase was estimated with FlowJo software following the acquisition of 30,000 events. Cell dissociation procedure does not affect fluorescence under the experimental conditions that were used in this study or in any others of which we are aware. Nuclei of viable cells were gated according FL-2 W × FL2-A relation.

2.7. Apoptosis Assay. Cells were resuspended in 400 μL of binding buffer containing 5 μL of annexin V FITC and 5 μL propidium iodide (Apoptosis Detection Kit II, BDBiosciences) for 15 min at room temperature. Annexin V binding was evaluated by flow cytometry (FACScalibur, BD Biosciences), and after acquisition of 30,000 events, the data were analyzed in CellQuest and FlowJo software.

2.8. Gene Expression Analysis. Total RNA was extracted from the studied cells using Trizol® Reagent (Invitrogen) according to the manufacturer's instructions. RNA yield and quality were determined by a spectrophotometer Nano-Drop ND-1000 V3.2 (Nanodrop Technologies, Wilmington, DE). Equal amounts (500 ng) of RNA from cells were reverse transcribed with cDNA synthesis kit "Superscript II First-Strand Synthesis System for RT-PCR" (Invitrogen) and Oligo (dT) primer (Invitrogen). The cDNA was used as a template for subsequent real-time polymerase chain reaction (RT-PCR). Quantitative RT-PCR was done in a StepOnePlus™ Real-Time PCR System (Life Technologies) using SYBR Green (Applied Biosystems, Grand Island, NY) following the manufacturer's instructions and using primers as shown in Table 1. The expression levels of ERBB2, GSTM1, $BRCA_1$, $BRCA_2$, PRAB (progesterone receptor isoform A and B), ERα (estrogen receptor α), and GPR30 (a G protein-coupled receptor for estrogen) mRNA were all normalized with β-actin and GADPH (glyceraldehyde-3-phosphate dehydrogenase) expression level. For the evaluation of the quality of RT-PCR products, analyses of the melt curve were performed after each assay. The expression is relative to the measure using the $\Delta\Delta$CT technique with β-actin and GADPH genes as the reference genes.

2.9. Statistical Analysis. The results presented are the mean and the corresponding standard deviation of three independent experiments performed in triplicate ($n = 9$). Data were analyzed using GraphPad Prism statistical software (version 5.04, GraphPad software, San Diego, CA). The univariate analysis of variance (ANOVA) with the Tukey posttest at a

Table 1: Primer sequences for the reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer	Reverse Primer
ERBB2	CCGTGCCACCCTGAGTGT	AGCCTCCGGTCCAAAACAG
GSTM1	TCCCTCTTCACTCCCCCTAAA	GGGTAGCTGAGGCTTCAAAGG
$BRCA_1$	CTGCTCAGGGCTATCCTCTCA	TGCTGGAGCTTTATCAGGTTATGT
$BRCA_2$	CCACAGCCAGGCAGTCTGTAT	AGAACACGCAGAGGGAACTTG
PRB	CCTGAAGTTTCGGCCATACC	CAGGGCCGAGGGAAGAGT
PRAB	GGCTACGAAGTCAAACCCAGTT	CAATTGCCTTGATGAGCTCTCTAA
ERα	CTGTTTGCTCCTAACTTGCTCTTG	TCCACCATGCCCTCTACACA
GAPDH	ATGGAAATCCCATCACCATCTT	CGCCCCACTTGATTTTGG

Table 2: Bioactive potential of pitaya evaluated by different methods.

Pitaya	2.0 mg/mL	5.0 mg/mL	10.0 mg/mL	R^2
ORAC assay (μM Trolox/g)	140.50 ± 1.90	560.00 ± 48.90	1079.70 ± 75.20	0.9943
FRAP assay (μmol Fe_2SO_4/g)	909.20 ± 68.46	1698.64 ± 33.17	2519.36 ± 53.99	0.9621
DPPH assay (% reduction)	33.05 ± 0.32	73.01 ± 0.38	83.99 ± 0.30	0.8892
	Pulp	Peel	Total anthocyanins (fruit)	Total anthocyanins (pitaya extract)
Total anthocyanins (mg/g)	19.14 ± 0.52	8.36 ± 2.70	27.50 ± 1.61	74.65 ± 2.18

Results expressed in mean ± standard error.

95% confidence level was used to test cell viability, cell cycle, and apoptosis rate.

3. Results

3.1. Bioactive Properties of Red Pitaya. Natural and synthetic antioxidants are widely used in modern medicine. In the comparison of the antioxidant assays, an important bioactive potential in pitaya (10 mg/mL) was identified in ORAC values (1079.70 ± 75.20 μM Trolox/g), FRAP assay (2519.36 ± 53.99 μmol sulfate ferrous/g), and DPPH reduction (83.99 ± 0.30%) (Table 2). There is a need for screening studies in order to identify the mode of action of different antioxidant compounds (enzymatic and nonenzymatic in addition, comparing between synthetic and natural antioxidant compounds) by different assays [26].

Pitaya contained significant levels of total anthocyanins (Table 2). The pulp showed a significantly higher anthocyanin content (19.14 ± 0.52 mg/g) in comparison with peel (8.36 ± 2.70 mg/g).

3.2. Effect of Pitaya Extract (PE) on Cell Viability

3.2.1. MTT Assay. The treatment with PE for 24 h decreased MCF-7 cell viability from the concentration of 250–1000 μg/mL, showing a mean reduction around 25.15% ($p < 0.05$) (Figure 1(a)). After 48 h, PE induced a higher inhibition of cell viability from the concentration of 2.5 μg/mL (by 29.33% compared with the control group, $p < 0.05$), and the maximum inhibition was obtained with 1000 μg/mL (40.22%, $p < 0.05$) (Figure 1(b)). Our data showed an important cell growth inhibition on MCF-7 cell after PE treatment (500 μg/mL and 1000 μg/mL) (Figure 1(c)).

As shown in Figures 1(d) and 1(e), a slight decrease in MDA-MB-435 cell viability was observed only in high concentrations of PE (500 and 1000 μg/mL) with maximum inhibition of 20% compared with control group after 48 h ($p < 0.05$).

3.2.2. Test of Colony Formation (CFU). The next step was to analyze the effect of PE on the clonogenic property of MCF-7 and MDA-MB-435 cells. According to the literature, cell groups with fewer than 50 cells were not considered as colonies [27]. Our data showed that the clonogenic ability of MCF-7 cells was inhibited in the presence of PE (500 and 1000 μg/mL) (Figure 2). Maximum reduction of clonogenic ability was obtained when 1000 μg/mL of PE (about 70%, $^{**}p < 0.001$) was used (Figure 2). No effect in colony formation was observed in MDA-MB-435 cell line after PE incubation.

3.2.3. Trypan Blue Exclusion. Another assay for cell proliferation was used to confirm the effect of PE in breast cancer cell lines. PE induced an inhibition of proliferation in MCF-7 cell line after 24 and 48 h from the concentration of 500 μg/mL (by 50% compared with the control group, $p < 0.05$), and the maximum inhibition was obtained with 1000 μg/mL (80%, $p < 0.05$). Corroborating with other methods used, when MDA-MB-435 cells were treated with PE for 24 h and 48 h, no changes in cell proliferation were detected when compared to untreated cells (Figure 3).

3.3. Effect of Pitaya Extract on Cell Cycle Progression. We next questioned whether PE would have any effect on cell cycle arrest in breast cancer cell lines. After 24 h and 48 h of treatment, PE caused an increase in the percentage of cells in the G_0/G_1 phase, with a corresponding decrease in the G_2/M

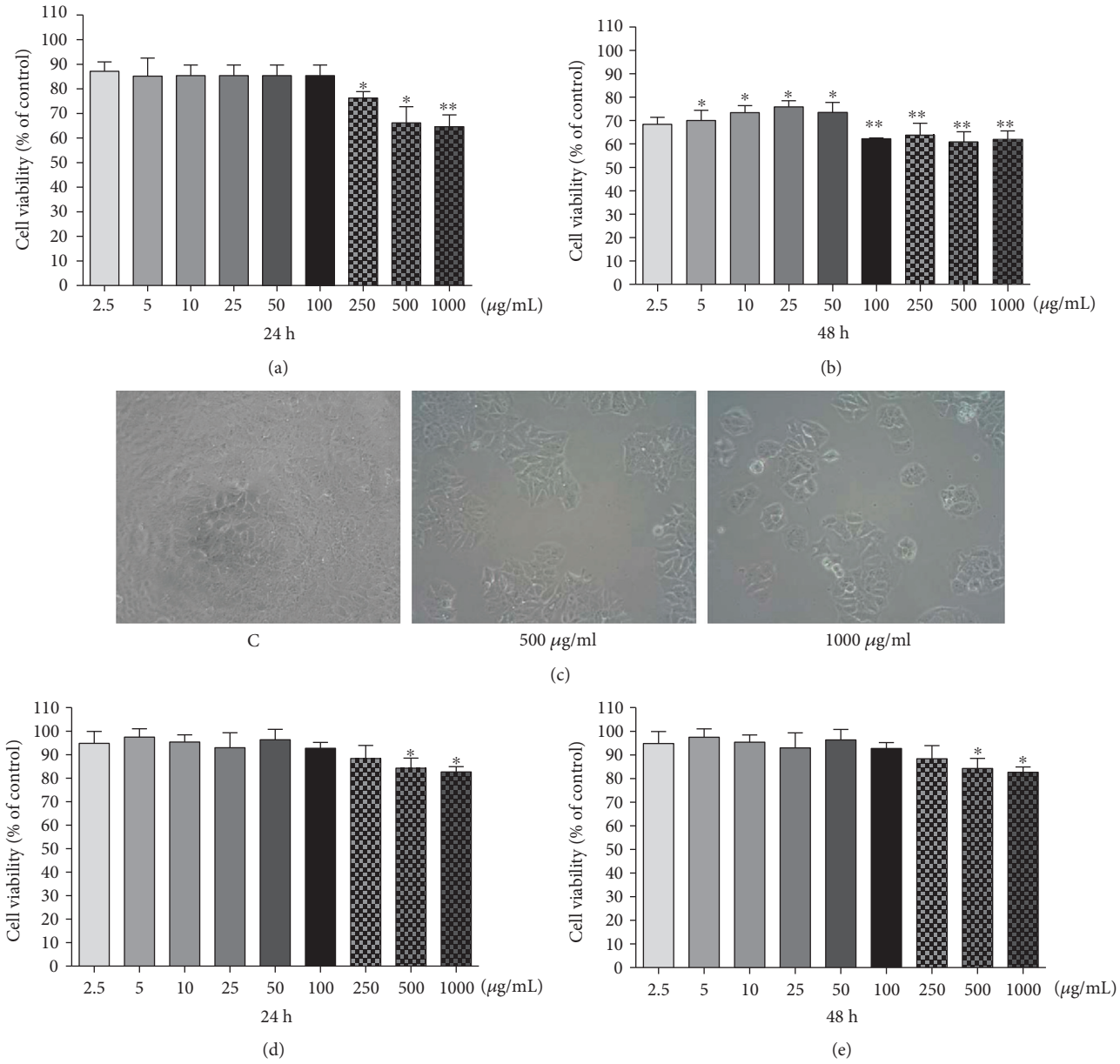

Figure 1: Effect of PE (2.5–1000 μg/mL) on viability of MCF-7 (a, b) and MDA-MB-435 (d, e) cells at different time intervals after exposure using MTT assays. The experiment is expressed as mean ± standard error, and differences significant between treated cells with PE were compared using the Tukey test ($^{*}p < 0.05$; $^{**}p < 0.01$). Phase contrast microscopy of MCF-7 cells (treated for 48 h with 500 and 1000 μg/mL of PE) was observed on 96-well culture plates (c).

phase, indicating a growth arrest of MCF-7 cells after that time (Figure 4 and Table 3). Corroborating with the data from cell proliferation, after 24 h and 48 h of treatment with PE, no changes in cell cycle profile of MDA-MB-435 cells were detected when compared to untreated cells (Figure 4 and Table 3).

3.4. Effect of Pitaya Extract on Apoptosis Assay. Flow cytometry analysis showed that treatment for 24 h and 48 h with PE at concentrations of 500 and 1000 μg/mL did not induce apoptosis in MDA-MB-435 cells. However, when MCF-7 cells were treated under the same conditions for 24 and 48 h, an increase in the number of apoptotic cells was detected (Figure 5).

3.5. Gene Expression Profile. The role of $BRCA_1$, $BRCA_2$, PRAB, and Erα genes as an oncogene responsible for the downregulation of the incidence of cancer progression is well established in a wide variety of tumors, including breast tumors. To study molecular mechanisms by which PE interferes in breast cancer progression, we investigated expression profile of several related genes (Figure 6). In MCF-7 cell line, PE treatment promoted a downregulation of $BRCA_1$, $BRCA_2$, PRAB, and Erα genes. Conversely, in MDA-MB-435 cells, no

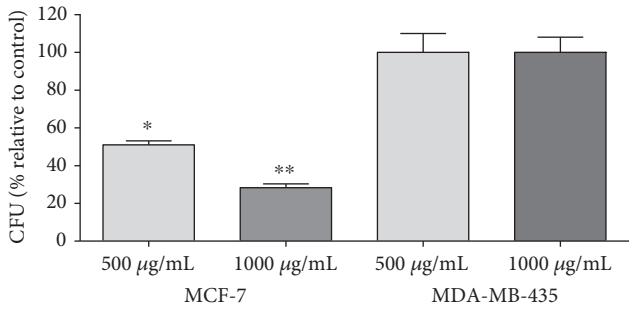

Figure 2: Formation of MCF-7 and MDA-MB-435 colonies. The number of MCF-7 and MDA-MB-435 colonies was determined after 18 days of culture in DMEM supplemented with 10% FCS containing PE at concentrations of 500 and 1000 μg/mL. Data are presented as mean ± standard deviation of 3 independent experiments, each performed at least in duplicate. $*$ indicates significant differences from the control group ($^{*}p < 0.05$; $^{**}p < 0.01$).

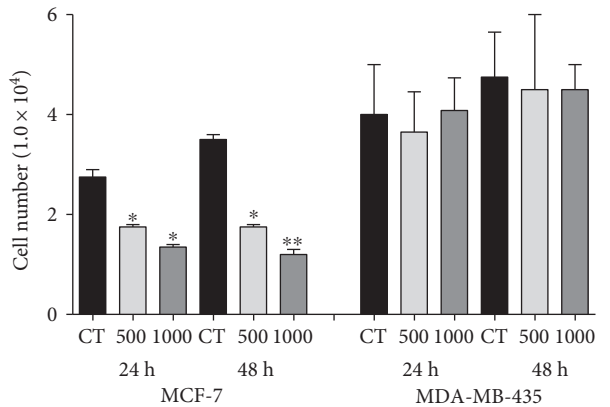

Figure 3: Effect of PE on cell proliferation of MCF-7 and MDA-MB-435 after 24 hours and 48 hours treatment using trypan blue exclusion. Data are presented as mean ± standard deviation of 3 independent experiments, each performed at least in duplicate. $*$ indicates significant differences from the control group ($^{*}p < 0.05$; $^{**}p < 0.01$).

changes in gene expression profile cells were detected when compared to untreated cells (Figure 6).

4. Discussion

The present study provided several sets of information on the antioxidant activity of PE and their effects on the cell viability, cell cycle, and apoptosis of MCF-7 and MDA-MB-435 cells. Breast cancer is the most common cause of cancer in women and the large international variation in breast cancer rates, coupled with the rapidly increasing rates observed in secular trend studies. Although dietary factors have long been suspected to be implicated in breast cancer etiology, few convincing dietary risk factors have been identified [6]. Fruits and vegetables contain numerous constituents that may reduce breast cancer risk, including antioxidants and several vitamins which can prevent cancer [28].

The red pitaya features functional potential related to its high antioxidant activity [21]. Hylocereus species were responsible for the major antioxidant capacity [29], and some studies showed that the peels also contain more or less antioxidant properties due to their color. Thus, both the peels and the pulps could be beneficial especially in food and pharmaceutical industry [30]. The main mechanism of antioxidant action in foods is radical scavenging activity. Therefore, many methods had been developed in which the antioxidant activity was evaluated by the scavenging of synthetic radicals in polar organic solvents such as ethanol [17].

In previous studies evaluating extracts of other fruits by ORAC assay, it reported lower ORAC values than those found in this study. The antioxidant capacity of the hydroalcoholic concentrated extract of red grape pomace showed 22.94 μM of Trolox/g for the ORAC assay. Already concentrate pitaya extract (PE) showed high antioxidant capacity with a reduction of up to 1000 μmol Trolox/g^{-1} [31]. The US Department of Agriculture [32] published, as part of the National Programme for Food and Nutrient Analysis, a study containing data on the antioxidant capacity of concentrated fruit extracts, using the ORAC method. Among the tested fruits were the blackberry (88.57 μM of Trolox/g), raspberries (37.98 μM of Trolox/g), and a strawberry (32.26 μM of Trolox/g).

FRAP is the only assay that directly measures antioxidants in a sample. The other assays are indirect because they measure the inhibition of reactive species (free radicals) generated in the reaction mixture, and these results depend strongly on the type of reactive species used. Mancini-Filho et al. [33] showed that those with average FRAP values higher than those found in the literature for other fruit extracts are also considered high potential antioxidants. The reducing potential of PE in this study was higher than the antioxidant capacity of some concentrated extracts of nontraditional Brazilian fruits such as camu-camu and uvaia jambolan. The fruits of camu-camu showed the highest antioxidant capacity, with a value of 2501.5 ± 74.5 μmol sulfate ferrous/g. Acerola and the netting-black are also significant because the camu-camu showed the highest values, 1995.8 ± 47 and 28.4 ± 908.95 μmol sulfate ferrous/g, respectively. The fruits of jambolan (172.8 ± 10.8 μmol sulfate ferrous/g) and uvaia (407.5 ± 34.9 μmol sulfate ferrous/g) showed lower values than those of pitaya.

Breast cancer cell lines MCF-7 and MDA-MB-435 are well known and widely used in studies on growth properties, regulatory mechanisms, and therapy of breast cancers. Our results showed for the first time that PE shows antitumorigenic effects on hormonal receptor-positive breast cancer MCF-7 cells. The epithelial cell line MCF-7 shows estrogen and progesterone receptors and low metastatic potential. Holliday and Speirs classified MCF-7 as cell line luminal with ER^{+}, $PR^{+/-}$, $HER2^{-}$, and Ki67 low endocrine responsive and often chemotherapy responsive [34].

Recently, Wang et al. [35] demonstrated that differences between MCF-7 and MDA-MB-435 in 229 genes were mainly implicated in the biological functions related to cell adhesion and motion, antigen processing and presentation (via MHC class II), hormone response, extracellular structure

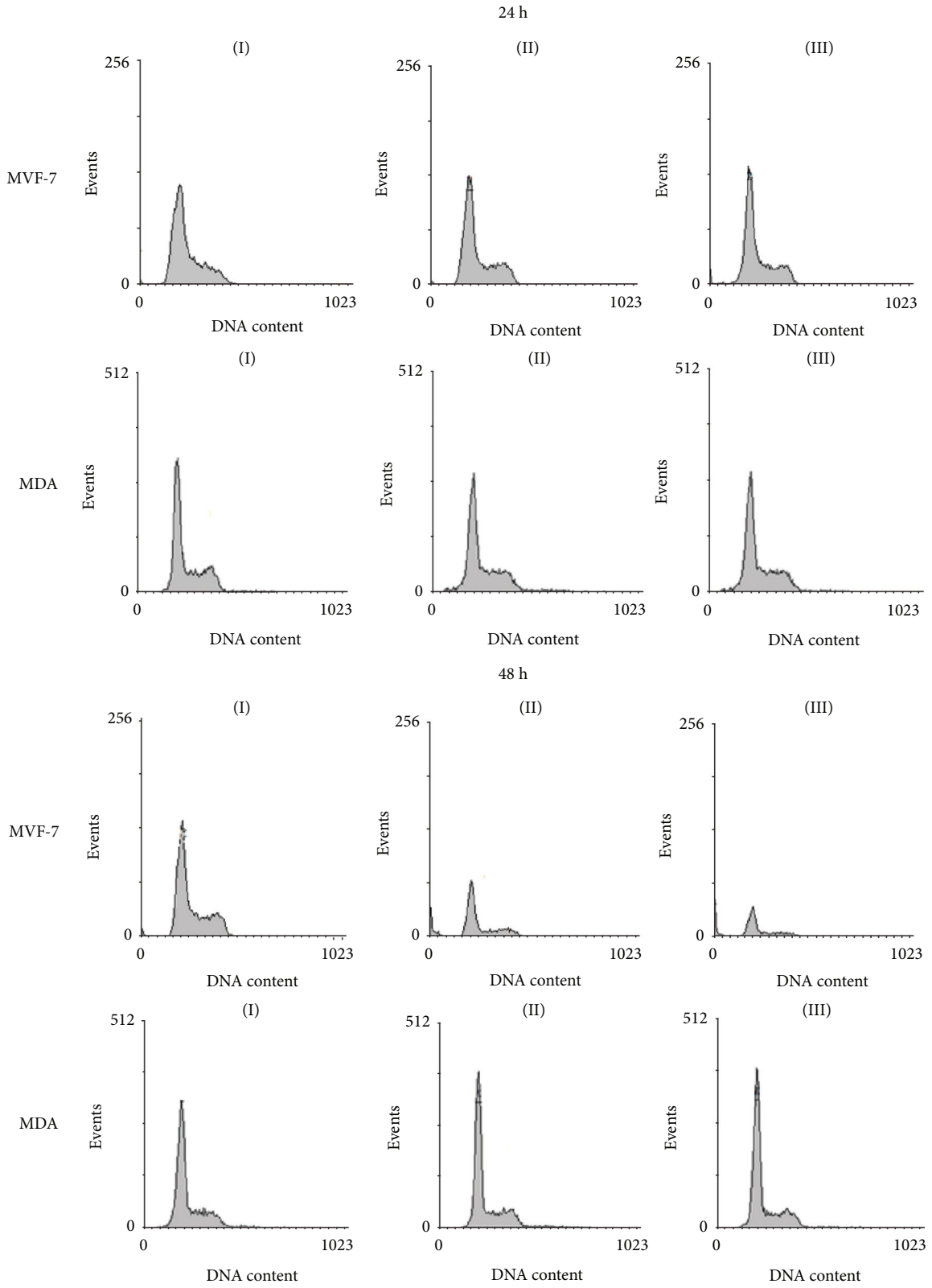

Figure 4: Effect of PE on cell cycle progression in MCF-7 and MDA-MB-435 cells after 24 and 48 h exposure. Data are presented as mean ± standard deviation of 3 independent experiments, with significant differences between the untreated cells (I) and treated with PE 500 (II) and 1000 (III) μg/mL compared by the Tukey test.

Table 3: Effect of PE on cell cycle progression in MCF-7 and MDA-MB-435 cells after 24 h and 48 h exposure.

Cell line	Incubation time	Cell cycle phases	Control (CT)	500 μg/mL	1000 μg/mL
MCF-7	24 h	G_0/G_1	59.59 ± 0.16	63.47 ± 2.07*	65.02 ± 0.23*
		S	16.91 ± 1.20	14.03 ± 0.61	14.80 ± 2.36
		G_2/M	19.28 ± 1.87	19.77 ± 1.42	17.48 ± 3.22
	48 h	G_0/G_1	58.49 ± 0.45	65.40 ± 1.10*	69.61 ± 3.90*
		S	16.45 ± 0.55	10.63 ± 0.25	13.64 ± 1.71
		G_2/M	22.28 ± 0.93	20.19 ± 0.04*	15.66 ± 3.72**
MDA-435	24 h	G_0/G_1	62.30 ± 1.12	61.99 ± 1.99	61.60 ± 0.64
		S	14.84 ± 0.43	14.93 ± 0.24	14.87 ± 0.51
		G_2/M	19.90 ± 1.29	19.83 ± 2.04	18.88 ± 0.68
	48 h	G_0/G_1	69.64 ± 1.18	70.64 ± 0.80	70.01 ± 1.85
		S	11.88 ± 0.89	11.30 ± 0.62	11.50 ± 0.53
		G_2/M	15.17 ± 1.03	15.52 ± 0.52	15.76 ± 0.83

The cell cycle phases and quantitative results are illustrated in accordance with the exposure time and PE concentration. The experiment is expressed as mean ± error standard. * indicates significant differences from the control group (*$p < 0.05$; **$p < 0.01$).

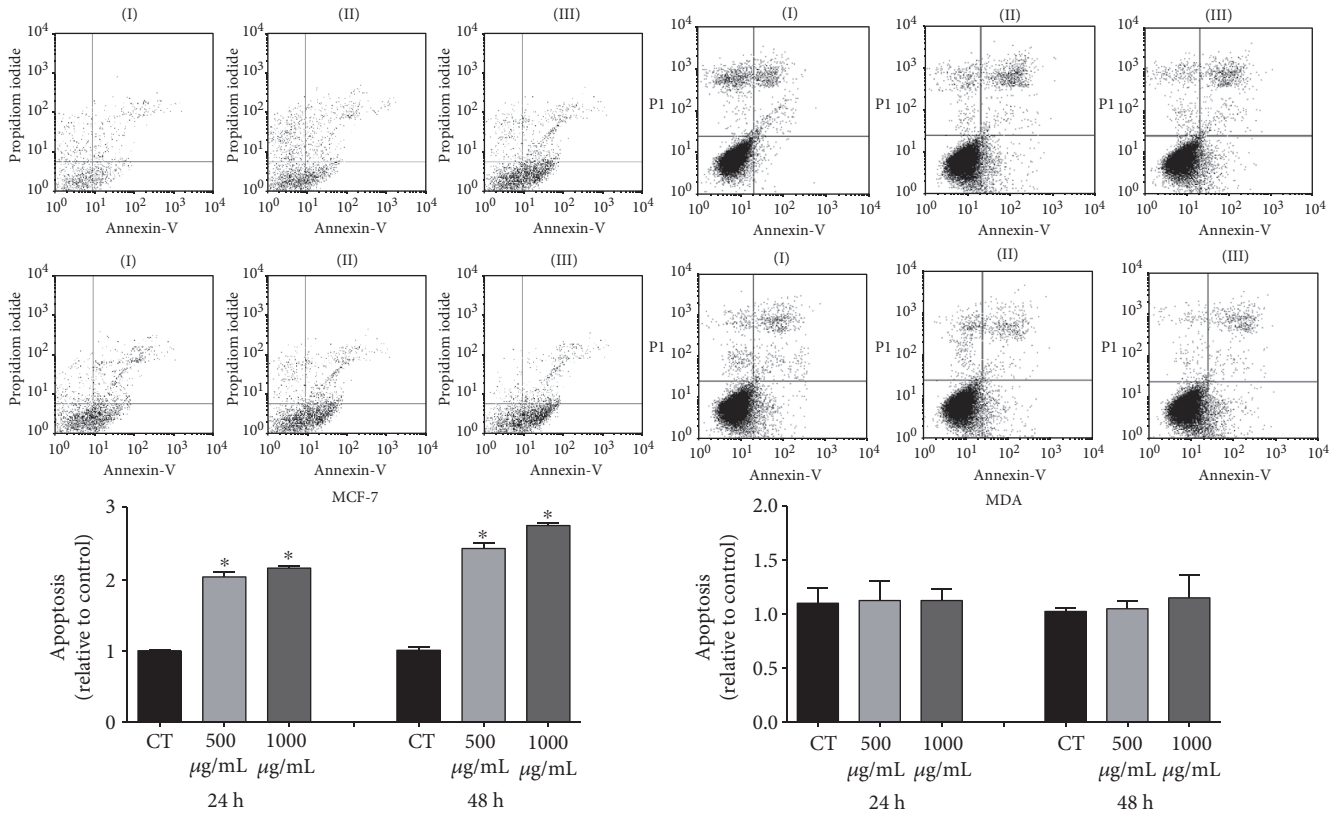

Figure 5: Detection of apoptotic MDA-MB-435 and MCF-7 cells by flow cytometry under PE stimulation at the concentrations of 500 and 1000 μg/mL for 24 h and 48 h. Data are expressed as mean ± standard deviation relative to the control, of 3 independent experiments, each performed with at least 3 replicates. * indicates significant differences from the control group (*$p = 0.05$).

organization, tissue remodeling, and cell proliferation regulation. A microarray analysis has indicated that the gene expression pattern of the human MDA-MB-435 [4] resembles that of human melanoma cell lines [5, 36]. This cell line has fusiform morphology and is considered luminal with low degree of invasion in Matrigel. The epithelial cell line MDA-MB-435 does not express hormone receptors and has a high metastatic potential and high tumorigenicity [37].

According to Ge et al. [38], MDA-MB-435 cell line is resistant to drugs in vitro breast cancer, due to the presence of high levels of *GSTP1* mRNA expression when compared to the levels expressed in MCF-7. Patients with breast cancer

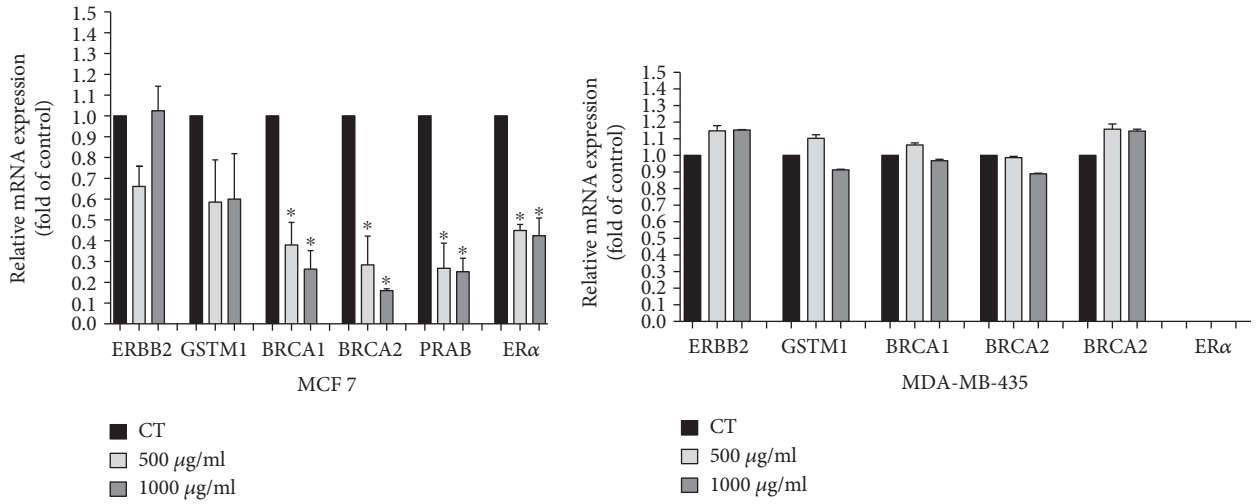

Figure 6: Profile of gene expression in MCF-7 and MDA-MB-435 cells. Quantitative analysis of real-time PCR in different genes associated with cancer progression, after 48 h incubation with PE. Data are presented as mean ± standard deviation of 3 independent experiments, each performed at least in triplicate. Differences significant between treated cells with PE (500 and 1000 μg/mL) were compared using the Tukey test ($^*p < 0.05$).

with the allele *GSTP1 105Val* are more likely to have a tumor with advanced histological grade, lymph node metastases, and negative estrogen receptor. The toxic damage to the genomic DNA in somatic cells not only induces carcinogenesis but also means that there is the development of tumors with more aggressive features, with poor differentiation, independent growth hormones, and metastatic potential. Probably, this is due to difference in the characteristic of aggressiveness between MCF-7 and MDA-MB-435 cell lines, since the MCF-7 cell line has hormone receptors and is more sensitive to the action of therapeutic drugs.

Pitaya has recently drawn much attention, not only because of their striking color and economic value as food products but also for their health properties [39]. For example, red pitaya was reported to offer many health benefits including chemoprevention of cancer, anti-inflammatory and antidiabetic effects, and a reduction in the mortality risk of cardiovascular disease [40], as well as antioxidative properties conferred by its betacyanin content [41]. Asmah et al. [42] reported that a red and white pitaya pulp are rich in polyphenols and a methanol extract showed promising antioxidant and antiproliferative capacity when used to treat cervix cancer cells (HeLa) and cytotoxic effect on human oral cancer cell metastases induced by B16-F10 melanoma.

Cell cycle deregulation is a fundamental aspect in cancer development. Deregulation of cell cycle has been linked with cancer initiation and progression [43]. Thus, cell cycle has emerged as one of the attractive therapeutic targets in the treatment of cancer [44].

Neoplastic cells contained in cell proliferation with a large proportion of cells in S phase and G_2/M [45]. The efficiency of a bioactive compound in food cancer control can be judged by its ability to block the cell cycle phases G_0/G_1 and G_2/M, reducing the proportion of cells in S phase [46]. PE promoted an increase in the percentage of cells in the G_0/G_1 phase, followed by reduction of cells in the G_2/M phase, indicating an arrest in the growth and proliferation of MCF-7 cells after this period. One of the important and limiting aspects of the cell cycle is cell progression in the first phase (G_1) of the S phase, which has its control affected in cancer [47].

There is an urgent need to develop innovative ways to treat breast cancer that has become resistant to apoptosis therapies. Apoptosis in clinical practice is a potential target for therapeutic use of programmed cell death or to understand the mechanisms of resistance to radiotherapy and chemotherapy. When cells become old or damaged, they die by apoptosis, necrosis, or a combination of the two and are replaced with new cells. On the other hand, cancer cells are immortal since they are resistant to apoptosis. Chemotherapy kills cancer cells through apoptosis and/or necrosis [48].

According Sreekanth et al. [49], pitaya extract compounds (betacyanin and anthocyanin) and pigments act on K562 cells that lead to human chronic myeloid leukemia altering the integrity of the mitochondrial membrane, leading to leakage of cytochrome c, caspase activation, and nuclear disintegration. These biochemical changes are reflected in structural changes typical of cells undergoing apoptosis (programmed cell death).

In this regard, the findings presented here coupled to the dragon fruit extract inhibited the viability and proliferation of human breast adenocarcinoma MCF-7, and it was found that these bioactive compounds present in the dragon fruit also interfere in the distribution phases of the cell cycle. However, we did not find studies of pitaya extract effects on tumoral breast cells in the literature.

Other components have already been well characterized in pitaya and, along with anthocyanins, have been described with substances potentially beneficial to human health. Esquivel et al. [29] found out that betalains containing both phenolic and nonphenolic structures were responsible for the major antioxidant capacity of purple Hylocereus juices evaluated, while nonbetalainic phenolic compounds contributed only to a minor extent. It was

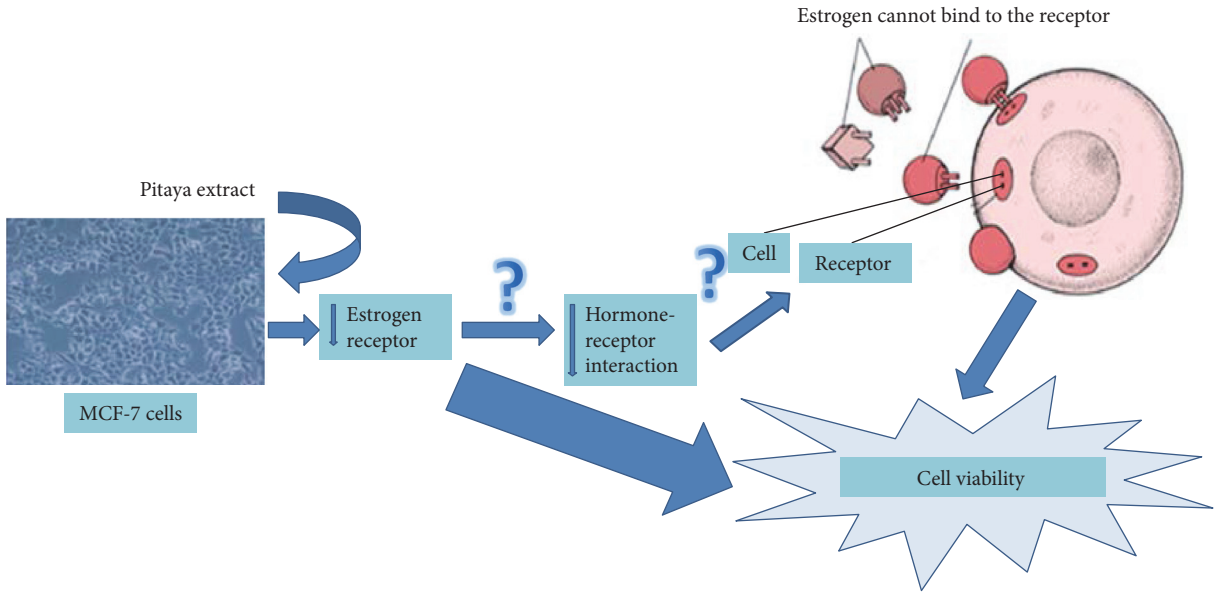

Figure 7: The proposed mechanism of action of PE in MCF-7 cells associated with decreased estrogen receptor expression.

once thought that betalains were related to anthocyanins (i.e., a flavonoid derivative), the reddish pigments found in most plants [50].

Estrogen stimulates proliferation of various breast cancer cells via estrogen receptors (ER). Studies show that different compounds present in food matrix could bind to estrogen receptors and mediate estrogen responses [51, 52]. The majority of authors show that there is a positive association between the presence of hormone receptors and a more favorable prognosis. The presence of hormone receptors indicates a functional state closest of normal breast cell. In other words, these tumors are similar in morphology to the cells of origin and thus are less aggressive to the body. The estrogen receptor expression by tumor cells suggests that at least part of cell proliferation depends on stimulation by estrogen. Therefore, it is possible to stop cell growth by blocking hormone [53]. The activity of PE was evaluated in this study to identify potential signaling pathways by real-time PCR analysis; the observations indicate that the PE showed antitumor activity in MCF-7 cell line by probably suppressing ERα.

$BRCA_1$ and $BRCA_2$ are human genes that produce tumor suppressor proteins. These proteins help repair damaged DNA and, therefore, play a role in ensuring the stability of the cell's genetic material. Genetic susceptibility to breast cancer comprises inherited mutations of the $BRCA_1$ and $BRCA_2$ genes related to hereditary breast cancers. In addition, some studies reported that vegetable and fruit intakes were modifiers in developing breast cancer in BRCA mutation carriers [54].

It is known that BRCA-related tumorigenesis is mainly caused by increased genome instability and DNA damage, but it is unclear why patients who have a mutation in $BRCA_1$ $BRCA_2$ are at higher risk of developing estrogen-responsive cancer. Literature suggests that $BRCA_1$ and estrogen and estrogen receptor signaling regulate cell proliferation and differentiation of breast cells, synergistically [55].

$BRCA_1$ and $BRCA_2$ were downregulated upon pitaya treatment, indicating that DNA damage and repair pathways were affected. Proteins (PRAB, $BRCA_1$, and $BRCA_2$) playing role in DNA damage response pathway were deregulated upon pitaya treatment [56]. Downregulation of PRAB, $BRCA_1$, and $BRCA_2$ imply that uncontrolled proliferation was to some extent normalized and DNA damage was accumulated leading to apoptosis. Our results on pitaya extract can be reconciled with more general findings in cancer biology that tumors activate DNA damage response pathways such as $BRCA_{1/2}$ upon exposure to DNA-damaging agents [57]. It is worth speculating that pitaya may be even more cytotoxic, if combined with other DNA-damaging drugs such as doxorubicin and cisplatin.

Thomson and Thompson [58] support the emphasis of public messages for greater vegetable and selective fruit intake by extending a potential benefit for ER-negative breast cancer. On the other hand, tumors with positive hormone receptors have a more favorable prognosis and respond better to hormonal therapy. This is because the strategies of treating a malignant tumor sensitive to hormones involve, on the one hand, the reduction of estrogen produced normally by the body and, on the other, the inhibition of the links between receptors and hormones. The first group has use drugs which inhibit the synthesis of the hormone, such as those that reduce the activity of the aromatase enzyme responsible for the synthesis of estrogens in various tissues, such as adipose tissue. Another option, more drastic and in selected cases, would be the surgical removal of the ovaries, which produce estrogens in premenopausal women. In the second group are drugs that aim to disrupt and/or compete with estrogens in its binding to the receptor.

Studies have shown that polymorphisms in the ERα gene (ER-alpha) are associated with diseases such as breast and prostate cancer, osteoporosis, Alzheimer's disease, and cardiovascular diseases [59]. The probable mechanisms of pitaya's proliferative action appear to be dependent on

decreased ERα expression that can directly trigger mechanisms of inhibition of cell viability or perhaps decreasing hormone binding to the receptor and thereby inhibiting cell growth (Figure 7). More studies are needed to conclude that the effects of pitaya extract are truly ER-dependent.

5. Conclusion

We conclude that pitaya may act on selective ER-responsive breast cancer cells by targeting multiple tumorigenic pathways leading to cell cycle arrest and apoptosis and probably suppress the expression of estrogen and progesterone receptors. Our data indicate that pitaya possesses therapeutic potential against breast cancer. Further preclinical and clinical studies are warranted to clarify the therapeutic potential of pitaya in the prevention and adjuvant treatment of breast cancer.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 5987082, 6 pages
https://doi.org/10.1155/2017/5987082

Review Article

Omega-3 Polyunsaturated Fatty Acids in Critical Illness: Anti-Inflammatory, Proresolving, or Both?

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Received 10 February 2017; Accepted 25 May 2017; Published 14 June 2017

Academic Editor: Gian L. Russo

Prognosis and outcomes of critically ill patients are strictly related with inflammatory status. Inflammation involves a multitude of interactions between different cell types and chemical mediators. Omega-3 polyunsaturated fatty acids (PUFAs), mainly represented by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are able to inhibit different pathways including leukocyte chemotaxis, adhesion molecule expression and interactions, and production of inflammatory cytokines, through the action of specialized proresolving mediators (SPMs). SPMs from omega-6 fatty acids, such as lipoxins, and from omega-3 fatty acids such as resolvins, protectins, and maresins, act in reducing/resolving the inflammatory process in critical diseases, stimulating the phases of resolution of inflammation. In this light, the resolution of inflammation is nowadays considered as an active process, instead of a passive process. In critical illness, SPMs regulate the excessive posttrauma inflammatory response, protecting organs from damage. This review focuses on the role of omega-3 PUFAs as pharma nutrition agents in acute inflammatory conditions, highlighting their effects as anti-inflammatory or proresolving agents.

1. Introduction

Polyunsaturated fatty acids (PUFAs) of the omega-3 series are essential nutrients since they cannot be produced by humans [1] and whose dietary intake, with food and/or supplements, is associated with several health benefits [2, 3]. Omega-3 PUFAs, primarily found in dietary fish oils [4], are derived also from plants [5] and are substrates able to reduce or limit inflammation in critical illness [6]. The underlying molecular mechanisms responsible for omega-3 PUFAs' biological effects are mediated by the production of proresolving mediators, which have been proposed to modulate and likely resolve inflammatory responses [7]. Lipid mediators synthesized from omega-6 and omega-3 fatty acids are known not only as anti-inflammatory molecules but also to have a key role in inducing active resolution of inflammation. These molecules are defined as specialized proresolving mediators (SPMs) [8]. In this light, PUFAs may be considered as potent modulators of the mechanisms regulating the onset, prolongation, and resolution of inflammation and, therefore, considered to be protective against uncontrolled inflammatory response. The omega-3 PUFAs, as docosahexaenoic acid (DHA) and eicosapentaeinoic acid (EPA), and the omega-6 PUFAs, as arachidonic acid, are available at sites of acute inflammation where they are converted into bioactive SPMs. These SPMs are distinguished in different distinct families, including the omega-6 PUFA-derived lipoxins, and the omega-3 PUFA-derived D-series resolvins, E-series resolvins, protectins, and maresins [8]. The SPMs play a direct key role in the resolution of inflammation, including inhibition of neutrophil migration, enhancement of macrophage phagocytosis of apoptotic neutrophils, and suppression of proinflammatory cytokines and chemokines, in particular during acute illness [1, 9].

Prognosis and outcomes of critically ill patients are directly related to inflammatory status and with the extent and duration of the inflammatory response based on increases in host SPMs, such as resolvin E1, resolvin D5, and 17-epiresolvin D1, and based on survival [10]. Gene expression for SPMs correlated with outcomes in acutely ill patients, and SPM pattern in human tissues was related to outcome in trauma patients [10, 11]. Orr et al. [11] described

in trauma patients the blood leukocyte expression of 18 genes involved in the synthesis, signaling, and metabolism of specialized proresolving lipid mediators and proinflammatory lipid mediators. An association was documented between clinical outcomes and gene expression of lipid mediators' pathway, documenting that trauma patients with uncomplicated hospitalization had higher gene expression of resolvin pathway (and lower gene expression of the ratios leukotriene/resolvin pathways), suggesting a potential protective and therapeutic role for SPMs during posttraumatic multiple organ failure [11]. Since EPA and DHA derived from fish oil have shown several health benefits, including improvements in inflammatory conditions and during chronic diseases, as well as a reduction in cardiovascular disease morbidity and mortality and positive neurological effects, scientific interest has been developed in determining whether plant-derived omega-3 PUFA precursors may also present those benefits [5]. Several studies have indicated the potential of plant-derived omega-3 PUFAs to resolve inflammation and to protect against inflammatory diseases, showing that an increased consumption of alpha-linolenic acid (mainly found in flaxseeds, flaxseed oil, and canola oil, also known as rapeseed oil) or stearidonic acid (mainly found in borage and borage seeds and in Corn Gromwell) tends to increase the proportion of EPA and DHA in membranes of inflammatory cells, including neutrophils, monocytes, and lymphocytes [5, 12–15]. It has been documented that parenteral nutrition based on soybean oil, which has also a high content of omega-6 PUFAs and has been largely used over the last decades, may adversely affect the inflammatory response and promote immunosuppressive effects in critical illness [6]. For this reason, alternative lipid emulsion with lower soybean oil content has been used, showing important improvements in clinical outcomes, such as ICU length of stay and mortality [6].

This review focuses on the most recent data on the role of omega-3 PUFAs as pharma nutrition agents in critical illness, and we specifically highlighted their role as anti-inflammatory and proresolving agents.

2. Genetic Signature and Inflammation in Critical Illness

Lipid mediators and SPM expression, their biosynthetic isomers, and their biosynthetic pathway reflect the patient's specific genetic background [9]. In particular, Colas et al. recently identified the SPM pathways, including resolvins, protectins, and maresins, in lymphoid tissues, blood, and tissues that were proportional with their regeneration functions and protective and proresolving effects [9]. Endogenous lipid specialized mediators and their pathway of action, involved in regulating/resolving inflammation, are of wide interest, and thus, their genetic signature profiles may provide a functional tool for characterizing health and disease states, as well as in monitoring the impact of treatments [9].

Trauma patients showing complications and worse clinical outcomes have higher expression ratios between leukotriene pathway genes and resolvin pathway genes [11], and in these patients, PUFAs may determine proresolving effects through the modulation of the expression of the genes regulating proinflammatory cytokines [16]. Severe traumatic injury itself may contribute to the dysregulation of lipid mediator pathway gene expression [11]. Clària et al. [17] documented that subcutaneous adipose tissue in patients with peripheral vascular disease had deficient levels of SPMs with potent protective actions in vascular inflammation, indicating phenotypic differences in the capacity and levels of SPMs between adipose tissue from patients with end-stage vascular disease and healthy control subjects.

High omega-3 PUFA circulating concentrations, or the shift in circulating omega-6/omega-3 ratios, might modulate the expression of genes known to be critical during inflammatory processes [16], although stronger clinical evidences are warranted. Experimental evidences have shown that EPA or DHA decrease the expression of genes for interleukin-1-beta and tumor necrosis factor-alpha and their mRNA levels [18–20]. In addition, clinical studies have documented that plant-derived omega-3 PUFAs and combination of long-chain omega-3 PUFAs and a short-chain omega-6 PUFAs were effective in critically ill patients suffering from sepsis, reducing the amount of ventilation time, the number of ICU hospitalization days, and increasing the overall survival [21, 22]. Altering the circulating levels of omega-6 and omega-3 PUFAs may influence the inflammatory responses in part by the capacity of the fatty acids, and specifically their metabolites, to regulate the expression of the early signal transduction genes and to downregulate, at a transcriptional level, the expression of proinflammatory genes involved in inflammatory responses essentially cytokines, chemokines, and NFκB pathway [16, 23]. NFκB is a key transcription factor involved in the upregulation of cyclooxygenase gene, adhesion molecules, and inflammatory cytokines. The study by Allam-Ndoul et al. showed a dose effect of omega-3 PUFAs in inhibiting the gene expression of selected inflammatory cytokine and of genes involved in the NFκB pathway [23].

Additional studies in critical setting support the potential protective and therapeutic role for SPMs in reducing complications in posttraumatic conditions. Experimental evidences showed that survival improved significantly after administration of resolvins to septic and burn-injured animals [24]. Similarly, clinical randomized controlled trials documented that intravenous administration of oil, containing DHA and EPA, may decrease mortality and ventilator days in critically ill patients [25, 26], although most recent randomized controlled trials, conducted in critically ill adult patients, did not document an effect in improving overall mortality [27].

It appears important to assess whether pathological conditions, characterized by excessive inflammation, such as critical illness, result from failed resolution mechanisms because of lack or block of specific SPM pathways and whether these mechanisms may be modulated either by EPA or DHA supplementation, or by therapies mimicking SPMs [28].

Patient's profiling of SPM pathway(s) may allow for the identification of metabolites possibly serving as proresolving mediators [9].

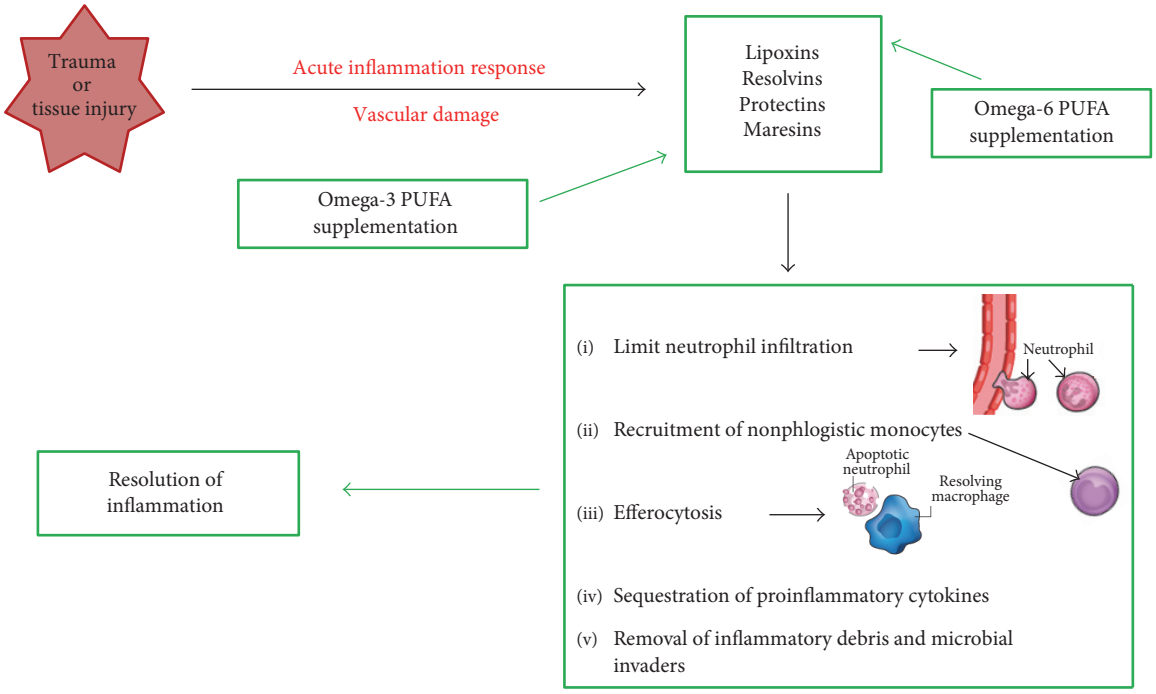

Figure 1: After a trauma or tissue injury, there is a vascular damage inducing the acute inflammatory response. The specialized lipid mediators (SPMs), derived from omega-6 and omega-3 fatty acid storage, act as proresolving mediators. The SPM class which initiates to resolve inflammation is represented by lipoxins that are able to limit neutrophil infiltration. Lipoxins and resolvins stimulate the recruitment of nonphlogistic monocytes. Resolvins and protectins stimulate the resolving macrophages to clear apoptotic neutrophils in the efferocytosis process. Signs of resolution include sequestration of proinflammatory cytokines and removal of inflammatory debris and microbial invaders. Maresins stimulate reepithelialization, wound healing, and tissue regeneration. Omega-3 fatty acid supplementation may enhance proresolving inflammatory responses via their capacity to regulate the expression of proinflammatory cytokines through the production of SPMs. PUFAs, polyunsaturated fatty acids.

3. How Inflammation Is Biologically Resolved in ICU?

An old mechanistic vision considered the resolution of inflammation as a passive process at the end characterized by decreased levels of cytokines, prostaglandins, and reactive oxygen species. During sepsis or other major inflammatory stresses, there is a balance within the host organism. The proinflammatory pathways rise to eliminate pathogens and dead tissue, often causing injury to the host. The anti-inflammatory responses, such as the systemic inflammatory response syndrome (SIRS) and the compensatory anti-inflammatory response syndrome (CARS) (coexistence of both is referred to as mixed antagonist response syndrome (MARS)), seem to limit the damage not interfering with the pathogen elimination. Nevertheless, CARS response may be dangerous when its effects are poorly timed, causing leukopenia, susceptibility to infection, and failure to clear infection [29].

Conversely, during the past few years, the resolution of inflammation has been more clearly identified as an active process where lipid mediators specifically participate in the resolution process by switching their phenotype [30].

Lipid mediators have a crucial role in the vascular damage and leukocyte recruitment, from initiation to resolution of inflammation [28]. Eicosanoids derived from omega-6 PUFAs are potent proinflammatory mediators, except for lipoxins, which are able to perform local proresolving actions in association with lipid mediators obtained from omega-3 PUFAs [9]. Lipoxins, resolvins, protectins, and maresins are produced during response to inflammation, as signaling molecules, and play a role in resolving inflammatory exudates (Figure 1). As part of the neutrophil-monocyte sequence, the lipoxin signals promote the blocking of the acute inflammatory response. Lipoxins and resolvins stimulate the recruitment of nonphlogistic monocytes. So, the resolving macrophages clear the apoptotic neutrophils. In this light, SPMs regulate the actions of the classic proinflammatory initiators prostaglandins and leukotrienes [9], reducing the duration of inflammation, and stimulate reepithelialization, wound healing, and tissue regeneration as signs of resolution [28].

Epidemiological studies and several randomized control trials demonstrate a positive relationship between consumption of omega-3 PUFAs (specifically EPA and DHA) and improvements of different inflammatory conditions [5]. Serhan et al. [7] demonstrated that increasing cellular uptake of omega-3 PUFAs causes an enhancement in the production of resolvins and protectins, which are crucial in resolving inflammatory responses (Figure 1).

An increasing interest is nowadays present in the study of the metabolism, functional effects, and health benefits of omega-3 PUFAs derived from plants [5].

In particular, DHA and EPA act through their enzymatic conversion to the potent lipid-derived mediators [31, 32]. DHA is enzymatically converted to the D-series resolvins through transcellular biosynthesis [1] and intermediates to the protectin family through 15-lipoxygenase action via an epoxide-containing intermediate [33]. The third major family of DHA-derived SPMs is represented by maresins, with a potent proresolving and tissue regenerative action, synthetized by macrophage involving an epoxide-maresin intermediate [28]. EPA can be enzymatically converted into the E-series resolvin family of SPMs through transcellular mechanisms [1].

Several reports in experimental models demonstrated important roles for SPMs in promoting a return to homeostasis after infection or injury leading to improved outcomes and survival [1].

SPMs have shown positive effects in decreasing pain and risk of sepsis, increasing epithelialization and wound healing, inducing tissue regeneration, potentiating the effects of antibiotics, and enhancing adaptive immunity [8]. Nevertheless, further and strong evidences are needed to clarify the effects and potential benefits of the use of SPMs in critical care, including a comparison with the effects obtained by the administration of oil-derived omega-3 PUFAs.

4. Clinical Aspect of the Anti-Inflammatory or Proresolving Effect of PUFAs

Since long time, several authors have discussed the anti-inflammatory effect of omega-3 PUFAs, acting through different mechanisms, including pathways via the cell membrane (G-protein coupled receptor 120) and intracellular (peroxisome proliferator-activated receptor (PPAR) gamma) receptors that control inflammatory cell signaling and gene expression patterns [34]. Therefore, EPA and DHA were considered as anti-inflammatory agents, whereby they compete with omega-6 arachidonic acid, reducing proinflammatory molecules [28]. In fact, in the last year's research, focus has shifted from inhibiting inflammation to accelerating resolution of inflammation using SPMs [8]. The anti-inflammatory process is not the same as proresolution, which involves the SPMs in activating the nonphlogistic responses and cell resolution programs [28]. The proresolving actions include inhibition of neutrophil tissue infiltration, counter-regulation of chemokines and cytokines, reduction in pain, and stimulation of actions mediated by macrophages, known as efferocytosis and phagocytosis of microbes [35] (Figure 1) (Table 1).

Supplementation of omega-3 PUFAs alters the profile of proinflammatory cytokine expression and production and provides significant modifications in inflammatory response aimed at resolving this process [16]. In critical illness, SPMs seem to regulate the excessive posttrauma inflammatory response and to protect organs from collateral damage [28], offering a potential therapeutic option modulating inflammation with minimal side-effects in contrast to currently available anti-inflammatory therapies.

The use of parenteral omega-3 PUFA-based lipid emulsions was considered safe and effective in ICU patients and in the postoperative period in terms of reduction in the infection rate lengths of ICU and in-hospital overall stay, while no significant difference in the mortality rate was documented between patients receiving omega-3 PUFA-enriched parenteral emulsions and those receiving standard lipid emulsions [36]. The latest clinical meta-analysis results in ICU patients documented a reduction of infections and a reduction in hospital length of stay in cardiac surgery spatients [37].

More recently, Grau-Carmona et al. investigated the effects of omega-3 PUFAs on the prevalence of nosocomial infections and clinical outcomes in medical and surgical critically ill patients [38]. The number of patients with nosocomial infections was significantly reduced in the group receiving the omega-3 PUFA supplementation and the predicted time free of infection was prolonged, showing that the administration of fatty acids was safe and well tolerated and that it reduced the risk of nosocomial infections and increased the predicted time free of infections in critically ill medical and surgical patients (Table 1). However, no statistically significant differences were observed

Table 1: Key points: proresolving effects of omega-3 fatty acids in critical illness.

(i) Omega-3 fatty acids, principally present in dietary fish oils, are derived also from plants and are able to reduce or limit inflammation during disease, including acute and critical illness [4–6].
(ii) The biological effects of omega-3 fatty acids are mediated by the production of specialized proresolvin mediators (SPMs) [8, 9].
(iii) Gene expression of SPMs in human tissues correlates with outcomes in critically ill patients [10, 11, 17].
(iv) Lipoxins, resolvins, protectins, and maresins are SPMs produced in response to inflammation, able to accelerate resolution of inflammation rather than inhibiting inflammation [4, 7–9].
(v) Key biologic actions of SPMs are to limit neutrophil infiltration, promote efferocytosis of apoptotic cells, enhance microbial clearance, counter-regulate cytokines and chemokines, and downregulate prostanoids [10, 30, 33, 34].
(vi) Administration of omega-3 fatty acids in surgical and acutely ill patients may be associated with better outcome and reduced health costs.

on the length of ICU and hospital stay, days of ventilation, and mortality [38].

In addition, Pradelli et al. evaluated the cost-effectiveness of the addition of omega-3 PUFAs to standard parenteral nutrition regimens in four European countries (from the healthcare provider perspective) [39]. The authors concluded that, according to their results, the supplementation of parenteral nutrition regimens with omega-3 PUFAs would be cost-effective in Italian, French, German, and United Kingdom hospitals [39].

In conclusion, resolution of inflammation is an active process, mainly driven by the synthesis of PUFA-derived SPMs. It is becoming increasingly clear that omega-3 PUFAs are both anti-inflammatory and proresolving nutrients. In fact, EPA and DHA not only act as anti-inflammatory agents, according to the classical view (i.e., by competing for the synthesis of proinflammatory, omega-6-derived mediators) but also actively promote the resolution of inflammation through the synthesis of SPMs. Administration of fatty acids affects SPM levels in plasma, the immune function, and it may be associated with better outcome and reduce health costs in surgical and acutely ill patients (Table 1). However, controversy still exists on the indications for the use of specific lipid emulsion(s) in ICU patients [6, 36, 38]. In the near future, the possibility to assess circulating SPM levels before, during, and after omega-3 PUFA supplementation, as well as the administration of SPMs, will possibly allow to assess the efficacy of the treatment and to better clarify the mechanisms through which omega-3 PUFAs and PUFA-derived mediators may confer clinical benefit in critically ill patients.

Conflicts of Interest

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

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 3831750, 11 pages
https://doi.org/10.1155/2017/3831750

Research Article

Red Yeast Rice Protects Circulating Bone Marrow-Derived Proangiogenic Cells against High-Glucose-Induced Senescence and Oxidative Stress: The Role of Heme Oxygenase-1

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Received 13 January 2017; Revised 28 March 2017; Accepted 2 April 2017; Published 7 May 2017

Academic Editor: Gian L. Russo

The inflammation and oxidative stress of bone marrow-derived proangiogenic cells (PACs), also named endothelial progenitor cells, triggered by hyperglycemia contributes significantly to vascular dysfunction. There is supporting evidence that the consumption of red yeast rice (RYR; *Monascus purpureus*-fermented rice) reduces the vascular complications of diabetes; however, the underlying mechanism remains unclear. This study aimed to elucidate the effects of RYR extract in PACs, focusing particularly on the role of a potent antioxidative enzyme, heme oxygenase-1 (HO-1). We found that treatment with RYR extract induced nuclear factor erythroid-2-related factor nuclear translocation and HO-1 mRNA and protein levels in PACs. RYR extract inhibited high-glucose-induced (30 mM) PAC senescence and the development of reactive oxygen species (ROS) in a dose-dependent manner. The HO-1 inducer cobalt protoporphyrin IX also decreased high-glucose-induced cell senescence and oxidative stress, whereas the HO-1 enzyme inhibitor zinc protoporphyrin IX and HO-1 small interfering RNA significantly reversed RYR extract-caused inhibition of senescence and reduction of oxidative stress in high-glucose-treated PACs. These results suggest that RYR extract serves as alternative and complementary medicine in the treatment of these diseases, by inducing HO-1, thereby decreasing the vascular complications of diabetes.

1. Introduction

Endothelial dysfunction-related atherosclerosis is typically multifactorial. It is most often dependent on inflammatory risk factors such as hyperglycemia, hypercholesterolemia, hypertension, smoking, and obesity [1, 2]. Complications from atherosclerotic-related diseases remain the leading cause of mortality and morbidity in various industrialized countries [3].

Hyperglycemia, which is associated with endothelial dysfunction, is a primary cause of vascular complications in diabetes [4]. Evidence shows that the repair of endothelium involves bone marrow-derived proangiogenic cells (PACs), also known as endothelial progenitor cells (EPCs), in vasculogenesis [5]. The impaired function and reduced number of EPCs were found to be associated with vascular complications in both type I and type II diabetes [6, 7]. In addition, our previous studies have shown that hyperglycemia directly

impairs the biological functions of angiogenesis, induces cellular aging (senescence), and produces reactive oxygen species (ROS) in EPCs [8–11].

The definition of EPCs has changed over the years as many studies have revealed the true face of the majority of EPC heterogeneity, which are in fact not endothelial precursors but can be described as myeloid-lineage-derived cells with proangiogenic properties. EPCs were classically described as cells that expressed a combination of endothelial and progenitor markers; however, none of these markers is fully specific [12–14]. Thus, other names, such as bone-marrow-derived PACs, have been suggested for EPCs. Nevertheless, despite their history and controversy, EPCs have been applied to different cell types that play roles in the regeneration of the endothelial lining in vasculature. EPCs in all their forms remain a promising target of regenerative medicine.

Red yeast rice (RYR; *Monascus purpureus* Went-fermented rice) has been used for many centuries to make rice wine in China, to maintain food taste and color, and for its medicinal properties. Biological and epidemiological evidence support that the intake of RYR may reduce the incidence of atherosclerosis. RYR contains naturally occurring statins that have serum lipid-modulating effects. Thus, RYR has a lipid-lowering effect in subjects with hyperlipidemia. Pharmacological RYR-related products are marketed in China, Taiwan, and in the United States. RYR has also been shown to have free radical scavenging abilities and can protect the function of endothelium through antioxidative and anti-inflammatory mechanisms [15]. Moreover, a previous study showed that RYR inhibited homocysteine-induced endothelial adhesion via intracellular ROS reduction [16].

Heme oxygenase-1 (HO-1) is a member of the heat shock protein family. The expression of HO-1 is triggered by various stressors, including oxidative stress, heavy metals, UV radiation, and hypoxia [17]. HO-1 expression is mediated through accumulation of the nuclear factor erythroid-2-related factor (Nrf2) in the nucleus [18]. HO-1 was found to be a pivotal antioxidative, anti-inflammatory, and antiapoptotic molecule [19]. Various medicinal plant-derived chemical substances may induce HO-1 activation and can maximize the intrinsic antioxidative abilities [20].

In this study, we explored the potency of RYR extract as an HO-1 inducer in PACs and investigated whether it contributed to the beneficial effects against PAC senescence and oxidative stress.

2. Materials and Methods

2.1. Materials. Glucose, mannitol, and other chemicals were obtained from Sigma Chemical Co. (MO, USA). RYR (LipoCol Forte) was obtained from NatureWise Biotech & Medicals Corporation (Taipei, Taiwan) and extracted at room temperature [15, 16]. Final concentration of solvents in following studies was always less than 0.5% to avoid potential interference. The cobalt protoporphyrin IX (CoPPIX) and zinc protoporphyrin IX (ZnPPIX) used (10 μM) did not significantly influence cell viability (>90%).

2.2. PAC Isolation, Cultivation, and Identification. The protocol conforms to the Helsinki declaration. China Medical University (Taichung, Taiwan) Institutional Review Board approved the study by expedited review. Peripheral blood mononuclear cells (MNCs) were isolated (gradient centrifugation) from volunteers by Histopaq-1077 (Sigma, USA). Isolated MNCs were plated in endothelial growth medium (EGM-2 MV; Cambrex, USA), with supplements (hydrocortisone, R^3-insulin-like growth factor 1, human epidermal growth factor, VEGF, human fibroblast growth factor, gentamicin, amphotericin B, vitamin C, and 20% fetal bovine serum) on fibronectin-coated plates. After culturing, medium was replaced and nonadherent cells were removed. Culture medium was changed every 3 days, and a number of cells can continue to grow into late outgrowth cells. Late outgrowth PACs under passage 3 were used for the study [8].

PACs were further characterized by immunofluorescence for CD34, kinase insert domain receptor (KDR, also named vascular endothelial growth factor receptor 2), and CD31 (platelet endothelial cell adhesion molecule; PECAM-1) (Santa Cruz, USA) expressions [8].

2.3. Western Blot Analysis. Cell lysates were prepared in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5). The concentration of protein was determined by the Bio-Rad Protein Assay reagent.

Nuclear protein extracts were prepared as previously described [8]. In brief, after being washed with ice-cold PBS, cells were scraped off the plates with a cell scraper in 1 mL of ice-cold buffer A (10 mmol/l HEPES/NaOH, pH 7.9; 10 mmol/l KCl; 1.5 mmol/l $MgCl_2$; 1 mmol/l DTT; l0.5 mmol/l PMSF; 2 μg/ml aprotinin; 2 μg/ml pepstatin; and 2 μg/ml leupeptin). After centrifugation at 300g for 10 minutes at 4°C, cells were resuspended in 80 μl of buffer B (buffer A containing 0.1% Triton X-100) by gentle pipetting. Cell lysates were allowed to stand on ice for 10 minutes and then centrifuged at 12,000g for 10 minutes at 4°C. Nuclear pellets were resuspended in 70 μL of ice-cold buffer C (20 mmol/l HEPES/NaOH, pH 7.9; 1.5 mmol/ $MgCl_2$; 1 mmol/l DTT; 0.2 mmol/l EDTA; 420 mmol/l NaCl; 25% glycerol; 0.5 mmol/l PMSF; 2 μg/ml aprotinin; 2 μg/ml pepstatin; and 2 μg/ml leupeptin), incubated on ice for 30 minutes with intermittent mixing, and then centrifuged at 15,000g for 30 minutes at 4°C. Nuclear protein extracts prepared as described above were determined by protein assay.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. The membranes were probed with goat anti-HO-1 antibody (R&D Systems, MN, USA) or rabbit anti-Nrf2 antibody (Abcam, Cambridge, MA, USA) and then incubated with horseradish peroxidase-conjugated secondary antibodies, and the proteins were visualized with a chemiluminescence detection kit (Amersham Biosciences, NJ, USA). Mouse anti-β-actin (Labvision/NeoMarkers, CA, USA) or anti-lamin B (Abcam, Cambridge, MA, USA)

antibodies were used as loading controls. Protein expression levels were quantified using ImageQuant (USA) software [20].

2.4. PAC Viability. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA) assay was used for cell viability. Briefly, PACs were grown in plates and incubated with various concentrations of agents. Medium containing MTT (0.5 mg/ml) was added. Finally, dimethyl sulfoxide was added to each well and the absorbance of blue formazan read at 540 nm using a microplate reader (Multiskan Ex, Thermo Lab systems, USA). Cells incubated in control medium were considered 100% viable [8].

2.5. PAC Senescence Assay. The senescence of PACs was determined by the senescent cell staining kit (Sigma, USA). Briefly, PACs were fixed for 6 min in 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline and then incubated for 12 h at 37°C with fresh X-gal staining solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$; pH 6). Then, blue-stained and total cells were counted for calculating the β-galactosidase-positive cell percentage [8].

2.6. ROS Production. Effect of RYR extract on ROS production was determined by a fluorometric assay by probe dichloro-dihydro-fluorescein diacetate (DCFH-DA). Confluent cells in 48-well plates were pretreated with RYR extract. HBSS containing 10 μM DCFH-DA was added, and the cells were incubated with it for 30 min. The relative fluorescence unit was measured at 485 nm excitation and 530 nm emission by a fluorescence microplate reader [21].

2.7. Measurement of Glutathione (GSH), Glutathione Reductase (GR), and Thiobarbituric Acid Reactive Substances (TBARS). GSH levels were measured by a colorimetric assay (Bioxytech GSH-400; OxisResearch, Portland, OR, USA). Metaphosphoric acid (5%) was added to the cells and then scraped off it. The mixture was centrifuged at 3000g for 5 min at 4°C, and the supernatant was measured at 400 nm after a chemical reaction with reagent R1 (4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate) and reagent R2 (30% NaOH) with a GSH standard curve [22].

The GR activity was determined with a protocol described previously [23]. In brief, GR activity was expressed as a rate of decrease in absorbance at 340 nm/min due to the NADPH oxidation by GR, and the enzyme activity was normalized with mg protein.

Lipid peroxidation was quantified by TBARS determination by spectrophotometric assay (Beckman Coulter, DU 640 spectrophotometer, Germany). The lipid peroxide levels, expressed as nmol malondialdehyde/mg protein, were calculated from the absorbance at 532 nm by external standard tetraethoxypropane [22].

2.8. RNA Extraction and Real-Time PCR. Total RNA was isolated from lung cancer tissues and adjacent normal lung tissues of the NSCLC patients and, subsequently, analyzed by real-time PCR. The following primers were designed using Primer Express software (RealQuant, Roche) based on published sequences: human HO-1 sense primer: 5′-TTC TTC ACC TTC CCC AAC TA-3′; HO-1 antisense primer 5′-GCA TAA AGC CCT ACA GCA AC-3′. Human GAPDH sense primer: 5′-AGC CAC ATC GCT CAG ACA-3′; GAPDH antisense primer 5′-GCC CAA TAC GAC CAA ATC C-3′. Fluorescence data were acquired after the final extension step. A melt analysis was conducted for all products to determine the specificity of the amplification [24].

2.9. Small Interfering RNA. A specific double-stranded 21-nucleotide RNA sequence homologous (small interfering RNA (siRNA)) to the target gene was used to silence HO-1 expression. The computer software and Silencer™ siRNA construction kit from Ambion (Austin, TX, USA) designed and synthesized siRNA for HO-1 (sequences of the ribonucleotides were 5′-rGAC UGC GUU CCU GCU CAA CdTdT-3′ and 5′-rGUU GAG CAG GAA CGC AGU CdTdT-3′) and negative control number 1 siRNA. HO-1 protein inhibition was assessed by immunoblot analysis following transfection of cells with HO-1-siRNA. Briefly, cells were transiently transfected with 20 nM siRNA using 8 μl of siPORT Amine (Ambion) [24, 25].

2.10. Statistical Analyses. Data were expressed as means ± standard deviation (SD). Statistical evaluation was performed using Student's t-test or one-way analysis of variance, followed by Dunnett's test. A P value of <0.05 was considered significant.

3. Results

3.1. Isolation and Characterization of Circulating Bone Marrow-Derived PACs. Cells originated from peripheral blood MNCs of healthy volunteers. MNCs initially seeded on fibronectin-coated wells were round-shaped. Late outgrowth PACs with cobblestone-like morphology were grown to confluence (Figure 1).

Cell characterization was performed by fluorescent stain. CD34, KDR, and CD31 may be considered markers of late outgrowth PACs. CD34 and KDR double positive may be important markers of these cells in vitro (Figure 1) [8, 26]. Endothelial marker CD31 was also used for characterization of the outgrowth cells.

3.2. RYR Extract Is Toxic Only in High Concentrations. Incubation of PACs with 0–50 μg/ml RYR extract for 24 h and 48 h did not result in cellular toxicity; however, high doses of RYR extract ($\geq$200 μg/ml for 24 h and $\geq$100 μg/ml for 48 h) significantly reduced cell viability (Figure 2). These data indicate that the significant cytotoxic effects of RYR extract on PACs were found in high doses. Thus, the noncytotoxic doses of RYR extract ($\leq$50 μg/ml) in the following experiments were used to avoid potential interference of cell survival.

3.3. RYR Extract Induces Nrf-2 Activation and HO-1 Expression in PACs. We further tested the effects of RYR extract on Nrf2 signaling pathway and HO-1 expression in PACs. 50 μg/ml RYR extract time-dependently induced Nrf2 nuclear translocation in PAC cells (Figure 3(a)). In

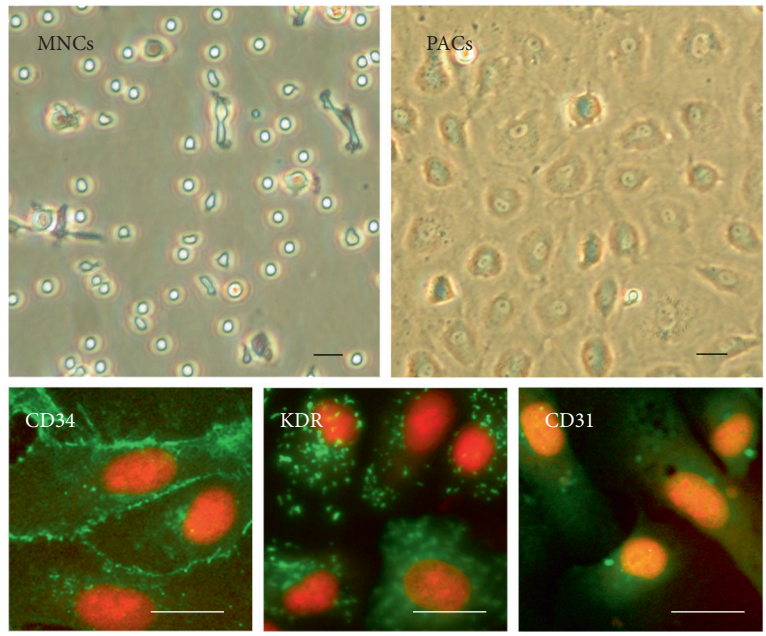


Figure 1: Characterization and morphology of PACs. MNCs were plated on fibronectin-coated plate on the first day (upper left). Late outgrowth PACs with cobblestone-like morphology were reseeded and grown to confluence (upper right). Immunofluorescence staining (green) of CD34, KDR, and CD31 for late outgrowth PACs. Cell nucleus was counterstained with propidium iodide (red). Scale bar = 50 μm.

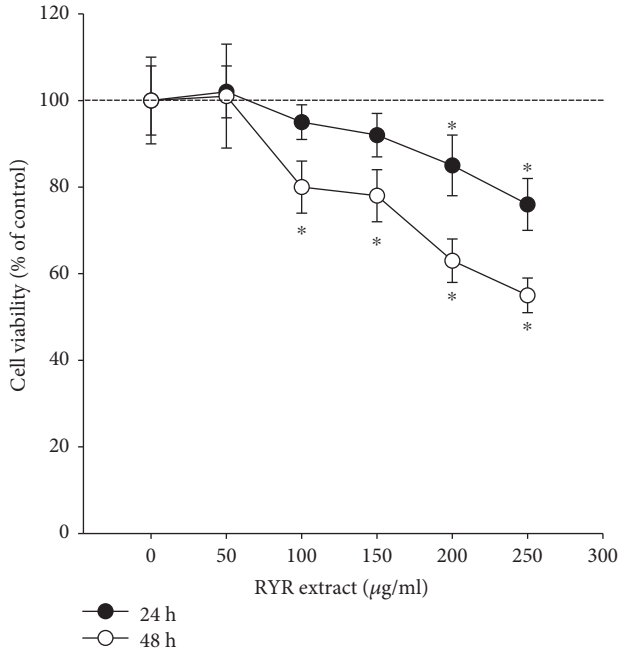


Figure 2: PAC viability after incubation with RYR extract for 24 h and 48 h is determined by MTT assay. Data are expressed as percentage (mean ± SD) of survival cells by the control group. The results are from six separate experiments, $^*P < 0.05$ compared to that of the control group.

addition, RYR extract (12.5, 25, and 50 μg/ml) was added to culture medium with PACs, and real-time PCR and Western blot were performed for HO-1 mRNA (12 h) and protein (48 h) expressions, respectively. As shown in Figures 3(b) and 3(c), RYR extract increased HO-1 mRNA and protein expression in a dose-dependent manner. Moreover, 50 μg/ml of RYR extract increased HO-1 protein expression in PACs in a time-dependent manner (12, 24, and 48 h) (Figure 3(d)).

3.4. RYR Extract Inhibits High-Glucose-Induced Senescence and Oxidative Stress. Our previous study demonstrated that high-glucose-caused (30 mM) senescence and oxidative stress in PACs as compared with the control (5 mM of glucose) or osmotic control (extra 25 mM of mannitol) groups [8]. To investigate whether RYR extract inhibited senescence of PACs induced by high glucose, PACs were coincubated with high glucose (30 mM) and RYR extract (12.5–50 μg/ml) for 48 h, and a β-galactosidase assay was performed. RYR extract had a dose-dependent effect to reduce senescence in high-glucose-treated PACs (Figure 4(a)).

In addition, to directly determine the effect of RYR extract on ROS generation, we analyzed the ROS level in high-glucose-treated PACs. As shown in Figure 4(b), treatment with high glucose for 48 h caused a higher increase of fluorescence compared with the control and mannitol groups. Coincubation of PACs with RYR extract inhibited high-glucose-induced ROS generation in a dose-dependent manner.

In addition, Table 1 shows that RYR extract treatment caused a significant increase of GSH content and GR activity but a significant decrease of thiobarbituric acid reactive substance (TBARS) content relative to high-glucose-treated PACs.

3.5. RYR Extract Inhibits High-Glucose-Induced Senescence and Oxidative Stress via HO-1. HO-1 siRNA was used to confirm the effects of RYR extract mediated through HO-1. Figure 5 shows a reduction of RYR extract-induced HO-1 by HO-1 siRNA. We further explored the effect of HO-1 on

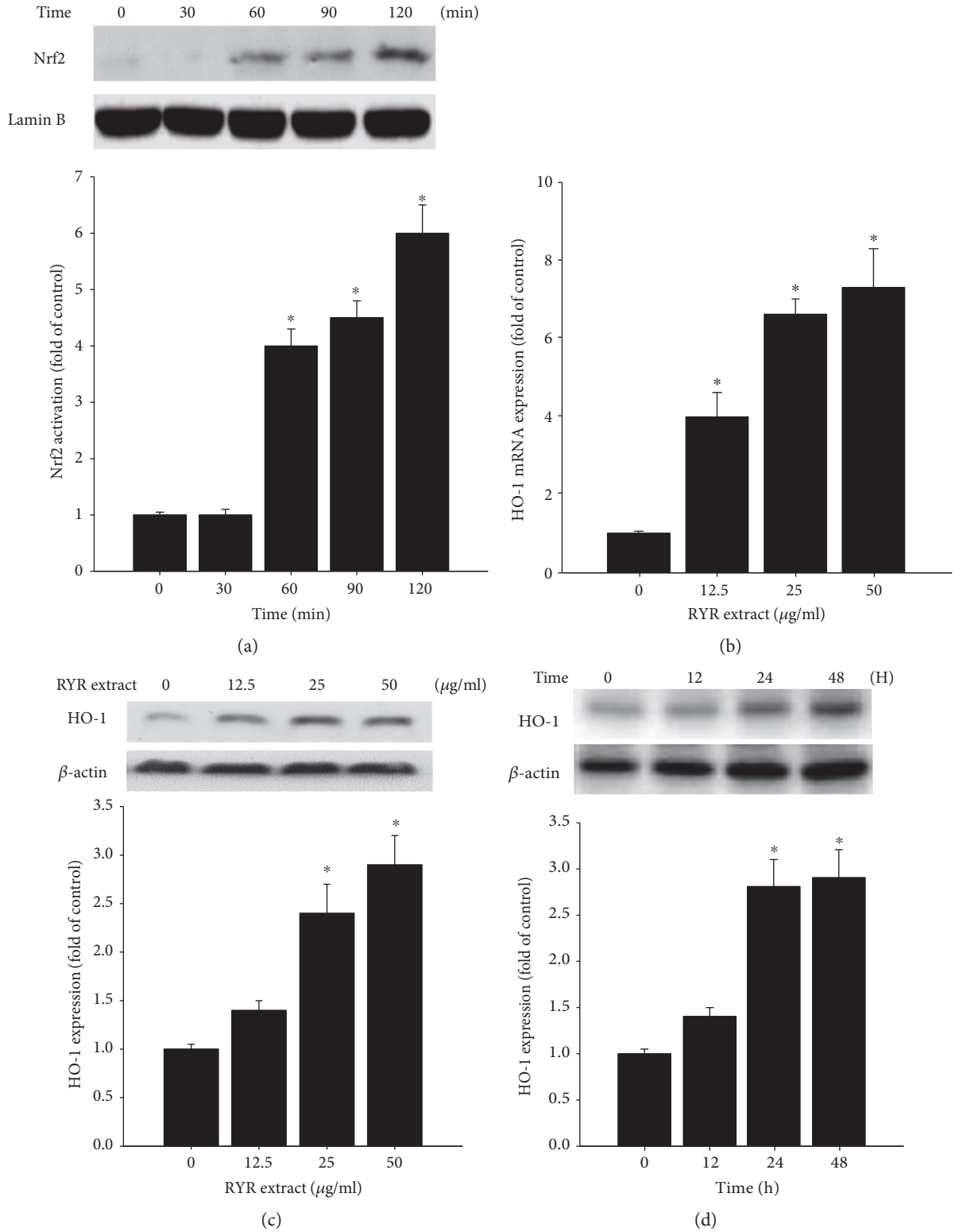
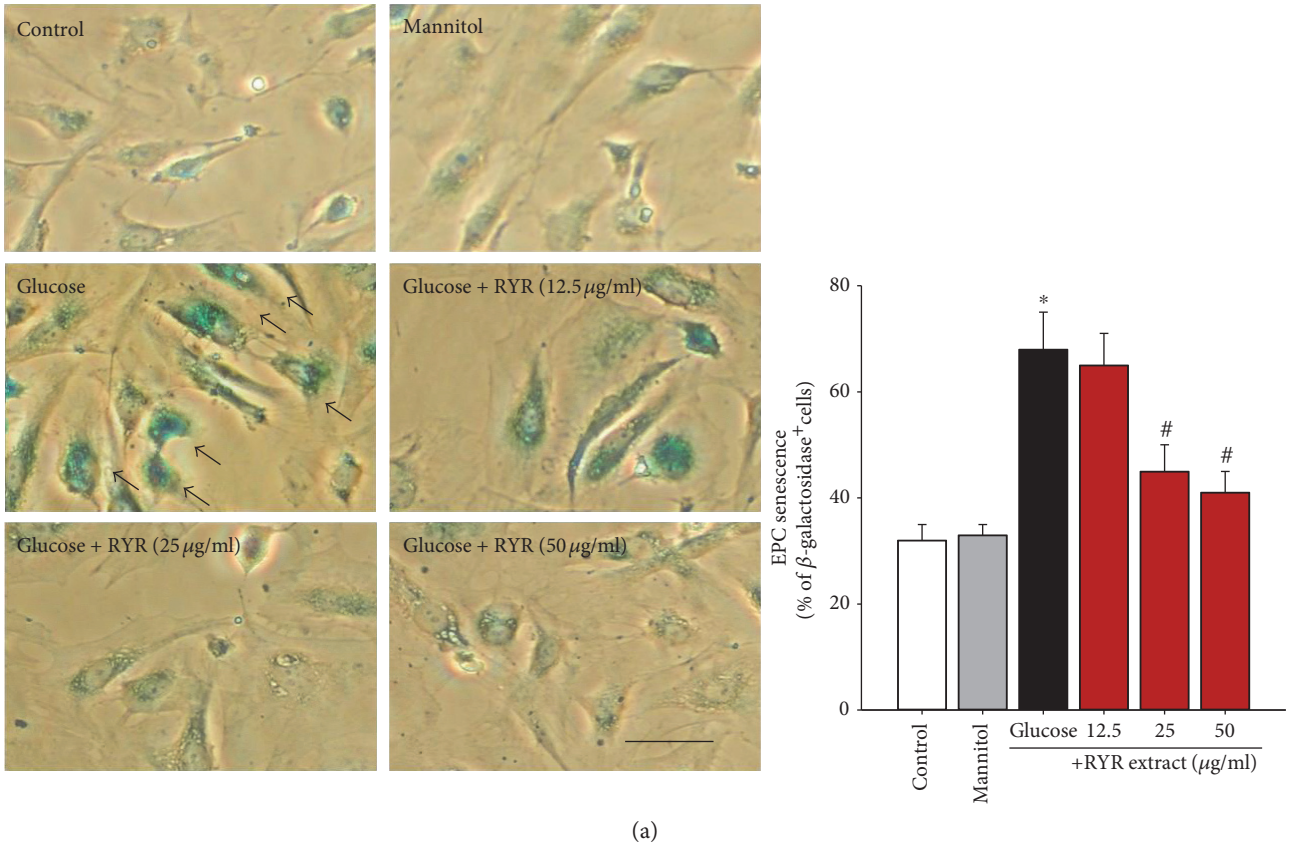
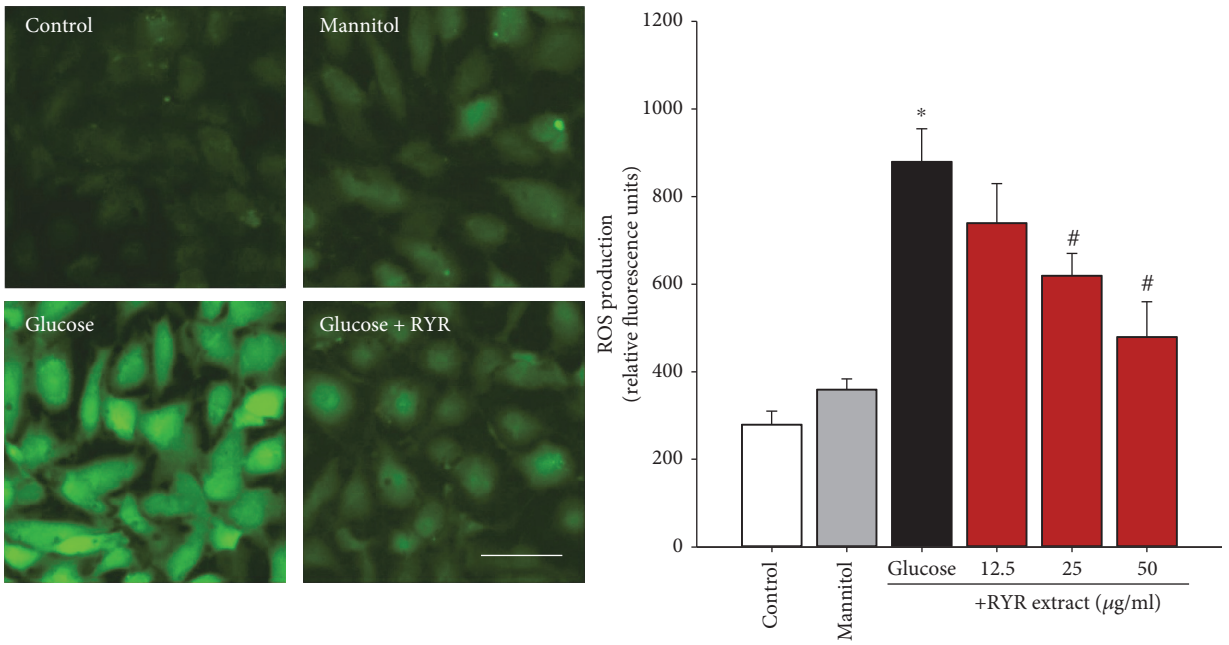


FIGURE 3: (a) RYR extract time-dependently induces Nrf2 nuclear translocation in PAC cells after RYR extract (50 μM) incubation. (b) RYR extract dose-dependently induces HO-1 mRNA expression (12 h) in PACs. RYR extract (c) dose- and (d) time-dependently induces HO-1 protein expression in PACs. Data are expressed as mean ± SD of three independent experiments. $^*P < 0.05$ compared with that of the medium alone control group.



(a)



(b)

Figure 4: RYR extract dose-dependently inhibits (a) cell senescence and (b) ROS production in high-glucose-treated PACs. Arrows: strong blue-stained β-galactosidase-positive cells. Data are expressed as mean ± SD of three independent experiments. $^{*}P < 0.05$ compared to that of the control group; $^{\#}P < 0.05$ compared to that of the high-glucose-treated group.

high-glucose-induced PAC senescence and oxidative stress. As shown in Figures 6(a) and 6(b), we found that the HO-1 inducer cobalt protoporphyrin (CoPPIX) also significantly decreased high-glucose-induced PAC senescence and oxidative stress, whereas the HO-1 enzyme inhibitor, zinc protoporphyrin IX (ZnPPIX), and HO-1 siRNA significantly

Table 1: The GSH, GR, and TBARS levels in PACs.

	Control	Mannitol	Glucose	Glucose + RYR extract
GSH (nmol/mg protein)	52.6 ± 8.3	46.3 ± 3.9	$29.3 \pm 2.6^{*}$	$48.5 \pm 5.5^{\#}$
GR (unit/mg protein)	1.8 ± 0.2	1.6 ± 0.2	$1.1 \pm 0.1^{*}$	$1.6 \pm 0.1^{\#}$
TBARS (nmol/mg protein)	2.8 ± 0.5	3.2 ± 0.6	$8.2 \pm 1.1^{*}$	$3.6 \pm 1.9^{\#}$

$^{*}P < 0.05$ compared to that of the control group; $^{\#}P < 0.05$ compared to that of the glucose group.

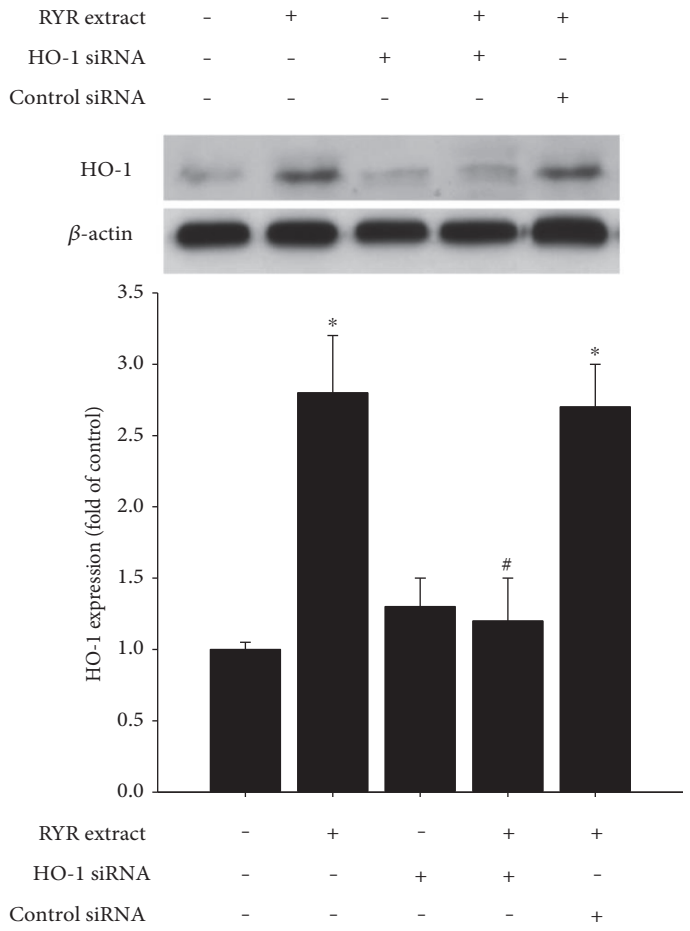


Figure 5: HO-1 siRNA inhibits RYR extract-induced HO-1 protein expression. PACs were transfected with HO-1 siRNA or control siRNA and then stimulated with RYR extract (50 μg/ml). Cell lysates were subjected to Western blotting to determine levels of HO-1 and β-actin. Data are expressed as mean ± SD of three independent experiments. $^{*}P < 0.05$ compared to that of the control group; $^{\#}P < 0.05$ compared to that of the RYR extract-treated group.

reversed RYR extract-caused inhibition in high-glucose-treated PACs. Oxidative stimulator H_2O_2 (50 μM) was also used to confirm the antioxidative effect of RYR extract.

4. Discussion

The present study showed, for the first time, that RYR extract attenuated high-glucose-induced senescence and oxidative stress of PACs. Our data also suggest that HO-1 activation may play a pivotal role in the anticellular senescence and antioxidative effects of RYR extract on PACs.

As the incidence of myocardial infarction and stroke increases as the population ages, there must be an increased focus on the fundamental processes and mechanisms of vascular aging. An advanced understanding of the molecular pathways leading to vascular aging may contribute to the design of therapeutic strategies to prevent vascular senescence. More recently, Paneni et al. reviewed the advances in the pathology of age-related vascular dysfunction including dysregulation of epigenetic modifications, inflammatory genes, and mechanisms of vascular calcification [27]. Oxidative stress contributes to the progression of endothelial-dysfunction-related clinical diseases through luminal narrowing in the brain (ischemic stroke), heart, and peripheral vessels [28]. Epidemiological studies indicate that RYR consumption is associated with reduced coronary heart disease risk [29, 30].

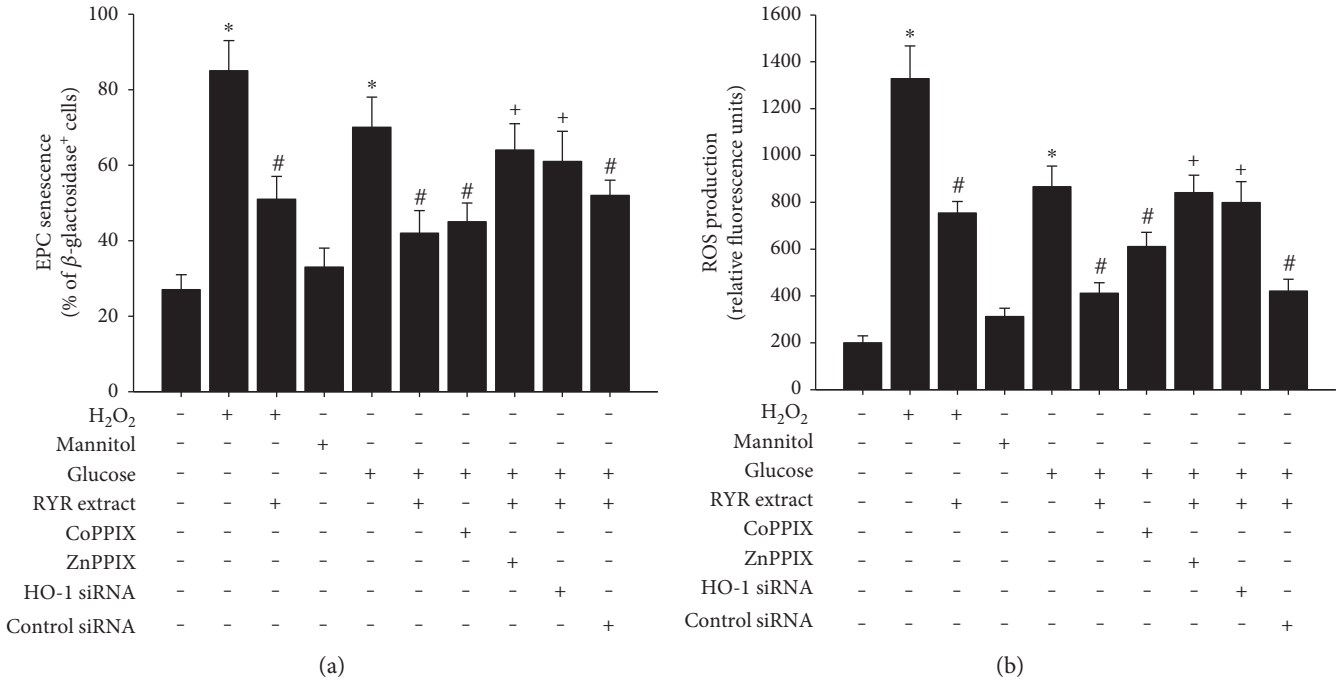


Figure 6: CoPPIX (10 μM), ZnPPIX (10 μM), and HO-1 siRNA modulate the inhibitory effect of RYR extract on high-glucose-caused (a) senescence and (b) oxidative stress in PACs. Oxidative stimulator H_2O_2 (50 μM) was used to confirm the antioxidative effect of RYR extract. Data are expressed as mean ± SD of three independent experiments. $^*P < 0.05$ compared to that of the control group; $^\#P < 0.05$ compared to that of the H_2O_2 or high-glucose-treated group; $^+P < 0.05$ compared to that of the RYR extract and high-glucose-treated group.

In vitro investigations have indicated that RYR can inhibit several key events of the atherogenic process, such as vascular smooth muscle and endothelial cell dysfunction by redox-sensitive mechanisms [15, 16, 31]. RYR contains chemicals that are similar to prescription statin medications including monacolin K (the same structure as the drug lovastatin). Statin-mediated HO-1 induction has been shown to occur in vascular smooth muscle cells [32, 33], endothelial cells [34–40], macrophages [41, 42], neurons [43–46], liver cells [47, 48], and pulmonary cells [49, 50]. In this study, we showed, for the first time, that RYR extract induces HO-1 activation in bone-marrow-derived PACs. HO-1 expression is mediated through accumulation of Nrf2 in the nucleus. We also found that RYR extract induces Nrf2 nuclear translocation in PACs. Moreover, the antioxidant properties of RYR extract were further reported to protect against cellular senescence by inhibiting high-glucose-induced oxidative stress in PACs via HO-1 induction. Thus, our results elucidated the relationship with hyperglycemia, oxidative stress, and endothelial dysfunction, regulation of atheroprotective genes HO-1, and how the regulation of these activities by RYR can lead to the prevention of diabetes-related vascular complications.

During atherogenesis, hyperglycemia-mediated chronic oxidative stress plays an important role in PAC dysfunction [51]. In the present study, RYR extract induced HO-1 expression in PACs in a dose- and time-dependent manner. Consistent with the present results, various vascular protective agents such as atorvastatin [52], estradiol [53], and oleuropein/oleacein (phenolic compounds from olive oil) [54] also increase HO-1 and display anti-inflammatory effects in EPCs. All these data suggest that RYR is a strong inducer of HO-1, and such induction may be independent of various vascular protective agents.

ROS have been implicated in the pathogenesis of most stages of atherosclerosis [11, 55, 56]. ROS, especially hydrogen peroxide and superoxide, are important intracellular signaling molecules in cells. ROS participate in the growth and death of PACs; these events play crucial roles in cardiovascular diseases, suggesting that the sources of ROS and the intracellular signaling pathways may be important therapeutic targets [57]. Evidence has shown that ROS influences cellular processes in vascular remodeling by activating various intracellular signaling cascades [57]. Our previous in vitro study demonstrated the activity of RYR extract on the radical-scavenging abilities of the probe-based ultraweak chemiluminescence technique and showed that RYR exhibited major radical-scavenging abilities on superoxide and hydroxyl radicals [16]. The present study further provides direct evidence that RYR extract maintained GSH amounts and upregulated GR activity in high-glucose-stressed PACs resulting in decreased TBARS, suggesting that RYR could maintain the intracellular antioxidant concentrations in biological systems. It should be further examined whether RYR upregulates other GSH-related enzymes, such as glutamate cysteine ligase and glutathione peroxidase, which catalyze GSH biosynthesis.

Our study has limitations. The composition of the various compounds from the RYR extract, in particular those that might be responsible for the protective effects in the RYR mixture and may specifically induce HO-1, is not clearly defined in this manuscript; only the crude extract of RYR

was studied. Moreover, the molecular mechanism underlying HO-1 induction by RYR is unclear. For example, it is unknown if nuclear factor erythroid 2-related factor 2, a major transcriptional regulator of HO-1 expression [58], or the HO products carbon monoxide and bilirubin [59] were involved in the protective effects of RYR on PACs. Finally, the measurement of some direct inflammation markers could provide more evidence on the anti-inflammatory role of HO-1 after RYR treatment. It is important to explore the effective compounds and mechanisms of RYR for further direction in the field of agricultural product research.

5. Conclusions

The present study demonstrated that RYR extract induced HO-1 expression in PACs in a dose- and time-dependent manner. RYR extract inhibited high-glucose-induced β-galactosidase activation and reduced high-glucose-induced oxidative stress in PACs in a dose-dependent manner. HO-1 expression might play a pivotal role in the atheroprotective effects of RYR on PACs. Thus, RYR may fulfill the definition of a pharmacological preconditioning agent for preventing cerebrovascular and cardiovascular diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported in part by China Medical University (CMU104-S-37), Chung Shan Medical University Hospital (CS08110), Taiwan Ministry of Science and Technology (MOST 104-2320-B-039-016-MY3), Taiwan Ministry of Health and Welfare Clinical Trial and Research Center of Excellence (MOHW106-TDU-B-212-113004), and CMU under the Aim for Top University Plan of the Ministry of Education, Taiwan.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 1583098, 10 pages
https://doi.org/10.1155/2017/1583098

Research Article

Cinnamon Polyphenol Extract Inhibits Hyperlipidemia and Inflammation by Modulation of Transcription Factors in High-Fat Diet-Fed Rats

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Received 13 January 2017; Accepted 26 February 2017; Published 15 March 2017

Academic Editor: Jasminka Giacometti

We evaluated the effects of cinnamon polyphenol extract on hepatic transcription factors expressions including SREBP-1c and LXR-α in rats fed high fat diet (HFD). Twenty-eight Wistar rats were allocated into four groups: (i) normal control: animals fed with normal chow; (ii) cinnamon: animals supplemented with cinnamon polyphenol; (iii) HFD: animals fed a high-fat diet; and (iv) HFD + cinnamon: animals fed a high-fat diet and treated with cinnamon polyphenol. Obesity was linked to hyperglycemia, hyperlipidemia, and oxidative stress as imitated by elevated serum glucose, lipid profile, and serum and liver malondialdehyde (MDA) concentrations. Cinnamon polyphenol decreased body weight, visceral fat, liver weight and serum glucose and insulin concentrations, liver antioxidant enzymes, and lipid profile ($P < 0.05$) and reduced serum and liver MDA concentration compared to HFD rats ($P < 0.05$). Cinnamon polyphenol also suppressed the hepatic SREBP-1c, LXR-α, ACLY, FAS, and NF-κB p65 expressions and enhanced the PPAR-α, IRS-1, Nrf2, and HO-1 expressions in the HFD rat livers ($P < 0.05$). In conclusion, cinnamon polyphenol reduces the hyperlipidemia, inflammation, and oxidative stress through activating transcription factors and antioxidative defense signaling pathway in HFD rat liver.

1. Introduction

Obesity is an important health problem that characterized excessive fat accumulation in the body resulting from an imbalance in energy intake and expenditure [1]. It is known to be a risk factor for numerous metabolic complaints such as diabetes, atherosclerosis, hyperlipidemia, and cancer [2, 3]. Consumption of high-fat diet causes a leading obesity and obesity-related complications including hyperlipidemia and oxidative stress [4, 5]. Liver fat synthesis is an extremely modified metabolic pathway which is vital for actual low-density lipoprotein production and is, therefore, important for energy delivery to other tissues [6]. Transcription factors such as the sterol regulatory element-binding protein 1 (SREBP-1) and liver X receptors (LXRs) and several enzymes including ATP-citrate lyase (ACL), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) have vital roles in this process [7–9]. SREBP-1 may control the ectopic accumulation of fat and may set the target gene FAS, an important enzyme that controls the amount of fatty acid synthesis [10, 11]. Obesity-induced insulin resistance triggers inflammation in the liver through the accumulation of reactive oxygen species that trigger nuclear factor kappa beta (NF-κB) pathway [12]. Besides systemic and hepatic fat metabolism deterioration, inflammation is a major factor underlying liver damage in diabetes [13, 14]. The peroxisome proliferator-activated receptor α (PPARα), which is extremely expressed in the liver, shows a vital role in the modulation of liver lipid metabolism [15].

Some potent drugs carry the risk of side effects on the central nervous system and the cardiovascular system in the treatment of obesity [16, 17]. Natural products can show an obvious role in the prevention of obesity and associated metabolic diseases. Cinnamon has been used as

spice for a long time [18]. In addition it is gained from the inner bark of tropical evergreen cinnamon plants [18]. There are two main types of cinnamon: True or Ceylon cinnamon *(Cinnamomum verum and C. zeylanicum)* and cassia cinnamon (*C. aromaticum* and *C. burmannii*). That cinnamon as a dietary supplement has antioxidant, anti-inflammation, and presently discovered antihyperlipidemia and antiobesity effect properties [19, 20]. Experimental and clinical studies have shown that cinnamon could be attributed to its beneficial effects on hyperlipidemia and glucose utilization [21, 22]. A study reported that cinnamon increases insulin sensitivity and liver glycogen by modulating insulin signaling and glycogen synthesis in insulin-resistant rats [22]. Research in animal models has also proven that cinnamon effectively prevents obesity caused by high-fat diets [23]. However, the underlying exact mechanisms are still unclear. Hence, in this study, the effects of cinnamon polyphenol extract on PPARα-mediated target genes involved in glucose and lipid metabolism, including SREBP-1c, LXRs, ATP-citrate lyase (ACLY), and FAS, and expression of molecular targets of inflammation (NF-κB) and antioxidant status (Nrf2 pathway) in the liver were examined to investigate more detailed mechanisms in the improvement of fatty liver with high-fat diet.

2. Materials and Methods

2.1. Animals. Twenty-eight Wistar rats (weighing 180 ± 20 g) were kept under a 12 h light/dark cycle at 22 ± 2°C, with feed and water ad libitum. Rats had free access to diet and water. Rats received humanitarian care according to standards defined in the "Guidelines for the Care and Use of Laboratory Animals" delivered by the National Academy of Sciences and published by the National Institutes of Health and permitted by the Ethical Commission of the Firat University, Elazig, Turkey. The composition of diets (control and HFD) is shown in Table 1. For obesity induction, animals were fed with HFD for 12 weeks and compared with rats fed normal diet.

2.2. Experimental Diets and Design. After acclimatization for 2 weeks, 28 rats were randomly allocated into four groups, with 7 rats in each group: (i) normal control group: animals fed with normal chow (12% of calories as fat) throughout the experimental period of 12 weeks; (ii) cinnamon group: rats fed with normal chow and administered with cinnamon polyphenol (100 mg/kg b.wt.) throughout the experimental period of 12 weeks; (iii) HFD group: rats fed with high-fat diet [42% of calories as fat] throughout the experimental period of 12 weeks; and (iv) HFD + cinnamon group: rats fed a high-fat diet and administered with cinnamon polyphenol throughout the experimental period of 12 weeks. Rats were orally treated with cinnamon polyphenol extract [100 mg/kg b.wt. dissolved in 5% dimethyl sulfoxide (DMSO)] daily by oral gavage in olive oil (1 ml/kg b.wt./day) to the end of the experiment. The amount of cinnamon polyphenol extract used was based upon an earlier study presenting a positive result of 100 mg of cinnamon per kilogram on diabetic rats [24]. The control rats in this study received similar amounts of sunflower oil by gavage. Cinnamon product (Product Code: 33002; Lot Number: CINP10001b) obtained from *Cinnamomum zeylanicum* by the aqueous-alcoholic extraction used in this study was provided by OmniActive Health Technologies Ltd. (Pune, India). The test compound contains 18.41% total polyphenols and it is light to dark reddish brown free flowing powder with an astringent taste. The quality of cinnamon polyphenol extract was confirmed to comply with strict quality control measures and found free of endotoxin and heavy metals.

At the end of the study, the blood was collected by cardiac puncture after an overnight fast and all rats were sacrificed by cervical dislocation. The visceral fat and liver samples were removed and weighed after sacrificing the animals.

2.3. Biochemical Estimations. Serum was prepared by centrifuging the blood at 3,000 ×g for 10 minutes and used for biochemical parameters and malondialdehyde (MDA) analyses. Sera samples were prepared by centrifuging the blood at 3,000 ×g for 10 min and used for the analyses of biochemical parameters and MDA. Serum parameters were determined using an automated analyzer (Samsung LABGEOPT10, Samsung Electronics Co., Suwon, Korea). Repeatability and device/method exactness of LABGEOPT10 were documented according to the IVR-PT06 guideline. The concentration of serum insulin was measured with the Rat Insulin kits (Linco Research Inc., St. Charles, MO, USA) by ELISA (Elx-800, Bio-Tek Instruments Inc., Vermont, USA). The sensitivity of the assays for insulin was 0.36 ng/ml. The interassay and intra-assay coefficients of variation were 5.3% and 7.5% for insulin. Liver MDA levels were determined according to the method described by Karatepe [25] by HPLC with a Shimadzu UV-Vis SPD-10 AVP detector, a CTO-10 AS VP column, and a mobile phase comprised of 30 mM KH_2PO_4 and methanol (82.5 : 17.5, v/v, pH 3.6) at a flow rate of 1.2 ml/min. Column effluents were monitored at 250 nm. Liver homogenate (10%, w/v) was prepared in 10 mM phosphate buffer (pH 7.4) and centrifuged at 13,000 ×g for 10 minutes at 4°C. The resulting supernatant was collected and kept at −80°C for MDA estimation.

Total antioxidant capacity (TAC) was determined by dark blue-green color reduction 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonate (ABTS) by antioxidants to its colorless form via the antioxidants in the sample [26]. In this analysis, ABTS is incubated with potassium persulfate to produce ABTS oxidation. Briefly, 10 mg of ABTS was dissolved in 10 mL of an aqueous solution containing 2.5 mmol/L potassium persulfate and allowing the mixture to stand in the dark at room temperature for one to four hours before use. For the study of samples, ABTS oxidized stock solution was diluted with deionized water to an absorbance of 0.70 at 734 nm. After addition of 1 mL diluted ABTS with 10 μL of serum oxidized, the absorbance readout was taken ten minutes after the first mixing. The results were expressed in mmol Trolox E/L.

Activity of total superoxide dismutase (SOD) in the homogenized liver tissue (in 20 mM HEPES (N-2 hydroxyethyl piperazine-N′-2-ethanesulfonic acid) buffer, 1 mM

Table 1: Composition of diets (g/kg diet) fed to rats.

	Control	HFD
Casein	200.0	200.0
Starch	579.5	150.0
Sucrose	50.0	149.5
Soybean oil	70.0	—
Beef tallow	—	400.0
Cellulose	50.0	50.0
Vitamin-mineral premix*	45.0	45.0
L-Cysteine	3.0	3.0
Choline bitartrate	2.5	2.5

*The vitamin-mineral premix provides the following (per kg): all-*trans*-retinyl acetate, 1.8 mg; cholecalciferol, 0.025 mg; all-*rac*-a-tocopherol acetate, 12.5 mg; menadione (menadione sodium bisulfate), 1.1 mg; riboflavin, 4.4 mg; thiamine (thiamine mononitrate), 1.1 mg; vitamin B-6, 2.2 mg; niacin, 35 mg; Ca-pantothenate, 10 mg; vitamin B-12, 0.02 mg; folic acid, 0.55 mg; *d*-biotin, 0.1 mg; manganese (from manganese oxide), 40 mg; iron (from iron sulfate), 12.5 mg; zinc (from zinc oxide), 25 mg; copper (from copper sulfate), 3.5 mg; iodine (from potassium iodide), 0.3 mg; selenium (from sodium selenite), 0.15 mg; choline chloride, 175 mg.

ethylene glycol tetraacetic acid, 210 mM mannitol, and 70 mM sucrose, pH 7.2, per g of tissue) was determined by a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The supernatant was collected after centrifugation at 12.000g for 20 min at 4°C. The supernatant was purified from the salt by passing through a Sephadex G-25 column. The samples were also treated with a mixture of ethanol-chloroform (2 : 1, v/v) and distilled water to remove hemoglobin and red blood cells and the absorbance plate was read on a reader (Bio-Tek Instruments, Inc., Vermont, USA) at 450 nm. The results were expressed as units per mg protein (U/mg protein) using standard calibration curve. Catalase (CAT) activity was also determined in homogenized tissue (50 mM potassium phosphate, 1 mM EDTA, pH 7, in cold buffer, per tissue) using a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The supernatant was collected after centrifugation at 12,000g for 20 minutes at 4°C. A formaldehyde solution was used as standard. The absorbance of standard and samples was taken at 540 nm using a plate reader (Bio-Tek Instruments, Inc. Vermont, USA). Catalase activity was expressed as nmol/min/mg protein using standard calibration curve. The activity of glutathione peroxidase (GSHPx) was analyzed according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Liver tissue was homogenized with the Polytron Homogenizer in cold buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM dithiothreitol) per tissue and then subjected to centrifugation at 10,000g for 15 minutes at 4°C. This method is based on the oxidation of NADPH to $NADP^+$, which is accompanied by an absorbance drop at 340 nm and GSHPx activity was measured by initiating the reaction with 2.4 mM cumene hydroperoxide. One unit is defined as the amount of enzyme that oxidizes 1 μmol of NADPH per min at 25°C. The absorbance was read every minute at 340 nm using a plate reader (Bio-Tek Instruments, Inc., Vermont, USA) to obtain at least 5 time points. The GSHPx activity was calculated in nmol/min/mg of protein using standard calibration curve.

2.4. Western Blot Analyses. Protein extraction was performed by standardizing the liver in 1 ml of ice-cold hypotonic buffer (buffer A) containing 10 mM HEPES (2-(4-(2-hydroxyethyl)-1-piperazinyl) ethane sulfonic acid, PH 7.8, 10 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for Western blot analysis. The homogenate was mixed with 80 μl of 10% Nonidet P-40 (NP-40) solution and then centrifuged at 14,000 ×g for 2 minutes. The precipitates were washed once with 500 μL buffer A and 40 μL 10% NP-40, centrifuged, and resuspended in a 200 μL buffer containing 50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 20% glycerol) and recentrifuged at 14,800 ×g for 5 min. The concentration of the protein was determined according to the procedure described by Lowry using a protein assay kit (Sigma, St. Louis, MO, USA). The supernatant was collected and used for the determination of SREBP-1c, LXRs, ACLY, FAS, NF-κB p65, PPARα, p-IRS-1, Nrf-2, and HO-1 according to the previously described method [27]. Briefly, 50 μg of proteins was electrophoresed and then transferred to a nitrocellulose membrane (Schleicher and Schuell Inc., Keene, NH, USA). The phosphorylated form of antibodies against SREBP-lc, LXRs, ACLY, FAS, NF-κB p65, PPARα, p-IRS-1, Nrf-2, and HO-1 (Abcam, Cambridge, UK) was diluted (1 : 1000) in the same buffer containing 0.05% Tween-20. Protein loading was controlled using monoclonal mouse antibody against β-actin (A5316; Sigma). The bands were examined densitometrically using ImageJ, an image analysis system (National Institute of Health, Bethesda, USA).

2.5. Statistical Analysis. Data were stated as mean ± SE. The alteration among groups was analyzed using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test (SAS Institute: SAS User's Guide: Statistics), and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Cinnamon Extract on Body Weight and Visceral Fat in HFD-Fed Rats. The effect of cinnamon polyphenol extract treatment on the final body weight, feed consumption, and visceral fat and liver mass was shown in Table 2. HFD feeding increased final body weight, visceral fat, and liver weight by 33.1%, 258.3%, and 34.8% and decreased feed intake by 16.9% as compared to the control rats ($P < 0.001$). Although the cinnamon polyphenol extract treatment decreased body weight, visceral fat, and liver weight by 8.4%, 36.6%, and 17.7% in the HFD-fed rats ($P < 0.001$), the HFD-fed rats treated with cinnamon still had a final body weight and visceral fat higher than those of the control rats ($P < 0.05$). No significant difference was found in the feed intake between HFD-fed rats and HFD-fed rats treated with cinnamon polyphenol extract ($P > 0.05$).

Table 2: Effect of cinnamon polyphenol extract supplementation on the body weight, visceral fat, and the liver weight in rats fed with HFD for 12 weeks.

Item	Groups			
	Control	Cinnamon	HFD	HFD + cinnamon
Final BW (g)	301.43 ± 5.27^{C}	298.86 ± 6.50^{C}	401.29 ± 5.07^{A}	367.71 ± 2.75^{B}
Feed intake (g/d)	22.77 ± 0.44^{A}	22.96 ± 0.40^{A}	18.93 ± 0.44^{B}	19.91 ± 0.41^{B}
Visceral fat (g)	6.62 ± 0.45^{C}	6.35 ± 0.33^{C}	23.72 ± 1.58^{A}	15.04 ± 0.48^{B}
Liver (g)	11.63 ± 0.25^{C}	11.79 ± 0.42^{C}	15.68 ± 0.38^{A}	12.90 ± 0.38^{B}

HFD, high-fat diet; data are expressed as mean ± SEM of 7 rats from each group. A, B, and C: means in the same row with different superscripts are significant ($P < 0.05$).

Table 3: Effects of cinnamon polyphenol extract biochemical parameters levels in rats fed with HFD for 12 weeks.

Item	Groups			
	Control	Cinnamon	HFD	HFD + cinnamon
Glucose (mg/dl)	75.86 ± 2.62^{C}	76.57 ± 3.34^{C}	200.86 ± 3.97^{A}	158.43 ± 2.07^{B}
Insulin (ng/mL)	1.61 ± 0.04^{C}	1.55 ± 0.04^{C}	8.21 ± 0.29^{A}	4.47 ± 0.23^{B}
FFA (mM)	1.74 ± 0.11^{C}	1.48 ± 0.06^{C}	5.03 ± 0.14^{A}	2.25 ± 0.07^{B}
T-C (mg/ml)	66.51 ± 5.58^{B}	53.26 ± 1.90^{B}	91.71 ± 2.28^{A}	61.43 ± 1.81^{B}
HDL-C (mg/dl)	15.29 ± 0.57^{BC}	13.57 ± 0.77^{C}	22.57 ± 0.53^{A}	18.57 ± 0.43^{AB}
TG (mg/dl)	25.86 ± 1.26^{C}	24.14 ± 1.81^{C}	57.57 ± 2.08^{A}	41.85 ± 1.49^{B}
AST (U/L)	146.413 ± 4.40	142.71 ± 4.30	157.00 ± 8.28	154.22 ± 5.38
ALT (U/L)	83.65 ± 6.59	81.86 ± 4.18	89.14 ± 4.39	86.43 ± 5.16

HFD, high-fat diet; FFA, free fatty acids; T-C, total cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine transferase. Data are expressed as mean ± SEM of 7 rats from each group. A, B, and C: means in the same row with different superscripts are significant ($P < 0.05$).

3.2. Effect of Cinnamon Extract on Biochemical Parameters in HFD-Fed Rats. Table 3 shows the effect of cinnamon polyphenol extract on supplementation on carbohydrate and lipid profile in HFD-fed rats. As seen in the table, HFD feeding increased the serum levels of glucose and insulin, free fatty acid (FFA), total cholesterol, HDL-C, and LDL-C, as well as TG in HFD rats ($P < 0.001$). The hypertriglyceridemia and elevated lipid indicators in HFD-fed rats were reduced with cinnamon polyphenol extract supplementation. HFD did not cause a significant increase in aspartate transaminase (AST) and alanine transaminase (ALT) in the duration of the treatment, and the levels remained more or less unaffected in cinnamon polyphenol extract supplemented rats ($P > 0.05$).

3.3. Effect of Cinnamon Extract on Antioxidant Status in HFD-Fed Rats. Serum and liver MDA levels increased by 158.8% and 81.7% ($P < 0.001$; Table 4) and serum TAC, liver SOD, CAT, and GSHPx decreased by 67.6, 54.7, 34.4%, and 56.4% upon obesity induction. The cinnamon polyphenol extract treatment caused 23.3% and 25.4% reduction in serum and liver MDA concentration and elevation in serum TAC, liver SOD, CAT, and GSHPx of 91.2, 62.6%, 21.9%, and 36.0% in the HFD-fed rats ($P < 0.001$), which was like the control group ($P > 0.05$).

3.4. Effect of Cinnamon Extract on Protein Levels in HFD-Fed Rats. SREBP-1c, LXRs, ACLY, and FAS expression in the HFD-fed rats increased by 75.1%, 98.7%, 106.0%, and 81.7% in liver (Figure 1), respectively ($P < 0.0001$ for all). SREBP-1c, LXRs, ACL, and FAS expression decreased by 18.1%, 27.9%, 22.7%, and 15.8%, respectively ($P < 0.05$ for all), when the HFD rats were treated with cinnamon polyphenol extract. All remained lower as compared to the control rats ($P > 0.05$ for both).

PPARα and IRS expression in liver in the HFD group were 71.3% and 67.0% lower than those in the control group (Figure 2; $P < 0.001$ for both). Despite the respective 1.72- and 1.73-fold increase in PPARα (Figure 2(a)) and IRS (Figure 2(b)) expression with cinnamon polyphenol extract treatment ($P < 0.001$ for both), PPARα and IRS expression levels still remained lower compared to the control group ($P < 0.001$ for both).

Expression of NF-κB increased by 92.2% in the liver in the HFD rats (Figure 3(a); $P < 0.001$). The cinnamon polyphenol extract treatment partially restored NF-κB expression levels in liver (by 23.3%; $P < 0.05$; Figure 3(a)) as compared to the control group. The induction of obesity was associated with 68.7 and 63.0% reduction in expression of Nrf2 and HO-1 in liver ($P < 0.001$; Figures 3(b) and 3(c)), respectively. The cinnamon polyphenol extract treatment partially elevated the expression of Nrf2 and HO-1 in the liver (by 111.7% and 72.1%; $P < 0.001$; Figures 3(b) and 3(c)).

4. Discussion

High-fat dietary intake leads to insulin resistance (IR) and altered glucose and lipid metabolism [28]. Cinnamon polyphenols can respond to IR and are therefore useful

Table 4: Effects of cinnamon polyphenol extract on oxidative stress and antioxidant status rats fed with HFD for 12 weeks.

Item	Groups			
	Control	Cinnamon	HFD	HFD + cinnamon
Serum MDA (nmol/mL)	0.68 ± 0.04^{C}	0.64 ± 0.03^{C}	1.76 ± 0.03^{A}	1.35 ± 0.03^{B}
Liver MDA (nmol/mg protein)	1.97 ± 0.06^{C}	1.90 ± 0.13^{C}	3.58 ± 0.09^{A}	2.67 ± 0.04^{B}
Serum TAC (nmol Trolox Equiv. per mg protein)	1.76 ± 0.06^{A}	1.88 ± 0.12^{A}	0.57 ± 0.07^{C}	1.09 ± 0.10^{B}
Liver SOD (U/mg protein)	202.29 ± 5.80^{A}	206.14 ± 7.31^{A}	91.71 ± 3.98^{C}	149.14 ± 2.03^{B}
Liver CAT (U/mg protein)	349.86 ± 11.97^{A}	353.85 ± 14.06^{A}	229.57 ± 4.30^{C}	279.82 ± 9.62^{B}
Liver GSHPx (U/mg protein)	53.67 ± 4.25^{A}	54.86 ± 3.56^{A}	23.42 ± 2.56^{C}	31.86 ± 2.48^{B}

HFD, high-fat diet; MDA, malondialdehyde; TAC, total antioxidant capacity; SOD, superoxide dismutase; CAT, catalase; GSHPx, glutathione peroxidase. Data are expressed as mean ± SE of 7 rats from each group. A, B, and C: means in the same row with different superscripts are significant ($P < 0.05$).

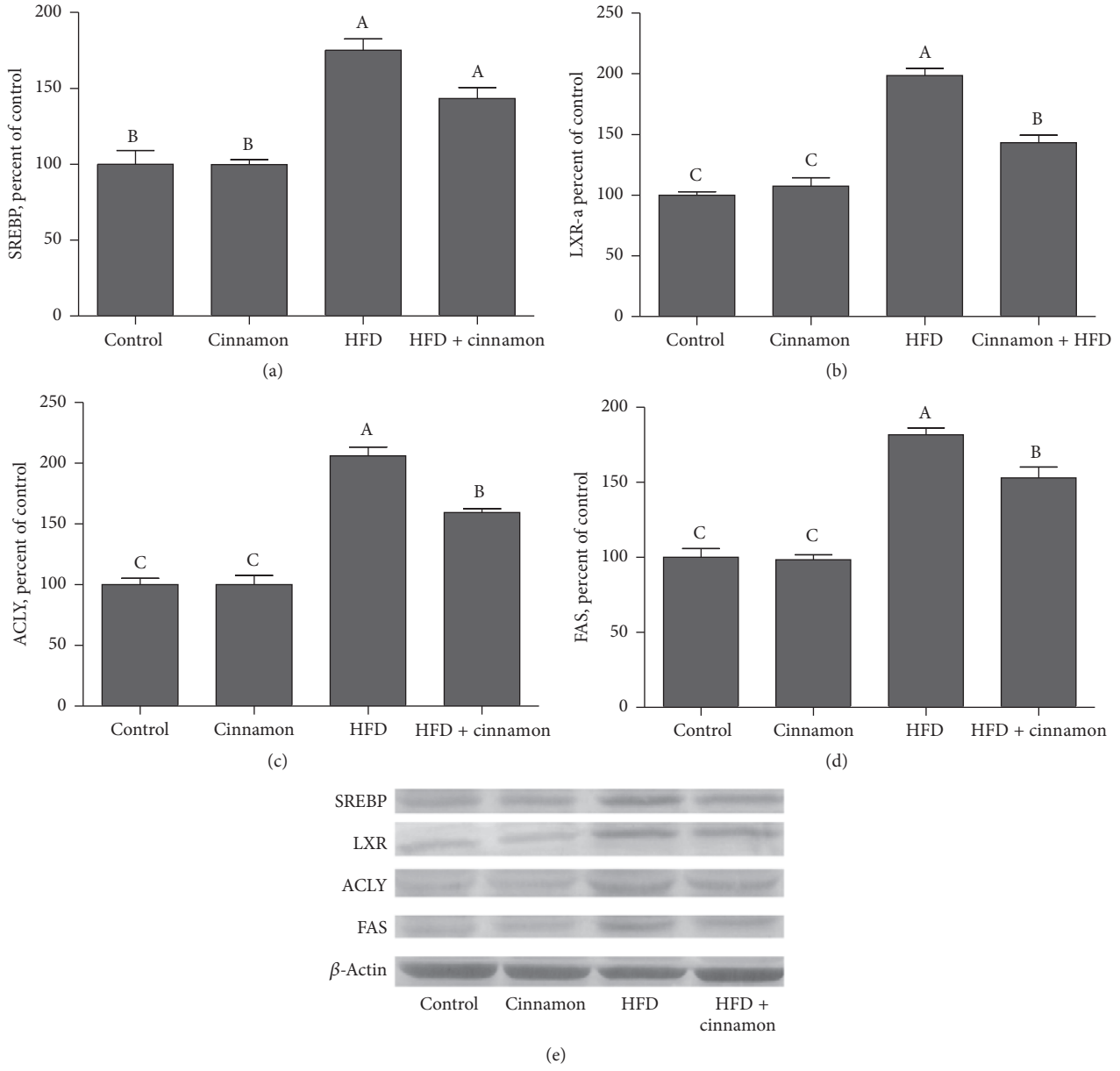

Figure 1: Hepatic SREBP-1c, LXRs, ACLY, and FAS expression levels in cinnamon polyphenol supplemented high-fat diet- (HFD-) fed rats and control groups. (a)–(d) show the expression level of SREBP-1c, LXRs, ACLY, and FAS in various groups. The intensity of the bands shown in (e) was quantified by densitometric analysis. Data are expressed as a ratio of normal control value (set to 100%). Each bar represents the mean and standard error of mean. Blots were repeated at least 3 times ($n = 3$) and only a representative blot is shown in (e). β-Actin was included to ensure equal protein loading.

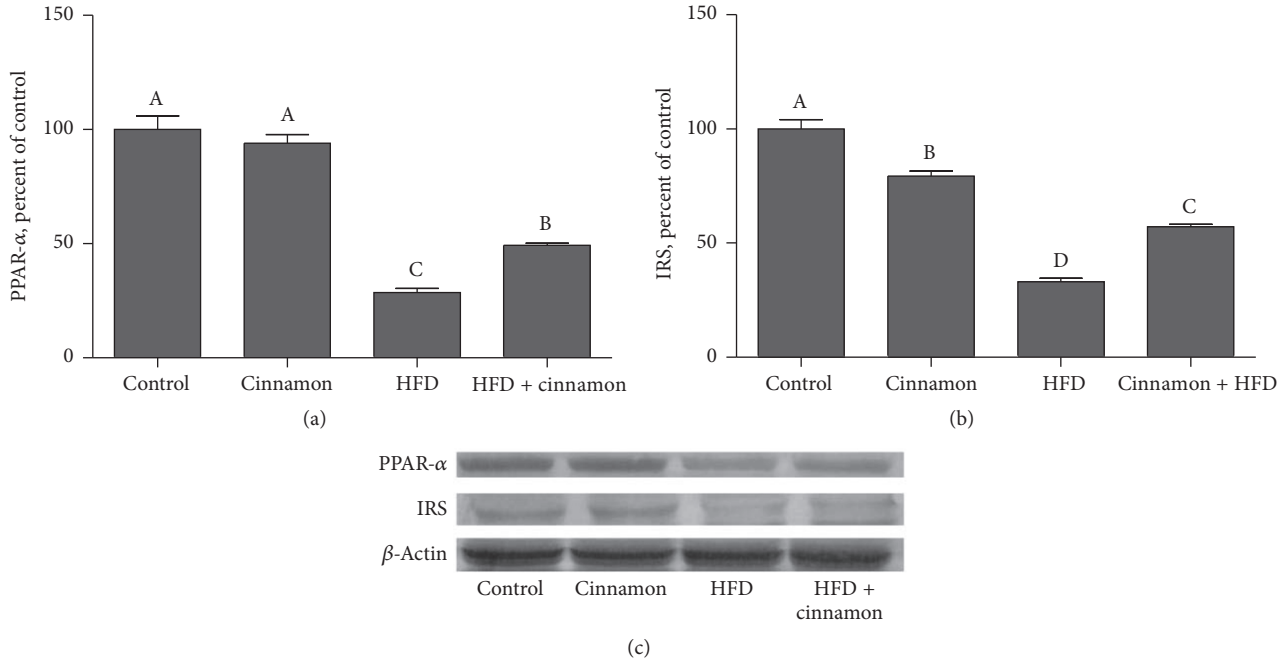

Figure 2: Hepatic PPARα and IRS expression levels in cinnamon polyphenol supplemented high-fat diet- (HFD-) fed rats and control groups. (a) and (b) show the expression level of PPARα and IRS in the groups. The intensity of the bands shown in (c) was quantified by densitometric analysis. Data are expressed as a ratio of normal control value (set to 100%). Each bar represents the mean and standard error of mean. Blots were repeated at least 3 times ($n = 3$) and only a representative blot is shown in (c). β-Actin was included to ensure equal protein loading.

because of their insulin-enhancing and antioxidant properties [29]. Cinnamon extracts have been recognized as in vitro and in vivo insulin sensitizers [22, 30]. The adverse effects of HFD/HFD on brain insulin signal changes were alleviated by the use of cinnamon, which suggests that cinnamon is associated with whole body insulin sensitivity and related changes, including hippocampal synaptic plasticity and cognition in the brain of neuroprotective effects [31, 32]. Consistent with previous studies, our results demonstrated that cinnamon polyphenol extract supplementation improved body weight, visceral fat, and carbohydrate metabolism including glucose, insulin, and free fatty acid and lipid profiles (TC, TG, and HDL-C) and lipid peroxidation and antioxidant enzymes in the HFD-fed rats [19, 33–36]. Qin et al. [28] reported that cinnamon polyphenol extract increased the use of insulin-regulated glucose in rats. In addition, Mang et al. [21] reported that cinnamon prevents IR by partially increasing insulin signaling pathway with high fructose diet.

Cinnamon extracts have also been shown to be useful in decreasing fasting plasma glucose, cholesterol, and triglycerides in diabetic patients [37]. Similarly, application of cinnamon extract reduced liver MDA levels in carbon tetrachloride-poisoned rats and improved SOD, CAT, and GSHPx activities [38]. Cinnamon has been shown to prevent hyperlipidemia and improved glucose tolerance in rats fed fructose/high fat [22, 39]. However, a direct association between cinnamon polyphenol intake and regulated SREBP-1c, LXRs, ACLY, and FAS expression by cinnamon polyphenol in the HFD-fed rats has yet to be established. Previous studies have shown that SREBP-1c has a regulatory role in the synthesis of lipogenic enzymes such as FAS, which inhibits TG accumulation in the liver, in fatty acid synthesis and lipid metabolism [8]. LXRs are also transcription factors that regulate fatty acid and cholesterol homeostasis and are expressed mainly in the liver and other tissues involved in lipid metabolism [40]. ACLY play a crucial role in obesity-related complications in glucose and lipid homeostasis of mice liver [9]. An animal study has shown activation of LXR protection effects in obesity induced by high-fat diet [41]. In the present study, we demonstrated for the first time that cinnamon polyphenol intake significantly reduced the expression of hepatic SREBP-1c, LXRs, ACLY, and FAS. There are no earlier studies associated with examining the effects of cinnamon polyphenol treatment on the expression of SREBP-1c, LXRs, ACL, and FAS in rats fed HFD to compare with this study. Nevertheless, it was reported that cinnamon prevented the hyperlipidemia in fructose-fed rats and improved glucose tolerance [39].

Peroxisome proliferator-activated receptors (PPARs), transcriptional factors complicated in the modulation of IR and adipogenesis, play key roles in regulating carbohydrate and lipid metabolism [42]. Activation of PPAR reduces serum triglycerides and raises serum HDL-cholesterol concentrations [43], whereas activation of PPARγ increases insulin sensitivity and causes antidiabetic effects [44]. IRS-1 plays an essential role in the pathway of insulin-stimulated signal transduction and binds the insulin receptor to its ultimate biological activities by a series of intermediates [45]. In a prior report, we showed that HFD in diabetic rats decreased PPARγ expression in the adipose tissue and reduced expression of

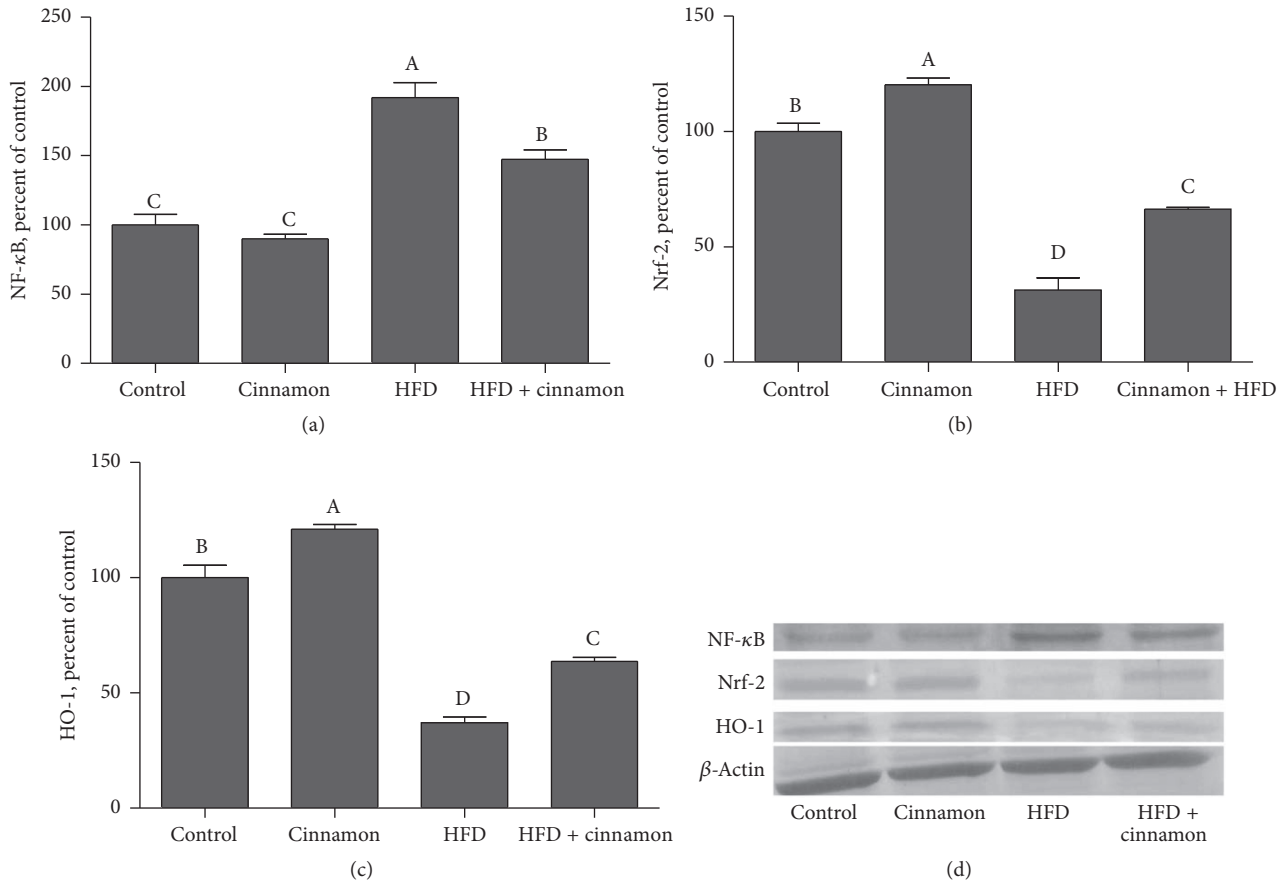

Figure 3: Hepatic NF-κB p65, Nrf2, and HO-1 expression levels in cinnamon polyphenol supplemented high-fat diet- (HFD-) fed rats and control groups. (a)–(c) show the expression level of NF-κB p65, Nrf2, and HO-1 in the groups. The intensity of the bands shown in (d) was quantified by densitometric analysis. Data are expressed as a ratio of normal control value (set to 100%). Each bar represents the mean and standard error of mean. Blots were repeated at least 3 times ($n = 3$) and only a representative blot is shown in (d). β-Actin was included to ensure equal protein loading.

IRS-1 in the liver and kidney [46]. In this study, cinnamon polyphenol increased PPARα and IRS expression in the liver; this may have potential insulin sensitizing effect and may increase IR in a rat obesity model. In accordance with our findings, Sheng et al. [42] showed that the cinnamon extract could induce expression of PPARγ and PPARα both in vitro and in vivo in mouse adipose cells. Similarly, Qin et al. [47] found that cinnamon extract supplementation resulted in reduced expression of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) mRNA while increasing expression of IR, IRS1, and IRS2 in hamster enterocytes.

NF-κB is a transcription factor that is responsible for controlling a DNA transcription and comprises cellular responses to various stimuli including free radicals. Kuhad and Chopra [48] reported that the signal transduction pathway for the activation of transcription factor NF-κB was evoked by reactive oxygen species associated with hyperglycemia and by advanced glycosylated end products [48]. In the situation of oxidative stress and numerous cytokines, NF-κB is quickly released from IκB in order to stimulate the expression of chemotactic and matrix proteins of various cytokines involved in inflammation, immunological responses, and/or proliferation [49]. In the present study, cinnamon polyphenol reduced liver of NF-κB expression in rats fed HFD (Figure 3). In a previous study, we have reported that HFD consumption enhances inflammation and NF-κB activation [50]. Fan et al. [51] showed that activity of NF-κB increased in rats fed HFD. But there was no earlier study studying the effects of cinnamon polyphenol on the NF-κB p65 in the liver with which to compare this study. Nevertheless, in a previous study, it was shown that cinnamon-based treatment induced inhibition of NF-κB and neuroinflammation and supported our present findings [52].

Another important mechanism contributing to cinnamon antiobesity is the upregulation of antioxidant-dependent proteins. We found that expression of the proteins Nrf2 and HO-1 increased in HFD rats with cinnamon intake, indicating that this antioxidant mechanism may underlie reduced levels of lipid peroxidation in liver tissues. Nrf2 transcription factor is one of the most important antioxidant defense mechanisms that protect cells and tissues from various oxidative stresses [53]. Specifically, Nrf2 induces the expression of genes encoding antioxidant proteins, including HO-1, by binding to the antioxidant response element [54].

HO-1 is reported to be a highly effective therapeutic target for protection against oxidative stress and damage. HO-1 also is one of phase II detoxifying enzymes and exerts a strong antioxidant effect, and it is regulated by the redox-sensitive transcription factors. In addition, Nrf2 likely interferes with lipogenic and cholesterolemic pathways, inhibiting lipid accumulation and oxidative stress in the mouse liver after administration of HFD [55]. In the current study, cinnamon polyphenol d increased Nrf2 and HO-1 expression in liver of rats fed by HFD (Figure 3). Tuzcu et al. [50] showed similar reductions in Nrf2 and HO-1 expressions as increased serum MDA in HFD-fed rats. In addition, cinnamaldehyde, an important flavor component in cinnamon essential oil upregulated Nrf2 expression, stimulated its translocation to the nucleus, and increased HO-1, NQO1, CAT, and GPx1 expression under high glucose conditions [7]. Wondrak et al. [56] reported that cinnamaldehyde and cinnamon extract upregulated cellular protein levels of Nrf2 in human colon cancer cells and recognized Nrf2 targets involved in the antioxidant response including HO-1 and gamma-glutamyl-cysteine synthetase.

5. Conclusions

In conclusion, cinnamon polyphenol has been reported to have several beneficial effects on obesity through the modulation of transcription factors including SREBP-1c, LXRs, NF-κB, and Nrf2 and several enzymes such as ACLY and FAS and insulin resistance, glucose, and lipid metabolism and antioxidant status. Cinnamon polyphenol may have a potential use in the management of hyperglycemia and hyperlipidemia.

Disclosure

Vijaya Juturu is employee of OmniActive Health Technologies Inc. (NJ, USA).

Conflicts of Interest

Authors have no competing conflicts of interest.

Acknowledgments

This study was sponsored by OmniActive Health Technologies Inc. (NJ, USA). This work was also supported in part by the Turkish Academy of Sciences.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 2163053, 14 pages
https://doi.org/10.1155/2017/2163053

Review Article

A Preclinical Systematic Review of Ginsenoside-Rg1 in Experimental Parkinson's Disease

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Received 23 November 2016; Revised 24 January 2017; Accepted 14 February 2017; Published 13 March 2017

Academic Editor: Elena Azzini

To date, no drug has been proven to be neuroprotective or disease-modifying for Parkinson's disease (PD) in clinical trials. Here, we aimed to assess preclinical evidence of Ginsenosides-Rg1 (G-Rg1), a potential neuroprotectant, for experimental PD and its possible mechanisms. Eligible studies were identified by searching six electronic databases from their inception to August 2016. Twenty-five eligible studies involving 516 animals were identified. The quality score of these studies ranged from 3 to 7. Compared with the control group, two out of the 12 studies of MPTP-induced PD showed significant effects of G-Rg1 for improving the rotarod test ($P < 0.01$), two studies for improving the swim-score values ($P < 0.01$), six studies for improving the level of TH protein expression ($P < 0.01$), and two studies for increasing the expression of TH mRNA in the substantia nigra of mice ($P < 0.01$). The studies reported that G-Rg1 exerted potential neuroprotective effects on PD model through different mechanisms as antineuroinflammatory activities ($n = 10$), antioxidant stress ($n = 3$), and antiapoptosis ($n = 11$). In conclusion, G-Rg1 exerted potential neuroprotective functions against PD largely by antineuroinflammatory, antioxidative, and antiapoptotic effects. G-Rg1 as a promising neuroprotectant for PD needs further confirmation by clinical trials.

1. Introduction

Parkinson's disease (PD) is the second most frequent neurodegenerative disease after Alzheimer's disease characterized by the loss of dopamine-containing cells in the substantia nigra (SN) [1]. The clinical symptoms of PD are a wealth of motor symptoms and nonmotor symptoms. The treatment is divided into two directions: symptomatic therapy for motor symptoms and modifying the underlying disease process through neuronal protection or restoration. However, current treatments of PD are mainly symptomatic therapies and no treatment has yet been proven to be truly neuroprotective [2]. Dopamine replacement therapy (L-DOPA and dopamine agonists) is still the most effective symptomatic treatment of PD, but this treatment frequently induces therapy-related motor complications such as dyskinesia, choreoathetosis, and fluctuations in motor function [3]. Thus, a number of PD patients resort to various kinds of complementary or alternative medicine (CAM) to improve their motor and/or nonmotor symptoms [4]. Traditional Chinese medicine (TCM), as one of the most important parts in CAM, has played a vital role in the medical care of PD patients for thousands of years [5]. Ginseng, the root of *Panax* species (C.A. Meyer Araliaceae), is a well-known traditional Chinese herbal medicine that has been used for various kinds of diseases in China, Japan, and Korea for thousands of years and is still a popularly and worldwide used natural medicine in modern time [6]. The major pharmacologically active ingredients of ginseng are Ginsenosides and they are responsible for most of the activities of ginseng [7]. Ginsenosides are divided into two categories as follows: (1) the protopanaxadiol (PPD) type: Rb1, Rb2, Rb3, Rc, Rd, and Rg3; (2) the protopanaxatriol (PPT) type: Rg1, Re, Rf, and Rg2 [8]. It has been reported that Ginsenosides-Rg1 (G-Rg1) might have neuroprotective

effects and little toxicity both in vitro and in vivo [9]. It also has beneficial effects on many neurological conditions, including the progressive neurodegenerative diseases such as PD [10]. The mechanisms of the neuroprotective effect of G-Rg1 include potentiating nerve growth factors, increasing anti-inflammation, antioxidation, and antiapoptosis, inhibiting excite toxicity and Ca^{2+} overinflux into neurons, maintaining cellular ATP levels, and preserving structural integrity of neurons [11]. However, no systematic review has been conducted to assess the effect of G-Rg1 on experimental PD models to date. Systematic review of all available evidence from animal experiments before clinical trials can provide us adequate interpretation of the limitations and potential of a novel treatment strategy [12]. Therefore, in the present study, we conducted a systematic review of all available animal studies to evaluate the preclinical evidence of G-Rg1 for experimental PD.

2. Methods

2.1. Search Strategy. Two trained researchers independently searched studies on the effects of G-Rg1 on PD from their inception to August 2016 in the following databases: PubMed, the Cochrane Database, Excerpta Medica (EMBASE), Chinese National Knowledge Infrastructure (CNKI), Wanfang database, and VIP Information Database. The following search terms were used: (Ginseng OR Ginsenoside OR Ginsenoside-Rg1 OR G-Rg1 OR Ginseng saponin) AND (Parkinson disease OR Parkinson's disease OR PD) in both English and Chinese.

2.2. Inclusion Criteria. Studies that were included met all of the following criteria: (1) all studies should test the effect of G-Rg1 on animal models of PD, regardless of language, blinding, or publication status; (2) in the treatment group, any intervention that used G-Rg1 for PD should be included irrespective of the frequency, dose, the method of injection, and intensity; (3) in the control group, animals were treated with normal saline or nothing.

2.3. Exclusion Criteria. Prespecified exclusion criteria were the following: (1) not reporting the efficacy of G-Rg1 on animal experiment of PD; (2) another neuroprotective agent being administered in the treatment group in addition to G-Rg1; (3) no control group; (4) reviews, case reports, abstracts, letters, comments, study protocol, editorials, and clinical guidelines; (5) duplicate publication.

2.4. Outcome Measurements. The primary outcome of the interest was the behavioral assessments, including rotarod test, pole test, wire suspension test, and the values of swim-score. Secondary outcomes were the number of Tyrosine Hydroxylase- (TH-) positive dopamine neurons in the substantia nigra pars compacta (SNpc), levels of TH protein expression in the SNpc, and the mechanisms of G-Rg1.

2.5. Data Extraction. Two investigators independently extracted information from each study, including (1) the first author's name and publication year; (2) individual data obtained for experimental animals including species, sex, number, weight, and anesthetic used; (3) experimental model; (4) information on treatment group including route of administration, dosage, and time for treatment; (5) data of control groups extracted as well as route of administration, dosage, and time of administration; (6) intergroup difference of each outcome measure; (7) outcome measures, including the behavioral exhibition of PD animal models, the number of TH neurons, and possible mechanisms of neuroprotective effects of G-Rg1 against PD. If outcomes were presented at different time points, data from the last time point were extracted. If the outcome data for meta-analysis were only expressed graphically or missing, we made attempt to contact authors for further information. When a response was not received, we used digital ruler software to measure the data from the graphs. We extracted data of mean value and standard deviation for each comparison from every study. Any disagreements were resolved through consultation with a corresponding author (Guo-qing Zheng).

2.6. Quality Assessment. The methodological quality of the included studies was assessed based on a nine-item modified scale from the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) [13]. The modified CAMARADES includes the following criteria: (1) peer reviewed publication; (2) control of temperature; (3) random allocation to groups; (4) blinded assessment of behavioral outcome; (5) use of anesthetic without significant intrinsic neuroprotective activity; (6) calculation of the sample size necessary to achieve sufficient power; (7) appropriate animal model which uses animals without relevant comorbidities (aged, diabetic, or hypertensive); (8) compliance with animal welfare regulations; (9) statement of potential conflict of interests. For quality assessment score, the interquartile range of score across studies was reported.

2.7. Statistical Analysis. We conducted statistical analysis using Cochrane's Review Manager (version 5.3) software. Data extracted from each study were considered as continuous data. WMD (weighted mean difference) is a standard statistic that measures the absolute difference between the mean values in two groups. Meanwhile, standardized mean difference (SMD) is also used as a summary statistic in meta-analysis when all the studies assess the same outcome but measure it in a variety of ways [14]. Heterogeneity among studies was estimated using Cochran's Q test (reported with χ^2 value and P value) and I^2 statistic. I^2 values of 75, 50, and 25% correspond to high, medium, and low levels of heterogeneity, respectively; I^2 values less than 50% indicated an acceptable degree of heterogeneity between studies [15]. Probability values of 0.05 were considered significant. Sensitivity analyses omitting each study at a time from the original analysis were conducted to verify our main results to be robust.

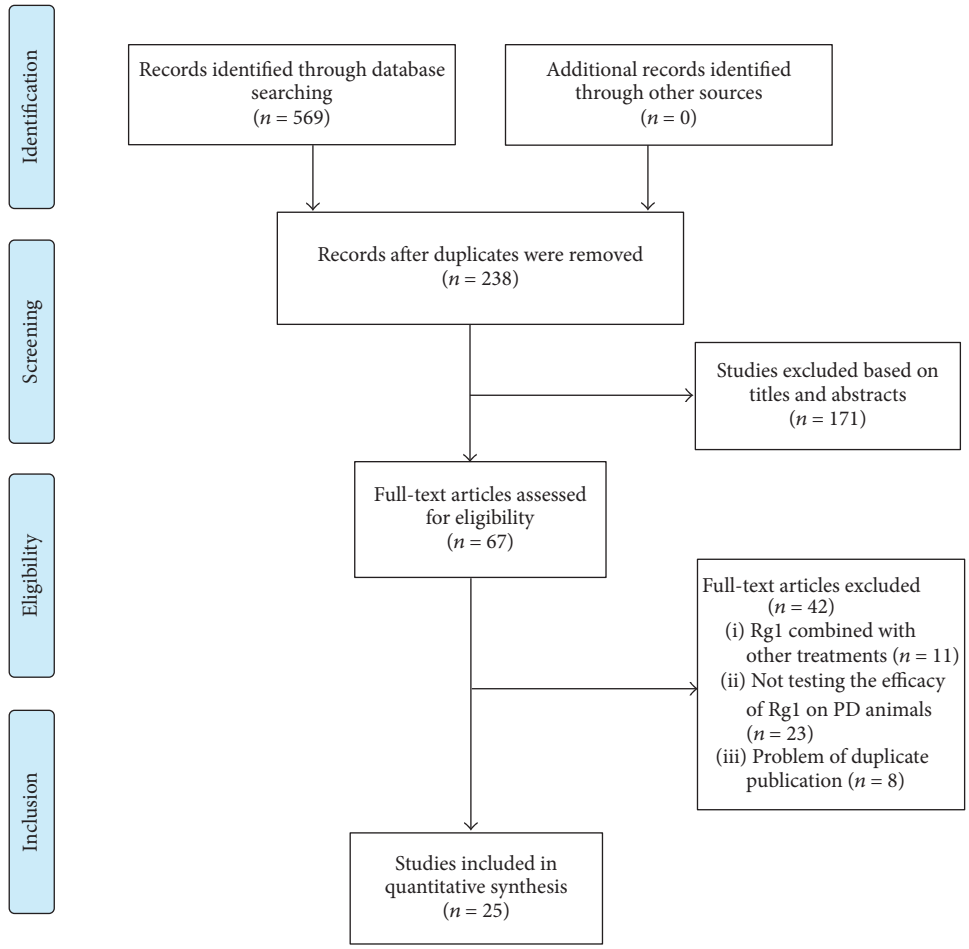

Figure 1: Summary of the process for identifying candidate studies.

3. Results

3.1. Description of Studies. We identified 569 potentially relevant articles from six electronic databases. After removing duplicates, 238 references remained. Through screening titles and abstracts, 171 studies were excluded. After full-text evaluation on the remaining 67 articles, 11 articles were removed because of combination with other treatment drugs in the experimental group; 23 articles were excluded because they did not test the efficacy of G-Rg1 on PD animals; 8 articles were excluded because of duplicate publication. Eventually, 25 eligible studies [16–40] were identified (Figure 1).

3.2. Study Characteristics. The 25 eligible studies included 516 animals from two species: 415 C57BL/6 mice and 111 ovariectomized Wistar rats. The weight of C57BL/6 mice varied from 16 g to 30 g, and the weight of ovariectomized Wistar rats varied from 200 g to 250 g. Eight articles [16–20, 35, 39, 40] were published in English academic journals and 17 articles [21–34, 36–38] were published in Chinese academic journals from 2001 to 2016. As for experimental animal model, twenty studies used 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP-) induced PD model [16–34, 39], 4 studies [35–38] used 6-hydroxydopamine (6-OHDA) induced PD model, and 1 study used lipopolysaccharide- (LPS-) induced PD model [40]. In terms of gender, five studies [25, 35, 37, 38, 40] used merely female animals and eighteen studies [16, 18–20, 22–24, 26–34, 36, 39] used merely male animals, while the remaining 2 studies [17, 21] did not report gender. Among 25 included studies, one study used ether cotton balls to induce anesthesia [33], two studies used urethane [17, 26], eleven studies used chloral hydrate [16, 23, 27, 28, 30, 32, 35–38, 40], four studies used pentobarbital sodium [18, 20, 31, 39], three studies did not report anesthesia [24, 29, 34], and the remaining four studies did not report the method of executing the animals [19, 21, 22, 25]. For all included studies, the intervention measures for experimental groups were injection with G-Rg1 before injection of MPTP, 6-OHDA, or LPS. Fifteen studies used behavioral assessments as primary outcomes [16, 17, 22–24, 26, 28, 29, 32–37, 39]. TH-positive dopamine neurons in the SNpc were observed in 16 studies [16–20, 24, 26, 28–32, 34, 35, 38, 39]. Nine studies reported the levels of TH protein expression [16, 18, 22–24, 29, 30, 32, 34]. Meanwhile, the indexes related to the mechanisms of G-Rg1 were used as outcomes as the anti-inflammatory activities in 10 studies [16–18, 21–23, 29, 33,

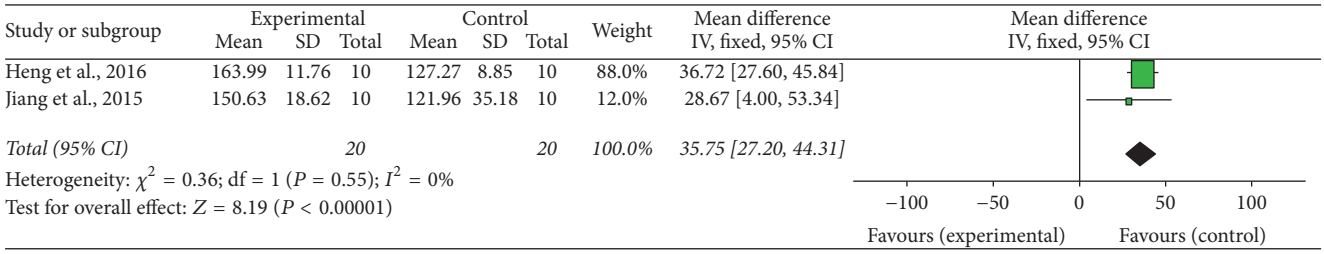

Figure 2: The forest plot: effects of G-Rg1 for improving the rotarod test compared with control group. Note: G-Rg1: Ginsenosides-Rg1.

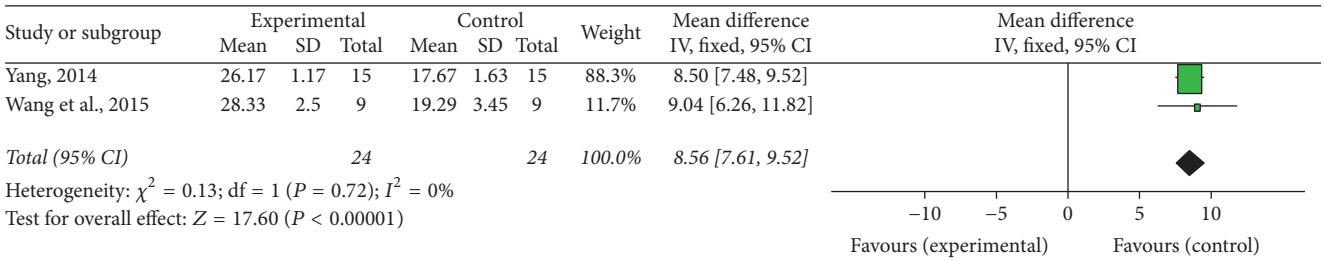

Figure 3: The forest plot: effects of G-Rg1 for improving the swim test compared with control group. Note: G-Rg1: Ginsenosides-Rg1.

34, 40], antioxidant stress activities in 3 studies [19, 30, 36], and antiapoptosis in 11 studies [19, 20, 22–24, 26, 30–32, 35, 39]. The detailed characteristics of included studies are summarized in Table 1.

3.3. Risk of Bias in Included Studies. According to the nine-item modified CAMARADES checklist, the mean quality score of the 25 included studies was 5.12 (interquartile range: 4.75–6.0), with scores ranging from 3 to 7 (Table 2), of which one study [21] got 3 points; five studies [22, 24, 25, 29, 31] got 4 points; eleven studies [19, 20, 23, 26–28, 32–34, 36, 40] got 5 points; six studies [16, 17, 30, 37–39] got 6 points; and two studies [18, 35] got 7 points. All studies were published in peer reviewed journals and described random allocation to groups. None of the studies reported blinded assessment of behavioral outcome. Eighteen studies [16–18, 22–26, 28–30, 32–35, 37–39] reported the control of temperature. Six studies [19, 21, 22, 24, 25, 29] did not use anesthetic without significant intrinsic neuroprotective activity. All of the studies do not have formal sample size calculation. Thirteen studies [17–22, 25, 27, 30, 35–38] reported using of animals without relevant comorbidities (such as aging, diabetes, or hypertension). Three studies [21, 22, 25] did not report the compliance with animal welfare regulations. Seven studies [16–19, 35, 39, 40] stated potential conflict of interests.

3.4. Effectiveness

3.4.1. Behavioral Assessments. Fifteen studies, including twelve MPTP-induced PD [16, 17, 22–24, 26, 28, 29, 32–34, 39] and three 6-OHDA induced PD [35–37] studies, used behavioral assessments as primary outcome measures. For the 12 studies on the motor dysfunction of MPTP-induced PD model, 2 studies [16, 17] provided clear data of rotarod test, 2 studies [22, 33] provided clear data of swim-score values, 4 studies [16, 17, 23, 39] provided graphical data of pole test, and the other 6 studies [24, 26, 28, 29, 32, 34] were descriptive studies without any data. Meta-analysis of 2 studies [16, 17] reported that the G-Rg1 group significantly improved rotarod test compared with MPTP-injected group ($n = 40$; WMD: 35.75; 95% CI: 27.20 to 44.31; $P < 0.00001$; heterogeneity: $\chi^2 = 0.36$; df = 1; $P = 0.55$; $I^2 = 0\%$) (Figure 2). Meta-analysis of 2 studies [22, 33] showed that the G-Rg1 group significantly improved the swim-score values compared with the MPTP-induced PD group ($n = 48$; WMD: 8.56; 95% CI: 7.61 to 9.52; $P < 0.00001$; heterogeneity: $\chi^2 = 0.13$; df = 1; $P = 0.72$; $I^2 = 0\%$) (Figure 3). Four studies [16, 17, 23, 39] indicated that mice treated with G-Rg1 spent less time descending the pole compared with mice treated with MPTP ($P < 0.01$ or $P < 0.05$ at different time point). There are two time durations that should be recorded in the pole test: one is the time it took the mouse to turn completely downward (T-turn) and the other one is the time it took the mouse to descend to the floor (T-total). But only one study recorded the two time durations; other studies did not clearly record them in detail. Meanwhile, the climbing pole time of mice in each study was conducted at different days. Owing to the above reasons, meta-analysis for this pole test could not be performed. The other 6 studies [24, 26, 28, 29, 32, 34] described that G-Rg1 group significantly improved the motor symptoms of PD induced by MPTP in mice, including the symptoms of thrilling, piloerection, raising tail, activity decrease, postural bradykinesia, and staggering gait but also failed to make a meta-analysis because they were just descriptive studies without any data. For the 3 studies on the motor dysfunction of 6-OHDA induced PD model, 2 studies [35–37] indicated that G-Rg1 group showed significant improvement in the rotational behavior in 6-OHDA-lesioned rats compared with

Table 1: Characteristics of included studies.

Study (years)	Species (n)	Weight	PD model	Anesthetic	Experimental group	Control group	Outcome measure (experimental: G-Rg1/control)	Intergroup difference (ID)
Heng et al., 2016	Male, C57BL/6 mice (10/10)	22–25 g	MPTP-induced PD	Chloral hydrate (400 mg/kg, ip)	G-Rg1 (10, 20, 40 mg/kg, ip) for 49 d + MPTP (25 mg/kg, ip) for 4 d	MPTP (25 mg/kg, ip) for 4 d	(1) Behavioral tests: rotarod test, pole test (2) Numbers of TH(+) cells ↑ (3) TH protein expression ↑ (4) GFAP and IBA-1 expression ↓ (5) IBA-1 and GFAP(+) cells ↓ (6) Concentrations of TNF-α, IL-1β ↓ (7) Oligomeric α-synuclein expression ↓	(1) $P < 0.01$ (2) $P < 0.01$ (3) $P < 0.05$ (4) $P < 0.01$ (5) $P < 0.01$ (6) $P < 0.05$
Jiang et al., 2015	C57BL/6 mice (10/10)	25–30 g	MPTP-induced PD	Urethane (Sigma) (1.5 g/kg)	G-Rg1 (10 mg/kg, ip) for 15 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Behavioral tests: rotarod test, pole test, wire suspension test (2) TH(+) neurons ↑, TH protein expression ↑ (3) α-Synuclein expression ↓	(1) $P < 0.01$, $P < 0.05$ (2) $P < 0.01$ (3) $P < 0.05$
Zhou et al., 2016	Male, C57BL/6J mice (10/10)	16–25 g	MPTP-induced PD	2% pentobarbital sodium (40 mg/kg)	G-Rg1 (5, 10, 20 mg/kg, ip) for 10 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Behavioral tests: pole test (2) Numbers of TH(+) neurons ↑ (3) The protein levels of Wnt-1 and β-catenin ↑; GSK-3β and p-GSK-3β ↓ (4) Caspase-3 expression ↓, Bcl-xL ↑ protein levels of Wnt-1 ↑ and β-catenin ↑; GSK-3β ↓ and p-GSK-3β ↓	(1) $P < 0.01$ (2) $P < 0.01$ (3) $P < 0.01$ (4) $P < 0.01$
Zhou et al., 2015	Male, C57BL/6J mice (5/5)	16–25 g	MPTP-induced PD	2% pentobarbital sodium (40 mg/kg)	G-Rg1 (5, 10, 20 mg/kg, ip) for 10 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) The TH(+) neurons, levels of TH protein expression ↑ (2) T cell subsets: $CD3^+CD4^+$ T cells ↑, $CD3^+CD8^+$ T cells ↓ (3) Concentrations of TNF-α, IFN-γ, IL-1β, and IL-6 ↓ (4) Microglial cells not activated ↓	(1) $P < 0.01$ (2) $P < 0.01$, $P < 0.05$ (3) $P < 0.01$ or $P < 0.05$
Chen et al., 2005	Male, C57BL mice (8/8)	18–22 g	MPTP-induced PD	NR	G-Rg1 (5, 10, 20 mg/kg, ip) for 8 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Numbers of TH(+) ↑ and Nissl(+) neurons ↑; TUNEL(+) neurons ↓ (2) GSH level ↑; T-SOD activity ↓ (3) Phospho-JNK ↓ and phospho-c-Jun protein levels ↓	(1) $P < 0.01$ (2) $P < 0.01$
Chen et al., 2002	Male, C57BL mice (6/6)	20 ± 2 g	MPTP-induced PD	2% pentobarbital sodium	G-Rg1 (2.5, 5, 10 mg/kg, ip) for 8 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) TH(+) neurons ↑; TUNEL(+) neurons ↓ (2) Bcl-2 cells ↑; Bcl-xL cells ↑; Bax cells ↓ (3) Caspase-3 cells ↓ (4) iNOS cells ↓	(1) $P < 0.01$ (2) $P < 0.01$ (3) $P < 0.01$ (4) $P < 0.01$

Table 1: Continued.

Study (years)	Species (n)	Weight	PD model	Anesthetic	Experimental group	Control group	Outcome measure (experimental: G-Rg1/control)	Intergroup difference (ID)
Wei et al., 2015	C57BL/6 mice (9/9)	22–30 g	MPTP-induced PD	NR	G-Rg1 (10 mg/kg, ip) for 7 d + MPTP (20 mg/kg, ip) for 4 d	MPTP (20 mg/kg, ip) for 4 d	(1) EphA4 mRNA expression ↓ (2) EphA4 protein expression ↓	(1) $P < 0.01$ (2) $P < 0.01$, $P < 0.05$
Wang et al., 2015	Male, C57BL/6 mice (9/9)	22–30 g	MPTP-induced PD	NR	G-Rg1 (10 mg/kg, ip) for 8 d + MPTP (20 mg/kg, ip) for 5 d	MPTP (20 mg/kg, ip) for 5 d	(1) Behavioral tests: the swim-score ↑ (2) TH protein expression ↑ (3) P-c-Jun protein expression ↓ (4) Numbers of EphB1(+) cells ↓	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.05$
Zhu et al., 2014	Male, C57BL/6 mice (9/9)	22–30 g	MPTP-induced PD	6% chloral hydrate (30 mg/kg)	G-Rg1 (10 mg/kg, ip) for 8 d + MPTP (20 mg/kg, ip) for 5 d	MPTP (20 mg/kg, ip) for 5 d	(1) Behavioral tests: pole test (2) TH mRNA ↑ (3) Ephrin-B2(+) cells ↓ (4) P-c-Jun(+) cells ↓	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.01$
Wang et al., 2014	Male, C57BL/6 mice (9/9)	25–30 g	MPTP-induced PD	Anesthetized	G-Rg1 (10 mg/kg, ip) for 8 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Behavioral exhibition (2) Number of TH(+), FLIP(+), FADD(+), and caspase-3(+) cells (3) Expression level of TH, FLIP, FADD, and caspase-3 protein	(1) Not found (2) $P < 0.01$ (3) $P < 0.01$
Yan et al., 2014	Female, C57BL mice (10/10)	20 ± 2 g	MPTP-induced PD	NR	G-Rg1 (10 mg/kg, ip) for 8 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (15 mg/kg, ip) for 5 d	(1) DA ↑ (2) Number of TH neurons ↑	(1) $P < 0.01$ (2) $P < 0.01$
Liu et al., 2008	Male, C57BL/6 mice (15/15)	18–23 g	MPTP-induced PD	20% urethane	G-Rg1 for 8 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Behavioral exhibition (2) Number of TH(+) cells ↑ (3) Number of caspase-3(+) cells ↓ (4) Number of TUNEL(+) cells ↓ (5) Caspase-3 protein ↓	(1) Not found (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.05$ (5) $P < 0.05$

Table 1: Continued.

Study (years)	Species (n)	Weight	PD model	Anesthetic	Experimental group	Control group	Outcome measure (experimental: G-Rg1/control)	Intergroup difference (ID)
Yang et al., 2009	Male, C57BL/6 mice (12/12)	17 ± 5 g	MPTP-induced PD	10% chloral hydrate (0.04 mg/kg)	G-Rg1 (70 mg/kg, ip) for 20 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Number of BrdU(+) cells ↑ (2) Number of Nestin(+) cells ↓ (3) BrdU+/Nestin+ cells ↑	(1) $P < 0.01$ (2) P < 0.01 (3) $P < 0.05$
Ji, 2008	Male, C57BL/6 mice (9/9)	25 ± 2 g	MPTP-induced PD	10% chloral hydrate (0.3 ml/kg)	G-Rg1 (5 mg/kg, ip) for 8 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Behavioral exhibition (2) Number of TH(+) cells ↑	(1) Not found (2) Not found
Wang et al., 2008	Male, C57BL/6 mice (10/10)	25–30 g	MPTP-induced PD	Anesthetized	G-Rg1 (10 mg/kg, ip) for 8 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Behavioral exhibition (2) Number of TH(+) cells ↑; number of COX-2 and PGE2(+) cells ↓ (3) Number of p-P38(+) cells ↓ (4) p-P38, COX-2, and PGE2 proteins ↓; TH proteins expression ↑	(1) Not found (2) $P < 0.01$ (3) $P < 0.01$ (4) $P < 0.01$
Yang et al., 2007	Male, C57BL/6 mice (6/6)	21 ± 2 g	MPTP-induced PD	Chloral hydrate	G-Rg1 (5 mg/kg, ip) for 8 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Number of TH(+) cells ↑; TH mRNA ↑ (2) DA, DOPAC, and HVA ↑ (3) Number of Fe(+) cells ↓ (4) Bcl-2 cells ↑, Bcl-2 mRNA ↑ (5) Caspase-3 and Bax(+) cells ↓, Bax and caspase-3 mRNA proteins ↓	(1) $P < 0.01$, $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.01$, $P < 0.05$ (5) $P < 0.01$, $P < 0.05$
Zhou et al., 2003	Male, C57BL mice (8/8)	20 ± 2 g	MPTP-induced PD	2% pentobarbital	G-Rg1 (5, 10, 20 mg/kg, ip) for 8 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Number of TH(+) and Nissl(+) cells ↑ (2) Number of caspase-3(+) and TUNEL(+) cells ↓ (3) p-JNK and p-c-Jun protein expression ↓	(1) $P < 0.01$ (2) $P < 0.01$ (3) $P < 0.01$
Meng et al., 2001	Male, C57BL/6 mice (15/15)	25–30 g	MPTP-induced PD	10% chloral hydrate (0.3 ml/kg)	G-Rg1 (10, 20 mg/kg, ip) for 9 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Behavioral exhibition: pole test (2) Number of TH(+) cells ↑; number of GRP78(+), caspase-l2(+), and caspase-3(+) cells ↓ (3) TH protein expression ↑; GRP78, caspase-l2, and caspase-3 protein expression ↓	(1) Not found (2) $P < 0.01$ (3) $P < 0.01$

Table 1: Continued.

Study (years)	Species (n)	Weight	PD model	Anesthetic	Experimental group	Control group	Outcome measure (experimental: G-Rg1/control)	Intergroup difference (ID)
Yang, 2014	Male, C57BL/6 mice (15/15)	22–30 g	MPTP-induced PD	Ethyl ether	G-Rg1 (10 mg/kg, ip) for 9 d + MPTP (30 mg/kg, ip) for 7 d	MPTP (20 mg/kg, ip) for 7 d	(1) Behavioral assessments: the swim-score (2) EphB6 and Ephrin-B1 mRNA expression ↓ (3) EphB6 and Ephrin-B1 protein expression ↓	(1) $P < 0.01$ (2) $P < 0.01$ (3) $P < 0.01$
Wang et al., 2009	Male, C57BL/6 mice (15/15)	25–30 g	MPTP-induced PD	Anesthetized	G-Rg1 (10 mg/kg, ip) for 8 d + MPTP (30 mg/kg, ip) for 5 d	MPTP (30 mg/kg, ip) for 5 d	(1) Behavioral assessments (2) Number of TH(+) cells ↑; number of COX-2(+) cells ↓ (3) TH proteins expression ↑; COX-2 protein expression ↓	(1) Not found (2) $P < 0.01$ (3) $P < 0.01$
Xu et al., 2009	Female ovariec-tomized Wistar rats (12/12)	220–250 g	6-OHDA induced PD	Chloral hydrate (400 mg/kg)	G-Rg1 (10 mg/kg, ip) for 14 d + 6-OHDA (3.6 g/l, ip)	6-OHDA (3.6 mg/ml, ip)	(1) Reduced apomorphine-induced rotarod test (2) Number of TH(+) neurons ↑ (3) Gene expression of TH ↑ (4) The Bcl-2 protein and gene expression ↑	(1) $P < 0.01$ (2) $P < 0.01$ (3) $P < 0.01$ (4) $P < 0.01$
Jie, 2010	Male ovariec-tomized Wistar rats (10/10)	200–250 g	6-OHDA induced PD	10% chloral hydrate (3 ml/kg)	G-Rg1 (3 μg/μl, ip) + 6-OHDA (2 mg/ml, ip) for 7 d	6-OHDA (2 mg/ml, ip) for 7 d	(1) Behavioral tests: decreased apomorphine-induced rotarod test (2) SOD ↑, GSH level ↑, MDA ↓, LDH ↓	(1) Not found (2) $P < 0.01$
Xu et al., 2008	Female ovariec-tomized Wistar rats (6/6)	200–250 g	6-OHDA induced PD	8% chloral hydrate	G-Rg1 (10 mg/kg, ip) for 14 d + 6-OHDA (3.6 g/l, ip)	6-OHDA (3.6 mg/ml, ip)	(1) Behavioral tests: decreased apomorphine-induced rotarod test (2) DA ↑, DOPAC ↑	(1) $P < 0.01$ (2) $P < 0.01$
Xu and Chen, 2007	Female ovariec-tomized Wistar rats (12/12)	200–250 g	6-OHDA induced PD	8% chloral hydrate	G-Rg1 (10 mg/kg, ip) for 14 d + 6-OHDA (3.6 mg/ml, ip)	6-OHDA (3.6 mg/ml, ip)	(1) Number of TH(+) neurons ↑ (2) TH gene expression ↑	(1) $P < 0.01$
Sun et al., 2016	Female ovariec-tomized Wistar rats (18/18)	250–300 g	LPS-induced PD	400 mg/kg chloral hydrate	G-Rg1 (10 mg/kg, ip) for 14 d + LPS (5.0 μg, ip)	LPS 5.0 μg dissolved in 2 μl of 0.9% saline	(1) DA, DOPAC, and HVA ↑ (2) TNF-α and IL-1β ↓	(1) $P < 0.01$ (2) Not found

Table 2: Quality assessment of included studies.

Study	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	Score
Heng et al., 2016	√	√	√		√			√	√	6
Jiang et al., 2015	√	√	√		√		√	√	√	6
Zhou et al., 2015	√	√	√		√		√	√	√	7
Chen et al., 2005	√		√				√	√	√	5
Chen et al., 2002	√		√		√		√	√		5
Wei et al., 2015	√		√				√			3
Wang et al., 2015	√	√	√				√			4
Zhu et al., 2014	√	√	√		√			√		5
Wang et al., 2014	√	√	√					√		4
Yan et al., 2014	√	√	√				√			4
Liu et al., 2008	√	√	√		√			√		5
Yang et al., 2009	√		√		√		√	√		5
Ji, 2008	√	√	√		√			√		5
Wang et al., 2008	√	√	√					√		4
Yang et al., 2007	√	√	√		√		√	√		6
Zhou et al., 2003	√		√		√			√		4
Meng et al., 2001	√	√	√		√			√		5
Yang, 2014	√	√	√		√			√		5
Wang et al., 2009	√	√	√		√			√		5
Xu et al., 2009	√	√	√		√		√	√	√	7
Jie, 2010	√		√		√		√	√		5
Xu et al., 2008	√	√	√		√		√	√		6
Xu and Chen, 2007	√	√	√		√		√	√		6
Zhou et al., 2016	√	√	√		√		√	√		6
Sun et al., 2016	√		√		√		√	√		5

Note: (1) peer reviewed publication; (2) control of temperature; (3) random allocation to groups; (4) blinded assessment of behavioral outcome; (5) use of anesthetic without significant intrinsic neuroprotective activity; (6) calculation of the sample size necessary to achieve sufficient power; (7) appropriate animal model (aged, diabetic, or hypertensive); (8) compliance with animal welfare regulations; (9) statement of potential conflict of interests.

control group; one study [17] reported that, in comparison with G-Rg1, mice treated with MPTP spent much more time reaching the platform during the wire suspension test ($P < 0.05$).

3.4.2. The Number of TH-Positive Dopamine Neurons. Sixteen studies, including fourteen MPTP-induced PD [16–20, 24, 26, 28–32, 34, 39] and two 6-OHDA induced PD [35, 38] studies, demonstrated the number of TH-positive dopamine neurons in the SNpc by immunohistochemistry analysis. Eleven out of 14 MPTP-induced PD studies provided raw data to make meta-analysis. Meta-analysis of 11 studies showed that G-Rg1 significantly improved the number of TH-positive neurons when compared with that in the MPTP-induced group ($n = 180$; WMD: 36.78; 95% CI: 35.27 to 38.28; $P < 0.00001$; heterogeneity: $\chi^2 = 368.15$; df = 10; $P < 0.00001$; $I^2 = 97\%$). Meanwhile, there was obvious heterogeneity for the analysis of TH-positive neurons between studies. Several factors were found to make significant influence on the outcome measure. When the authors counted the number of TH-positive neurons, the different types of microscopes, various magnification (such as ×10, ×40, ×100, and ×200), different sample drawing areas of the substantia nigra, different numbers of specimens of brain glass (such as 3 brain slices or 5 brain slices), different slices of brain tissue thickness (such as cut into 20 μm and 30 μm), and use of diverse anesthetics (such as chloral hydrate, pentobarbital sodium, and urethane) in different studies may contribute to this discrepancy. Thus, those reasons were considered as the potential sources of the heterogeneity. Seven studies [16–18, 22, 24, 32, 34] which reported the level of TH protein expression were qualified to perform a meta-analysis, and the random-effect model was applied for statistical analysis account for the heterogeneity ($n = 82$; SMD: 5.56; 95% CI: 3.56 to 7.56; $P < 0.00001$; heterogeneity: $\chi^2 = 18.24$; df = 6; $P = 0.006$; $I^2 = 67\%$) favouring G-Rg1 when compared with controls. We used sensitivity analyses omitting each study at a time from the original analysis. After removing 1 study [22] which was considered to be the potential source of the heterogeneity, the remaining 6 studies reported that G-Rg1 significantly improved the level of TH protein expression compared with control group ($n = 64$; SMD: 4.46; 95% CI: 3.15 to 5.76; $P < 0.00001$; heterogeneity: $\chi^2 = 6.54$; df = 5; $I^2 = 23\%$) (Figure 4). Two studies [23, 30] showed that G-Rg1 significantly increased the expression of TH mRNA in the substantia nigra of mice compared with the control group ($n = 30$; WMD: 2.07; 95% CI: 1.13 to 3.01; $P < 0.00001$; heterogeneity: $\chi^2 = 0.01$; df = 1; $P = 0.93$; $I^2 = 0\%$) (Figure 5).

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Std. mean difference IV, random, 95% CI
Heng et al., 2016	0.65	0.06	4	0.36	0.06	4	13.4%	4.20 [1.00, 7.41]
Jiang et al., 2015	99.01	10.73	5	52.07	14.2	5	22.6%	3.37 [1.10, 5.63]
Meng et al., 2001	0.77	0.066	6	0.54	0.05	6	25.0%	3.63 [1.53, 5.73]
Wang et al., 2009	9.5	0.8	6	5.4	0.5	6	15.0%	5.67 [2.68, 8.67]
Wang et al., 2014	26.92	1.34	6	12.33	1.45	6	6.6%	9.65 [4.80, 14.49]
Zhou et al., 2015	0.93	0.06	5	0.69	0.04	5	17.6%	4.25 [1.56, 6.95]
Total (95% CI)			*32*			*32*	*100.0%*	*4.46 [3.15, 5.76]*

Heterogeneity: $\tau^2 = 0.62$; $\chi^2 = 6.54$; df = 5 ($P = 0.26$); $I^2 = 23\%$
Test for overall effect: $Z = 6.71$ ($P < 0.00001$)

Std. mean difference IV, random, 95% CI

−100 −50 0 50 100
Favours (experimental) Favours (control)

Figure 4: The forest plot: effects of G-Rg1 for improving the level of TH protein expression compared with control group. Note: G-Rg1: Ginsenosides-Rg1; TH: Tyrosine Hydroxylase.

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Std. mean difference IV, fixed, 95% CI
Zhu et al., 2014	0.65	0.04	9	0.55	0.05	9	60.7%	2.10 [0.90, 3.31]
Yang et al., 2007	0.78	0.14	6	0.44	0.17	6	39.3%	2.02 [0.52, 3.51]
Total (95% CI)			*15*			*15*	*100.0%*	*2.07 [1.13, 3.01]*

Heterogeneity: $\chi^2 = 0.01$; df = 1 ($P = 0.93$); $I^2 = 0\%$
Test for overall effect: $Z = 4.31$ ($P < 0.0001$)

Std. mean difference IV, fixed, 95% CI

−100 −50 0 50 100
Favours (experimental) Favours (control)

Figure 5: The forest plot: effects of G-Rg1 for improving number of TH mRNA compared with control group. Note: G-Rg1: Ginsenosides-Rg1; TH: Tyrosine Hydroxylase.

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Std. mean difference IV, fixed, 95% CI
Heng et al., 2016	79.87	2.39	10	86.95	6.16	10	65.6%	−1.45 [−2.46, −0.44]
Zhou et al., 2015	323.93	60.98	5	396.36	53.89	5	34.4%	−1.14 [−2.53, 0.26]
Total (95% CI)			*15*			*15*	*100.0%*	*−1.34 [−2.16, −0.53]*

Heterogeneity: $\chi^2 = 0.13$; df = 1 ($P = 0.72$); $I^2 = 0\%$
Test for overall effect: $Z = 3.22$ ($P = 0.001$)

Std. mean difference IV, fixed, 95% CI

−100 −50 0 50 100
Favours (experimental) Favours (control)

Figure 6: The forest plot: effects of G-Rg1 for decreasing the concentrations of IL-1β compared with control group. Note: G-Rg1: Ginsenosides-Rg1; IL-1β: cytokine interleukin-1β.

3.4.3. The Mechanisms of Neuroprotective Function of G-Rg1 in PD

Anti-Inflammatory Activities. Ten studies [16–18, 21–23, 29, 33, 34, 40] reported the anti-inflammatory effect of G-Rg1 on the PD mice induced by MPTP ($n = 7$), 6-OHDA ($n = 2$), and LPS ($n = 1$) in the SNpc. Among them, only 2 studies [16, 18] reported the change of concentrations of cytokine interleukin-1β (IL-1β), whereas the other 8 studies failed to be pooled for analysis due to use of different anti-inflammatory indicators once or the absence of data. Meta-analysis of 2 studies [16, 18] showed that the concentrations of cytokine interleukin-1β (IL-1β) in the G-Rg1 groups significantly decreased compared with the control group ($n = 40$; SMD: −1.32; 95% CI: −2.02 to −0.62; $P = 0.0002$; heterogeneity: $\chi^2 = 0.12$; df = 1; $P = 0.73$; $I^2 = 0\%$) (Figure 6). Three studies [16, 18, 40] also showed that tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and IL-6 in the G-Rg1 groups significantly decreased compared with the control group ($P < 0.01$ or $P < 0.05$). Three studies [21, 22, 33] reported significant effects of G-Rg1 for decreasing the expression of erythropoietin-producing hepatocellular cell line such as EphA4, EphB6, and EphB1 compared with the control group in the SNpc ($P < 0.01$ or $P < 0.05$). One study [16] showed that G-Rg1 groups significantly decreased the expression of IBA-1 and GFAP proteins and the number of IBA-1- and GFAP-positive cells compared with the control group

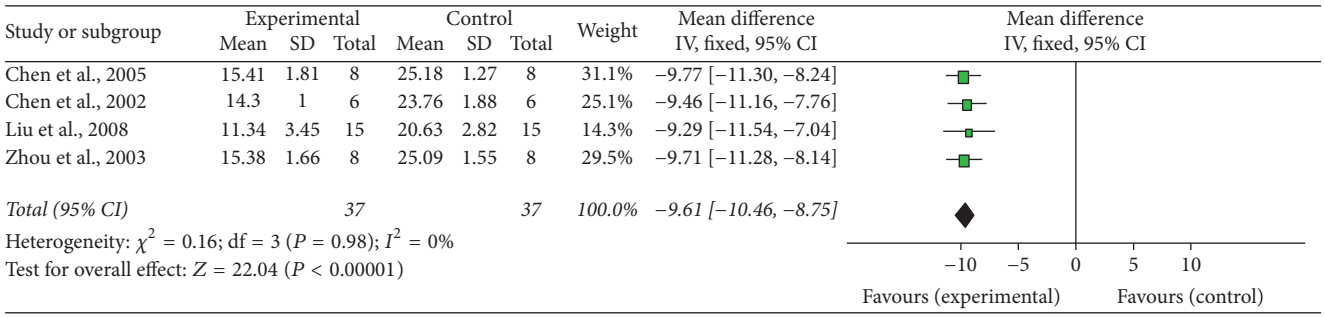

Figure 7: The forest plot: effects of G-Rg1 for improving TUNEL expression compared with control group. Note: G-Rg1: Ginsenosides-Rg1.

($P < 0.01$). Two studies [29, 34] showed that G-Rg1 reduced COX-2 expression in the SN and might act on the P38 signaling pathway to protect the DA neurons in PD ($P < 0.01$).

Antioxidant Stress. Three studies including two MPTP-induced PD [19, 30, 36] and one 6-OHDA induced PD [36] studies reported the antioxidant stress effect of G-Rg1 on PD models. Two studies [19, 36] detected that G-Rg1 significantly increased glutathione (GSH) level and decreased total superoxide dismutase (T-SOD) activity and lactate dehydrogenase (LDH) levels in the SN compared with the control group ($P < 0.01$). The remaining study [30] showed significant effects of G-Rg1 for reducing the numbers of iron-staining cells compared with the control group ($P < 0.01$).

Antiapoptosis. Eleven studies [19, 20, 22–24, 26, 30–32, 35, 39] reported the effect of G-Rg1 against MPTP-induced ($n = 10$) or 6-OHDA ($n = 1$) induced apoptosis in mouse SN neurons. Four studies [19, 20, 26, 31] used the number of TUNEL-positive neurons as one of the indicators of antiapoptotic activities mechanisms. Meta-analysis of 4 studies [19, 20, 26, 31] showed that pretreatment with G-Rg1 remarkably decreased the TUNEL-positive neurons in the SN compared with the control ($n = 74$; WMD: −9.61; 95% CI: −10.46 to −8.75; $P < 0.00001$; heterogeneity: $\chi^2 = 0.16$; df = 3; $P = 0.98$; $I^2 = 0\%$) (Figure 7). The other 7 studies failed to be pooled for analysis due to lack of the data of TUNEL-positive neurons. Three studies [20, 30, 35] showed that G-Rg1 significantly increased the number of Bcl-2 and Bcl-xL cells compared with the control ($P < 0.01$ or $P < 0.05$). Six studies [20, 24, 26, 30–32, 39] reported that G-Rg1 remarkably decreased the number of caspase-3 positive cells in the SN compared with the control ($P < 0.01$ or $P < 0.05$). Three studies [22, 23, 31] reported that G-Rg1 dramatically decreased phospho-JNK and phospho-c-Jun protein expression compared with the control ($P < 0.01$ or $P < 0.05$).

4. Discussion

4.1. Summary of Evidence. Twenty-five studies with 516 animals were identified. This study found that G-Rg1 could improve the neurobehavioral abnormality and exert potential neuroprotective effects on PD model through different mechanisms such as antineuroinflammation, antioxidant stress, and antiapoptosis. However, we should treat the preclinical evidences cautiously because the methodological flaws undermine the validity of outcomes.

4.2. Methodological Considerations. This systematic review has a number of weaknesses. Firstly, animal studies with neutral or negative results may be more likely to remain unpublished and will be missed. Therefore, the effect size may be overstated. Secondly, our search strategy includes only Chinese or English databases, which may cause a certain degree of selective bias [41]. Thirdly, previous meta-analyses have suggested that animal studies that are less rigorously designed may overestimate treatment effects [42]. In the present study, all the studies failed to mention the blinded assessment of behavioral outcome. It may lead to performance bias and detection bias [43]. Sufficient size is essential to determine the efficacy of a new therapy or drug [44]. No study reported the calculation of the sample size that was necessary to achieve sufficient power, which indicated the lack of statistical power to ensure suitable estimation of the therapeutic effect [45]. Finally, the results from individual studies were inconsistent, and most of the studies used the graph rather than original data to present the outcomes. Therefore, we could not synthesize these data into the quantity.

4.3. Possible Neuroprotective Mechanism. The possible mechanisms of neuroprotective activity of G-Rg1 in PD are summarized as follows. (i) Inhibiting oxidative stress: high reactive iron levels can yield excess hydrogen peroxide and other reactive oxygen species (ROS), which will lead to mitochondrial dysfunction and increased dopamine metabolism. G-Rg1 could reduce the number of iron-staining cells in the SN of MPTP treated mouse [30] and showed protective effect. As one of the most important antioxidant molecules, GSH could clear H_2O_2 and prevent its reaction with iron to form the highly reactive $^{\bullet}OH$ radical in the Fenton reaction. The present study showed that pretreatment with G-Rg1 could protect antioxidant defense system through attenuating the loss of GSH and increasing activity of T-SOD (including Cu/Zn-SOD and Mn-SOD) following MPTP treatment [19]. (ii) Inhibiting neuroinflammation: animal,

human, epidemiologic, and therapeutic studies all revealed that the neuroinflammatory cascade plays a key role in the pathogenesis of PD. Recent studies demonstrated that G-Rg1 notably decreased neuroinflammation levels in the SNpc induced by MPTP. G-Rg1 could decrease the level of IBA-1, GFAP, EhpA, and EhpB protein expression, IBA-1, GFAP, EhpA, and EhpB positive cells, phosphorylated p38, COX-2, and PGE2 proteins, TNF-α, IL-1β, and the oligomericα-synuclein expression in the SNpc [16–18, 21, 22]. (iii) Decreasing toxin-induced apoptosis: the protective effect of G-Rg1 against neurons apoptosis was related to enhancing Bcl-xL immunoreactive cells, Bcl-2 expression, TH^+ neurons, reducing the level of caspase-3 cells, Bax, TUNEL neurons, and iNOS expression, and preventing c-Jun NH2-terminal kinase (JNK) signaling cascade [19, 20, 24, 26, 30, 31]. Therefore, G-Rg1 exerts beneficial effects on multiple aspects of the pathophysiology in PD.

4.4. Implications. It is well known that animal experiments have contributed to our understanding of mechanisms of diseases, but the translation of preclinical experiment which results in a prediction of the effectiveness of treatment strategies in clinical trials is still challenging [46, 47]. Previous studies [13] suggested that the quality of the research design is an important factor affecting the outcome. The main causes for the failure of translation of animal studies to human clinical trials include inadequate animal data and overoptimistic conclusions about efficacy drawn from methodologically flawed animal studies. Thus, it is essential to improve the methodological standards in the design, execution, and reporting of preclinical PD studies in the future.

Quantitative and statistical analysis of Ginsenosides in plasma indicates that PD type exhibits higher concentration and longer half-life than PT type [48]. Due to the low membrane permeability, active biliary excretion, and biotransformation, the oral bioavailability of G-Rg1 is very low [49–51]. After an oral administration of G-Rg1, the experiment in rats indicated that the area under the curve of G-Rg1 is 28.93 $\mu g \cdot h \cdot L^{-1}$ and the mean value of half-life is 15.26 hours. The peak concentration is 7.15 $\mu g \cdot L^{-1}$, while T_{max} is 2.19 hours. In clinic, the use of ginseng for the suggestive symptoms of PD could date back to 1623–1670 AD recorded in Yizong Jiren Bian (Compiled Texts on Self Duty of Medicine) by Gao Gufeng who discussed the pathogenesis of tremor syndrome in the chapter shiver, shake, tremble: "Pathogenesis is mainly due to deficiency of Qi and Blood. The bones and muscles could not get enough nourishment, causing tremble that could not be controlled." Ginseng Tonic Decoction should be used for treatment to invigorate Qi and Blood [5]. In fact, ginseng was one of most commonly used herbs for tremor syndrome from the Han Dynasty to the end of the Qing Dynasty (206 BC–1911 AD) in China by using the frequency statistics according to 232 prescriptions involving 193 herbs and 2529 total frequency of herbs [5]. In modern time, several clinical studies have been conducted to assess the efficacy and safety of ginseng prescription for PD, and the results indicated that ginseng prescriptions could significantly ameliorate the motor symptoms and improve the quality of life [52, 53]. However, no clinical study of G-Rg1 for PD has been yet conducted. In the present study, the findings indicated that G-Rg1 exerted potential neuroprotective functions against PD and its mechanisms are involved with on multiple aspects of the pathophysiology in multiple PD models. Thus, G-Rg1 may be a promising candidate neuroprotectant from bench to bedside. In addition, high-quality randomized controlled trials (RCTs) and a systematic review of those RCTs are commonly regarded the highest level of evidence in judging the treatment efficacy and safety of interventions [54]. Given the huge gap between the animal studies and the clinical trials, seeking and developing innovative neuroprotectants and further rigorous RCTs are urgently needed. In the present study, the findings indicated that G-Rg1 exerted potential neuroprotective functions against PD and its mechanisms are involved with multiple aspects of the pathophysiology of PD. Thus, G-Rg1 may be a promising candidate neuroprotectant from bench to bedside.

5. Conclusion

G-Rg1 exerted potential neuroprotective functions against PD despite of the methodological flaws. In addition, we identified an important area, which is worthy of further study. G-Rg1 as a promising clinical candidate neuroprotectant for PD needs to be further confirmed by clinical trials.

Competing Interests

None of the authors have potential competing interests to be disclosed.

Authors' Contributions

Liang Song, Meng-Bei Xu, and Xiao-Li Zhou contributed equally to this work.

Acknowledgments

This project was supported by the grant of the Administration of Traditional Chinese Medicine of Zhejiang Province (2010ZQ010), Wenzhou Municipal Science and Technology Bureau in Zhejiang province (Y20130337), Young and Middle-Aged University Discipline Leaders of Zhejiang Province, China (2013277), and Zhejiang Provincial Program for the Cultivation of High-Level Health Talents (2015).

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 1038153, 12 pages
https://doi.org/10.1155/2017/1038153

Research Article

Antioxidant, Cytotoxic, and Toxic Activities of Propolis from Two Native Bees in Brazil: *Scaptotrigona depilis* and *Melipona quadrifasciata anthidioides*

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Received 10 November 2016; Revised 27 January 2017; Accepted 1 February 2017; Published 9 March 2017

Academic Editor: Jasminka Giacometti

Propolis is a natural mixture of compounds produced by various bee species, including stingless bees. This compound has been shown to exhibit antioxidant, antiproliferative, and antitumor activities. The present study aimed to determine the chemical constituents as well as the antioxidant, cytotoxic, and toxic activities of ethanol extracts of propolis obtained from the stingless bees *Scaptotrigona depilis* and *Melipona quadrifasciata anthidioides*, which are found in Brazil. Phytosterols, terpenes, phenolic compounds, and tocopherol were identified in the ethanol extracts of propolis (EEPs) in different concentrations. The compounds stigmasterol, taraxasterol, vanilic acid, caffeic acid, quercetin, luteolin, and apigenin were found only in EEP-M. The EEPs were able to scavenge the free radicals 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and protected human erythrocytes against lipid peroxidation, with the latter effect being demonstrated by their antihemolytic activity and inhibition of malondialdehyde formation. The EEPs showed cytotoxic activity against erythroleukemic cells and necrosis was the main mechanism of death observed. In addition, the concentrations at which the EEPs were cytotoxic were not toxic against *Caenorhabditis elegans*. In this context, it is concluded that EEP-S and EEP-M show antioxidant and cytotoxic activities and are promising bioactive mixtures for the control of diseases associated with oxidative stress and tumor cell proliferation.

1. Introduction

Stingless bees, also known as meliponini, belong to the tribe Meliponini and are distributed across more than 32 genera [1]. Most species in this group exhibit eusocial habits and are found in tropical and subtropical regions, and 244 species have been described in Brazil [2, 3].

This group of bees plays an important ecological role, contributing to the preservation of plant species through pollination. Moreover, they produce pollen, honey, wax, and propolis, which are used in the hive and are consumed by humans as nutraceuticals [4–6]. Among these compounds, propolis is obtained through the collection of exudates from different parts of plants and combined with salivary enzymes from bees, resulting in a resinous material that is used to repair cracks and damage to the hive, defend against microorganisms, and mummify the bodies of other insects [7, 8].

Propolis is generally composed of 50% to 60% resins and balsams, 30% to 40% waxes, 5% to 10% essential oils, and 5% pollen grains and micronutrients, with small amounts of vitamins B1, B2, B6, C, and E [9]. The color and chemical composition of this resin vary depending on the plant species

from which bees collect the raw material and the bee species that produces it [8].

Therefore, studies on propolis collected from different geographical regions and bee species are of great importance because these elements affect the chemical composition and, consequently, the biological properties of propolis.

The therapeutic activity of propolis from stingless bees has been widely investigated in recent decades, including descriptions of its antioxidant activity [10–12], antimicrobial activity [5, 13, 14], anti-inflammatory activity [15, 16], and antitumor activity [7, 12, 17].

Among bee species, *Scaptotrigona depilis*, popularly known as "mandaguari" and *Melipona quadrifasciata anthidioides*, known as "mandaçaia," are stingless species found in South American countries including Paraguay, Argentina, and Brazil [1] and their genetic and behavioral characteristics have been well described [18–21]. However, studies on the biological activity of propolis in these species are scarce in the literature, particularly in view of the difficulty in finding colonies in their natural environment, where species are disappearing because of anthropogenic activity.

The pharmacological properties of propolis in these two species were evaluated by Velikova et al. [13], who described the antimicrobial activity of propolis extracts from *M. q. anthidioides*, and by Sawaya [10] who described the antioxidant activity of propolis extracts from *S. depilis*. In this context, the present study aimed to determine the chemical constituents as well as the antioxidant, cytotoxic, and toxic activities of ethanol extracts of propolis from the stingless bee species *S. depilis* and *M. q. anthidioides* from the state of Mato Grosso do Sul in Midwest Brazil.

2. Materials and Methods

2.1. Research Ethics. No specific permits were required for the described field studies. All field works to collect the propolis samples were conducted on private land and with owner permission. The field studies did not involve endangered or protected species. The protocol to collect of human peripheral blood was approved by the Research Ethics Committee (Comitê de Ética em Pesquisa; CEP) of the University Center of Grande Dourados (Centro Universitário da Grande Dourados; UNIGRAN), Brazil (CEP process number 123/12). All subjects provided written informed consent for participation.

2.2. Preparation of the Ethanol Extract of Propolis (EEPs). Propolis samples from *S. depilis* (83.81 g) and *M. q. anthidioides* (36.42 g) were collected from the state of Mato Grosso do Sul (22°13′12″S–54°49′2″W), in the Midwest Region of Brazil, with a total of seven collections being performed for each species. The ethanol extract of propolis (EEPs) was prepared using 4.5 mL of 80% ethanol per 1 g of propolis. This mixture was incubated in a water bath at 70°C in a sealed container until total dissolution and subsequently filtered in filter paper qualitative 80 g/m^2 (Prolab, São Paulo, Brazil) to obtain the EEPs of *S. depilis* (EEP-S) and *M. q. anthidioides* (EEP-M) [22]. After preparation of the extracts, they were kept at a temperature of −20°C until analysis.

2.3. Chemical Analysis

2.3.1. Preparation of the Samples. The samples (1 mg) was fractionated with hexane and water in proportion 1 : 1 v : v and fraction soluble in hexane was analyzed by GC-MS and fraction in water by HPLC.

2.3.2. GC-MS. Samples were injected and analyzed by gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis was performed on a gas chromatograph (GC-2010 Plus Shimadzu Kyoto Japan) equipped with a mass spectrometer detector (GC-MS Ultra 2010) using LM-5 (5% phenyl dimethyl poly siloxane) capillary column (15 m length × 0.2 mm i.d. and 0.2 μm film thickness) with initial oven temperature set at 150°C and heating from 150°C to 280°C at 15°C min^{-1} and a hold at 280°C for 15 min. The carrier gas was helium (99.99%) supplied at a flow rate of 1.0 mL/min, with split ratio 1 : 20, 1 μL injection volume. The injector temperature was 280°C and the quadrupole detector temperature was 280°C. The MS scan parameters included an electron-impact ionization voltage of 70 eV mass range of 45–600 m/z and scan interval of 0.3 s. The identifications were completed by comparing the mass spectra obtained in the NIST21 and WILEY229 libraries. In some cases, the compound was confirmed by comparison of standards. Standards of the stigmasterol, β-sitosterol, β-amyrin, α-amyrin, β-amyrin acetate, and tocopherol (Sigma-Aldrich with purity ≥97%) were prepared in the concentration initial of 1000 μg/mL. The concentrations of compounds were determined by extern calibration after dilutions appropriated in the range of 0.1–50 μg/mL. The quantification of taraxasterol was performed in relation to stigmasterol. The procedure was performed in triplicate.

2.3.3. HPLC. The extracts were analyzed in an analytical HPLC (LC-6AD, Shimadzu, Kyoto, Japan) system with a diode array detector (DAD) monitored at λ = 200–600 nm. The HPLC column was a C-18 (25 cm × 4.6 mm; particle size, 5 μm; Luna, Phenomenex, Torrance, CA, USA), with a small precolumn (2.5 cm × 3 mm) containing the same packing, used to protect the analytical column. In each analysis, the flow rate and the injected volume were set as 1.0 mL min^{-1} and 20 μL, respectively. All chromatographic analyses were performed at 22°C. Elution was carried out using an binary mobile phase of water with 6% acetic acid and 2 mM sodium acetate (eluent A) and acetonitrile (eluent B). The following applied gradients are as follows: 5% B (0 min), 15% B (30 min), 50% B (35 min), and 100% B (45 min). Standards of the vanilic acid, caffeic acid, ferulic acid, p-coumaric acid, benzoic acid, cinnamic acid, quercetin, luteolin, apigenin, and vanillin (Sigma-Aldrich, ≥97%) were prepared in the concentration initial of 1000 μg/mL. The concentrations of compounds were determined by extern calibration after dilutions appropriated in the range of 0.01–10 μg/mL. The procedure was performed in triplicate.

2.4. Antioxidant Activity

2.4.1. DPPH Free Radical Scavenging Activity. The free radical-scavenger activity was determined by the DPPH

(2,2-diphenyl-1-picrylhydrazyl) assay, as described previously by D. Gupta and R. K. Gupta [23] with some modifications. The antiradical activity of extracts was evaluated using a dilution series, in order to obtain a large spectrum of sample concentrations. This involved the mixing of 1.8 mL of DPPH solution (0.11 mM DPPH in 80% ethanol) with 0.2 mL of EEP-S or EEP-M (1–300 μg/mL), followed by homogenization. After 30 min, quantification of the remaining DPPH radicals was recorded by using absorption set at 517 nm. Ascorbic acid and butylated hydroxytoluene (BHT) were used as reference antioxidants. The tests were performed in duplicate in 2 independent experiments. DPPH solution without the tested sample was used as control. The percentage inhibition was calculated from the control with the following equation:

$$\text{Scavenging activity } (\%) = \left(1 - \frac{\text{Abs sample}}{\text{Abs control}}\right) \times 100. \quad (1)$$

2.4.2. ABTS Free Radical Scavenging Activity. Free radical scavenging capacity for EEP was studied as described by Re et al. [24], through the evaluation of the free radical scavenging effect on 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical. The stock solutions included 7 mM ABTS solution and 140 mM potassium persulfate solution. The $ABTS^{\bullet+}$ radical was then prepared by mixing the two stock solutions (5 mL of ABTS solution and 88 μL potassium persulfate solution) and left for 12–16 h at room temperature in the dark. The solution was then diluted by mixing 1 mL $ABTS^{\bullet+}$ radical with ethanol absolute to obtain an absorbance of 0.70 nm ± 0.05 units at 734 nm using a spectrophotometer. Then, 20 μL of EEP-S or EEP-M (1–300 μg/mL) was mixed with 1980 μL of the $ABTS^{\bullet+}$ radical and the absorbance was taken at 734 nm after 6 min using a spectrophotometer. Ascorbic acid and butylated hydroxytoluene (BHT) were used as positive controls. Three independent experiments were performed in triplicate. The percentage of inhibition of the ABTS radical was calculated according to the following equation, where $\text{Abs}_{\text{control}}$ is the absorbance of $ABTS^{\bullet+}$ radical without the tested sample:

$$\%\ \text{inhibition of ABTS} = \left(\frac{\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right)}{\text{Abs}_{\text{control}}}\right) \times 100. \quad (2)$$

2.4.3. Antioxidant Assay Using the Human Erythrocyte Model

(1) Preparation of Erythrocyte Suspensions. Following approval by the Research Ethics Committee, 20 mL of peripheral blood was collected from healthy donors into sodium citrate-containing tubes and was subsequently centrifuged at 1500 rpm for 10 min. After centrifugation, the blood plasma and leukocyte layers were discarded, and the erythrocytes were washed 3 times with saline solution and centrifuged at 1500 rpm for 10 min. Finally, 10% erythrocyte suspensions were prepared in saline solution to obtain 2.5% after the treatment.

(2) Oxidative Hemolysis Inhibition Assay. The protective effect of the propolis extracts was evaluated according to the method described by Campos et al. [12], with minor modifications. The assays were conducted with erythrocyte suspensions. The erythrocytes were preincubated at 37°C for 30 min in the presence of different concentrations of EEP or ascorbic acid (50–125 μg/mL). Then, 50 mM 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) solution was added. A sample of 1 % ethanol was used as a negative control. Total hemolysis was induced by incubating erythrocytes with distilled water. Basal hemolysis caused by EEP was assessed by incubating erythrocytes with the extract without the presence of AAPH, and the control was assessed in erythrocytes incubated only with 0.9% NaCl. This mixture was incubated at 37°C for 240 min, with periodical stirring. Hemolysis was determined after every 120, 180, and of 240 minutes of incubation; specifically, sample were centrifuged at 1500 rpm for 10 min and aliquots of there were transferred to tubes with saline, after which the absorbance of the supernatant was read spectrophotometrically at 540 nm. The percentage hemolysis was measured with the formula $A/B \times 100$, where (A) is the sample absorbance and (B) is the total hemolysis. Five independent experiments were performed in duplicate.

(3) Inhibitory Efficiency against Lipid Peroxidation. A 2.5% erythrocyte suspension was used to assess the protective effects of EEP against lipid peroxidation as described by Campos et al. [12] with some modifications. Erythrocytes were preincubated at 37°C for 30 min with different concentrations of EEPs or ascorbic acid (50–125 μg/mL). A sample of 1% ethanol was used as a negative control. Next, 50 mM AAPH was added to the erythrocyte solution, which was then incubated at 37°C for 4 hours with periodical stirring. At 120, 180, and 240 minutes of incubation, the samples were centrifuged at 1500 rpm for 10 min, and 500 μL aliquots of the supernatant were transferred to tubes with 1 mL of 10 nmol thiobarbituric acid (TBA). As a standard control, 500 μL of 20 mM malondialdehyde (MDA) solution was added to 1 mL of TBA. The samples were incubated at 96°C for 45 min. The samples were then cooled, 4 mL of n-butyl alcohol was added and the samples were centrifuged at 3000 rpm for 5 min. The absorbance of supernatants sample was read at 532 nm. Three independent experiments were performed in duplicate. MDA levels in the samples were expressed in nmol/mL, obtained with the following formula:

$$\text{MDA} = \text{Abs sample} \times \left(20 \times \frac{220.32}{\text{Abs standard}}\right). \quad (3)$$

2.5. Cytotoxic Activity and Cell Death Profile. K562 erythroleukemia cells line was grown is suspension in RPMI 1640 media (Cultilab, Campinas, São Paulo, Brazil) supplemented with 10% fetal bovine serum (FBS; Cultilab), 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a humidified atmosphere at 37°C in 5% CO_2. The cytotoxic activity and

cell death profile were evaluated according to the method described by Paredes-Gamero et al. [25], with minor modifications. Cells were seeded into 96-well plates (2×10^4 cell/well) and cultured in medium with 10% FBS in the absence or presence of EEP-S or EEP-M (31–500 μg/mL) for 24 h. As negative controls were used cells were incubated with 0.2% ethanol (highest concentration of ethanol in extract). All effects of the EEPs were compared with negative controls. After this period, the K562 cells were washed with PBS and resuspended in annexin-labeling buffer (0.01 M HEPES, pH 7.4, 0.14 M NaCl, and 2.5 mM $CaCl_2$). The suspensions were stained with annexin-FITC and propidium iodide (PI) (Becton Dickinson, Franklin Lakes, NJ, USA), according to the manufacture's instructions. The cells were incubated at room temperature for 15 min. Three thousand events were collected per sample, and the analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson) with CellQuest software (Becton Dickinson).

2.6. In Vivo Toxicity

2.6.1. Rearing and Maintenance of Caenorhabditis elegans. To perform the in vivo toxicity assay, we used the wild-type N2 strain of the nematode *Caenorhabditis elegans*. The specimens were incubated at 20°C in Petri dishes containing nematode growth medium (NGM) agar and fed with *Escherichia coli* strain OP50-1. The nematode culture was synchronized through treatment of pregnant hermaphrodites with 2% sodium hypochlorite and 5 M sodium hydroxide.

2.6.2. Assessment of Toxicity in C. elegans. A toxicity assay for the EEPs was performed in *C. elegans* [26] in 96-well plates. Each well contained 10 nematodes at the L4 stage, which were incubated for 24 hours at 20°C with EEP-S and EEP-M at different concentrations (250–1000 μg/mL) in M9 medium. After this period, nematode viability was evaluated by repeatedly touching the worms with a microspatula. For the manipulation and examination of nematodes, a model Motic SMZ-140 & W10X/23 (British Columbia, Canada) stereomicroscope was used. The data were calculated from two independent experiments in duplicate.

2.7. Statistical Analyses. All data are shown as the mean ± standard error of mean (SEM) and for statistical significant differences between the groups, using the analysis of variance (ANOVA) and posttest Dunnett, comparing the treatment with the control, using the Prism 6 GraphPad software. The results were considered significant when $p < 0.05$.

3. Results

3.1. Chemical Composition. The compounds identified in EEP-S and EEP-M are shown in Tables 1 and 2. Phytosterols, terpenes, phenolic compounds, and tocopherol were identified in the two extracts in different concentrations. EEP-S presented a higher content of amyrins (triterpenes) and β-sitosterol (phytosterols), whereas EEP-M exhibited a higher concentrations of tocopherol, amyrins, and apigenin (flavonoid). The compounds stigmasterol, taraxasterol, vanilic acid, caffeic acid, quercetin, luteolin, and apigenin were found only in EEP-M.

3.2. Antioxidant Activity

3.2.1. DPPH and ABTS Free Radical Scavenging Activity. EEP-S and EEP-M were observed to scavenge free radicals in vitro. In both of the evaluated methods, EEP-M showed better antioxidant activity compared with EEP-S. In the DPPH assay, EEP-M showed 50% inhibition of free radicals (IC_{50}) at a concentration of 60.91 ± 2.01 μg/mL. The IC_{50} was not calculated for EEP-S. The maximum activity of EEP-M was achieved at a concentration of 300 μg/mL (Table 3).

In the assay with the ABTS radical, IC_{50} values of the EEPs were 80.04 ± 0.31 μg/mL (EEP-S) and 13.45 ± 1.81 μg/mL (EEP-M), and they showed maximal activity at concentrations of 200 μg/mL and 100 μg/mL, respectively. The antioxidant activity of EEP-M was similar to that of the synthetic antioxidant BHT (Table 3).

3.2.2. Oxidative Hemolysis Inhibition Assay. The standard antioxidant ascorbic acid and the EEPs showed concentration- and time-dependent antihemolytic activity. EEP-S decreased hemolysis for 120 min, with hemolysis inhibition reaching 63.5 ± 10.7% at a concentration of 125 μg/mL, when compared with the AAPH sample. At the same concentration, ascorbic acid and EEP-M protected erythrocytes against hemolysis induced by the oxidant 2,2′-azobis(2-aminopropane) hydrochloride (AAPH) for up to 240 min, with hemolysis inhibition reaching 56.5 ± 12.8% and 37.7 ± 10.4% at 240 min, respectively, compared with erythrocytes treated with AAPH (Figure 1). At the various concentrations tested, the basal hemolysis observed using ascorbic acid and EEPs without the AAPH inducer was similar to the control treatments with saline and ethanol (data not shown).

3.2.3. Efficiency of EEPs in the Inhibition of AAPH-Induced Lipid Peroxidation. The effectiveness of EEPs in inhibiting lipid peroxidation induced by AAPH in human erythrocytes can be determined by measuring malondialdehyde (MDA) levels. Ascorbic acid and EEPs decreased MDA levels in a concentration- and time-dependent manner. EEP-S inhibited lipid peroxidation for 180 min. The ascorbic acid control solution inhibited lipid peroxidation by 65.6 ± 8.9%, whereas EEP-M inhibited peroxidation by 74.4 ± 6.1% for 240 min at a concentration of 125 μg/mL, when compared with the AAPH sample (Figure 2).

3.3. Cytotoxic Activity and Cell Death Profile. The ethanol extracts of propolis showed concentration-dependent cytotoxicity. At the highest concentration tested (500 μg/mL), the cell growth of erythroleukemic cells (K562) were 32.6 ± 3.2% (EEP-S) and 21.2 ± 4.1% (EEP-M) (Figure 3). At this concentration, after 24 h of treatment, EEP-S promoted death by necrosis in 52.9 ± 4.1% of cells and death by late apoptosis in 12.1 ± 0.6% of cells (Figures 4(a) and 4(b)), whereas EEP-M promoted death by necrosis in 57.5 ± 3.8% of cells and death by late apoptosis in 19.4 ± 1.6% of cells (Figures 5(a) and 5(b)).

Table 1: Compounds identified in unpolar fraction of the EEPs from *Scaptotrigona depilis* and *Melipona quadrifasciata anthidioides* by GC-MS.

Peak	Retention time (min)	Compounds	Molecular mass	EEP-S (mg/g)	EEP-M (mg/g)
1	17.02	Stigmasterol*	412	—	4.8 ± 0.1
2	17.72	β-Sitosterol*	414	9.6 ± 0.2	5.4 ± 0.2
3	17.93	β-Amyrin*	426	14.3 ± 0.3	11.6 ± 0.3
4	18.09	Taraxasterol	426	—	3.0 ± 0.1
5	18.45	α-Amyrin*	426	10.5 ± 0.3	5.0 ± 0.1
6	19.65	β-Amyrin acetate*	468	21.5 ± 0.4	13.7 ± 0.4
7	24.56	Tocopherol*	430	3.6 ± 0.1	15.0 ± 0.5

*Compound was confirmed by comparison of standard.
Data are shown as media ± standard deviation.

Table 2: Compounds identified in polar fraction of the EEPs from *Scaptotrigona depilis* and *Melipona quadrifasciata anthidioides* by HPLC.

Peak	Retention time (min)	Compounds	Molecular mass	EEP-S (mg/g)	EEP-M (mg/g)
1	7.95	Vanilic acid	168	—	5.9 ± 0.1
2	8.64	Caffeic acid	180	—	6.1 ± 0.2
3	10.44	Vanillin	152	5.5 ± 0.2	5.7 ± 0.1
4	13.48	p-Coumaric acid	164	6.3 ± 0.2	6.1 ± 0.2
5	17.28	Ferulic acid	194	5.4 ± 0.2	6.1 ± 0.2
6	19.99	Benzoic acid	122	6.8 ± 0.2	6.9 ± 0.1
7	35.33	Quercetin	302	—	9.9 ± 0.2
8	36.68	Luteolin	286	—	1.3 ± 0.1
9	40.01	Cinnamic acid	148	13.4 ± 0.4	13.2 ± 0.3
10	42.62	Apigenin	270	—	15.6 ± 0.4

*Compound was confirmed by comparison of standard.
Data are shown as media ± standard deviation.

3.4. Toxicity in C. elegans. EEP-S and EEP-M were not toxic to the nematodes after 24 h of incubation at any of the concentrations evaluated compared with the control group (Figure 6).

4. Discussion

Propolis is a bee product that is widely used in the cosmetics and food industries and is considered a functional food (nutraceutical) able to prevent diseases when included in food products [5]. The chemical constituents present in propolis are responsible for its therapeutic properties [7, 11, 27], including its antibacterial, antifungal, and antiviral activities [5, 14] as well as its anti-inflammatory and antitumor activities [15, 16, 28, 29].

The major compounds identified in the EEP-S were β-amyrin, β-amyrin acetate, and α-amyrin and in the EEP-M were tocopherol, β-amyrin acetate, and apigenin. Both extracts show similar amounts of β-amyrin, vanillin, p-coumaric acid, ferulic acid, cinnamic acid, and benzoic acid; however, the EEP-S showed higher content of amyrins than EEP-M. By contrast, EEP-M exhibited approximately four times the amount of tocopherol found in EEP-S and other compounds which were found exclusively on the EEP-M. Despite presenting the same chemical constituents, variations in the concentrations of these compounds may influence the biological activities of the extracts.

The compounds phenolic and flavonoid are correlated with the antioxidant and antitumor activity of propolis [8, 10–12, 30]. Additionally, other compounds identified in the propolis such as caffeic acid, apigenin, and triterpenes are descript with important blockers of oncogenic kinase PAK1, well known to be responsible for a variety of diseases such as infectious diseases, Alzheimer's disease, diseases inflammatory, diabetes, hypertension, obesity, and cancer [31].

Phenolic compounds and terpenes have been found in propolis extracts of other species of stingless bees from the same geographical region [12, 29], which may be related to the plant species from which the bees collect raw materials for propolis production.

The terpenes and phenolic compounds found in EEPs have been described as compounds responsible for the antioxidant activities of various plant species [32–34]. Antioxidants are compounds that, when present at low concentrations, retard or prevent the oxidation of substrates and are highly beneficial to health due to protecting cells and macromolecules from oxidizing agents [35].

The most common oxidants in the body include the superoxide (O_2^-), hydroxyl ($OH^\bullet$), peroxyl (ROO), alkoxyl (RO), and hydroperoxyl (HO_2) radicals, which are collectively known as reactive oxygen species (ROS). These free radicals are produced via gradual reduction of molecular oxygen and generate unpaired electrons, which cause oxidative stress when they are out of equilibrium [36].

Table 3: IC_{50} and maximum DPPH and ABTS radical scavenging activity of standard antioxidants, EEP-S and EEP-M.

Sample	DPPH			ABTS		
	IC_{50} (μg/mL)	Maximum inhibition		IC_{50} (μg/mL)	Maximum inhibition	
		%	μg/mL		%	μg/mL
Ascorbic acid	3.32 ± 0.65	96.75 ± 0.41	50	2.50 ± 0.48	97.37 ± 1.55	10
BHT	22.84 ± 7.87	89.36 ± 2.30	200	20.46 ± 2.78	95.36 ± 1.80	100
EEP-S	ND	14.91 ± 1.73	300	80.04 ± 0.31	73.42 ± 3.47	200
EEP-M	60.91 ± 2.01	97.47 ± 0.03	300	13.45 ± 1.81	99.31 ± 0.12	100

Values are means ± SEM. DPPH ($n = 2$) and ABTS ($n = 3$). ND: not determined.

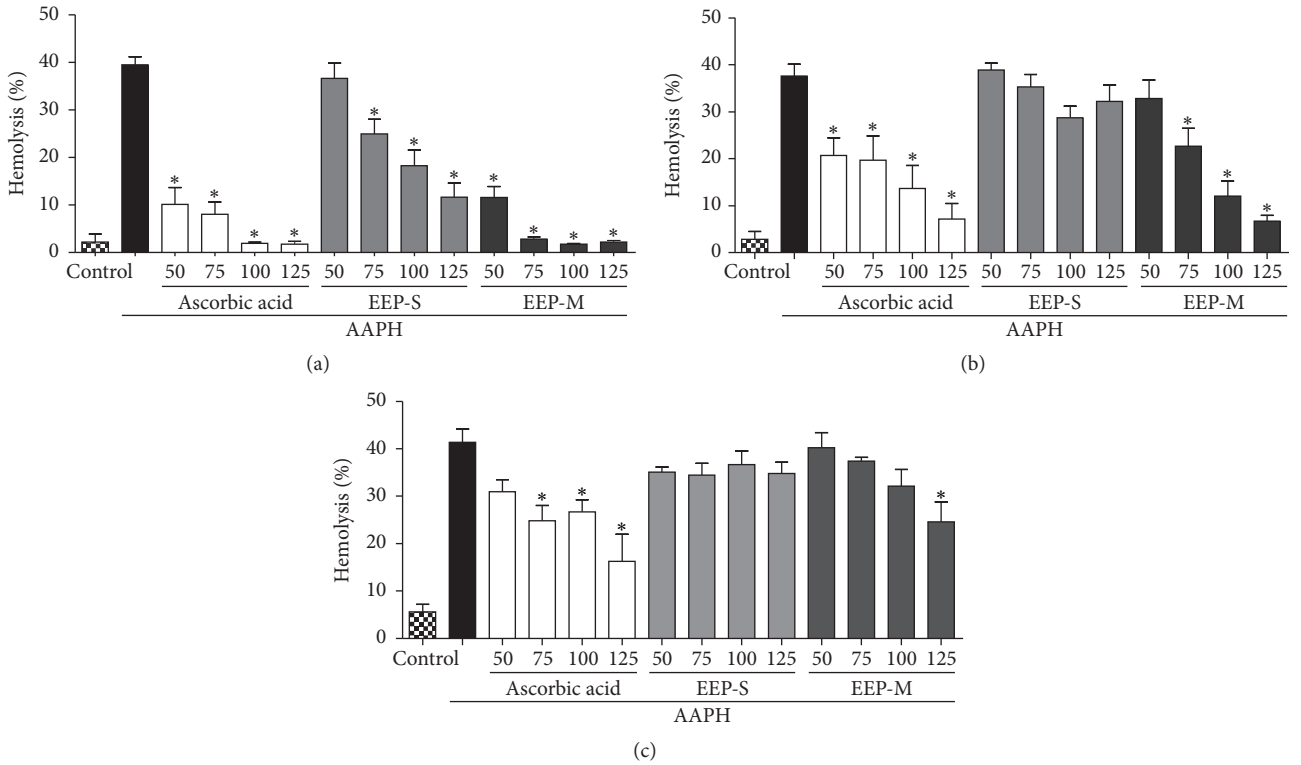


Figure 1: Protective effects of ascorbic acid (standard antioxidant) and ethanol extracts of propolis from *S. depilis* and *M. q. anthidioides* against AAPH-induced hemolysis determined using a human erythrocyte suspension at 120 min (a), 180 min (b), and 240 min (c). Ethanol was employed as a negative control. The results are expressed as the mean ± SEM (standard error of the mean), $n = 5$. * Significantly different ($p < 0.05$) compared with the AAPH group.

Both EEPs stabilized the free radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). However, EEP-M showed higher antioxidant activity than EEP-S, which may be related to the different concentrations of tocopherol in the extracts. Some studies have reported the importance of tocopherols as antioxidants [37, 38].

In addition, the amyrins may be associated with the antioxidant activity of the extracts. Tocopherols and these triterpenes are fat-soluble antioxidants that scavenge ROS [34, 39, 40]. These compounds may have been responsible for the increased antioxidant activity of the EEPs observed in the assay with the free radical ABTS, as this method is applied to hydrophilic and lipophilic antioxidant systems [41]. Therefore, the higher solubility of these compounds in the solvent used in this assay produced greater antioxidant activity.

These results corroborate those obtained in the assays involving the inhibition of lipid peroxidation, in which the EEPs presented antihemolytic activity and protective activity against lipid peroxidation when incubated with human erythrocytes in the presence of an oxidizing agent. EEPs may also inhibit the peroxyl radical (ROO), which induces peroxidation of lipids and proteins present in human erythrocyte membranes [42].

Oxidative stress leads to lipid peroxidation and, consequently, cell damage due to the oxidation of essential cellular compounds, including lipids, proteins, and nucleic acids. An

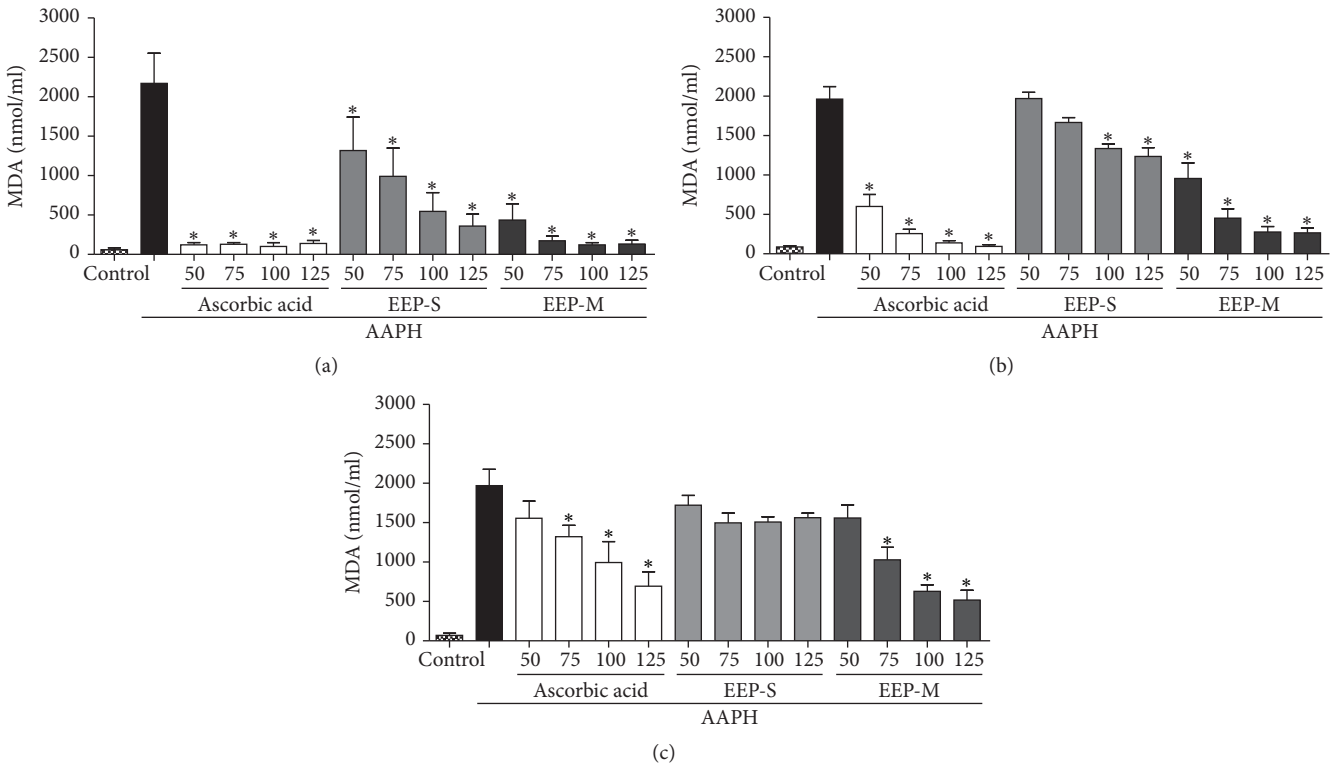


Figure 2: Protective effects of ascorbic acid (standard antioxidant) and ethanol extracts of propolis from *S. depilis* and *M. q. anthidioides* against the production of malondialdehyde (MDA)–a byproduct of lipid peroxidation–in a human erythrocyte suspension at 120 min (a), 180 min (b), and 240 min (c). Ethanol was used as a negative control. The results are expressed as the mean ± SEM (standard error of the mean), $n = 3$. *Significantly different ($p < 0.05$) compared with the AAPH group.

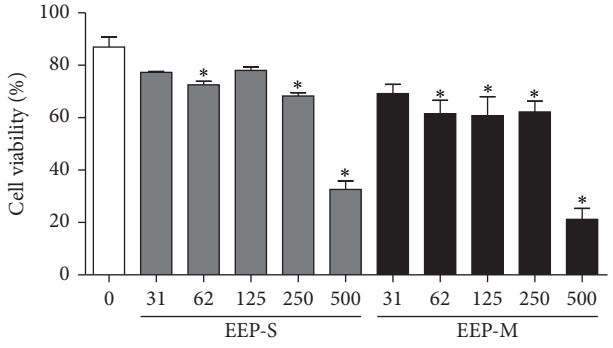


Figure 3: Cytotoxic activity of EEPs from *S. depilis* (EEP-S) and *M. q. anthidioides* (EEP-M) against the K562 erythroleukemia cell line. * $p < 0.05$ for the treated group versus control viable cells.

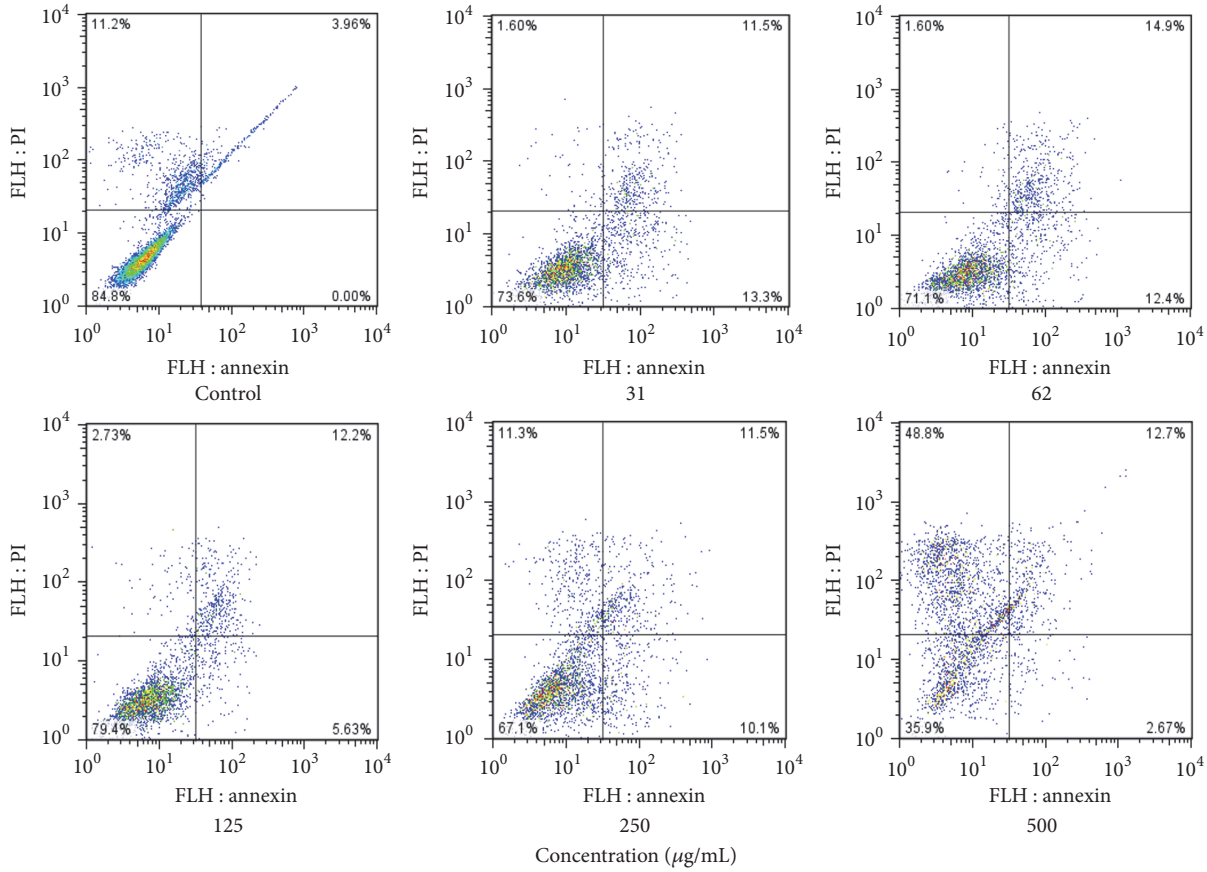
excess of these free radicals can promote cell aging and the development of various diseases, including Alzheimer's, cancer, arthritis, and diabetes, and can increase the risk of cardiovascular disease [36, 43].

Therefore, the evaluated EEPs contain important antioxidant compounds that can limit the spread of oxidative stress-related diseases. The free radicals scavenging and antihemolytic ability demonstrated by the EEP-M were more efficient than results observed for propolis from the stingless bee *Tetragonisca fiebrigi* [29] and *Melipona orbignyi* [12] from Midwest Region of Brazil and some extracts of *Apis mellifera* [44, 45].

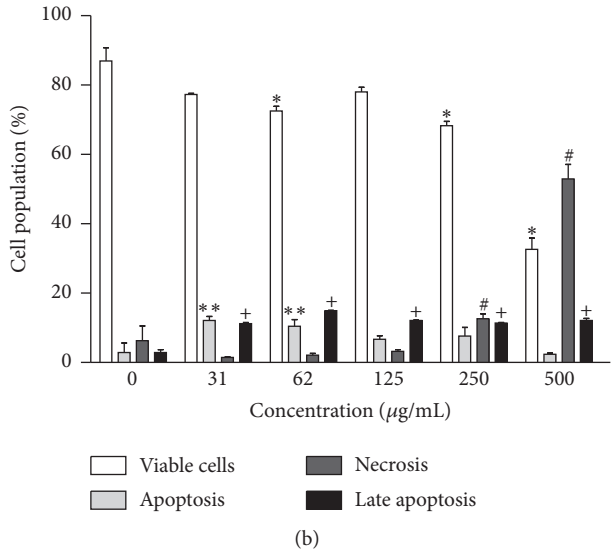
In the present study, EEP-S and EEP-M exhibited cytotoxic activity against K562 erythroleukemic cells. In addition, the decrease in cell viability was greater in cells treated with EEP-M than in those treated with EEP-S. However, both EEPs caused necrosis in most of the cells at a concentration of 500 μg/mL. The cytotoxic effect of propolis was also observed in other cell lines as human lung adenocarcinoma epithelial (A549), human cervical adenocarcinoma (HeLa), and human breast adenocarcinoma (MCF-7) but the mechanisms involved in the death of these tumor cells were apoptosis [46–48]. Therefore, the use of EEP-S and EEP-M may constitute an alternative treatment for chronic myeloid leukemia, as K562 cells are resistant to apoptosis induced by various agents [49].

Some compounds found in EEPs may play an important role in anticancer activity, including tocopherol, which shows antitumor activity in esophageal cancer cells [50] and breast cancer in vitro and in vivo [51]. Furthermore, caffeate derivatives are cytotoxic against human carcinoma cell lines [52].

Other phenolic compounds present in propolis exhibit antiproliferative and cytotoxic effects against various tumor cell lines, including those obtained from renal cell carcinomas [53] and the colon [30], pancreas [54], skin [55], and lungs [56]. Amyrins can be isolated from plants and is known as natural potent anticancer; its compounds induces tumor cell



(a)



(b)

FIGURE 4: Cytotoxic action of EEP from *S. depilis* against the K562 erythroleukemia cell line. (a) Representative diagrams obtained via flow cytometry of cells stained with annexin V-FITC/PI: the lower left quadrant (PI−/An−) represents viable cells; the lower right quadrant (PI−/An+) represents apoptotic cells; the upper left quadrant (PI+/An−) represents cells undergoing necrosis; and the upper right quadrant (PI+/An+) represents cells in late apoptosis. (b) Frequency of cell death, obtained from the corresponding diagrams for the tested concentrations. $^{*}p < 0.05$ for the treated group versus control viable cells. $^{**}p < 0.05$ for the treated group versus control apoptosis. $^{\#}p < 0.05$ for the treated group versus control necrosis. $^{+}p < 0.05$ for the treated group versus control late apoptosis.

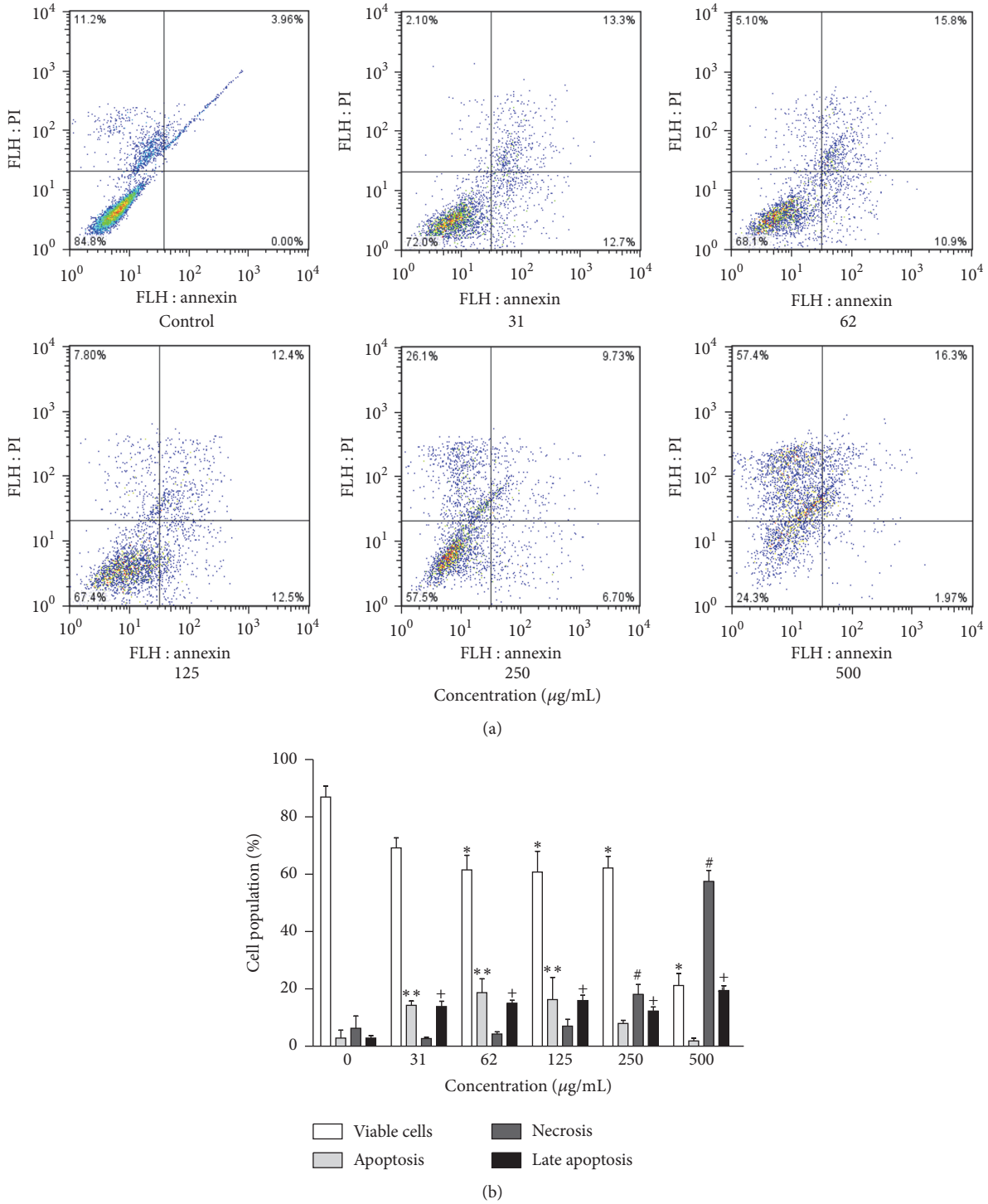


Figure 5: Cytotoxic action of EEP from *M. q. anthidioides* against the K562 erythroleukemia cell line. (a) Representative diagrams obtained via flow cytometry of cells stained with annexin V-FITC/PI: the lower left quadrant (PI−/An−) represents viable cells; the lower right quadrant (PI−/An+) represents apoptotic cells; the upper left quadrant (PI+/An−) represents cells in necrosis; and the upper right quadrant (PI+/An+) represents cells in late apoptosis. (b) Frequency of cell death, obtained from the corresponding diagrams for the tested concentrations. $^{*}p < 0.05$ for the treated group versus control viable cells. $^{**}p < 0.05$ for the treated group versus control apoptosis. $^{\#}p < 0.05$ for the treated group versus control necrosis. $^{+}p < 0.05$ for the treated group versus control late apoptosis.

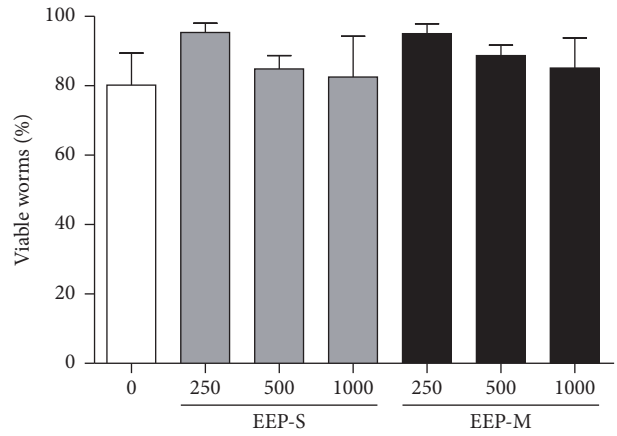


Figure 6: Toxicity of EEPs from *S. depilis* EEP-S and *M. q. anthidioides* (EEP-M) against *C. elegans*. $^{*}P < 0.05$ for the treated group versus the control with untreated nematodes.

death as human bladder carcinoma (NTUB1) [34, 57] and leukemia cells (HL-60) [58].

Although the EEPs presented cytotoxic activity against K562 cells, no toxic or lethal effects were observed against the nematode *C. elegans*.

In vivo experimental models serve as a tool to understand effects of natural products in whole organisms. These results suggest that the evaluated propolis samples show specificity against leukemic cells, considering that these nematodes were not affected. This specificity may be important for the treatment of leukemia because drug toxicity and low specificity are among the major difficulties in the treatment of this disease [37].

Corroborating with the toxicity data of the EEPs, recent study showed that the crude extract of propolis presented anticancer effects in human lung cancer cell and is antimelanogenic in the melanoma cell line; additionally it was able to prolong the life of *C. elegans* [48]. In addition, the caffeic acid, the major constituent of propolis, does no present toxic effects and also was able to increase the survival of the nematode *C. elegans* after infection with the fungal pathogen [59]. The ability of propolis or caffeic acid to extend lifespan in *C. elegans* was associated with inactivation of oncogenic kinase PAK1 [48, 59].

Previous studies have shown that *C. elegans* can be used as an experimental model for obtaining rapid results in toxicity studies for pharmacological compounds [60, 61] because it is a multicellular organism with a high reproduction rate and short life cycle, which makes it an excellent in vivo model for complementing cell culture-based systems [61].

Therefore, we conclude that the tested EEPs exhibit antioxidant and cytotoxic activities, attributed to their chemical composition, which includes phytosterols, terpenes, phenolic compounds, and tocopherol, and possibly to the synergy between different compounds present in propolis. Moreover, these EEPs show therapeutic potential for use in the prevention and treatment of diseases associated with oxidative stress and the proliferation of tumor cells.

Abbreviations

AAPH: 2,2′-Azobis-(2-amidinopropane) dihydrochloride
Abs: Absorbance
An: Annexin V-FITC
BHT: Butylhydroxytoluene
DAD: Diode array detector
DPPH: 2,2-Diphenyl-1-picrylhydrazyl
CG-MS: Gas chromatography-mass spectrometry
HPLC: High performance liquid chromatography
EEPs: Ethanol extract of propolis
EEP-S: Ethanol extract of propolis of *Scaptotrigona depilis*
EEP-M: Ethanol extract of propolis of *Melipona quadrifasciata anthidioides*
MDA: Malondialdehyde
NaCl: Sodium chloride
PI: Propidium iodide
SEM: Standard error of the mean
TBA: Thiobarbituric acid.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The authors thank Marcelo Mori, Ph.D., for intellectual assistance. This work was supported by grants from Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Edson Lucas dos Santos, Cláudia Andréa Lima Cardoso, and Edgar J. Paredes-Gamero were recipient of fellowship from CNPq, Brazil.

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Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 8374295, 7 pages
https://doi.org/10.1155/2017/8374295

Research Article

Phytoconstituents and Nutritional Properties of the Fruits of *Eleutherococcus divaricatus* and *Eleutherococcus sessiliflorus*: A Study of Non-European Species Cultivated in Poland

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Received 23 November 2016; Revised 8 December 2016; Accepted 28 December 2016; Published 15 January 2017

Academic Editor: Elena Azzini

Eleutherococcus fruits have been consumed in Russia and Asia throughout the centuries. Currently, there is an increasing interest in these products by the community of Western Europe. Many people suffer from micronutrient deficiencies, known as malnutrition, which consequently influences body condition. The aim of this study was to investigate pharmaconutrition, proximate, mineral, and fatty acid composition, total phenolics content, and total flavonoids content of *Eleutherococcus divaricatus* and *Eleutherococcus sessiliflorus* fruits cultivated in Poland. *Eleutherococcus divaricatus* and *E. sessiliflorus* contain a high amount of protein and fibres (16.70% and 12.28%; 61.41% and 45.63%, resp.). The fruits were generally high in K (21 g/kg) and low in sodium (0.001 g/kg). In terms of fatty acid composition, both species had a high amount of monounsaturated fatty acids (54.84–57.95%) and polyunsaturated fatty acids (36.22–37.0%). Using LC-ESI-MS/MS, protocatechuic acid has been identified as the most abundant compound, ranging from 260 to 810 μg/100 g DE. Among flavonoids, hyperoside was found to be in the highest amount (120–780 μg/100 g DE). Considering a rich chemical composition of the fruits, a better understanding of their health benefits is important in order to increase their utility and to enrich dietary sources of health promoting compounds. Because of a high amount of protein and a low calorific value, the fruits should be considered food for vegans or vegetarians.

1. Introduction

Species from the *Eleutherococcus* Maxim. genus are a valuable source of eleutherosides, phenolic acids, flavonoids, anthocyanins, triterpenoids, and biopolymers. One of the most known species of that genus is *E. senticosus*, which is very popular as a dietary supplement in Asia and the United States. Its products are used in the form of capsule, powder, and teabag, as health foods and drugs [1, 2]. The main compounds, including eleutherosides, phenolic acids, and flavonoids, were characterised by HPLC, HPTLC, and LC-MS methods [3]. Apart from the aforementioned compounds, these species contain oleanolic and betulinic acids, chiisanoside, lipid acids, essential oil, and sesamin [4, 5].

Several reports have revealed that *E. divaricatus* and *E. sessiliflorus* fruits are beneficial for human health, and currently there has been a growing research interest with regard to the products of these fruits used for consumption. According to Załuski's previous studies, the fruits of species cultivated in Poland act as antioxidants, induce apoptosis in Jurkat 45 leukemic cell line, and inhibit the activity of MMP-1, MMP-2, MMP-3, and MMP-9. It is thought that chiisanoside, which was identified in the leaves, decreases the absorption of lipids. The roots and stems of *E. sessiliflorus* have been

shown to have antipancreatic lipase and anti-inflammatory activities. With regard to its pharmacological aspects, *E. sessiliflorus* has been used in traditional medical protocols as a tonic, analgesic, antihypertensive, and antidiabetic agent. Załuski's previous studies have shown that the fruits may reduce DPPH* radical, inhibit lipid peroxidation, and have an ion-chelating ability [6–10]. In food industry, only species native to Asia are used, while species cultivated in Europe are not yet used commercially. It is important that tested species are cultivated in other geographical zones (Poland) than native ones (Asia). The Polish climate conditions may have an influence on the chemical profile and nutritional value of fruits. They were successfully cultivated at the botanical garden in Rogów, which lies in the Central Polish Lowlands region with geographic data 51°49′N and 19°53′E. The vegetative period lasts for 212 days and the average annual precipitation is 596 mm, of which 80% occurs during the vegetative period. The average annual air temperature is 7.2°C. The average long-term temperature is −20.1°C, which classified the garden to the 6bth subclimate (according to "USDA Frost Hardiness Zones") and to the second zone according to Kórnik's category. These plants are grown on the acidic, luvic, and sandy soils [11].

Phenolic compounds, known as nonnutritional ingredients in food, constitute one of the most widely occurring groups of phytochemicals with a wide range of physiological properties [12]. They are components of many fruits and vegetables, which are associated with health benefits after their consumption [13]. Clinical trials and epidemiological studies have established that dietary intake of fruits is strongly associated with a reduced risk of the civilization diseases. In the human body, they act as antiallergenic, antiatherogenic, anti-inflammatory, antimicrobial, antioxidant, and antithrombotic agents [12, 14].

A study of the pharmaconutrients panel of fruits can give us a better understanding of their potential beneficial effects on human health. It is a common opinion that the origin of most illnesses is due to inappropriate nutrition. Keeping in mind their long-term use by the Asians, we have decided to evaluate the quality of *E. divaricatus* and *E. sessiliflorus* cultivated in Polish climate conditions as a pharmaconutrient material and potential dietary and/or pharmaceutical product. Because those species are cultivated in another geographical zone than a native one, it is necessary to study the composition of the raw material, to ensure product quality. No comprehensive data have been reported on the proximate, mineral, fatty acids, phenolics, and flavonoids composition and caloric value of the fruits. Apart from the raw extracts, we investigated also the infusions, as very popular homemade beverages, in many cases containing different fruits.

2. Experimental

2.1. Plant Material. The fruits of *E. divaricatus* (Siebold et Zucc.) S. Y. Hu and *E. sessiliflorus* (Rupr. & Maxim.) S. Y. Hu were collected at the arboretum in Rogów (Poland) in October 2016. All plant samples were deposited at the Department of Pharmacognosy, Collegium Medicum, Bydgoszcz, Poland (Cat. number ED 01-2016; ES 02-2016).

2.2. Chemicals. Folin-Ciocalteau reagent, DMSO, gallic acid, quercetin, and hesperetin were purchased from ChromaDex. Standards of gallic, protocatechuic, gentisic, 4-OH-benzoic, vanillic, caffeic, syringic, p-coumaric, ferulic, salicylic, veratric, sinapic, 3-OH-cinnamic, and rosmarinic acid, luteolin 7-glucoside, luteolin 3,7-diglucoside, rutin, hyperoside, isoquercetin, naringin, naringenin 7-glucoside, quercitrin, apigenin 7-glucoside, and LC grade acetonitrile were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Kaempferol 3-rutinoside and astragalin were from Carl Roth (Karlsruhe, Germany). Luteolin 4′-O-glucoside was obtained from LGC Standards (Dziekanów Leśny, Poland). 2,4-DNPH, ethanol, $FeCl_3$, and HNO_3 were obtained from POCH (Lublin, Poland). LC grade methanol (MeOH) was purchased from J. T. Baker (Phillipsburg, USA). LC grade water was prepared using a Millipore Direct-Q3 purification system (Bedford, MA, USA). All others reagents were of analytical grade.

2.3. Accelerated Solvent Extraction (ASE). The air-dried and powdered fruits (5 g each) were placed in an extraction cell with 30 g of neutral silica gel. The ASE cell was placed into ASE for the extraction process (Dionex system). During the extraction process, 75% ethanol was delivered into the extraction cell. Pressure (1000 psi) was applied to maintain the solvent in its liquid state. The extraction process was repeated three times using 10 mL ethanol. The extraction temperature was 40°C, and the extraction time was 15 min. Following extraction, the extract containing the target analytes was purged from the cell using nitrogen into a collection vial for analysis. After the three extraction cycles, 30 mL of extracts was obtained. The solvents were dried with an evaporator under vacuum conditions at 45°C and subjected to lyophilisation.

2.4. Infusion Preparation. The infusion was prepared by adding 50 mL of distilled water (95°C) to 5 g of fruits. The infusions were brewed for 15 minutes and were then filtered over Whatman No. 1 paper. The aqueous extracts were frozen and lyophilised.

2.5. Proximate Composition. The fruits were analysed for proximate composition: moisture by air drying at 105°C for 2 h, total fat by extraction with hexane in Soxhlet's apparatus, protein by Kjeldahl's method, and ash by direct analysis at 550°C for 6 h. Total carbohydrates were calculated by difference. Values represent averages of triplicate determinations performed for all analyses.

2.6. AAS of Minerals. 0.5 g of the dried and ground fruits was put into a burning cup, and 2 mL of pure HNO_3 was added. The samples were incinerated in a MARS 5 microwave oven (Manufactured by CEM Corporation, USA) at a temperature of 90°C for 15 min and next at 120°C for 10 min and 210°C for 30 min, and the solution was diluted to 100 mL with water. Minerals content was determined with a Varian SpektrAA 280FS + Autosampler SPS 3 spectrometer. Minerals and trace elements were determined using the instrumental conditions recommended for each mineral and were calculated based on the respective standard curve.

2.7. GC-FID Fatty Acid Analysis. Fatty acids were extracted with hexane in Soxhlet's apparatus. A highly sensitive and accurate multiplex gas chromatography-linear ion trap technique was used to identify components of lipid fraction. GC/MS/MS was performed using Varian 4000 GC/MS/MS chromatograph. The GC conditions were as follows: VF-5 ms fused silica capillary column (30 × 0.32 mm, film thickness: 0.25 μm), with the oven temperature programmed at a rate of 3°C from 200 (held for 10 min) to 240°C (held for 4.67 min) and injector kept at 250°C and detector at 300°C, with split ratio 1 : 50. Helium was used as the carrier gas with a constant flow rate of 2.5 mL/min. The sample size was 1 μL in hexane. Compounds were identified using Galaxie™ Chromatography Data System. The following acids were analysed: C6:0 caproic; C8:0 caprylic; C10:0 capric; C11:0 undecanoic; C12:0 lauric; C13:0 tridecanoic; C14:0 myristic; C14:1 myristoleic; C15:0 pentadecanoic; C15:1 cis-10-pentadecenoic; C16:0 palmitic; C16:1 palmitoleic; C17:0 heptadecanoic; C17:1 cis-10-heptadecenoic; C18:0 stearic; cis-C18:1n–9 oleic; trans-C18:1n–9 elaidic; cis-C18:2n–6 linoleic; trans-C18:2n–6 linolelaidic; C18:3n–6 γ-linolenic; C18:3n–3 α-linolenic; C20:0 arachidic; C20:1 cis-5-eicosenoic; C20:2 cis-11,14-eicosadienoic; C20:3n–6 cis-8,11,14-eicosatrienoic; C20:4n–6 arachidonic; C20:5n–3 eicosapentaenoic; C21:0 heneicosanoic; C22:0 behenic; C22:1n–9 erucic; C22:2 cis-13,16-docosadienoic; C23:0 tricosanoic; C24:0 lignoceric; C24:1n–9 nervonic. The data of total lipids were statistically analysed and expressed as mean ± standard deviation.

2.8. Total Phenolic Content (TPC). The total phenolic content of extracts was determined using the method of Singleton and Rossi [15]. TPC was expressed as gallic acid equivalents (20–100 μg/mL; $y = 0.0026x + 0.044$; $r^2 = 0.999$; GAE/g dry extract). The experiments were done in triplicate.

2.9. Total Flavonoid Content (TFC). The TFC in investigated samples was determined using aluminium chloride and 2,4-dinitrophenylhydrazine colorimetric methods [16]. TFC were expressed as means (±SE) mg of quercetin equivalent (20–100 μg/mL; $y = 0.0041x + 0.236$; $r^2 = 0.999$; QEs/g dry extract for $FeCl_3$ method) and as means (±SE) mg of hesperetin equivalent (HEs/g dry extract for DNPH method; 250–1000 μg/mL; $y = 6.374x - 0.098$; $r^2 = 0.988$). The experiments were done in triplicate.

2.10. LC-ESI-MS/MS Conditions of Analysis of Phenolic Acids and Flavonoids. To evaluate phenolic acids content, the samples were analysed using a modified LC-ESI-MS/MS version of Nowacka et al. [17], with the levels of flavonoid glycosides as reported below. An Agilent 1200 Series HPLC system (Agilent Technologies, USA) equipped with a binary gradient solvent pump, a degasser, an autosampler, and column oven connected to 3200 QTRAP mass spectrometer (AB Sciex, USA) was used. Chromatographic separation was carried out at 25°C, on an Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm particle size; Agilent Technologies, USA) with a mobile phase consisting of water containing 0.1% HCOOH (solvent A) and acetonitrile containing 0.1% HCOOH (solvent B), using 5 μL injections. The flow rate was 450 μL min^{-1} and the gradient was as follows: 0-1 min, 18% B; 1.5–5.5 min, 20% B; 7–10 min, 25% B; 13–15 min, 60% B; 17–21 min, 18% B. The QTRAP-MS system was equipped with electrospray ionisation source (ESI) operated in the negative-ion mode. ESI worked at the following conditions: capillary temperature of 500°C, curtain gas at 25 psi, nebulizer gas at 50 psi, and negative-ionisation mode source voltage of −4500 V. Nitrogen was used as curtain and collision gas. For each compound, the optimum conditions of Multiple Reaction Mode (MRM) were determined in the infusion mode. The data was acquired and processed using Analyst 1.5 software (AB Sciex, USA). Triplicate injections were made for each standard solution and sample. The analytes were identified by comparing retention time and *m/z* values obtained by MS and MS^2 with the mass spectra from corresponding standards tested under the same conditions. The calibration curves obtained in MRM mode were used for quantification of all analytes. The identified phenolic acids were quantified on the basis of their peak areas and comparison with a calibration curve obtained with the corresponding standards. Linearity ranges for calibration curves were specified. The limit of detection (LOD) and limit of quantification (LOQ) for phenolic compounds were determined at a signal-to-noise ratio of 3 : 1 and 10 : 1, respectively, by injecting a series of dilute solutions with known concentrations.

2.11. Statistical Analysis. All determination was performed in triplicate. The obtained data were subjected to statistical analysis using Statistica 7.0. (StatSoft, Cracow). The evaluations were analysed for one-factor variance analysis. Statistical differences between the treatment groups were estimated by Spearman's (R) and Pearson's (r) test. All statistical tests were carried out at significance level of $\alpha = 0.05$.

3. Results and Discussion

3.1. Proximate Composition and Calorific Value. The results of proximate analysis are presented in Table 1. *Eleutherococcus divaricatus* contains high proportions of protein and fibres compared to *E. sessiliflorus*, whereas *E. sessiliflorus* has a higher level of carbohydrates. The carbohydrates content in *E. sessiliflorus* (25.7%) is quite high when compared to *E. divaricatus* (2.5%), which can be appropriate in formulating high carbohydrate diets. The ash content of 5.53 and 4.89% makes the fruits a good source of minerals for consumers.

Suárez-Martínez et al. reported on the content of protein in *Phaseolus angularis*, *P. lunatus*, *P. vulgaris*, and *P. mungo* in the range of 22.9–26.2%, respectively. Overall, the content of bean proteins is said to be at the level of 20–30%. In turn, soybean proteins represent about 35–40% on a dry weight basis. Despite the fact that the soybean is receiving increasing attention with respect to its health effects, it is also a well-recognized allergenic food for sensitive people. Additionally, soybean should not be ingested in a high amount by males because soy food and soy isoflavones are associated with lower sperm concentration [18, 19]. In this case, particular attention should be directed to the vegetarian or vegan males. Taking into account the proximate composition, it can be pointed out that the fruits of *E. divaricatus* should enrich any

Table 1: Proximate composition (%) of the fruits of *E. divaricatus* and *E. sessiliflorus*[*].

Nutrient [%]	*E. divaricatus*	*E. sessiliflorus*
Moisture	7.72	8.21
Ash	5.53	4.89
Protein	16.70 ± 0.53	12.28 ± 0.39
Fat	6.9	3.26
Carbohydrates	2.5	25.7
Fibres	61.41 ± 20.26	45.63 ± 15.06
Calorific value	1046 kJ/100 g	1132 kJ/100 g
	255 kcal/100 g	273 kcal/100 g

[*]Results are means ± standard deviation of triplicates.

diet where high protein and fibre content is needed. Taking into account the calorific value, the fruits may be used in slimming diet.

3.2. Mineral Content. Antioxidant activity of plants is, very often, associated with the amount of mineral constituents. Some of them (Se, Zn, Mn, and Cu) are often thought to be a dietary antioxidant protecting cells from oxidative damage [20]. The concentrations of the mineral components of the fruits according to the mineralisation and identification methods are reported in Table 2. The variation in the amount of minerals between two species has been noticed. The main elements of both species were Ca and K. The fruits of *E. divaricatus* have a high level of Mn and Zn compared to *E. sessiliflorus*. *Eleutherococcus divaricatus* contains a high amount of Fe.

Other studies indicated that the *E. senticosus* fruits, collected in Korea, contain 465, 1433, 199, and 13 mg/kg dry weight of Ca, Mg, Mn, and Zn, respectively [21]. Compared with species cultivated in Poland, there is a difference in the level of Ca and Mn. Both species contain higher level of Ca and lower level of Mn. A major finding is that the species native to Asia does not contain Fe, a factor that excludes these fruits as an ingredient of antianemic diet. It is worth noting that *E. divaricatus* has higher Fe content than the *Rosa canina* L. fruits (27.0 mg/kg), which is very popular in the diet of the Europeans. Moreover, the results obtained in the present study indicated a higher content of Mn, Zn, and Cu than that in the *Rosa canina* L. and *Rosa damascena* Mill. fruits [22]. It should be mentioned that *E. divaricatus* contains more Zn and Mn than walnut of kernels (from 17.9 to 20.6 and 17.5 to 22.2 mg/kg). According to a WHO report, over 2000 million people in developing countries have iron deficiency anemia [23]. The global burden of disease estimates showed that, among the 26 major risk factors of the global burden of disease, iron deficiency ranks ninth overall, while zinc deficiency is eleventh [24]. In that case, the fruits, especially of *E. divaricatus*, should be considered a new dietary ingredient that may be included in the antianemic diet. In order to receive the best results, a combination of these fruits has to be included in the diet.

3.3. Fatty Acid Composition. A total of 36 different fatty acids were identified by GC-FID analysis. 16 different fatty acids were identified in *E. sessiliflorus*, and 14 were identified in *E. divaricatus*, contained at various concentrations in all the analysed fruits (Table 3). The fruits of *E. sessiliflorus* have a threefold higher amount of α-linolenic acid (ALA) than *E. divaricatus* (2.27 and 0.79%), the acid whose occurrence is limited. The α-linolenic acid is recognized as a promising therapeutic agent for numerous health disorders acting as the preventive and neuroprotective constituent of the human diet. The predominant unsaturated fatty acids obtained were oleic acid and elaidic acid up to 57%. Overall, in the present study, a majority of the fatty acids were monounsaturated (57.95% and 54,84% in *E. divaricatus* and *E. sessiliflorus*, resp.). The MUFA content was higher than that of walnut, which is an important ingredient of the European diet. Another popular product is virgin coconut oil, which is suggested as a functional food with a high amount of MUFA and PUFA. However, this oil contains a lower amount of C18:1 (6.5%) than the analysed samples. It has been established that MUFA has a beneficial effect on health by raising high-density lipoprotein cholesterol (HDLC) [25]. Apart from MUFA, the high content of PUFA and omega-6 has also been assayed.

3.4. TPC and TFC in the Fruits. As it was shown in Table 4, all ethanol extracts contain a higher amount of TPC than the infusions (45.3 and 52.3 mg/g DE for *E. divaricatus* and *E. sessiliflorus*, resp.). In turn, the summed amount of flavonoids ranged from 18.4 to 23.0 mg/g DE for *E. divaricatus* and *E. sessiliflorus*. We found that flavonoid content obtained by aluminium chloride reaction was much higher than those obtained by DNPH reaction. This can indicate that the species contain more compounds with the OH group which are responsible for the high antioxidant activity of the extracts.

The contents of phenolic compounds were within the range of those previously reported for the various *Eleutherococcus* species, cultivated in Poland. Załuski and Janeczko [26] showed that the fresh fruits of *E. divaricatus* and *E. sessiliflorus* contain 6.1 and 6.9 mg/g dry sample of polyphenols. Shohael et al. [27] studied *E. sessiliflorus* growing in Korea but reported a lower concentration of phenols than that now estimated. The TPC content found in the 75% ethanolic extracts from the spring leaves ranged from 20.3 to 37.2 mg/g, followed by the fresh fruits (6.1–19.7 mg/g) and the roots (6.9–10.6 mg/g). Jang et al. [28] revealed that the fruits of *E. senticosus* collected in Korea contained from 197.9 to 334.3 mg/g of polyphenols, while the TFC ranged from 41.2 to 203.7 mg/g.

Our findings showed that the investigated *Eleutherococcus* fruits contain more TPC than the blueberries fruits, which in Poland or other European countries are very widely used in food products and for medical purposes and are recognized as rich sources of polyphenols. According to Grace et al. [29], blueberries contain from 22.7 to 39.3 mg/g extract of polyphenols. According to Załuski and Janeczko [26], the content of TPC and TFC in the fruits is not changed during storage and quantified between 4.11 and 4.35 g/100 g for the freshly dried fruits from *E. senticosus* and *E. henryi*. After 1-year storage, the amount did not change significantly and was between 3.85 and 4.13 for *E. senticosus* and *E. henryi*. Heo

Table 2: Mineral compositions of the fruits [mg/kg].

	Fe	Ca	Mg	K	Na	Cu	Zn	Mn	Se
E. divaricatus	30 ± 3.5	3310	1460	2100	1	5.3 ± 0.8	27.5 ± 2.8	105.5	0.84
E. sessiliflorus	20 ± 2.9	1730	1240	2790	25	3 ± 0.62	10 ± 1.8	4	0.41

Table 3: Fatty acid composition of the fruits of *E. divaricatus* and *E. sessiliflorus* [%].

Fatty acid	*E. divaricatus**	*E. sessiliflorus**
C10:0	—	0.06
C12:0	0.06	0.15
C13:0	0.08	—
C14:0	0.16	0.19
C15:0	0.05	0.13
C16:0	3.76 ± 0.22	4.96 ± 0.29
C16:1	—	0.76
C17:0	0.06	0.10
C17:1	—	0.10
C18:0	0.85 ± 0.09	1.27 ± 0.14
C18:1n9c + C18:1n9t	**57.61** ± 1.86	**53.73** ± 1.74
C18:2n6c + C18:2n6t	**35.43** ± 0.43	**34.73** ± 0.42
C18:3n3 (alpha)	0.79 ± 0.06	2.27 ± 0.17
C20:0	0.19 ± 0.01	0.23 ± 0.01
C20:1	0.34	0.26
C22:0	0.35	0.63
C24:0	0.23	0.41
SFA	5.77	8.11
MUFA	57.95	54.84
PUFA	36.22	37.00
Omega-3	0.79	2.27
Omega-6	35.43	34.73

*Results are means ± standard deviation of triplicates.

et al. [21] reported a lower concentration of polyphenols and flavonoids in ethanol, methanol, and water extract of the *E. senticosus* fruits growing in Korea (0.3, 0.6, and 0.6% and 0.20, 0.23, and 0.3%, resp.) than that now estimated.

3.5. LC-ESI-MS/MS Analyses for Phenolic Acids and Flavonoids. Results of the optimization of conditions of LC-ESI-MS/MS analysis are given in Tables S1, S2, S3, and S4 in Supplementary Material available online at https://doi.org/10.1155/2017/8374295. The concentrations of individual compounds, which were quantified by comparison of peak areas with the calibration curves obtained for the corresponding standards, are reported in Table 5. Among fourteen phenolic acids (gallic, protocatechuic, gentisic, 4-OH-benzoic, 3-OH-benzoic, vanillic, syringic, *p*-coumaric, ferulic, veratric, salicylic, 3-OH-cinnamic, sinapic, and rosmarinic), just five were qualitatively and quantitatively determined in the fruits (protocatechuic, caffeic, *p*-coumaric, ferulic, and salicylic). Protocatechuic acid occurs in the highest amount (260–810 μg/100 g DE). In turn, LC-ESI-MS/MS analysis of flavonoids revealed the presence of six flavonoids, of which hyperoside was determined in the highest amount (120–780 μg/100 g DE). This is the first time astragalin is found in *Eleutherococcus* spp. Overall, smaller quantities of phenolic acids and flavonoids were present in the infusions than in the 75% ethanol extracts.

Only a few studies have focused on the assessment of phenolic acids and flavonoids present in roots, leaves, and fruits of species native to Asia and Russia. Data in the literature indicated that Kurkin et al. [30] identified free phenolic acids (syringic, *p*-coumaric, vanillic, *p*-hydroxybenzoic, caffeic, and ferulic acids) and depside (chlorogenic acid) in the roots of *E. senticosus* growing in Russia. Kurkin et al. [30] identified protocatechuic, chlorogenic, and caffeic acids in the roots of a Chinese sample. Bączek identified rosmarinic, chlorogenic, ferulic, and caffeic acids in the roots, fruits, and stem barks of six species [31, 32].

Protocatechuic acid has been identified in *Hibiscus sabdariffa* L. (2.8 and 11.9 mg/g aqueous and ethanol extracts from roselle calyx) and *Euterpe oleracea* Mart. (630 mg/L of oil) [33, 34]. In turn, the content of protocatechuic acid in *Allium cepa* L. was dependent on a type of raw material. The highest content was determined in a dried material (76.3 μg/g) contrary to a fresh material (5.8 μg/g) [35]. Comparing the results obtained in this work with the cited ones, it is concluded that *Eleutherococcus* spp. contain a higher amount of protocatechuic acid. This acid also occurs in various fruits such as berries (raspberry, blueberry, mulberry, cranberry, and gooseberry), wine, honey, and soybean. Protocatechuic acid has been found to have various activities such as antibacterial, antidiabetic, antiulcer, anti-inflammatory, and cardiac effects [36].

Data related to flavonoids in *Eleutherococcus* spp. are scarce and mainly relate to Załuski et al.'s previous studies [9, 26]. Zhao et al. [37] reported on the presence of hyperin in the green and mature fruits of *E. sessiliflorus* (630.7 and 430.1 μg/g). Lee at al. [38] have detected rutin, hyperin, quercetin, and kaempferol in *E. divaricatus*, planted in Korea. Other studies on the content of rutin, hyperin, quercetin, and kaempferol, in different species of *E. divaricatus* and *E. sessiliflorus* collected in South Korea, have shown the differences dependent on the plant part. The roots and stems were rich in rutin (1.9, 3.5 and 4.2, 0.8 mg/g for *E. divaricatus* and *E. sessiliflorus*) [39].

4. Conclusions

Our results demonstrate that the fruits of *Eleutherococcus* species, rich in polyphenols and nutrients, have promising potential as a new income source of agriculture and industry in natural products and foods. The fruits may become ingredients of herbal teas or natural products where a high amount of phytochemicals and nutrients is needed.

Table 4: TPC and TFC in extracts from the fruits of *E. divaricatus* and *E. sessiliflorus* (mg GAE/g and QEs/g dry extract*).

Species	TPC	Flavonoid content		TFC
		$FeCl_3$	DNPH	
^*E. divaricatus*	52.3 ± 0.5	12.3 ± 0.11	6.0 ± 0.04	18.4 ± 0.02
^*E. sessiliflorus*	45.3 ± 0.5	17.3 ± 0.08	5.7 ± 0.01	23.0 ± 0.03
•*E. divaricatus*	41.1 ± 0.5	12.0 ± 0.5	6.1 ± 0.5	18.2 ± 0.5
•*E. sessiliflorus*	40.6 ± 0.5	10.0 ± 0.5	5.9 ± 0.5	15.0 ± 0.5

*Results are means ± standard deviation of triplicates.
^The 75% ethanol extracts; •the infusions.

Table 5: Phenolic acid contents expressed in μg per 100 g of dry weight of extracts.

	75% ethanol		Infusion	
	E. divaricatus	*E. sessiliflorus*	*E. divaricatus*	*E. sessiliflorus*
		Phenolic acids		
Protocatechuic acid	693	818	270	267
Caffeic acid	133	0.8	22.8	19.8
p-Coumaric acid	Trace	Trace	5.2	5.3
Ferulic acid	11.1	6.1	4.1	5.9
Salicylic acid	0.15	Trace	Trace	Trace
		Flavonoids		
Rutin	90	Trace	0.002	Trace
Hyperoside	780	360	120	280
Isoquercetin	Trace	100	Trace	Trace
Naringin	Trace	Trace	0.9	1.0
Astragalin	30.0	10	—	—
Naringenin 7-glucoside	5.0	3.0	—	4.0

—: not detected; Trace: trace amounts. Mean values of three replicate assays with standard deviation.

Abbreviations

TPC: Total phenolics content
TFC: Total flavonoids content
MUFA: Monounsaturated fatty acids
PUFA: Polyunsaturated fatty acids
DNPH: 2,4-Dinitrophenylhydrazine
$FeCl_3$: Aluminium chloride
GA: Gallic acid
HE: Hesperetin equivalent
QE: Quercetin equivalent
AAS: Atomic absorption spectroscopy.

Competing Interests

The authors declare no competing financial interests.

Acknowledgments

The paper was developed using the equipment purchased within the project The Equipment of Innovative Laboratories Doing Research on New Medicines Used in the Therapy of Civilization and Neoplastic Diseases within the Operational Program Development of Eastern Poland 2007–2013, Priority Axis I Modern Economy, Operations I.3 Innovation Promotion.

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