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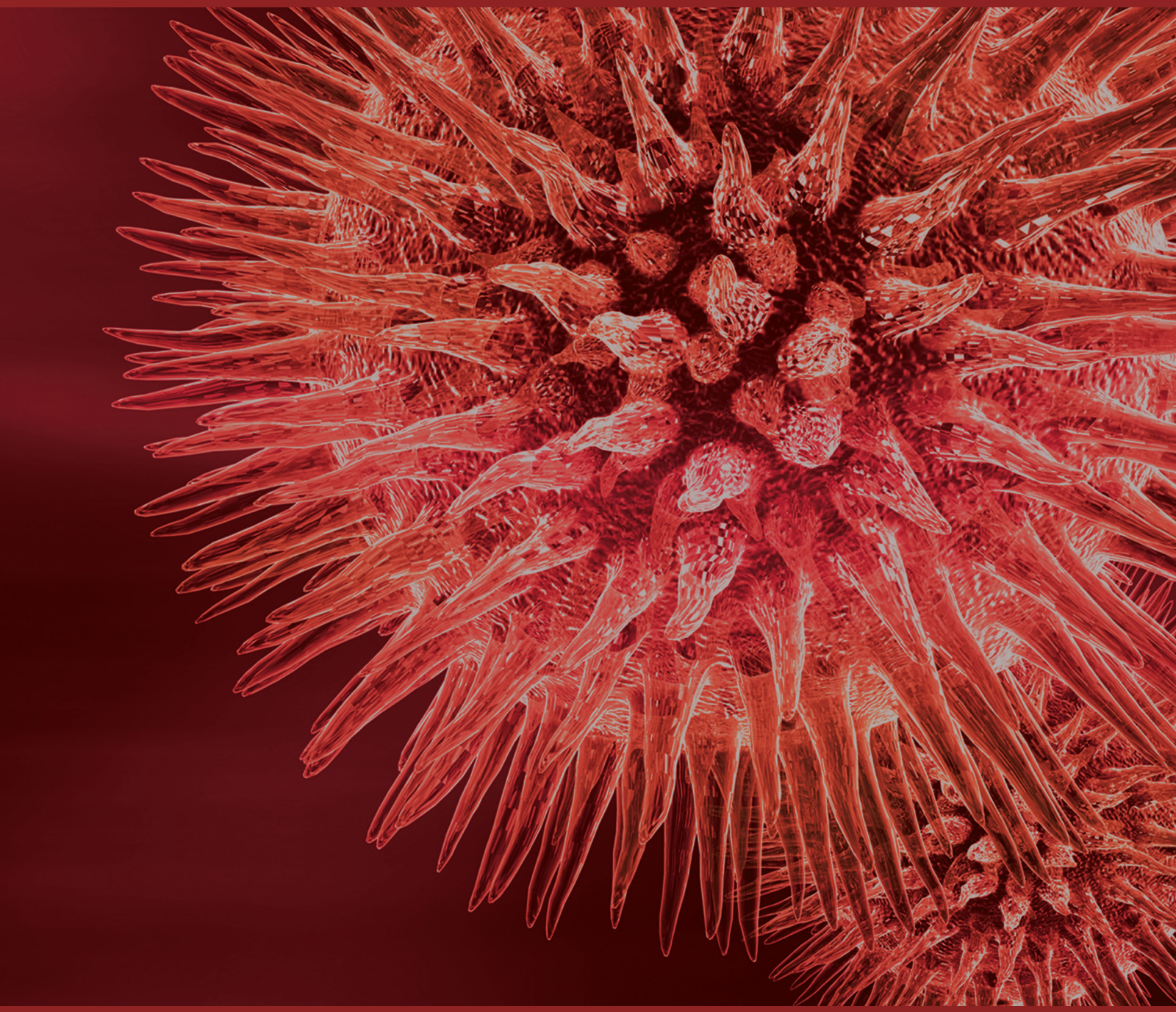
Food Bioactive Compounds against Diseases of the 21st Century

Guest Editors: Chia-Chien Hsieh, Juliana Maria Leite Nobrega de Moura Bell, and Blanca Hernández-Ledesma



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Editorial

Food Bioactive Compounds against Diseases of the 21st Century

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Increasing incidence of metabolic disorders has been evidenced in the past decades. Common metabolic disorders include obesity, diabetes, metabolic syndrome, and other chronic diseases, including cardiovascular and neurological diseases, stroke, cancers, immune disorder, and chronic respiratory diseases. Despite clear evidence of the impacts of those diseases on human health, their incidence is reaching epidemic proportions in industrialized countries, with similar trend being observed in developing countries. Understanding genetic and environmental factors that might promote those diseases became a crucial research topic. Diet and lifestyle are two environmental factors having a significant impact on those diseases. Thus, modifications of diet and lifestyle are becoming a new strategy for prevention/treatment of metabolic disorders. Foods contain a wide range of bioactive compounds with multiple physiological properties. This research area has increased in the last years, with growing numbers and a wide variety of functional foods available in the worldwide market. Because of the importance of a healthy diet on metabolic disorders prevention, the present special issue summarizes the most recent advances on applied science to foods considered as a source of bioactive compounds that could be potentially useful to prevent/treat different diseases of the 21st century. The selected papers represent a rich and many-facet knowledge, which we have the pleasure of sharing with the readers.

Potential health benefits of naturally occurring α-linolenic acid have been described in a review entitled "α-Linolenic Acid, an Omega-3 Fatty Acid with Neuroprotective Properties: Ready for Use in the Stroke Clinic?" In this review, N. Blondeau et al. highlighted some protective effects of α-linolenic acid on neurological disorders and possible mechanisms of action. Sources of naturally occurring α-linoleic acid, intake recommendations, and current state of *in vitro* and *in vivo* tests have been reported.

In a paper entitled "Antioxidant/Prooxidant and Antibacterial/Probacterial Effects of a Grape Seed Extract in Complex with Lipoxygenase" by V. S. Chedea et al. The biological activities of flavan-3-ols and procyanidins from grape seeds, pure catechin, and an aqueous grape seed extract were evaluated in the presence of lipoxygenase or in extract. Those fractions were applied to leucocyte culture, *Escherichia coli B41* and *Brevibacterium linens* where lipid peroxidation, cytotoxicity, and growth rate of exposed cells were evaluated.

In a paper entitled "Role of Feed Forward Neural Networks Coupled with Genetic Algorithm in Capitalizing of Intracellular Alpha-Galactosidase Production by *Acinetobacter sp.*" S. Edupuganti et al. enhanced the production of intracellular alpha-galactosidase (7.5 to 10.2 U/mL) from *Acinetobacter sp.* isolated from sugar cane waste by using hybrid artificial neural networks and genetic algorithm (ANN-GA).

In a paper entitled "Angiotensin I Converting Enzyme Inhibitory Peptides Obtained after *in Vitro* Hydrolysis of Pea *(Pisum sativum var. Bajka)* globulins" A. Jakubczyk and B. Baraniak evaluated the potential antihypertensive effect of peptides released from pea seed globulins under conditions simulating gastrointestinal digestion. These peptides are demonstrated to act as uncompetitive inhibitors of angiotensin-converting enzyme activity.

In a paper entitled "Anti-Inflammatory Effects of *Siegesbeckia orientalis* Ethanol Extract in *in Vitro* and *in Vivo* Models" Y.-H. Hong et al. demonstrated that *Siegesbeckia orientalis* ethanol extract attenuated local and systemic acute inflammation in both *in vitro* and *in vivo* studies by inhibiting inflammatory mediators through suppression of MAPKs and NF-κB dependent pathways.

In a paper entitled "Evaluation of the Antioxidant Activity and Antiproliferative Effect of the *Jaboticaba (Myrciaria cauliflora)* Seed Extracts in Oral Carcinoma Cells" C.-G. Lin et al. pointed to the potential of *Jaboticaba* seed extract as a chemopreventive agent against oral carcinoma cells. The antioxidant activity and the apoptosis-inducing properties are responsible for the observed effects.

In a paper entitled "The Study of Interactions between Active Compounds of Coffee and Willow *(Salix sp.)* Bark Water Extract" A. Durak and U. Gawlik-Dziki demonstrated that both coffee and willow bark are sources of multidirectional antioxidant compounds. The extracts from willow as an ingredient in coffee beverages can provide health promoting may be benefit to consumers.

In a paper entitled "Oral and Intraperitoneal Administration of Quercetin Decreased Lymphocyte DNA Damage and Plasma Lipid Peroxidation Induced by TSA *in Vivo*" S.-T. Chan et al. pointed quercetin administered intraperitoneally decreased trichostatin A-induced lymphocyte DNA damage and plasma lipid peroxidation, but no effect on tumor growth was observed, indicating the potentially auxiliary protection and used pathway of quercetin in chemotherapy.

In a paper entitled "Food-Derived Bioactive Peptides on Inflammation and Oxidative Stress" F. Jahandideh and J. Wu showed the roles of various food-derived bioactive peptides in inflammation and oxidative stress and discussed the potential benefits and limitations of using these compounds against the burden of chronic diseases.

In a paper entitled "Milk Proteins, Peptides, and Oligosaccharides: Effects against the 21st Century Disorders" C.-C. Hsieh et al. summarized the impact of proteins, derived-peptides, and oligosaccharides present in milk on human health, with special emphasis on their effects against most common chronic disorders nowadays.

Acknowledgment

We would like to warmly thank all authors for their excellent contribution and the reviewers for their fundamental work that have made possible the publication of this special issue.

Chia-Chien Hsieh
Juliana Maria Leite Nobrega de Moura Bell
Blanca Hernández-Ledesma

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Review Article

Alpha-Linolenic Acid: An Omega-3 Fatty Acid with Neuroprotective Properties—Ready for Use in the Stroke Clinic?

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Alpha-linolenic acid (ALA) is plant-based essential omega-3 polyunsaturated fatty acids that must be obtained through the diet. This could explain in part why the severe deficiency in omega-3 intake pointed by numerous epidemiologic studies may increase the brain's vulnerability representing an important risk factor in the development and/or deterioration of certain cardio- and neuropathologies. The roles of ALA in neurological disorders remain unclear, especially in stroke that is a leading cause of death. We and others have identified ALA as a potential nutraceutical to protect the brain from stroke, characterized by its pleiotropic effects in neuroprotection, vasodilation of brain arteries, and neuroplasticity. This review highlights how chronic administration of ALA protects against rodent models of hypoxic-ischemic injury and exerts an anti-depressant-like activity, effects that likely involve multiple mechanisms in brain, and may be applied in stroke prevention. One major effect may be through an increase in mature brain-derived neurotrophic factor (BDNF), a widely expressed protein in brain that plays critical roles in neuronal maintenance, and learning and memory. Understanding the precise roles of ALA in neurological disorders will provide the underpinnings for the development of new therapies for patients and families who could be devastated by these disorders.

1. Introduction

Dietary approaches for stroke prevention and rehabilitation hold promise to improve outcomes in individuals at risk of stroke and those who have had a stroke [1–4]. Although there is abundant literature that connects reduction in stroke risk to certain dietary elements and increase in stroke risk to other certain dietary components, there is a paucity of clinical trial data to direct the public and clinicians in this important area of clinical need. Compounds with pleiotropic effects aimed at reducing infarct size by one or more mechanisms and improving outcome would be advantageous in reducing the devastating effects of stroke on patients and their families [1–3]. One compound that has been demonstrated to exert neuroprotective, anti-inflammatory, and antidepressant properties is α-linolenic acid (ALA), an 18-carbon, essential omega-3 polyunsaturated fatty acid (PUFA) (Figure 1). In this review we discuss beneficial effects of α-linolenic acid and

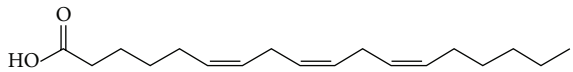

Figure 1: Structure of α-linolenic acid. α-Linolenic acid is an 18-carbon, polyunsaturated fatty acid that is essential for normal health. Because humans do not possess the enzymes to synthesize the compound, it must be obtained from dietary sources.

clinically relevant data to suggest that further exploration of this dietary component might be useful in stroke prevention and recovery.

Omega-3 fatty acids are required for normal health, especially for the brain development and function [16]. Prior work has shown that a seafood-rich diet was associated with low rates of coronary heart disease and autoimmune disorders in Greenland Eskimos which has been generally ascribed to the intake of eicosapentaenoic acid (EPA) and docosahexaenoic acid [DHA] [4]. A change in diet over the last century toward a higher total fat and saturated fat content and a sedentary lifestyle has been associated with an increase in the incidence of chronic disorders such as hypertension, diabetes, and atherosclerosis [17–20], all of which are stroke risk factors or risk markers. In addition, omega-6 fatty acids are consumed at a higher level compared with omega-3 fatty acids in a standard western diet and they have been implicated in coronary atherogenesis [21]. The distinction between the two types of PUFAs is underscored by the fact that a higher ratio of omega-6 fatty acids (linoleic acid) to omega-3 fatty acids (alpha-linolenic acid) increases platelet aggregation [22], is prothrombotic, and increases vasoconstriction [1–4, 20, 23]. These effects are presumably due, at least in part, to being integral components of the cell membrane [1, 24]. A large body of evidence from experimental, clinical, and epidemiologic research reports a cardioprotective role of long-chain omega-3 fatty acids EPA and DHA derived primarily from fatty fish. While prospective observational cohort investigations indicated that consumption of fatty fish twice or more a week significantly lowers risk of cardiovascular death [25], the findings from randomized clinical trials examining the effects of fish oil supplementation on cardiovascular disease morbidity and mortality in secondary prevention settings were inconsistent. Fourteen randomized clinical trials were evaluated by both Messori et al. [26] and Kwak et al. [27]. These two groups adopted different statistical methods, but neither found a benefit associated with omega-3 fatty acid supplements versus placebo [26, 27]. Importantly, however, the 14 randomized clinical trials so far reported have been small and short-term studies that were not specifically designed to evaluate CVD end points and, of note, the 2 large open-label trials that report a benefit with omega-3 supplementation [28, 29] were excluded from their analysis. While awaiting more definitive results that include a standardized dose and a formulation maximizing bioavailability, the American Heart Association has released dietary guidelines that recommend intake of fatty fish twice a week, underscoring the view that a cardioprotective diet needs to be rich in omega-3 fatty acids [30–32]. There is extensive literature on the effects of EPA and DHA in cardiovascular disease compared to α-linolenic acid, the precursor of EPA and DHA (see [1, 24] and the references therein).

2. Cardiovascular Disease (CVD) and α-Linolenic Acid

In the absence of definitive evidence, several sources imply, rather than directly state, that the high ratio of omega-6/omega-3 that constitutes the typical western diet may promote the pathogenesis of many diseases, including cardiovascular disease, cancer, inflammatory and autoimmune diseases. It is therefore a widely held belief that restoring the balance omega-6/omega-3 to a ratio of 5 : 1 is important, but this "ratio theory" remains controversial. Indeed, a high omega-6 intake may not be characteristic of many western countries and a focus on the omega-6/omega-3 ratio risk diverts attention away from simply increasing the absolute intake of omega-3 fatty acids, which alone has been shown to have beneficial effects, especially on cardiovascular health [33]. Interestingly, only the daily intake of EPA and DHA was promoted while the absolute and relative change of omega-6/omega-3 in the food between the late paleolithic period and the current US western diet seems mainly mediated by the pronounced change in the linolenic acid (LA): α-linolenic acid (ALA) ratio of the diet [34]. This points out that the importance of ALA as a particularly bioactive component from vegetables food source has been underestimated, especially because humans, like all mammals, cannot synthesize α-linolenic acid (e.g., we do not possess the enzymes for *de novo* synthesis. ALA must therefore be obtained from the diet and excellent sources of ALA include rapeseed and walnuts [35, 36]. In fact, interest in omega-3 in CVD has mainly focused on EPA and DHA rather than ALA because ALA bioconversion to EPA and DHA is minimal and therefore a diet rich in ALA might not fulfill DHA requirements (for review, [37, 38]). Since a wide variety of protective mechanisms were ascribed directly to DHA (for review, [39, 40]), diet supplementation with high levels of ALA has been seen of little interest as compared to supplementation with preformed EPA or DHA. This might have been an unfortunate outcome in view of the growing evidence that dietary ALA may also protect against CVD.

First, ALA-enriched diets have been shown in some animal studies to influence the concentration of lipoprotein in plasma. This ability to decrease low density lipoprotein (LDL) may be of importance as increased levels of LDL in plasma are strikingly correlated with the risk of developing atherosclerosis and CHD. Unfortunately, this plasmatic LDL reduction has not been found in studies in humans, although consumption of ALA-enriched sources affected LDL content in ALA, EPA, and DHA that were increased [41–43]. Second, consumption of ALA-enriched sources and of fish oils rich in EPA/DHA has similar antiarrhythmic properties [44, 45], which are known to reduce the human risk of myocardial infarction and fatal ischemic heart disease. Nevertheless, the conclusion of prospective cohort studies that dietary ALA is beneficial against CVD [46–48] has been recently challenged by a meta-analysis concluding that increasing ALA intake

may only produce modest cardioprotection [49]. In addition to the modification of ionic channels currents induced by the incorporation of these polyunsaturated fatty acids into the cardiomyocytes membrane phospholipid bilayer, which could account for the antiarrhythmic effects, omega-3 PUFAs are paradoxical antioxidant and anti-inflammatory compounds and therefore could indirectly decrease oxidation and inflammation associated with CVD [50–52]. A diet rich in ALA reduces proinflammatory cytokines which in turn is related to the omega-6/omega-3 ratio (i.e., a lower ratio reduces the proinflammatory mediators [7]; inflammation is considered to play an important role in atherosclerosis, a major risk factor for cardiovascular disease and stroke [53]). In a recent study, de Goede and colleagues [54] have examined the 10-year incidence of CHD and stroke in relation to ALA intake in a Dutch population-based cohort of over 20,000 adults. While no association between ALA intake and incident coronary heart disease was observed, their study revealed that ALA intake lowered the risk of stroke. Compared to an Eskimo population where the omega-6/omega-3 ratio is 1, the ratio of a typical western diet is 10/1–25/1 [34]. Thus, increasing the intake of ALA may be beneficial in reducing stroke risk.

3. Stroke and α-Linolenic Acid

A typical western diet is severely deficient in omega-3 fatty acids and this may elevate the risk for stroke [1, 3, 24, 54]. During an ischemic stroke, glutamate excitotoxicity through overactivation of N-methyl-D-aspartate (NMDA) receptors is the major mechanism of neuronal cell death within the core and surrounding ischemic area called the penumbra. Neuronal necrosis driven by glutamate excitotoxicity occurs within minutes to hours following cerebral ischemia. This creates an extremely reduced time window of intervention for administration of therapeutics aimed at inhibiting glutamate-mediated cell death pathways [55]. This time constraint of acute neuroprotection will probably be difficult to achieve in clinical practice drawing attention to the importance of prevention. The common view of prevention of the risk factors is to reduce the occurrence of stroke. Nevertheless an emerging concept in the field is that nutritional factors may exert a protective role against stroke-induced damage, a field of study of potentially major relevance but still poorly addressed (see [1, 3]).

There is a great deal of evidence that ALA is a potent neuroprotective agent against focal and global ischemia in animal models [11, 56–62]. This same mechanism appears to underlie clinical findings, where, in adult men, serum levels of ALA were independently associated with a 37% reduction in stroke risk [63]. Also, the higher the intake of α-linolenic acid, the lower the prevalence of a carotid plaque [64], and similar results were reported in mice [35]. ALA activates a neuronal background rectifying potassium channel [65] leading to membrane hyperpolarization which in turn increases the magnesium block of the calcium channel associated with NMDA receptors which play a predominant role in mediating glutamate-mediated excitotoxic neuronal cell death [58, 61]. In this rodent model of global ischemia where hippocampal pyramidal neuronal death is mainly driven by glutamate excitotoxicity, we found that ALA exerted a profound protective effect that was more pronounced and reproducible than with EPA and DHA [61]. Additional studies in rodents revealed an essential role for the transcription factor, nuclear factor kappaB, in the ability of ALA to protect neurons against ischemia [11] and to induce tolerance [57], a phenomenon where neurons become resistant to a stressful environment such as ischemia [66]. ALA was shown to increase levels of brain-derived neurotrophic factor (BDNF), a widely distributed protein that [59] in the brain carries out diverse functions, including neuronal maintenance, learning and memory, neuronal survival, and neurogenesis [67–72]. Other proteins, such as HSP70, a heat shock protein [57, 60], which acts as a protein chaperone, also have roles in regulating programmed cell death (i.e., apoptosis) [73]. While some features are known, the precise mechanisms by which α-linolenic acid exerts its pleiotropic properties in brain are still not clear. Omega-3 fatty acids act via multiple mechanisms such as through the alteration of plasma membrane fluidity, lipid rafts, and signal transduction mechanisms in addition to effects on gene expression [74]. Delineating ALA-mediated mechanisms may increase the number of cellular and molecular targets that lead to enhanced therapeutic efficacy.

4. Stroke and Brain-Derived Neurotrophic Factor (BDNF)

Of the known gene targets of ALA, BDNF shows promise as a therapy for stroke. In many studies, BDNF has been shown to reduce infarct size and improve outcome (see [75–77] and the references therein) whereas blocking endogenous BDNF worsens ischemia [78]. Administration of BDNF via the intravenous route as well as the intracerebroventricular route reduced infarct size and improved outcome in the transient middle cerebral artery occlusion model of stroke [79, 80]. However, in humans, anticipated pharmacokinetic challenges make it difficult to develop BDNF itself as a therapy to the clinic [81]. This problem, however, creates opportunities to discover compounds that increase endogenous expression of BDNF in brain. To this end, chronic ALA treatment increases BDNF mRNA and protein levels in the cortex and hippocampus (Figure 2), two brain regions that are susceptible to ischemia but are also involved in plasticity responses. ALA increases neurogenesis, synaptogenesis, and synaptic function in the rodent brain [82]. The ability to increase neurogenesis in the brain is critical because it has been shown that neural stem cells improve neurological function in stroke [83–87]. Neural stem cells can modulate the ischemic environment via the upregulation of survival-promoting/neurotrophic factors such as BDNF and/or by restoring neurotransmitter function by integrating in existing networks and improving network circuitry. Taken together, these findings indicate that ALA induces tolerance and reduces infarct size in animal models of stroke. ALA was also demonstrated to exert antidepressant activity and increase BDNF mRNA and protein levels in the brain which in turn likely stimulates neurogenesis, synaptogenesis, and synaptic function. The benefit between the intake of ALA and the

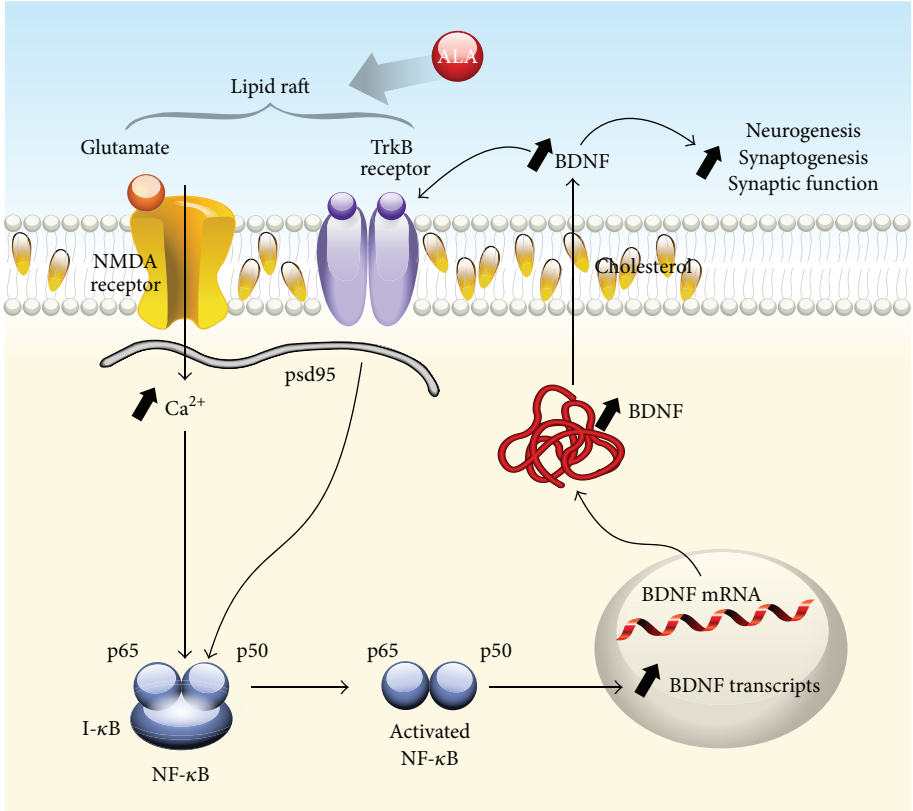

Figure 2: Interplay between NMDA and TrkB receptors mediated by ALA-induced lipid rafts in neuronal plasma membranes. An increase in the nutraceutical ALA is hypothesized to markedly increase membrane fluidity leading to the efficient formation of lipid rafts [5] in neuronal plasma membranes. Lipid rafts are the functional domains of the plasma membrane and play a crucial role in the regulation of transmembrane signaling [6]. TrkB receptors and some NMDA receptors are constituents of lipid rafts [7–10] and one of the major nonprotein components of lipid rafts is cholesterol [6]. The enhanced formation and/or efficiency of transmembrane signaling is hypothesized to result in enhanced activation (phosphorylation) of NMDA and TrkB receptors via the binding of BDNF to its cognate receptor, TrkB. Activation of NMDA receptors results in enhanced calcium influx and activation of signal transduction pathways leading to activation of nuclear factor kappa B (NF-κB) via the canonical pathway (phosphorylation of I-κB leads to its dissociation from the dimer (p65/p50) which then translocates to the nucleus where it binds to κB sites to regulate gene expression) which in turn increases BDNF mRNA and protein levels [11–14]. Enhanced intracellular BDNF protein expression would lead to an increase in secretion, thereby maintaining its availability to bind to TrkB in an autocrine fashion [14, 15] as well as to stimulate neurogenesis, synaptogenesis, and synaptic function at distant sites (paracrine function).

reduction in stroke risk in humans, the substantial evidence that ALA reduces infarct size, improves outcome and survival in animal models and the fact that ALA exhibits a wide safety margin provides a strong rationale for the systematic study of ALA administration in stroke.

5. Stroke, Depression, ALA, and BDNF

Poststroke depression is a common occurrence and can adversely affect outcome after stroke [88]. Stroke and depression are complex and multifaceted diseases but both disorders have common pathological substrates that could be targeted by therapeutic intervention. For example, there is growing evidence that neuroplasticity plays a crucial role in both pathologies. Consequently, compounds that increase neuroplasticity in the brain could ameliorate or prevent an infarct and reduce downstream consequences such as poststroke depression.

A longitudinal study of 50,000 women found that increased intake of ALA reduced depressive symptom [89]. Earlier studies showed similar results [90–92]. In normal mice, ALA treatment (given intravenously or in the diet) exerted an antidepressant effect. This effect was associated with increased synaptogenesis and an increase in BDNF mRNA levels in brain (Figure 3; [82, 93]). Evidence has shown that antidepressant drugs enhance the activation of TrkB receptors, the high affinity receptor that binds BDNF [94] and is a key event in exerting antidepressant properties [82, 94, 95]; BDNF has been implicated in mediating the antidepressant effects in brain [96].

6. Conclusion

In common with several others groups, we have demonstrated the broad neuroprotective and neuroplastic potential of omega-3 injection in animal models of neurodegenerative

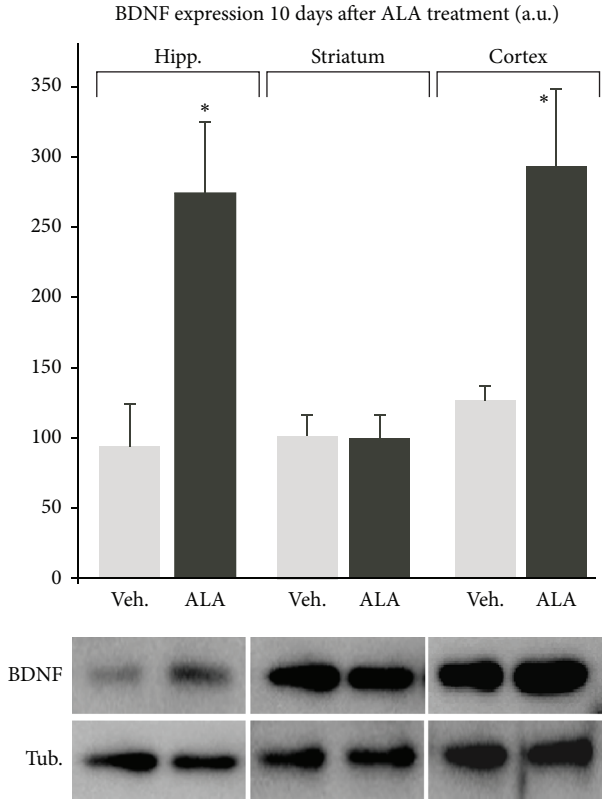

Figure 3: *In vivo* subchronic ALA treatment increases mature BDNF levels in neurons of the cortex and hippocampus, but not in striatum. BDNF increase in these specific brain regions is consistent with well-known properties for the efficiency of antidepressant drugs and with the level of brain protection offered by the subchronic ALA treatment. Mature BDNF expression was measured 10 days after the subchronic treatment by Western blots in cortex, hippocampus ($^*P > 0.05$), and striatum ($P < 0.05$) of mice injected with ALA or vehicle. Subchronic treatment consisted of three i.v. injections of 500 nmol/kg of α-linolenic acid on days 1, 3, and 7.

conditions, including acute neurological injuries such as stroke and spinal cord injury (for review, see [1, 3, 97]. In addition, intravenous perfusion of omega-3 fatty acid—in the form of 10% fish oil emulsion supplementing parenteral nutrition—has been shown to improve organ failure-related outcomes [98]. Although the impact of omega-3 fatty acid intravenous supplementation in human neurological conditions has not been addressed, it is tempting to speculate that this approach may offer significant benefit in human ischemic conditions. With regard to omega-3 consumption, a maximum dose of 3 g/day of long chain omega-3 fulfills the Generally Recognized as Safe status in the United States and the French recommendation not to exceed more than 15 times the Daily Recommended Intake [99]. Therefore, we believe that, in light of the currently available data, the conventional recommendations of omega-3 at a dose of 1 g/day of ALA, or 0.750–1 g/day of EPA + DHA, may offer therapeutic benefit in patients at risk of cardiovascular diseases. It is also noteworthy that these doses are without adverse effects. General consensus on the importance of eating for health may turn as a particular commitment for prevention, recovery, and rehabilitation from stroke. Healthy eating after stroke may be important for recovery though additional formal testing is needed, as it could be to improve outcome and reduce reoccurrence. Choosing healthy foods may be a challenge, underlying the importance of identifying natural products with health benefit, like ALA that is a non-proprietary, naturally occurring omega-3 fatty acid contained in foodstuffs. ALA has anti-inflammatory and other potential beneficial properties and, based on the weight of available data, may reduce stroke risk, size, and/or consequences. Sources of α-linolenic acid include but are not limited to flaxseed, rapeseed, and walnuts. ALA is well tolerated and can be supplemented into the diet in a variety of food sources including muffins. The potential benefits of ALA are supported by both animal studies and human observational epidemiologic studies. Early phase clinical trials evaluating α-linolenic acid are justified, and if these indicate benefit, larger scale studies of this agent in stroke prevention should follow.

Conflict of Interests

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

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Review Article

Milk Proteins, Peptides, and Oligosaccharides: Effects against the 21st Century Disorders

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Milk is the most complete food for mammals, as it supplies all the energy and nutrients needed for the proper growth and development of the neonate. Milk is a source of many bioactive components, which not only help meeting the nutritional requirements of the consumers, but also play a relevant role in preventing various disorders. Milk-derived proteins and peptides have the potential to act as coadjuvants in conventional therapies, addressing cardiovascular diseases, metabolic disorders, intestinal health, and chemopreventive properties. In addition to being a source of proteins and peptides, milk contains complex oligosaccharides that possess important functions related to the newborn's development and health. Some of the health benefits attributed to milk oligosaccharides include prebiotic probifidogenic effects, antiadherence of pathogenic bacteria, and immunomodulation. This review focuses on recent findings demonstrating the biological activities of milk peptides, proteins, and oligosaccharides towards the prevention of diseases of the 21st century. Processing challenges hindering large-scale production and commercialization of those bioactive compounds have been also addressed.

1. Introduction: Role of Milk in Human Health

Milk, as the first food for mammals, supplies all the energy and nutrients needed for the proper growth and development of the neonate. For all mammalians, the consumption of milk ends at the weaning period with the exception of humans that continue consuming milk throughout their life. Milk and derived dairy products are considered an important constituent of a balanced diet. Moreover, it is a source of many bioactive components, such as high-quality proteins, lipids, carbohydrates, lactose, vitamins, minerals, enzymes, hormones, immunoglobulins, and growth factors, among others. These components not only help meeting human nutritional requirements, but also play a relevant role in preventing various disorders such as hypertension and cardiovascular diseases [1], obesity [2], osteoporosis [3], dental caries [4], poor gastrointestinal health [5], colorectal cancer [6], ageing [7], and others [8].

Milk proteins supply nitrogen and amino acids to young mammals and possess multiple physiological properties in the intact form. Moreover, studies carried out in the past decades have demonstrated the role of these proteins as a source of biologically active peptides. Bioactive peptides are inactive within the sequence of the parent protein but, once released by *in vitro* processing conditions or by *in vivo* gastrointestinal digestion, are capable of acting as regulatory compounds exerting a positive impact on body functions and ultimately promoting health benefits to the consumer [9].

Human milk is undoubtedly the most complete source of nourishment for the newborn. Breastfed infants have been

Table 1: Commercial milk products containing peptides with proven antihypertensive activity.

Commercial name	Obtention process	Protein source	Active sequence(s)	Publication number [reference]
Peptide C12	Hydrolysis with trypsin	α_{s1}-Casein	FFVAPFPEVFGK	JP62270533 [31]
Biozate	Hydrolysis with trypsin	Whey proteins	Whey peptides	US6998259 [157]
Lowpept	Hydrolysis with pepsin	α_{s1}-Casein	RYLGY, AYFYPEL	WO2005012355 [158]
Calpis	Fermentation	β-Casein	VPP, IPP	US5449661A [37, 38]
Evolus	Fermentation	β-Casein	VPP, IPP	US6972282 [159]

shown to be less susceptible to diseases (i.e., diarrhea and respiratory diseases) than those that were not breastfed. This protective effect, which was previously attributed to human milk antibodies, is today strongly correlated with the presence of complex oligosaccharides (OS), the third most abundant component of human milk [10]. Human milk is composed of OS in concentrations varying according to different stages of lactation: 20–23 g/L in colostrum and 12–14 g/L in mature milk [11], being even more abundant than proteins (12 g/L) [12]. Human milk oligosaccharides (HMO) are complex sugars having 3 to 20 monosaccharide units [13] that are not digestible by human enzymes [14]. These compounds have important functions related to the newborn's development and health at local and systemic levels, including prebiotic probifidogenic effects and antiadherence of pathogenic bacteria [15], brain development [16], and immunomodulatory properties [17], among others.

In the last fifty years, chronic disorders have become the leading cause of morbidity and mortality in industrialized countries, with increasing incidence also observed in developing countries. Chronic disorders include cardiovascular and neurological diseases, stroke, cancers, immune disorder and chronic respiratory disease, obesity, diabetes, and metabolic syndrome [18]. In Europe, 87% of all deaths occur due to chronic diseases and the number of people affected is expected to rise considerably over the next few decades. The majority of chronic diseases are caused by risk factors which are mostly preventable. Diet and lifestyle are two environmental factors that strongly affect these diseases; thus modifications of these habits are becoming a new strategy for disease prevention/treatment.

The aim of this paper is to review the recent literature on the physiological effects of proteins, peptides, and oligosaccharides with special emphasis on animal and human trials. Other aspects such as the limited availability of *in vivo* studies demonstrating the biological activities of OS from bovine and caprine milk and the current challenges associated with the recovery and commercial production of these compounds have also been addressed.

2. Impact of Milk Proteins and Peptides on the 21st Century Diseases

2.1. Milk-Derived Peptides against Cardiovascular Diseases. Cardiovascular diseases (CVD) have become the leading cause of morbidity and mortality worldwide, representing an important medical and public health issue [19]. Although earlier studies associated the consumption of whole milk with higher incidence of CVD, it has been demonstrated that milk contains a plethora of bioactive substances which may contribute to the prevention of most of the risk factors of CVD [20]. Recently, bioactive milk peptides have gained interest because of their notable antihypertensive, antioxidant, anti-inflammatory, and hypocholesterolaemic effects. In this section, the most current scientific information regarding *in vitro* and *in vivo* studies on the role of milk proteins-derived peptides on CVD is summarized and discussed.

2.1.1. Milk Peptides with Antihypertensive Activity. Epidemiological studies suggest that the dietary intake of milk and dairy foods is related to decreased risk of hypertension [21]. In addition to their high mineral content (e.g., calcium, potassium, and magnesium) that can lower blood pressure [22], other milk components, such as proteins and their hydrolyzed products, have been also linked to the antihypertensive effect of milk and dairy products. Angiotensin-converting enzyme (ACE) is a multifunctional enzyme that acts as one of the main regulators of blood pressure. Thus, ACE inhibition is currently considered as one of the best strategies for hypertension treatment. Most biologically active peptides generated from milk proteins have demonstrated ACE inhibitory activity. In the last two decades, antihypertensive effects of some of these peptides have been evaluated in spontaneously hypertensive rats (SHR) and hypertensive humans, and the peptide sequences, doses, and maximum decreases of systolic blood pressure (SBP) have been summarized in several reviews [23–25]. The hydrolyzate obtained by the action of pepsin on casein, containing the α_{s1}-casein-derived peptides RYLGY and AYFYPEL, has been patented and commercialized under the name of Lowpept by its antihypertensive properties demonstrated in both SHR [26] and hypertensive humans [27] (Table 1). Pepsin has been also used to hydrolyze whey protein lactoferrin, with the release of peptides containing ACE activity and ACE-dependent vasoconstriction inhibitory properties [28]. Antihypertensive effects in SHR after short-term and long-term treatments have been also observed for those peptides [29, 30]. Trypsin is another gastrointestinal enzyme used to release the antihypertensive peptide α_{s1}-casein peptide f(23–34) from casein during the manufacture of the commercial

ingredient peptide C12 [31, 32] (Table 1). In addition to the use of gastric and pancreatic enzymes, alone or in combination, to produce antihypertensive peptides, the use of food-grade enzymes derived from microorganisms has become common for the release of peptides with demonstrated SBP lowering effects in SHR [33–36].

Milk fermentation is another strategy to produce antihypertensive peptides by the proteolytic action of lactic acid bacteria on milk proteins. The most representative peptides are those derived from β-casein and identified in sour milk fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Calpis, Table 1). These tripeptides, with sequences VPP and IPP, have demonstrated an ability to exert potent decreasing effects on the SBP of SHR [37, 38]. A number of clinical trials have been conducted to confirm their antihypertensive properties in humans although controversial results have been found. Three meta-analyses performed with the published data of 17 [39], 12 [40], and 28 [41] clinical trials have reported an average decrease in SBP of 5.1, 4.8 mm, and 1.7 mm of Hg, respectively. However, no effects were found in Dutch and Danish subjects consuming fermented milk containing peptides VPP and IPP [42, 43]. A recent meta-analysis including 18 trials has reported higher antihypertensive effects for these two tripeptides in Asian than in Caucasian people [44]. Those findings suggest that genetics and/or dietary patterns might exert an important influence on the antihypertensive effects of peptides IPP and VPP. Similarly, the age has been described as another major influencing factor [45]. With the evidence presented to date, the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA) [46] concluded that there are no sufficient data to establish a cause/effect relationship between the consumption of peptides VPP and IPP and the control of hypertension, and further studies are thus required. Other peptides derived from β-casein during milk fermentation with *Enterococcus faecalis*, in which sequences are LHLPLP and HLPLP, have also shown antihypertensive effects in SHR [47]. In recent studies, fermented milk with *Lactococcus lactis* NRRLB-50571 and NRRLB-50572 has presented important SBP, diastolic blood pressure (DBP), and heart rate-lowering effects in SHR [48, 49] although the peptides responsible for the activity have not been identified.

Accumulating evidence built in animal and clinical studies is currently available on the antihypertensive activity of milk-derived peptides. However, much work is still needed. Identification of the active form reaching the target organs and elucidation of its bioavailability after oral ingestion and its complete mechanism of action are two of the main aspects required to be deeply investigated in the future to support health claims.

2.1.2. Antioxidant and Anti-Inflammatory Milk-Derived Peptides. Oxidative stress is one of the main responsible factors for the initiation or evolution of CVD. The search of natural antioxidants providing additional benefits to the endogenous antioxidant defense system is gaining interest [50]. Among food-derived peptides with antioxidant properties without harm side effects, those derived from milk proteins are most frequently studied. The majority of the studies carried out to characterize antioxidant peptides derived from casein and whey proteins have only used *in vitro* chemical assays [51, 52]. However, their limited similarity to physiological conditions makes the *in vitro* assays very restrictive, and reported effects need to be confirmed by animal models and/or human trials. Nevertheless, to date, just few *in vivo* trials have been carried out to demonstrate the antioxidant effects of milk-derived peptides related to benefits on cardiovascular health. Zommara et al. [53] reported the antiperoxidative action of fermented milk on rats fed a vitamin-E deficient diet. The consumption of fermented milk by healthy subjects has been also demonstrated to lower the levels of oxidized low-density lipoprotein, isoprostanes, and the glutathione redox ratio. Improvements of total plasma antioxidant activity and of the resistance of the lipoprotein fraction to oxidation have resulted in enhanced antiatherogenicity [54]. The compounds responsible for the observed effects have not been identified yet, although milk peptides liberated during fermentation process might have a crucial role. Thus, further studies focused on evaluating the potential of milk-derived peptides as antioxidant at cardiovascular level should be of great relevance.

Chronic inflammation is another responsible factor for the development of CVD. The downregulation of cytokines involved in the inflammation-associated endothelial dysfunction by food components, including peptides, may delay or alleviate inflammation, thus exerting favorable effects against CVD [55]. A recent study using lipopolysaccharide-(LPS-) stimulated mouse macrophages has reported the ability of a yak casein hydrolyzate to reduce the secretion of proinflammatory cytokines and the production of nitric oxide and to scavenge free radicals, suggesting a potential role as preventive agent against inflammation related disorders [56]. To date, only one human trial has been conducted to demonstrate the anti-inflammatory properties of milk peptides. This study reported an improvement in the vascular function through modulation of the glucose levels and inflammation and oxidative stress biomarkers after the consumption of the commercial whey derived peptide NOP-47 by healthy individuals [57]. This finding opens a new door towards searching of new milk-derived peptides with antioxidant and anti-inflammatory activity.

2.1.3. Hypocholesterolaemic Milk Peptides. Blood lipids are represented in various forms including total cholesterol, triglycerides, lipoproteins (high-density lipoproteins or HDL, low-density lipoproteins or LDL, and very-low-density lipoproteins or VLDL), and free fatty acids. An inappropriate ratio of these lipids is one of the most important risk factors for developing CVD. Therefore, CVD therapy/prevention strategies focus on reaching an optimal lipid balance in order to achieve a positive cardiovascular health. Those therapies aim at increasing the physiological levels of desirable lipids (e.g., HDL cholesterol) while reducing the others associated with atherogenic functions (e.g., LDL cholesterol, triglycerides). Milk proteins, mainly whey proteins

and derived hydrolyzates or peptides, have been reported to exert hypocholesterolaemic effects in different animal models. The ingestion of whey protein was correlated with a significant reduction of total cholesterol levels in rats fed with cholesterol-free and cholesterol-enriched diets [58, 59]. Nagaoka et al. [60] have reported similar effects for a β-lactoglobulin tryptic hydrolyzate administered to rats fed with a diet rich in cholesterol. The hydrolyzate reduced total cholesterol and increased HDL cholesterol and fecal steroid excretion. The fragment f(71–75) of this whey protein, known as lactostatin, with sequence IIAEK, has been reported as the main factor responsible for the observed effects [60]. β-Lactotensin, another β-lactoglobulin peptide, released by chymotrypsin hydrolysis, decreased total cholesterol, LDL, and VLDL cholesterol content in mice fed with a cholesterol-enriched diet [61]. Although the mechanism of action of those peptides has not been completely elucidated, preliminary results suggest a key role played by the amino acid composition [50]. Further studies are clearly needed to corroborate those results. The exact mode of this hypocholesterolaemic action needs to be determined in clinical trials.

2.2. Milk-Derived Hydrolyzates and Peptides on Intestinal Health. The gastrointestinal tract (GIT) serves as a specialized interface between the body and the external environment. The GIT is strategically covered by a monolayer of specially designed epithelial cells continually exposed to a high concentration of food components and substances along the gut luminal surface. Hence, the modulator effect of the diet on GIT functions has been accepted as essential for maintaining and improving the general health of the host [62]. Interestingly, more than 70% of the current "food for specified health uses products" (FOSHU) are related to GIT functions [63].

Dairy proteins, hydrolyzates, and peptides have been demonstrated to transform the dynamics of mucus mainly via influencing the mucin secretion and expression and the number of goblet cells. In *ex vivo* preparations of rat jejunum, casein hydrolyzates increased mucin secretion [64, 65]. The β-casein derived peptide β-casomorphin 7 produced the same effects which have been suggested to be mediated by interaction with opioid receptors. Also, this peptide has been reported to stimulate the expression of mucin *Muc2* and *Muc3* genes in rat intestinal DHE cells and *MUC5AC* gene in human intestinal HT29-MTX cells [66]. Another β-casein fragment, f(94–123), identified in commercial yoghurt, also had the ability to increase the mucin output and the mRNA levels of *MUC2* and *MUC4* genes in HT29-MTX cells [67]. Casein and whey proteins hydrolyzates have been reported to be a source of peptides with capacity to induce mucin secretion and *MUC5AC* gene expression in HT29-MTX cells [68]. Among these peptides, the α_{s1}-casein fragments f(143–149) and f(144–149) and the β-lactoglobulin fragment f(102–105) known as β-lactorphin were suggested as the major peptides responsible for the observed effects.

A few *in vivo* studies have also pointed out the regulation of the protective mucus layer by dairy proteins and products thereof. Rats fed with a diet based on casein hydrolyzates, as the exclusive source of nitrogen, were found to enhance their endogenous nitrogen flow and expression of mucin genes *Muc3* and *Muc4* in the small intestine and colon, respectively [69]. Plaisancié et al. [67] reported the capacity of the β-casein fragment f(94–123), once orally ingested by rats, to upregulate the *Muc2, Muc4, rat defensing 5* and *lysozyme* mRNA transcripts expression, the goblet cells recounts, and the number of crypts containing Paneth cells in the rat small intestine. In the dextran sulphate sodium- (DSS-) induced model of rat colitis, the studies of Sprong et al. [70] and Faure et al. [71] demonstrated the gut-protective effects exerted by a cheese whey protein diet and a diet supplemented with Thr, Ser, Cys, and Pro residues, respectively. Moreover, this protection has been reported for a whey protein isolate and α-lactalbumin hydrolyzate against chemical-induced ulcerative gastric lesions [72, 73].

Enhancement of the mucosal immune response is also a dietary modulating strategy of the defense systems protecting the GIT. Animal models have proved the improvement of the mucosal immunity by promotion of gut-related immunoglobulin (Ig) levels after ingestion of lactoferrin or its derived peptides, lactoferricin and lactoferrampin [74, 75]. Likewise, immunomodulatory effects have been reported for a trypsin casein hydrolyzate in newborn calves [76] and casein phosphopeptides (CPPs) and peptides released from *Lactobacillus helveticus* R389-fermented milk in mice [77, 78]. Furthermore, Kitamura and Otani [79] demonstrated that ingestion by healthy humans of CPPs-enriched cakes induced an increase in the faecal IgA content, suggesting a positive effect on mucosal immunity.

Oxidative and inflammatory imbalances are both involved in the etiology of several human chronic gut-related disorders such as ulcerative colitis and Crohn's disease. The search of natural preventive treatments against these imbalances is being prompted [80, 81]. Whey protein has been suggested to exert beneficial effects through enhancement of antioxidant enzymes and downregulation of both oxidative markers and proinflammatory cytokines [82]. These protective findings were found in animal [83, 84] and humans trials [85, 86]. The whey-derived peptide caseinomacropeptide has been proven to have protective properties in the 2,4,6-trinitrobenzene sulphonic acid (TNBS) and DSS-induced model of rat ileitis and colitis, through immunomodulation of the regulatory T helper cells activation and interleukin secretions [87, 88]. Turbay et al. [89] demonstrated, in the TNBS-induced murine colitis model, the anti-inflammatory effects exerted by β-casein hydrolyzates generated by the cell envelope-associated proteinase of *Lactobacillus delbrueckii* ssp. *lactis* CRL 581. However, peptides released and responsible for the observed bioactivity have not been identified yet.

2.3. Milk Proteins and Peptides against Metabolic Disorders. Diabetes mellitus is considered one of the most common metabolic disorders and one of the major health problems worldwide. It affects almost 6% of the world's population, with type 2 diabetes representing approximately 90–95% of the diagnosed cases [90]. Diet and lifestyle interventions

are the preferred treatment strategies for this metabolic disorder, with pharmacotherapy being prescribed only if supervised lifestyle intervention fails [91]. Epidemiological evidence supports that consumption of milk and dairy foods is associated with a lower incidence of type 2 diabetes. These beneficial effects on metabolic and inflammation factors linked to diabetes and insulin resistance have been also demonstrated by cell and animal models, being multiple milk components, such as calcium, medium-chain fatty acids, linoleic conjugated acid, lactose, citrate, proteins, and peptides characterized as the main responsible factors for the observed effects acting through different mechanisms of action [92].

During the ingestion of a meal, the presence of nutrients at gastrointestinal level stimulates the secretion of two incretins hormones, the glucagon-like peptide-1 (GLP-1) and the glucose-dependent insulinotropic polypeptide (GIP). Both hormones are implicated in the stimulation of the insulin secretion from the pancreatic β-cells, secretion of gastric and pancreatic enzymes, and modulation of gut motility and nutrient absorption, allowing the clearance of the absorbed glucose [93]. Type 2 diabetes is characterized by different disorders including progressive dysfunction of pancreatic cells, insulin resistance, and augmented production of hepatic glucose [94]. Continuous intravenous administration of GLP-1 has been demonstrated to normalize blood glucose levels in diabetic subjects [95]. However, the rapid degradation of this hormone by the enzyme dipeptidyl peptidase-IV (DPP-IV) and its consequent inactivation makes this type 2 diabetes treatment strategy impracticable. Currently, specific DPP-IV inhibitors are thus incorporated to GLP-1 analogues in new oral therapies against this metabolic disease [96].

Diet supplementation with whey protein is currently under preclinical and clinical trials as a promising alternative in the prevention and/or treatment of type 2 diabetes and related diseases [97, 98]. Several mechanisms of action have been suggested for whey protein, including the stimulation of insulin release, improvement of glucose tolerance in diabetic patients, reduction of body weight, and modulation of gut hormones such as cholecystokinin, leptin, and GLP-1 [99]. In the last years, the role of peptides released during the transit of whey proteins through the GIT on the observed effects has been hypothesized [100]. Cell culture and animal models have been used to confirm this hypothesis. A dose-dependent insulinotropic activity of whey protein hydrolyzates has been observed in a cell-based coculture using pancreatic BRIN-BD11 cells and Caco-2 cells monolayers [101]. These authors also observed that the oral administration of the hydrolyzates to obese mice evoked an improvement of blood glucose clearance, reduction of hyperinsulinemia, and restoration of the pancreatic capacity to secrete insulin in response to glucose. The main mechanism of action suggested for these hydrolyzates is the DPP-IV inhibitory activity exerted by the peptides contained in them [102]. Among the bioactive peptides described to date, sequences derived from β-lactoglobulin IPA and IPAVF are the most potent as DPP-IV inhibitors [103, 104]. Another β-lactoglobulin fragment with sequence VAGTWY has been also demonstrated to exert hypoglycemic effects in the oral glucose tolerance test in mice [105]. Likewise, both *in vitro* DPP-IV inhibitory and *in vivo* hypoglycemic effects have been reported for peptides released from caseins [106]. Recent *in silico* studies have shown that both caseins and whey proteins might serve as precursors of DPP-IV inhibitory peptides because of the high number of fragments contained within them that match DPP-IV inhibitory sequences [107, 108]. Thus, this research area holds a great potential, and currently a number of investigations are focused on the identification of new milk proteins-derived peptide with capacity to prevent diabetes and associated metabolic syndromes.

2.4. Chemopreventive Role of Milk Proteins and Peptides. Cancer is the second leading cause of mortality worldwide, and its incidence will continue rising in the next few years in spite of the important advances achieved in the development of cancer therapies. It has been estimated that, by 2020, approximately 15 million new cancer cases will be diagnosed, and 12 million cancer patients will die [109]. It is well known that 35% of cancer deaths are attributed to diet and its food components [110]. However, cell culture and animal and human trials results have shown that an important number of food constituents can lower cancer risk and even sensitize tumor cells against anticancer therapies [111]. In the last few years, food proteins and derived peptides have become one of the food components with the most promising preventive properties against cancer initiation, promotion, and progression stages [112].

Among the milk proteins, lactoferrin and its derived peptide lactoferricin are the most studied. For both compounds, their antioxidant, immunomodulatory, and anti-inflammatory activities are closely linked to their protective effects against cancer (Table 2). Lactoferrin acts by inducing apoptosis, inhibiting angiogenesis, and modulating carcinogen metabolizing enzymes, in addition to its antioxidant and immunomodulatory properties [113]. Moreover, lactoferricin has shown potent anticancer properties in different cell lines, including breast, colon, fibrosarcoma, leukemia, and oral and ovarian cancer cells, without harming normal lymphocytes, fibroblasts, or endothelial or epithelial cells [114]. Also, animal models have confirmed the beneficial properties of this milk-derived peptide. The possible mechanism of bovine lactoferricin in anticarcinogenesis has been shown to be related to its ability to induce apoptosis. It is its strongly cationic nature that allows this peptide to target negatively charged cancer cells with the outer membrane [115]. The suppressed ability in angiogenesis of bovine lactoferricin was *in vitro* and *in vivo* demonstrated to contribute to its chemopreventive properties [116]. A significant inhibition of tumor growth and of liver and lung metastasis was reported after subcutaneous administration of bovine lactoferricin in both spontaneous and experimental metastasis mice models [117]. Similar results were observed after subcutaneous treatment and repeated injections of this peptide on Meth A fibrosarcoma xenografts mice and established neuroblastoma xenografts, respectively [118, 119].

α-Lactalbumin is a whey protein with anticancer properties which has been reported when it forms a complex with

Table 2: Chemopreventive properties of lactoferrin and its derived peptide lactoferricin against cancer demonstrated by cell culture experiments and animals models.

Type of cancer	Animal species/protein-peptide	Cell line/animal model	Effects/mechanisms of action	Reference
Breast cancer	Human lactoferrin	MDA-MB-231 cells	Inhibition of cell growth Cell cycle arrest	[160]
	Bovine lactoferrin	4T1 xenograft Balb/c mice	Improvement of tamoxifen chemopreventive effects Downregulation of proinflammatory cytokines	[161]
	Bovine lactoferrin-oleic acid complex	MCF-7 cells	Inhibition of proliferation Induction of apoptosis	[162]
	Bovine lactoferricin	MCF-7, T-47D, and MDA-MB-435 cells	Cytotoxic activity Induction of apoptosis	[163]
Colon cancer	Camel lactoferrin	HCT-116 cells	Inhibition of cell proliferation Antioxidant activity Inhibition of DNA damage	[164]
	Bovine lactoferrin	Caco-2 xenograft mouse model	Inhibition of tumor growth	[165]
	Bovine lactoferrin-oleic acid complex	HT-29 cells	Inhibition of proliferation Induction of apoptosis	[166]
		C26 cells	Cytotoxic activity	[118]
		Caco-2 cells	Inhibition of cell proliferation Cell cycle arrest by downregulation of cyclin E1	[166]
	Bovine lactoferricin	Ultraviolet-irradiated Caco-2 cells	Reduction of DNA damage Cell cycle arrest by downregulation of cyclin E1	[167]
		Colo-35 and HT-29 cells	Cytotoxic activity/induction of apoptosis	[163]
Cervical cancer	Bovine lactoferrin	HeLa cells	Inhibition of cell growth Induction of nuclear accumulation of Smad-2	[168]
Fibrosarcoma	Bovine lactoferricin	Meth A cells	Cytotoxic activity Tumor cell membrane disruption	[118]
Head and neck cancer	Human lactoferrin	Squamous carcinoma O12 tumor bearing mice	Reduction of tumor Immunomodulatory effects	[169]
Hepatocarcinoma	Bovine lactoferrin-oleic acid complex	HepG2 cells	Inhibition of proliferation Induction of apoptosis	[162]

Table 2: Continued.

Type of cancer	Animal species/protein-peptide	Cell line/animal model	Effects/mechanisms of action	Reference
Leukemia	Bovine lactoferricin	THP-1 human monocytic leukemic cells	Induction of apoptosis Activation of ROS generation and Ca^{2+}/Mg^{2+}-dependent endonucleases	[170]
		Jurkat T leukemia cells	Induction of apoptosis by triggering mitochondrial swelling and release of cytochrome c Induction of cell membrane permeabilization	[163, 171]
			Activation of ROS generation and caspase-3 and caspase-9 activity Reduction of DNA methyltransferases expression	[172]
Lymphoma	Bovine lactoferricin	Raji and Ramos Burkitt's B-lymphoma cells	Induction of apoptosis Stimulation of DNA fragmentation, chromatin condensation, and nuclear disintegration	[114]
		Ramos B-lymphoma cells xenografts in SCID/beige mice	Extension of survival of mice	[114]
		A20 cell lymphomas in syngeneic Balb/c mice	Tumor necrosis and regression of the tumors Induction of long-term specific cellular immunity	[173]
		B16-BL6 melanoma and L5178Y-ML25 lymphoma cells metastasis models in syngeneic mice	Inhibition of tumor metastasis in lung	[117]
Lung cancer	Bovine lactoferrin	A549 cells Transgenic mice overexpressing hVEGF-A165	Downregulation of proinflammatory cytokines Suppression of tumor development	[174]
Melanoma	Bovine lactoferricin	B16F10 cells	Cytotoxic activity	[118]
		Spontaneous B16-BL6 metastasis models in syngeneic mice	Inhibition of tumor metastasis in lung	[117]
Nasopharyngeal carcinoma	Human lactoferrin	5–8F, CNE2, and HONE1 cells Xenograft Balb/c mice	Suppression of tumorigenesis through inhibition of the AKT pathway	[175]
Neuroblastoma	Bovine lactoferricin	Human MYCN-amplified and non-MYCN-amplified neuroblastoma cells	Cytotoxic activity Destabilization of the cytoplasmic membrane Activation of caspase-6, caspase-7, and caspase-9	[119]
		SH-SY-5Y neuroblastoma xenografts in nude rats	Reduction of the tumor growth	[119]
Oral cancer	Bovine lactoferricin	Oral squamous carcinoma SAS cell	Induction of apoptosis Cleavage of caspase-3 and poly-ADP ribose polymerase Phosphorylation of extracellular signal-regulated kinase and c-Jun N-terminal kinase/stress activated protein kinase	[176]
Ovarian cancer	Bovine lactoferricin	Skov3 and Caov3	Cytotoxic activity Induction of apoptosis	[163]

oleic acid known as "human alpha-lactalbumin made lethal to tumor cells, HAMLET" or "bovine alpha-lactalbumin made lethal to tumor cells, BAMLET." It has been recognized that both protein and fatty acid are required to show cytotoxic activity against cancer cells [115]. Treatment of cancer cells with HAMLET provokes morphological changes typical of apoptotic cells through caspase activation and causes mitochondrial permeability transition resulting in mitochondrial swelling, loss of mitochondrial membrane potential, and cytochrome c release [120]. These authors also found that this complex induced autophagic cell death and changes in the proteasome structure and function. Similar effects resulting from chromatin condensation and cell shrinkage have been observed after treatment of cancer cells with the complex BAMLET. The efficacy of both complexes has been shown to be influenced by the type of cancer cell line [120]. In the last years, the therapeutic effects against bladder cancer have been studied in animal models as preliminary step for BAMLET use in human trials. It has been demonstrated that intravesical administration of HAMLET delays tumor progression in a murine bladder cancer model although no preventive effects on tumor formation were observed [121].

Intact caseins have not been characterized as chemopreventive proteins but they have been suggested as an important source of peptides with anticancer properties. CPPs are able to bind calcium, to inhibit cell proliferation, and to induce apoptosis of intestinal tumor HT-29 and AZ-97 cells through activation of voltage-activated calcium channels, which mediate the calcium flood according to the depolarization state of the cell [122]. However, in differentiated epithelial intestinal cells, a protective effect from programmed cell death is observed after treatment with these peptides [123]. β-Casomorphin 7 and β-casomorphin 5, two casein-derived sequences with opioid properties, have shown antiproliferative and cell cycle arresting activities on breast and colon cancer cells [115, 124, 125]. It has been suggested that these effects are mediated by interaction with specific opioid and somatostatin receptors although further studies confirming this mode of action are needed.

3. Impact of Milk Oligosaccharides on Human Health

Despite the important role of HMO in infant health, the limited supply of human milk has hindered its use in commercial infant formula [126] and in large-scale clinical trials. Presumably, the health benefits provided by HMO to infants could be extended to humans of all ages if alternative sources of these complex OS are identified [127]. In that view, the need of finding other sources of human-like OS has prompted the identification, characterization, and quantification of unknown OS present in many other types of milk and their respective industrial streams [128, 129].

3.1. Alternative Sources of Oligosaccharides: Major Sources of Nonhuman Milk Oligosaccharides and Their Industrial Effluents. Increasing interest on plant- and lactose-derived OS has been observed in the past decade as an alternative source for complex HMO. Some of these OS include galacto-OS (GOS), fructo-OS (FOS), and lactulose, among others [130]. These indigestible OS are considered prebiotics due to their ability to confer health benefits to the host through the selective growth and activity of commensal bacteria [131]. One such example is inulin, an oligofructan with D-fructofuranosyl β(1-2) links that cannot be broken down by human digestive enzymes, thus exerting several intestinal physiological effects that contribute to the host health. GOS, commonly produced by transgalactosylation of lactose by β-galactosidases, are another example of a current available source of OS for use by the infant formula industry.

Despite the fact that some health promoting effects, such as improved bifidogenic activity, have been attributed to some of those OS [131], little similarity has been observed between commercially available GOS and HMO, except that they are both built on a lactose core [127]. GOS and FOS are composed of a simple linear core, being devoid of structures having high biological activity such as fucose, sialic acid, and *N*-acetyl glucosamine. Because GOS and FOS do not possess the intrinsic structural complexity observed in HMO, it is expected that domestic farm animals and their processing streams, such as whey permeate from cheese manufacturing, can be a source of OS more similar to the ones present in human milk [132].

World milk production is almost entirely derived from cattle (83%), buffaloes (13%), goats (2%), sheep (1%), and camels (0.3%) (http://www.fao.org/agriculture/dairy-gateway/milk-production/dairy-animals/en/#.VA95gvldXXs). Considering that cow milk accounts for 83% of the world milk production, the enormous interest of the scientific community to identify, quantify, and characterize the OS present in cattle milk and their industrial byproducts is not surprising. A comprehensive review by Urashima et al. [132] shows that approximately 25 bovine milk OS (BMO) structures had been characterized before 2011. The development of advanced analytic techniques, such as several mass spectrometric methods and hydrophilic interaction liquid chromatography-high performance liquid chromatography, has enabled significant improvement in the identification of new BMO; as many as 40 BMO have been characterized [133, 134].

The low concentration of BMO makes it challenging to identify and characterize these compounds when compared with HMO. The OS concentration can reach values as high as 0.7–1.0 g/L in bovine colostrum or can be detected as just trace amounts in bovine milk [135], being much lower than the OS concentration in human milk. Caprine milk is another type of milk, which contains complex OS similar to HMO. The discovery of the presence of fucosylated and sialylated OS that are considered as prebiotics and which have the ability to reduce pathogen adherence to the intestine wall has opened up translational opportunities to human health [136]. Approximately 37 caprine milk OS (COS) have been identified, of which nearly half of them have had their structural complexity elucidated. Similar to bovine milk, COS are present in very small concentrations when compared with HMO. However, they have been found in concentrations of 0.25–0.3 g/L, which is 4-5 times higher than BMO [137].

From those two alternative sources of HMO-like OS (BMO and COS), industrial streams arising from cheese manufacturing and production of whey protein concentrates (WPC) and isolates (WPI) have been considered as a more realistic source of OS for future commercialization [129, 138]. Considering the enormous worldwide production of whey (180–190 $\times$ 10^6 tonnes/year; http://www.adpi.org/Portals/0/PDF/09Conference/TAGEAFFERTSHOLT.pdf) and the fact that the major industrial application of whey to produce WPC and WPI generates a new byproduct containing the target OS, the development of economically feasible processes to recover these compounds represents a key step in enabling the large-scale production of OS.

3.2. Biological Activities of Oligosaccharides. While a wide range of biological functions has been attributed to HMO, less information is available regarding the biological activities of BMO and COS. The limited availability of large quantities of OS with high degree of purity can be inferred by the limited number of *in vivo* studies with those compounds, with the majority of milk OS biological activities being described by *in vitro* studies. Recent reports of some of the biological activities of HMO, BMO, and COS are reported in Table 3.

3.2.1. Prebiotic Activity. One of the main features of HMO is that they can only be consumed by very specific bacteria strains that possess the appropriate set of enzymes to cleave their complex structure. This prebiotic effect is associated with improved health outcomes. A prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or in the activity in the gastrointestinal microflora, conferring benefits upon host well-being and health" [139]. Because HMO are only partially digested in the small intestine, they can reach the colon intact where they selectively stimulate the development of bifidogenic flora. A recent study has demonstrated the bifidogenic effect of major fucosylated and sialylated HMO when fed as a sole source of carbon to 25 major isolates of the human intestinal microbiota [140]. Most of the *Bifidobacteria* spp. and *Bacteroides* spp. were able to consume those OS and to produce short chain fatty acids, while common pathogenic bacteria were not able to grow on those OS. *In vitro* biological activities of HMO have been supported by *in vivo* studies. One of the newest publications in this topic demonstrated the ability of 2-fucosyllactose and 3-fucosyllactose to selectively increase some intestinal bacteria populations like *Barnesiella*, the major bacterial genus in mice [141], being this effect correlated with reduced level of colitis.

Prebiotic activities of COS, recovered from caprine whey, have been evaluated by *in vitro* studies [142]. The purified COS fraction favored the development of *Bifidobacterium* spp. and produced short chain fatty acids such as lactic and propionic acids but presented no inhibition of *Staphylococcus aureus* and *Escherichia coli* grown in human faeces.

3.2.2. Antipathogenic Activity. A second feature of OS is the ability to reduce pathogen biding to the intestinal mucosa. The intestinal mucosa is heavily glycosylated and covered with complex glycans including glycoproteins, glycolipids, and mucins, among others [143, 144]. Bacteria and viruses are able to recognize certain types of fucosylated and sialylated OS and adhere to them [130], therefore acting as anti-infective agents. Milk OS are also fucosylated and sialylated so bacteria and viruses, in presence of OS, will attach less to intestinal cells. The ability of pathogens to bind to specific OS seems to be intrinsically correlated with their structure. Neutral OS containing HexNAc block adhesion of pathogens that cause diarrhea (*Vibrio cholerae*) and pneumonia (*Streptococcus pneumoniae*) [15, 145], while neutral fucosylated OS have been shown to inhibit adhesion of other pathogens (i.e., *Campylobacter jejuni* and diarrheagenic *E. coli*) that cause gastrointestinal disorders [146]. Acidic OS containing sialic acid are able to block adhesion of *Helicobacter pylori*, which causes peptic ulcers and other gastric diseases [147], *Staphylococcus aureus*, and *Clostridium botulinum* [148].

Recent *in vitro* studies have demonstrated that BMO also possess antibacterial properties as observed for HMO. BMO from colostrum permeate proved to be effective in protecting HEp-2 cells from enteropathogenic *E. coli*, *Cronobacter sakazakii*, and *Salmonella enterica* serovar *typhimurium* [149]. It has also been demonstrated that BMO can inhibit the pili-mediated adhesion of *Neisseria meningitidis in vitro* [150]. Several studies have demonstrated the inhibition of the attachment of enteric pathogens such as *E. coli* and *Campylobacter jejuni* and noroviruses with HMO [151]. This effect has also been demonstrated by *in vivo* studies in which isolated HMO were fed to suckling mice before and after infection with enteropathogenic *E. coli*. Mice that received HMO significantly reduced colonization of this species compared with untreated controls [152].

3.2.3. Anti-Inflammatory Activity. OS have been also considered as anti-inflammatory agents due to their prebiotic activities and their ability to act as receptors of microorganisms. *In vivo* studies have demonstrated that COS possess anti-inflammatory properties towards the development of experimental colitis in rats. Pretreatment of the rats with isolated COS reduced the typical signs of induced colitis, including less anorexia, better body weight gain, and less macroscopic intestinal lesions, among others [153]. Similar results were observed by Lara-Villoslada et al. [154], where COS were shown to play an important role in intestinal protection and repair after a damage caused by DSS in rats.

4. Future Prospects

Milk has long been recognized as a source of macro- and micronutrients. Recent identification of many important biologically active substances on milk and its derivatives has attracted much attention from the scientific community. Not only are many of these bioactive compounds associated with growth, but they also confer many health benefits that might support disease prevention. Milk proteins and peptides are usually well tolerated and demonstrate oral bioavailability. In this view, they have the potential to act as health promoting ingredients and to participate in auxiliary therapies to boost

Table 3: Biological activities of human, bovine, and goat oligosaccharides.

Microorganisms/animals	Molecule used	Dose	Duration/details	Outcome measured	Reference
Bifidobacterium spp., *Bacteroides* spp., *Clostridium* spp., *Lactobacillus* spp., *Enterococcus* spp., *Streptococcus* spp., *Staphylococcus* spp., *Enterobacter* spp., and *Escherichia coli*	HMO (2′-FL, 3′-FL, LDFT, 3′-SL and 6′-SL)	0.5–2 g/L	48 hrs OS incubation	SCFA quantification, bacterial growth, and OS consumption	[140]
Mice	HMO (2′-FL and 3′-FL)	500 mM, starting with 5 mL, increasing by 2.5 mL every 3 d reaching a daily amount of 25 mL on day 20	From day 1 to day 20 after birth	Bacterial amount, colitis signs	[141]
Bacteria from human feces	Pooled GOS		During incubation	Bacterial amount	[142]
Mice	Pooled HMO	15 mg/day	One day before and after infection with EPEC	Intestinal colonization of EPEC	[152]
HEp-2 cells	Pooled BMO from colostrum	20 mg/L of total carbohydrate in culture	During incubation	Adherence inhibition	[149]
Bovine thyroglobulin and human salivary agglutinin glycoproteins	Pooled HMO and BMO	40 g/L	During incubation	*Neisseria meningitidis* Pili attachment	[150]
Rats	Pooled GOS	500 mg/(kg∗d)	2 days before and 6 days after induced colitis	Colonic damage	[153]

HMO: human milk oligosaccharides; FL: fucosyllactose; LDFT: lacto-difucosyl-tetraose; SL: sialyllactose; GOS: galacto-oligosaccharides; BMO: bovine milk oligosaccharides.

overall success in chronic diseases. However, this research area is only at its beginning and more peptides with physiological effects are to be discovered in the future. Confirming the health benefits of these bioactive compounds requires the design of clinical trials based on metabolomic genomics, proteomics, transcriptomics, and epigenetic data, in order to explore new biomarkers related to the observed health benefits.

While larger data for *in vivo* biological activities of milk and peptides is observed, the same is not observed for OS. To date, few studies have demonstrated the safety and efficacy of OS supplementation [155, 156]. The reduced number of biological activities evaluated for BMO and COS reveals the challenges associated with the production of OS in adequate quantities and purity needed for clinical trials. The development of new synthetic pathways to produce highly purified OS and of large-scale processes to recover those OS from their respective industrial streams will likely improve the elucidation of their biological activities and determine their safety and efficacy in clinical trials with humans. Moreover, the development of more environmentally friendly processes that are also economically feasible not only will enable the production of a new generation of prebiotics but will address environmental issues associated with the disposal of OS-containing waste streams and poor economic viability of our food industry.

Conflict of Interests

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

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Research Article

Antioxidant/Prooxidant and Antibacterial/Probacterial Effects of a Grape Seed Extract in Complex with Lipoxygenase

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In an attempt to determine the antioxidant/prooxidant, antibacterial/probacterial action of flavan-3-ols and procyanidins from grape seeds, pure catechin (CS), and an aqueous grape seed extract (PE), were applied in the absence and presence of pure lipoxygenase (LS) or in extract (LE) to leucocyte culture, *Escherichia coli* B_{41} and *Brevibacterium linens*, and observed whether there was any effect on lipid peroxidation, cytotoxicity, or growth rate. Short time periods of coincubation of cells with the polyphenols, followed by the exposure to LS and LE, revealed a high level of lipid peroxidation and a prooxidative effect. Longer coincubation and addition of LS and LE resulted in the reversal of the prooxidant action either to antioxidant activity for CS + LS and PE + LS or to the control level for CS + LE and PE + LE. Lipid peroxidation was significantly reduced when cells were exposed to polyphenols over a longer period. Longer exposure of *E. coli* to CS or PE followed by addition of LS for 3 h resulted in bactericidal activity. Significant stimulatory effect on microbial growth was observed for PE + LS and PE + LE treatments in *B. linens*, illustrating the potential probacterial activity in *B. linens* cultures. Lipoxygenase-polyphenols complex formation was found to be responsible for the observed effects.

1. Introduction

During grape processing, it is estimated that 20% of total weight of grape fruits used results in grape pomace that presents a challenging waste disposal problem for the winery and grape juice industry [1]. Winemaking by-products are of particular interest because grape is the world's largest fruit crop, with more than 68 million tons produced per year. The European Union affords approximately 24.5 million tons per year, and Romania produces 740.118 tons of grapes ranking the 19th place in the world (in 2010). It also produces 125.450 tons of wine [2]. Therefore, solutions involving further processing of the grape pomace to provide useful products that may balance out waste treatment costs are very important [3]. An alternative utilisation of the grape pomace could involve the isolation of the grape seeds and extraction of the polyphenols. Among the total extractable phenolics from grapes, approximately 60–70% comprises catechins, epicatechins, procyanidins, and proanthocyanidins, a group of important polyphenols that exert a beneficial effect on human health [4–6]. Grape seeds contain lipid, protein, carbohydrates, and 5–8% polyphenols, depending on the variety.

The most abundant phenolic compounds isolated from grape seeds are catechin, epicatechin, and procyanidins [4]. Grape seed proanthocyanidins constitute a complex mixture that consisted of procyanidins and procyanidin gallates [5].

Grape seed extract (GSE) has been reported to possess a broad spectrum of pharmacological and therapeutic effects including anti-inflammatory activity and can reduce apoptotic cell death [7, 8]. The proanthocyanidins from GSE have shown promising chemopreventive and/or anticancer properties in various cell culture and animal models [9]. The findings of Feng et al. [10] indicate that grape seed extract has neuroprotective properties in the neonatal rat hypoxia-ischemic brain injury model. The results also indicate that the suppression of free radicals after hypoxic ischemia by grape seed extract is one potential mechanism of this neuroprotection. Oxidative stress, the consequence of an imbalance of prooxidants and antioxidants in the organism, has rapidly gained recognition as a key phenomenon in chronic diseases: cardiovascular disease, hypertension, diabetes mellitus, and cancer [11]. The harmful effects of oxidative processes in living organisms can be reduced by the dietary intake of flavan-3-ols and procyanidins [12].

Lipoxygenase (LOX, EC 1.13.11.12), a dioxygenase known to be widely distributed in plants, animals, and microorganisms, catalyses the oxidation of polyunsaturated fatty acids to hydroperoxides [13]. Peroxyl radical complexes have been reported to exist during the catalytic cycle of LOX and can serve as sources of free radicals [14]. Thus, lipoxygenase can be seen as an oxidative stress inducer and also oxidative stress may favor a concerted package of lipoxygenase-mediated enzymatic and no-enzymatic lipid peroxidation and cooxidative processes [11]. Considering the various detrimental effects of imbalances or perturbations in fatty acid oxidation, a considerable interest in the development and characterization of LOX inhibitors was reported [15, 16]. Antioxidants such as flavonoids, which act as free radical quenchers, may act also as LOX inhibitors [17]. Schewe et al. [18, 19] studying the inhibitory effect of (−) epicatechin and of related oligomers, procyanidins, towards mammalian lipoxygenase, suggest that the general lipoxygenase inhibitory potency of flavanols and procyanidins may contribute to their beneficial effects on the cardiovascular system in man.

Phenolic compounds from grape seeds have pharmacological and nutraceutical benefits showing antiviral and antimutagenic actions [20] that are closely related to their antioxidant and singlet oxygen quenching ability. Recognition of such health benefits of catechins and procyanidins has led to the use of grape seed extract as a dietary supplement [21, 22]. Besides its antioxidant activity, the grape seed extract proved to act also as antibacterial agent [23–25].

In an attempt to determine the beneficial properties for the human health of the flavan-3-ols and procyanidins from grape seeds—through food or immediate house medical care—and also the exploitation of the potential added-value of this by-product, the effects of pure catechin and of an aqueous grape seed extract was assessed in this study. The evaluation was done in the absence and presence of pure lipoxygenase or in extract, on the lipid peroxidation and cytotoxicity on leucocyte culture as well as on the growth rate of *Brevibacterium linens* and *Escherichia coli* B_{41}.

E. coli is the most common cause of infections by Gram-negative bacilli and the bacterial organism most often isolated from blood cultures. It is a frequent cause of outpatient urinary tract infections in women worldwide, of hospitalization due to pyelonephritis and septicemia, and of nosocomial infections among hospitalized patients. Meningitis caused by *E. coli* in neonates is frequently fatal. Resistance to recommended first- and second-line agents, such as penicillins, cephalosporins, sulfa drugs, and fluoroquinolones, is high in many countries and is commonly associated with treatment failure [26]. For observing and comparing the differences on Gram-positive bacteria, *B. linens* was used in this study.

Based on previous studies [25, 27] using the UV-Vis spectroscopy, interactions between LOX soybean extract and the catechin-type compounds from grape seed extract in leukocyte and bacterial culture were monitored addressing the issue of possible inhibition of lipoxygenase by catechins as well as the complexion of the enzyme with these polyphenols in inducing an antioxidant/prooxidant or antibiotic/probacterial action.

As a prooxidant inducer, the standard soybean lipoxygenase as well as a raw extract from soybean containing LOX-1 and LOX-3 isoenzymes were used to determine potential inhibitory activity of different classes of polyphenols towards LOX enzymes [28]. Flavonoids, in particular those containing a catechol group, are known to chelate iron and other transition metal ions, and lipoxygenases contain an iron moiety at the active site [29]. This possible chelation through a prooxidant/antioxidant effect was evaluated by the TBARS (thiobarbituric acid reactive substances) and MTT assays on the leucocyte culture.

Lipid peroxidation is a classic indicator of oxidative stress whereby free radicals extract electrons from cell membranes inflicting damage. Quantification of lipid peroxidation uses malondialdehyde (MDA), the end product of lipid peroxidation, and uses the reaction between MDA and thiobarbituric acid (TBA), which yields thiobarbituric acid reactive substances (TBARS) which can be quantified by visible or fluorescence spectrophotometry [30]. Although the thiobarbituric acid assay is not specific for MDA and several other aldehydic products of cellular molecules can react with TBA, it is the most commonly used method to determine lipid peroxidation [10].

2. Materials and Methods

2.1. Chemicals. Catechin standard ((±)-catechin hydrate) and pure soybean lipoxygenase-1 were purchased from Sigma Chemical Co., St. Louis, MO. The standard enzyme contained 46.000 units/mg protein. Other chemicals used were all analytical grade. RPMI medium, fetal serum, penicillin, streptomycin, L-glutamine, Triton, Hanks salt containing MTT ((3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide), and DMSO (dimethyl sulfoxide) were from Sigma, Cluj-Napoca, Romania.

2.2. Microbial Strains. Catechin standard (CS), LOX-1 standard (LS), and two extracts (grape seed polyphenolic extract (PE) and LOX soybean extract (LE) were tested against *Escherichia coli* B_{41}-a reference strain and *Brevibacterium linens*. *Escherichia coli* B_{41} was obtained from the culture collection of microbial strains of the Department of Microbiology, Faculty of Veterinary Medicine, Cluj-Napoca, Romania. *Brevibacterium linens* was isolated from the fermented cheese Năsal, a Romanian brand of maturated cheese, and maintained in the same collection as above.

Bacterial strains cultured overnight at 37°C in agar were transferred into 250 mL peptone broth (Difco Laboratories) kept in a reciprocal shaker at 37°C for 24 h yielding a stock preparation with a log-phase cell density of approximately 10^7 colony forming units (CFU)/mL as evaluated initially by measurements of the optical density at 630 nm.

2.3. Standard Solutions Preparation (CS and LS). Catechin was solubilized in pure Milli-Q water (CS). CS was then added to the cell or microbial culture to a final concentration of 310 μM. Lipoxygenase standard was solubilized in physiological saline buffer (PBS) pH = 7, as a stock solution of 1 mg/mL protein corresponding to 46.000 enzymatic units/mL in PBS (LS). In the experiments, 575 e.u. LOX/mL leucocyte or bacterial culture medium was used.

2.4. Extraction of Polyphenols from Grape Seeds (PE) and LOX from Soybeans (LE). The polyphenols from grape seeds were extracted and characterized as previously described [31]. The total content of the catechin type compounds in this extract as determined by HPLC was 1956 mg total catechins/Kg dry grape seeds [31].

To obtain LE, an aliquot of 5 g soybean meal was mixed with 30 mL of PBS, pH 7.0, stirred for 1 h at room temperature, filtered through cheesecloth, and then centrifuged at 16,000 rpm for 10 min. The resultant supernatant of the raw extract was designated as the lipoxygenase extract, LE. The total protein content of LE, determined as described by Gornall et al. [32], was 27 μg total protein/mL extract.

2.5. Total Polyphenol Content of PE and LE. The total polyphenol content measured by the Folin-Ciocalteu method [33] was 3.2 g gallic acid equivalents (GAE)/kg grape seeds and 1.8 g gallic acid equivalents/kg soybean.

2.6. Leukocytes Isolation and Treatment. Viable leukocytes were obtained from sterile, whole horse blood drawn with plastic syringe containing cold heparin solution in physiological saline buffer at final concentration of 50 UI/mL. Once drawn, gravitational sedimentation of erythrocytes was performed by centrifugation for 10 min at 1500 rpm, (blood is separated into plasma or "buffy coat" phase of leukocytes above the erythrocyte phase). The plasma phase was then transferred to a fresh tube and centrifuged again for 10 min at 3500 rpm. The supernatant was discarded and the pellet was washed with PBS. After a further centrifugation of 10 min at 3500 rpm, the pellet was suspended in media to a concentration of 1×10^6 cells/mL. Leukocytes were cultured in RPMI medium supplemented with 10% fetal serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine under standard culture condition (37°C, 95% humidified air and 5% CO_2) [27].

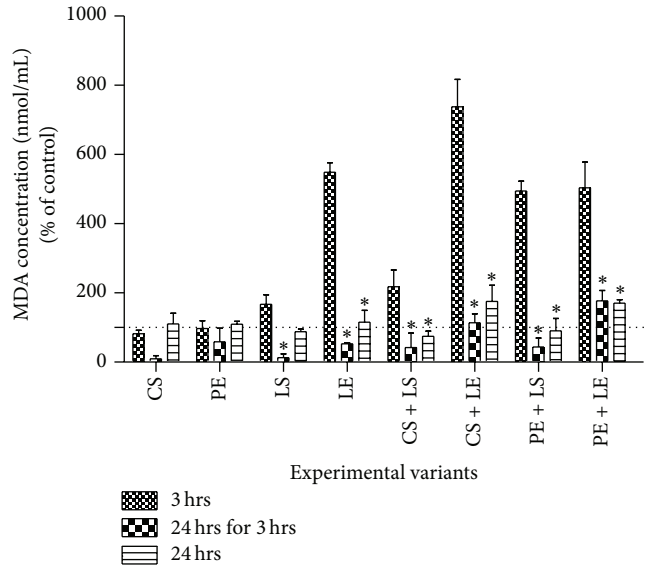

Figure 1: Effect of grape seed extract on leucocytes thiobarbituric acid reactive substances (TBARS). Treatment with the pure catechin (CS to a final concentration of 310 μM/mL medium) and grape seed extract (PE to a final concentration of 35 μg polyphenols/mL medium) was administrated at the initiation of the culture (3 hrs and 24 hrs for 3 hrs) and at 24 h (24 hrs). Leucocyte thiobarbituric acid reactive substances were assessed 4 h (3 hrs) and 24 h (24 hrs for 3 hrs) after adding the polyphenols (CS and PE) for the same cell sample and at 25 h (24 hrs) for another one. Data are presented as mean ± S.E.M. Values are expressed as percent of control for the experimental variants tested: LS-standard lipoxygenase, LE-lipoxygenase extract, CS-catechin standard, and PE-polyphenolic extract.

Then, 36 μL of CS or PE was added on the cell culture. Two incubation protocols were used for the polyphenols and leucocyte: 3 h (short incubation time) and 24 h (long incubation time). After incubation, 250 μL of LS or LE was added and incubated for 1 hour before TBARS and MTT assays were performed.

2.7. Determination of Thiobarbituric Acid Reactive Substances (TBARS Assay). The influence of the polyphenols and lipoxygenase interaction on leucocyte's lipid peroxidation was evaluated by TBARS assay, using suspension of 2×10^5 cells per well in 12-well plates. Two incubation protocols were used for the polyphenols and leucocyte: 3 h (short incubation time) and 24 h (long incubation time). After incubation (3 h or 24 h) with polyphenol (CS and PE) and while in logarithmic growth cell phase, cells were treated with lipoxygenase (LS and LE). TBARS assay was then performed at 4 h (3 hrs in Figure 1) and 24 h (24 hrs for 3 hrs in Figure 1) for the experimental variant when LS and LE were added after 3 h. At 25 hours after the antioxidant treatment the assay was done

for the experimental variant when LOX (LS and LE) was added after 24 h (24 hrs in Figure 1).

For determining lipid peroxidation, 1 mL of leucocyte culture homogenate was mixed with 2 mL working solution containing 15% (w/v) thiobarbituric acid, 0.25 N HCl. The mixture was heated for 15 min in boiling water. After cooling, the precipitate was removed by centrifugation at 3500 rpm for 10 min. Absorbance was determined at 535 nm using a spectrophotometer JASCO V-500, Cluj-Napoca, Romania. The MDA concentration was determined from a calibration curve. Results were expressed as the percentage (%) of MDA concentration, assuming the absorbance of control cells as 100%.

2.8. MTT Assay. The influence of interaction between polyphenols and lipoxygenase on leucocyte's mitochondrial respiration was evaluated by MTT assay, using suspension of 2×10^3 cells per well in 96-well plates. Two incubation protocols were used for the polyphenols and leucocyte: 3 h (short incubation time) and 24 h (long incubation time). After incubation with polyphenol (CS and PE), while in logarithmic growth cell phase, cells were treated with lipoxygenase (LS and LE). MTT assay was performed at 4 h (3 hrs in Figure 2) and 24 h (24 hrs for 3 hrs in Figure 2) for the experimental variant when LS and LE were added after 3 h. At 25 h after the antioxidant treatment the assay was done for the experimental variant when LOX (LS and LE) was added after 24 h (24 hrs in Figure 2).

For the MTT assay, cells were pelleted and washed with PBS and 150 μL Hanks salt containing MTT (455 μg/mL) was added into each well. After 2-hour incubation under standard conditions the MTT solution was removed and 200 μL of DMSO was added into each well. Absorbance was measured at 490 nm using a Biotek Synergy HT Microplate Reader (Cluj-Napoca, Romania). Results were expressed as the percentage (%) of MTT reduction, assuming the absorbance of control cells as 100%.

2.9. Interactions between Lipoxygenase (LS or LE) and Polyphenols (CS and PE) on E. coli and B. linens Cultures: Growth Inhibition Assay (Turbidimetry and Spectrophotometry). Ten microliters of logarithmic-phase bacterial cultures (10^7 CFU/mL) in 200 μL nutrient broth was added in each well, in 48 wells plates. CS, to a final concentration of 310 μM or 36 μL and PE, to a final concentration of 35 μg polyphenols/mL medium, were cocultivated with the microorganisms (Figures 3 and 4 experimental variants CS and PE). LS and LE were also incubated with bacteria, at a final concentration of 575 e.u. LOX/mL buffer for LS and 27 μg total protein/mL extract for LE (Figures 3 and 4 experimental variants LS and LE). The same final concentration of LS and LE was added after 3 h to the microbial cultures treated with CS and PE (Figures 3 and 4, treatments CS + LS 3 h, CS + LE 3 h, PE + LS 3 h, and PE + LE 3 h).

In a volume of 2 mL PBS pH = 7, 36 μL CS (to a final concentration of 310 μM) or 36 μL PE (to a final concentration of 35 μg polyphenols/mL medium) was added. Each experimental variant (CS or PE in PBS) was mixed with 250 μL LS diluted 1 : 10 (to a final conc. of 575 e.u. LOX/mL buffer) and also with 250 μL LE (27 μg total protein/mL extract). The mixture was kept for 3 h at 37°C and then added on the bacterial culture (Figures 3 and 4 experimental variants CS + LS 0 h, CS + LE 0 h, PE + LS 0 h, and PE + LE 0 h).

The cultures were incubated at 37°C for 26 h and growth inhibition was measured by determination of the absorbance at 630 nm. Absorbance readings (630 nm) were taken periodically (at 2 h, 7 h, 20 h, and 24 h).

2.10. Statistical Analysis. Data were presented as the mean percentages of control ± standard deviation from at least three independent experiments. Experimental data were analysed with the program Graph Pad Prism 5, performing two-way analysis of variance (ANOVA) and Bonferroni posttest was used to compare the experimental variants (a $P < 0.05$ was considered significant).

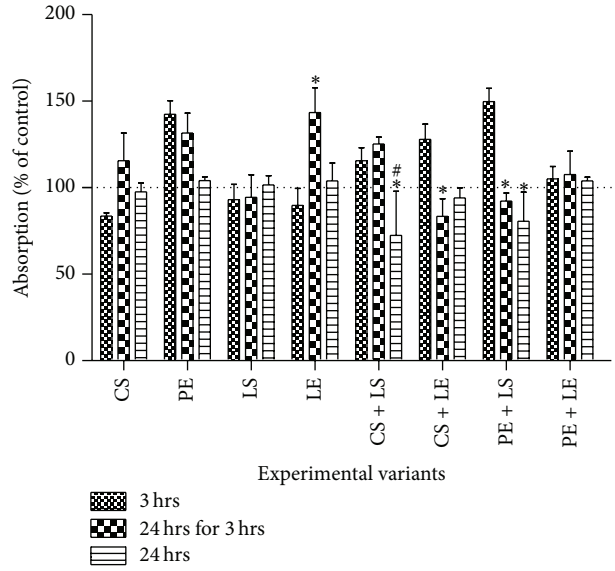

Figure 2: Cellular respiration (as measured by the mitochondrial MTT conversion to formazan) after coincubation with different mixtures of polyphenols (CS and PE) and lipoxygenases (LS and LE) for different periods (3 hs and 24 hs). Treatment with the pure catechin (CS to a final concentration of 310 μM/mL medium) and grape seed extract (PE to a final concentration of 35 μg polyphenols/mL medium) was administrated at the initiation of the culture (3 hrs and 24 hrs for 3 hrs) and at 24 h (24 hrs). Leucocyte respiration was assessed 4 h (3 hrs) and 24 h (24 hrs for 3 hrs) after adding the polyphenols (CS and PE) for the same cell sample and at 25 h (24 hrs) for another one. Data are presented as mean ± S.E.M. Values are expressed as percent of control for the experimental variants tested: LS-standard lipoxygenase, LE-lipoxygenase extract, CS-catechin standard, and PE-polyphenolic extract.

3. Results and Discussion

The aqueous extract of polyphenols from the grape seeds was analyzed through LC-UV-DAD and LC-ESI-MS and the quantitative analysis of its components was performed as previously reported [31]. The composition of polyphenols of the extracts tested (PE and LE) as evaluated using

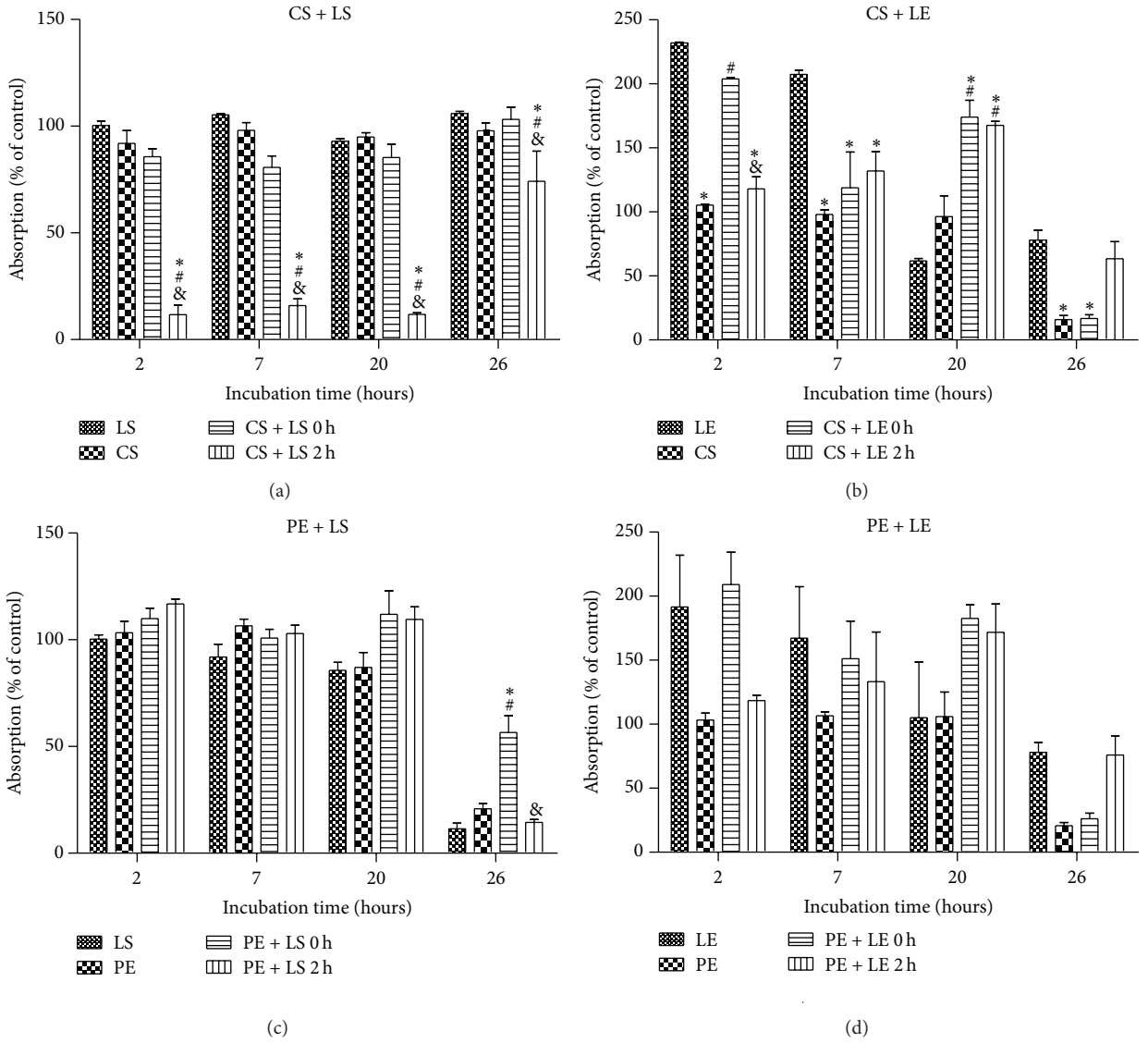

Figure 3: Growth rate, as referred to control, of *E. coli* B_{41} measured by UV-Vis spectroscopy at 630 nm at different time intervals (2, 7, 20, and 26 h). ∗: statistically different when compared with LS or LE, #: statistically different when compared with CS or PE, and &: statistically different when compared with CS + LS 0 h, CS + LE 0 h, PE + LS 0 h, or PE + LE 0 h for $P < 0.05$.

the LC-MS technique revealed that epicatechin and catechin were the major compounds in PE, representing together with epicatechin gallate (ECG) 60% of total polyphenols, followed by procyanidin dimers (28%) and trimers (12%) [28]. In LE, isoflavones daidzein and genistein were the main polyphenolic compounds identified, although in very low quantity consistent with our previous study [34].

3.1. Influence of Polyphenols (CS and PE) on Leukocytes Lipid Peroxidation (TBARS Assay) in Presence and Absence of LOX (LS or LE). In this study, very high levels of TBARS were observed after 3-hour incubation of polyphenols and 1-hour incubation with LS or LE in the case of the experimental variants LE, CS + LE, PE + LS, and PE + LE (Figure 1). When LOXs and polyphenols were coincubated with the leucocyte culture for 21 h (24 hrs for 3 hrs), there was a significant decrease of TBARS levels lower than control with statistically significant values measured for LE, CS + LE, and PE + LS treatments. In contrast, coincubation of cells with polyphenols for 24 h followed with the addition of LS or LE for 1 h resulted in no significant antioxidant activity for any of the experimental variants tested.

In Figure 1, it can also be observed that longer incubation time (24 h versus 3 h) of polyphenols (CS or PE) correlated well with lower lipid peroxidation for LS, LE, CS + LS, CS + LE, PE + LS, and PE + LE treatments. However, the addition of LE after CS or PE elicited prooxidant activity rather any antioxidant tendency for all the incubation times tested.

3.2. The Effect of Polyphenols (CS and PE) on Cellular Respiration (MTT Test) in the Presence of LOX (LS or LE). The percentage of inhibition or stimulation of respiration

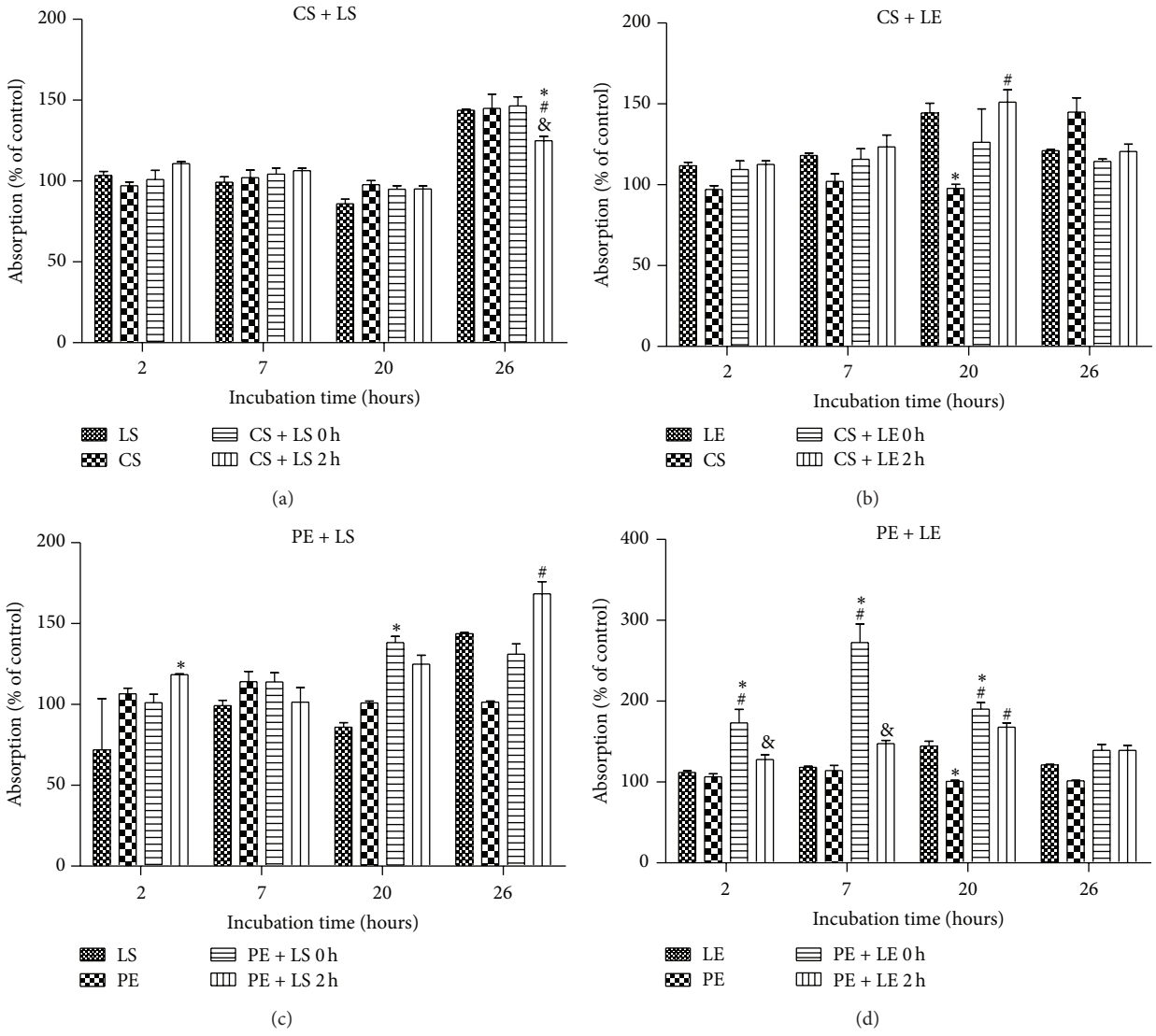

Figure 4: Growth rate, as referred to control, of *B. linens* measured by UV-Vis spectroscopy at 630 nm at different time intervals (2, 7, 20, and 26). ∗: statistically different when compared with LS or LE, #: statistically different when compared with CS or PE, and &: statistically different when compared with CS + LS 0 h, CS + LE 0 h, PE + LS 0 h, or PE + LE 0 h for $P < 0.05$.

(measured by the MTT reduction to formazan) for cells coincubated with CS, PE with/without LS, or LE is illustrated (Figure 2). Respiration is generally stimulated under oxidative stress, if a similar number of cells are cultivated (same protein content). Consequently, high values of MTT correlate with a prooxidative activity, while lower values correspond to antioxidant action [27].

As shown in Figure 2, shorter incubation of cells with CS (3 h) was associated with antioxidant effect (experimental variant *3* hrs in Figure 2). However, CS incubation for 24 h (*24* hrs *for 3* hrs) resulted in the slight observation of prooxidant effect, although no effect was observed at 25 h (*24* hrs). This observation is consistent with argument that longer incubation time leads to increased mitochondrial respiration due to the formation of the oxidation products [27]. MTT test also revealed that LS for all the incubation times and LE incubated for 1 h (*3* hrs and *24* hrs) with the cells exhibited no significant effect on the mitochondrial respiration (Figure 2).

During coincubation of cells with the polyphenols for 3 h followed by the exposure to either LS or LE, the measured MTT after 4 h (*3* hrs) revealed a prooxidative effect of CS + LS, CS + LE, PE + LS, and PE + LE samples. This prooxidative intensity was highest for PE + LE, while PE + LS showed the lowest intensity. In contrast, longer coincubation of cells with polyphenols for 24 h and addition of LS and LE for 1 h (*24* hrs) prior to analysis resulted in the reversal of the prooxidant action either to antioxidant activity for CS + LS and PE + LS or to the control level for CS + LE and PE + LE. For longer LOX-polyphenol interaction times (leucocytes cells initially incubated with CS and PE for 3 h followed by the addition of LS and LE and further incubation for total of LOX-polyphenol interaction time of 21 h (*24* hrs *for 3* hrs))

antioxidant activities were detected for CS + LE and PE + LS, whereas prooxidant tendency was observed for PE + LE and CS + LS. The activities for the later reaction mix were slightly higher than those observed for 3 h exposure experiments.

3.3. Interactions between Lipoxygenase (LS or LE) and Polyphenols (CS and PE) on E. coli and B. linens Cultures. In the experiments involving mixing CS or PE with LS or LE prior to the addition to *E. coli* and *B. linens* culture, two points of view were taken into consideration: the formation of enzyme-polyphenol complex that may have influence on bacteria and the inhibition of lipoxygenase by polyphenols, thus reducing LOX prooxidant activity. In addition, LOX oxidative catalytic modification of polyphenols may lead the production of o-quinones and other electrophilic prooxidative by-products [35] which by themselves could be toxic to and/or inhibitory to microorganisms [36]. LOX-polyphenol complexion and LOX inhibition by polyphenols and their influence on *E. coli* and *B. linens* culture were analyzed by treating the cell culture with polyphenols for 3 hours before the addition of LOX and the bacterial growth monitored by $OD_{630\,nm}$ after 1 h post-incubation with LOX. Under these conditions, there is initial interaction of polyphenols with bacteria cell culture implying their possible oxidative modification prior to addition of the prooxidant LOX.

Figure 3 shows the bacterial growth monitored after 2, 7, 20, and 26 h for *E. coli* cocultivated with LS, LE, CS, and PE alone or in combinations. The addition of CS was associated with instant cessation of growth in *E. coli* culture. *CS + LS 3* h also exhibited statistically significant antibacterial activity for up to 20 h, when compared with other experimental treatments. However, no significant effect was observed when CS and LS were mixed and added on the *E. coli* culture (*CS + LS 0* h).

For CS + LE treatment, there was an intense bacterial growth observed upon addition of LE after 7 h, which however decreased after 20 hours of incubation. *CS + LE 0* h also improved the bacterial growth but proved to be bactericidal at 26 h, a property observed also for CS treated cells. Similarly, the best antibacterial action for PE + LS was recorded after 26 hours of cocultivation and when PE was added 3 h before the LS (*PE + LS 3* h) or when the two extracts were mixed prior to being added to the bacterial culture (*PE + LE 0* h).

Figure 4 presents the bacterial growth measured at 2, 7, 20, and 26 h for *B. linens* cocultivated with LS, LE, CS, and PE alone or in mixtures as described in the legends. *Brevibacterium linens* was chosen in this study for comparative analysis on the influence of the polyphenols between a Gram-positive bacteria and a Gram-negative *E. coli* B_{41}. In contrast to varied inhibitory activity observed in *E. coli* cell cultures cocultivated with LS, LE, CS, and PE, no antibacterial or bacteriostatic action was observable in the case of *B. linens* (Figure 4).

Interestingly, significant stimulatory effect on microbial growth was observed for PE + LS and PE + LE treatments, illustrating the potential probacterial activity of the polyphenols and LOX in *B. linens* cultures. These results are consistent with the previous study where CS and PE exhibited different effect on the Gram-negative *E. coli* in comparison to the Gram-positive bacteria *B. linens* [25].

Detailed physicochemical studies suggest that the bactericidal activities of galloylated tea catechins at the cell membrane level may be due to their specific perturbations of the ordered structure of phosphatidylcholine and phosphatidylethanolamine bilayers constituting bacterial cell wall membranes [37, 38]. Differential effects of catechins on bacterial cell walls compared to membranes of human cells may be due to differences in structures of the respective walls (membranes) [39]. The bactericidal action of EGCG (epigallocatechin gallate) may depend on hydrogen peroxide derived from the reaction of EGCG with oxygen (prooxidative activity) [40, 41]. These observations suggest that antimicrobial effects arise from the interactions of catechins with oxygen, genes, cell membranes, and enzymes [39]. Postulated antimicrobials mechanisms for botanicals investigated may be disruption of microbial cell membranes and chelation to essential trace elements such as zinc and iron that the bacteria need for growth [42].

4. Conclusions

In our present study, the effect of the different oxidation products of grape seed polyphenols in presence of LOX on leucocyte culture was monitored by the TBARS and MTT assays. The TBARS assay revealed that the action of the tested extracts due to the different molecular interactions proves to be time-dependent; longer incubation time of polyphenols and LOX with leucocyte culture generally displayed ant-lipidic peroxidation effect, probably via the complexing of the two classes of molecules and/or LOX inhibition by either CS or PE. While the presence of LOX (LS and LE) was generally associated with higher lipid peroxidation activity of leukocyte cells that was detectable within 3 h, longer incubation time (24 hrs versus 3 hrs) of the same samples in presence of GSE polyphenols resulted in the decrease of peroxidation. In particular, coincubation with polyphenols alone (CS) and coincubation in combination with LS (CS + LS and PE + LS) for 21 h were most effective in inhibiting lipid peroxidation of the cells. However, longer coincubations (24 h) prior to the addition of the LOX enzymes did not significantly lower the lipid peroxidation activity, indicating that autooxidized CS and PE were not effective inhibitors of LOX activity.

Based on the current findings, it can be concluded that either the unoxidized GSE polyphenols and/or their intermediates after 21 h incubation form a complex with LOX thereby suppressing the LOX-mediated lipid peroxidation. It is also plausible that unoxidized GSE polyphenols may participate in the quenching of the highly oxidative free radical species and hydroperoxides liberated during LOX-catalyzed reactions, thus conferring protection to the cells against lipid peroxidation.

Similar to TBARS assay results, MTT test also revealed that longer coincubation time of cells (>24 h) with polyphenols prior to addition of LS resulted in the highest cytotoxicity observed in leukocyte cells. It is generally believed that longer exposure of the cells to polyphenols may lead to

polyphenol oxidation to form toxic by-products. Thus, it can be concluded that higher cytotoxicity observed above could be attributable to the effect of polyphenol oxidative intermediates or byproducts rather polyphenol-lipoxygenase interaction. Comparatively, this effect was more pronounced for LS than for LE, indicating a weaker oxidative capacity of LE towards leukocyte cells. CS was also found to be slightly cytotoxic; however, the effect was reversible on addition of LS and LE illustrating the possible beneficial action of catechin-lipoxygenase complex formation.

On the study of the effect of potential lipoxygenase-polyphenols complex formation *in vivo* on bacterial culture, longer exposure (up to 26 hrs) of *E. coli* to CS or PE followed by addition of LS for 3 h resulted in bactericidal activity. Thus, different types of cells may account to different intermolecular interactions involving LOX and polyphenols *in vitro*. Furthermore, Gram-negative bacteria *E. coli* was affected differently from positive *B. linens* in presence of LOX and GSE polyphenols.

In conclusion, our studies have highlighted the beneficial effect of lipoxygenase-polyphenols complex formation in the protection of leucocytes against LOX-mediated lipid peroxidation and cytotoxicity on as wells as imparting antibacterial and probacterial activities on *E. coli* and *B. linens*, respectively. Grape seeds represent an important source of health promoting polyphenols, and thus cost effective technologies will be important in the future for their large scale processing to tap into the high polyphenol content with the beneficial effects on human health.

Conflict of Interests

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

Authors' Contribution

Veronica Sanda Chedea and Cornelia Braicu contributed equally to this work.

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Research Article

Role of Feed Forward Neural Networks Coupled with Genetic Algorithm in Capitalizing of Intracellular Alpha-Galactosidase Production by *Acinetobacter* sp.

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Alpha-galactosidase production in submerged fermentation by *Acinetobacter* sp. was optimized using feed forward neural networks and genetic algorithm (FFNN-GA). Six different parameters, pH, temperature, agitation speed, carbon source (raffinose), nitrogen source (tryptone), and K_2HPO_4, were chosen and used to construct 6-10-1 topology of feed forward neural network to study interactions between fermentation parameters and enzyme yield. The predicted values were further optimized by genetic algorithm (GA). The predictability of neural networks was further analysed by using mean squared error (MSE), root mean squared error (RMSE), mean absolute error (MAE), mean absolute percentage error (MAPE), and R^2-value for training and testing data. Using hybrid neural networks and genetic algorithm, alpha-galactosidase production was improved from 7.5 U/mL to 10.2 U/mL.

1. Introduction

Alpha-galactosidases (3.2.1.22) belong to the family of glycosyl hydrolases or glycosidases. These enzymes catalyze the hydrolysis of terminal alpha 1–6 linked galactose residues from simple and complex oligosaccharides and polysaccharides [1]. They are widely distributed in plants, animals, and microorganisms. Alpha-galactosidases find potential applications in food, pharmacological, and chemical industries. The enzyme has been used in food industry for enhancing the nutritional quality of legumes by degrading galactooligosaccharides that cause gas or flatulence [2]. It is also used to improve crystallization of sugar by removing raffinose from molasses in beet sugar industry [3] and in guar gum processing [4] and for enhancing bleaching of softwood along with mannanase in paper and pulp industry [5] and in processing of animal feed [6]. In humans, mutations in *gfA* gene lead to Fabry's disease, a rare X-linked recessive lysosomal storage disorder. Enzyme replacement therapy with α-galactosidase is considered a potential treatment for Fabry's patients [7]. In addition, the enzyme can also convert type "B" erythrocytes to type "O" erythrocytes [8] and is also used in xenotransplantation [9]. Microbial sources for alpha-galactosidase are being explored because of ease of cultivation and fermentation conditions. However, for cost-effective production, fermentation medium plays a vital role in the commercial production of enzymes. The nutritional requirements of each microorganism are varied and are regulated by physiological, biochemical, and genetic makeup of the organism [10]. Therefore optimization of fermentation medium is considered a crucial step for cost-effective production of the desired product. Traditional methods use one at a time method of approach that is laborious and time-consuming and it does not reflect interactions between different variables [11]. Experiments based on statistical methods are considered to be more economical and effective than traditional methods in

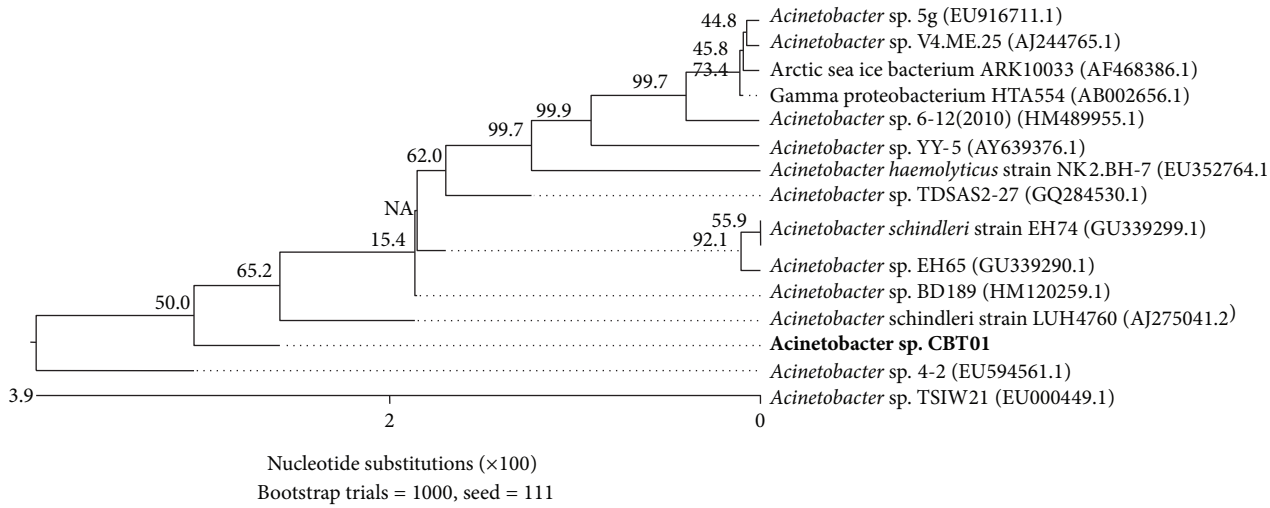

Figure 1: Phylogenetic analysis of *Acinetobacter* sp. CBT01 by ClustalW software (Accelrys, San Diego, CA, USA). The branching pattern was generated by the neighbor joining method.

understanding interaction between variables and minimizing the number of experiments. Response surface methodology (RSM) is one of the widely used statistical methods for the optimization of medium parameters [12–17]. RSM-based models can predict the relationship between a limited number of input and output parameters and hence cannot be applied for highly nonlinear processes [18]. Artificial neural networks (ANNs) and genetic algorithms (GAs) are termed as artificial intelligence that mimics the human with a given set of experimental criteria; GA enables identification of best alternative with goodness of fit by performing multiple random searches. ANN along with GA is widely used in various optimization studies, even in cases where the primary function under study is discontinuous, nondifferentiable, stochastic, or highly nonlinear [19, 20]. Currently, hybrid ANN-GA is being applied in optimizing various physical and nutritional fermentation parameters. This method has been applied to enhance the production of alkaline protease from *Bacillus circulans* and glutaminase from *Bacillus subtilis* [21, 22].

The present study focuses on enhancing the production of intracellular alpha-galactosidase from *Acinetobacter* sp. isolated from sugar cane waste by using hybrid artificial neural networks and genetic algorithm (ANN-GA). This is the first report of optimization of intracellular alpha-galactosidase using ANN-GA. A feed forward neural network (FFNN) together with back propagation was used for nonlinear modelling in this study to reduce the experimental error and subsequent optimization of enzyme production using genetic algorithm (GA).

2. Materials and Methods

2.1. Microorganisms. Microorganisms were isolated from sugar cane waste. The isolate was observed to be gram-negative, short-rods, nonmotile, and nonspore forming bacteria. The isolate was positive for catalase and citrate but negative for nitrate reduction, H_2S production, and oxidase and indole production. Based on morphological, biochemical, and 16s rRNA sequencing analysis, the isolate showing maximum intracellular alpha-galactosidase activity was identified as *Acinetobacter* sp. (Figure 1). The organism was grown at 36°C for 12 hours and maintained on agar slants at 4°C and was subcultured at 4-week interval.

2.2. Inoculum Preparation and Cell Lysis. A 24-hour-old culture 0.5% (w/v) inoculum was taken and inoculated into a 250 mL Erlenmeyer flask containing 100 mL sterile production media containing raffinose 25, tryptone 10, K_2HPO_4 10, $MgSO_4{\cdot}7H_2O$ 1, and $FeSO_4{\cdot}7H_2O$ 1 in $g\,l^{-1}$ (pH 7.0). The inoculated culture media were incubated at 36°C for 24 hours in shaking incubator at an agitation speed of 170 rpm. Cells were harvested from broth by centrifugation at 10,000 g and washed with 20 mM Tris buffer (pH 7.0). The cells were suspended in the same buffer containing 0.3% (w/v) lysozyme, 0.1% (w/v) Triton X 100, and 1 mM PMSF and incubated for 1 hour at 30°C. The cells were further disrupted by sonication. Cell debris was removed by centrifugation (10,000 g, 20 minutes, 4°C). Alpha-galactosidase activity was measured in the supernatant.

2.3. Alpha-Galactosidase Activity. Alpha-galactosidase activity was measured according to Dey and Pridham [1] in a reaction system containing 550 μL of 20 mM Tris buffer (pH 7.2), 100 μL of supernatant (enzyme preparation), and 250 μL of 2 mM ρ-nitrophenyl-alpha-D-galactopyranoside (ρNPGal). The reaction mixture was incubated at 50°C for 10 minutes and the reaction was stopped by addition of 1 mL of 0.2 mM Na_2CO_3. The absorbance was read at 405 nm. One enzyme unit (U) is defined as the amount of enzyme required to produce one μmol of ρ-nitrophenol per minute under the above assay conditions.

Table 1: Selected factors and their minimum and maximum range chosen for intracellular alpha-galactosidase production.

Parameter	Low	High
Temperature (°C)	32	40
pH	6	8
Agitation speed (rpm)	150	190
Tryptone (g/100 ml)	0	2
Raffinose (g/100 ml)	1.5	3.5

2.4. Modelling and Optimization of Enzyme Production

2.4.1. Data Sets. In the present study, the most promising factors which influence the alpha-galactosidase production were optimized by using the neural networks and genetic algorithms. Based on the preliminary studies (data not shown), temperature, pH, agitation speed, raffinose, tryptone, and K_2HPO_4 concentrations were found to be the most important parameters that influence alpha-galactosidase production from the isolated bacterial strain. The list of selected variables with their minimum and maximum concentrations was given in Table 1. A central composite design with 50 experiments was employed in the present study (Table 2). The data was divided into two sets comprising 40 observations used for training the network and 10 data sets used as testing data. The training data was used to compute the network parameters. The testing data was used to ensure robustness of the network parameters.

2.4.2. Artificial Neural Networks. In the present study a multilayer perceptron (MLP) neural network was used. A feed forward neural network, which uses error backpropagation learning algorithm (BPNN), was constructed for modelling alpha-galactosidase production. The network consists of three layers of neurons, namely, an input layer, a hidden layer, and an output layer. All three layers are connected to the subsequent layers in the forward direction; the connections are termed as weights. The weights play a vital role in optimizing the data. Experimental conditions were chosen as inputs for the network whereas output is alpha-galactosidase activity. The number of the neurons in the hidden layer was optimized based on the trial and error method (examined from 3 to 18). All the data were normalized to −1 to +1. Scaled data are passed through the input layer and then data is propagated from input layer to hidden layer and finally to reach the targets (output layer) of the network. Every node in input and hidden layer is connected to the nodes in the subsequent layer. Each neuron in the hidden and output layer acts as a summing junction, which combines and modifies the inputs from the previous layer using the following equation:

$$Y_i = \sum_{j=1}^{n} x_i w_{ij} + b_j, \tag{1}$$

where Y_i is net input to node j in hidden or output layer, X_i is outputs of previous layer, W_{ij} is weights between the ith node and jth node, n is number of neurons, and b_j is the bias associated with node j.

Sigmoid transfer function was used for the hidden layer and linear transfer function was used for the output layer to avoid error between observed and predicted values. During this process, Marquardt-Levenberg algorithm was used for training the network. Initially weight and bias values were taken randomly. However, in subsequent training steps, the weights and biases, in hidden and output layers, were adjusted in accordance with a convergence criterion to get the similarity in training and testing experimental values.

In order to evaluate the ANN output error, the coefficient of determination (R^2) was used, which describes the extent of variance in the modelled variables. The error was calculated based on difference between the experimental and predicted values. A popular measure such as mean squared error (MSE) or root mean squared error (RMSE), mean absolute error (MAE), and mean absolute percentage error (MAPE) was used to evaluate the ANN simulated data:

$$\begin{aligned}
\text{MSE} &= \frac{1}{n}\sum_{i=1}^{n}\left(y_p - y_e\right)^2, \\
\text{RMSE} &= \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(y_p - y_e\right)^2}, \\
\text{MAE} &= \frac{1}{n}\sum_{i=1}^{n}\left|y_p - y_e\right|, \\
\text{MAPE} &= \frac{1}{n}\sum_{i=1}^{n}\frac{\left|y_p - y_e\right|}{y_e},
\end{aligned} \tag{2}$$

where n is number of experiments, y_p is ANN predicted value, and y_e is experimental value.

2.4.3. GA Optimization. Genetic algorithm was used to search in different subspace and to locate the global maximum on the objective function surface. Optimization was performed with FFNN output values of weights and bias using fitness function:

$$\begin{aligned}
Y_{\text{Output}} &= \text{Weight}^O \\
&\times \left(\frac{2}{1 + e^{(-2\times \text{Weight}^H \times \text{Input vector} + \text{Input bias}(b^I))}} - 1\right) \\
&+ \text{Hidden layer bias}\left(b^H\right).
\end{aligned} \tag{3}$$

Weight^H is weight on connections between input and hidden nodes. Weight^O is weight on connections between hidden and output nodes.

In this study, different parameters of GA optimization such as chromosome length (L_{chr}) as 36, population size (N_{pop}) as 36, crossover probability (C_p) as 0.8, and mutation

Table 2: Experimental design and alpha-galactosidase activity (experimental and predicted) and error values.

Serial number	Temperature (°C)	pH	Agitation (rpm)	Tryptone (g/100 mL)	Raffinose (g/100 mL)	K_2HPO_4 (g/100 mL)	Alpha-galactosidase activity		
							Observed	Predicted	Error
1	34	6.5	160	0.5	2	0.75	2.80	2.5	0.28
2	34	6.5	160	0.5	3	1.25	4.70	4.78	−0.08
3	34	6.5	160	1.5	2	1.25	5.10	5.00	0.09
*4	34	6.5	160	1.5	3	0.75	5.40	5.26	0.13
5	34	6.5	180	0.5	2	1.25	5.70	5.79	−0.09
6	34	6.5	180	0.5	3	0.75	5.80	5.75	0.04
7	34	6.5	180	1.5	2	0.75	5.90	6.12	−0.22
8	34	6.5	180	1.5	3	1.25	6.60	6.69	−0.09
9	34	7.5	160	0.5	2	1.25	4.80	4.78	0.01
10	34	7.5	160	0.5	3	0.75	4.60	4.54	0.05
11	34	7.5	160	1.5	2	0.75	5.20	4.96	0.23
12	34	7.5	160	1.5	3	1.25	5.80	5.88	−0.08
*13	34	7.5	180	0.5	2	0.75	3.60	3.45	0.14
14	34	7.5	180	0.5	3	1.25	5.70	5.92	−0.22
15	34	7.5	180	1.5	2	1.25	6.50	6.54	−0.04
*16	34	7.5	180	1.5	3	0.75	5.30	5.30	0
17	38	6.5	160	0.5	2	1.25	5.30	5.26	0.03
18	38	6.5	160	0.5	3	0.75	6.50	6.42	0.07
19	38	6.5	160	1.5	2	0.75	5.80	5.54	0.25
20	38	6.5	160	1.5	3	1.25	4.70	4.81	−0.11
21	38	6.5	180	0.5	2	0.75	5.40	5.28	0.11
22	38	6.5	180	0.5	3	1.25	5.90	6.10	−0.20
*23	38	6.5	180	1.5	2	1.25	5.40	5.42	−0.02
24	38	6.5	180	1.5	3	0.75	5.60	5.58	0.01
25	38	7.5	160	0.5	2	0.75	5.20	5.07	0.12
26	38	7.5	160	0.5	3	1.25	6.50	6.24	0.25
27	38	7.5	160	1.5	2	1.25	5.60	5.61	−0.01
28	38	7.5	160	1.5	3	0.75	5.70	5.57	0.12
*29	38	7.5	180	0.5	2	1.25	4.80	4.90	−0.10
30	38	7.5	180	0.5	3	0.75	4.50	4.56	−0.06
31	38	7.5	180	1.5	2	0.75	4.20	4.08	0.11
*32	38	7.5	180	1.5	3	1.25	3.30	3.55	−0.25
33	32	7	170	1	2.5	1	5.10	5.11	−0.01
34	40	7	170	1	2.5	1	5.10	5.20	−0.10
35	36	6	170	1	2.5	1	5.70	5.74	−0.04
*36	36	8	170	1	2.5	1	5.00	5.07	−0.07
37	36	7	150	1	2.5	1	5.60	6.23	−0.63
38	36	7	190	1	2.5	1	7.10	6.58	0.51
39	36	7	170	0	2.5	1	5.80	5.92	−0.12
40	36	7	170	2	2.5	1	6.50	6.49	0
*41	36	7	170	1	1.5	1	5.50	5.89	−0.39
42	36	7	170	1	3.5	1	7.00	6.72	0.27
43	36	7	170	1	2.5	0.5	5.00	5.65	−0.65
44	36	7	170	1	2.5	1.5	7.10	6.56	0.53
45	36	7	170	1	2.5	1	7.50	7.32	0.17
*46	36	7	170	1	2.5	1	7.20	7.32	−0.12
47	36	7	170	1	2.5	1	7.40	7.32	0.07
48	36	7	170	1	2.5	1	7.30	7.32	−0.02
*49	36	7	170	1	2.5	1	7.40	7.32	0.07
50	36	7	170	1	2.5	1	7.40	7.32	0.07

*Data used for testing.

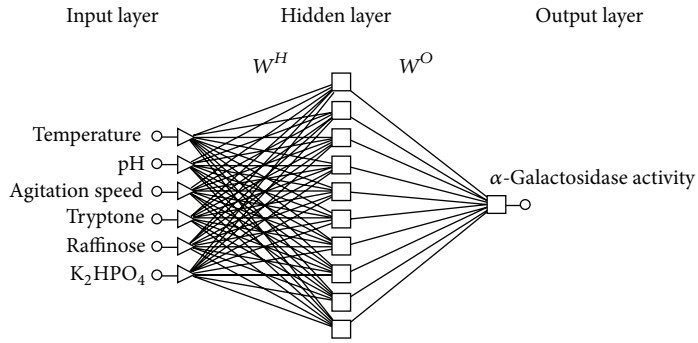

FIGURE 2: Feed forward neural network design used for optimization of alpha-galactosidase production. W^H is weight of connection between input and hidden layer and W^O is weight of connection between hidden and output layer.

probability (P_{mut}) as 0.01 were taken. Optimum conditions were selected after evaluation of GA for 500 generations ($Ng_{max} = 500$) to achieve fine-tuned fermentation conditions in the given range of input parameters. Neural networks and genetic algorithm toolboxes of MATLAB 7.0 (The Mathworks, USA) were used in modeling studies.

3. Results and Discussion

In the present study, both physical and nutritional factors were chosen to optimize the enzyme production in shake flask. Table 2 depicts the experimental design along with experimental and predicted values of alpha-galactosidase production from *Acinetobacter* sp. From Table 2, it was observed that the enzyme production varied from the 3.3 to 7.5 U/mL under the various selected conditions. The observed minimum and maximum enzyme production indicate that the selected parameters have a greater influence on the alpha-galactosidase production. The data was further modelled with ANN and the conditions were optimized using the GA. The network was constructed by using the selected parameters and alpha-galactosidase production as input and output neurons. The selected six variables such as incubation temperature, pH, agitation speed, raffinose, tryptone, and K_2HPO_4 concentrations were chosen as input neurons in the input layer. Similarly the alpha-galactosidase production was set as output neuron in the output layer. The number of neurons in the hidden layer plays a vital role in the training time and generalization property of neural networks. Lesser number of neurons in the hidden layer would increase the training time whereas higher number of neurons in the hidden layer would cause overtraining and saturation of the network, which leads to false results. The number of neurons in a hidden layer depends on the complexity of the system being modelled. According to Sathish and Prakasham [22] the best approach to finding the optimal number of neurons in hidden layer is by trial and error method. In this study, the number of neurons in the hidden layer was varied from 3 to 18 and the optimal number chosen by the crossvalidation criterion with the number of epochs fixed at 1000 for all the structures studied. The neural network with 10 hidden neurons was found to have highest correlation and lowest MAPE and RMSE values. Figure 2 depicts the constructed

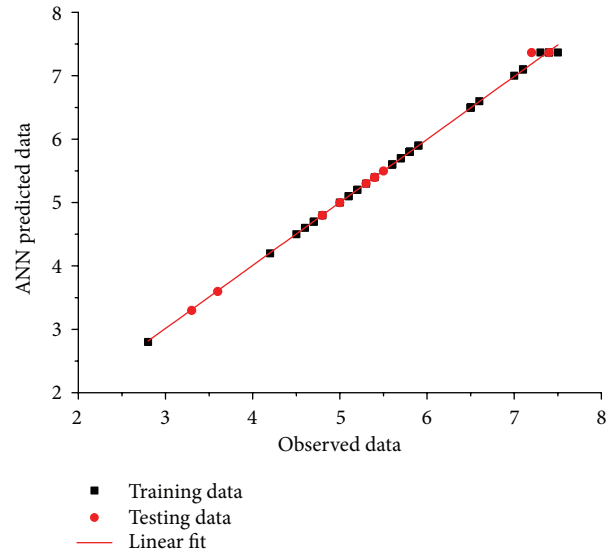

FIGURE 3: Correlation chart for experimental and predicted values of alpha-galactosidase activity.

neural network topology "6-10-1" neurons in input, hidden, and output layers.

The accuracy of the neural network based prediction can be calculated using the R^2 value based on the measured and predicted outputs in the training and test data. The calculated R^2 value was found to be 0.9994 indicating the model accuracy of the constructed ANN (Figure 3). Figure 2 depicts good correlation between the experimental values and ANN predicted values, suggesting the accuracy of the ANN predictability of the nonlinear systems.

Further, the predictability of the neural networks was analyzed based on the MSE, RMSE, MAE, and MAPE of the training and testing data. The overall MSE (6.1×10^{-4}), RMSE (2.47×10^{-2}), MAE (3.4×10^{-3}), and MAPE (4.4×10^{-4}) of the training data suggested that the constructed network is suitable for the alpha-galactosidase production. This was further confirmed by testing data. The resultant data indicates a value of 2.8×10^{-4}, 1.673×10^{-2}, 1.4×10^{-3}, and 1.8×10^{-3} for MSE, RMSE, MAE, and MAPE, respectively.

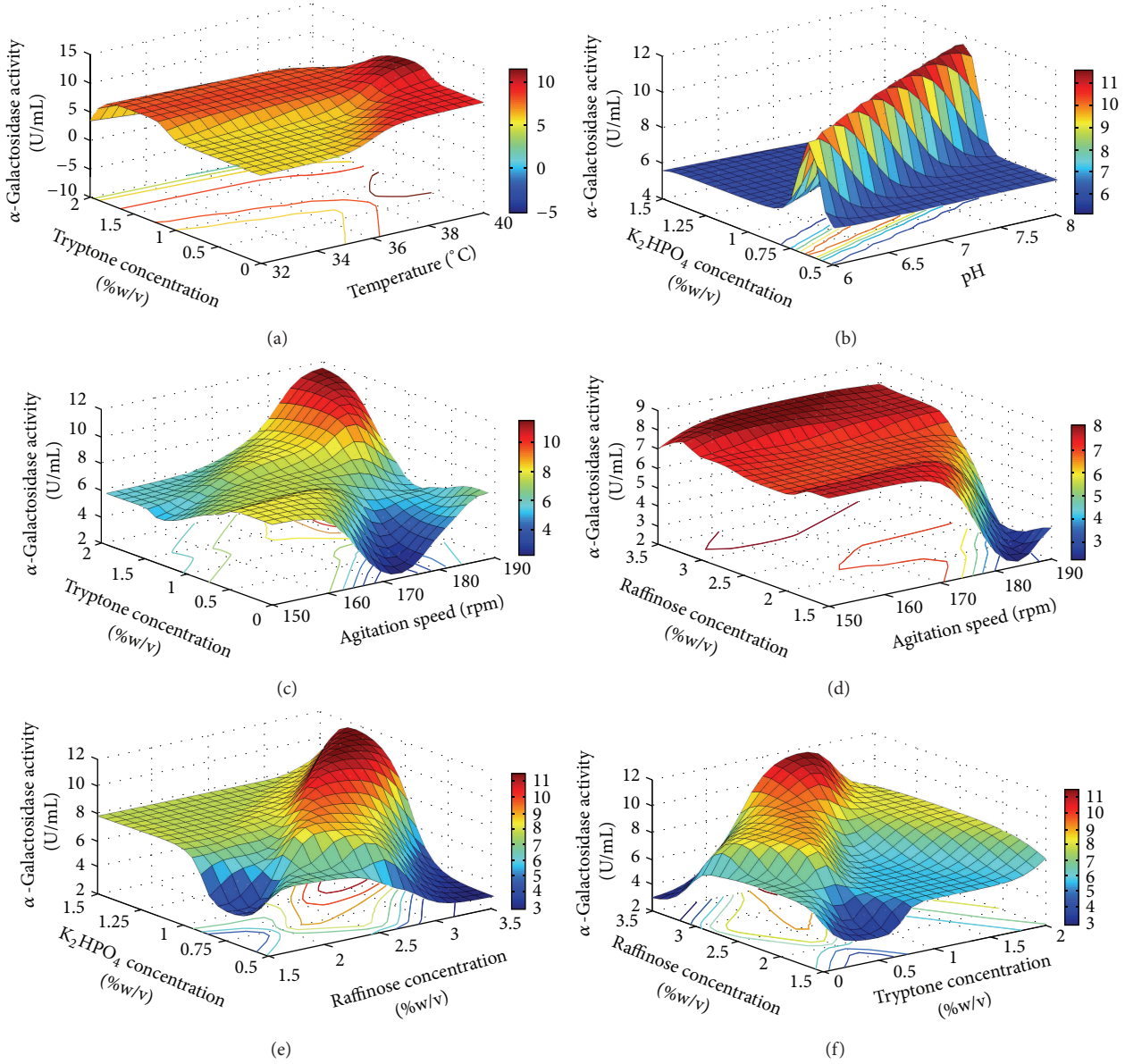

FIGURE 4: Effect of selected parameters interactions on alpha-galactosidase activity. (a) Tryptone versus temperature; (b) K_2HPO_4 versus pH; (c) tryptone versus agitation; (d) raffinose versus agitation; (e) K_2HPO_4 versus raffinose; (f) raffinose versus tryptone.

3.1. Interaction Influence of Selected Variables on the Alpha-Galactosidase Production. Analysis of interactions between different selective process parameters provides information on the concentration mediated regulatory role of alpha-galactosidase production. Figure 4 shows the interactive influence of selected variables on alpha-galactosidase production. Figure 4(a) depicts the influence of temperature with tryptone concentration indicating that alpha-galactosidase production increases with temperature up to 37°C. Similarly, studies on enzyme production at different pH values indicated that the production is better at neutral pH or pH slightly above neutral pH (Figure 4(b)). Similar findings have been reported in the literature (temperature 34 to 38°C and pH 6.8 to 7.5) for mesophilic bacteria. Mixing of the components in the media has a significant role in the microbial enzyme synthesis and secretion into external environment [22]. Figures 4(c) and 4(d) show the interaction influence of the agitation speed with tryptone and K_2HPO_4 concentration. From these graphs it can be concluded that, to achieve higher yields of the galactosidase, higher concentration of nutrients and higher agitation speed are needed. Figures 4(a), 4(c), and 4(f) depict the interaction influence of the tryptone with other process parameters as well as other nutrients. From these surface plots, it was observed that tryptone at 1–1.5% is favourable for the alpha-galactosidase production. The interaction influence of the carbon source (raffinose) and nitrogen source (tryptone) is depicted in Figure 4(f). It was observed that tryptone at 1% is suitable for the enzyme production. Figures 4(b) and 4(e) show the interaction influence of K_2HPO_4 with pH and raffinose.

Table 3: Best possible fermentation conditions and predicted and observed yields of enzyme alpha-galactosidase.

Serial number	Temperature (°C)	pH	Agitation (rpm)	Tryptone (g/100 mL)	Raffinose (g/100 mL)	K_2HPO_4 (g/100 mL)	Alpha-galactosidase activity	
							Predicted	Observed
1	35.1	6.8	180	1.2	2.5	1.1	9.5	9.6
2	36	7	180	1.4	2.8	1.3	10	9.9
3	37	7.2	183	1.1	2.4	1.7	10.5	10.2
4	36.5	7	175	1.4	2.5	1.3	9.4	9.8

Table 4: List of statistical methods used to enhance alpha-galactosidase production in various microorganisms.

Serial number	Organism name	I/E	Type of fermentation	Design	Design variables	Activity U/mL	Reference
1	*Streptomyces griseoloalbus*	E	Submerged	RSM (Box-Behnken Design)	pH, temperature, inoculum size, inoculum age, incubation period, agitation speed, carbon source, yeast extract, $MgSO_4 \cdot 7H_2O$, $FeSO_4$, and salinity	50 U/mL	[23]
2	*Streptomyces griseoloalbus*	E	Solid-state	RSM	Inoculum size, moisture, and galactose	117 U/g of Fermented dry substrate of soyabean flour	[25]
3	*Aspergillus foetidus* ZU-G1	E	Solid-state	RSM	Wheat bran, soybean meal, KH_2PO_4, $MnSO_4 \cdot H_2O$, and $CuSO_4 \cdot 5H_2O$	2207.19 U g(−1) dry matter	[26]
4	*Aspergillus foetidus* ZU-G1	E	Submerged	RSM	Soybean meal, wheat bran, KH_2PO_4, $FeSO_4 \cdot 7H_2O$, and the medium initial pH	64.75 U/mL	[27]

3.2. GA Optimization and Validation Studies. The ANN output data was further optimized using GA. In order to obtain the best suitable conditions for alpha-galactosidase production, an objective function with weights and bias was used. Among 500 conditions generated by GA, four best suitable conditions were chosen and the validation experiments performed under these conditions (Table 3). From Table 3, it could be seen that the maximum intracellular alpha-galactosidase production was 10.2 U/mL which is 36% more than the maximum enzyme production in Table 2.

Alpha-galactosidase production titres vary for different microorganisms and are also influenced by microbial strain, enzyme localization, and physical and nutritional factors of fermentation medium. In the present study, the enzyme yield was increased from 7.5 (Table 2) to 10.2 U/mL (Table 3). Similar increase in enzyme production was reported in the case of *Streptomyces griseoloalbus* when optimized using RSM [23]. List of statistical methods and activity yield using various microorganisms is presented in Table 4. Similar trend was reported by several researchers working with alkaline protease [21], L-glutaminase production [22], and rifamycin production [24].

4. Conclusion

Alpha-galactosidase production by *Acinetobacter* sp. was optimized by using feed forward ANN-GA approach, selecting six different medium parameters. A cogent correlation of 0.9994 was obtained for observed and predicted values. Interactions of raffinose and temperature with other variables are considered to be significant for maximum enzyme production. The hybrid FFNN-GA approach showed excellent predictable accuracy and can also be used for other bioprocess methods.

Conflict of Interests

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

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Research Article

Angiotensin I Converting Enzyme Inhibitory Peptides Obtained after *In Vitro* Hydrolysis of Pea (*Pisum sativum* var. Bajka) Globulins

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Pea seeds represent a valuable source of active compounds that may positively influence health. In this study, the pea globulins were digested *in vitro* under gastrointestinal condition and potentially bioaccessible angiotensin I converting enzyme (ACE) inhibitory peptides were identified. The degree of hydrolysis after pepsin, 14.42%, and pancreatin, 30.65%, were noted. The peptides with the highest ACE inhibitory properties were separated using ion exchange chromatography on DEAE-cellulose. Thirteen peptides fractions were obtained but only four showed potential antihypertensive properties. The highest inhibitory activity was determined for the fraction F8 (IC_{50} = 0.0014 mg/mL). This fraction was separated on Sephadex G10 and two peptide fractions were obtained. The peptides fraction (B) with the highest ACE inhibitory activity (IC_{50} = 0.073 mg/mL) was identified by ESI-MS/MS. The sequences of ACE inhibitory peptides were GGSGNY, DLKLP, GSSDNR, MRDLK, and HNTPSR. Based on Lineweaver-Burk plots for the fraction B, the kinetic parameters as K_m, V_{max}, and K_i and mode of inhibition were determined. This fraction belongs to uncompetitive inhibitor of ACE activity. The seeds of pea are the source of precursor protein, which releases the ACE inhibitory peptides as a result of enzymatic hydrolysis.

1. Introduction

In recent years, food has become the subject of research not only as a source of energy and basic nutrients, which are essential for proper development and functioning of the body, but also as a source of bioactive compounds that may have the positive influence on health condition [1–3]. The proteins are very important component of diet providing necessary amino acids for the proper functioning of the body and may be precursors of active peptides, which as a result of food processing or release during gastrointestinal digestion have physiological activity [4].

One of the most important parts of human homeostasis is the renin-angiotensin-aldosterone system (RAS) whose influence on the regulation and functioning of the circulatory system and water-electrolyte has been known for many years. This system is associated with the maintenance of normal blood volume, blood pressure stability, and proper amount of sodium ions [5]. Disorder in its functioning is the basis for the pathogenesis of diseases such as heart failure, coronary artery disease, hypertension, diabetes complications, or kidney disease [6, 7]. Angiotensin converting enzyme (ACE, EC 3.4.15.1), zinc metalloprotease, plays the most important role in this system and converts the inactive angiotensin I (NRVY-IHPFHL) into the potent vasoconstricting angiotensin II (NRVYIHPF) and the vasodilator bradykinin into an inactive peptide leading to an increase in blood pressure [8, 9]. The beneficial effect of inhibitors of ACE is the reduction of angiotensin II in plasma, as well as increased levels of bradykinin. This results in preventing the contraction of the heart and blood vessels, positive effect on endothelial function, and an anti-arteriosclerosis and regulation of blood clotting mechanism [10]. Nowadays, many synthetic drugs (such as captopril, quinapril, enalapril, or ramipril) whose action is based on the inhibition of ACE activity which affects the proper functioning of the body and protects the heart muscle are used. Unfortunately, they may cause serious side effects and therefore the search of alternative

inhibitory of this enzyme has increased. One of them can be peptides-natural compounds in the free state as secondary metabolites or released during the enzymatic hydrolysis of plant or animal proteins [11]. Many ACE inhibitory peptides have been purified and identified from plant foods protein such as protein from some legume species, cowpea, soybean, chickpea, and pea [12–14], or other plants and products, fermented pea [15], cottonseed [16], or garlic [17]. Legume seeds are becoming increasingly frequent part of the diet due to not only the taste qualities but also nutrition.

The aim of this study was the identification of potentially bioaccessible ACE inhibitory peptides obtained by digestion under simulated gastrointestinal conditions of pea globulins. Pea seeds are a valuable source of protein, minerals, carbohydrates, vitamins, or active compounds: peptides or antioxidants. To data, no study has conducted the investigation of primary structure of angiotensin converting enzyme inhibitors from pea globulins after *in vitro* digestion.

2. Materials and Methods

2.1. Materials. Pea seeds (*Pisum sativum* var. Bajka) were obtained from Company of Horticulture Seeds and Nursery in Ożarów Mazowiecki, Poland. HHL (hippuryl-L-histidyl-L-leucine), pepstatin A, PMSF (phenylmethanesulfonyl fluoride), α-amylase from hog pancreas (50 U/mg, 10080, Sigma), pepsin from porcine gastric mucosa (250 U/mg, P7000, Sigma), pancreatin from porcine pancreas (P7545, Sigma), enalapril, bile extract, and TNBS (2,4,6-trinitrobenzenesulfonic acid) were purchased from Sigma-Aldrich Company, USA, and Amino Acid Standard from Pierce, USA. Any other chemicals were of analytical grade.

2.2. Preparation of ACE from Pig Lung. Angiotensin converting enzyme was prepared according to the procedure of Hayakari et al. [18] with slight modifications. Pig lungs were purchased in a local market and used as a starting material. Lung tissues (100 g) were homogenized in 0.1 M borate buffer pH 8.3 containing pepstatin A (0.1 mM) and PMSF (0.1 mM) at 4°C in ratio 1 : 2 (w/v). The homogenate was centrifuged at 8000 ×g, 4°C, for 20 min. The purification of ACE was initiated by the addition of solid ammonium sulphate at 80% saturation and next dialyzed (molecular weight cut-off 12 kDa) for 24 h at 4°C against 20 volume of 0.1 M borate buffer pH 8.3. The dialysate sample was centrifuged at 8000 ×g, 4°C, for 20 min. The ACE activity of the dialysate was assayed with the method below, frozen, and used for further analysis.

2.3. Isolation of Globulins. Globulins were isolated from pea flour according to Gupta and Dhillon [19] with modification: endoproteases and their inhibitors were inactivated by heating in laboratory oven at 100°C for 10 min to inactivate endoproteases and their inhibitors. Flour was dispersed in 0.2% NaOH in ratio 1 : 10 (w/v) and protein extraction was carried out under stirring for 1 h at room temperature. The solution was centrifuged for 20 min at 8000 ×g, 4°C. After that, pH of supernatant was adjusted to the isoelectric point of pea globulins (pH 4.3) with 0.1 M HCl. Precipitated globulins were centrifuged for 20 min, 4°C, 8000 ×g and were washed with distilled water. Globulins were stored at −18°C until further use.

2.4. In Vitro Digestion and Absorption. *In vitro* digestion of pea globulins was carried out according to the method described by Gawlik-Dziki [20] with slight modification. Briefly, the lentil globulins (4%, w/v) were resuspended in stimulate saliva solution with final concentration 7 mM $NaHCO_3$ and 0.35 mM NaCl, pH 6.75, and stirred for 5 min at 37°C in darkness. After that, α-amylase (50 U/mg) was added (the ratio of enzyme to substrate was 1 : 10; w/w) and the mixture was stirred for 10 min at 37°C in darkness. For the gastric digestion, solution was adjusted to pH 2.5 with 1 M HCl and pepsin (250 U/mg) was added (the ratio of enzyme to substrate was 1 : 100; w/w). Reaction was carried out for 2 h at 37°C. Solution was neutralized to pH 7.0 with 1 M NaOH and successively a mixture containing 0.7% solution of pancreatin and 2.5% solution of bile extract (1 : 2.5, v/v) was added (simulated intestinal digestion). The incubation was carried out for 1 h at 37°C in darkness and reaction was stopped by heating at 100°C for 5 min.

Hydrolysates were dialyzed with the dialysis sacks (D9777-100FT, molecular weight cut-off 12000, Sigma-Aldrich) against phosphate buffered saline (PBS) at the physiological concentration 1 : 4, v/v (simulated absorption process). The process was carried out in darkness for 1 h at 37°C. After this stage, the samples were concentrated tenfold using a vacuum evaporator; the parameters were set as follows: 40°C, pressure 0.8 mPa.

2.5. Determination of Free Amino Groups Content. Free amino groups content was determined by the trinitrobenzenesulfonic acid (TNBS) method using L-leucine as the standard [21]. All assays were performed in triplicate.

2.6. Determination of Degree of Hydrolysis (DH). The degree of hydrolysis (DH) after *in vitro* digestion was estimated by determination of free amino groups by reaction with TNBS according to the method by Adler-Nissen [21]. The total number of all amino groups was determined in a sample by complete hydrolysis with 6 M HCl at 120°C for 24 h. All assays were performed in triplicate and the degree of hydrolysis was calculated from the formula

$$\mathrm{DH} = \frac{h}{h_{\mathrm{tot}}} \times 100\%, \quad (1)$$

where DH is degree of hydrolysis, h are peptide bounds in the sample, h_{tot} are the total peptide bounds.

2.7. Assay for ACE Inhibitory Activity. ACE inhibitory activity was measured with 5 mM HHL as a substrate by spectrophotometric method according to Jakubczyk et al. [15]. Briefly, 50 μL of peptide sample was added to 50 μL of 5 mM HHL solution and 50 μL of 3 mU/mL ACE (one unit of ACE activity was defined as an increase absorbance of 0.001 per minute at 390 nm). The reaction mixture was incubated at

37°C for 60 min. The reaction was terminated by adding 0.7 mL of the 0.1 M borate buffer with 0.2 M NaOH. The absorbance was measured at 390 nm and the ACE inhibition was determined as follows:

$$\text{ACE inhibition } (\%) = \left[1 - \left(\frac{A1 - A2}{A3}\right)\right] * 100\%, \quad (2)$$

where $A1$ is the absorbance of sample with ACE and peptide inhibitor, $A2$ is the absorbance of sample with peptide inhibitor, without ACE, and $A3$ is absorbance of sample with ACE and without peptide inhibitor but with 0.1 M borate buffer pH 8.3.

The IC_{50} (inhibitory concentration) was determined by assessing the ACE inhibition of several dilutions of each hydrolysate sample and interpolating the peptides concentration at which the inhibition percentage reached 50%. The IC_{50} value was calculated from the graph plotting inhibition for the five different peptide concentrations. Enalapril, applied in hypertension and chronic failure treatment as inhibitory of ACE, was used as the positive control.

2.8. Purification of ACE Inhibitory Peptide

2.8.1. Ion Exchange Chromatography. The sample obtained after absorption process was separated using ion exchange chromatography on DEAE-cellulose (the column 1.5 × 14 cm). The concentration of peptides for ion exchange chromatography was calculated by soluble peptide content by the trinitrobenzenesulfonic acid (TNBS) method using L-leucine as the standard [21]. 2 mL of solution (3.19 mg/mL) was loaded on the column previously equilibrated with 0.1 M borate buffer pH 8.3 and was eluted with linear gradient of NaCl in 0.1 M borate buffer pH = 8.3 (0–0.8 M NaCl) at a flow rate of 0.8 mL/min. Two milliliters fractions were collected and the absorbance was monitored at 220 nm. Fractions with the highest absorbance were combined and concentrated on the vacuum evaporator (40°C, 0.8 mPa) and ACE inhibitory activity was determined. Fraction with the highest ACE inhibitory activity was taken to the next step of purification.

2.8.2. Gel Filtration Chromatography. Peptide fraction with the highest activity of ACEinhibiting properties was subsequently separated on Sephadex G10. 2 mL of fraction was loaded on the column (1 × 120 cm) previously washed with distilled water that was used also as an eluent, with flow rate of 0.8 mL/min. One milliliter of each fraction was collected and the absorbance was monitored at 220 nm. Fractions with the highest absorbance were combined and ACE inhibitory activity was determined. Fractions with the highest ACE inhibitory activity were lyophilized and used in further analysis.

2.9. Kinetics of ACE Inhibition. Hippuryl-His-Leu solutions (1.0, 2.0, 3.0, 4.0, and 10.0 mM) were prepared and used to determine the Michaelis constant (K_m), the inhibition constant (K_i), and the maximum velocity (V_{max}) of ACE. The kinetic parameters were evaluated by Lineweaver-Burk's method. The reaction conditions were the same as ACE inhibitory activity assay. Briefly, 50 μL of peptide sample (or 0.1 M borate buffer pH 8.3 for enzyme activity) was added to 50 μL HHL solution (1.0, 2.0, 3.0, 4.0, or 10.0 mM) and 50 μL of 3 mU/mL ACE (one unit of ACE activity was defined as an increase absorbance of 0.001 per minute at 390 nm). The reaction mixture was incubated at 37°C for 60 min. The reaction was terminated by adding 0.7 mL of the 0.1 M borate buffer with 0.2 M NaOH. The absorbance was measured at 390 nm.

2.10. Amino Acid Composition. The samples (50 μg) were hydrolyzed in gas phase using 6 M HCl at 115°C for 24 h. The liberated amino acids were converted into phenylthiocarbamyl (PTC) derivatives and analyzed by high-pressure liquid chromatography (HPLC) on a PicoTag (3.9 × 150 mm) column (Waters, Milford, MA, USA) according to method of Boogers et al. [22].

2.11. Mass Spectrometry Analysis (ESI/MS-MS). The molecular mass andpeptide sequencing were estimated by positive ion mode using electrospray ionisation-mass spectrometry. Lyophilized samples (0.1 mg) were dissolved in 0.3 mL of 50% acetonitrile containing 0.1% formic acid (v/v) and analysed using Applied Biosystems mass spectrometer, type Star XL MS-MS with nanoelectrospray ionization (nano-ESI). The analysis was carried out in duplicate in the positive ion mode under the following conditions: the potential of the cone (DP): 60 V, the voltage on the needle during sputtering (IS): 900–1500 V, and scan mode: TOF-MS (product ion). Ions were monitored in the field: 100–3500 m/z (TOF-MS), 50–2500 m/z (product ion), and collision energy (CE): 51.2 V. Acquired raw data were processed by Mascot Distiller followed by Mascot Search (Matrix Science, London, UK, onsite license) against NCBInr database (20120224). Search parameters for precursor and product ions mass tolerance were 40 ppm and 0.6 Da, respectively, enzyme specificity was semitrypsin, missed cleavage sites allowed were 0, and fixed modification of cysteine was by carbamidomethylation and variable modification of lysine carboxymethylation and methionine oxidation. Peptides with Mascot Score exceeding the threshold value corresponding to <5% false positive rate, calculated by Mascot procedure, and with the Mascot score above 30 were considered to be positively identified.

2.12. Statistical Analysis. Each treatment was conducted in triplicate and the results were presented as mean ± standard deviation. STATISTICA 7.0 was used for statistical analysis. Tukey's test was used to estimate significant differences among the mean values at the 5% probability level ($\alpha < 0.05$).

3. Results and Discussion

Most studies of bioavailability of peptides released during protein hydrolysis process relate to changes caused by a single proteolytic enzyme or subsequent hydrolysis resulted in proteases of the digestive system, bypassing the step α-amylase hydrolysis. The α-amylase is not a proteolytic enzyme, but, in order to reflect the processes occurring in

the gastrointestinal tract more accurately, it was also used in this study. The globulins hydrolysates obtained from pea were digested under simulated gastrointestinal digestion condition (α-amylase, pepsin, and pancreatin). The degree of hydrolysis was noted as follows: after pepsin, 14.42 ± 0.14%, and after pancreatin, 30.65 ± 0.33%. Similar results were obtained by Lo et al. [23] for soybean protein isolate; the value of DH after pepsin digestion was 11.0%. According to Barbana and Boye [12], DHs of proteins two varieties of chickpea (Xena kabuli and Myles desi) and the yellow pea var. Golden after simulating human gastrointestinal digestion were noted to be 34.41% ± 0.15, 40.78% ± 0.03, and 31.08% ± 0.05, respectively. The differences in results may be due to the fact that the preparation of samples, the method used for isolation of peptides, different protein fraction source, or varieties of pea could have an influence on the DHs value. It should be noted that the research about pea proteins hydrolysates obtained under gastrointestinal condition did not include stimulated saliva solution.

Potentially bioactive fragments of proteins with structural motifs corresponding to the bioactive peptides are inactive precursor protein in the sequence and only released by the action of proteolytic enzymes which may exhibit diverse biological activity [24, 25]. In this study, nonhydrolyzed protein showed no ACE inhibitory activity. Moreover, after *in vitro* digestion and absorption, the IC_{50} ACE peptides inhibitory value was noted to be 0.72 mg/mL, as enalapril with IC_{50} value of 7.46 mg/mL was used as a control. This result corresponds well with results obtained by Barbana and Boye [12], where IC_{50} values were determined for two varieties of chickpea and yellow pea for nonhydrolyzed proteins and after gastrointestinal digest. The results showed that only hydrolysates have ACE inhibitory activity and IC_{50} were noted for chickpeas to be 0.229 mg/mL and 0.14 mg/mL and for yellow pea 0.159 mg/mL [12]. Akillioğlu and Karakaya [13] reported that common bean and green lentils proteins hydrolyzed under 50 minutes of intestine digestion had ACE inhibitory activity with IC_{50} = 0.77 mg/mL and 0.26 mg/mL, respectively. As it was previously indicated, the legumes food proteins are a good source of ACE inhibitory peptides released after digestion and they must enter the circulatory system to have benefit to health.

For the purification of ACE inhibitory peptides, potentially bioavailable ion exchange chromatography with DEAE-cellulose and Sephadex G10 was used. As shown in Figure 1(a), the hydrolysates were fractionated into thirteen peptides fractions. For each fraction, peptides content and ACE inhibitory activity were determined (Table 1). Only five fractions showed ACE inhibitory activity. Although the highest peptides content (L-leucine as a standard) was noted in the first fraction (5.24 ± 0.42 mg/mL), the potentially highest ACE inhibitory peptide activity was determined for the eighth fraction (F8), where IC_{50} value was noted to be 0.0014 mg/mL. It should be noted that in the peptide content in most peptide fractions no significant difference was determined. F8 was not characterized by the highest peptides content; it suggests that the ACE inhibitory activity depends on the amino acids sequences. This fraction was separated using gel filtration on Sephadex G10 (Figure 1(b)).

Table 1: Peptides concentration and IC_{50} value of peptides inhibitory activity fractions obtained after separation on DEAE-cellulose.

Fraction number	Peptides content (mg/mL)	IC_{50} (mg peptide/mL)
F1	5.24 ± 0.42^{a}	0.573 ± 0.017^{A}
F2	0.14 ± 0.01^{b}	ND
F3	0.006 ± 0.0006^{b}	0.0045 ± 0.0009^{B}
F4	0.009 ± 0.0003^{b}	ND
F5	0.014 ± 0.0006^{b}	ND
F6	0.018 ± 0.0005^{b}	ND
F7	0.015 ± 0.0014^{b}	0.0026 ± 0.0004^{C}
F8	0.024 ± 0.008^{b}	0.0014 ± 0.0003^{D}
F9	0.022 ± 0.004^{b}	ND
F10	0.024 ± 0.005^{b}	ND
F11	0.027 ± 0.007^{b}	ND
F12	0.024 ± 0.0007^{b}	ND
F13	0.022 ± 0.0013^{b}	0.0018 ± 0.0003^{E}

ND: not noted.
All values are mean ± standard deviation for triplicate experiments.
Values with different letters superscripts are significantly different at $\alpha < 0.05$.

After this step, two peptides fractions were obtained but only the second fraction (B) exhibited potential hypertensive properties, IC_{50} = 0.073 mg/mL, and the fraction was used for further analysis. Higher IC_{50} value at this stage than after ion chromatography separation can be caused by synergistic action of peptides in the mixture.

An important step in the identification of peptides with high ACE inhibitory properties is determination of their amino acid composition. In this study, amino acid composition of peptide fractions with the highest ACE inhibition activity obtained after the separation on Sephadex G10 was determined by hydrolyzing them using 6 M HCl and then analyzed by HPLC (Table 2). Due to the fact that during hydrolysis process some amino acids as asparagine and glutamine are partially converted to aspartic acid and glutamic acid, respectively, the data for asparagine and/or aspartic acid were shown as Asx while those for glutamine and/or glutamic acid were reported as Glx. Hydrophilic amino acids were the main part in composition of fraction B. The highest content amino acids were Glx (20.78 ± 0.03%), alanine (16.69 ± 0.02%), glycine (11.14 ± 0.02%), serine (7.23 ± 0.01), Asx (10.41 ± 0.02), and arginine (7.47 ± 0.02). However, between Asx and argininine, no statistical significant difference was noted. On the other hand, threonine and phenylalanine were not detected. This corresponds well with results obtained by Wu and Ding [26]. Cited investigators also reported amino acid composition of potent ACE inhibitory peptides fraction obtained after separation of the hydrolysates on a cation exchange resin. The hydrophilic amino acids content was determined: arginine 19.67%, lysine 11.85%, and Glx 10.54% [26]. Important aspect in the explanation mechanism of ACE inhibition is to understand the construction of peptide inhibitors. The most common technique for the identification of active peptides is mass spectrometry. Figure 2

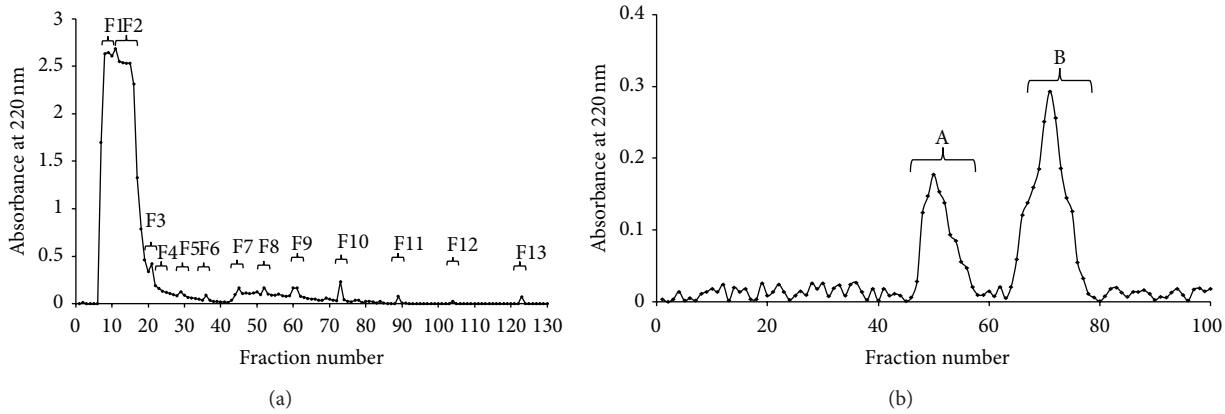

Figure 1: Purification of ACE inhibitory peptides from pea globulins hydrolysates separation on DEAE-cellulose (a) and F8 separation on Sephadex G10 (b).

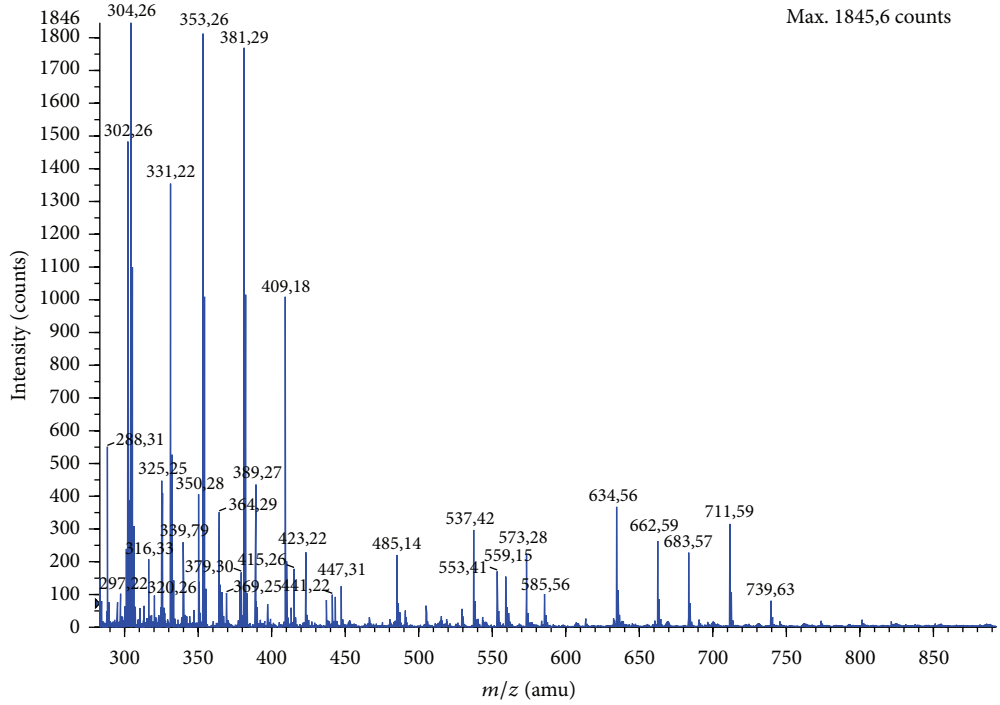

Figure 2: Nano-ESI-MS/MS spectrum of fraction B.

illustrates the mass spectrum of fraction B. The analysis of ion sequencing was made using the Mascot database. In this study, the sequence of potential ACE inhibitory peptides was determined as follows: GGSGNY (553.41 m/z), DLKLP (585.56 m/z), GSSDNR (634.56 m/z), MRDLK (662.59 m/z), and HNTPSR (711.59 m/z). Figure 3 illustrates representative fragmentation spectra. The relationship between structure and activity of peptide inhibitors of ACE indicates that binding of the enzyme molecule is dependent on the type of amino acid C-terminal tripeptide sequence of the peptide. The strongest inhibitors of ACE contain hydrophobic (aromatic or branched side chains containing) amino acid residues at the C-terminus; the most preferred is proline [27]. In this study, one peptide containing C-terminal proline was identified as DLKLP. Some studies indicate that the occurrence of certain amino acids at the C-terminus or in a peptide does not necessary mean that the relationship had a strong hypotensive effect. Guang and Phillips [28] isolated a peptide with ACE inhibitory activity from hazelnut with sequence KAFR. Another example of a peptide without proline at the C-terminus is pentapeptide with sequence LVQGS isolated from the extract of fermented soybean seeds [29]. Moreover, Li et al. isolated from alcalase hydrolysates of mung bean proteins ACE inhibitory peptides with sequence KDRL, VTPALR, and KLPAGTLF [30].

Lineweaver-Burk plots of the ACE inhibition pattern of the fraction B were shown in Figure 4. The data (Table 3) revealed that K_i of the peptide fraction resulted in reduction of the value of K_m and the maximum rate of enzymatic reaction (V_{max}); therefore, this fraction was uncompetitive

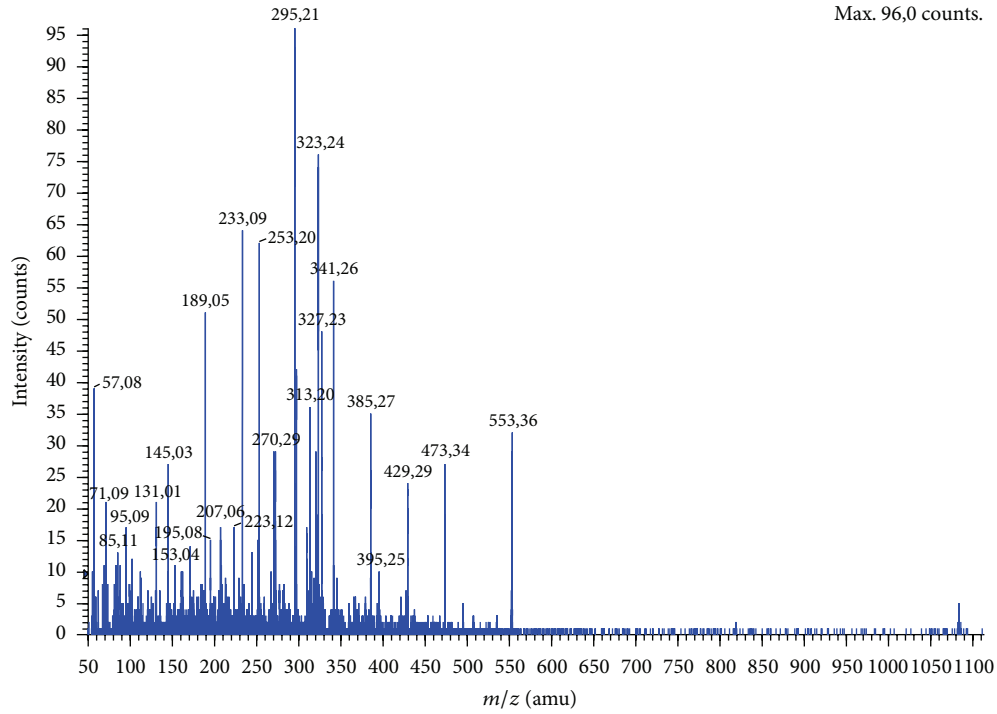

Figure 3: Representative fragmentation spectra of 553.41 *m/z*.

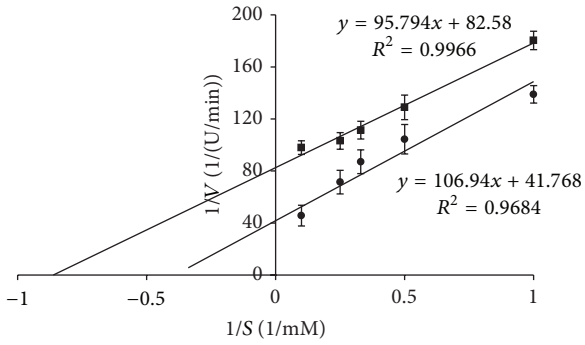

Figure 4: The Lineweaver-Burk plot for the inhibition of ACE (●—without inhibitor; ■—with fraction B of *in vitro* digested pea globulins).

Table 2: Amino acid composition (%) of fraction B.

Amino acid	% of amino acid composition
Asx*	10.41 ± 0.02^{e}
Glx*	20.78 ± 0.03^{g}
S	$7.23 \pm 0.01^{d,e}$
G	11.14 ± 0.02^{e}
H	$1.02 \pm 0.004^{a,b}$
R	$7.47 \pm 0.02^{d,e}$
T	ND
A	16.69 ± 0.02^{f}
P	$5.18 \pm 0.01^{c,d}$
Y	$2.08 \pm 0.002^{a,b,c}$
V	$4.25 \pm 0.006^{b,c,d}$
M	0.32 ± 0.002^{a}
I	$3.93 \pm 0.005^{a,b,c,d}$
L	$5.18 \pm 0.009^{c,d}$
F	ND
K	$4.32 \pm 0.008^{b,c,d}$

*Asx: D + N; Glx: E + Q.
ND: not detected values.
All values are mean ± standard deviation for triplicate experiments.
Values with different letters superscripts are significantly different at $\alpha < 0.05$.

inhibitor of ACE. This means that inhibitor binds only to the enzyme-substrate complex (E-S) and not to the free enzyme. Although ACE inhibitory peptides have been reported as competitive inhibitors [11, 25], most ACE inhibitory peptides belong to uncompetitive inhibitors. According to Pedroche et al. [31], two ACE inhibitory peptides fractions from chickpea hydrolysates were uncompetitive inhibitors. Furthermore, alcalase hydrolysates of soybean proteins were also a source of peptides that inhibit ACE activity as uncompetitive inhibitors [26, 32].

4. Conclusions

As a result of the conducted research, it can be stated that the seeds of pea are the source of precursor protein, which releases the ACE inhibitory peptides as a result of enzymatic hydrolysis. In this study, ACE inhibitory peptides were identified by ESI-MS/MS. The sequences of novel ACE inhibitory peptides were GGSGNY, DLKLP, GSSDNR, MRDLK, and HNTPSR. Based on Lineweaver-Burk plots for the fraction

Table 3: ACE inhibitory constants for fraction B.

	Control	Fraction B
IC_{50} (mg/mL)	—	0.073 ± 0.002
K_i (mg/mL)	—	0.039 ± 0.003
K_m (mM)	2.56 ± 0.32^{a}	1.16 ± 0.11^{b}
V_{max} (U/min)	0.024 ± 0.004^{A}	0.012 ± 0.002^{B}

All values are mean ± standard deviation for triplicate experiments.
Values with different letters superscripts are significantly different at $\alpha < 0.05$.

B, the kinetic parameters as K_m, V_{max}, and K_i and mode of inhibition were determined. This fraction belongs to uncompetitive inhibitor of ACE activity.

Conflict of Interests

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

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Research Article

Anti-Inflammatory Effects of *Siegesbeckia orientalis* Ethanol Extract in *In Vitro* and *In Vivo* Models

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This study aims to investigate the anti-inflammatory responses and mechanisms of *Siegesbeckia orientalis* ethanol extract (SOE). In cell culture experiments, RAW264.7 cells were pretreated with SOE and stimulated with lipopolysaccharide (LPS) for inflammatory mediators assay. In animal experiments, mice were tube-fed with SOE for 1 week, and s.c. injected with λ-carrageenan or i.p. injected with LPS to simulate inflammation. The degree of paw edema was assessed, and cytokine profile in sera and mouse survival were recorded. Data showed that SOE significantly reduced NO, IL-6, and TNF-α production in LPS-stimulated RAW264.7 cells. *In vivo* studies demonstrated that mice supplemented with 32 mg SOE/kg BW/day significantly lowered sera IL-6 level and resulted a higher survival rate compared to the control group ($P = 0.019$). Furthermore, SOE inhibited LPS-induced NF-κB activation by blocking the degradation of IκB-α. The SOE also reduced significantly the phosphorylation of ERK1/2, p38, and JNK in a dose-dependent manner. In summary, the *in vitro* and *in vivo* evidence indicate that SOE can attenuate acute inflammation by inhibiting inflammatory mediators via suppression of MAPKs- and NF-κB-dependent pathways.

1. Introduction

Growing evidence suggests that systemic inflammation is associated with increased risk of chronic diseases [1, 2]. The mechanisms of inflammation may involve activation of macrophages and T lymphocytes, as well as the release of proinflammatory mediators, including tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1, IL-6, nitric oxide (NO), and prostaglandin E_2 (PGE_2) that amplify the inflammatory activity [3]. Appropriate production of these mediators promotes effective innate immune response; however, excessive inflammation may cause such conditions as chronic inflammation, sepsis, and even death [4].

Notably, mitogen-activated protein kinases (MAPKs) play an important role in the regulation of proinflammatory mediators on cellular responses [5, 6]. Lipopolysaccharide (LPS) stimulation on macrophages can mimic inflammatory responses [7]. The MAPK cascade, the key downstream pathway for LPS-induced signaling events, leads to several functional responses. Activated MAPKs are responsible for phosphorylating and activating numerous transcription factors, including ERK1/2, p38, SAPK/JNK, and NF-κB, which then translocate into the nucleus of cells and induce the transcriptional activation of various inflammatory and immune genes [8]. Beside LPS, sulfated polysaccharide and carrageenan can also act as the proinflammatory agent. Recently, experimental evidence has shown that carrageenan-activated inflammatory cascades are related to generation of reactive oxygen species and may be integrated at the level of IκB kinase (IKK) signalosome, leading to degradation of

IκB-α and translocation of NF-κB to nuclei [9]. Accordingly, modulating effectively aberrant production of proinflammatory mediators can reduce inflammatory response. Thus, analyzing the expression of proinflammatory mediators may facilitate identification of anti-inflammatory substances.

The dietary application of natural products, including food materials and Chinese medicinal herbs, has been proposed that may prevent inflammatory diseases [10, 11]. Herba Siegesbeckiae, one of common Chinese medicinal foods, has been used to treat rheumatoid arthritis, malaria, and snakebite based on its ability to dispelling wind, eliminating dampness, and strengthening the sinews [12]. To date, three originals, *Siegesbeckia orientalis* L. *Siegesbeckia pubescens* Makino, and *Siegesbeckia glabrescens* Makino have been identified. Among them, *S. orientalis* has been reported to have antirheumatic [13], antiallergic [14], and immunosuppressive activities [15]. *S. orientalis*'s pure component, kirenol, was also found to have a topical application on the attenuation of skin inflammation in murine models [16]. However, *S. orientalis* via oral administration has not been reported regarding its *in vivo* anti-inflammatory activity and related mechanisms. Therefore, this study investigates the anti-inflammatory responses and their related mechanisms in inflammatory cells or mice pretreated with ethanol extract from *S. orientalis* (SOE).

2. Materials and Methods

2.1. Reagents and Plant Materials. The reagents lipopolysaccharide (LPS, *E. coli* serotype O55:B5), indomethacin (IND), and ammonium pyrrolidinedithiocarbamate (PDTC), obtained from Sigma-Aldrich (St. Louis, MO, USA), were all of analytical grade and dissolved in phosphate buffer saline as a stock. The *S. orientalis* L. samples were purchased from a local herbal store (Yuanshan Company, Kaohsiung, Taiwan). The sample's original was identified and its DNA polymorphism had been reported [17]. In this study, the samples were freeze-dried and then ground into powder. The dried powder (9.3 kg) was extracted with a 5-fold volume of 95% ethanol by stirring at room temperature for 1 day. This step was repeated three times. The extracted solutions were collected and filtered through filter paper (Whatman number 1; Whatman Paper Ltd., Maidstone, Kent, UK). The SOE was acquired by removing solvent via a rotary evaporator and dried in a freeze dryer. The dry weight of this extract was 489 g, and the yield was 5.3%. The SOE was dissolved in dimethyl sulfoxide (DMSO; the final DMSO concentration never exceeded 0.1% in medium) for cell culture test and was dissolved in sunflower oil for tube feeding in the mice experiment.

2.2. Cell Culture. The RAW264.7 cells (Bioresource Collection and Research Center; Hsinchu, Taiwan) were cultured in Dulbecco's modified Eagle's medium (DMEM-F12) supplemented with 10% inactivated fetal bovine serum, 0.01% glutamine, 1% penicillin/streptomycin, 0.02% sodium bicarbonate, and pH 7.2–7.4. The cells were cultivated in a humidified incubator at 37°C with 5% CO_2 and 95% air. In this experiment, the RAW264.7 cells were seeded on 6 cm dishes at a cell density of 7×10^5 cells/mL for Western blotting assay, or on 96-well plates at a cell density of 5×10^4 cells/well for culture medium test. The cells were then pretreated with various concentrations of SOE for 1 h before adding 1 μg/mL LPS for the indicated assay. In the Western blotting assay, the cell lysate was collected for detection of target proteins after 1 h (MAP kinase family) or 12 h (iNOS) stimulation. In the culture medium test, culture supernatants were harvested for analysis of the production of proinflammatory mediators after stimulation for 48 h. The cell viability was evaluated using the MTT method. The medium solution was removed after cultivation. An aliquot of 100 μL of DMEM medium containing 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was loaded to the plate. The cells were incubated for 3 h, and then the medium solution was removed. An aliquot of 100 μL of DMSO was added to the plate, which was shaken until the crystals dissolved. The cytotoxicity against cells was determined by measuring the absorbance of the converted dye at 570 nm in an ELISA reader (Model 550, Bio-Rad Laboratories, Hercules, CA, USA).

2.3. NO Determination. Griess reagent was freshly prepared from reagents A (1% sulfanilamide in 2.5% phosphoric acid) and B (0.1% N-1-naphthylethylenediamide dihydrochloride in water) at a ratio of 1 : 1. An equal volume of Griess reagent was added to supernatants from cells treated with test samples in a 96-well plate for 10 min. Absorbance was measured by an ELISA reader at 540 nm. The NO concentrations were determined using a $NaNO_2$ standard curve.

2.4. Cytokine Production Assay. Production of cytokines TNF-α and IL-6 in cell supernatants and mice serum was assayed using a commercial ELISA kit (eBioscience, Minneapolis, MN, USA). Briefly, primary anti-IL-6 or TNF-α antibodies were coated onto 96-well plates. After overnight incubation, plate wells were washed with washing buffer and blocked with blocking solution for 1 h. After washing, diluted supernatants or sera were added to wells for 2 h incubation. Next, wells were washed with washing buffer and biotin-conjugated anti-IL-6 or TNF-α antibody was added for 1 h. The wells were then washed and horseradish peroxidase-conjugated streptavidin was added for 30 min, washed, and incubated with tetramethylbenzidine (TMB; Clinical Science Products, Mansfield, MA, USA). Absorbance was measured by an ELISA reader at 620 nm. Data were calculated according to standard curves of cytokines.

2.5. Western Blot Analysis. The cells were trypsinized, washed twice with phosphate buffered saline (PBS), and lysed with lysis buffer (modified RIPA buffer) at 4°C. The pellet cellular debris was removed by centrifugation at 12500 rpm for 30 min and the supernatants were then either analyzed immediately or stored at −80°C. Protein concentrations were measured by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Lysates in sample buffer (2% SDS, 10%

glycerol, 80 mM Tris-base, 720 mM *DL*-dithiothreitol, and 0.001% bromophenol blue) were denatured at 95°C for 5 min. Equivalent amounts of protein (25 μg) from total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Temecula, CA, USA). Nonspecific binding was blocked by soaking the membrane in Tris-buffered saline (TBS, 20 mM Tris-base, and 300 mM NaCl) containing 5% fat-free milk for 1 h. The membrane was incubated with primary antibodies (anti-p38, anti-ASPK/JNK, anti-ERK, anti-IκB-α and anti-NF-κB at 1 : 5000 in TBS; anti-actin at 1 : 7500 in TBS) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membrane was then incubated with a secondary antibody, a goat antirabbit IgG, or goat antimouse IgG conjugated to horseradish peroxidase. The protein levels were determined by using enhanced chemiluminescence (ECL) plus western blotting detection reagents (Amersham Bioscience, Uppsala, Sweden) and the bands intensities were scanned. Densitometric analyses were conducted using the Quantity One software (Bio-Rad). Incubation with polyclonal mouse antihuman β-actin antibody was performed for comparative control.

2.6. Experimental Animals. Six-week-old female BALB/c mice and 7-week-old female ICR mice were purchased from the National Animal Center (Taipei, Taiwan). These mice were maintained in an air-conditioned room at 23 ± 2°C on a regulated 12 h light-dark cycle. They were fed a nonpurified diet (Lab Rodent Chow 5001, Ralston Purina Inc., St. Louis, MO, USA) for adaptation. At age of 9 weeks, the mice were started on dietary treatment. Animal care and handling conformed to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals [18].

2.7. SOE Treatment Prior to λ-Carrageenan-Induced Paw Edema. To identify the effects of the SOE on local acute inflammation, 28 9-week-old ICR mice were divided randomly into four groups: the control group (n = 8), the LSOE group (n = 8), the HSOE group (n = 8), and the IND group (n = 4, positive control). The control and the IND groups were tube-fed daily with 100 μL sunflower oil, while the LSOE and the HSOE groups were tube-fed daily with 10 and 32 mg SOE/kg BW in 100 μL sunflower oil, respectively. All mice also had free access to chow diet and water. These oral doses of SOE were derived from the effective dose of 50 μg/mL in RAW264.7 cells and primary macrophages based on a previous report [19]. After tube feeding with either sunflower oil or SOE for 1 week, 50 μL of 2% λ-carrageenan (in saline) and 50 μL saline was injected subcutaneously (s.c.) into the right paw and left paw plantar of each mouse, respectively. Mice in the IND group were s.c. injected with 20 mg indomethacin/kg BW 3 h before λ-carrageenan challenge. After each λ-carrageenan injection, paw volume was measured at 1-hour intervals using a plethysmometer (Apelex 7150, Massy, France). The stimulation index (S.I.) was used to express the degree of murine paw edema, which was calculated as

$$\text{S.I.} = \frac{\text{the volume of right paw (carageenan injection)}}{\text{the volume of left paw (PBS injection)}}. \quad (1)$$

2.8. SOE Treatment Prior to LPS-Induced Systemic Inflammation. Forty-five 9-week-old BALB/c mice were divided randomly into four groups: the control group (n = 13), the LSOE group (n = 12), the HSOE group (n = 12), and the PDTC group (n = 8, positive control). All mice were fed chow diet and supplemented with 100 μL sunflower oil daily. Their experimental doses and treatment durations were the same as those in the λ-carrageenan experiment. After 1 week of tube-feeding, all mice were injected intraperitoneally (i.p.) with 15 mg LPS/kg BW to induce systemic inflammation. Mice in the PDTC group were i.p. injected with 50 mg PDTC/kg BW, a dose with anti-inflammatory effects, 1 h before LPS challenge. Sera were collected at 2 and 9 h after LPS challenge for cytokine assay. The life spans of all mice were also recorded.

2.9. Gas Chromatography-Mass Spectrometry. GC-MS analysis was performed using Varian 450-GC and 240-MS system (Varian, Salt Lake City, UT, USA) with the electron impact mode (70 eV) injector and a Varian data system. The GC column was VF-5 ms capillary column (30 m × 0.25 mm, film thickness 0.25 μm, FactorFour, USA). Injector and detector temperatures were set at 250°C and 290°C, respectively. Oven temperature was kept at 50°C for 5 min, then raised to 120°C by a rate of 5°C/min, kept at 120°C for 8 min, and then raised to 300°C by a rate of 10°C/min. The carrier gas was helium at a flow rate of 1 mL/min. Diluted samples of 1.0 μL were injected manually and in the splitless mode. The percentages of the compounds were calculated by the area normalization method. The components were identified by comparison of their mass spectra with the NIST MS 2.0 database (Gaithersburg, MD, USA). Caryophyllene oxide, hexadecanoic acid ethyl ester, and caryophyllene were purchased from Sigma-Aldrich. The compound 6,10,14-trimethyl-2-pentadecanone was purchased from Apollo Scientific Co. (Stockport, Cheshire, UK).

2.10. High Performance Liquid Chromatography (HPLC) Analysis for Kirenol. The content of kirenol was determined by HPLC (LC-20AT, Shimadzu, Tokyo, Japan). The sample was dissolved in methanol and filtered with a 0.22 mm filter. The diluted sample was analyzed by an Ascentis C18 column (number 581325-U, 5 mm, 250 × 4.6 mm; Supelco, Bellefonte, PA, USA). The mobile phase consisted of acetonitrile/methanol (90 : 10, v/v) and water with a linear gradient elution, 0–25 min for 20–60% acetonitrile/methanol (90 : 10, v/v) and 25–50 min for 60–20% acetonitrile/methanol (90 : 10, v/v) at a flow rate of 1.0 mL/min. The sample injection size was 10 μL. The detection was carried out at 215 nm. The residence time of kirenol was 15.4 min.

2.11. Statistical Analysis. Each experiment was performed at least three times. The data are expressed as the means ± SD. The significant difference compared to the control group was statistically analyzed by Student's t-test using the SAS software program (SAS/STATA version 8.2; SAS Institute, Cary, NC, USA). Statistical comparison between different survival curves was analyzed by Cox's proportional hazards regression test (STATA version 9.0; Stata Corp., TX, USA). The relationship was analyzed by the simple correlation of the SAS program. Statistical significance is expressed as $P < 0.05$.

3. Results

3.1. In Vitro Anti-Inflammatory Effects of SOE. Notably, NO is endogenously synthesized by inducible nitric oxide synthase (iNOS) through activated NF-κB and MAPK and is strongly related to inflammatory responses. Because formation of NO can induce inflammation, this study first determined whether SOE suppresses NO generation in LPS-stimulated RAW264.7 cells. As presented in Figure 1(a), SOE suppressed NO production dose-dependently. Compared to the Control, NO production was inhibited by 57 ± 7% at the SOE concentration of 50 μg/mL. Next, the inhibitory effects of SOE on the production of proinflammatory cytokines were examined. Experimental data shown in Figure 1(a) indicate that LPS-stimulated IL-6 production was inhibited markedly by SOE pretreatment in a dose-dependent fashion. Consistent with the IL-6 result, SOE significantly inhibited the production of TNF-α dose-dependently. The SOE significantly reduced iNOS protein expression (Figure 1(b)). These data indicate that the inhibitory effect of SOE on NO production is related to its suppression on iNOS protein expression. The cytotoxicity of SOE on LPS-induced RAW264.7 cells was also assessed using the MTT assay. Cell viability did not decrease after incubation for 48 h with SOE up to 50 μg/mL, indicating that SOE is not cytotoxic to cells within this concentration range (Figure 1(c)).

3.2. In Vivo Effects of SOE on Inflammatory Conditions. Animal experiments were conducted to determine whether 1-week gavage of SOE at the indicated dose ameliorates λ-carrageenan-induced and LPS-stimulated inflammation. Experimental results shown in Figure 2(a) indicate that the high dose of SOE (HSOE, 32 mg/kg BW/day) reduced the degree of paw edema at 4 h after λ-carrageenan challenge. For systemic inflammation, only half of the control group mice survived at 26 h after LPS challenge, but approximately 75% of mice in the HSOE group were alive at the same time point. At 36 h after LPS challenge, no mouse in the control group survived, but around 30% of those in the HSOE group survived (Figure 2(b)). The survival rate of the HSOE group was significantly higher than that of the control group according to the COX proportional hazards regression test ($P = 0.019$).

Our previous studies examined sera cytokine profile of BALB/c mice with 15 mg/kg BW LPS challenge [19, 20], which is a similar animal model to this investigation. In those studies, the life span was found to be negatively correlated with sera IL-6 and TNF-α level at the early stage (2 h) and the late stage (9 h) of the acute-inflammation period. Therefore, this study examined the level of IL-6 and TNF-α at 2 h and 9 h after LPS challenge. The HSOE group had significantly lower serum IL-6 levels at 2 and 9 h after LPS challenge (Table 1). The PDTC group, a positive control group, had significant lower levels of cytokines than the control group.

3.3. In Vitro Effects of SOE on NF-κB Activation and MAPK Phosphorylation. As NF-κB pathway is closely related to the expression of iNOS and proinflammatory cytokines, modulation of SOE on NF-κB activation was examined. Figure 3 shows Western blot results for LPS-induced RAW264.7 cells under treatment with different SOE concentrations. The SOE significantly suppressed phosphorylation of IκB-α and induced IκB-α expression in a dose-dependent manner. Consistently, SOE reduced the degree of phosphorylation of NF-κB when treatment dose increased. These analytical results suggest that SOE inhibits LPS-induced NF-κB activation by blocking the degradation of IκB-α. To further investigate whether inhibition of NF-κB activation and inflammatory mediators by SOE is modulated by the MAPK pathway, the effects of SOE on LPS-induced phosphorylation of ERK1/2, p38, and JNK were examined. The SOE significantly reduced phosphorylation of ERK1/2, p38, and JNK in a dose-dependent fashion (Figure 4).

3.4. Chemical Compositions of SOE. The GC-MS analytical results show that at least 20 compounds exist in SOE (Figure 5), of which a total of 10 constituents were identified using mass spectrometry (Table 2). The mass spectra of these compounds were matched with those found in the NIST spectral database. The major compounds in SOE were quantified as caryophyllene oxide (46.9%), [−]-spathulenol (25.7%), and hexadecanoic acid ethyl ester (9.6%) based on the results obtained from GC-MS analysis. Caryophyllene oxide, hexadecanoic acid ethyl ester, caryophyllene, and 6,10,14-trimethyl-2-pentadecanone were confirmed by comparing their mass spectral data with the NIST mass spectral library and commercially available products. Inhibition of NO production in LPS-induced RAW264.7 cells shows that the anti-inflammatory effects of these four compounds were insignificant (data not shown). Therefore, these compounds were not the bioactive anti-inflammatory ingredients of SOE.

The content of kirenol, an ingredient isolated from ethanolic extract of *S. orientalis* and was demonstrated to exhibit significant anti-inflammatory activity [16], in SOE was determined by HPLC analysis. By matching the retention time (RT = 15.4 min) with authentic standards, good linearity was obtained ($R^2 = 0.9995$) and the quantity of kirenol was determined as 4.2 ± 0.08 mg/g SOE.

4. Discussion

Inflammation, a complex process, is regulated by various immune cells and effector molecules, such as NO and proinflammatory cytokines. Inhibition of these mediators with pharmacological modulators has been proved as an effective

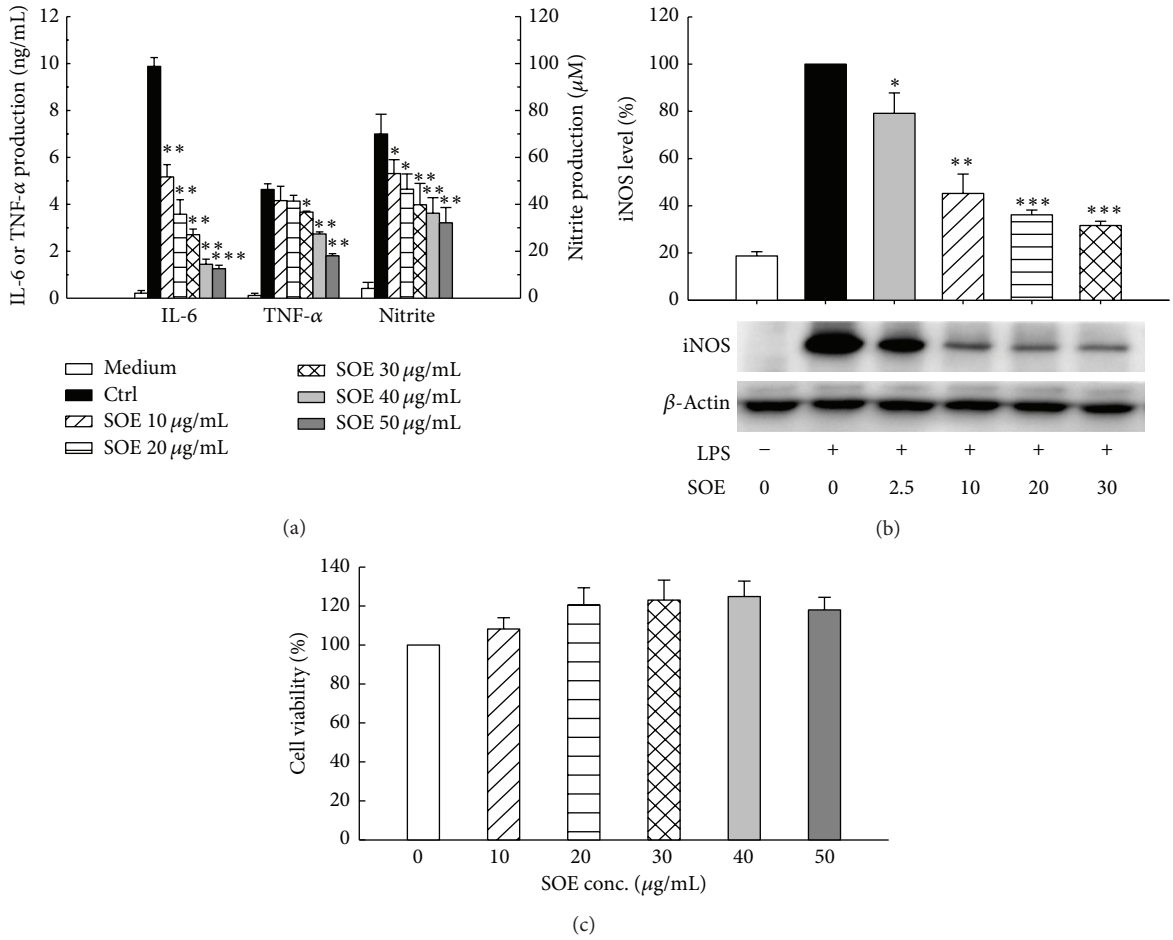

Figure 1: Effects of SOE on proinflammatory mediators production, iNOS expression, and cell viability in LPS-stimulated RAW264.7 macrophages. Cells were pretreated with 0 (control) or the indicated concentration of SOE for 1 h and then stimulated with 1 μg/mL LPS for 12 h (iNOS) or 24 h (proinflammatory mediators). The negative control (medium) is that cells were cultured with medium for the indicated time. (a) Production of IL-6 and TNF-α was measured by an ELISA kit. The nitrite concentration was analyzed using Griess reagent. (b) The expression of the iNOS protein was determined by Western blotting analysis. The iNOS level was quantified by densitometric analysis using the Quantity One software (Bio-Rad). (c) Cell viability was determined by MTT assay. Bar values are means ± SD of three independent experiments in these assays. A significant difference from the control (LPS alone) was indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$ by Student's t-test.

therapeutic strategy for reducing inflammatory reactions and risk of inflammatory diseases [21–23]. Macrophages are crucial to host-defense against infections and in inflammation processes through the release of molecules such as NO, PGE_2, TNF-α, and IL-6. Overproduction of these mediators has been implicated in several inflammatory diseases and cancer [24]. Thus, inhibition of activation of these cells appears to be an important target when treating inflammatory diseases. Stimulation of macrophages with LPS induces high production of NO by iNOS and PGE_2 by cyclooxygenase- (COX-) 2 [25]. Therefore, a reagent that prevents the release of these mediators or downregulates iNOS or COX-2 expression may possess anti-inflammatory activities.

S. orientalis, the most common used original of Herba Siegesbeckiae, and its ethanol extract (SOE), which mimics formulas in medicinal foods, were used to explore its preventive effects against inflammation. In cell culture test, LPS induced-cellular production of IL-6, TNF-α, and NO via iNOS activity was dose-dependently reduced by the SOE in RAW264.7 macrophages (Figure 1). However, decreased PGE_2 production and COX-2 expression were insignificant within the tested SOE concentration range (data not shown). Moreover, the cell viability rose slightly as the SOE dose increased (Figure 1(c)), implying that SOE exhibited significant anti-inflammatory activity without causing cytotoxicity. These results imply that SOE has potential as an anti-inflammatory agent. With this *in vitro* inhibitory effect of SOE on the production of proinflammatory mediators, the *in vivo* anti-inflammatory potential of SOE was then evaluated.

First, a λ-carrageenan-induced paw edema model, which is considered as a highly sensitive tool for evaluating the efficacy of acute inflammation [26], was adopted to assess the preventive effect of SOE on local acute inflammation. It has also been reported that this paw edema would be

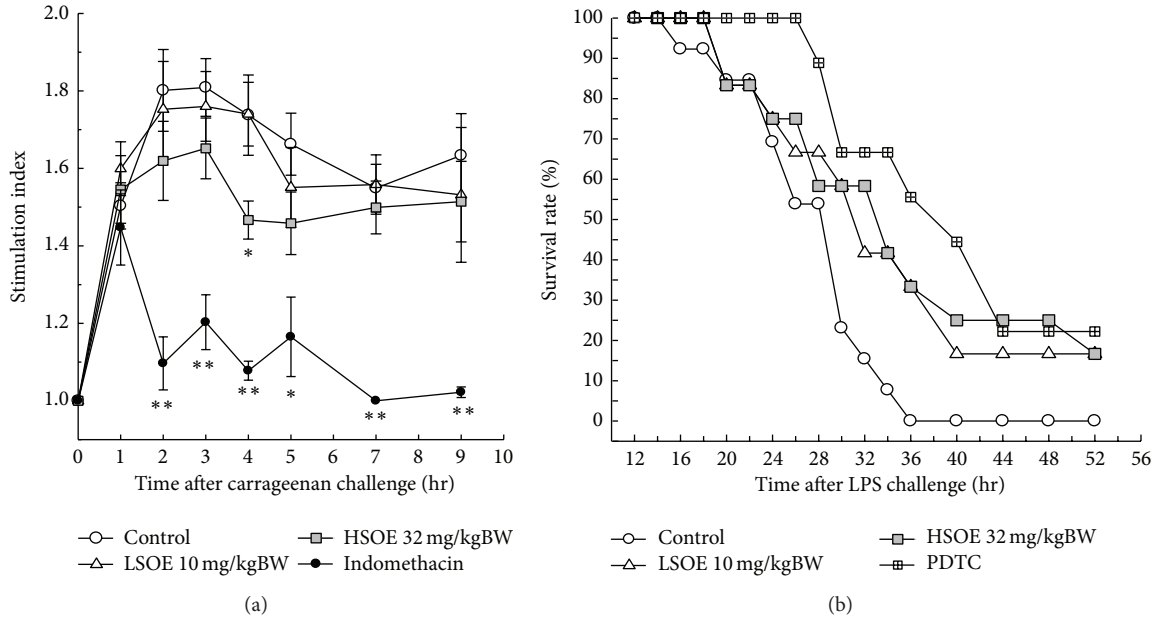

Figure 2: Effects of SOE pretreatment on λ-carrageenan-induced paw edema and mouse survival in LPS-challenge model. (a) The ICR mice tube-fed without (control or IND) or with the indicated dose of SOE (10 or 32 mg/kg BW/day) for 7 days were s.c. injected with λ-carrageenan into right paw plantar, and the degree of mice paw edema was recorded. The positive control mice were s.c. injected with indomethacin (20 mg/kg BW) at 3 h before λ-carrageenan challenge. S.I. = the volume of right paw/the volume of left paw. A significant difference from the control was indicated as $^{*}P < 0.05$ or $^{**}P < 0.01$ by Student's t-test. (b) The BALB/c mice tube-fed without (control or PDTC) or with the indicated dose of SOE (10 or 32 mg/kg BW/day) for 7 days were i.p. injected with LPS, and mouse survival was recorded. The positive control mice were i.p. injected with PDTC (50 mg/kg BW) at 1 h before LPS challenge. The LSOE, HSOE, and PDTC positive control groups had increased survival using the COX proportion hazards regression test ($P = 0.027$, 0.019, and 0.006, resp.).

Table 1: Effects of SOE pretreatment on sera cytokine production in LPS-challenged mice[a].

Group	IL-6 (ng/mL)	TNF-α (ng/mL)
At 2 h after LPS challenge		
Control	318 ± 102	2.99 ± 1.52
LSOE	257 ± 89.5	4.32 ± 2.62
HSOE	205 ± 102*	3.51 ± 2.04
PDTC	202 ± 117*	1.47 ± 0.32*
At 9 h after LPS challenge		
Control	151 ± 125	0.63 ± 0.50
LSOE	139 ± 88.7	0.56 ± 0.26$^{\#}$
HSOE	81.3 ± 46.5*	0.52 ± 0.35$^{\#}$
PDTC	27.9 ± 15.4**	0.25 ± 0.12*

[a]Sera at 2 h and 9 h after LPS injection were collected for cytokines assay. The cytokine production in serum was assayed by ELISA kits. Values are means ± SD. $^{\#}0.05 < P < 0.1$, $^{*}P < 0.05$, or $^{**}P < 0.01$, significantly different from the control group analyzed by Student's t-test.

ameliorated by reducing the levels of IL-1, IL-6, TNF-α, and NO [27]. In this study, SOE at a dose of 32 mg/kg BW/day (HSOE) suppressed significantly λ-carrageenan-induced paw edema (Figure 2(a)). Second, a LPS-challenge model, which mimics systemic endotoxemia, was applied to determine whether SOE pretreatment could reduce systemic chronic inflammation. Previous studies had indicated that reduction of sera TNF-α and/or IL-6 levels in LPS-challenged mice could benefit their survival [19, 20]. Our data show that pretreatment with HSOE significantly decreased the sera IL-6 level in mice but did not significantly reduce the level of sera TNF-α (Table 1). However, the HSOE mice had a higher survival rate than the control mice (Figure 2(b)), implying that reduction of IL-6 only remains useful in increasing mice survival in LPS-induced systemic inflammation. These data suggest that the oral administration of SOE has favorable effects on prevention of local and systemic acute inflammation by downregulating production of inflammatory mediators.

The RAW264.7 macrophage cell line has been used as a rapid *in vitro* screening method when studying anti-inflammatory agents [7, 28, 29]. The cytokine profile in the cell model in this study was resembled that in the LPS-induced *in vivo* model. The SOE has similar significant effects on reduction of IL-6 expression in both *in vitro* and *in vivo* studies. However, the effect of SOE on the reduction of the TNF-α level in cell cultures was significant but insignificant in mouse sera. This difference is likely due to the SOE dose used in the cell-model experiment and animal-model experiment. The high dose of SOE of 50 μg/mL in the cell-model test inhibited 84% of IL-6 production and 58% of TNF-α production (Figure 1). In comparison, the high dose of SOE of 32 mg/kg BW/day in the animal-model test inhibited 46%

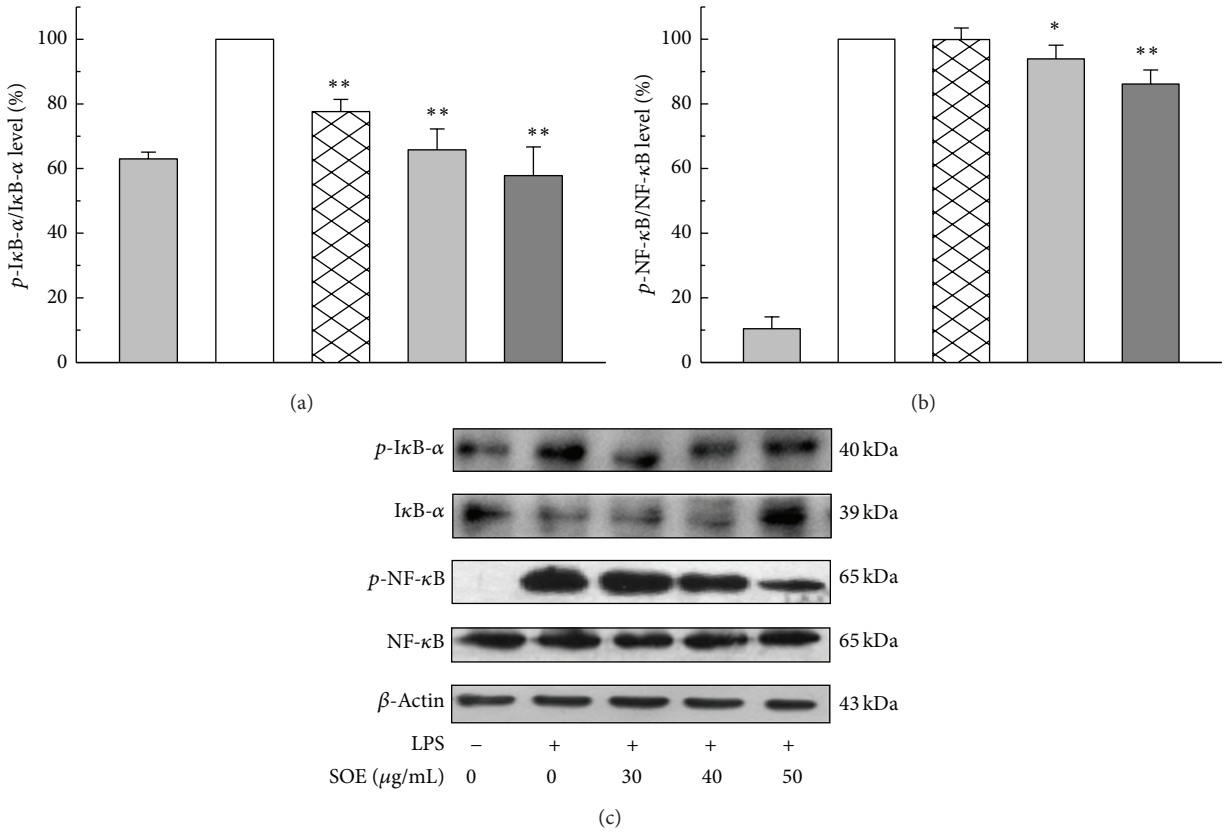

Figure 3: Effects of SOE on the expression of *p*-IκB-*α* and *p*-NF-*κ*B in LPS-stimulated RAW264.7 cells. Cells were pretreated with the indicated doses of SOE for 1 h and then stimulated with 1 *μ*g/mL LPS for 1 h. Bar values are means ± SD of three independent experiments. The electrophoresis experiment was repeated three times, and one representative result is shown here. A significant difference from the control (LPS alone) was indicated as $^{*}P < 0.05$ or $^{**}P < 0.01$ by Student's *t*-test.

Table 2: Chemical compositions of SOE analyzed by GC-MS.

No.	Component	Rt (min)[a]	R. match	Percentage (%)[b]
1	2-Oxabicyclo[2,2,2]octane-6-ol	20.72	763	1.8
2	2-tert-Butyl-1,4-dimethoxy-benzene	23.13	805	3.8
3	Caryophyllene	23.59	866	3.1
4	*cis*-*α*-Bisabolene	26.57	860	4.1
5	[−]-Spathulenol	27.62	853	25.7
6	Caryophyllene oxide	27.95	858	46.9
7	*cis*-Lanceol	29.28	719	1.7
8	[Z,Z,Z]-9,12,15-Octadecatrienoic acid ethyl ester	32.49	736	1.2
9	6,10,14-Trimethyl-2-pentadecanone	33.53	790	2.1
10	Hexadecanoic acid ethyl ester	36.93	794	9.6

[a]Retention time (min).
[b]Relative percentage calculated by integrated peak area.

of IL-6 formation and 17% of TNF-*α* formation (Table 1). Therefore, a higher dose of SOE in the animal-model test would be worthy of further work.

Notably, NF-*κ*B plays an important role in the regulation of cell survival genes and induction of the expression of inflammatory enzymes and cytokines. Therefore, blocking the NF-*κ*B transcriptional activity in the nuclei of macrophages may reduce the expression of iNOS, COX-2, and proinflammatory cytokines and has been considered to be an effective therapy for treating inflammation-related diseases [30]. Under unstimulated conditions, NF-*κ*B is an inactive complex bound to I*κ*B*α* in cytosol. After stimulation with LPS, NF-*κ*B is activated through phosphorylation and degradation of I*κ*B*α* by increasing IKK or Akt kinase activity [31]. Phosphorylation of NF-*κ*B, regulated by MAPK pathway, plays a vital role in modulating transcriptional activity

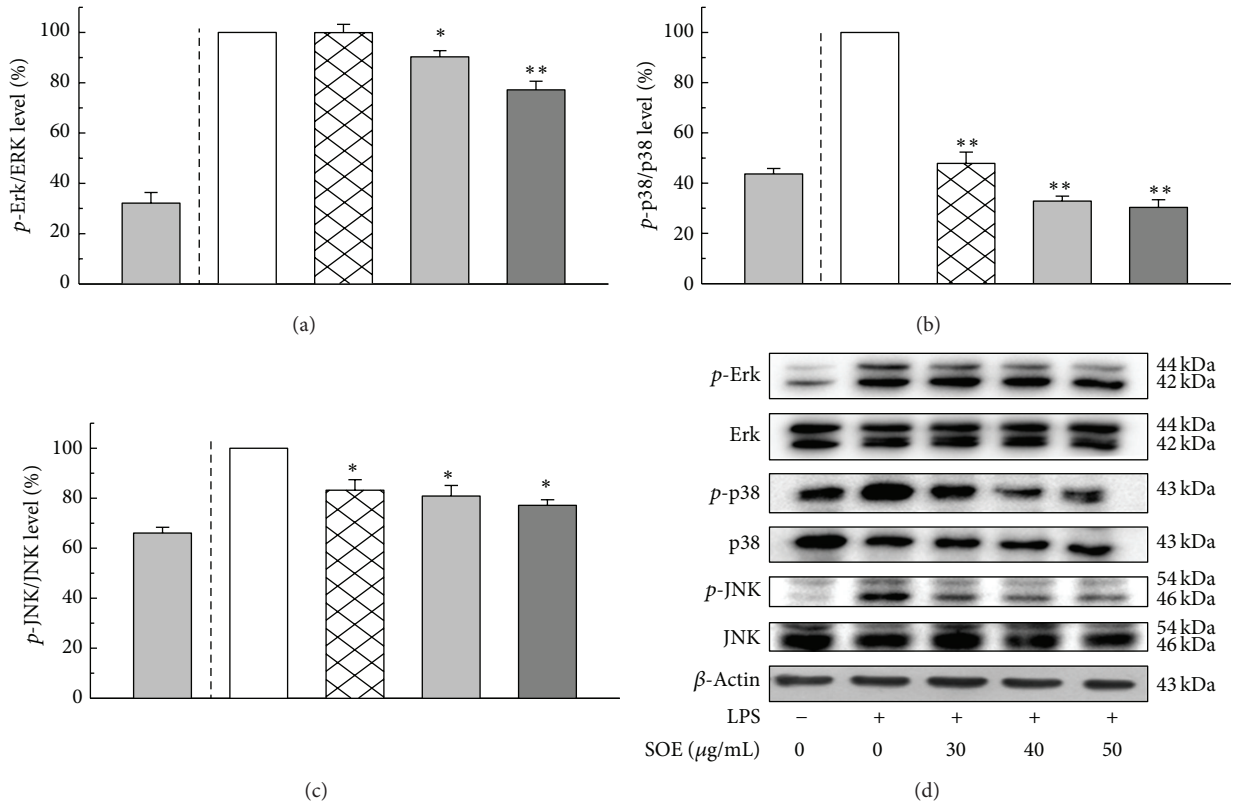

Figure 4: Effects of SOE on the expression of *p*-Erk, *p*-p38, and *p*-JNK in LPS-stimulated RAW264.7 cells. Cells were pretreated with the indicated doses of SOE for 1 h and then stimulated with 1 μg/mL LPS for 1 h. Bar values are means ± SD of three independent experiments. The electrophoresis experiment was repeated three times, and one representative result is shown here. A significant difference from the control (LPS alone) was indicated as $^{*}P < 0.05$ or $^{**}P < 0.01$ by Student's *t*-test.

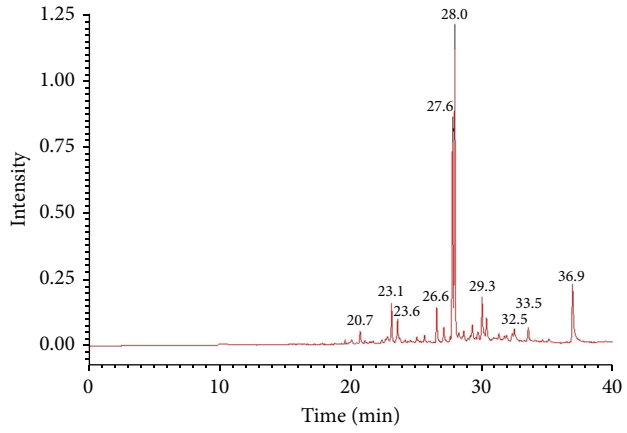

Figure 5: Gas chromatography-mass spectrometry profile of SOE.

of NF-κB and is independent of IκBα proteins [32]. In this study, Western blotting results show that SOE decreased phosphorylated NF-κB level but unaffected total NF-κB level in LPS-stimulated RAW264.7 cells. It was also observed that SOE dose-dependently decreased *p*-IκBα/IκBα and SOE at 50 μg/mL significantly increased IκBα level (Figure 3). These suggest that regulatory effects of SOE on NF-κB activation are partly through modulating the degradation of IκBα.

The MAPKs are a family of serine/threonine kinases that are involved in a variety of cellular processes. Three MAPK molecules, ERK, p38, and JNK, are activated in response to certain extracellular stimuli such as LPS or carrageenan challenge. These kinases have different downstream targets and mediate diverse cellular responses, including regulation of apoptosis, proliferation, and inflammation [33]. This study shows that treatment by SOE significantly inhibited LPS-induced ERK1/2, p38, and JNK phosphorylation in LPS-stimulated macrophages (Figure 4), which may contribute to the inhibitory effect of SOE on the production of proinflammatory mediators in LPS-induced macrophages.

In GC-MS analysis, 10 SOE constituents were identified using mass spectrometry (Table 2). Among them, 4 compounds, caryophyllene oxide, hexadecanoic acid ethyl ester, caryophyllene, and 6,10,14-trimethyl-2-pentadecanone, were obtained from commercial sources. However, none of these 4 compounds had a significant inhibitory effect on NO production in LPS-induced RAW264.7 cells. Our laboratory has separated SOE into several fractions using a partition procedure with *n*-hexane, ethyl acetate (EA), and methanol. The IC_{50} values of the SOE, *n*-hexane fraction, and EA fraction on the inhibition of NO production in LPS-activated

macrophages were 41.8, 53.7, and 5.3 μg/mL, respectively, while the methanol fraction was insignificant. Due to the effectiveness of the EA fraction, the bioactive components from this fraction will be screened and separated in our next study.

5. Conclusions

In summary, *in vivo* evidence suggests that SOE has significant inhibitory effects on local and systemic acute inflammation, while *in vitro* data reveal that SOE could block activation of NF-κB and MAPKs, thereby inhibiting the induction of iNOS expression and the release of inflammatory cytokines. Taken together, this study demonstrates that SOE is a medicinal food material capable of preventing inflammation. Further studies are still needed to evaluate the detailed molecular mechanisms and define the main bioactive phytochemicals in the SOE.

Conflict of Interests

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

Acknowledgments

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Research Article

Evaluation of the Antioxidant Activity and Antiproliferative Effect of the Jaboticaba (*Myrciaria cauliflora*) Seed Extracts in Oral Carcinoma Cells

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It is becoming increasingly evident that certain phytochemicals possess cancer chemopreventive properties. In this study, the antiproliferative activity of extracts from different parts of the jaboticaba (*Myrciaria cauliflora*) plant was evaluated for its effect on human oral carcinoma cell lines. The cytotoxicities of various plant extract concentrations were examined and the 50% maximal inhibitory concentration (IC_{50}) was determined. Water extracts of jaboticaba seeds showed concentration-dependent antiproliferative effects. Annexin V/propidium iodide positivity with active caspase-3 induction indicated that the treated cells underwent apoptosis. Several important regulatory proteins (Bcl-2, Bcl-xL, Bid, and survivin) involved in apoptosis were also evaluated. The antioxidant activity of jaboticaba was investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays, and the drug concentration eliciting 50% maximum stimulation (SC_{50}) was determined. The present findings suggest that water extracts of jaboticaba seeds exhibit an antiproliferative effect against oral cancer cells by inducing apoptosis through downregulating survivin expression and thereby activating caspase-mediated Bid cleavage.

1. Introduction

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. Reactive oxygen species are produced naturally in mammalian systems as a result of oxidative metabolism. Free radicals can lead to a variety of physiological and biochemical lesions and induce degenerative illnesses such as coronary artery disease and cancer [1]. Oxidative damage is balanced by endogenous antioxidants; however, the additional protection provided by nutritive and nonnutritive elements in food is critical for chemoprevention of diseases [2]. There has been increasing

interest in finding naturally occurring antioxidants in foods and medicines to replace synthetic antioxidants, as their use is being restricted due to undesirable side effects [3].

Jaboticaba (*Myrciaria cauliflora*), belonging to the family Myrtaceae, is a purple-black plant with plum-sized fruit clusters that grow directly around the stem and main branches. It is known colloquially as the "Brazil grape tree" [4], is native to Brazil, and is distributed throughout the Atlantic Forest biome. The fruit has an appearance and texture similar to that of grapes, but with thicker, tougher, purple-colored skin. Each jaboticaba fruit contains one to four seeds in the white jelly-like flesh. Common in Brazilian markets, jaboticaba fruits are consumed fresh as well as in processed forms in jams, juices, and liqueurs. Their popularity has been likened to that of grapes in other countries [5].

Jaboticaba has been reported to be rich in phenolic constituents, including resorcinol, p-hydroxybenzoic acid, anthocyanins, hydroxycinnamic acids, flavonoids, coumarins, and ellagitannins. Phenolic compounds are well-known to be potent deactivators of reactive species, strong antioxidant and anti-inflammatory biological properties [5, 6], and anticancer potency [7, 8]. Gallic acid, one constituent of jaboticaba, a naturally occurring plant phenol, which can be abundantly found in natural plants, tea, and red wines [6, 9], has been demonstrated to have various biological properties, including antioxidant, anti-inflammatory, and anticancer activities [10]. Jaboticabin, a depside which is isolated from the fruit of jaboticaba, exhibits antiradical and anti-inflammatory activity and showed potential benefits in chronic obstructive pulmonary disease [5, 11]. A newly identified component of jaboticaba is syringin and its glucoside [6]. Syringin, the active principle of *Eleutherococcus senticosus*, can lower plasma glucose by increasing the release of acetylcholine from nerve terminals [12]. However, the antioxidant and anticancer potential of syringin remains to be elucidated.

Survivin is a member of the family of inhibitors of apoptosis proteins (IAPs) [13–15]. The integral role of survivin in cancer cell division and survival makes it an attractive therapeutic target to inhibit cancer cell growth [13–15]. It was originally suggested that survivin inhibits cell death induced through the extrinsic and intrinsic apoptotic pathways, conferring resistance to apoptosis by directly suppressing caspase activity [16]. Bid is a proapoptotic protein that upon activation by cleavage translocates to the mitochondria and binds there as a truncated Bid [17]. It can be cleaved by caspase-8 [14, 15], caspase-2 [18], and caspase-3 [19]. In the present study, we demonstrated that water extract of jaboticaba seeds exerts apoptotic activity on oral cancer cells through the suppression of survivin, thereby, activating caspase-mediated Bid cleavage.

2. Materials and Methods

2.1. Plant Extract Preparation. Jaboticaba fruits and stems were purchased from Tien-Yi Treegrape Farm in Chang-Hua County, Taiwan. The peels, stems, and seeds were isolated, washed, and air-dried thoroughly. For the ethanol-extraction part, peels, stems, and seeds were soaked in 95% ethanol with continuous shaking at room temperature for three days, respectively. The extracts were concentrated and strained through a 0.45 μm filter, and the entire extraction process repeated twice. For the water-extraction part, the jaboticaba seeds were soaked in double-distilled water (ddH_2O) at 50°C for three days and then concentrated and filtered. This water-extraction procedure was also performed twice.

2.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay. The antioxidant capacity was determined by the DPPH radical-scavenging method according to Brand-Williams et al. [20]. Various 100 μL concentrations of the extracts in ethanol were added to 750 μL 0.0025% ethanol DPPH solution. After a 30 min incubation period at room temperature, absorbance was read against a blank at 517 nm. DPPH is a purple-colored stable free radical which when reduced becomes yellow-colored diphenylpicrylhydrazine. The water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a positive control. The inhibition ratio was calculated as % of inhibition using the following formula: [(absorbance of control − absorbance of test sample)/absorbance of control] × 100%. The extract concentration providing 50% inhibition (IC_{50}) was calculated using a graph and plotting inhibition % against extract concentration [21].

2.3. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonic Acid) (ABTS) Assay. The free radical-scavenging activity of the examined essential oils was determined by the ABTS radical cation decolorization assay described by Pellegrini et al. [22]. ABTS radical cation was produced by reacting ABTS solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The incubation mixture in a total volume of 5 mL contained 0.54 mL of ABTS radical cation, 0.5 mL of phosphate buffer, and varying concentrations of the extracts. Appropriate solvent blanks were run with each assay. The absorbance was read by spectrophotometer at 734 nm and compared with the Trolox control.

2.4. Cell Line Maintenance. The human oral squamous cell carcinoma cell line HSC-3 (Japan Health Science Research Resources Bank) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The cells were maintained in the appropriate growth medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and used over a restricted culture period of 10 passages.

2.5. Cell Viability Analysis. The effect of test extracts on cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in four to six replicates. HSC-3 cells were grown in 10% FBS-supplemented DMEM, in 96-well plates for 24 h, and then exposed to various concentrations of extracts in the same medium for predetermined time intervals. Controls received dimethyl sulfoxide (DMSO) and ddH_2O vehicle at a concentration equal to that in drug-treated cells. At the end

of the treatment, the medium was removed and replaced with 200 μL of 0.5 mg/mL MTT in the same medium. Cells were then incubated in a CO_2 incubator at 37°C for 2 h. Supernatants were removed from the wells and the reduced MTT dye was solubilized in DMSO 200 μL/well. Absorbance was determined at 595 nm using a plate reader.

2.6. Annexin V/Propidium Iodide (PI) Staining and Flow Cytometry Analysis. After treating with extracts for 3 h, 5 $\times$ 10^5 cells were collected and washed in cold phosphate-buffered saline (PBS). The supernatant was aspirated, leaving the pellet undisturbed. The procedure for staining with the ApoAlert Annexin V Kit (Clontech Laboratories Inc., Mountain View, CA, USA) was based on the manufacturer's protocol. In brief, the cells were resuspended in 100 μL binding buffer. To each tube, 5 μL Annexin V-fluorescein isothiocyanate (Annexin V-FITC, 20 μg/mL) and 10 μL PI (50 μg/mL) were added. Each tube was then gently mixed and incubated at room temperature for 15 min in the dark. Cells were analyzed with a FACScan flow cytometer (Becton, Dickinson and Company, Palo Alto, CA, USA).

2.7. Caspase-3 Activity Assay. Caspase-3 activity was determined using the BD ApoAlert Caspase-3 assay according to the manufacturer's instructions (Becton, Dickinson and Company) as previously described. In brief, cell lysates were mixed with 10 mM dithiothreitol- (DTT-) rich reaction buffer containing 50 μM DEVD-pNA, a caspase-3 substrate, and incubated for 1 h at 37°C. Enzyme-catalyzed release of pNA was monitored using a microplate reader at 405 nm. A total of 10,000 events were acquired for each sample and analyzed with a FACScan flow cytometer.

2.8. Protein Extraction and Western Blot Analysis. Biomarkers of apoptosis were assessed by Western blotting as follows. Treated cells were washed in PBS, resuspended in sodium dodecyl sulfate (SDS) sample buffer, sonicated for 5 s, and then boiled for 5 min. After brief centrifugation, equal amounts of total protein from each sample were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The transblotted membrane was washed thrice with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 60 min, the membrane was incubated with an appropriate primary antibody at 1 : 1000 dilution in TBST-5% low-fat milk at 4°C overnight and then washed thrice with TBST. The membrane was probed with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugate (1 : 10000) for 1 h at room temperature and washed thrice with TBST. The hybridized immunocomplex was detected with Renaissance Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA, USA). The quantitative analysis of Western blotting was carried out using the ImageQuant-TL-7.0 software, version 2010 (Amersham Biosciences).

2.9. Statistical Analyses. All analyses were run in triplicate and expressed as mean ± standard deviation (SD). Statistical significance was evaluated by Student's t-test and confidence limits were set at $P < 0.05$.

3. Results

3.1. Scavenging Activity of Jaboticaba. The results of the determination of antioxidant activity of different extracts using two methods (DPPH and ABTS) are presented in Figure 1. For DPPH scavenging activity the water extract of jaboticaba seed was more active than the ethanol extract of seed and other parts of jaboticaba. The SC_{50} of ethanol extracts of peel, stem, seeds, and water extract of seeds were 0.049, 0.017, 0.027, and 0.0059, respectively. The SC_{50} of water extract of seed was close to the positive control, Trolox (0.0053).

In addition, water extract of the seed showed much more active antioxidant potential in ABTS scavenging. The SC_{50} of ethanol extracts of peel, stem, and seed and water extract of seed were 0.038, 0.01, 0.091, and 0.0027, respectively, whereas the SC_{50} of Trolox was only 0.0052. These data indicate that the water extract of seed possesses very potent antioxidant activity.

3.2. Antiproliferative Activity of Plant Extracts from Different Parts of Jaboticaba. To evaluate which part of jaboticaba possesses the most potent antiproliferating effect on oral cancer cells, we treated HSC-3 cells with jaboticaba water or ethanol extracts from different parts of the plant. Cytotoxicity on HSC-3 cells determined using the MTT assay is shown in Figure 2. Of the four types of extracts (jaboticaba seed/water, seed/ethanol, stem/ethanol, and peel/ethanol), only seed/water showed a marked inhibition effect on HSC-3 cell viability and it was in a dose-dependent manner. After a 24 hr treatment, the IC_{50} of jaboticaba seed/water extract was approximately 15 μg/mL. These data suggest that the seed/water extract of jaboticaba is the most potent of all of the extracts we examined.

3.3. Induction of Apoptosis in Jaboticaba Seed Extract-Treated HSC-3 Cells. To further understand whether the decrease in cell numbers observed with the MTT assay was due to the slowdown of the cell cycle or the increase of apoptosis, we examined cell behavior after treatment using Annexin V/PI staining. Figure 3(a) shows the Annexin V (+) population change after adding jaboticaba seed/water extract to HSC-3 cells. In the control group, the Annexin V (+) population was less than 5%. When HSC-3 cells were treated with the extract, the population increased to 15.2% and 57.1% in 10 and 50 μg/mL treatments. In addition, the induction of caspase-3 activity was also examined by flow cytometry (Figure 3(b)). The increasing level of active caspase-3 with the elevated concentration of the extract showed that the apoptosis of HSC-3 cells was induced by the jaboticaba seed water extract.

3.4. Decrease of Survivin and Induction of Bid Cleavage Induced the Apoptosis Caused by Water Extracts of Jaboticaba Seeds. Treatment of HSC-3 cells with different concentrations of water extracts of jaboticaba seeds for 24 h promoted

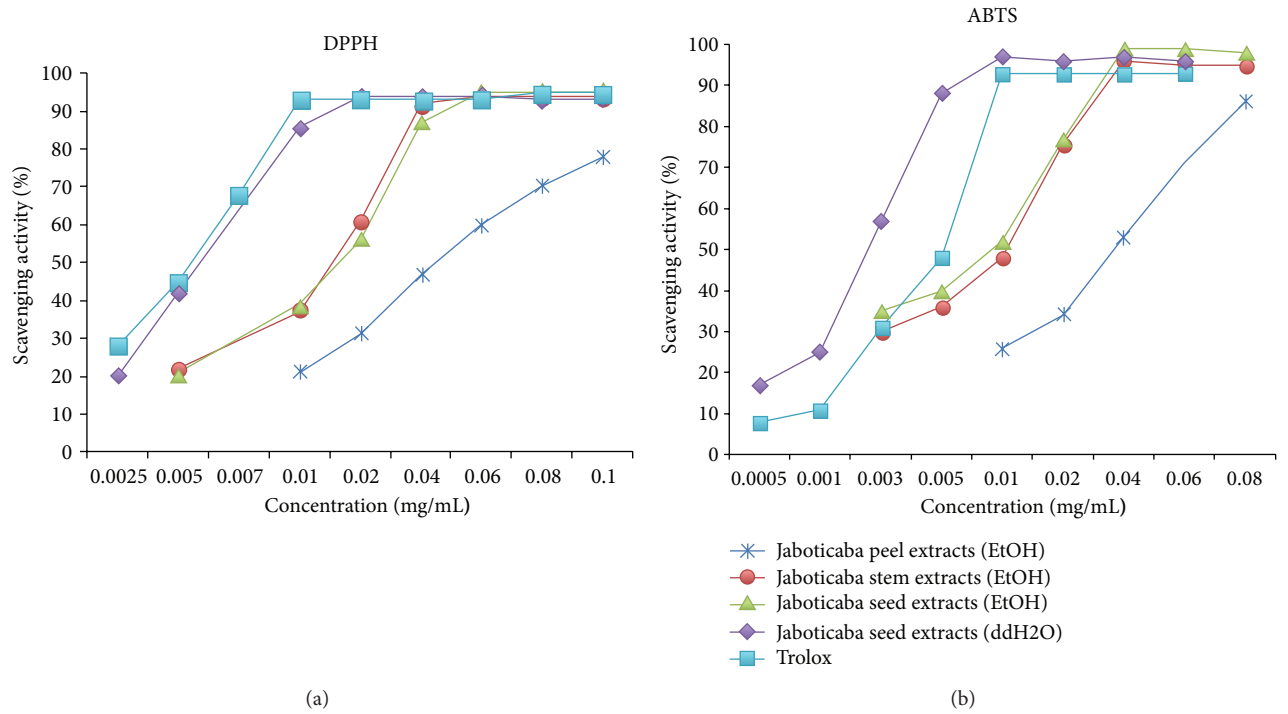

Figure 1: Antioxidative activity of different parts of jaboticaba.Using DPPH and ABTS assays, the water extract of jaboticaba seeds was found to have the best scavenging activity compared to other portions of the plant. Trolox served as a positive control.

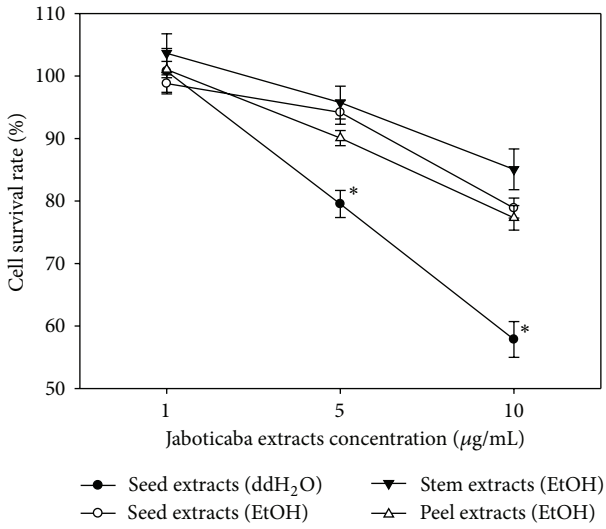

Figure 2: Water extract of jaboticaba seeds possessed the most potent cytotoxic activity. Cell survival rate of HSC-3 cells after 24 h treatment with various extracts showed that the water extract of jaboticaba seeds had the most significant cytotoxic activity compared to extracts from other portions of the plant. The IC_{50} of water extract of jaboticaba seeds in HSC-3 cells was approximately 15 μg/mL. Each point represents the mean and SD of six determinations.

dose-dependent cleavage of poly (ADP-ribose) polymerase (PARP) from the full length 116-kDa to an inactive 85-kDa form by activating caspases (Figure 4), which is another indicator of apoptosis. In order to further understand the apoptotic phenomenon, we evaluated the protein level of various key regulators in the apoptosis pathway through Western blot analysis. It is known that caspase-3 activity can be inhibited by a group of proteins that are collectively termed "inhibitors of apoptosis proteins," of which survivin is one. We particularly determined the expression of survivin because it was shown to directly bind and inhibit caspase-3 [14, 15]. The dramatic abolishment of survivin thereby activated Bid cleavage, indicating that the water extract of jaboticaba seeds induced cell death by lowering the inhibition of apoptosis. Remarkably, the conventional intrinsic apoptosis pathway controlled by the Bcl-2 family did not show an unbalanced change.

4. Discussion

Jaboticaba has been reported to contain anthocyanins, flavonoids, phenolic acids, and tannins, which are well-known antioxidants with anti-inflammatory properties that are believed to play an important role in the prevention of certain diseases [2]. Traditionally, the jaboticaba fruit has been used as a treatment for hemoptysis, asthma, and diarrhea and gargled for chronic inflammation of the tonsils [4]. Recently, intake jaboticaba peels have been found to be able to attenuate oxidative stress in tissues and reduce circulating saturated lipids of rats with high-fat diet-induced obesity [23]. It was suggested that jaboticaba may have the potential to be developed as a functional food. Most studies have focused on the peel or the flesh extract of the fruit. However, the

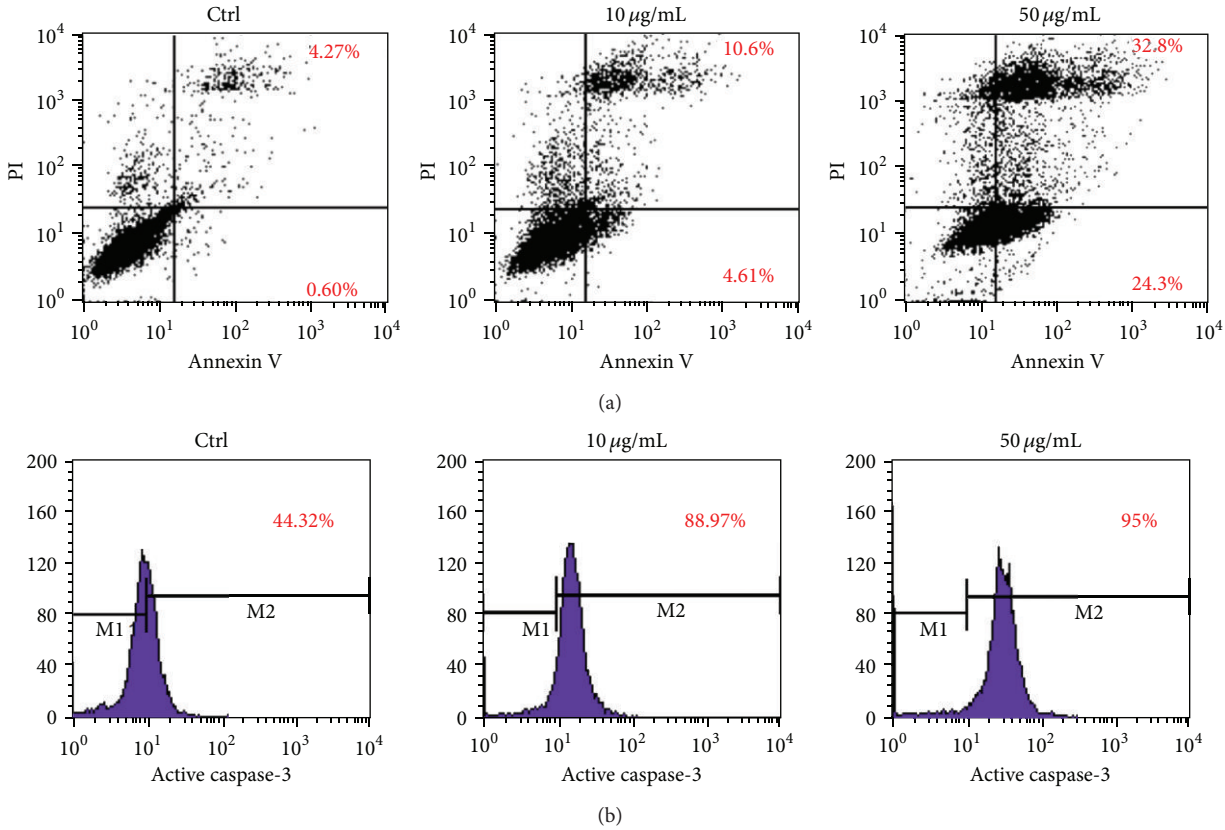

Figure 3: Apoptosis induction by jaboticaba seed water extract treatment of HSC-3 cells. (a) HSC-3 cells treated with jaboticaba seed water extract for 24 h at desired concentrations were stained with Annexin V-FITC and propidium iodide (PI). The Annexin V-FITC signal is shown on the *x*-axis and PI signal is shown on the *y*-axis. The apoptotic populations of cells were significantly increased in treatment groups in a dose-dependent manner. (b) Flow cytometric analysis was performed after a 48 h treatment to determine caspase-3 activity, which was found to be activated in a dose-dependent manner.

cancer chemopreventative activities of this fruit have not been extensively reported in the literature. In this study, extracts from different parts of jaboticaba were investigated for their antioxidant activity and water extract of jaboticaba seeds was further evaluated for antitumor activity against human oral cancer cell lines. Here we provide the first report revealing that the best antioxidant and cancer chemopreventative activity exists in the water-soluble seed extract.

In the present study, we examined different portions of jaboticaba including stem, peel, and seeds. The most notable cytotoxic activity against oral cancer cells appeared to be in seed extracts, particularly, water extracts. In terms of the extraction method, previous studies used methanol or ethanol as solvents to conduct extraction and, undoubtedly, the water extract composition will be changed as a result of the alcohol used. In addition, previous studies focused on analyzing the constituents of freeze-dried fruit, fresh fruit, peel, and pulp, but not seeds. Freeze-dried jaboticaba peels have been found to be rich in fiber and anthocyanins (delphinidin and cyanidin 3-glucoside) and showed high antioxidant activity. The jaboticaba peels extract showed antiproliferative effects against leukemic cells and prostate cancer cells [24]. Many phenolic constituents, like anthocyanins, were found to exist exclusively in the dark-colored fruit skin and not in the pulp or seeds [25, 26]. We are the first to demonstrate the appreciable anticancer activity of water extract of jaboticaba seeds. Purification and identification of the active compounds in the water extract of jaboticaba seeds are required for a better understanding of the protective mechanisms involved and to deduce possible applications in medicine.

Evasion of cell death is a characteristic feature of human cancers and represents a key source of resistance to current treatment approaches [27, 28]. Therefore, reactivation of cell death programs in cancer cells is a promising strategy to overcome resistance to treatment, which is one of the major unsolved problems in clinical oncology [29]. IAP proteins comprise a family of antiapoptotic proteins that promote prosurvival signaling pathways and prevent the activation of the effector phase of apoptosis by interfering with the activation of caspases [29]. Survivin, an IAP member [13], is an antiapoptotic protein that is basally expressed in normal tissue and overexpressed in nearly all human cancers. The expression of survivin in tumor cell lines increases with the proliferation rate and resistance to therapy [30]. Therefore, survivin is an emerging target for the development of novel anticancer therapies. The water extract of jaboticaba seeds induced oral cancer cell apoptosis by decreasing the

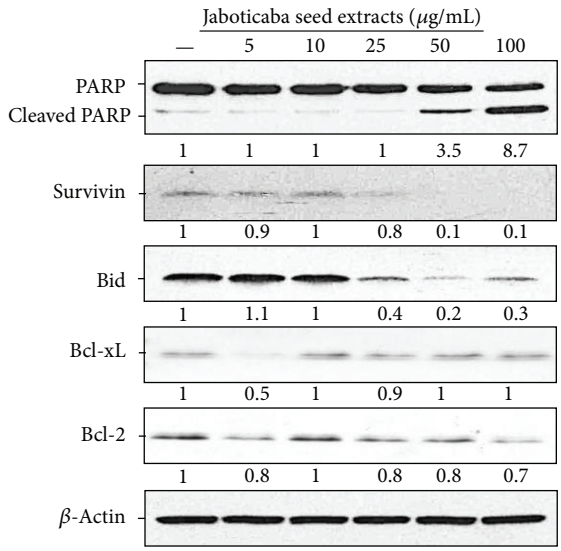

Figure 4: Effects of jaboticaba seed water extract on apoptosis regulatory proteins in HSC-3 cells. HSC-3 cells were treated with 5, 10, 25, 50, and 100 μg/ml of jaboticaba seed extract or ddH_2O as a control for 48 h, and then total proteins were isolated. Equal amounts of cell lysates were analyzed for PARP, survivin, Bid, Bcl-xL, and Bcl-2 expression by Western blotting with corresponding antibodies. β-actin served as the loading control. The fold change was calculated as the ratio of the target proteins in the presence of indicated concentration of Jaboticaba seed extract after normalization of the target proteins to β-actin in each lane.

expression of survivin. These results point to the potential of jaboticaba seed extract as a chemopreventive agent.

5. Conclusions

Dark-colored fruits like jaboticaba are a potentially rich source of many dietary phenolic antioxidants and are believed to play an important role in the prevention of many oxidative and inflammatory diseases. Most previous studies have focused on the alcohol extracts of flesh or peel. Here we showed that the water extract of jaboticaba seeds possesses appreciable antioxidant activity as well. Moreover, this is the first study to show the strong chemopreventative effects of the jaboticaba seed water extract. Our results show that the decrease of survivin and activated Bid cleavage is responsible for the ability of the jaboticaba seed water extract to induce apoptosis. Only a few studies have associated the consumption of jaboticaba with cancer protection. Based on these results, jaboticaba is promising not only as a source of antioxidants but also as a chemopreventative agent.

Conflict of Interests

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

Authors' Contribution

Wen-Hung Wang and Yu Chang Tyan contributed equally to this work as first authors.

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Research Article

The Study of Interactions between Active Compounds of Coffee and Willow (*Salix* sp.) Bark Water Extract

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Coffee and willow are known as valuable sources of biologically active phytochemicals such as chlorogenic acid, caffeine, and salicin. The aim of the study was to determine the interactions between the active compounds contained in water extracts from coffee and bark of willow (*Salix purpurea* and *Salix myrsinifolia*). Raw materials and their mixtures were characterized by multidirectional antioxidant activities; however, bioactive constituents interacted with each other. Synergism was observed for ability of inhibition of lipid peroxidation and reducing power, whereas compounds able to scavenge ABTS radical cation acted antagonistically. Additionally, phytochemicals from willow bark possessed hydrophilic character and thermostability which justifies their potential use as an ingredient in coffee beverages. Proposed mixtures may be used in the prophylaxis or treatment of some civilization diseases linked with oxidative stress. Most importantly, strong synergism observed for phytochemicals able to prevent lipids against oxidation may suggest protective effect for cell membrane phospholipids. Obtained results indicate that extracts from bark tested *Salix* genotypes as an ingredient in coffee beverages can provide health promoting benefits to the consumers; however, this issue requires further study.

1. Introduction

Multitarget therapy is a new therapy concept which tries to treat diseases with a multidrug combination in a more causally directed manner. Physicians practicing phytotherapy recognized very early that a greater efficacy can be achieved with the application of a combination of plant extracts than with a (usually high dosed) monodrug. They noticed that this therapy concept at the same time has the advantage of reducing or eliminating side effects due to the lower doses of the single compounds or drug components within the extract mixtures [1, 2].

Results of synergy effects have been described using two mathematical equations. According to the first equation: $E(d_a, d_b) > E(d_a) + E(d_b)$, "a total effect of a combination is greater than expected from the sum of the effects of the single components." The second equation states that "synergy is deemed present if the effect of a combination is greater than that of each of the individual agents" (i.e., $E(d_a, d_b) > E(d_a)$ and $E(d_a, d_b) > E(d_b)$; E = observed effect and d_a and d_b are the doses of agents a and b) [3].

Coffee is a popular beverage that is widely consumed around the world [4, 5]. Recently, scientific studies have pointed out the positive effect of coffee on human health [6]. However, there are some reports with little evidence of health risks and considerable evidence of health benefits for healthy adults as a result of moderate coffee consumption [7]. The beverage also stands out as a dietary source of potential antioxidants, such as caffeine [8, 9], chlorogenic acids [10, 11], hydroxycinnamic acids [12], and Maillard reaction products, such as melanoidins [13, 14]. Thus, the antioxidant capacity of coffee is related to the presence of both natural constituents and compounds formed during processing. Most studies in the literature refer to the antioxidant activity of roasted coffee. Despite the great economic importance of soluble coffee, little has been reported about its antioxidant potential or the influence of processing conditions. To become solubilised, coffee undergoes an extraction process. The beans are roasted, ground, and subjected to successive percolation with water at temperatures ranging from 100 to 180°C. Chemically, percolation results in the selective solubilisation of coffee

solids. Depolymerisation and degradation of coffee solids may occur during high-temperature extraction [15], and process variations may affect the product's characteristics.

Willow bark is included in the Polish Pharmacopeia and in the European monograph as a constituent of many herbal drugs, dietary supplements, and weight loss enhancement remedies [16–18]. Willow bark preparations, better tolerated by patients than synthetic derivatives, can be used for the traditional symptomatic indications of fever, infections, mild rheumatic complaints, headache, and chronic pain syndromes and extracts of bark have antioxidant abilities, thus many people have begun to turn back to willow bark as an alternative to aspirin [18–22].

Salix purpurea L., *S. daphnoides* Vill., and *S. alba* L. are a very popular herbal species affirmed in the natural habitats and field-cultivated in Poland [23, 24]. Bark of this species contains phenolic glicosides, ρ-hydroxybenzoic, vanillic, cinnamic, ρ-coumaric, ferulic, and caffeic acids and naringenin known for their prohealth properties [19, 25, 26]. However, little is as yet known about synergic effects of coffee and willow bark bioactive compounds.

It has been hypothesized that interactions between the active compounds may improve the health promoting properties of both materials. Thus, the aim of the study was to determine the interaction between antioxidant compounds contained in soluble coffee and present in the extracts from the bark of some species of willow (*Salix purpurea* and *Salix myrsinifolia*).

2. Materials and Methods

2.1. Chemicals. ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), Folin-Ciocalteu reagent, gallic acid, linoleic acid, and potassium ferricyanide were purchased from Sigma-Aldrich company (Poznan, Poland). Acetonitrile and methanol gradient HPLC grade and formic acid LC-MS grade for LC-UV-MS separations were purchased from J. T. Baker (Phillipsburg, New Jersey, USA). Water was purified in-house with a Milli-Q water purification system Simplicity-185 (EMD Millipore Corporation, Billerica, Massachusetts, USA). All other chemicals were of analytical grade.

2.2. Materials. *Salix purpurea* and *Salix myrsinifolia* plants were cultivated on the sandy soil (heavy loamy sand) of experimental fields at the University of Life Sciences in Lublin (51°33′N; 22°44′E). Willow shoots were harvested in November (2012) in three replicates. The shoots were washed with deionized water. Bark was separated from the wood by peeling and subsampled for chemical analysis. The bark material sampled for salicylate analysis was dried at room temperature and intensively mixed and homogenized. After drying, the phenolic glycosides content calculated on salicin was determined by means of the HPLC technique in the laboratory of Labofarm in Starogard Gdański according to methods in Polish Pharmacopoeia VI (2002) and expressed as mg/g dry mass (DM).

The experimental material consisted of soluble coffee available on the Polish market (Lublin, Poland), the typical average quality coffee.

2.3. Willow Bark Preparation. Two g of raw material (willow bark) were poured with 15 mL of hot water; the samples were shaken for 60 min at room temperature. After centrifugation (10 min, 20°C, 4000 g), the supernatant was decanted from the precipitate, and extraction procedure was repeated. The supernatants were combined andthen the extracts were evaporated to dryness in a vacuum evaporator (50°C, under reduced pressure). Thus, obtained preparation was used for further analysis.

For extraction of water-soluble phenolic compounds 50 mg of the sample (soluble coffee, *S. myrsinifolia,* and *S. purpurea* bark preparations) were dissolved in 5 mL of warm water and were used for further analysis in order to determine the antioxidant properties of the individual raw materials. The final extracts concentration was 10 mg dry weight (DW)/mL.

2.4. Ultraperformance Liquid Chromatography. Compounds of interest were analyzed using a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA), consisting of a binary pump system, sample manager, column manager, and PDA detector (also from Waters Corp.). Waters MassLynx software v.4.1 was used for acquisition and data processing. The samples were separated on a BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm), which was maintained at 40°C. The flow rate was adjusted to 0.40 mL/min. The following solvent system: mobile phase A (0.1% formic acid in Milli-Q water, v/v) and mobile phase B (0.1% formic acid in MeCN, v/v) was applied. The gradient program was as follows: 0-1.0 min, 5% B; 1.0–24.0 min, 5–50% B; 24.0-25.0 min, 50–95% B; 25.0–27.0 min, 95% B; 27.0-27.1 min, 95–5% B; 27.1–30.0 min, 5% B. Samples were kept at 8°C in the sample manager. The injection volume of the sample was 2.0 μL (full loop mode). Strong needle wash solution (95 : 5, methanol-water, v/v) and weak needle wash solution (5 : 95, acetonitrile-water, v/v) were used. UV-PDA data was acquired from 220 nm to 480 nm, at 5 point/s rate, 3.6 nm resolution. The separation was completed in 30 min. Peaks were assigned on the basis of their UV spectra, mass to charge ratio (m/z), and ESI-MS/MS fragmentation patterns.

The MS analyses were carried out on a TQD mass spectrometer (Waters Corp.) equipped with a Z-spray electrospray interface. The following instrumental parameters were used for ESI-MS analysis of phenolic compounds (negative ionization mode): capillary voltage, 2.8 kV; cone voltage, 40 V; desolvation gas, N_2 800 L/h; cone gas, N_2 100 L/h; source temperature 140°C; desolvation temperature 350°C. Compounds were analyzed in full scan mode (mass range of 100–1600 amu was scanned).

For ESI-MS/MS, selected ions were fragmented using collision energy of 15 V (phenolic acids derivatives) or 25 V (flavonoids derivatives) and collision gas (argon) at 0.1 mL/min.

2.5. Total Phenolic Analysis. Total phenols were estimated according to the Folin-Ciocalteu method [27]. A 0.1 mL of the extract was mixed with 0.1 mL of H_2O, 0.4 mL of Folin reagent (1 : 5 H_2O), and after 3 min with 2 mL of 10% Na_2CO_3. After 30 min, the absorbance of mixed samples was measured at a wavelength of 720 nm. The amount of total phenolics was expressed as gallic acid equivalents (GAE).

2.6. Determination of ABTS Radical Scavenging Activity. Free radical-scavenging activity was determined by the ABTS method according to Re et al. [28]. This reaction is based on decolourization of the green colour of the free ABTS radical cation ($ABTS^{\bullet+}$). The radical solution was prepared with ABTS and potassium persulfate, diluted in ethanol, at final concentration of 2.45 mM and left at dark for 16 h to allow radical development. The solution was diluted to reach absorbance measures around 0.70–0.72 at 734 nm. 1.8 mL $ABTS^{\bullet+}$ solution was mixed with 0.04 mL of each sample. The absorbance was measured after one minute of reaction at 734 nm. Distilled water was used as blank. Percentage inhibition of the $ABTS^{\bullet+}$ radical was then calculated using the equation

$$\text{scavenging } \% = \left[1 - \left(\frac{A_s}{A_c}\right)\right] \times 100, \quad (1)$$

where A_s is the absorbance of sample; A_c is the absorbance of control (ABTS solution).

All assays were performed in triplicate.

2.7. Inhibition of Linoleic Acid Peroxidation [29]. Ten microliters of sample was added into a test tube together with 0.37 mL of 0.05 M phosphate buffer (pH 7.0) containing 0.05% Tween 20 and 4 mM linoleic acid and then equilibrated at 37°C for 3 min. The peroxidation of linoleic acid in the above reaction mixture was initiated by adding 20 μL of 0.035% hemoglobin (in water), followed by incubation at the same temperature in a shaking bath for 10 min and stopped by adding 5 mL of 0.6% HCl (in ethanol). The hydroperoxide formed was assayed according to a ferric thiocyanate method with mixing in order of 0.02 M ferrous chloride (0.1 mL) and 30% ammonium thiocyanate (0.1 mL). The absorbance at 480 nm (A_s) was measured with a spectrophotometer for 5 min. The absorbance of blank (A_0) was obtained without adding hemoglobin to the above reaction mixture; the absorbance of control (A_{100}) was obtained with no sample addition to the above mixture. Thus, the antioxidant activity of the sample was calculated as

$$\% \text{ inhibition} = 1 - \left[\frac{(A_s - A_0)}{(A_{100} - A_0)}\right] \times 100\%. \quad (2)$$

2.8. Determination of Reducing Power. Reducing power was determined by the method of Oyaizu [30]. A 0.5 mL of extract was mixed with 0.5 mL (200 mM) of sodium phosphate buffer (pH 6.6) and 0.5 mL potassium ferricyanide (1% v/v) and samples were incubated for 20 min at 50°C. After that, 0.5 mL of TCA (10% v/v) was added and samples were centrifuged at 650 g for 10 min. Upper layer (1 mL) of supernatant was mixed with 1 mL of distilled water and 0.2 mL of ferric chloride (0.1% v/v). The absorbance was subsequently measured at 700 nm in the spectrophotometer.

Table 1: Composition of samples used for isobolographic analysis.

Coffee [mg DW]	Willow bark preparation [mg DW]	Weight ratio [w/w]
100	150	2 : 3
125	125	1 : 1
150	100	3 : 2
200	50	4 : 1

Antioxidant activities (except reducing power) were determined as EC_{50}-extract concentration (mg/mL) provided 50% of activity based on a dose-dependent mode of action. Reducing power determined as EC_{50} is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis.

2.9. The Isobolographic Analysis of Interactions. In order to have isobolographic analysis of interaction between the active compounds of coffee and willow bark the following samples were prepared (Table 1).

To the thus prepared mixtures 6 mL of H_2O was added. Each type of extract was prepared in duplicate.

An isobole is an "iso-effect" curve, in which a combination of constituents (d_a, d_b) is represented on a graph, the axes of which are the dose-axes of the individual agents (D_a and D_b). If the agents do not interact, the isobole will be a straight line. If synergy is occurring, the curve is said to be "concave." The opposite applies for antagonism, in which the dose of the combination is greater than expected and produces a "convex" isobole.

2.10. Theoretical Approach. In accordance with the definition, the half-maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of inhibitors. It is commonly used as a measure of antagonist drug potency in pharmacological research. The IC_{50} value is reliable for determining the activity of a single- or two-compound mixture (e.g., isobolographic analysis) [3]. Further, the EC_{50} index quantitatively measures the amount of extractor extracts mixture that is required to exhibit half of the measured activity.

The following factor was also determined [31]:

the interaction factor (IF), which provides an explanation for the mode of interaction:

$$\text{IF} = \frac{A_M}{A_T}, \quad (3)$$

where A_M = measured activity of a mixture of samples and A_T = theoretically calculated mixture activity (based on the dose response of single components at various concentrations).

IF value <1 indicates synergistic interaction; IF > 1 indicates antagonism; IF ≈ 1 indicates additional interactions.

2.11. Statistical Analysis. The experimental results were mean ± S.D. of three parallel experiments ($n = 9$). Statistical analysis was performed using Statistica 7.0 software (StatSoft, Inc., Tulsa, USA) for mean comparison using Tukey's test at the significance level $\alpha = 0.05$.

3. Results and Discussion

3.1. Identification of Coffee Phenolic Compounds. New beneficial properties of the coffee beverage are being continuously discovered [32]. Coffee brew contains many of the most important functional ingredients known, like flavonoids (catechins and anthocyanins), caffeic, and ferulic acid [33]. In addition, other biologically active compounds found in coffee are nicotinic acid, trigonelline, quinolinic acid, tannic acid, pyrogallic acid, and caffeine [34]. The beverage is also known for the antioxidant properties of its components caffeine, CGA, hydroxycinnamic acids, and melanoidins [35, 36]. Antioxidants of the hydroxycinnamic acids group, such as combined or conjugated forms of caffeic, chlorogenic, coumaric, ferulic, and sinapic acids, are also found in coffee beverage [37].

UPLC/MS analyses allowed the identification of 11 phenolic compounds (primarily phenolic acids) in extract of used soluble coffee (Figure 1). The main phenolic acids were compounds from hydroxycinnamic acids family such as caffeoylquinic acid and its isomers. Furthermore, after detection at the wavelength at 250 nm we can observe a significant peak at retention time (Rt) 4.20 min characteristic for caffeine. Therefore, in addition to caffeine, phenolic acids are the main bioactive constituents of coffee responsible for its potential health benefits.

The potential properties of coffee, for which the caffeine is responsible, should be mentioned. Caffeine can exert potent pharmacologic effects that can generate or alleviate headache, depending on the site of action, dosage, and timing of drug exposure. Caffeine is currently implicated in mechanisms of generation of chronic daily headache and analgesic-overuse headache. The likely target of caffeine in mediating these effects is the antagonism of adenosine receptors. Clinicians should regard caffeine as they would any other analgesic in the induction of chronic headache, and patients should be counseled to limit dietary and pharmaceutical caffeine consumption accordingly [38]. The coffee used in our experiment contained 15.27 mg/g DM of caffeine (Figure 2).

A bioactive compound is often characteristic of a plant species or even of a particular organ or tissue of the plant. This makes the dominant active compound responsible for health-promoting properties of food of plant origin. Coffee is usually associated with caffeine and its properties, and willow bark is considered a source of salicylates. In our study, *Salix* bark samples were characterized by a diverse content of phenolic glycosides (Figure 2). The purple willow (*Salix purpurea*) bark is the most important natural source of salicylic glycosides. These compounds are easily disintegrated in the gastrointestinal tract, releasing salicylic alcohol, which is in the liver oxidized to salicylic acid thereby causing no damage to the gastric mucosa [39].

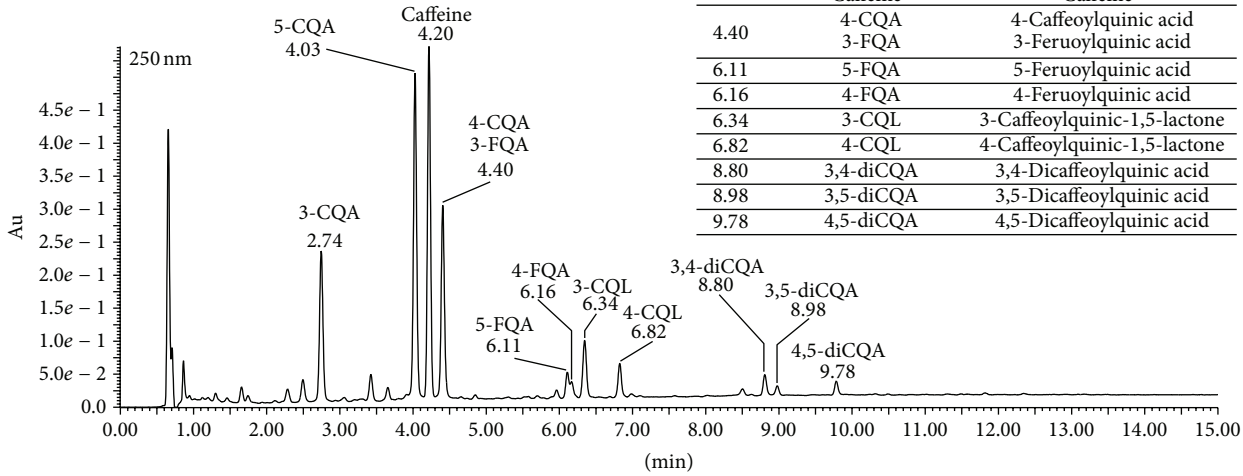
3.2. Total Phenolics Content. According to current literature data the most condensed source of polyphenols among all beverages consumed in the world is coffee [40]. It is also confirmed by research of Svilaas et al. [41], which indicate a high coffee position among food products providing antioxidants. According to the literature 180 mL of brewed coffee provides an average of 936 mg of polyphenols [40], while other literature data indicate that one cup of coffee contains 200–500 mg of polyphenols [41]. Ramirez-Coronel et al. [42] using the HPLC method showed that one kilogram of coffe fruit pulp contains 37.9 g of polyphenolic compounds including: 11.8 g of chlorogenic acid, 20.01 g of proanthocyanidins and 0.6 g of flavonoids. The results obtained in this study indicate that the polyphenol content of the freeze-dried coffee, commercially available, is 26.71 mg/g DM (Table 2).

The reasons for the differences in the content of polyphenolic compounds in the extracts of coffee, presented in different scientific studies, are few. Researchers have used different varieties of coffee to their analysis, with different degrees of maturity and originating from different countries. The concentration of polyphenols in coffee beans depends on the species, variety, and roasting procedures [43], resulting in the decrease in the contents of polyphenols [44].

Willow's active chemical constituent, salicin, was identified in 1829 by the French pharmacist H. Leroux [45]. Salicin and salicylic acid were widely used by 19th century European physicians to treat rheumatic fever and as an antipyretic, gout remedy, and analgesic, particularly for joint pain [46]. Acetylsalicylic acid, firstly synthesized by a French chemist in 1853, was rediscovered by Felix Hoffman at the Bayer Company who created acetylsalicylic acid from the spiric acid (aspirin) found in meadowsweet in the 1890s [47]. However, the high doses used (8–10 grams daily) routinely led to vomiting and gastric irritation, and the search was on for a less noxious salicylate. Some herbalists recommend willow bark extract as a natural substitute for aspirin to achieve these same benefits. In Germany, willow bark is often taken along with aspirin to enhance the therapeutic effects while minimizing side effects [48]. The European Scientific Cooperative on Phytotherapy (ESCOP) has approved willow bark extract to treat fever, pain, and mild rheumatic complaints [49].

The healing properties of willow due to presence of salicylic glycosides, the content of which based on salicin, is strictly defined and is a minimum of 1%. Among salicylic glycosides five compounds were detected: salicin, salicortin, populin, fragilin, and tremulacin; only salicin is in the form of glycosides, and the others are in the form of glycosides, also esterified with different acyl groups [19, 50]. It is considered that the effect of anti-inflammatory and analgesic willow bark determines not only the salicylic derivatives but also other compounds present in the raw material, such as flavonoids, proanthocyanidins, and phenolic acids, which have antioxidant properties and the ability to "scavenge" free radicals [19].

Knowledge about the glycoside content in *S. myrsinifolia* is insufficient. The studies of Sugier et al. [23] show that the bark of the dark-leaved willow was characterized by the phenolic glycoside content ranging from 14.34 to 30.08% and



Rt (min)	Abbreviation	Full name
2.74	3-CQA	3-Caffeoylquinic acid
4.03	5-CQA	5-Caffeoylquinic acid
4.20	Caffeine*	Caffeine*
4.40	4-CQA 3-FQA	4-Caffeoylquinic acid 3-Feruoylquinic acid
6.11	5-FQA	5-Feruoylquinic acid
6.16	4-FQA	4-Feruoylquinic acid
6.34	3-CQL	3-Caffeoylquinic-1,5-lactone
6.82	4-CQL	4-Caffeoylquinic-1,5-lactone
8.80	3,4-diCQA	3,4-Dicaffeoylquinic acid
8.98	3,5-diCQA	3,5-Dicaffeoylquinic acid
9.78	4,5-diCQA	4,5-Dicaffeoylquinic acid

Figure 1: UPLC coffee extract phenolic profile.

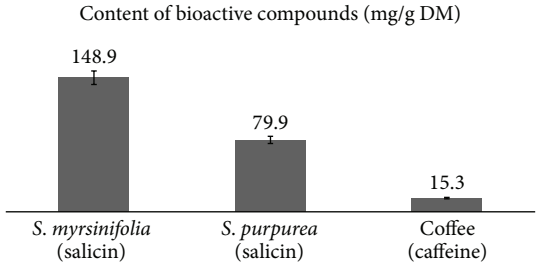


Figure 2: Content of distinctive bioactive compound in plant material.

Table 2: Total phenolic content of coffee, *S. myrsinifolia*, and *S. purpurea* samples.

Sample	Total phenolic content [mg/g DM]
Coffee	26.71 ± 1.3
S. myrsinifolia	23.10 ± 1.2
S. purpurea	20.04 ± 1.0

mean value 22.38% in the year 2007, 23.67% in the year 2008, and 24.27% in 2009. The mean content of phenolic glycosides in the willow bark was higher in 2009 than in 2008 and 2007, but the differences were not statistically significant.

From among the several willow species used as herbal raw material, the bark of *S. myrsinifolia* was characterized by the highest concentration of phenolic glycosides. The content of salicylates in the bark of *S. myrsinifolia* was higher in comparison to other herbal willows, such as *S. purpurea*, *S. daphnoides*, *S. alba*, and *S. pentandra* L. [50–57]. In our study we also observed a higher level of phenolic compounds in the bark of willow *S. myrsinifolia* (23.10 mg/g DM) compared to the *S. purpurea* (20.04 mg/g DM).

Among the *Salix* spp. only *S. purpurea* and *S. alba* are recognized as medicinal plants in Poland [58]. The concentration of salicylates recorded in study of Sugier et al. [23] was many times higher than the minimum reported in Polish Pharmacopoeia VI (2002). Therefore, this species should certainly be treated as yet another taxon which can be recognized as a medicinal plant and commonly used in the pharmaceutical industry.

Most willow species constitute a basic floristic element of vegetation and are frequent in river valleys and peatlands [23, 59, 60]. Due to the high concentration of salicylates and rapid growth, many of them are recommended for herbal production and are a promising source of herbal drugs in the pharmaceutical industry [23, 54, 55, 61]. In Poland, such species as *Salix alba*, *S. daphnoides*, and *S. purpurea* originating from natural habitats and field-cultivated are mainly used to produce *Salicis cortex* [19, 54, 55].

3.3. Antioxidant Potential of Extracts. The antioxidant potential of plant extracts and pure compounds can be measured using numerous *in vitro* assays. Each of these assays is based on one feature of antioxidant activity, such as antiradical ability or to inhibit lipid peroxidation. However, the total antioxidant activities of food of plant origin cannot be evaluated by any single method, due to the complex nature of phytochemicals. Two or more methods should always be employed in order to evaluate the total antioxidant effects [62]. In this study ABTS decolorization assay, ferric reducing antioxidant power, and ability to lipid peroxidation inhibition were used for screening of the antioxidant activities of analyzed samples. Several phenolic acids, including salicylic and caffeic acids, possess anti-inflammatory and analgesic activity which has been associated with their antioxidant activity. The scavenging of oxygen free radicals decides about the anti-inflammatory activity of gallic and protocatechuic

acids [63]. Antioxidant activity was also shown for caffeic, ferulic, and chlorogenic acids [26].

Preparations of both *Salix* species bark showed significant antiradical activity (Figure 3) and the highest ability to neutralize free radicals was noted for *S. myrsinifolia* (EC_{50} = 5.65 mg/mL). Taking into account coffee extract, its antiradical activity was lower than noted for *S. myrsinifolia* but better than for extract of *S. purpurea*. *In vitro* studies on the ability of the compounds contained in the coffee extract to neutralize free radicals have shown that the effect is mainly responsible for ferulic acid, caffeic acid, and then chlorogenic acid (CGA) [10]. The studies of this group of researchers suggest that two of the most important colonic metabolites of CGA: *m*-coumaric and 3-(hydroxyphenyl) propionic acid have a high antioxidant activity. Both compounds showed antioxidant values only slightly lower than that of chlorogenic acid. Furthermore, it has been well established that CGA derivatives are the predominant antioxidants in coffee brews. Chlorogenic acids are a family of esters formed between *trans*-cinnamic acids and quinic acid. The most usual and widespread individual chlorogenic acid is formed between caffeic acid and quinic acid and the most abundant CGAs in coffee are caffeic acid including 5-caffeoylquinic acid (5-CQA) and together with two major positional isomers, 4-CQA and 3-CQA [64].

Iron salts in a biological system attach to biological molecules, where they cause site-specific formation of $^{\bullet}OH$ radicals and consequent damage to lipid, protein, and DNA formation of hydroxyl and peroxyl radicals (*via* the Fenton reaction) which can be delayed by chelating iron ions [65–67].

Reducing power assay measures the electron-donating capacity of an antioxidant [68]. Presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex to the ferrous form which serves as a significant indicator of its antioxidant activity [69]. In general, the reducing power observed in the present study was in the following order: coffee ≥ *S. myrsinifolia* > *S. purpurea*. The data presented here indicate that the marked reducing power was the highest for coffee extract and EC_{50} value was the lowest: 2.21 mg/mL. However, for *S. myrsinifolia* extract EC_{50} was very similar (2.35 mg/mL). It is presumed that the phenolic compounds may act in a similar fashion as reductones by donating electrons and reacting with free radicals to convert them to more stable products and terminating the free radical chain reaction [70].

Cell membrane phospholipids are very sensitive to oxidation and have been found to be frequent targets of radical-induced damage that enables them to participate in free radical chain reactions. Many of the fatty acids are polyunsaturated, containing a methylene group between two double bonds that makes the fatty acid more sensitive to oxidation. The high concentration of polyunsaturated fatty acids in phospholipids enables them to participate in free radical chain reactions [71]. In particular, the inhibition of lipid peroxidation (LPO) by extracts of both willows is similar (Figure 3): EC_{50} = 8.06 mg/mL for *S. purpurea* and EC_{50} = 8.31 mg/mL for *S. myrsinifolia*, respectively. Furthermore, bioactive compounds of coffee have the best ability to inhibit

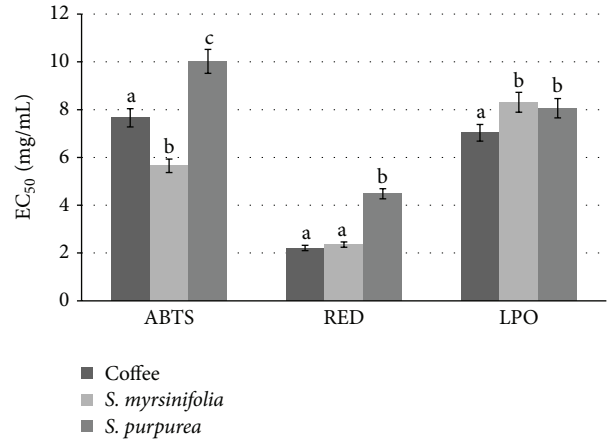


Figure 3: Antioxidant activities of extracts from soluble coffee, *S. myrsinifolia*, and *S. purpurea*. ABTS: antiradical activity; RED: reducing power; LPO: inhibition of lipid peroxidation. Means with different letter within a same activity are significantly different ($a < 0.05$).

lipid peroxidation. In the scientific publication of Meletis [33] it has been determined that water is the best method for general antioxidant extraction. When four solvents were used, water, methanol, ethanol, and n-hexane-water extracts of coffee produced the highest yields of antioxidants and the best lipid-peroxidation protection. The water extract demonstrated a particularly high protective effect against oxidative damage to proteins. The water extract also showed superior free-radical scavenging, generally reducing the ability and capacity to bind ferrous ions thus reflecting its dynamic capacity as both a primary and secondary antioxidant. In our work, we have also analyzed activity of water-soluble bioactive compounds of coffee.

It has been proposed that although willow extracts have been traditionally used as anti-inflammatory compounds for their salicin content, the presence of high amounts of phenolic compounds can contribute to the beneficial effects seen with the consumption of commercial willow extracts [72]. We therefore propose that extracts from this species of plants may provide substantial amounts of a combination of antioxidants and thereby provide health promoting benefits to the consumers.

3.4. Interaction Assay. Unlike in the case of synthetic pharmaceuticals based on an activity of single (chemical) active compounds, numerous phytochemical compounds act in a beneficial manner by an additive of synergistic activity in one or numerous target sites connected to physiological processes. This idea has found an application in pharmacology during investigations on combinations of few metabolites in multidirectional therapy [73]. The method usually used for identification of interactions between active compounds is isobolographic analysis. Isobole method is independent of the mechanism of action and applies under most conditions [3].

The antioxidant capacity of the water-soluble compounds of coffee extract in combination with willow bark extract

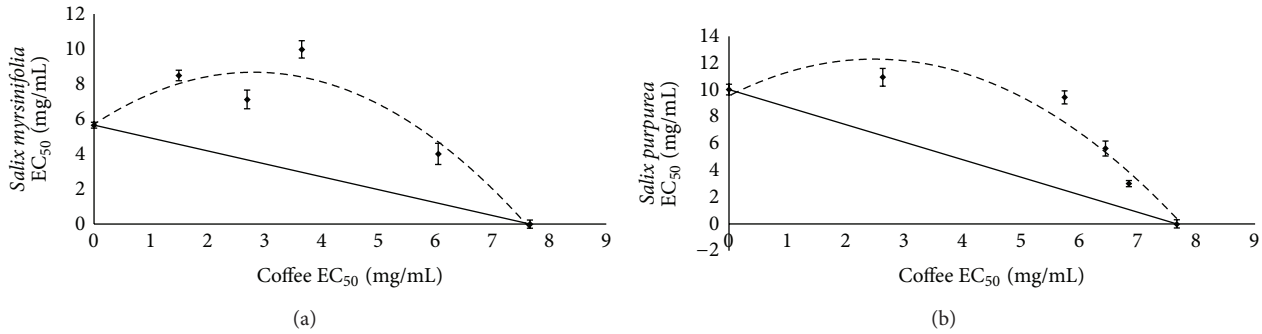


Figure 4: Isobole curves for 50% ABTS radical scavenging activity of coffee and willow mixtures: (a) coffee with *S. myrsinifolia*; (b) coffee with *S. purpurea*. EC_{50}-extract concentration (mg/mL) provided 50% of activity based on a dose-dependent mode of action.

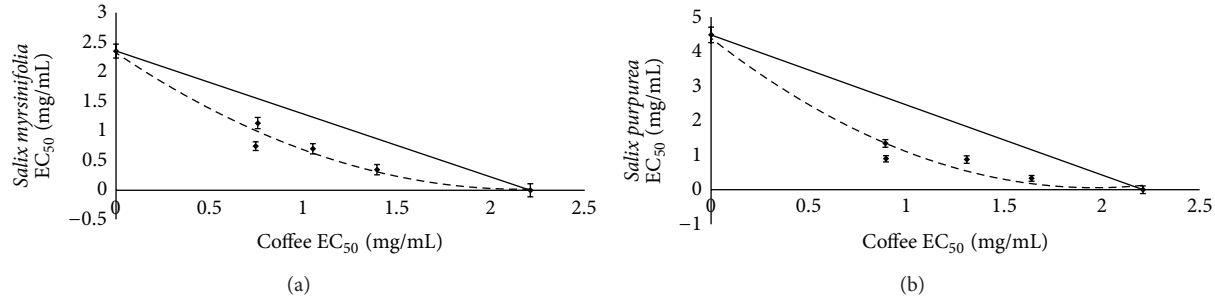


Figure 5: Isobole curves for 50% reducing power activity (RED) of coffee and willow mixtures: (a) coffee with *S. myrsinifolia*; (b) coffee with *S. purpurea*. EC_{50}-extract concentration (mg/mL) provided 50% of activity based on a dose-dependent mode of action.

has been revealed by ABTS method. As in Figures 4(a) and 4(b), isoboles took the convex form. This result indicates that antiradical scavengers included in coffee and willow acted as antagonists in the case of both studied species of the *Salix* family.

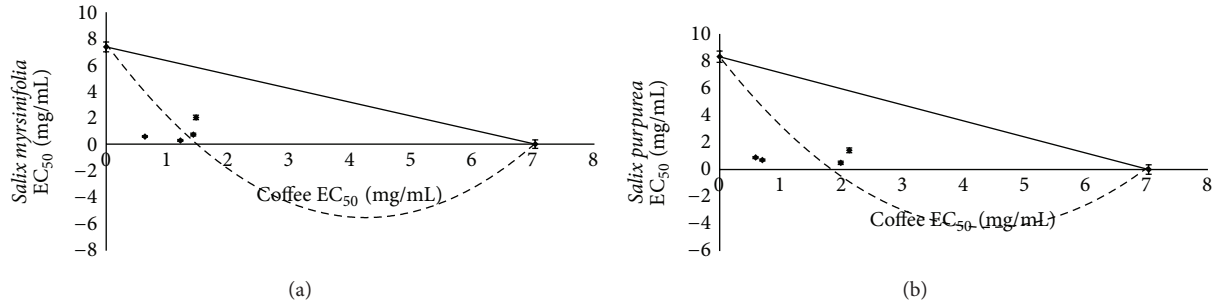
In examining the reducing power activity, we observed a synergistic interaction between coffee and both willow extracts. As Figures 5(a) and 5(b) show, isoboles have a concave shape. The results indicate that simultaneous using of drugs and/or other preparation containing studied material could give better effect than expected.

The next analysis was evaluation of ability to inhibition lipid peroxidation (LPO). As in Figures 6(a) and 6(b) isoboles took the concave form. This result indicates that bioactive compounds included in coffee and willow bark acted synergistically and effect of the combination is greater than expected from their individual dose-response curves; the dose of the combination needed to produce the same effect will be less than that for the sum of the individual components.

The isobolographic analysis is quite time-consuming and complicated; that is why we use interaction factor (IF), which provides an explanation for the mode of interaction. It is a simple way to make an assessment of type of interactions between the examined extracts or chemical compounds. As Table 3 presents, isobole curves shown in Figures 4, 5, and 6 are confirmed by the IF, calculated by the ratios of measured activity of samples and theoretically calculated mixture activity (based on the dose response of single components at various concentrations), expressed as EC_{50}.

Determination of interaction factor (IF), like isobolographic analysis, is independent on mechanism of active compounds activity and requires linear relationship between an activity and sample concentration. Its crucial advantage is possibility of studies of interactions between any number of components and the fact that this is definitely less complicated. Moreover, the "strength" of interaction may be estimated approximately based on IF value. Therefore, as mentioned above, this index can be used for the rapid assessment of the interaction between the two active ingredients [31].

Phytotherapy is one of the oldest branches of conventional medicine, which is experiencing continuous growth in popularity. Using a combination of plant extracts, we can achieve better results than with one drug, often a greater dose. The key issue seems to be also minimizing or eliminating the side effects resulting from the use of medicines in smaller but therapeutically effective dose [3]. The synergism of medicinal plants is considered in three categories: synergism in the individual extracts, synergistic in herbal mixtures, and synergy in combining herbal medicine with synthetic drug. An example of synergism in herbal mixtures might be the interaction of ingredients of nettle root extract and bark of the African plum tree. The combined use of both raw extracts inhibits 5α-reductase and aromatase a greater extent than it would result from the sum of the activity of the individual components. In turn, in order to confirm the synergism between herbal



(a) (b)

Figure 6: Isobole curves for 50% inhibition of lipid peroxidation (LPO) of coffee and willow mixtures: (a) coffee with *S. myrsinifolia*; (b) coffee with *S. purpurea*. EC_{50}-extract concentration (mg/mL) provided 50% of activity based on a dose-dependent mode of action.

Table 3: Comparison of interaction factors (IF) of mixtures of coffee with willow bark preparation.

Sample	Willow bark	Activity	A_M*	A_T**	IF***
Coffee/willow bark preparation mixture (1 : 1 [w/w])	*S. myrsinifolia*	Antiradical potential	9.97	6.66	**1.50**
		Reducing power	1.49	2.28	**0.65**
		Inhibition of lipid peroxidation	1.27	7.55	**0.17**
	S. purpurea	Antiradical potential	5.67	8.84	**1.48**
		Reducing power	1.79	3.35	**0.54**
		Inhibition of lipid peroxidation	1.40	7.67	**0.18**

*Measured activity (A_M) of a mixture of samples (expressed as EC_{50} [mg/mL]).
**Theoretically calculated mixture activity (A_T) (based on the dose response of single components at various concentrations) (expressed as EC_{50} [mg/mL]).
***Interaction factor (IF) value < 1 indicates synergistic interaction; IF > 1 indicates antagonism; IF ≈ 1 indicates additional interactions.

remedy and synthetic drug, green tea with antibacterial agent ciprofloksacin was tested. Inhibition of bacterial growth and inflammation were significantly higher after administration of the drug in combination with green tea than in the case of the drug itself. This indicates a synergistic interaction of the active ingredients of tea (mainly catechins) with ciprofloksacin [74]. Williamson [3] in his work describes synergistic interactions of many herbs, including licorice (*Glycyrrhiza gabbros*), ginkgo biloba (*Ginkgo Biloba*), pepper (*Kava-Kava*), and valerian (*Valeriana officinalis*). So far in the available literature, there are no studies on the properties of coffee and willow mixtures.

4. Conclusion

Presented preliminary study clearly showed that both coffee and willow bark are sources of multidirectional antioxidant compounds. Additionally, phytochemicals from willow bark possessed hydrophilic character and thermostability which justifies their potential use as an ingredient in coffee beverages. It has been found that the biologically active compounds contained in the analyzed raw materials interact with each other, thus affecting their activity. Antagonism is demonstrated with respect to the ABTS radical neutralizing capacity, while with other determinations performed synergistic interaction between the active compounds, the coffee extract, and the individual willows was observed.

Proposed mixtures may be used in the prophylaxis or treatment of some civilization diseases linked with oxidative stress. Most importantly, especially strong synergism was observed for phytochemicals able to prevent lipids against oxidation, which may suggest protective effect for cell membrane phospholipids. Obtained results indicate that extracts from bark tested *Salix* genotypes as an ingredient in coffee beverages can provide health promoting benefits to the consumers; however, this issue requires further study.

Conflict of Interests

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

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Research Article

Oral and Intraperitoneal Administration of Quercetin Decreased Lymphocyte DNA Damage and Plasma Lipid Peroxidation Induced by TSA In Vivo

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Our previous study showed that quercetin enhances the anticancer effect of trichostatin A (TSA) in xenograft mice given quercetin intraperitoneally (10 mg/kg, 3 times/week). Herein, we investigate whether quercetin administered orally exerts such an effect and prevents the cytotoxic side effects of TSA. We found that quercetin given orally (20 and 100 mg/kg, 3 times/week) failed to enhance the antitumor effect of TSA although it increased the total quercetin concentration more than quercetin administered intraperitoneally in the plasma. The compound quercetin-3-glucuronide (Q3G) increased the most. However, quercetin administered intraperitoneally increased the total quercetin level in tumor tissues more than oral quercetin. Oral and intraperitoneal administration of quercetin similarly decreased lymphocyte DNA damage and plasma lipid peroxidation level induced by TSA. Furthermore, we found that the enhancing effect of Q3G on the antitumor effect of TSA and the incorporation of Q3G was less than that of quercetin in A549 cells. However, we found that A549 cells possessed the ability to convert Q3G to quercetin. In conclusion, different from quercetin administered intraperitoneally, quercetin administered orally failed to enhance the antitumor effect of TSA because of its metabolic conversion. However, it prevented TSA-induced DNA damage and lipid peroxidation.

1. Introduction

Quercetin is a common flavonoid found in various vegetal foods, such as onions, apples, and green leafy vegetables, and studies suggest that quercetin possesses various physiological properties including antioxidative and anti-inflammatory properties [1, 2]. For example, oral quercetin effectively decreases carbon tetrachloride-induced oxidative liver injury in mice and suppresses the depletion of glutathione peroxidase and superoxide dismutase [1]. Bureau et al. [2] demonstrated that quercetin reduces the inflammation-induced apoptotic neuronal cell death in a cell culture system. Our recent study showed that quercetin inhibits benzo[a]pyrene-induced lung inflammation in gerbils and in A549 cells which may be due to the downregulation of the JNK pathway [3]. In addition, growth evidence shows that quercetin may regulate intracellular signaling pathways, which are associated with cell proliferation and apoptosis, and then prevent cancer development [4, 5]. Quercetin may also enhance the effects of anticancer drugs [6, 7]. For example, quercetin significantly increases the anticancer effect of doxorubicin in breast cancer cells through several mechanisms and reduces

the cytotoxic side effects of doxorubicin in nontumor cells [7]. Chen and Kang [8] found that quercetin in combination with trichostatin A (TSA), a histone deacetylase inhibitor with antiproliferation effects on various cancer cells [9, 10], cooperatively induces cell death in human leukemia HL-60 cells. Our previous study demonstrated that quercetin synergistically enhances the antitumor effect of TSA on human lung carcinoma cells (A549) through upregulation of p53 protein, at least in part [11]. We also found that quercetin administered by intraperitoneal (i.p.) injection decreases tumor size and upregulates the expression of p53 in tumor in xenograft mice, indicating that activating p53 may play an important role in the effect of i.p. injection quercetin [11]. However, whether oral administration of quercetin enhances the antitumor effect of TSA is unclear.

It has been shown that after quercetin intake, conjugated metabolites, such as quercetin glucuronides and quercetin sulfates, rather than quercetin aglycone are prevalent in human plasma due to its efficient phase II metabolism [12]. Similar results have been observed in animal studies [13, 14]. The biochemical and biophysical properties among quercetin and quercetin metabolites may be different because of structure modification [15], although some metabolites remain physiologically active [16, 17]. In addition, the incorporation efficiency of quercetin and its metabolites into cells may be different because of the different polarity of each compound. Thus, it is reasonable to assume that the influence of quercetin administered orally or intraperitoneally is different on the antitumor effect of TSA or other anticancer drugs.

The aim of this study was to investigate the effect of oral administration of quercetin on the antitumor effect and toxic effect of TSA in tumor bearing mice, compared with intraperitoneal administration. We also determined the distribution of quercetin and its metabolites in the plasma and tumor tissues to investigate the mechanisms that contributed to the effects of oral administration of quercetin. The doses of quercetin administered orally were at 20 and 100 mg/kg body weight 3 times/week, which were tenfold higher than the intraperitoneal doses. Besides, in our previous study we found that the plasma, which was obtained from Mongolian gerbils 2 h after quercetin feeding by gavage at 100 mg/kg body weight/week, induced A549 cell growth arrest in vitro [14]. In addition, we also used A549 cells to compare the intracellular accumulation and the enhancing effect of quercetin and quercetin-3-glucuronide (Q3G) on the antiproliferation effect of TSA.

2. Materials and Methods

2.1. Reagents. All chemicals used were reagent grade or higher. Quercetin and TSA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI medium 1640, fetal bovine serum, trypsin, penicillin, streptomycin, sodium pyruvate, and nonessential amino acids were purchased from GIBCO/BRL (Rockville, MD, USA). Q3G and quercetin-3′-sulfate (Q3′S) was synthesized and purified according to the methods described previously [18, 19]. Mass spectrometric analysis was performed with electrospray ionisation in the negative-ion mode and in the range of m/z 100–700. The purity of Q3G and Q3′S was checked by HPLC and was found to be about 95% and ≧99%, respectively. The level of free quercetin in each of the synthetic compounds was undetectable (the limit of detection ≦6 pmole/injection).

2.2. The Animal Study. All study protocols were approved by the Institutional Animal Care and Use Committee at Chung Shan Medical University, and animal care followed the guidelines of the National Research Council [20]. Male nude mice aged 5 to 6 weeks were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The animals were housed in specific pathogen-free conditions with an alternating 12-hour light:dark cycle. After being acclimated for 1 week, the animals were subcutaneously injected in the right flank with A549 cells at a dose of 5×10^6 cells (in 200 μL of matrigel; BD Biosciences, Franklin Lakes, NJ, USA). Tumor nodule volumes were measured once a week with the following formula: $(L1 \times L2^2)/2$ [21], where $L1$ is the long axis and $L2$ is the short axis of the tumor. Three weeks after cell injection, tumor nodules were palpable. The mean tumor volume was not significantly different among the groups at this time. The animals were then randomly assigned to the following six groups ($n = 6$/group) for 16 weeks: control, TSA, OL+TSA, OH+TSA, IL+TSA, and IH+TSA for TSA alone or in combination with quercetin treatment. TSA was given twice a week (0.5 mg/kg body weight) by i.p. injection, while quercetin was given 3 times a week by oral gavage (OL and OH, 20 and 100 mg/kg body weight, resp.) or by i.p. injection (IL and IH, 2 and 10 mg/kg body weight, resp.). Stock solutions of ethanol-TSA and ethanol-quercetin were freshly prepared before each injection and were diluted with 0.9% saline solution. The injection or gavage volume was 200 μL (containing 25 μL of stock solution and 175 μL 0.9% saline solution) each time. The control group was given 200 μL of vehicle (25 μL ethanol and 175 μL 0.9% saline solution) orally and intraperitoneally, respectively. All animals were allowed free access to a standard rodent diet (Lab 5001, Purina Mills, St. Louis, MO) and water during the study. During the 18-week experimental period, the body weights of the mice were recorded weekly. Blood samples were collected at weeks 4–10 (for determining quercetin concentration) and at week 17 (for determining lipid peroxidation level) from the retro-orbital plexus of the nude mice under deep isoflurane anesthesia. After the experiment, the animals were sacrificed. Blood and tissue samples were collected and were stored at −80°C until analyzed.

2.3. Measurement of Total Quercetin and Its Metabolites. We measured total or individual concentrations of quercetin and its metabolites, Q3G and Q3′S, in plasma and tumor tissues according to previously described methods [13, 18]. To determine the total concentration of quercetin, plasma (100 μL) or homogenized tissue samples (0.03 g tissue in 300 μL PBS) were incubated with a 5 μL *Helix pomatia* enzyme mixture (~7500 U β-glucuronidase and ~750 U sulfatase in 0.5 M sodium acetate with 28 mM ascorbic aicd, pH 5.0) at 37°C for 2 h. To determine the individual concentration of quercetin

metabolites, samples were added to the same volume of sodium acetate buffer but without hydrolyzed enzyme. Then 200 μL of acetonitrile and 100 μL of 20% H_3PO_4 were added to the mixture to deproteinize. After centrifugation at 2300 ×g and 10°C for 10 min, the supernatant was filtered and analyzed by HPLC with spectrophotometric detection at 370 nm. For HPLC analysis, an ODS3 reverse-phase silica column (250 × 4.6 mm, Phenomenex, Macclesfield, UK) was used. Solvents A (water : tetrahydrofuran : trifluoroacetic acid, 98 : 2 : 0.1, *v* : *v* : *v*) and B (acetonitrile) were employed to run a gradient program at a flow rate of 1.0 mL/min. The gradient program was as follows: 17% B (2 min), increasing to 25% B (5 min), 35% B (8 min), 50% B (5 min), and then to 100% B (5 min). To clean up the column, the solvent was maintained at 100% B for 5 min and then was returned to 17% B for the following 15 min.

2.4. Lipid Peroxidation and DNA Damage Assay. To determine the oxidative stress in the mice, we measured lipid peroxidation levels by determining thiobarbituric acid-reactive substances (TBARS) in the plasma using the fluorimetrical method which has been described previously [22]. The lymphocytes in the plasma, which were collected when the animals were sacrificed, were separated using Ficoll-Paque (GE Healthcare, Uppsala, Sweden). The DNA damage in the lymphocytes was determined by comet assay [23]. In brief, the lymphocytes were suspended in low-melting-point agarose in PBS at 37°C and were then placed on a frosted glass microscope slide precoated with a layer of 1% normal-melting-point agarose. After the application of a third layer of 1% normal-melting-point agarose, the slides were immersed in cold-lysing solution (10 mM Tris, 2.5 M NaCl, 100 mM Na_2EDTA, 1% sodium N-laurylsarcosine, 1% Triton X-100, and 10% dimethylsulphoxide) for 1 h at 4°C. The slides were then placed in an electrophoresis tank, and the DNA was allowed to unwind for 15 min in the alkaline solution. Electrophoresis was performed using the method described by Collins et al. [24]. The images were analyzed by the Interactive Image Analysis Comet Assay III (Perceptive Instruments, Haverhill, UK), and DNA strand breaks were expressed as a percentage of DNA in the tail (% DNA in tail).

2.5. The Cell Culture. A549 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI medium 1640 containing 10% (*v*/*v*) FBS, 0.37% (*w*/*v*) $NaHCO_3$, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified incubator under 5% CO_2 and 95% air. An equal number (2.5×10^4/mL) of cells were incubated for 24 h before the various treatments. After being washed twice with PBS (pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4), the cells were incubated in fresh culture medium containing TSA (25 ng/mL equal to 82.5 nM) alone or in combination with quercetin, and Q3G (5 μM) for indicated time. Stock solutions of ethanol-TSA (100 μg/mL) were freshly prepared before each experiment. Quercetin and its metabolites were prepared in methanol at a stock concentration of 20 mM. The final solvent concentration in the medium was ≦0.05%. The medium was replaced every day. Cell growth was mainly measured by MTT colorimetric assay. The concentration of quercetin and Q3G incorporated into the cells was determined by the method mentioned above. In addition, according to the method described by Kawai et al. [25], we determined the intracellular β-glucuronidase activity by a colorimetric assay with phenolphthalein mono-β-glucuronide as the substrate. After 30 min of incubation of the cell lysate with a substrate, the formation of phenolphthalein aglycone was determined by monitoring the absorbance of samples at 550 nm.

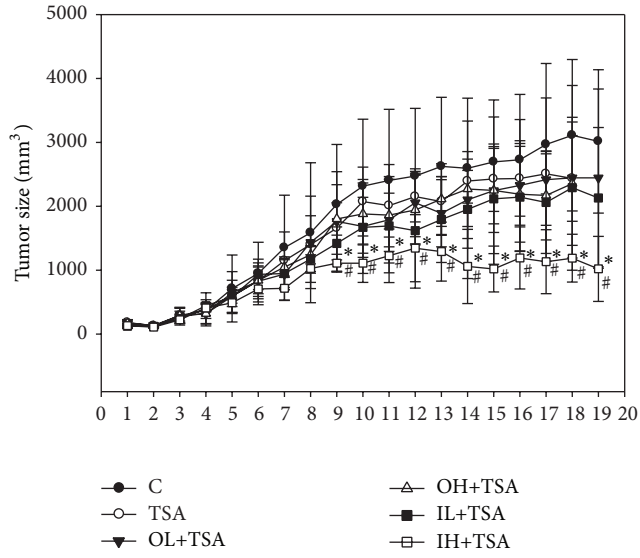

Figure 1: Effects of trichostatin A (TSA) alone or in combination with quercetin on tumor growth in tumor-bearing mice. Thirty-six male nude mice were injected with A549 cells and after 3 weeks were treated with TSA alone (0.5 mg/kg body weight) or in combination with quercetin (OL and OH, quercetin administered orally at 20 and 100 mg/kg body weight, resp.; IL and IH, quercetin administered intraperitoneally at 2 and 10 mg/kg body weight, resp.) for 16 weeks as described in Section 2. The control group was administered the vehicle only. Values (means ± SD, $n = 6$) with a ∗ denote a significant different from the control group, while a # is significantly different from the TSA group ($P < 0.05$).

2.6. Statistical Analysis. Values are expressed as means ± SD. We used one-way factorial analysis of variance followed by Duncan's multiple range test for comparisons of group means or Student's *t*-test for two-group comparisons. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Tumor Volume in Tumor Bearing Mice. Consistent with our previous study, TSA at 0.5 mg/kg body weight did not significantly decrease tumor size compared to the control group (Figure 1). OL+TSA, OH+TSA, and IL+TSA treatments also had no significant effect on tumor size in A549 tumor-bearing nude mice compared to the control group ($P > 0.05$). Only IH+TSA significantly decreased tumor growth in A549

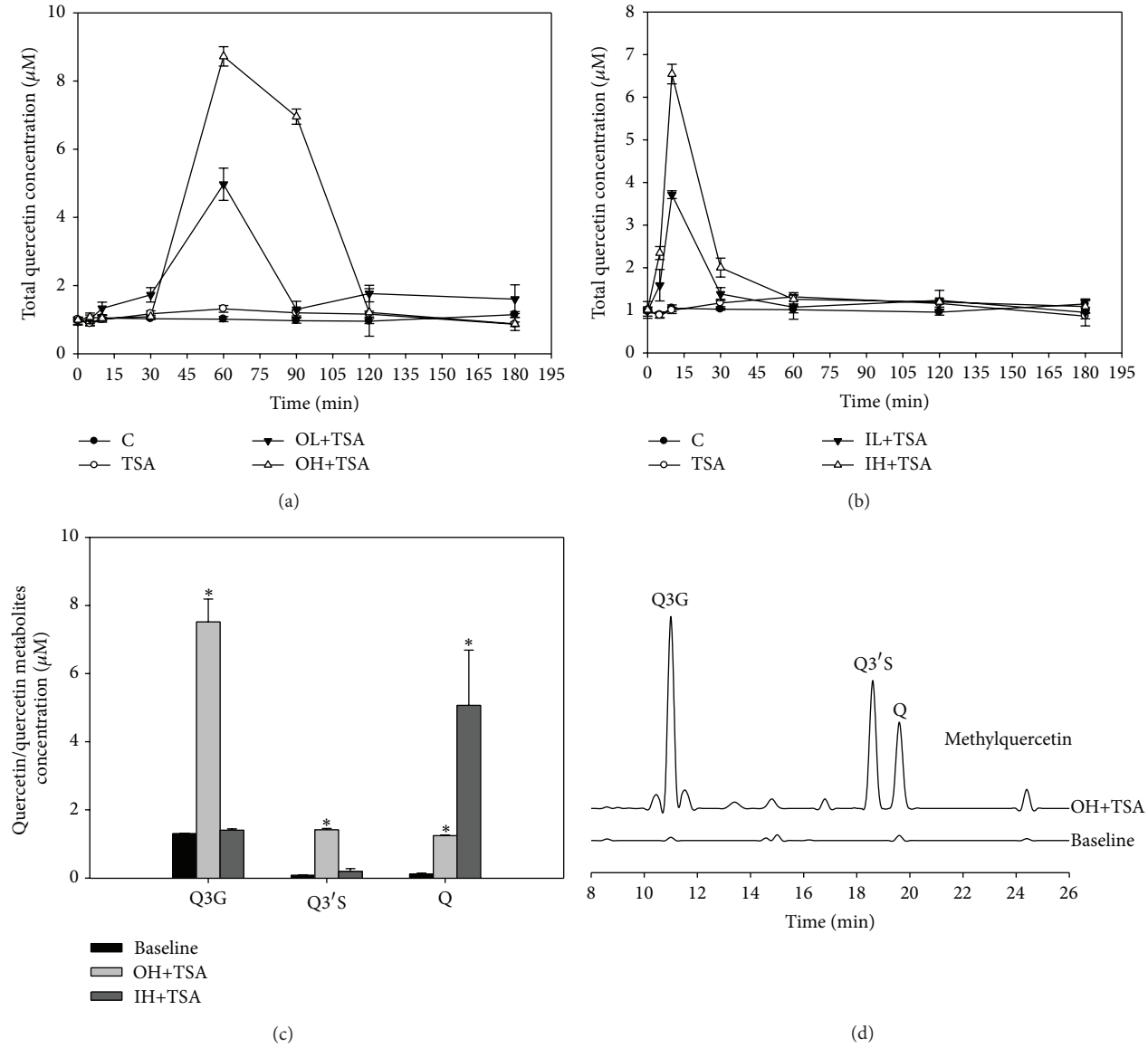

Figure 2: Total (a and b) and individual (c) concentrations of quercetin (Q) and its metabolites in plasma of tumor-bearing mice with various treatments as well as the HPLC chromatograms in the plasma (d). Thirty-six male nude mice were injected with A549 cells and after 3 weeks were treated with TSA alone (0.5 mg/kg body weight) or in combination with Q (OL and OH, Q administered orally at 20 and 100 mg/kg body weight, respectively; IL and IH, Q administered intraperitoneally at 2 and 10 mg/kg body weight, resp.) for 16 weeks as described in Section 2. The control group was administered the vehicle only. After various treatments for 4–10 weeks, total Q concentrations were determined after Q administration. Individual concentration of Q, quercetin-3-glucuronide (Q3G), and quercetin-3′-sulfate (Q3′S) in the plasma in OH+TSA and IH+TSA groups were determined at 1 h and 10 minutes after Q administration, respectively. Values are expressed as means ± SD ($n = 6$) and those of the same compound not sharing a common letter are significantly different ($P < 0.05$).

tumor-bearing nude mice since week 9 compared with the control or TSA alone group. Quercetin alone, given orally and intraperitoneally, did not affect the tumor size; body weight of mice among groups was not significantly different ($P > 0.05$) (data not shown).

3.2. The Levels of Quercetin and Its Metabolites in Plasma and Tumor Tissues. After treatment with TSA alone or in combination with quercetin for 4–10 weeks, the time course of the total quercetin concentration (the sum concentration of glucuronidated and sulfated quercetin and quercetin aglycone) after quercetin administration was determined by HPLC. The results showed that both oral and intraperitoneal administration of quercetin increased the total plasma concentration of quercetin in a dose dependent manner. The total quercetin level in the OL+TSA and OH+TSA groups reached a maximum approximately 1 h after quercetin administration (Figure 2(a)), while the maximum levels in the IL+TSA and IH+TSA groups appeared as early as 10 minutes (Figure 2(b)). We further determined individual levels of Q3G, Q3′S, and quercetin aglycone in the plasma at 1 h and at 10

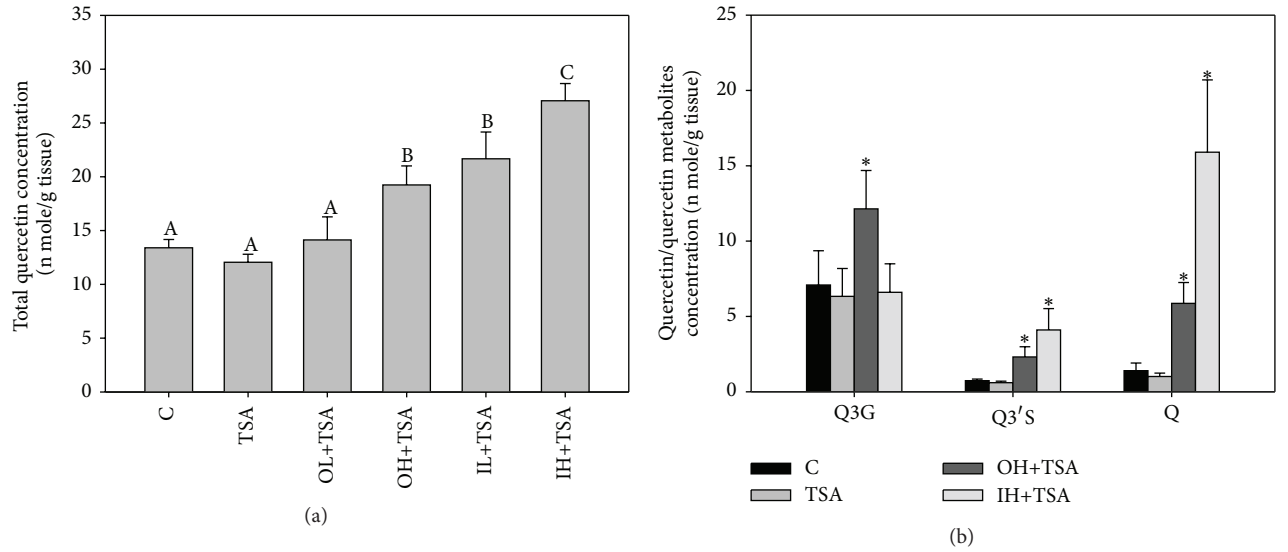

Figure 3: Total (a) and individual (b) concentrations of quercetin (Q) and its metabolites in tumor tissues of tumor-bearing mice with various treatments. Thirty-six male nude mice were injected with A549 cells and after 3 weeks were treated with TSA alone (0.5 mg/kg body weight) or in combination with Q (OL and OH, Q administered orally at 20 and 100 mg/kg body weight, respectively; IL and IH, Q administered intraperitoneally at 2 and 10 mg/kg body weight, resp.) for 16 weeks as described in Section 2. The control group was administered the vehicle only. After the mice were sacrificed, total and individual concentrations of Q, quercetin-3-glucuronide (Q3G), and quercetin-3′-sulfate (Q3′S) in the tumor tissues were determined. Values are expressed as means ± SD ($n = 6$) and those of the same compound not sharing a common letter are significantly different ($P < 0.05$).

minutes in the OH+TSA and IH+TSA groups, respectively. As shown in Figure 2(c), all Q3G, Q3′S, and quercetin levels in the OH+TSA groups increased significantly compared with those at the base line. However, Q3G was the major compound present in the plasma in the OH+TSA group and its level was about 5–6-fold of quercetin and Q3′S, meanwhile, in the IH+TSA group only the concentration of quercetin was significantly higher than that at the base line. Figure 2(d) shows the HPLC chromatograms in the plasma of the OH+TSA group and at baseline. Methylated quercetin also increased in the OH+TSA group, but the concentration was markedly lower than Q3G and Q3′S ($0.07 \pm 0.01\,\mu$M).

After the animals were sacrificed, we determined the levels of quercetin and its metabolites in the tumor tissues. The results showed that the total quercetin level in the tumor tissues increased significantly in the OH+TSA, IL+TSA, and IH+TSA groups compared with the control group. The total quercetin levels were in the following order: IH+TSA > IL+TSA, OH+TSA > OL+TSA (Figure 3(a)). All Q3G, Q3′S, and quercetin levels increased significantly in the OH+T group compared with the control group (Figure 3(b)). On the other hand, the quercetin level increased significantly to about 11-fold in the IH+TSA group compared with the control group. In addition, in the IH+TSA group, the Q3′S level also increased significantly.

3.3. DNA Damage and Lipid Peroxidation. TSA alone significantly increased lymphocyte DNA damage up to 8.2-fold compared to the control group (Figure 4(a)). TSA also significantly increased the lipid oxidative product level in the plasma in tumor bearing mice (Figure 4(b)). In contrast, oral and intraperitoneal administration of quercetin significantly and similarly decreased TSA-induced lymphocyte DNA to the base line level ($P < 0.05$). In addition, quercetin administered orally and intraperitoneally decreased lipid peroxidation induced by TSA. The TBARS levels in the groups with quercetin administered intraperitoneally were even lower than in the control group ($P < 0.05$).

3.4. The Effect and the Incorporation of Q3G in A549 Cells Exposed to TSA. Because Q3G was the dominant metabolite present in the plasma after oral administration of quercetin, we compared the enhancing effect of Q3G and quercetin at $5\,\mu$M (this concentration is comparable to the maximum level in the plasma after quercetin administration) on the antiproliferation effect of TSA (25 ng/mL) in A549 cells. TSA alone inhibited the growth of A549 cells by 35% after incubation for 72 h (Figure 5(a)). Both quercetin and Q3G significantly enhanced the antiproliferation effect of TSA. However, the enhancing effect of quercetin was significantly better than Q3G (76.7% versus 38.2%, $P < 0.05$). Furthermore, we compared the levels of quercetin and its metabolites in A549 cells incubated with quercetin or Q3G. After incubation with quercetin for 1 and 4 h, the intracellular quercetin concentration increased significantly in a time-dependent manner (Table 1). Q3G incubation also significantly increased the intracellular Q3G concentration in a time-dependent manner. However, the increased level of quercetin + Q3G induced by Q3G incubation was only 43% of that induced by quercetin incubation at 4 h. In addition, we surprisingly found that Q3G incubation for 4 h also increased the intracellular quercetin level. To investigate

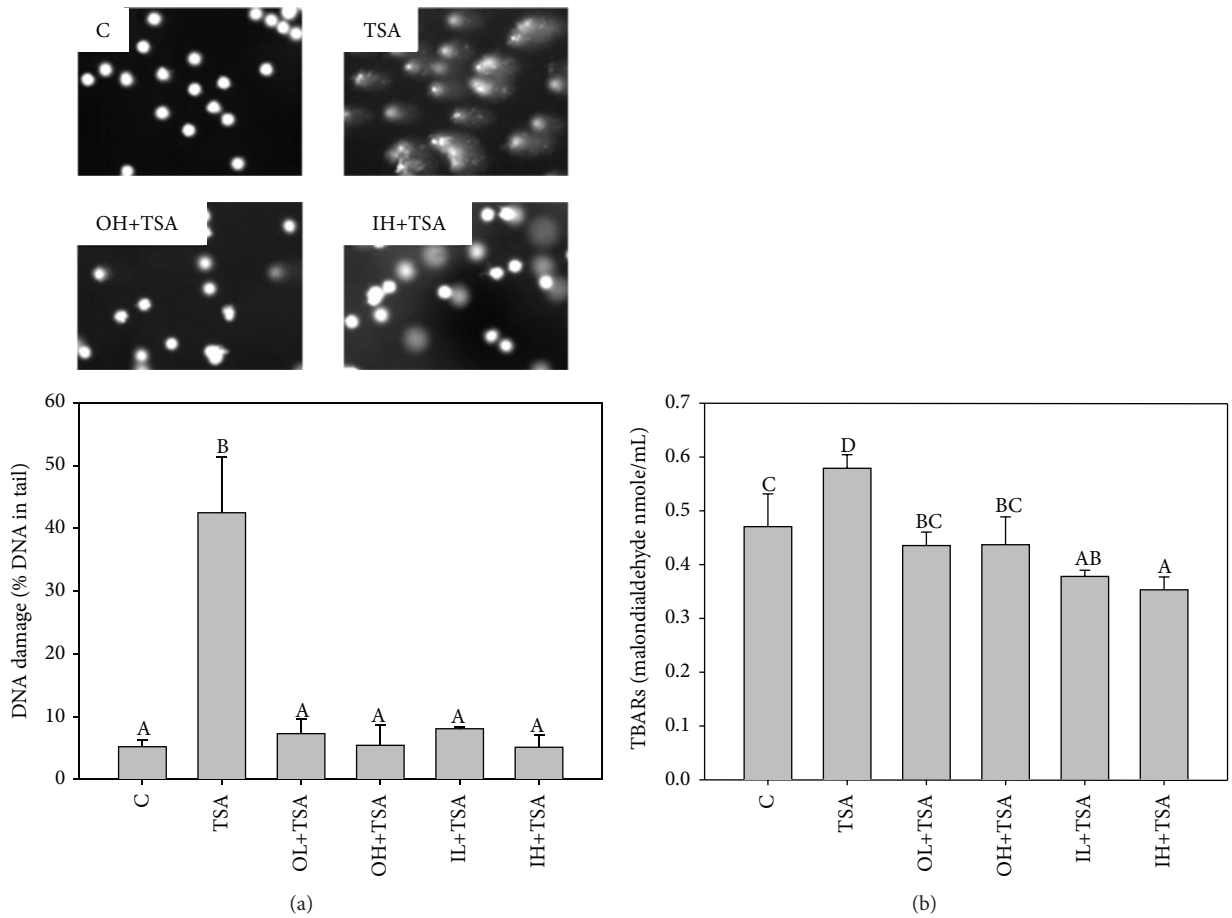

Figure 4: Lymphocyte DNA damage (a) and lipid peroxidation (TBARS level) (b) in tumor-bearing mice with various treatments. Thirty-six male nude mice were injected with A549 cells and after 3 weeks were treated with TSA alone (0.5 mg/kg body weight) or in combination with quercetin (OL and OH, quercetin administered orally at 20 and 100 mg/kg body weight, resp.; IL and IH, quercetin administered intraperitoneally at 2 and 10 mg/kg body weight, resp.) for 16 weeks as described in Section 2. The control group was administered the vehicle only. Values (means ± SD, $n = 6$) not sharing a common letter are significantly different ($P < 0.05$).

whether A549 cells possessed the ability to converted Q3G to quercetin, we determined intracellular β-glucuronidase activity. As shown in Figure 5(b), after incubation with Q3G for 4 h, β-glucuronidase activity increased significantly in A549 cells compared with the control and quercetin groups.

4. Discussion

Quercetin, an antioxidative flavonoid widely distributed in plants, has been shown to possess cancer preventive effects on various cancer cells. In addition, growing evidence shows that quercetin may enhance the effect of anticancer drugs [7, 11]. In our previous study [11] we found that quercetin administered intraperitoneally at 10 mg/kg body weight significantly enhanced the antitumor effect of TSA in tumor bearing mice which may be due to the upregulation of p53 expression. However, in the present study, the results demonstrated that quercetin (20 and 100 mg/kg body weight, 3 times per week) given by gavage failed to exert such an effect, despite the level of total quercetin (quercetin plus its metabolites) in the plasma being higher and the duration time being longer in the OH+TSA group compared to the IH+TSA group. The contributing factor for the ineffectiveness of oral quercetin administration could be associated

Table 1: Accumulation of quercetin (Q) and quercetin-3-glucuronide (Q3G) in A549 cells[1].

Group	Compound	
	Q3G (*n* mole/g protein)	Q (*n* mole/g protein)
	1 h	
C	1.39 ± 0.01^{a}	0.70 ± 0.35^{a}
Q	1.40 ± 0.01^{a}	2.36 ± 0.69^{b}
Q3G	1.99 ± 0.03^{b}	0.77 ± 0.21^{a}
	4 h	
C	1.39 ± 0.01^{a}	0.70 ± 0.35^{a}
Q	1.88 ± 0.09^{b}	10.70 ± 1.14^{c}
Q3G	2.48 ± 0.01^{c}	2.97 ± 0.10^{b}

[1]The cells were incubated with 5 μM quercetin Q or Q3G for 1 and 4 h. Values (means ± SD, $n = 3$) of the same compound and the same time point not sharing a common letter are significantly different ($P < 0.05$).

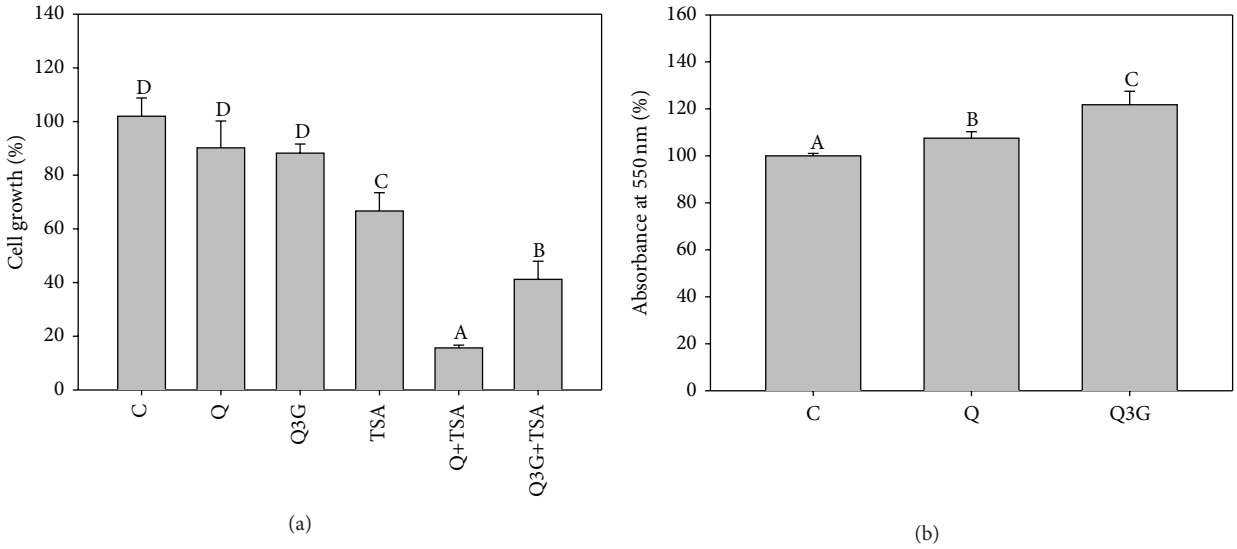

Figure 5: Effects quercetin (Q) or quercetin-3-glucuronide (Q3G) on the growth (a) or intracellular β-glucuronidase activity (b) of A549 cells exposed to trichostatin A (TSA) or not. To determine cell growth, the cells were incubated with TSA (25 ng/mL), 5 μM Q, or Q3G alone or combined for 72 h. Meanwhile, the cells were incubated with 5 μM Q or Q3G only for 1 or 4 h to study the intracellular β-glucuronidase activity which was assayed by determining the formation of phenolphthalein after incubation of cell lysates with phenolphthalein monoglucuronides. Values (means ± SD, $n = 3$) not sharing a common letter are significantly different ($P < 0.05$).

with the metabolic conversion of quercetin in vivo. A lot of chemopreventive compounds including phytochemicals may have different biological effects between oral administration and intraperitoneal administration because the absorbance and metabolism of these compounds affect their concentrations and structures in vivo [26, 27]. For example, although curcumin, a phytochemical present in herbs and spices, possesses various biological activities and inhibits the growth of various cancer cells in vitro, its low systemic bioavailability following oral dosing has been suggested to limit access of sufficient concentrations for pharmacological effect in certain tissues [26]. However, in the gastrointestinal tract, curcumin may attain biologically active levels.

Consistent with previous studies [13, 14], the present study also showed that oral administration of quercetin mainly increased quercetin metabolites, especially Q3G, in the plasma of mice. In contrast, i.p. injections of quercetin mainly increased the level of quercetin itself. These data suggest that the overall enhancing effects of quercetin metabolites on the antitumor effect of TSA are less than quercetin itself in vivo. Our in vitro study supports this postulation because we found that Q3G had little effect on the antiproliferation effect of TSA in A549 cells than quercetin itself (Figure 5). We cannot rule out that the other quercetin metabolites, such as Q3′S and methylated quercetin, may be more efficient than Q3G. However, because Q3G is the dominant product found in plasma in nude mice (the concentrations of Q3G, Q3′S, and methylated quercetin: 7.5 μM, 1.4 μM, and 0.07 μM, resp.), we speculated that the effects of quercetin metabolites are mainly attributed to the effect of Q3G. Growing evidence shows that the biochemical and biophysical properties among quercetin and quercetin metabolites are different [4]. However, few studies address the effect of Q3G on cancer development. A recent study using doses of 21–210 μM, which, however, were 4–40-fold of those used in our study, found that Q3G suppresses MCF-7 breast cancer cell migration induced by plasmin [28].

Furthermore, we determined the levels of quercetin and its metabolites in tumors. To our knowledge, this is the first report showing the presence of quercetin and its metabolites in tumor tissue. In contrast to the findings in plasma samples, the level of total quercetin in tumor tissues in the IH+TSA group was higher than in the OH+TSA group. Quercetin was still the dominant compound present in tumor tissues in the IH+TSA group while Q3G was the major compound present in tumor tissues in the OH+TSA group. These findings suggest that quercetin entered cells more efficiently than its metabolites which may be due to the higher polarity of Q3G and Q3′S than that of quercetin. Our in vitro study also supports the in vivo finding; that is, after incubation with Q3G, the total level of quercetin in cells was lower than that of quercetin incubation (Table 1).

An unexpected finding herein was that oral administration of quercetin and Q3G exposure increased quercetin levels in tumor tissues and A549 cells, respectively, suggesting that A549 cells may produce glucuronidase and deconjugated Q3G into quercetin. It has been suggested that quercetin metabolites may convert to quercetin due to some physiological conditions [25, 29]. Kawai et al. [25] demonstrated that Q3G was selectively incorporated into activated macrophages and then deconjugated to quercetin. Our further study with cell culture experiments demonstrated that Q3G incubation in A549 cells significantly induced intracellular β-glucuronidase activity, indicating the possibility that Q3G converts to quercetin and increases its bioactivity in lung tumor cells. However, more studies are

warranted to investigate whether other lung cancer cells possess such a feature.

TSA has been shown to induce oxidative stress and leads to apoptosis of HeLa cervical cancer cells [30]. Our previous study showed that TSA increases oxidative stress and DNA damage in A549 cells [31]. In the in vivo study, we found that TSA also increased lipid peroxidation and lymphocyte DNA damage in tumor-bearing mice. In fact, various chemotherapy drugs exert toxic effects on normal cells which lower the treatment tolerance of patients. Although quercetin administered orally at 20 and 100 mg/kg body weight failed to enhance the antitumor effect of TSA, the present study demonstrated that oral supplementation with quercetin decreased the TSA-induced toxic effects without influencing its antitumor effect. The protective effects of quercetin given orally were comparable to that given intraperitoneally. A recent study also demonstrated that quercetin (50 mg/kg/day, intraperitoneally) reduces cisplatin nephrotoxicity in rats without compromising its antitumor activity [32]. The study showed that the underlying mechanisms are associated with the antioxidative and anti-inflammatory activity of quercetin. Although the precise mechanisms under the protective effect of oral administration of quercetin remain unclear in our study, these results suggest that oral intake of quercetin has a potential protective compound against chemotherapy toxicity without compromising the efficiency of chemotherapy.

In conclusion, the present study showed that the oral administration of quercetin at doses of 20 and 100 mg/kg body weight, 3 times per week, failed to enhance the antitumor effect of TSA. This result was associated with the metabolic conversion of quercetin in vivo. However, quercetin administered orally diminished TSA-induced adverse effects in nude mice and the effect was similar to that of i.p. injections. Further studies are warranted to investigate the effect of quercetin administered orally at higher doses.

Conflict of Interests

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

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Review Article

Food-Derived Bioactive Peptides on Inflammation and Oxidative Stress

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Chronic diseases such as atherosclerosis and cancer are now the leading causes of morbidity and mortality worldwide. Inflammatory processes and oxidative stress underlie the pathogenesis of these pathological conditions. Bioactive peptides derived from food proteins have been evaluated for various beneficial effects, including anti-inflammatory and antioxidant properties. In this review, we summarize the roles of various food-derived bioactive peptides in inflammation and oxidative stress and discuss the potential benefits and limitations of using these compounds against the burden of chronic diseases.

1. Introduction

Chronic noncommunicable diseases such as cardiovascular diseases and cancer make up an increasing share of the global disease burden. With the increased longevity and improvement in global living standards, these "diseases of affluence" are now widespread in both developed and developing nations [1, 2]. Indeed, cardiovascular diseases such as atherosclerosis and its complications are now the leading cause of mortality and morbidity worldwide, closely followed by various cancers [3, 4]. Increased life spans have also meant corresponding increase in aging-related diseases in both developing and developed nations which may overwhelm their health care systems. Although atherosclerosis, cancers, and aging-related diseases can have diverse etiologies, they share many underlying pathological mechanisms including abnormalities in inflammatory responses and oxidative stress [5–7]. Thus targeting of the common pathological pathways has gained increasing attention in recent years for both prevention and treatment of chronic diseases.

While a number of commercially available anti-inflammatory and antioxidant drugs exist, none of these are free from side effects. Given the concerns about the side effects from prolonged usage of synthetic compounds, there is growing interest in the therapeutic applications of natural compounds and their derivatives as safer alternatives, either as functional foods or nutraceuticals. Food proteins from both plant and animal sources have been used to obtain a wide range of bioactive peptides [8]. Bioactive peptides are generally short peptides (3–20 amino acids) derived from proteins that can exert biological activities over and above their expected nutritional value [9]. These peptides are often functionally inactive within the native proteins and must be released by proteolysis (*in vivo* digestion, *in vitro* enzymatic hydrolysis, or bacterial fermentation) to achieve their specific "bioactive" roles. Many of these food-derived peptides demonstrate antihypertensive, anti-inflammatory, antidiabetic, and antioxidant properties under experimental conditions [10–12]. While some studies have observed the effects of single peptides, many others have examined protein hydrolysates composed of a mixture of diverse bioactive peptides [13–15]. Given their food-based sources and a perceived lack of serious side effects, bioactive peptides and peptide-rich protein hydrolysates can potentially provide a better alternative to synthetic pharmaceuticals for the prevention and treatment of chronic illnesses that affect an increasing number of people.

While bioactive peptides and peptide-rich protein hydrolysates can have a range of beneficial effects on diverse pathological conditions, this review would mainly focus on

their anti-inflammatory and antioxidant actions. We would also discuss the potential challenges that may limit the use of these compounds as novel therapies against the global burden of chronic diseases.

2. Bioactive Peptides on Inflammation

2.1. Inflammation and Chronic Disease. Inflammation is the body's response to nonlethal injury which is characterized by increased endothelial permeability, leakage of protein-rich exudates, and infiltration of leukocytes into extravascular tissues. While inflammation is essential for resistance to microbial infections and wound healing, excessive and uncontrolled inflammatory changes often lead to chronic diseases. Indeed, vascular inflammation is an early event in the development of atherosclerosis and its complications such as myocardial infarction and stroke. Increasing evidence also links chronic inflammation to many types of cancer which further highlights its key role as a mediator of noncommunicable illnesses. Despite the significance of inflammation, relatively few therapies have been devised to target the inflammatory component of cardiovascular and malignant diseases. The nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin are widely used to prevent and manage cardiovascular diseases, due to its antithrombotic as well as anti-inflammatory properties [16, 17]. Recent studies suggest that NSAIDs may also contribute to beneficial effects against cancers of the gastrointestinal system, further broadening the potential for anti-inflammatory therapies [18]. However, the presence of well-known side effects such as gastric bleeding and ulceration preclude the long-term use of NSAIDs for a large part of the population.

2.2. Pathways of Inflammatory Response. Inflammation is a complex and multisystem event affecting a wide range of cells, tissues, and organs. The vascular endothelium plays a key role as a gate keeper for the extravasation of leukocytes which is a hallmark of inflammation. However, tissue macrophages, epithelial cells, and fibroblasts are often involved in the generation of mediators which impinge upon and subsequently activate the endothelium through expression of leukocyte adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) which recruit leukocytes from the bloodstream and lead to their extravasation through a sequential cascade that involves tethering, rolling, activation, firm adhesion, and, finally, transmigration across the endothelial barrier. Mediators like various proinflammatory cytokines (such as tumor necrosis factor and interleukin (IL)-1β), chemokines (such as IL-8 and monocyte chemoattractant protein-1 (MCP-1)), and reactive oxygen species (ROS, such as superoxide and peroxynitrite) are involved in both the generation and propagation of the inflammatory response. A number of intracellular signaling pathways are activated in the participating cells which include nuclear factor-kappaB (NF-κB), mitogen activated protein (MAP) kinases, and activator protein-1 (AP-1), to name a few [19, 20]. Thus, the markers of inflammation include activation of proinflammatory signaling cascades, upregulation of leukocyte adhesion molecules, tissue infiltration of leukocytes, and increased levels of cytokines and chemokines in the circulation. Given the complexity and diversity of the inflammatory response, an investigation of potential anti-inflammatory agents involves the study of their effects on several of these markers, often using different cellular and intact animal systems for validation.

2.3. Bioactive Peptides on Inflammation in Cellular Systems. Much of the recent knowledge on bioactive peptides has been based on studies performed in cultured mammalian cells. Cell culture systems offer fast, economically feasible, and reproducible assays to analyze and validate the effects of many different compounds on a wide range of inflammatory markers. Peptides and protein hydrolysates derived from food sources such as milk, egg, fish, meat, and soybeans (to name a few) have all been tested for potential beneficial effects in these systems.

Bioactive peptides from milk have been among the first food-derived peptides studied. Milk is rich in caseins and whey proteins, both of which can give rise to a number of peptides with bioactive properties upon further processing such as enzymatic hydrolysis, digestion, and/or fermentation. The tripeptides VPP and IPP, derived from bacterial fermentation of casein, demonstrate inhibitory effects on angiotensin converting enzyme (ACE) in addition to stimulation of nitric oxide (NO) and bradykinin-mediated vasorelaxant pathways, thus suppressing the prohypertensive and proinflammatory mechanisms associated with hypertension and atherosclerosis [21]. Recently, a more direct anti-inflammatory role for VPP has been shown by its ability to attenuate leukocyte-endothelial interactions *in vitro*, largely through inhibition of proinflammatory c-Jun N-terminal kinase (JNK, a type of MAP kinase) pathway [22]. Casein hydrolysates generated by enzymatic digestion and containing a mixture of peptides have also been evaluated for anti-inflammatory properties. For example, digestion of casein with Corolase yields preparations that demonstrate anti-inflammatory effects on activated macrophages [23]. Hydrolysates of whey proteins also show promise in inhibition of inflammatory responses in respiratory and intestinal epithelial cells [24, 25]. Lactoferrin is a milk protein with antimicrobial properties which also exerts anti-inflammatory effects on activated macrophages [26]. Hydrolysis of lactoferrin yields the bioactive peptide lactoferricin, which demonstrates anti-inflammatory effects on human cartilage and synovial cells, suggesting potential benefits in arthritis management [27, 28]. In addition, both human and animal milk contain a number of anti-inflammatory and immunomodulatory compounds such as transforming growth factor-beta (TGF-beta), IL-10, and immunoglobulins which can further modulate the immune system of the gastrointestinal tract; however, these are not strictly "bioactive" peptides as they do not require processing from the native protein for their actions (reviewed in [11, 29, 30]).

Egg is another nutritious dietary component that is a source for many bioactive peptides [31]. Work from our lab

has demonstrated the generation of egg tripeptides (IRW and IQW) from ovotransferrin (an egg white component) which are effective in the downregulation of cytokine-induced inflammatory protein expression in vascular endothelium, at least partly through the modulation of NF-κB pathway [32, 33]. These anti-inflammatory properties are also observed in conjunction with antioxidant and ACE inhibitory effects, further enhancing the beneficial actions [34]. Interestingly, these beneficial effects require the presence of an intact tripeptide as the corresponding dipeptides and constituent amino acids alone failed to replicate the anti-inflammatory functions, indicating a structure-function relationship between the tripeptide structure and blockade of inflammation [32].

Fish and meat are important sources of dietary protein. Recent findings suggest they also contribute to human health through generation of bioactive peptides; however, detailed studies at the cellular and molecular level are still quite sparse. A fish hydrolysate preparation has been shown to induce proliferation and migration in intestinal epithelial cells, which may contribute to anti-inflammatory and healing properties [13].

Plant-derived foods are another important source for bioactive compounds including many peptides and protein hydrolysates. Soybean hydrolysates have yielded several bioactive peptides with anti-inflammatory effects on macrophage cell lines, with preparations from germinated beans eliciting the stronger responses [35]. Chungkookjang, a fermented soybean product from Korea, is rich in bioactive peptides and shows anti-inflammatory effects in breast cancer cells by downregulation of cytokine/chemokines expression and activation of transforming growth factor (TGF)-beta signaling [36]. One of the soybean-derived peptides, lunasin, appears to exert widespread anti-inflammatory effects including suppression of NF-κB activity, reduced cytokine expression, and reduction in cyclooxygenase-2 (COX-2) levels in addition to its antioxidant and anticarcinogenic properties [37, 38]. The presence of an RGD motif in lunasin and similar peptides is believed to contribute to their anti-inflammatory effects, potentially involving antagonism of integrin signaling and downstream proinflammatory cascades [39].

2.4. Bioactive Peptides on Inflammation In Vivo. Based on the encouraging findings from cell-based studies, several bioactive peptides and hydrolysates have now been tested in animal models of human diseases. A number of different inflammatory models, typically experimentally induced colitis, arthritis, atherosclerosis, and respiratory tract inflammation, have been used. As much of this work has been performed only within the last few years, large-scale human trials are still lacking, although a few smaller studies on humans have shown some therapeutic promise.

Not surprisingly, milk-derived peptides have been in the forefront of *in vivo* studies of anti-inflammatory properties. The tripeptides VPP and IPP appear to be beneficial in a model of intestinal enterocolitis by their mediation of anti-inflammatory effects [40]. In addition, these peptides offer protection against the development of atherosclerotic changes in the apolipoprotein E (ApoE) knockout mice through a concerted action that involves modulation of both inflammatory and hypertensive pathways [41]. Hydrolysates of whey proteins can attenuate the dermatitis in NC/Nga mice [42], while casein hydrolysates (such as those produced by *Aspergillus oryzae* protease or fermentation with thermophilic lactobacilli) have shown promise in treating adjuvant arthritis in rats and chemically induced colitis in mice through modulation of both chronic and acute inflammatory responses [43, 44].

Other peptides and protein hydrolysates from animal sources have been used in several animal models of disease. In our lab, the egg-derived tripeptide IRW has shown promise in controlling both the hyperactive renin-angiotensin system (RAS) pathway as well as the exaggerated proinflammatory phenotype in spontaneously hypertensive rats (SHRs), a widely used model of hypertension and cardiovascular disease [45]. Fish protein hydrolysates have demonstrated protective effects on different murine models of colitis, including those induced by dextran sulphate as well as by chronic NSAID usage, suggesting their potential applications in human disease [46–48]. A similar preparation also reduced markers of inflammation and improved the plasma lipid profile in high fat-fed mice, with potential implications for obesity-induced inflammation and vascular disease [49]. Chicken collagen hydrolysate (CCH) containing an array of bioactive peptides has been used in rodent models of cardiovascular diseases. In the ApoE deficient mice, CCH administration successfully reduced the plasma levels of inflammatory cytokines in addition to improving the plasma lipid profile [50]. CCH given to SHRs reduced blood pressure and circulating inflammatory markers while increasing the bioavailability of the beneficial vasorelaxant NO [51]. A pilot study on human volunteers has also confirmed the antihypertensive effects of CCH although the potential anti-inflammatory mechanisms, if any, remain to be determined [52].

A number of plant-derived bioactive peptides and peptide-rich hydrolysates have also been tested by *in vivo* studies. Feeding of soy protein isolate to rodents (which presumably generates bioactive short peptides through intestinal digestion) has shown beneficial effects on experimentally induced arthritis [53] and genetically predisposed atherosclerosis [54], through the induction of protective anti-inflammatory effects. Soybean peptides such as VPY and others have shown promise in controlling cytokine/chemokines levels, reduction of oxidative stress, and reversal of the tissue damage observed in animal models of colitis, suggesting potential applications in treatment of inflammatory bowel diseases [55, 56]. Oral intake of a corn gluten hydrolysate also reduced inflammatory injury in a rat model of experimental colitis [57]. Similarly, ingestion of pyro-glutamyl leucine (a bioactive peptide from wheat gluten hydrolysate) was shown to protect against dextran sulphate-induced colitis in mice [58] and chemically induced hepatitis in rats [14], further supporting the *in vivo* anti-inflammatory functions of plant-derived peptides.

These anti-inflammatory effects of bioactive peptides and hydrolysates have been summarized in Table 1. A schematic diagram of the potential anti-inflammatory mechanisms

of bioactive peptides is also shown (Figure 1) demonstrating the effects of these compounds on proinflammatory signaling kinases, pro- and anti-inflammatory cytokines, integrin-dependent signaling, ROS generation, and the renin-angiotensin system.

3. Bioactive Peptides on Oxidative Stress

3.1. Oxidative Stress and Chronic Disease. The term ROS encompasses a range of oxygen-containing highly reactive species including free radicals superoxide ($O_2^{-\bullet}$) and hydroxyl radicals ($HO^{\bullet}$) as well as nonradical form like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen, and peroxynitrite ($ONOO^-$) [59]. In low concentrations, ROS can be actually beneficial by induction of apoptosis in damaged/aged cells, detoxification of xenobiotics by cytochrome P450 system, and killing invading microorganisms by phagocytes and as regulatory mediators in cell signaling pathways [60, 61]. However, an excess of ROS, both due to excessive production or impaired antioxidant capacities or both, is harmful and leads to what is known as oxidative stress.

In pathological conditions, ROS attack nucleic acids (DNA or RNA), proteins, and unsaturated fatty acids and aggravate cellular damage (reviewed in [62, 63]). One example of DNA lesions is the conversion of guanine to 8-hydroxyguanine which affects the methylation of cytosine. Normal methylation of cytosine is considered as a critical step in regulation of gene expression and once it is altered, it may contribute to carcinogenesis [64]. Apart from DNA, peroxyl radicals ($ROO^{\bullet}$) can also initiate peroxidation of fatty acids. The final products of this reaction are malondialdehydes (MDA) which possess carcinogenic properties [65]. Proteins are another group of macromolecules affected by the ROS. Cleavage of the peptide bond, amino acid modification, and formation of cross-linked peptide aggregates happen during protein oxidation by ROS that leads to formation of protein derivatives possessing highly reactive carbonyl groups (ketones and aldehydes) which are involved in the complications of diabetes and many age-related diseases [66].

In addition to destructive effects on macromolecules, ROS also impair vasodilatory responses by reaction with NO. The reaction between NO and O_2^- results in the production of peroxynitrite ($ONOO^-$), which reduces the bioavailability of NO which is a potent vasorelaxant signaling messenger in vascular system. Increased oxidative stress and its downstream effects can lead to various conditions such as cardiovascular diseases [67], Alzheimer's disease [68], aging [69], and cancer [70]. Dietary intake of antioxidant compounds can reinforce the body's oxidant status and help to maintain a balanced condition in terms of oxidant/antioxidant in the body. Given this background, there is increasing interest in food proteins and their constituent peptides as potential candidates for use as antioxidants.

3.2. Bioactive Peptides as Antioxidants: Cell-Free Systems. Several chemical methods with different mechanisms of action have been developed to measure antioxidant potential of food proteins and peptides. This is because of complexity of oxidative reactions taking place in biological systems. Scavenging of stable free radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) methods), reduction of metal ions (ferric ion reducing antioxidant power (FRAP) and cupric ion reducing antioxidant capacity (CUPRAC) methods), protecting a target molecule by inhibition of its consumption (oxygen radical absorbance capacity (ORAC) and total radical-trapping antioxidant potential (TRAP) assays), and inhibition of low density lipoprotein (LDL) oxidation are some of the common strategies used in chemical-based antioxidant assays (reviewed in [71, 72]). While the DPPH-based assay was among the first ones to be used extensively [73–76], many such assays have been widely used for screening antioxidant peptides.

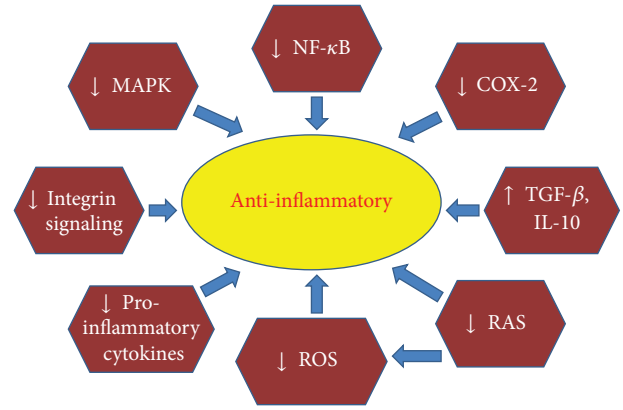

Figure 1: The potential mechanisms of action of anti-inflammatory bioactive peptides and peptide-rich protein hydrolysates. MAPK: mitogen activated protein kinase; NF-κB: nuclear factor-κB; COX-2: cyclo-oxygenase-2; TGF-beta: transforming growth factor-beta; IL-10: interleukin-10; RAS: renin-angiotensin system; ROS: reactive oxygen species.

A wide range of antioxidant peptides have been identified from marine organisms including oyster, shrimp, squid, blue mussel, and a variety of fish species (tuna, sardine, hoki, sole, and pacific hake) after hydrolysis with different enzymes. Puffer fish hydrolysate produced strong antioxidant action as shown by the ORAC assay compared to many other fish sources [77]. Both the salmon protein hydrolysate and peptide fractions inhibited the oxidation of linoleic acid [78]. Flounder fish muscle hydrolyzed with α-chymotrypsin has been also reported to possess strong antioxidant activities by scavenging free radicals *in vitro* [79]. Blue mussel (*Mytilus edulis*) is another source for the production of antioxidant peptides. Hydrolysis of this protein by the enzyme neutrase could scavenge 30% of DPPH radicals while further purification of this hydrolysate revealed the active peptide with the sequence of YPPAK with enhanced hydroxyl and superoxide anion radical scavenging activities [80].

Milk proteins also contribute much in the context of antioxidant peptides. YFYPEL, a hexapeptide isolated from pepsin hydrolysate of bovine casein, showed antioxidant

Table 1: Food-derived anti-inflammatory peptides/hydrolysates in cell-based and *in vivo* systems.

Protein source	Preparation	Active component	Cell/organism tested in	Observed effects	Reference
Casein	Bacterial fermentation	VPP	Endothelial cells and leukocytes	Reduced leukocyte recruitment	[22]
		VPP, IPP	Murine colitis	Anti-inflammatory	[40]
Casein	Corolase hydrolysis	Hydrolysate	Macrophages	Downregulation of COX-2, NF-κB inhibition	[23]
	Aspergillus oryzae protease hydrolysis	Hydrolysate	Rat adjuvant arthritis	Reduced arthritic score, anti-inflammatory	[43]
Whey protein	Enzymatic hydrolysis	Hydrolysate	Epithelial cells	Reduced cytokine expression	[24, 25]
			NC/Nga mouse	Reduced dermatitis	[42]
Lactoferrin	Enzymatic hydrolysis	Lactoferricin	Synovial cells	Anti-inflammatory, antiarthritis	[27, 28]
Ovotransferrin	Thermolysin and pepsin hydrolysis	IRW, IQW	Human endothelial cells	Reduced ICAM-1/VCAM-1 with cytokine treatment	[32]
Fish protein	Enzymatic hydrolysis	Hydrolysate	Intestinal epithelial cells (human and rat)	Anti-inflammatory, increased proliferation	[13]
			Murine colitis (DSS/NSAID induced)	Reduced cytokines, improved healing	[46]
			High fat-fed mouse	Improved lipid profile	[49]
Chicken collagen	Acid treatment followed by *Aspergillus oryzae* protease hydrolysis	Hydrolysate	ApoE knockout mouse	Reduced cytokines, improved plasma lipid profile	[50]
			SHR (rat)	Reduced ICAM-1 and decreased blood pressure	[51]
Soy protein	Fermentation	Chungkookjang	Breast cancer cells	Anti-inflammatory, increased TGF-beta	[36]
Soy protein	Enzymatic hydrolysis	Lunasin	Macrophage	Reduced cytokines, NF-κB inhibition	[37]
		VPY	Murine colitis	Reduced cytokines, reduced oxidative stress, and improved histology	[55]
Wheat gluten	*Aspergillus oryzae* protease hydrolysis and fractionation	Pyro-glutamyl leucine	Rat hepatitis	Anti-inflammatory, improved hepatic enzyme profile	[14]
			Mouse colitis	Improved mucosal histology and less weight loss	[58]

activity by scavenging superoxide, DPPH, and hydroxyl radicals *in vitro* [81]. In a recent study, whey protein concentrates (WPC) hydrolyzed by Corolase or thermolysin were investigated for antioxidant activity by ORAC assay. Thermolysin-hydrolyzed WPC (8 hrs at 80°C, enzyme/substrate ratio: 0.10 w/w) was the most potent hydrolysate with radical scavenging activity and several peptides were identified in this hydrolysate [82]. Ovine κ-casein antioxidant activity also increased by 3-fold upon hydrolysis with pepsin, trypsin, and chymotrypsin. This casein hydrolysate further inhibited lipid peroxidation and several peptides contributing to antioxidant activity were identified [83]. Goat milk casein also exhibited enhanced free radical scavenging and metal ion chelating activity following hydrolysis by a combination of neutral

and alkaline proteases. Further purification revealed five novel peptides in this hydrolysate with potential antioxidant properties [84].

Plants are known for antioxidant effects mostly because of their polyphenolic compounds. However recent research indicates the significance of many plant proteins and peptides as novel antioxidant agents. The potential of commercially available microbial proteases to enhance antioxidant potential of soy and corn proteins has been recently demonstrated [85, 86]. Corn protein hydrolysates were more effective than soy protein hydrolysate and inhibited lipid oxidation by 53% at lower incorporated dosage (200 μg/g) while soy protein hydrolysate reduced oxidation by 20% at much higher dosage (800 μg/g) [85]. In a recent study the antioxidant activity of chickpea albumin hydrolysate through *in vitro* radical scavenging and reducing power assays has been assayed. Further purification of the hydrolysate fraction with highest antioxidant activity identified RQSHFANAQP as the active component responsible [87]. All of the aforementioned peptides were evaluated for antioxidant activity through *in vitro* methods. Although these methods are good for screening and assessing preliminary data, there are drawbacks associated with these chemical-based assays including potential lack of relevance to biological systems and altered mechanisms of free radical generation [72, 88]. Therefore, it is preferable to use at least two different chemical assays prior to validation of antioxidant activity in more physiologically relevant systems like cells and whole organisms.

3.3. Bioactive Peptides as Antioxidants: Cellular Systems. Cell-based assays as intermediate methods have been used increasingly recently to evaluate the protective effects of antioxidants against oxidative stressors and to elucidate mechanism of action of peptides within cells [89]. Cell-based methods can be used to elucidate the mechanism/s of action of antioxidant agents within live cells. Moreover, cell culture assays are useful for the determination of peptide dosage to exert beneficial antioxidant effects without cytotoxicity for *in vivo* experiments. Among animal source peptides, those from flounder fish protein hydrolysates showed antioxidant and cytoprotective effects against 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) without cytotoxicity in the range of 12.5 to 200 μg/mL in Vero cells, a monkey kidney fibroblast line [79]. An antioxidant peptide from skin gelatin hydrolysate of hoki fish increased expression of cellular antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in human hepatoma cells (Hep3B) [90].

A recent study on bacterial fermentation in sourdough showed antioxidant effects on cultured fibroblasts [91]. Hempseed-derived antioxidant peptides also exhibited protective effects against oxidative apoptosis in rat pheochromocytoma line PC12 cells [92]. Similar effects were exerted by a hydrolysate of rice endosperm protein on mouse macrophages [93]. Results of these cell-based assays clearly show antioxidant potential of food peptides beyond the free radical scavenging in chemical assays.

3.4. Bioactive Peptides as Antioxidants: In Vivo Effects. *In vivo* studies such as animal experiments or human trials should be conducted after identification of an antioxidant peptide through *in vitro* and cell-based assays to demonstrate its antioxidant activity in an intact organism. Despite the importance of *in vivo* studies for verifying the antioxidant activity of bioactive peptides, only a few studies on the efficacy of antioxidant peptides have been conducted in animal models. Long-term administration (17 weeks) of egg white hydrolysate to SHRs has been shown to improve the plasma antioxidant capacity. Moreover MDA levels decreased significantly in the aortic tissue of rats receiving 0.5 g/kg/day and reverted to baseline levels five weeks after the treatment [94]. Similarly, recent studies on whey protein consumption also suggest a number of benefits including reduction of oxidative stress and better management of metabolic syndrome in both animal models and human subjects (reviewed in [12]). Among plant-derived sources, rapeseed crude protein hydrolyzed with Alcalase and Flavourzyme has shown to be a potential source for antioxidant peptides. Rapeseed hydrolysates were intraperitoneally injected to rats at 50 or 100 mg/kg/day for 16 days. At the end of the experiment, the serum was collected when 12.8% and 46.9% reduction in MDA levels were observed for 50 and 100 mg/kg/day dosages of rapeseed protein hydrolysate, respectively [95]. The findings from various cell-based and *in vivo* studies have been summarized in Table 2.

4. Potential Challenges and Opportunities

4.1. Limitations and Risks. While the field of bioactive peptides is an exciting and growing area of research, there are a few risks and limitations before the widespread use of such peptides in the general population. As previously discussed, many of the studies are still at an early stage and more *in vivo* data will be needed before applications to human health. In the absence of solid pharmacokinetic data, proper dosage and frequency of administration may be impossible to determine, leading to wide variability in intake and biological effects [29, 96]. While these bioactive peptides are considered to be relatively safe, there is always the risk of potential side effects if too high a dose is consumed. For example, antioxidant vitamins were traditionally considered safe even in high doses; yet recent evidence suggests potential toxic effects on excessive consumption [97, 98]. Another potential risk could be due to the presence of immunogenic proteins and peptides within the protein hydrolysates, which may induce and/or exacerbate allergic reactions in a minority of users [99, 100]. Proper screening prior to the ingestion of such hydrolysates might be necessary in subjects prone to allergies. While this lack of knowledge about specific compounds and their potential side effects is a limitation, it also provides opportunities for future research in several directions.

4.2. Future Directions. Future studies in the field of bioactive peptides would likely involve detailed studies on animals and human volunteers to better understand the pharmacokinetics of these compounds, testing for potential immunogenicity

Table 2: Food-derived antioxidant peptides/hydrolysates in cell-based and *in vivo* systems.

Protein source	Preparation	Active component	Cell/organism tested in	Observed effects	Reference
Ovotransferrin	Thermolysin and pepsin hydrolysis	IRW, IQW	Endothelial cells (human)	Reduced cellular superoxide (dihydroethidium staining)	[32]
Chicken egg white	Hydrolysis with pepsin	Egg white hydrolysate	SHR (rat)	Increase in plasma radical scavenging, reduction in aorta MDA levels	[94]
Hoki skin gelatin	Hydrolysis with trypsin	HGPLGPL	Hep3B (human hepatoma cells)	Increase in cellular antioxidant enzymes (catalase, SOD, GPx)	[90]
Flounder fish protein	Hydrolysis with α-chymotrypsin	CAAP, VCSV	Vero cells (monkey kidney fibroblast cell line)	Cytotoxic protective effects, scavenging intracellular ROS	[79]
Cereal flours	Fermentation of sourdough with lactic acid bacteria	25 peptides (8–57 amino acid residues)	Mouse fibroblasts (Balb 3T3)	Protective effects against oxidative stress in fibroblasts	[91]
Hempseed	Alcalase hydrolysate of hempseed protein isolate	NHAV HVRETALV	Rat pheochromocytoma line PC12 cells	Protective effects against cell death/oxidative apoptosis	[92]
Rice endosperm protein	Neutrase hydrolysate of defatted rice endosperm protein	FRDEHKK	Mouse macrophage (RAW 264.7)	Scavenging of intracellular ROS (DCFH-DA method)	[93]
Rapeseed	Hydrolysis with Alcalase and Flavourzyme	Rapeseed crude hydrolysate	Wistar rat	50% reduction in serum MDA levels	[95]

(to prevent allergies), characterizing individual components of complex peptide-rich hydrolysates to tease out their specific actions as well as basic biomedical research directed towards identifying specific receptors and signaling pathways involved in mediating some of these beneficial anti-inflammatory and antioxidant actions. Indeed, outside the renin-angiotensin system [101, 102], few receptors have been identified as involved in bioactive peptide actions. Further work on identifying specific peptide sequences and their corresponding receptors may provide opportunities for better targeting of inflammation and oxidative stress in a tissue- and organ-specific manner.

5. Conclusions

Bioactive peptides and peptide-rich protein hydrolysates represent a new direction in functional foods and nutraceuticals. While both types of preparations have shown promise as potential anti-inflammatory and antioxidant agents, further research is still needed to verify these beneficial effects in order to successfully translate the research from bench to the bedside to effectively control the growing burden of chronic noncommunicable illnesses with minimal side effects.

Conflict of Interests

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

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