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Bioactive Natural Products 2017

Lead Guest Editor: Yiannis Kourkoutas
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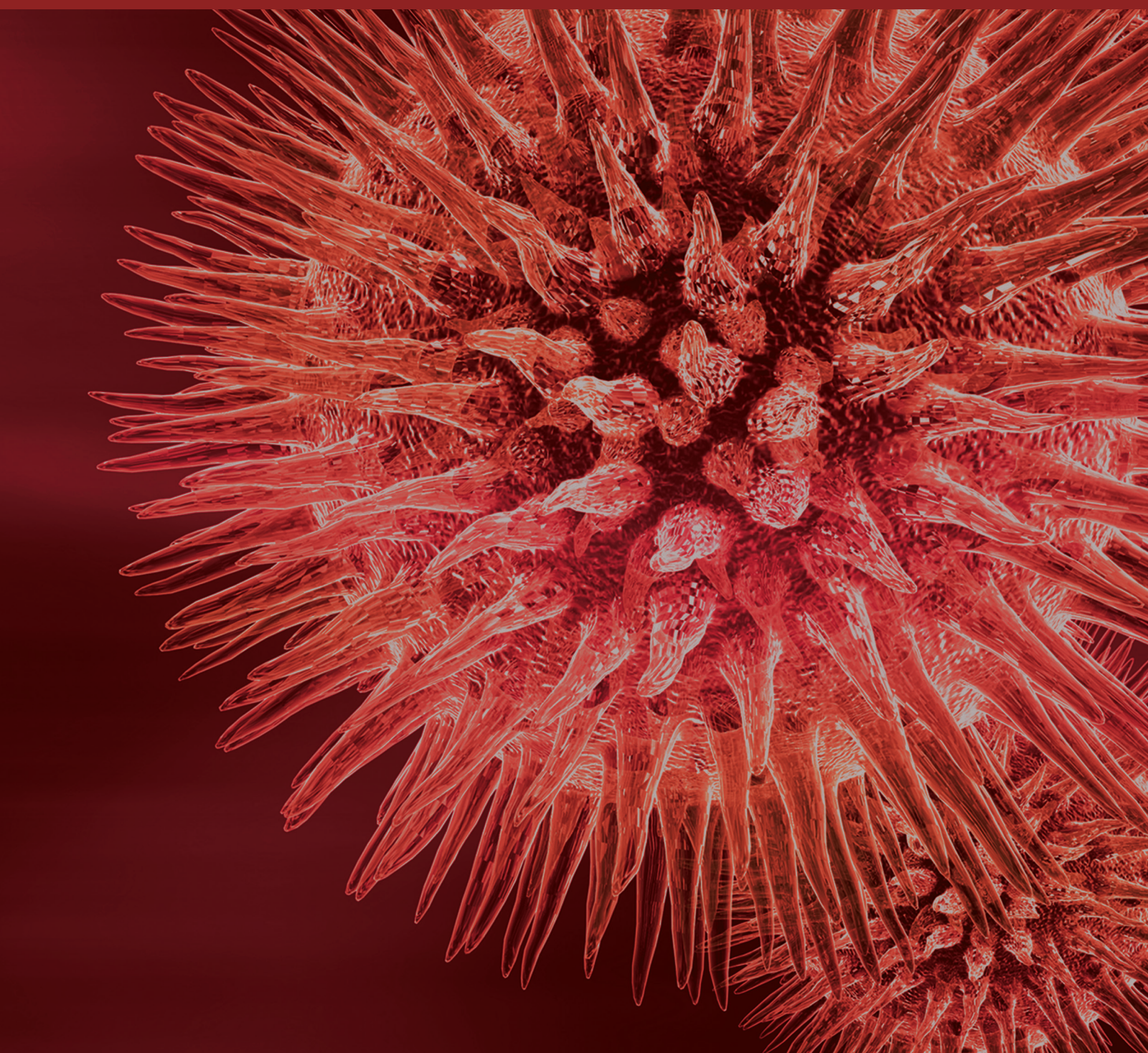

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Editorial

Bioactive Natural Products 2018

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The global phenomenon of antibioresistance, resistance genes pool (clinical and environmental reservoirs), and environmental pollution, especially by xenobiotics, including antibiotics which are considered water micropollutants, are acknowledged as some of the most important problems the world is facing today [1]. Thus, the necessity of antimicrobial agents that are new, efficient, non-toxic, and with no selective pressure activity is obvious.

Despite the huge scientific progress in vaccination and chemotherapy, infectious diseases remain a serious health issue. Under the selective pressure of therapeutical antibiotics, used excessively during the last decades, some bacterial species/strains harboring resistance genes (pre-existent to antibiotherapy) were selected and disseminated, developing other mechanisms of resistance. As a consequence, infectious diseases remain among the leading causes of morbidity worldwide and a top priority for the public health. However, little progress has been made in the development of new antimicrobial drugs. Moreover, the wide use of antibiotics has evolutionary and ecological effects, leading to the recruitment of more genes into the *resistome* and *mobilome*, with adverse consequences for human welfare and environment [2, 3]. There are also a lot of biofilm-associated infections and the biofilm embedded cells show a different form of resistance, called now tolerance. Biofilms cause great medical concerns, as they may be developed on medical devices, tissues, and organs (normal or damaged), but also industrial problems, since they could be formed on any device and industrial equipment. Microorganisms attached to a substratum and organized in biofilms exhibit a high tolerance to the current antibiotics, antiseptics, and biocides, as well as to the host defense mechanisms. Moreover, the resistance and virulence genes are easily achieved between biofilm's embedded cells by horizontal transfer, due to their proximity [4].

In the industrial environment, current anti-fouling agents are also far from being efficient. Hence, the adherent microorganisms on surfaces produce great economical losses caused by the uncontrolled development of biofilms on pharmaceutical or food industrial equipment. Therefore, numerous industrial technologies have to make a difficult choice: either to utilize a high amount of an efficient anti-fouling agent with the risk of developing side effects and impurifications on the final product or not to be able to control the microbial contamination and biofilm development within the technological processes. In these conditions, new, safe for health (without cytotoxicity), and eco-friendly biocides are necessary, because the consumers are currently informed, show great interest, and demand healthy food. In the coming years, it is estimated that the EU regulations will be changed and certain biocides will be banned, due to their biohazard effects.

Considering the high frequency of genetic antibioresistance in the most common pathogens, the huge public health burden of severe biofilm-associated infections (60–80% of all infections), and the great economical loses caused by

the uncontrolled development of biofilms on industrial equipment, alternative strategies are urgently needed to efficiently control their formation and their negative effects. Thus, the researchers are in a continuous quest for new antibacterial agents for resistant/multiresistant strains, able to penetrate the biofilms and with activity on adherent cells. Innovative approaches include the following: (1) the development of prophylactic antimicrobial peptides, able to interfere with the intercellular communication by quorum-sensing (QS) mechanism, involved in regulation of a series of genes, including virulence genes; such QS inhibitors (QSIs) belong to the antipathogenic strategies [5]; (2) enzymes able to degrade biofilm's matrix (dispersins) or the signal molecules (quenching enzymes). To date, none of the envisaged antibiofilm solutions has an absolute outcome, but only their combinations seem to be effective.

The use and abuse of antibiotics, especially those with large spectrum of activity, are the cause of the frequent condition of disbiosis or alteration of the intestinal microbiota's interspecific equilibrium. Such conditions are leading to opportunistic infections, metabolic disturbances, increased intestinal permeability, and chronic inflammation. Evidence obtained by animal models and clinical studies confirm the association of an altered gut microbiota with all corollary consequences, such as metabolic diseases from obesity to type-2 diabetes, tooth decay, cardiovascular diseases, and cancer [6].

All these recently high increased problems have catalyzed the research efforts to find new ways to fight against pathogens, with no side effects on the host and its normal microbiota, but also on the environment. A lot of studies are now focused on the investigation of bioactive natural products (BIONPs), mainly obtained from plants with a very wide range of biological activities: antimicrobial, anti-inflammatory, antioxidant, immunomodulatory, antidepressant, antihyperglycaemic (amylase activity), antihypertensive, anticarcinogenic, etc. These BIONPs are used as plant extracts or fractions, coupled or not with carriers (nanoparticles). Medicinal plants have now to be investigated at molecular level, in order to identify the mechanisms of action, efficiency, and lack of cytotoxicity, since their use has to be scientifically based, in definite amounts and for a specific target, in comparison with the allopathic drugs. The potential synergistic activity with antibiotics should be also explored [7, 8].

Thus, plants are an important source of BIONPs; all plants have immune defense mechanisms mediated by anti-infectious phytocompounds, such as phytoanticipins and phytoalexins and the more recently described QSIs. The QSIs exhibit, when used even in subinhibitory concentrations, an indirect antimicrobial effect, manifested by inhibiting the bacterial intercellular communication by QS mechanism and coordinated expression of virulence genes depending on cellular density. The use of QSIs could represent an efficient and intelligent strategy to control resistance/tolerance, virulence, and colonization/biofilm formation, without selective pressure and other side effects [5, 9–13].

However, the use of BIONPs has some limitations, due to their low availability and stability, high volatility, and a great diffusion ability that do not recommend their implementation in the current medical practice. These features lead to the necessity of developing vectorization and delivery agents for improving their efficiency and also optimized assay methods adapted for their specific properties. However, the research efforts are fully justified by their great potential.

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Review Article

Alternative and Natural Therapies for Acute Lung Injury and Acute Respiratory Distress Syndrome

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Introduction. Acute respiratory distress syndrome (ARDS) is a complex clinical syndrome characterized by acute inflammation, microvascular damage, and increased pulmonary vascular and epithelial permeability, frequently resulting in acute respiratory failure and death. Current best practice for ARDS involves "lung-protective ventilation," which entails low tidal volumes and limiting the plateau pressures in mechanically ventilated patients. Although considerable progress has been made in understanding the pathogenesis of ARDS, little progress has been made in the development of specific therapies to combat injury and inflammation. *Areas Covered*. In recent years, several natural products have been studied in experimental models and have been shown to inhibit multiple inflammatory pathways associated with acute lung injury and ARDS at a molecular level. Because of the pleiotropic effects of these agents, many of them also activate antioxidant pathways through nuclear factor erythroid-related factor 2, thereby targeting multiple pathways. Several of these agents are prescribed for treatment of inflammatory conditions in the Asian subcontinent and have shown to be relatively safe. *Expert Commentary*. Here we review natural remedies shown to attenuate lung injury and inflammation in experimental models. Translational human studies in patients with ARDS may facilitate treatment of this devastating disease.

1. Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are associated with high morbidity and mortality rates [1–3]. These disorders are characterized by rapid-onset respiratory failure, severe hypoxemia, and decreased static respiratory system compliance [2]. A recent consensus-based definition (i.e., the Berlin definition) has proposed the subdivision of ARDS into three categories based on degree of hypoxemia and has urged practitioners to drop the term "acute lung injury" [4, 5]. Increased severity of ARDS is associated with poorer prognosis and higher mortality [6]. ARDS results from uncontrolled acute inflammation and dysfunction of endothelial and epithelial barriers of the lung, and an excessive transepithelial leukocyte migration, leading to the loss of alveolar-capillary membrane integrity and overproduction of proinflammatory cytokines. The pathogenesis of ARDS involves activation of both immune and structural cell types. Immune cells implicated in ARDS include macrophages and neutrophils [7–9], as well as lymphocytes and platelets [7, 9]. The inflammatory response in ALI and ARDS is initiated, amplified, and modulated by a complex network of cytokines and other proinflammatory molecules produced by a variety of cell types in the lungs, including fibroblasts, epithelial cells, and inflammatory cells [10].

Endothelial injury is an underlying cause of increased permeability and pulmonary edema in ALI and ARDS, but epithelial injury also plays an important role in their

development. Endothelial activation may also lead to obstruction or destruction of the pulmonary vasculature [11]. Injury to alveolar type II cells contributes to surfactant abnormalities [8]. The hallmark of therapy for ALI and ARDS is supportive care [12]. Despite an increased understanding of its molecular pathogenesis, specific therapies have yet to be developed for ARDS [13, 14].

Contemporary approaches to develop drug therapies have not been productive. In particular, blockade of single cytokines and chemokines have failed to improve outcomes because of the complex pathogenesis and nature of ARDS. Therefore, defining the contribution of proximal signaling pathways that amplify the inflammatory response and developing therapies to specifically block them is an attractive approach, one that may limit injury and inflammation associated with this devastating disease. Intracellular signaling pathways triggered by diverse pattern-recognition receptors converge on signaling hubs, including transcription factors nuclear factor κB (NF-κB), interferon regulatory factor families, STAT, and AP-1. There is also simultaneous activation of oxidant and antioxidant pathways, particularly in innate immune cells.

Nuclear factor erythroid 2-related factor 2 (Nrf2), a member of the cap'n'collar family of basic leucine zipper transcription factors, provides a key antioxidant response. Most widely studied experimental models use lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria. More recently, the roles of other molecules (e.g., danger-associated molecular pattern molecules [DAMPS]), intracytoplasmic receptors (e.g., nod-like receptors [NLRs]), amplifiers (e.g., triggering receptors expressed on myeloid cells-1 [TREM-1]), and several others are being recognized. The detailed molecular mechanisms of lung injury and ARDS have been reviewed extensively in several recent publications [7, 9, 15].

Since its initial description in 1967, little progress has been made in the development of novel therapies for ARDS. To date, no pharmacological agents have demonstrated efficacy in preventing ARDS or improving its symptoms, and the morbidity and mortality continue to be significant [6, 16, 17]. Hence, ARDS represents an unmet medical need, and the need to develop new therapies to treat patients with this condition is urgent. Certain natural remedies have been shown to inhibit multiple inflammatory pathways associated with ALI/ARDS at a molecular level and therefore may be effective in ARDS treatment. Here we review some of the natural products that have been studied in lung inflammation. After summarizing some of the key inflammatory pathways that play a role in lung injury, we will discuss natural products that target these pathways.

2. Inflammatory Pathways That Contribute to Pathogenesis of Acute Lung Injury

Inflammation is an important component of ALI and ARDS, as inflammation is what damages the respiratory membrane. Most inflammatory cells, including macrophages and neutrophils, release inflammatory cytokines in response to various stimuli. LPS, a main component of the outer membranes of gram-negative bacteria, has been identified as a key risk factor for ALI and ARDS. LPS binds to Toll-like receptor 4 (TLR4), which induces activation of intracellular pathways. Ligand binding to TLR4 induces the recruitment and activation of adaptor proteins through the Toll/interleukin- (IL-) 1 receptor (TIR) domain. Recruitment of the adaptor protein myeloid differentiation primary response gene 88 (MYD88) to the receptor complex will only occur if the TIR domain contains adaptor protein (then called TIRAP, or MAL). MYD88 recruits IL-1 receptor-associated kinase 4 (IRAK4), which forms an active complex capable of recruiting the tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6).

Activation of TRAF6 leads to activation of IκB kinase (IKK) enzyme complex and regulatory scaffold proteins [18]. The associated pathway of mitogen-activated protein kinase (MAPK) also leads to activation of the nuclear transcription factor NF-κB. Three types of MAPKs have been studied: (1) extracellular signal-regulated protein kinase (ERK), (2) c-jun n-terminal kinase (JNK), and (3) p38 MAPK. Inhibition of any of these MAPK pathways significantly decreases TNF-α production by LPS (Figure 1).

NF-κB plays a central role in intracellular inflammatory pathways. The most predominantly characterized NF-κB complex is the p50/p65 heterodimer. In most cells, NF-κB remains inactive in the cytoplasm in a complex with any of the family of inhibitory IκB proteins [7]. Activation of inflammatory pathways induces phosphorylation, ubiquitination, and proteasome-mediated degradation of the IκB protein, followed by translocation of NF-κB to the nucleus and regulation of gene expression through binding to the *cis*-acting NF-κB element. The tyrosine phosphorylation of p65 NF-κB efficiently modulates transcription activity. Activation of NF-κB leads to expression of transcription of adhesion molecules, chemokines, colony-stimulating factors, and other cytokines necessary for inflammatory responses.

TREM-1 belongs to the TREM superfamily of receptors expressed on monocytes and neutrophils. Although the precise ligands for TREM-1 have not been identified, it is significantly upregulated by various TLR ligands, including lipoteichoic acid (ligand for TLR2), polyinosinic-polycytidylic acid (ligand for TLR3), and LPS (ligand for TLR4).

These receptors activate downstream signaling pathways with the help of an adaptor molecule, TYRO protein tyrosine kinase binding protein (DAP12). The activation of TREM-1 synergizes with the effects of the TLR ligands and amplifies the synthesis of inflammatory cytokines. This interaction then leads to the activation of NF-κB and to the release of proinflammatory TNF-α, IL-12, IL-1, IL-6, IL-8, and anti-inflammatory cytokines IL10 and TGF-β. Recent studies also suggest that activation of TREM-1 is modulated by prostaglandins [47] and that it prolongs survival of activated macrophages [48]. We recently used a novel nanomiceller approach to show that blocking TREM-1 attenuates LPS-induced lung injury in a murine model. Additionally, we have shown that curcumin inhibits the binding of p65 to TREM-1 promoter in response to LPS—which enhances the anti-inflammatory effects of curcumin [19].

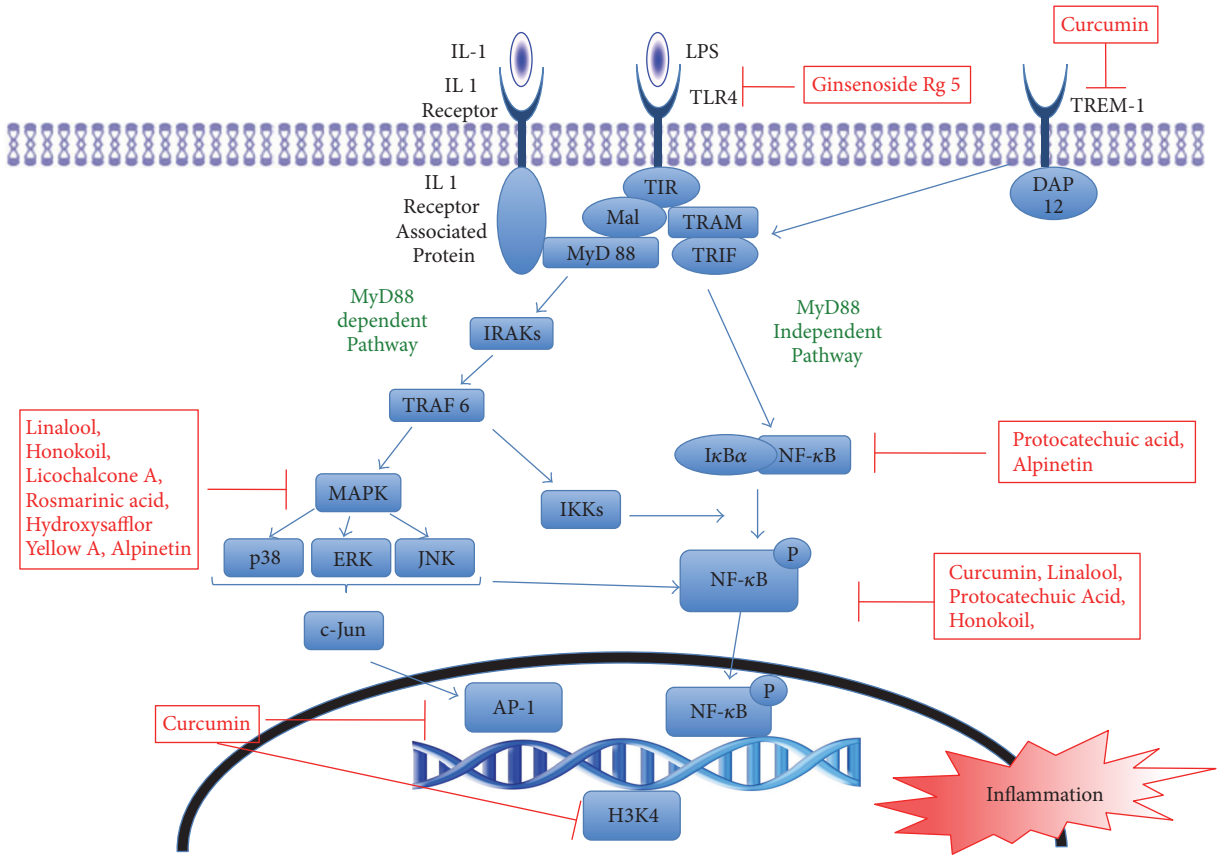

Figure 1: Intracellular signaling pathways associated with inhibition of the nuclear transcription factor-kappa B (NF-κB).

3. Natural Products That Target the Inflammatory Pathway

Clinically used anti-inflammatory drugs have several disadvantages, including adverse effects and a high cost of treatment. Since ancient times, traditional medicines and phytopharmaceuticals have been used to treat inflammation and other disorders, especially in the Asian subcontinent. Such treatments are natural products, and this affords us the valuable opportunity to identify their bioactive compounds, which could ultimately translate into development of new drugs for treatment of inflammatory diseases. The potential of these compounds to attenuate inflammation in the lungs has been studied in cell cultures and animal models. Several studies have focused on investigating natural compounds that can inhibit TLR signaling pathways, particularly through inhibition of NF-kB.

Tables 1 and 2 summarize some of the natural products that have been shown to attenuate inflammation and have been studied *in vitro* and *in vivo* in experimental models of lung injury. Kim et al. [21] showed that Ginsenoside Rg5, a rhizome extract, significantly decreased inflammation in ALI and ARDS models by interacting with TLR4 receptor. Alpinetin, derived from seeds of *Alpinia katsumadai Hayata*, inhibits phosphorylation of the IκBα protein, eventually decreasing activation of NF-κB [22]. Additional studies have shown that alpinetin specifically inhibits phosphorylation of p38 and ERK-associated pathways. An *in vitro* study showed that protocatechuic acid (PCA), a benzoic acid derivative, inhibits degradation and phosphorylation of IκBα, thereby decreasing NF-κB activation [23]. Several naturally occurring products have been shown to attenuate inflammation by inhibiting phosphorylation of p38 and ERK pathways. Chu et al. [24] reported that Licochalcone A (LicoA), found in the root of Xinjiang licorice, suppressed NF-κB and p38/ERK MAPK signaling in a dose-dependent manner.

LicoA has also been shown to inhibit vascular smooth muscle proliferation by suppressing platelet-derived growth factor-induced activation of the ERK1/ERK2 pathway. Rosmarinic acid, a natural prolyl oligopeptidase inhibitor, increases superoxide dismutase (SOD) activity and suppresses ERK/MAPK signaling [25].

Additionally, rosmarinic acid has other effects, such as inhibition of the complement cascade, which may also contribute to its protective effects. Hydroxysafflor yellow A (HSYA) inhibits MAPK, thereby inhibiting NF-κB activation [26, 27]. Linalool, a major volatile component of essential oils in several aromatic plant species, demonstrated anti-inflammatory capability in *in vitro* and *in vivo* models of ALI/ARDS [28]. Patchouli alcohol has also been shown to have anti-inflammatory effects on mouse ALI models by inhibiting IkB-alpha and p65 NF-κB phosphorylation

Table 1: Natural products that decrease inflammation through NF-κB pathway.

Natural product	Isolation	Study performed
Curcumin	Root of plant *Curcuma longa*	BMDM, mice [19, 20]
Ginsenoside Rg5	Rhizome of *Panax ginseng* C. A. Meyer	Macrophage, mice [21]
Alpinetin	Roots of *Alpinia Katsumadai Hayata*	RAW 264.7*, mice [22]
PCA	Major benzoic acid derivative found in vegetables, nuts, brown rice, fruits, and herbal medicines	Mice [23]
LicoA	Flavonoid found in licorice root *(Glycyrrhiza glabra)*	RAW 264.7*, mice [24]
Rosmarinic acid	Herbal plants including rosemary *(Rosmarinus officinalis)*, oregano *(Origanum vulgare)*, and spearmint *(Mentha spicata)*	Mice [25]
HYSA	Chinese herbal medication, *Carthamus Tinctorius L.* (safflower)	Mice [26, 27]
Linalool	Component of essential oils in several aromatic plants	RAW 264.7*, mice [28]
PA	Pogostemon cablin	Mice [29]
Shikonin	Napthoquinone pigment extracted from root of *Lithospermum erythrorhizon*	Mice [30]

BMDM, bone marrow-derived macrophage; HYSA, hydroxysafflor yellow A; LicoA, licochalcone A; PA, patchouli alcohol; PCA, protocatechuic acid; *RAW 264.7, mice macrophage cell line.

Table 2: Natural products that decrease inflammation in in vivo model of ALI/ARDS.

Natural product	Isolation	Study performed
Honokiol	Component of the genus *Magnolia*	Mice [31]
ISOF	*Coleus forskohlii* native of Yunnan	Mice, rats [32]
Sophorolipid	Fermentation of *Candida bombicola*	Rats [33]
CAPE	Extract of propolis	Rats [34]
Ruscogenin	*Ruscus aculeatus*	Mice [35]
Bark extract of *Bathysa cuspidata*	*Bathysa cuspidata* (A. St.-Hil.) Hook f.	Rats [36]
CJT	Herbal remedy	Mice [37]

CAPE, caffeic acid phenethyl ester; CJT, *Callicarpa japonica Thunb*; ISOF, isoforskolin.

induced by LPS [29]. Bai et al. [30] showed that shikonin, a natural pigment, suppressed LPS-induced COX 2 and iNOS activation by downregulating NF-κB activation.

Table 2 lists some of the natural products that inhibit inflammation in ALI or ARDS models. Honokiol, a component of a Chinese tree, decreases production of early-phase cytokines (e.g., HMGB1) in mice models. It also inhibits protein kinase C-α and MAPK [31]. Isoforskolin (ISOF) has been shown to prevent LPS-induced ALI development in pretreated animal models [32]. ISOF is an effective adenylyl cyclase activator that causes increased intracellular cyclic adenosine monophosphate (cAMP), which has attenuated in *in vitro* LPS-induced ALI. Caffeic acid phenethyl ester (CAPE), an extract of propolis, has exhibited antioxidant qualities [34], as well as anti-inflammatory effects by modulating the arachidonic acid (AA) cascade. It also inhibits $Na+/K^+$ ATPase activity in LPS-induced ALI models. Ruscogenin has been shown to inhibit tissue factor expression and iNOS and NF-κB activation [35]. In rats, the bark extract of *Bathysa cuspidata* attenuates ALI-induced by paraquat by reducing lipid and protein oxidation and preventing a reduction in catalase and SOD activity [36]. Shin et al. [37] showed that a traditional herbal remedy, *Callicarpa japonica Thunb* (CJT), inhibited LPS-induced inflammation by reducing iNOS expression and interleukin-6 *in vitro* and *in vivo*.

The Chinese herbal formula Huang-Lian-Jie-Du-Tang (HLJDT) comprises *Rhizoma coptidis, Radix scutellariae, Cortex phellodendri,* and *Fructus gardeniae*. In rats with LPS-induced ALI, HLJDT dose-dependently reduced the number of leukocytes adhering to the endothelium and decreased the expression of VCAM1 in lung venules. *In vitro*, HLJDT inhibited NF-κB nuclear translocation in endothelial cells [49].

As noted above, TREM-1 is a prolific amplifier of TLR-induced inflammatory responses. Curcumin (or diferuloylmethane), a natural product found in tumeric, has been shown to decrease inflammation by inhibiting multiple proinflammatory pathways and activating anti-inflammatory pathways [20]. We have shown that curcumin inhibits the expression of TREM-1 *in vitro* in primary bone marrow derived macrophages and *in vivo* in the lungs of mice with

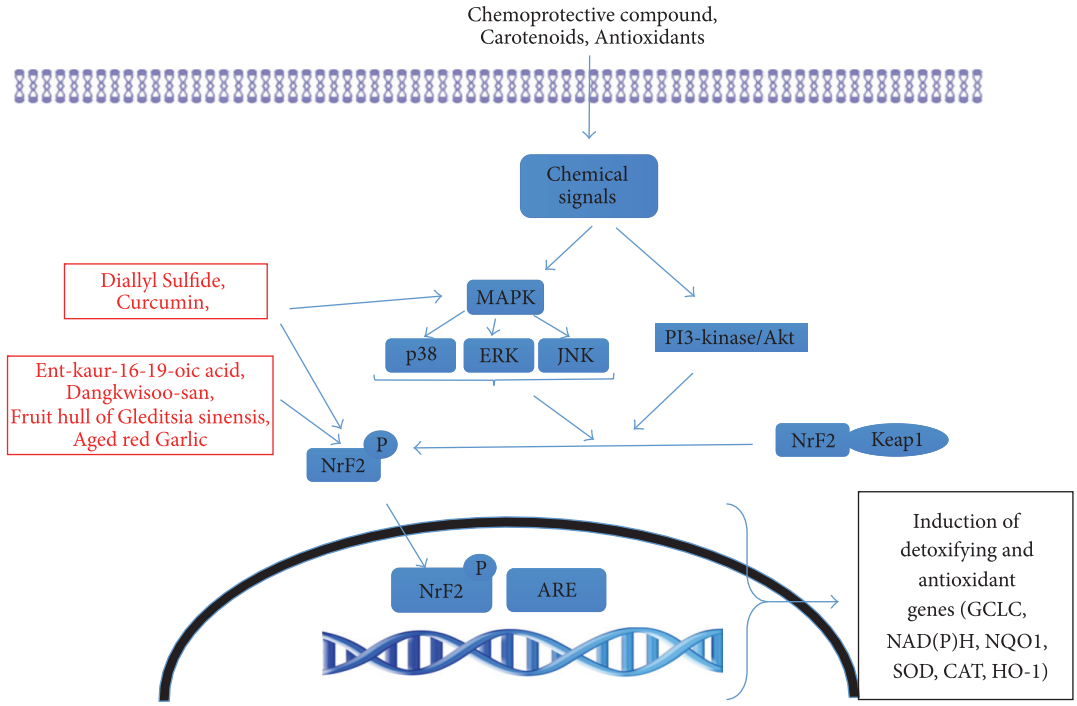

Figure 2: Cellular pathways involved in regulating the phosphorylation of nuclear factor erythroid 2-related factor 2 (Nrf2) and antioxidant response element- (ARE-) mediated antioxidant gene expression.

sepsis. Chromatin immunoprecipitation assay confirmed that curcumin inhibits the binding of p65 to TREM-1 promoter in response to LPS. Furthermore, we showed that curcumin attenuated methylation and acetylation of histone 3 and histone 4 (H3K4) by inhibiting p300-HAT, a key epigenetic element known to activate transcription of the genes that regulate inflammation [19].

Together these studies highlight the potential of several natural compounds that can attenuate lung inflammation by pleiotropic actions and that inhibit key signaling components and amplifiers of TLR pathways. Although some of these studies provide proof-of-principle data in cell and preclinical models, translation of these studies to human clinical trials is lacking.

4. Nuclear Factor Erythroid 2-Like 2 (Nrf2): An Anti-Inflammatory Transcription Factor

Oxidative stress also plays a key role in the development of ALI and ARDS. Tissue homeostasis requires that an intricate, delicate balance between oxidants and antioxidants be maintained. Any disruption in this checks-and-balances system can lead to harmful consequences, particularly in the setting of lung injury. Some cells, including pulmonary macrophages, express various proteins that scavenge reactive oxygen species. One of the key transcription factors that induces these proteins is Nrf2, a member of the cap'n'collar family of basic leucine zipper transcription factors. The inactive form of Nrf2 remains bound in cytosol by Kelch-like ECH-associated protein 1 (KEAP1). In pulmonary macrophage activation, Nrf2 binds to its *cis*-acting antioxidant response element (ARE) sequence, resulting in expression of various phase 2 detoxification genes, including glutamate-cysteine ligase, catalytic subunit (GCLC), NAD(P)H, quinone-1 (NQO1), SOD, catalase (CAT), glutathione peroxidase (GPx), and heme oxygenase-1 (HO-1). Studies have demonstrated that phosphatidylinositol 3-kinase/Akt and various MAPKs (e.g., ERK, JNK, and p38) are involved in regulating the phosphorylation of Nrf2 and ARE-mediated antioxidant gene expression (Figure 2) [50].

Table 3 lists natural products that have been shown to decrease inflammation through this pathway. Dangkwisoo-san (DS), a Korean herbal remedy, has been shown to activate Nrf2 and induce Nrf2-regulated genes (including GCLC, NQO1, and HO1) in *in vitro* studies [40]. DS is thought to activate Nrf2 by dissociating KEAP1 from Nrf2.

Three separate herbal product derivatives, ent-kaur-16-19-oic acid (KA) [41], the fruit hull of *Gleditsia sinensis* (FGS) [42], and Carthami Flos (CF) [43], have been shown to activate Nrf2 and to induce Nrf2-regulated gene expression in *in vitro* macrophage cell lines. CF in particular attenuated neutrophilic lung inflammation in mice, in the presence of Nrf2 [43]. Diallyl sulfide (DAS), a natural antioxidant found in garlic, induces Nrf2 activation and translocation in nuclei triggered by p38/ERK-signaling pathways in lung MRC-5 cells [44]. *In vitro* experiments show that DS, KA, FGS, and CF exert their anti-inflammatory effects by activating Nrf2 and inducing Nrf2-regulated genes, including GCLC, NQO1, and HO1. Activation of Nrf2 occurs without reactive oxygen species production [41–43].

Table 3: Natural products that decrease oxidative stress through Nrf2 activation.

Natural product	Isolation	Study performed
Curcumin	Root of plant *Curcuma longa*	BMDM, mice [38, 39]
DS	Herbal formula in Korea (combination of 9 species of herbal plants)	RAW 264.7*, mice [40]
KA	Dried roots of *Aralia continentalis*	RAW 264.7*, [41]
FGS	Herbal formula in Korea	RAW 264.7*, mice [42]
CF	Purified aqueous extract used in Asian medicine to treat blood stagnation	Mice [43]
DAS	Garlic extract *(Allium sativum)*	MRC-5 lung cells [44]
BE	Root of *Scutellaria baicalensis Georgi*, a Chinese herb	Rats [45]
ARGE	*Allium sativum*, a member of the lily family	RAW 264.7*, [46]

ARGE, aged red garlic; BE, baicalein; BMDM, bone marrow-derived macrophage; CF, carthami flos; DAS, diallyl sulfide; DS, Dangkwisoo-san; FGS, fruit hull of *Gleditsia sinensis*; KA, ent-kaur-16-19-oic acid; *RAW 264.7, mice macrophage cell line.

An oriental remedy called baicalein (BE) has shown to augment the Nrf2/heme oxygenase-1 (HO-1) pathway and to inhibit NF-κB activation in LPS-instilled rat ALI models, thereby attenuating the histopathological symptoms of ALI [45]. Garlic *(Allium sativum)*, a member of the lily family, is a known antioxidant. DAS, enriched in garlic, is a natural organosulfur compound that prevents oxidative stress. DAS induces Nrf2 activation and translocation in nuclei triggered by ERK/p38 signaling pathways in lung MRC-5 cells [44]. Aged red garlic extract (ARGE), which has a more powerful antioxidant effect without the intense taste and smell of regular garlic, reduces the production of LPS-induced nitric oxide in macrophage. The polyphenolic and organosulfur compounds in ARGE could cause activation of Nrf2, eventually increasing HO-1 [46]. In a recent study we showed that glycosylation of aesculin (3-O-β-d-glycosyl aesculin) significantly suppressed neutrophilic lung inflammation in a mouse model of ALI. The anti-inflammatory function of glycosylated aesculin was mediated through Nrf2. In a mouse model of sepsis, a major cause of ALI, 3-O-β-d-glycosyl aesculin significantly enhanced the survival of mice, compared with aesculin, suggesting that glycosylation could confer the ability to activate Nrf2 on aesculin, enhancing the anti-inflammatory function of aesculin. Kaurenoic acid (ent-kaur-16-en-19-oic acid: KA) is a key constituent found in the roots of Aralia continentalis Kitagawa (Araliaceae) and has shown to be an Nrf2 activator. In a murine model of lung injury, we showed that KA has therapeutic potential against inflammatory lung disease, the effect of which is associated with Nrf2 activation.

Curcumin (or diferuloylmethane), a natural product found in turmeric, has been shown to inhibit multiple proinflammatory pathways and to activate anti-inflammatory pathways [51]. Curcumin modulates the activity of several transcription factors (e.g., NF-κB, PPARγ [peroxisome proliferator-activated receptor gamma], and activator protein 1). It inhibits TREM-1 in bone marrow macrophages. Curcumin also inhibits p300-HAT, a key epigenetic element known to activate transcription of the genes that regulate inflammation. Curcumin exhibits antioxidant effects at the level of the KEAP1–Nrf2 complex, resulting in the dissociation of KEAP1 and Nrf2, followed by nuclear accumulation of Nrf2. Kang et al. [50] showed that curcumin activates PI3K and p38 and increases AR activity, which may be a meaningful cellular response against oxidative stress.

Six different phase 1 human trials found no toxicity from curcumin. Both human and laboratory studies have found evidence of anti-inflammatory properties of curcumin, and it inhibits a bevy of enzymes and mediators of inflammation [38, 52]. The benefits of curcumin in sepsis patients appear to be mediated by the upregulation of PPAR-γ, leading to the suppression of the expression and release of TNF-α [39].

5. Conclusions

Since its first description 50 years ago, there has been an increase in the understanding of molecular pathogenesis and pathophysiology for the development of ARDS. However, to date the best practice involves "lung-protective ventilation" in mechanically ventilated patients with ARDS with no specific therapies directed towards lung inflammation. The inflammatory response in patients with ARDS is initiated, amplified, and modulated by a complex network of proinflammatory signaling pathways and oxidant stress generated by a variety of cell types in the lungs. Here, we reviewed some natural products whose biological effects may be useful in the development of new therapies for ARDS. Many of these agents have pleiotropic effects, such as inhibiting proinflammatory signaling while activating antioxidant defense mechanisms. One of the benefits of these natural products is that they have been consumed in the Asian subcontinent for centuries with no significant toxicity. However, to be developed for therapies, systematic studies (including pharmacokinetics and pharmacodynamics) must be carried out in human trials.

Although great strides have been made in the last several decades in defining molecular pathways for ALI and ARDS, these discoveries have not been translated into actual changes in medical treatments for patients with ARDS. To date, supportive strategies and lung-protective ventilation are the only approaches that have been shown to improve outcomes in these patients. A major challenge in generating effective therapeutics has been the ability to develop reliable animal models of critical illness that allow for the generation and testing of novel hypotheses and, ultimately, the translation of these findings to the human condition [53]. Areas of potential study include (1) novel methods of administration

for better absorption (e.g., nanomicelles, lipid spheres) [54–56], (2) a combinatorial approach: multiple remedies could be administered simultaneously, as they are relatively low-toxicity products, and (3) use of natural products for prevention in high-risk patients (identified by lung injury prediction score).

6. Expert Commentary

The prognosis of patients with ARDS continues to be abysmal, with mortality rates ranging from 30% to 40%. Therefore, ARDS represents an unmet medical need and there is an urgent need to develop new therapies to treat patients with this condition. To date, treatment of the inciting event, lung-protective ventilation with lower tidal volumes, and optimal management of fluids remain the key therapeutic strategies for ARDS, but no specific therapies yet exist. Because of the complex nature of the disease (i.e., its involvement of multiple signaling pathways), neither blocking individual proinflammatory cytokines with antibodies nor the use of antioxidants has been rewarding. An interest in natural therapies as anti-inflammatory and antioxidative agents for systemic conditions has been growing. Given the complexity of the pathogenesis of ARDS, many natural products have been tested as pleiotropic agents that may help combat the inflammation and promote healing of the lung. We have reviewed the *in vitro* and *in vivo* data for many products that have been studied in preclinical models of ARDS.

The challenge lies in conducting translational studies to prove the efficacy and safety of these compounds in clinical trials. Although many of the aforementioned agents are widely consumed as herbal supplements or food additives in Asian countries, further study is needed before they can be adopted as therapies for ARDS. The pharmacokinetics and pharmacodynamics of these compounds in the setting of ARDS need to be established. The systemic administration of these compounds can also be challenging, so novel approaches to administer these compounds as nanomedicine or through aerosolization are other potential avenues for future study. Translational studies using these agents in patients with ARDS will provide potential opportunity to develop much-needed novel therapies for this devastating disease.

Abbreviations

AA: Arachidonic acid
ALI: Acute lung injury
AP1: Activating protein 1
AR: Aldose reductase
ARDS: Acute respiratory distress syndrome
ARE: Antioxidant response element
ARGE: Aged red garlic extract
BE: Baicalein
CF: Carthami flos
cAMP: Cyclic adenosine monophosphate
CAPE: Caffeic acid phenethyl ester
CAT: Catalase
CJT: *Callicarpa japonica Thunb*
COX 2: Cyclooxygenase 2
DAP12: TYRO protein tyrosine kinase binding protein
DAS: Diallyl sulfide
DS: Dangkwisoo-san
ERK: Extracellular signal-regulated protein kinase
FGS: Fruit hull of *Gleditsia sinensis*
GCLC: Glutamate-cysteine ligase, catalytic subunit
GPx: Glutathione peroxidase
HLJDT: Huang-Lian-Jie-Du-Tang
HMGB1: High-mobility group box 1
HO-1: Heme oxygenase- 1
HSYA: Hydroxysafflor yellow A
H3K4: Histone 3 and histone 4
IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKK: IκB kinase
iNOS: Inducible nitric oxide synthase
IRAK-4: Interleukin-1 receptor-associated kinase 4
ISOF: Isoforskolin
JNK: c-Jun-terminal kinase
KA: Ent-kaur-16-19-oic acid, or kaurenoic acid
KEAP1: Kelch-like ECH-associated protein 1
LicoA: Licochalcone A
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MCP-1: Monocyte chemoattractant protein-1
MYD-88: Myeloid differentiation primary response gene 88
NF-κB: Nuclear transcription factor-kappa B
NQO1: NAD(P)H quinine oxidoreductase- 1
Nrf2: Nuclear factor erythroid 2-related factor 2
PA: Patchouli alcohol
PCA: Protocatechuic acid
PPARγ: Peroxisome proliferator-activated receptor gamma
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TF: Tissue factor
TIR: Toll/interleukin-1 receptor
TIRAP: Toll/interleukin-1 receptor domain containing adaptor protein
TLR4: Toll-like receptor 4
TNF: Tumor necrosis factor
TRAF6: Tumor necrosis factor receptor-associated factor 6
TREM1: Triggering receptor expressed on myeloid cells 1
VCAM1: Vascular cell adhesion molecule 1.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Tannins and Bacitracin Differentially Modulate Gut Microbiota of Broiler Chickens

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Antibiotic growth promoters have been used for decades in poultry farming as a tool to maintain bird health and improve growth performance. Global concern about the recurrent emergence and spreading of antimicrobial resistance is challenging the livestock producers to search for alternatives to feed added antibiotics. The use of phytogenic compounds appears as a feasible option due to their ability to emulate the bioactive properties of antibiotics. However, detailed description about the effects of in-feed antibiotics and alternative natural products on chicken intestinal microbiota is lacking. High-throughput sequencing of 16S rRNA gene was used to study composition of cecal microbiota in broiler chickens supplemented with either bacitracin or a blend of chestnut and quebracho tannins over a 30-day grow-out period. Both tannins and bacitracin had a significant impact on diversity of cecal microbiota. Bacitracin consistently decreased *Bifidobacterium* while other bacterial groups were affected only at certain times. Tannins-fed chickens showed a drastic decrease in genus *Bacteroides* while certain members of order Clostridiales mainly belonging to the families Ruminococcaceae and Lachnospiraceae were increased. Different members of these groups have been associated with an improvement of intestinal health and feed efficiency in poultry, suggesting that these bacteria could be associated with productive performance of birds.

1. Introduction

For more than 50 years, antibiotic growth promoters (AGPs) have been used in agricultural animal production as a means to increase growth performance through maintained animal health and improved feed efficiency [1]. During the last decades, global concern about development and transference of antimicrobial resistance from animal to human strains is rising [2]. The benefits of AGPs use in production animals are often argued to be outweighed by their negative effects and this practice has been discontinued in the European Union since 2006 due to increasing concern over the spread of antibiotic resistance genes to human pathogens [3]. On the other hand, an important and growing consumer demand for antibiotic-free poultry products is pressing to use cost effective alternatives to AGPs [4, 5].

Although it is still unclear how AGPs enhance animal performance, it is speculated that they act mainly through modulation of gastrointestinal microbiota [6, 7]. The chicken intestinal microbiota plays an important role in digestion and conversion of food into body mass [8, 9] and also in protection from pathogens, detoxification, and modulation of the immune system [10, 11]. Many studies on poultry microbiota have used the cecum as sampling site due to its relationship with chicken productivity and the highly diverse bacterial communities that inhabit this section of

the intestine. The cecum is an important organ contributing to intestinal health and nutrition of birds where anaerobic fermentation of cellulose, starch, and other resistant polysaccharides is performed [12, 13].

Much research has been done in order to characterize the intestinal microbiota of poultry. Initially, most of these works have relied on culture-dependent approaches [14]; and more recently, culture-independent methods have been employed such as denaturing gradient gel electrophoresis, restriction fragment length polymorphisms, and clone libraries, in an effort to overcome the limitations and biases associated with culture-based techniques, since a large portion of the microorganisms comprising the microbiota are not cultivable [15–17]. The advent of high-throughput sequencing of 16S rRNA gene amplicons has enabled the study of bacterial communities at increased depth and resolution [18]. This technology has been used to describe the functional diversity [19] and natural variability of cecal microbiota [20, 21], as well as the temporal [22, 23] and spatial [24–26] variations that normally exist in the chicken gastrointestinal microbiota.

Bacitracin is a mixture of high molecular weight polypeptides that possess antimicrobial activity against gram-positive microorganisms interfering with formation of the bacterial cell wall [27]. Bacitracin is one of the most extensively used AGPs to improve productivity in poultry [1]. In calves, bacitracin has been shown to alter fecal microbiota composition but did not improve animal performance [28]. Some studies have reported alterations in the gut bacterial community of broiler chickens associated with dietary supplementation with bacitracin [17, 29].

Among the available alternatives to replace AGPs for poultry industry, phytogenic additives appear as candidates due to their ability to emulate the bioactive properties of conventional AGPs [30]. Tannins are polyphenolic compounds widely distributed in the plant kingdom, where they play a protective role [31]. Tannins added to the diet are being used in farm animals to improve nutrition and control enteric diseases [32, 33]. However, the effects of tannins on the chicken gut microbiota remain unclear since previous studies have often relied on *in vitro* observations or culture-dependent methods which fail to provide an accurate description of the taxonomic composition and bacterial community structure of chicken microbiota. The aim of the present study was to comparatively analyze the differential effects of dietary supplementation with tannins and bacitracin on chicken cecal microbiome by means of high-throughput sequencing of 16S rRNA gene amplicons.

2. Materials and Methods

2.1. Chicken Diets and Experimental Design. A total of 120 one-day-old unvaccinated male Cobb chicks were obtained from a local commercial hatchery and grown over a 30-day period in biosafety level 2 facilities located at Veterinary and Agriculture Research Center (CICVyA-INTA). Studies presented here were reviewed and approved by the CICVyA-INTA Institutional Animal Care and Use Committee under protocol number 20/2010.

Birds were randomly divided into three groups (40 chicks per group) corresponding to the following dietary treatments: (1) CON: control diet without any supplements; (2) BAC: diet supplemented with subtherapeutic levels of zinc bacitracin (1 g/kg of feed); (3) TAN: diet supplemented with a blend of tannins derived from chestnut *(Castanea sativa)* and quebracho *(Schinopsis lorentzii)* (1 g/kg of feed). Dietary treatments were prepared by thoroughly mixing commercial starter feed (3200 kcal/kg; 20% protein; Alimcer S.A., Buenos Aires, Argentina) with the corresponding supplements. Chickens had ad libitum access to feed and water. Each experimental group was housed in a floor pen (1.5 × 1.5 × 0.8 m) made of 0.55 mm wire mesh and hardboard pieces covering the lower part of the mesh, each containing a galvanized steel self-feeder and a waterer. Birds were raised under controlled environmental conditions and automated ventilation system with 18-hour lighting cycle and a temperature of 32°C on day 1, which was gradually diminished and maintained to 24°C on day 15. Prior to chick placement on pens, litter from a previous flock in which no supplements were used was thoroughly mixed with fresh commercial wood shavings and placed into all pens. On day 21, each group of birds was randomly split in two pens in order to avoid overcrowding and maintain animal density. Body weight (BW) of each animal and feed consumed by each treatment group were recorded on days 5, 12, 19, 26, and 30. Feed conversion ratio (FCR) was calculated as the ratio of feed intake (kg) and weight gained (kg) for each group.

2.2. Sample Collection and DNA Extraction. On days 12, 19, 26, and 30, five animals per group were euthanized by cervical dislocation and both cecal lobes were removed from each bird. The samplings were always carried out at 10 AM, six hours after the start of the light phase of the photoperiod. The tips of the cecal lobes were cut off, and cecal contents were aseptically squeezed out and pooled into sterile recipients for each group. Samples were immediately refrigerated on ice and stored at −80°C until DNA extraction. Total DNA was isolated from 300 mg of cecal contents using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following manufacturer instructions. DNA concentration and quality were assessed in NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA). DNA was stored at −20°C until further analysis.

2.3. 16S rRNA Gene Library Preparation and High-Throughput Sequencing. The 16S rRNA gene V3-V4 regions were amplified using Illumina primers (forward 5′ CCTACGGGNGGCWGCAG 3′, reverse 5′ GGACTACHVGGGTATCTAATCC 3′) with standard adapter sequences attached for barcoding and multiplexing. 16S rRNA gene libraries construction and high-throughput sequencing were performed at Macrogen Inc. (Seoul, South Korea) using the Illumina MiSeq platform following manufacturer's instructions for 2 × 300 bp paired-end sequencing protocol [34]. In order to reduce unbalanced and biased base compositions, 15% of PhiX control library was spiked into the amplicon pool. The datasets generated in this study are available under request.

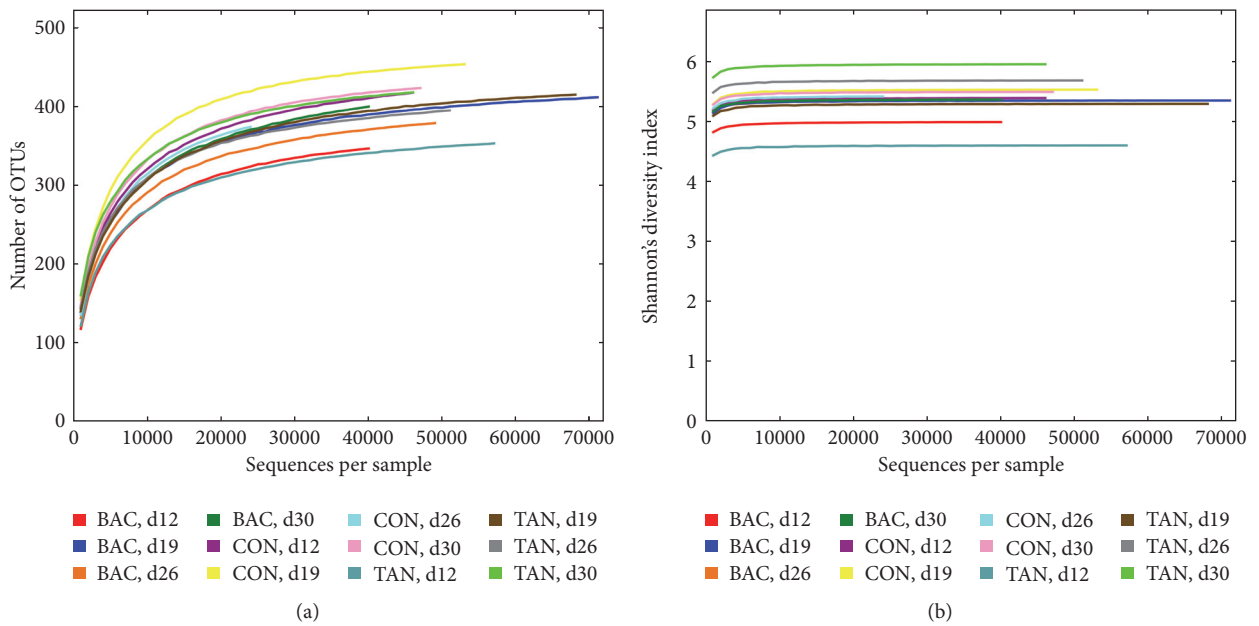

Figure 1: Rarefaction curves of (a) number of OTUs and (b) Shannon's index, obtained based on 16S rRNA gene V3-V4 sequences. OTUs were picked using the UCLUST method with 3% dissimilarity in QIIME. Each curve corresponds to a single pooled cecal sample.

2.4. Sequence Preprocessing. Primer and adapter sequences were trimmed using Trimmomatic v0.33 [35], also removing leading and trailing bases. Paired-end reads were merged into single contigs with FLASh v1.2.11 [36]. Reads were demultiplexed and filtered using a threshold Phred quality score of $Q > 20$. Chimeric sequences were filtered out using USERCH algorithm [37].

2.5. Microbial Community Analysis. Microbial composition and diversity were analyzed using Quantitative Insights into Microbial Ecology (QIIME) software v1.9.1 [38] with default parameters, unless specified. Open-reference operational taxonomic units (OTUs) picking was performed using UCLUST and USEARCH algorithms. Taxonomy was assigned against the Greengenes reference OTU build version 13.8, using a 97% sequence similarity threshold. OTUs with abundance below 0.005% were filtered out from the final OTU table. Normalization of OTU counts was done by performing multiple rarefactions with steps of 1.000 reads and 100 iterations at each rarefaction depth. Alpha diversity was calculated through richness (number of OTUs) and diversity (Shannon's index) estimators. Principal coordinate analysis (PCoA) plots were generated in QIIME based on unweighted UniFrac distance matrix. This method is a β-diversity measure that takes into account the phylogenic divergence between OTUs to identify differences in the overall microbial community structure between samples [39].

2.6. Statistical Analysis. The relative abundances of bacterial populations were analyzed using Statistical Analysis of Metagenomic Profiles (STAMP) software [40]. Relative abundances were compared by two-tailed Fisher's exact test with Storey's FDR correction at each level of classification (phylum, class, order, family, and genus). Additionally, when comparing pairs of cecal samples, STAMP was set to only consider taxa represented by at least 50 sequences and an effect size filter of 3.00. Comparisons on growth performance parameters and diversity estimators between groups of samples were calculated using nonparametric Kruskal–Wallis test and two-tailed Mann–Whitney test for pairs of groups (GraphPad Software, CA, USA), which were considered statistically significant if $p < 0.05$. Calculation of unweighted UniFrac β-diversity metric was subjected to nonparametric permutational analysis of variance (PERMANOVA) in QIIME with 1.000 permutations in order to assess significant differences between samples taken at different time points and between dietary treatments.

3. Results

A total of 1.129.286 paired-end reads were obtained from 12 cecal samples. After quality filtering and removal of chimeric reads, 619.152 sequences remained covering complete V3-V4 regions of the 16S rRNA gene, with a mean length of 452 ± 10 bp. The average number of reads per cecal sample was 51.596 ± 12.406 bp. A total of 513 operational taxonomic units (OTUs) with abundance greater than 0.005% were obtained from all samples.

3.1. Impact of Dietary Treatments on Diversity of Cecal Microbiota. Internal sample α-diversity was estimated through the number of OTUs (richness) and Shannon's index (diversity). Rarefaction curves of observed OTUs (Figure 1(a)) and Shannon's index values (Figure 1(b)) reached a plateau in all samples, demonstrating that sequencing depth was adequate to cover the bacterial diversity in poultry cecal samples.

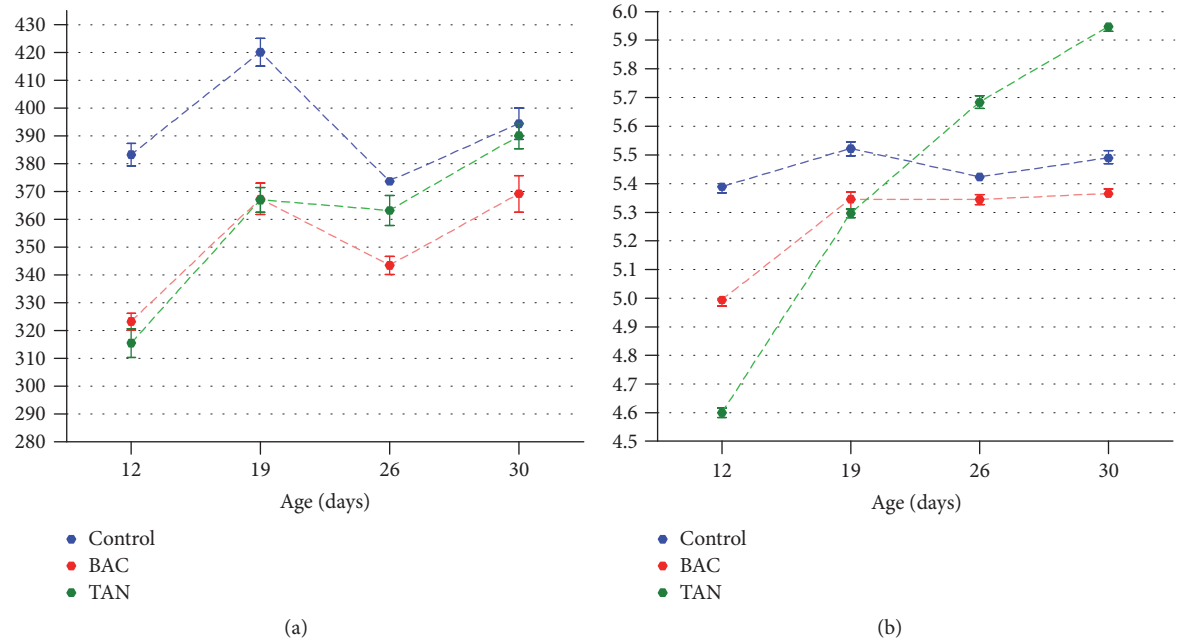

Figure 2: Effect of tannins and bacitracin supplementation on (a) the number of OTUs and (b) Shannon's diversity index of cecal microbiota over time. Bars indicate SD.

The overall average number of OTUs per sample was 368 ± 29, while average Shannon's index was 5.37 ± 0.32. Alpha diversity estimators varied significantly with both treatments as well as with the age of sampling ($p < 0.001$). Between days 12 and 26, animals treated with tannins and bacitracin showed significantly lower richness than the control group (Figure 2(a)). At day 30, tannins-supplemented birds reached a number of OTUs similar to that of the control, while cecal richness of bacitracin-treated animals remained significantly lower. Shannon's diversity index showed a similar profile, although more complex bacterial communities were evidenced in tannins-supplemented birds between days 26 and 30 (Figure 2(b)).

A principal coordinate analysis (PCoA) based on unweighted UniFrac distances was conducted to determine any separation into sample clusters (Figure 3). PCoA plots revealed that the samples corresponding to each dietary treatment form separate series, indicating that tannins and bacitracin differentially modulate cecal microbiota. The PERMANOVA analysis detected significant changes on β-diversity among dietary treatments ($p = 0.031$) and among sampling times ($p = 0.019$), which is consistent with the evident temporal structure of the data depicted in the PCoA plot.

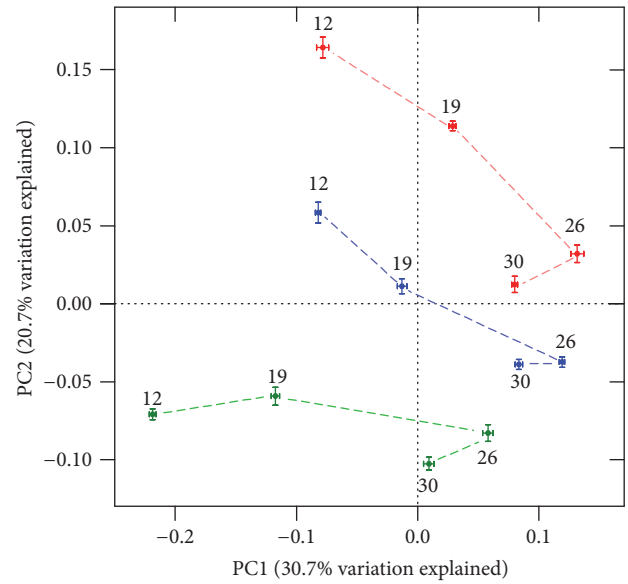

Figure 3: PCoA plot based on unweighted UniFrac metric. Each color represents a different dietary treatment (blue: control without additives; red: bacitracin; green: tannins). Numbers by each point indicate the age of sampling in days. Axes (PC1 = 30.7% and PC2 = 20.7%) account for 51.4% of total variation observed. Bars indicate SD.

3.2. Effects of Tannins and Bacitracin on Composition of Cecal Microbiota. At the phylum level, cecal microbiota was dominated by Firmicutes (CON: 49.29%, BAC: 46.28%, and TAN: 54.00%) and Bacteroidetes (CON: 45.03%, BAC: 48.57%, and TAN: 39.97%), followed by Proteobacteria (CON: 3.90%, BAC: 3.65%, and TAN: 3.16%) and Actinobacteria (CON: 1.58%, BAC: 1.09%, and TAN: 2.39%). The other two phyla, Deferribacteres (CON: 0.10%, BAC: 0.29%, and TAN: 0.37%) and Tenericutes (CON: 0.02%, BAC: not detected, and TAN: 0.03%), were detected in specific samples. Less than 0.10% of the sequences remained unclassified.

The abundances of the two predominant phyla, Firmicutes and Bacteroidetes, showed a strong inverse correlation (Spearman $R = -0.958$, $p < 0.0001$). The Firmicutes to

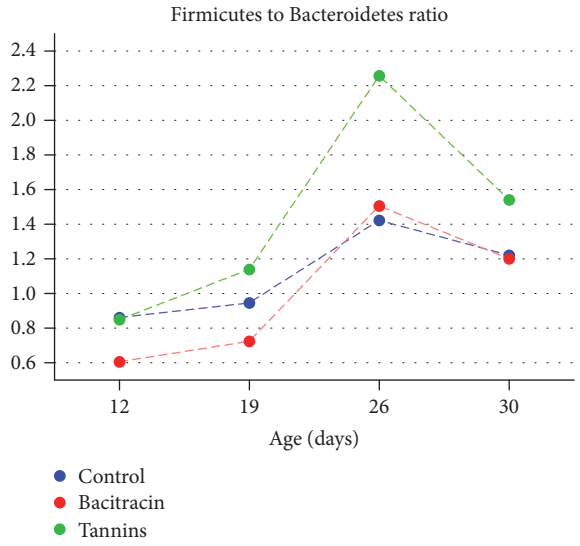

Figure 4: FBR of CON, BAC, and TAN treated chickens over time.

Bacteroidetes ratio (FBR) showed variations over time and across treatments (Figure 4). At day 12, Firmicutes were significantly more abundant in the CON and TAN groups (42.51% and 42.66%, resp.) than in BAC treated chicks (34.89%), while Bacteroidetes showed the opposite pattern. At day 19, BAC treated animals had a lower proportion of Firmicutes than the CON group (38.99% and 45.09%, resp.). At days 26 and 30, no significant differences in the abundance of Firmicutes or Bacteroidetes between the CON and BAC groups were detected. From days 19 to 30, the TAN group exhibited a significantly higher abundance of Firmicutes than CON and BAC treatments.

Different bacteria of the cecal microbiota were affected by tannins and bacitracin, and the impact of each treatment also varied depending on the age of sampling. Twenty bacterial taxa were significantly altered by the treatment with BAC or TAN at least at one time point (Figure 5 and Supplementary Figure 1).

Bacteroides was the most abundant genus on average (21.90%), but these bacterial taxa showed a drastic decrease in the TAN group at all the time points analyzed. Other less abundant bacterial species were significantly affected by the TAN treatment at day 12, including the genera *Phascolarctobacterium*, *Sutterella*, and *Faecalibacterium* and unclassified members of family Succinivibrionaceae. The decline of genus *Bacteroides* in TAN treated chicks was compensated by an increase of other Bacteroidetes belonging to the families Rikenellaceae and Barnesiellaceae and also by the increase of the Firmicutes, including members of order Clostridiales and family Ruminococcaceae (at all the times analyzed), and genus *Blautia* (at days 26 and 30). An increase was also observed in bacteria belonging to phylum Actinobacteria (*Bifidobacterium* at days 12 and 19 and members of the family Coriobacteriaceae between days 19 and 30) and phylum Proteobacteria (members of the family Enterobacteriaceae at days 12 and 19).

Table 1: Growth performance of broilers in different treatment groups.

Parameter	Treatments		
	CON	BAC	TAN
BW (g)			
Day 12	337 ± 28	348 ± 27	323 ± 43
Day 19	777 ± 68	821 ± 77	768 ± 106
Day 26	1444 ± 122	1481 ± 153	1452 ± 224
Day 30	1814 ± 222	1905 ± 232	1798 ± 310
FCR	1.83	1.92	1.99

Chickens supplemented with bacitracin showed a different cecal microbiota profile than those treated with tannins. Bacitracin did not significantly affect genus *Bacteroides* but increased them by 4% at day 30 with respect to the control group. At day 12, BAC treatment impacted on a group of bacteria that included genera *Mucispirillum*, *[Ruminococcus]*, *Ruminococcus*, and *Bifidobacterium* and an unclassified member of the family Coriobacteriaceae. The negative effect of bacitracin on Actinobacteria was repeatedly observed at subsequent sampling times, particularly for the genus *Bifidobacterium*. Genera *Ruminococcus* and *[Ruminococcus]* were also lowered by bacitracin between days 19 and 30. On the other hand, bacitracin favored different taxa at each age of sampling. In the first two sampling times, BAC treatment significantly enhanced members of phyla Proteobacteria (genus *Helicobacter* and families Enterobacteriaceae and Succinivibrionaceae) and Bacteroidetes belonging to the families Rikenellaceae and [Barnesiellaceae], as well as genera *Mucispirillum* and *Peptococcus*. At day 26, a strong increase of genus *Lactobacillus* was detected in BAC treated chicks (CON: 2.17%; BAC: 8.39%), although the opposite pattern was observed at day 30 (CON: 5.36%; BAC: 1.63%). Three taxa from different phyla were significantly enhanced in BAC treated animals at day 30: *Bacteroides*, *Mucispirillum*, and an unclassified member of family Ruminococcaceae.

3.3. Growth Performance. Results for production traits of broilers through the experimental period are shown in Table 1. The average body weight of chickens did not differ significantly among treatments throughout the breeding cycle.

4. Discussion

Detailed description about the effects of classic AGPs and alternative phytogenic compounds on chicken intestinal microbiota is necessary to understand the underlying mechanisms of growth promotion. Improvements in feed conversion associated with dietary supplementation with antibiotics are thought to involve gastrointestinal microbial communities, but this connection remains poorly understood. The establishment of an adult microbiota is a complex process that is influenced by numerous factors including host genetics, intestinal health, stress, age, breeding conditions, weather conditions, diet, litter composition, and the use of feed

Phylum	Class	Order	Family	Genus	Bacitracin d12	Bacitracin d19	Bacitracin d26	Bacitracin d30	Tannins d12	Tannins d19	Tannins d26	Tannins d30
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*								
	Coriobacteria	Coriobacteriales	Coriobacteriaceae	*Unclassified*								
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*								
	Bacteroidia	Bacteroidales	[Barnesiellaceae]	*Unclassified*								
	Bacteroidia	Bacteroidales	Rikenellaceae	*Unclassified*								
Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	*Mucispirillum*								
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*								
	Clostridia	Clostridiales	Unclassified	*Unclassified*								
	Clostridia	Clostridiales	Lachnospiraceae	*Blautia*								
	Clostridia	Clostridiales	Lachnospiraceae	*Dorea*								
	Clostridia	Clostridiales	Lachnospiraceae	*[Ruminococcus]*								
	Clostridia	Clostridiales	Peptococcaceae	*Peptococcus*								
	Clostridia	Clostridiales	Ruminococcaceae	*Unclassified*								
	Clostridia	Clostridiales	Ruminococcaceae	*Faecalibacterium*								
	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*								
	Clostridia	Clostridiales	Veillonellaceae	*Phascolarctobacterium*								
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Sutterella*								
	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	*Helicobacter*								
	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	*Unclassified*								
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Unclassified*								

Figure 5: Effects of tannins and bacitracin in the relative abundance of different bacterial groups of cecal microbiota over time. The heatmap in the right depicts the changes in the relative abundance of each of the taxa with respect to that observed in the control group (green: increased abundance; red: decreased abundance). Cells boxed with thick lines indicate statistically significant changes detected with STAMP.

additives [9, 18, 41, 42]. In the present study, high-throughput sequencing of 16S rRNA gene was used to monitor bacterial composition of the cecal microbiota in chickens supplemented with either bacitracin or tannins over a 4-week production cycle. We found that tannins and bacitracin have a distinct impact on cecal microbiota, each one affecting different bacterial groups at each sampling time.

Analyses of rarefaction curves and diversity indexes indicate that microbial richness and diversity significantly changed with age and dietary treatments. Previous reports found that bacterial diversity in the cecum increases with the age of the bird [18, 23, 43, 44]. This observation was corroborated by our data, since Shannon's diversity index was higher at day 30 than at day 12 in all groups, but the increase was more pronounced in tannins treated birds. There is evidence suggesting that higher diversity microbiota is beneficial in chickens but the cause and effect relationships have not been elucidated [8]. On the other hand, bacitracin-treated chicks showed lower diversity parameters than the control at all the time points analyzed. Lu et al. (2008) also detected a reduction in the gastrointestinal microbiota diversity when bacitracin was administered [29]. However, other authors found that the overall microbial diversity is not significantly disturbed by bacitracin and other AGPs [43, 45–48], although changes in the relative abundance of certain taxa were described in each case.

Although much research has been done regarding the effects of AGPs on the intestinal microbiota of poultry, detailed information about the impact of phytogenic feed additives on chicken microbiota is still lacking. Different tannins including those derived from chestnut and quebracho have shown activity against *Clostridium perfringens* and other poultry pathogens both *in vitro* and *in vivo* [30]. Chickens fed tannin-rich grape products showed increased diversity in the cecum, and these effects correlated with the detection of several potential tannins-degrading bacteria and higher counts of *Lactobacillus* and *Enterococcus* at 21 days of age [49]. Our results indicate that inclusion of tannins in the diet increased cecal diversity between days 26 and 30, although this effect was not associated with the abundance of members of class Bacilli but with bacteria belonging mainly to families Ruminococcaceae and Lachnospiraceae.

Previous studies showed that dietary supplementation with AGPs alters the composition of chicken microbiota, mainly affecting lactobacilli and other Firmicutes in the proximal section of the gastrointestinal tract [50, 51]. A recent work showed that virginiamycin supplementation increased the relative abundance of genus *Propionibacterium* in the ileum, which correlated with a higher concentration of propionate in the cecum, and the authors hypothesized that these bacteria may contribute to the reported growth promoting effects of AGPs [45]. Other authors found significant changes in the cecal microbiota composition of chickens treated with monensin in the presence of AGPs tylosin and virginiamycin, which reduced lactobacilli and enterococci and modulated the abundance of members of the families

Ruminococcaceae and Lachnospiraceae [44]. We found that bacitracin impacted on several members of the families Ruminococcaceae and Lachnospiraceae mainly represented by genera *Ruminococcus* and *[Ruminococcus]*, respectively, while the enrichment in an unclassified Ruminococcaceae was detected at day 30. On the other hand, tannins treatment consistently and strongly increased unclassified members of order Clostridiales and family Ruminococcaceae, as well as the levels of other classified genera of the families Ruminococcaceae and Lachnospiraceae at 26 and 30 days of age. Previous reports have found a cecal enrichment in different member of order Clostridiales after dietary supplementation with AGPs, including unclassified Clostridiales and members of the families Ruminococcaceae and Lachnospiraceae [46, 50, 52]. Moreover, some authors have suggested that these microorganisms could be developed as poultry probiotics [17, 47, 53].

The ratio between phyla Firmicutes and Bacteroidetes in the gut microbiota has been linked to the efficiency in energy harvesting in different animals including mice, pigs, cows, and humans [54–57], suggesting a correlation between growth performance and FBR. In all the analyzed samples, regardless of the dietary treatment, cecal microbiota was dominated by phyla Firmicutes and Bacteroidetes, comprising 94% of the sequences, which is in line with previous reports [12, 13, 52, 58, 59]. A significant increase in the FBR in tannins treated animals was observed in comparison with CON and BAC groups, but this parameter showed no correlation with BW in any of the treatments. Stanley et al. (2013) showed that FBR in the cecum is variable among individual chickens from the same flock but this parameter was not correlated with growth performance [58]. Similarly, other authors found no significant correlation between FBR in the cecum and BW [52, 60]. However, other studies have found a concomitant increase in FBR and FCR in both cecal [52] and fecal [61] microbiota of chicken. The lack of a significant performance response with BAC and TAN supplementation in this study is not surprising given the small number of birds employed and the highly sanitized experimental conditions used, which may not faithfully reproduce the productive conditions.

Interestingly, the higher abundance of members of order Clostridiales has been linked to improved performance of chicken when analyzing both cecal [53, 62, 63] and fecal microbiota [61]. Further research about the specific taxa of order Clostridiales that are associated with growth promotion is required in order to identify and develop new probiotics and prebiotics for poultry.

The presence of probiotic bacteria in the intestine of chicken is associated with an improvement in the performance parameters and a reduction of pathogen loads [8, 64, 65]. Many studies have documented a reduction in the chicken intestinal load of probiotic bacteria after administration of AGPs, including lactobacilli, bifidobacteria, and enterococci [16, 29, 43, 44, 50, 66].

Lactic acid bacteria, especially *Lactobacillus* strains, have been considered as excellent probiotic microorganisms because of their activities in reducing the enteric diseases and maintaining healthy poultry [64]. In this study, genus *Lactobacillus* showed an oscillating pattern in BAC treated chicks, with a strong increase at day 26 and a sharp fall at day 30 with respect to the control group. Our results show that the effect of bacitracin on lactobacilli populations may vary with the age of sampling, similarly to what has been described for other factors such as the analyzed section of the gut [26, 46, 51] or the rearing conditions [47, 48]. Other studies have also found that certain species of lactobacilli can be favored by AGPs [17, 46].

A clear difference was observed between the effects of bacitracin and tannins on the genus *Bifidobacterium*. Tannins increased the abundance of bifidobacteria in the first two sampling times while bacitracin lowered them throughout all the breeding cycle. Previous studies have described a reduction in the cecal counts of bifidobacteria in chickens fed bacitracin and other AGPs [16, 43, 66]. On the other hand, tannins-rich grape products have been found to favor lactobacilli and to a lesser extent bifidobacteria, and it has been suggested that tannins might act as prebiotics, stimulating the proliferation of probiotic bacteria [49]. In line with this, inclusion of mannanoligosaccharides [66] and xylooligosaccharides [45] prebiotics in the diet of chickens has been shown to increase the abundance of lactobacilli and bifidobacteria. Tannins did not affect the levels of *Lactobacillus* and *Bifidobacterium* at day 30 whereas bacitracin significantly affected both of these genera. This differential effect of tannins on probiotic bacteria could contribute to improvement of bird health and reduce pathogens burden at the end of the breeding cycle of poultry.

The breakdown of nondigestible plant carbohydrates originating from the diet of herbivores leads to the formation of fermentation short-chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate. The molar ratio between SCFAs has been linked to the composition of intestinal microbiota in poultry [67]. Previous studies have found that butyrate reduces shedding of acid-sensitive pathogens such as *Salmonella* in poultry and improves the growth of epithelial cells in piglets [68, 69].

Our results show that genus *Bacteroides* was drastically reduced by tannins but not by bacitracin, and this decline was mainly compensated by the increase of Bacteroidetes from the families Rikenellaceae and Barnesiellaceae, as well as the increase of Firmicutes from the families Ruminococcaceae and Lachnospiraceae. Nonadherent *Bacteroides* species have been shown to outcompete gram-positive bacteria such as Firmicutes for easily hydrolysable starch, while the latter are specialized in the degradation of a wide variety of recalcitrant substrates and persist as part of the fibrolytic communities [70]. *Bacteroides* are gram-negative saccharolytic and proteolytic microorganisms that play an important role in breaking down complex macromolecules and generate acetate and propionate as main fermentation products [71, 72]. Previous studies found that polyphenols can inhibit the growth of certain *Bacteroides* while other species within this genus are favored by polyphenols [73, 74]. Families Barnesiellaceae and Rikenellaceae belong to the order Bacteroidales which encompass gram-negative anaerobic coccobacilli, with saccharolytic and proteolytic activities. Barnesiellaceae is a proposed taxonomic group which has not been yet characterized.

Rikenellaceae have been found enriched in the ceca of mice with high-fat diet-induced obesity [75] and seem to be highly susceptible to perturbations in the gut microbiota such as those caused by antibiotics or probiotics supplementation [76, 77].

On the other hand, order Clostridiales encompasses mostly nonpathogenic commensal bacteria including members that have been associated with prevention of inflammatory bowel disease and maintenance of mucosal homeostasis, which has been attributed to the capacity of clostridia to produce butyrate [78]. Moreover, high-concentration butyrate-producing clostridia were isolated from the cecal content of chickens [79]. Laying hens fed tea polyphenols showed increased cecal concentration of butyrate, which protected the duodenal cells from apoptosis [80]. Mašek et al. (2014) reported an increase in the total SCFAs concentration in chickens supplemented with tannic or gallic acids [81]. It is possible that the increase of members of the families *Lachnospiraceae* and *Ruminococcaceae* observed in tannins-fed chickens could alter the SCFAs profile in the cecum towards butyrate production.

5. Conclusions

Taken together, our study indicates that tannins and bacitracin have a differential impact on the composition and diversity of cecal microbiota in poultry. An increase in FBR was observed in tannins-fed chickens at the end of the breeding cycle and at the same time a cecal enrichment in bacteria belonging to the order Clostridiales was detected. The abundance of different members of order Clostridiales has been linked to an improvement in the intestinal health and energy harvesting efficiency in poultry, suggesting that these taxa could be associated with growth performance. However, the mechanisms by which tannins modulate the gut ecology are still poorly understood. Further investigation utilizing full shotgun sequencing metagenomics as well as the measurement of SCFAs concentrations in the gut of chickens will shed light on this issue.

Abbreviations

AGPs: Antibiotic growth promoters
CON: Control
BAC: Bacitracin
TAN: Tannins
BW: Body weight
OTUs: Operational taxonomic units
PCoA: Principal coordinates analysis
FBR: Firmicutes to Bacteroidetes ratio
FCR: Feed conversion ratio
SCFAs: Short-chain fatty acids.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Supplementary Figure 1: effects of tannins and bacitracin in the relative abundance of different bacterial groups of cecal microbiota over time. Colored bars show the abundance of taxa for each treatment group (blue: control; red: bacitracin; green: tannins). For each triad of bars corresponding to a single taxonomic group at a given sampling age, different letters denote treatments with significantly different relative abundances. *(Supplementary Materials)*

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Research Article

Isolation and Characterization of Two New Antimicrobial Acids from *Quercus incana (Bluejack Oak)*

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Two new compounds [**1-2**] were purified from ethyl acetate fraction of *Quercus incana*. The structure of these compounds is mainly established by using advanced spectroscopic technique such as UV, IR, one-dimensional (ID) and two-dimensional (2D) NMR techniques, and EI mass. The structural formula was deduced to be 4-hydroxydecanoic acid [**1**] and 4-hydroxy-3-(hydroxymethyl) pentanoic acid [**2**]. Both isolated compounds were tested for their antimicrobial potential and showed promising antifungal activity against *Aspergillus niger* and *Aspergillus flavus*.

1. Introduction

The family Fagaceae is large family comprising 8 genera and about 800–1100 species. *Quercus* is the largest genus of family Fagaceae having huge medicinal importance and mostly found in dry conditions [1]. The genus *Quercus* have long been considered among the clades of woody angiosperms in terms of species diversity, horticultural merit, ecological dominance, and industrial and economic values [2]. The *Quercus robur* is the only cultivated species while other 600 known species are found in temperate regions of the Northern Hemisphere, Southward through Central America to Colombia and through Turkey to Pakistan [3].

The wood is durable, is attractively grained, and is mostly utilized for timber purposes; it is particularly important in shipbuilding, construction for flooring, furniture, railroad ties, and veneers. The bark of *Quercus* spp. has been used for medicinal purposes and is an important source of phenolic compounds like tannins which are used for tanning leather and wine production [4]. The fruit (acorn) of *Quercus* has husk coating which is edible and highly nutritious and rich in carbohydrates and protein. *Quercus* (oak) species are utilized in conventional pharmaceutical, as astringent, antiseptic, and hemostatic and in addition to the treatment of acute diarrhea, hemorrhoid, and oral, genital, and anal mucosa inflammation. Moreover, the decoction plants from this genus can be used against burns and added to ointments for the healing of cuts [5]. Oak seeds are a major source of sugar, amino acids, lipids, and different sterols [6]. *Quercus* species have been utilized against problems of skin, wounds and gastrointestinal illnesses [7], astringent, mellow germ-free, small cuts [8], and mouth washes [9] all suggesting their antimicrobial potential.

Genus *Quercus* is characterized by six species found mostly in Northern areas of Pakistan. The most promising timber specie is *Quercus incana* Roxb. (Blue jack oak or cinnamon oak) locally called Ban shindar, Kharpata serci (Punjabi), Rein (Hindko), and Serie (Pushto) [10]. The *Q. incana* has huge medicinal usage; it may be used as astringent [11], diuretic, and antidiarrheal agent and for treatment of asthma. Bark and leaves of *Q. incana* may be used as antipyretic, antirheumatism, antidiabetic, and antiarthritic

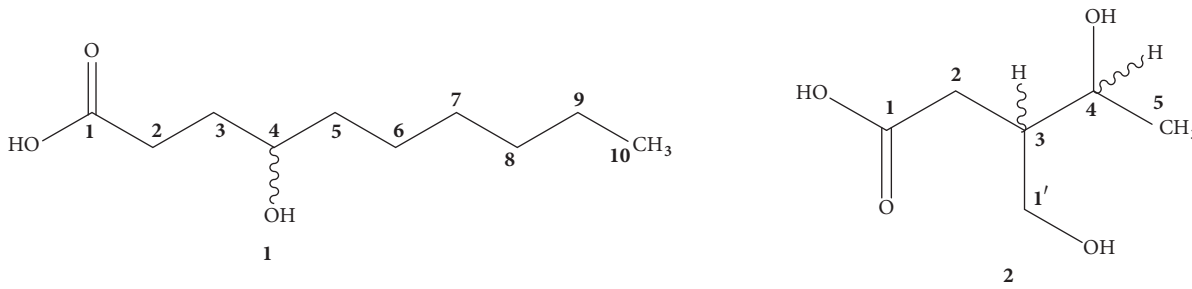


Figure 1: Structure of compounds [**1-2**].

purposes [12]. These medical applications and therapeutic potential of *Quercus incana* prompted us to carry out the phytochemical investigation to explore biologically active compounds.

2. Material and Methods

2.1. Experimental Procedures. The ethyl acetate soluble fraction was selected for isolation of bioactive compounds using column chromatographic analysis having column silica and flash silica gel as an adsorbent material. The column was eluted by using *n*-hexane and ethyl acetate with increasing polarity, which yield two new compounds 4-hydroxy decanoic acid [**1**] and 4-hydroxy-3-(hydroxymethyl) pentanoic acid [**2**] by increasing polarity. The purity of compounds [**1-2**] was checked by using precoated TLC plates. The IR spectrum was recorded by using spectrophotometer JASCO-320A. The EI mass was recoded on double focusing Varian MAT-312 Spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were measured by using advance Bruker AMX-300 spectrometer machine. The chemical shifts in parts per million (δ) relative to tetramethylsilane as an internal standard and scalar *(J)* were described in Hz.

2.2. Plant Extraction and Fractionation. Extraction and fractionation of *Quercus incana* were reported in our previous study [13]. Ethyl acetate soluble fraction was subjected to repeated column chromatography which yielded two pure compounds [**1**-**2**].

2.3. Antibacterial Activity of Compounds [1-2]. Antibacterial activity was performed by agar well diffusion method with some modifications [14]. Three Gram-positive (*Staphylococcus aureus, Micrococcus luteus,* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli, Pseudomonas pickettii,* and *Shigella flexneri*) pathogens were used in study. 10 μg of each compounds [**1**-**2**] was dissolved in 1 mL DMSO. Standard drug and each sample (20 μL) were poured in 6 mm well. The assay plates were incubated at 37°C for 24 hrs. The zone of inhibition was dignified in mm and DMSO was used as a negative control in the experiment.

2.4. Antifungal Assay. Disc diffusion methods were used for determination of antifungal effects by using two selected fungal strains such as *Aspergillus niger* and *Aspergillus flavus* [15]. DMSO was used as a solvent; before applying compounds on petri plates DMSO was completely evaporated.

Characterization of Compound 1. Colorless oil; IR (KBr) υ_{max} 3622 br (OH), 1714 (C=O) cm^{-1}. $[\alpha]_D{}^{25}$ + 34.80° (c = 0.78, $CHCl_3$). EI-MS m/z: (rel. int.) 188 $[M]^+$ (15), 176 (9), 157 (35), 128 (5), 115 (9). HR-EI-MS: m/z 188. 1420 (calcd. for 188.1412 for $C_{10}H_{20}O_3$). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 3.62 (1H, m, H-4), 0.88 (3H, t, J = 7.7 Hz, H-10), 2.08 (2H, m, H-2), 1.49, 1.62 (2H, m, H-3), 1.45 (2H, m, H-5), 1.47, 1.33 (2H, m, H-6), 1.30 (2H, m, H-7), 1.28 (2H, m, H-8), 1.29 (2H, m, H-9). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 179.6 (C-1), 72.1 (C-4), 13.9 (C-10), 34.6 (C-2), 34.9 (C-3), 37.9 (C-5), 26.1 (C-6), 29.8 (C-7), 32.0 (C-8), 22.1 (C-9).

Characterization of Compound 2. Colorless oil: IR (KBr) υ_{max} 3495 (OH), 1708 (C=O) cm^{-1}. $[\alpha]_D{}^{25}$ + 53.60° (c = 0.97, $CHCl_3$). EI-MS m/z: (rel. int.%) 131 $[M\text{-}OH]^+$ 131 (10), 115 (8), 86 (100), 71 (31). HR-EI-MS: m/z $[M\text{-}OH]^+$ 131.0744 (calcd. 131.0736 for $C_6H_{11}O_3$-OH). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 2.35 (1H, m, H-2), 2.04 (1H, m, H-2), 2.58 (1H, m, H-3), 3.92 (1H, m, H-4), 1.23 (3H, d, J = 6.9 Hz, H-5), 4.31 (2H, m, H-1′). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 177.5 (C-1), 27.3 (C-2), 45.9 (C-3), 68.1 (C-4), 20.9 (C-5), 65.5 (C-1′).

3. Result and Discussion

Ethyl acetate soluble fraction was subjected to repeated column chromatography on silica gel using *n*-hexane and ethyl acetate as a solvent with gradual increasing in polarity up to 100% ethyl acetate, which resulted in four subfractions (Fractions **A**–**D**). The fractions obtained based on TLC profile were resubjected to pencil column chromatography and eluted with *n*-hexane : EtOAc, 25 : 75 and *n*-hexane: EtOAc, 30 : 70 to purify compound **1** (10.5 mg) and compound **2** (9.8 mg) (Figure 1).

Compound **1** was isolated as a colorless oil and has molecular formula of $C_{10}H_{20}O_3$ as suggested by molecular ion peak at m/z 188 $[M]^+$ in HR-EIMS. The other fragment peaks were obtained at m/z 176, 157, 128, and 115. The HR-EIMS gave exact mass of compound **1** which was at m/z 188.1420 (calcd. m/z 188.1412). The IR spectrum displayed absorption bands for hydroxyl and carbonyl groups at 3622 and 1714 cm^{-1}, respectively. The ^{1}H-NMR spectrum of compound **1** exhibited typical signal for aliphatic acid skeleton, which was strongly supported by DEPT experiment. The

Table 1: ^{1}H-NMR ($CDCl_3$, 300 MHz) data of compounds [**1-2**] in ppm, J in Hz.

Position	**1**	**2**
1	-	-
2	2.08, m	2.04, m 2.35, m
3	1.62, m 1.49, m	2.58, m
4	3.62, m	3.92, m
5	1.45, m	1.23 (1H, d, J = 6.9 Hz)
6	1.47, m 1.33, m	-
7	1.30, m	-
8	1.28, m	-
9	1.29, m	-
10	0.88 (t, J = 7.7)	-
1′	-	4.31, m

Table 2: ^{13}C-NMR ($CDCl_3$, 75 MHz) of compounds [**1-2**] in ppm.

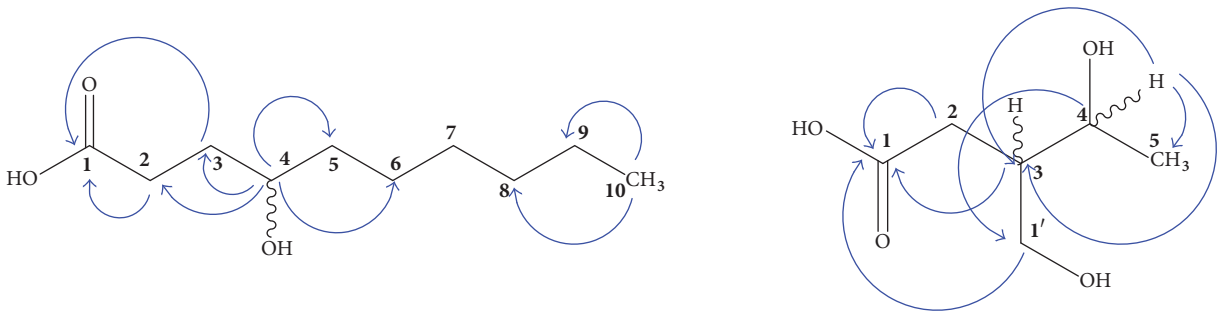
Position	**1**	**2**
1	179.6	177.5
2	34.6	27.3
3	34.9	45.9
4	72.1	68.1
5	37.9	20.9
6	26.1	-
7	29.8	-
8	32.0	-
9	22.1	-
10	13.9	-
1′	-	65.5

^{13}C-NMR spectrum revealed the presence of one methyl, one methine, seven methylene, and one quaternary carbon signals. The methine signal appearing at δ_H 3.62 (1H, m) was assigned to H-4 while methyl group resonated at δ_H 0.88 (3H, t, J = 7.7 Hz). Typical methylene signals were resonated as multiplet for seven methylene carbons at δ_H 2.08 (2H, m), δ_H 1.49 (1H, m), δ_H 1.45 (2H, m), δ_H 1.33 (1H, m), δ_H 1.47 (1H, m), δ_H 1.30 (2H, m), δ_H 1.28 (2H, m), and δ_H 1.29 (2H, m) assigned to H-2, H-3, H-5, H-6, H-7, H-8, and H-9, respectively (Table 1). The ^{13}C-NMR spectrum (BB and DEPT) corroborated the presence of seven methylene carbons, one methine carbon, one terminal methyl carbon, and one quaternary carbon. The carbonyl carbon showed signals at δ_C 179.6 whereas methine signal centered at δ_C 72.1. The ^{13}C-NMR chemical shift of CH_3-C10 was observed at δ_C 13.9 and seven methylene carbons appeared at δ_C 34.6, 34.9, 37.9, 26.1, 29.8, 32.0, and 22.1 for C-2, C-3, C-5, C-6, C-7, C-8, and C-9, respectively (Table 2). The HMBC and COSY spectra were quite helpful for accurate placement of various substituents in the molecule. The HMBC spectrum showed strong correlation of methine proton at δ_H 3.62 (H-4) with C-2, C-3, C-5, and C-6 [13]. The methyl proton at δ_H 0.88 showed strong HMBC correlation with C-9 (δ_C 22.1), C-8 (δ_C 32.0) which was quite supportive in the establishment of structure. Finally all spectral data confirmed, compound **1** as an aliphatic acid having straight chain of -$(CH_2)_7$-CH_3- moiety [16] and was proposed to be 4-hydroxy decanoic acid.

Compound **2** was isolated as colorless oil. Its structure was mainly established by ^{1}H-NMR and high resolution mass spectroscopy and supported by ^{13}C-NMR spectrum. Its molecular formula $C_6H_{12}O_4$ was concluded from the accurate mass measurement of peak at m/z [M-OH]$^+$ 131, corresponding to molecular composition $C_6H_{11}O_3$-OH. In addition to its molecular ion peak, it showed some characteristic fragments at m/z 115, 86, and 71. The HR-EIMS gave exact mass of compound **2** at m/z 131.0744 (calcd. 131.0736 for $C_6H_{11}O_3$-OH). The IR spectrum showed absorption bands at 3495 cm^{-1} and 1708 cm^{-1} indicating presence of the hydroxyl group and carbonyl carbon, respectively. Similarly, broad absorption centered at 2935 cm^{-1} suggested the presence of carboxylic acid. The ^{1}H-NMR showed a signal for a secondary methyl group at δ 1.23 (3H, d, J = 6.9 Hz, H-5), connected to a methine group resonated at δ_H 3.92 (1H, m, H-4) possessing hydroxyl group, while the other methine signal appeared at δ_H 2.58 (1H, m, H-3). The downfield methylene bearing hydroxyl group appeared at δ_H 4.31 (2H, m, H-1′) and the other methylene centered at δ_H 2.04 (1H, m, H-2) and δ_H 2.35 (1H, m, H-2) was directly connected to carboxylic acid (Table 1). The ^{13}C-NMR spectrum confirmed the presence of one methyl carbon, two methylene groups, two methine carbons, and one quaternary carbon in the structure.

In ^{13}C-NMR spectrum, signal for secondary methyl appeared at δ_C 20.9 whereas the methine signal bearing hydroxyl group was observed at δ_C 68.1 for C-4. The signal for another methine appeared at δ_C 45.9 for C-3. The side chain methylene having free hydroxyl group resonated at δ_C 65.5, while the second methylene group at position C-2 appeared at δ_C 27.3. Similarly, the quaternary carbon in the form of carboxylic acid showed signal at δ_C 177.5 (Table 2). Based on the HMBC and H-H COSY correlation (Figure 2), the connectivity of the C-1 to C-5 chain was found in agreement with literature [17]. The HMBC spectrum showed H-C correlation of CH_3-5 with that of C-4 and C-3. Similarly the position of hydroxyl group at C-1′ was confirmed by strong HMBC correlation of CH_2-1′ with C-3, C-2, and C-4 and weak interaction with C-1. The structure of compound **2** was mainly established by ^{1}H-NMR, high resolution mass spectrometry and supported by ^{13}C-NMR spectrum. From all spectral data it was evident that compound **2** was 4-hydroxy-3-(hydroxymethyl) pentanoic acid.

3.1. Antibacterial Activity. The antibacterial activity of isolated compounds [**1-2**] was determined by agar well diffusion method (Table 3). Compound **1** was significantly active against *Bacillus subtilis, Staphylococcus aureus,* and *Micrococcus luteus* (Gram-positive). Both compounds [**1-2**] showed promising antibacterial activity against *Staphylococcus aureus* with 16 mm and 13 mm zone of inhibition. Compound **2** was



Important HMBC correlation of **1**

Important HMBC correlation of **2**

Figure 2: HMBC correlation of compounds [**1-2**].

Table 3: Antibacterial activity of isolated compounds [**1-2**].

S. number	Culture	Zone of inhibition (mm)		
		1	**2**	Ciprofloxacin
1	*Bacillus subtilis*	8	5	8
2	*Staphylococcus aureus*	16	13	16
3	*Micrococcus luteus*	11	9	18
4	*Pseudomonas pickettii*	0	0	0
5	*Escherichia coli*	0	0	0
6	*Shigella flexneri*	6	9	14

Table 4: Antifungal activity of isolated compounds [**1-2**].

Extract	Pathogenic fungi	
	Aspergillus flavus	*Aspergillus niger*
1	12 mm ± 0.50	15 mm ± 0.70
2	17 mm ± 0.28	22 mm ± 0.57
Nystatin (standard)	16 mm ± 0.92	21 mm ± 0.28

Note. Each value in the table was obtained by calculating the average of three experiments.

moderately active against *Bacillus subtilis* and *Micrococcus luteus* with 5 mm and 9 mm zone of inhibition. Both compounds were inactive against *Escherichia coli* and *Shigella flexneri*.

3.2. Antifungal Activity. Antifungal activity of both compounds [**1-2**] was done against *Aspergillus flavus* and *Aspergillus niger*. Both compounds [**1-2**] showed immense activity against *Aspergillus niger* with 15 mm ± 0.70 and 22 mm ± 0.57 zone of inhibition (Table 4). Moderate activity was observed by compound **1** against *Aspergillus flavus* having 12 mm ± 0.50 zone of inhibition.

4. Conclusion

The current study describes the isolation, characterization, and antimicrobial activity of isolated compounds from ethyl acetate fraction of *Quercus incana*. Both compounds displayed promising antimicrobial activity against human bacterial and fungal strains. Therefore, these isolated compounds may be considered as the lead compounds as an antimicrobial agents.

Conflicts of Interest

There are no conflicts of interest regarding this paper.

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Research Article

Mineral and Phytochemical Profiles and Antioxidant Activity of Herbal Material from Two Temperate *Astragalus* Species

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Only a few species of the large *Astragalus* genus, widely used for medicinal purposes, have been thoroughly studied for phytochemical composition. The aim of our research was to investigate the rarely studied species *A. glycyphyllos* L. and *A. cicer* L. for the distribution of mineral elements and phytochemicals in whole plants at two growth stages and in morphological fractions. We also investigated the capacity of the plant extracts to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and to chelate ferrous ions. Chemical composition and antioxidant properties depended on species, maturity, and plant part. Herbal material of *A. glycyphyllos* was richer in Fe, total phenolics, and flavonoids, whereas extracts of *A. cicer* showed a higher antioxidant activity. Young plants had more isoflavones, showed greater quenching of DPPH radicals, and exhibited better mineral profiles than flowering plants. Among plant parts, leaves were the most valuable plant material according to most characteristics investigated. Isoflavone concentration in flowers was lower than in leaves and stems. None of the *Astragalus* samples contained detectable amounts of the alkaloid swainsonine. The study demonstrates the potential of plant material from two *Astragalus* species as a valuable source of iron, phenolic substances including isoflavones, free-radical scavengers, and Fe^{2+} chelators for pharmaceutical use.

1. Introduction

During the past few years, a revival of interest in botanical sources of natural drugs, cosmeceuticals, nutraceuticals, herbal teas, and other health-promoting products has been appreciable. The medicinal use of *Astragalus* species dates back to more than 2000 years ago [1]. A wide range of medicinal applications of remedial preparations have been demonstrated and described in many publications [2–4]. Researchers have demonstrated their immunostimulant activity, antiviral effects, and immunorestorative function in various cancers. Particular attention from the viewpoint of both medicinal use and scientific investigation has been and remains focused on several species of *Astragalus* genus, the largest one among flowering plants, which is comprised of about 3000 species [5]. Species *A. membranaceus* (Fisch.) Bge. and *A. membranaceus* var. *mongolicus* (Bge.) Hsiao are important herbs in traditional Chinese medicine. These species are indigenous to China, Korea, Mongolia, and Siberia and are commercially cultivated in northern China and Korea; their herbal material has been studied in detail for

chemical composition and pharmacological applications [3, 4, 6–8].

The two *Astragalus* species chosen for this study grow naturally in the temperate climatic conditions of Eurasia. *Astragalus cicer* L. (cicer milkvetch) is a perennial plant native to Central and Eastern Europe [9], which was introduced to areas in Southern Europe, North America, and South America. *Astragalus glycyphyllos* L., commonly known as liquorice milkvetch, is also a perennial species which is widespread throughout Europe and temperate Asia [10]. These two species have been used in traditional medicine as well as in food in several European countries [7, 11]. Application of remedies from roots and leaves of *A. glycyphyllos* relates to their refreshing, purifying, diuretic, and many other properties [7, 8, 12, 13]. Lysiuk et al. [14] quantified hydroxycinnamic acids in the plant material of *A. glycyphyllos* and stated that some of them might contribute to a positive impact on the urinary system due to their renoprotective effects. According to De Vos [15], the use of cicer milkvetch as a medicinal herb was listed in eight sources, beginning with the Hippocratic Corpus of the 5th century BC and ending with the Farmacopea Española of 1865. Although cicer and liquorice milkvetches have been in use for a long period of time, there are few data available on their chemical composition and bioactive properties.

Qualitative data on the occurrence of flavonoids in both above- and underground parts of *Astragalus* species were summarised in recent review articles [2, 16, 17]. Based on reviews, it appears that flavonoid-like compounds identified in *A. cicer* and *A. glycyphyllos* are species-specific. This species specificity may be related to the taxonomic differences between species. Based on phylogenetic analyses, species were attributed to the different taxonomic units [18–20].

There are numerous reports on the association between phytoestrogens and reduced osteoporosis and cardiovascular disease, prevention of cancer, antidiabetic effects, and relief of menopausal symptoms [21]. Li et al. [2] confirmed that several isoflavones and their derivatives were detected in *Astragalus* spp., including formononetin, biochanin A, and genistein. However, these data were related to species not included in this study. The information on isoflavone distribution in plant material of *A. glycyphyllos* and *A. cicer* is scarce and generally limited to qualitative data [16, 17]. In addition to valuable bioactive compounds and properties, *Astragalus* species contain toxic substances, including the neurotoxin indolizidine alkaloid, swainsonine [8, 22].

Phenolic substances are major contributors to the antioxidant properties of plants, acting as free-radical scavengers and chain breakers [23]. Despite that, iron deficiency results in impaired production of iron-containing proteins and inhibition of cell growth; excessive iron uptake has been related to hereditary hemochromatosis, leading to tissue damage [24]. According to Santos et al. [25], iron chelation may be useful in the prevention and treatment of microbial infections. However, we did not find published data on ferrous ion-chelating ability by extracts of *A. cicer* and *A. glycyphyllos* plants.

Diets of over two-thirds of the world's population lack one or more essential mineral elements [26]. The minerals, iron (Fe), zinc (Zn), calcium (Ca), potassium (K), and magnesium (Mg), are classified as components of high priority for dietary supplementation [27]. Currently, Fe and Zn are the most common microelements lacking in diets of young children and women of childbearing age, particularly in low- and middle-income countries [28]. Investigations of *Astragalus cicer* and *A. glycyphyllos* with respect to mineral and phytochemical compositions, as well as antioxidant activity, remain sporadic and mostly of a qualitative nature.

In this study, we investigated the distribution of minerals and bioactive substances in the aerial parts of two European *Astragalus* species and conducted an *in vitro* evaluation of antioxidant activity in plant extracts. The influence of growth stage and plant part on these parameters was examined.

2. Materials and Methods

2.1. Plant Materials. Five samples of each of two *Astragalus* species (*A. cicer* L. and *A. glycyphyllos* L.) were used in the current investigation (Figure 1, Supplementary Materials). Seeds of wild ecotypes of *A. glycyphyllos* and *A. cicer* were collected in natural habitats in Lithuania (55°22′51″N; 23°50′35″E) and Latvia (57°01′45″N; 21°25′23″E), respectively. Species were identified by Dr. Vaclovas Stukonis (Department of Grass Breeding, Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry, IA, LAMMC) according to the morphological descriptions [19, 29]. Species were catalogued at IA, LAMMC, under the respective numbers ŽI-13 and ŽI-71.

The following year, seeds were sown in the germplasm collection of the perennial legumes species in single rows, 2.5 m long and 0.5 m apart. Plots were set up in the experimental site of IA, LAMMC (Central Lowland of Lithuania, 55°23′49″N, 23°51′40″E). A randomised complete block design was used with four replications. No pesticides were applied. Samples of milkvetch species were collected during the fully flowering and vegetative stages. Aerial parts of each legume sampled at full flowering were divided into two subsamples: one was investigated as a sample of the whole aerial part; the other subsample was divided into three plant parts (stems, leaves, and flowers) (Figure 1).

The four replicate samples were pooled for chemical analyses. All samples were washed thoroughly with tap water, rinsed with distilled water, and blotted on filter paper. Samples were then chopped, immediately predried in an oven (105°C for 15 min) to rapidly stop the processes of metabolism, oven-dried (65 ± 5°C for 24 h), and then ground using a cyclone mill and passed through a 1 mm screen. Before analysis, a small portion (2-3 g) of each sample was dried to a constant mass in a forced-air oven at 105 ± 5°C so that data could be expressed per unit dry matter (DM). Chemical analyses were carried out in triplicate.

2.2. Analytical Standards and Reagents. Daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one; ≥98%), formononetin (7-hydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one; ≥99%), genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one; ≥98%), biochanin A (5,7-dihydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one; ≥98%), swainsonine

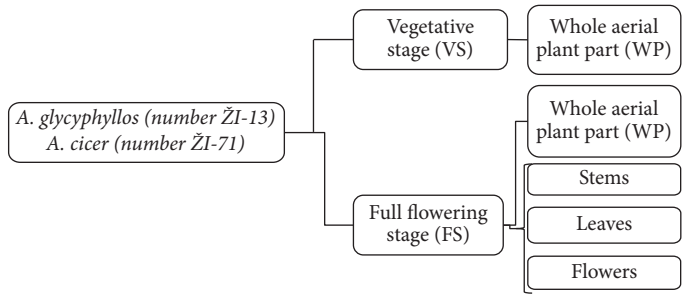

Figure 1: Sampling design.

(≥99%), Folin-Ciocalteu phenol reagent (2 N), gallic acid monohydrate (≥98.0%), sodium carbonate (anhydrous, 99.5–100%), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid monosodium salt (ferrozine; 97%), methanol (≥99.9%), acetone (≥99.8%), hexane (≥97.0%), acetic acid (≥99.7%), sulphuric acid (95–98%), LC-MS grade acetonitrile, formic acid, acetic acid, ammonium formate, and ammonium acetate were purchased from Sigma-Aldrich (UAB Labochema, Vilnius, Lithuania). LC-MS grade methanol, acetic acid, and formic acid were obtained from Fluka/Sigma-Aldrich (UAB Labochema). Stable free DPPH (2,2-diphenyl-1-picrylhydrazyl) radical (95%) and iron(II) chloride (anhydrous, 99.5%) were supplied by Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany). Acetic acid (100%), aluminium chloride hexahydrate (≥95%), hexamethylenetetramine (≥99%), and rutin trihydrate (≥95%) were obtained from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Ethanol (96.3% v/v) was purchased from Stumbras (Kaunas, Lithuania). Double-deionised water with conductivity lower than 18.2 MΩ was purified using a Milli-Q Direct 8 water purification system (Millipore, Bedford, MA, USA).

2.3. Determination of Minerals. Concentrations of potassium, calcium, magnesium, zinc, and iron were quantified after nitric acid plus hydrogen peroxide digestion followed by flame atomic absorption spectroscopy (AAS) using a PerkinElmer model AAnalyst 200 (USA). Parameters of the instrument were chosen in accordance with the manufacturer's instructions. Total phosphorus was determined after sulphuric acid digestion of the samples and reaction with molybdate vanadate. The absorbance was measured by a UV-Vis spectrophotometer (Cary 50, Varian, USA) at 430 nm. Mineral content was expressed as mg/100 g DM.

2.4. Preparation of the Extracts. Preliminary extraction studies were performed to determine the effect of the extraction method (maceration and ultrasonic agitation) and aqueous ethanol (40–80% v/v) on the recovery of total phenolic contents (data not shown). It was established that the highest recovery of phenolics was achieved using 70% (v/v) aqueous ethanol and ultrasonic extraction of botanical samples at 50°C for a 15-minute period of sonication. An ultrasonic bath Elmasonic S40H (Elma Schmidbauer GmbH, Germany) was used for sonication extractions. Samples (0.25 g) of powdered (oven-dried) plant material plus 25 mL of 70% (v/v) aqueous ethanol were sonicated at 50°C for 15 min. The suspension was filtered and the supernatant adjusted to 25 mL in a measuring flask.

Acid hydrolysis and extraction of isoflavones were both performed in a single step according to a slightly modified procedure described by Saviranta et al. [30]. The representative amount of samples (250 mg) was extracted with 10 mL of methanol/water (8 : 2, v/v) containing 2 M HCl using sonication for 30 min at room temperature before being hydrolysed at 80–85°C for 1.5 h. Extracts were filtered through a 0.2 μm nylon syringe filter and then analysed.

Swainsonine extraction was performed according to Gardner and Cook's [31] published procedure. Dried plant material (100 mg) was placed in a 10 mL screw-cap glass container and extracted with 5 mL of 2% acetic acid for 16 h with agitation. After extraction, the samples were centrifuged for 5 min. Aliquots (0.50 mL) of the extract were added to 1.00 mL of acetonitrile, thoroughly mixed, filtered through a 0.2 μm nylon syringe filter into a glass sample vial, and then analysed.

2.5. Total Phenolic Content. Total phenolic content (TPC) of plant extracts was determined spectrophotometrically by the Folin-Ciocalteu method [32]. Standard solutions of gallic acid were prepared at concentrations of 11–350 μg/mL in ethanol (96% v/v). Aliquots (1 mL) of the standards or appropriately diluted extract were combined with 0.2 N Folin-Ciocalteu's reagent (5 mL). After 5 min, sodium carbonate (4 mL of 7.5% w/v solution) was added to the mixture and shaken. Samples were left to stand for 60 min at room temperature and then absorbance was determined at 765 nm using a UV-Vis spectrophotometer, Spectronic Genesys 2 (Spectronic Instruments, USA). Quantification was based on the gallic acid standard curve. TPC concentration was expressed in mg of gallic acid equivalents (GAE) per g DM (mg GAE/g).

2.6. Total Flavonoid Content. Analysis of extracts for total flavonoid content (TFC) was performed by spectrophotometry, as described in the Lithuanian Pharmacopoeia [33]. A 1 mL aliquot of plant extract (Section 2.4) was added to a 25 mL volumetric flask containing 10 mL of 96% (v/v) ethanol. Then, 0.5 mL of 33% acetic acid, 1.5 mL 10% $AlCl_3$, and 2 mL of 5% hexamethylenetetramine solution were pipetted into the flask and made up to 25 mL with distilled

water. Absorbance was read at 407 nm after 30 min at 20°C versus the prepared blank. Blank samples were prepared from 1 mL of plant extract, 10 mL of 96% (v/v) ethanol, and 0.5 mL of 33% acetic acid and diluted to 25 mL with distilled water. The absorbance of the reference solution, which was prepared using 1 mL of rutin solution instead of plant extract, was measured simultaneously. Standard rutin solution was prepared by dissolving 0.05 g of rutin in 100 mL of 96% ethanol. TFC was expressed as milligrams of rutin equivalents (RE) per g on DM (mg RE/g).

2.7. Quantification of Isoflavones. Quantification of the four isoflavones (daidzein, genistein, and their 4′-methylated derivatives, formononetin and biochanin A) was performed by ultraperformance liquid chromatography (UPLC) using a Waters Acquity UPLC system (Waters, Milford, MA, USA) equipped with a binary pump, membrane degasser, autosampler, thermostated column compartment, and diode array detector (DAD). An Acquity UPLC BEH C18 column (100 × 2.1 mm i.d., 1.7 μm, Waters) was used in the experiments. Elution was performed using 0.25% aqueous acetic acid (mobile phase A) and 0.25% acetic acid in 80 : 20 v/v methanol/water (mobile phase B). The flow rate was 0.25 mL/min with a linear gradient from 2% to 100% B in 15 min, followed by reequilibration with the initial mobile phase for 5 min. Column temperature was maintained at 30°C, the mobile phase flow rate was 0.25 mL/min, and the injection volume was 5 μL. Data collection and management were performed using HyStar 3.2 software (Bruker Daltonics, Bremen, Germany). Isoflavones in extracts were identified according to our recently published procedure [34] and by comparing retention times with those of corresponding standards. Quantification was performed by external calibration and results are expressed as mg/100 g DM. The limits of quantification, defined as the concentration resulting in a signal ten times the noise level, were 0.15 mg/L (0.006 mg/g DM) for biochanin A and formononetin, 0.20 mg/L (0.008 mg/g DM) for genistein, and 0.25 mg/L (0.010 mg/g DM) for daidzein.

2.8. LC-MS/MS Conditions of Swainsonine Detection. Hydrophilic interaction chromatography (HILIC) combined with tandem mass spectrometry (MS/MS) was used for quantification of swainsonine [35]. All separation procedures were carried out using a 1290 Infinity UHPLC system connected to 6410 triple quadrupole mass spectrometer, equipped with electrospray ionisation (ESI) source (Agilent Technologies, USA). HILIC separation was performed on an Acquity UPLC BEH HILIC column (2.1 × 100 mm, 1.7 μm, Waters). The first and third quadrupoles were operated at unit resolution. Data were acquired and processed using MassHunter software (Agilent).

2.9. DPPH Radical-Scavenging Activity. The antioxidant activity of plant extracts was determined based on free-radical scavenging capacity using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [36]. A solution of DPPH in 96% (v/v) ethanol (6×10^{-5} M or 0.06 μmoles of pure DPPH per 1 mL solution) was prepared daily before analysis. 50 μL of the plant extract (Section 2.4) was mixed with 2 mL of DPPH solution and left to stand for 30 min (until the reaction reached a steady state) in the dark at room temperature. The decrease in absorbance due to scavenging of DPPH was monitored with a spectrophotometer at 515 nm. The absorption of a blank sample containing the same amount of 70% (v/v) ethanol and DPPH solution was determined each time before the analysis. The radical-scavenging capacity of plant extracts was calculated as the percentage of DPPH inhibition (DPPH%) according to the following equation:

$$\text{DPPH\%} = \frac{\left[\left(A_{\text{control}} - A_{\text{sample}}\right) \times 100\%\right]}{A_{\text{control}}}, \quad (1)$$

where A_{control} is the absorption of the blank sample (t = 0 min) and A_{sample} is the absorption of the solution containing the plant extract (t = 30 min). Finally, results were recalculated as μmoles of DPPH free radicals scavenged by the extract from 1 g of plant material DM (DPPH μmol/g) according to the following equation:

$$\text{DPPH } \mu\text{mol/g} = \frac{(0.12 \times \text{DPPH\%})}{(0.0005 \times 100)}, \quad (2)$$

where 0.12 is μmoles of pure DPPH in the aliquot and 0.0005 is the plant material mass (g) in the volume of extract used for the test.

2.10. Ferrous Ion-Chelating Activity. Ferrous ion-chelating (FIC) potential of the extract was investigated according to the modified method of Dinis et al. [37], wherein the $Fe2^{+}$-chelating ability was determined by measuring ferrous iron-ferrozine complex at 562 nm. Briefly, 50 μL of 2 mM $FeCl_2$ solution was added to a 1 mL aliquot of 70% (v/v) ethanolic plant extract (appropriately diluted). After 5 min, the reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine solution. The mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. A 1 mL aliquot of 70% (v/v) ethanol, containing no plant extract, was used as the control. The percentage of ferrous ion-chelating activity (FIC%) of the extracts was calculated according to the following equation:

$$\text{FIC\%} = \frac{\left[\left(A_{\text{control}} - A_{\text{sample}}\right) \times 100\%\right]}{A_{\text{control}}}, \quad (3)$$

where A_{control} is the absorbance of the control and A_{sample} is the absorbance of the reaction mixture containing the plant extract.

The final FIC capacity was calculated as the amount of Fe^{2+} μmoles bound by chelating agents in the extract from 1 g of plant material DM (FIC μmol/g) according to the following equation:

$$\text{FIC } \mu\text{mol/g} = \frac{(0.1 \times \text{FIC\%})}{(0.01 \times 100)}, \quad (4)$$

where 0.1 is Fe^{2+} μmoles in the aliquot of $FeCl_2$ solution and 0.01 is the plant material mass (g) equivalent to the volume of extract used for the test (1 mL).

Table 1: Mineral profile of *A. glycyphyllos* and *A. cicer* plant material; whole aerial part (WP) of plants harvested at the vegetative stage (VS) and fully flowering stage (FS) and the separate parts of fully flowering plants (mean ± standard deviation).

Mineral	WP VS	WP FS	Stems	Leaves	Flowers
			A. glycyphyllos		
Ash, g/100 g	10.82 ± 0.396	7.66 ± 0.007	4.11 ± 0.014	8.97 ± 0.233	7.88 ± 0.375
K, g/100 g	2.78 ± 0.060	2.18 ± 0.092	1.78 ± 0.115	2.22 ± 0.042	2.94 ± 0.045
Ca, g/100 g	1.55 ± 0.141	1.10 ± 0.042	0.701 ± 0.021	1.91 ± 0.113	0.463 ± 0.008
Mg, g/100 g	0.531 ± 0.008	0.511 ± 0.001	0.272 ± 0.003	0.596 ± 0.011	0.341 ± 0.052
P, g/100 g	0.320 ± 0.010	0.276 ± 0.017	0.198 ± 0.003	0.256 ± 0.003	0.428 ± 0.016
Zn, mg/100 g	2.44 ± 0.045	3.47 ± 0.113	2.30 ± 0.113	2.70 ± 0.141	4.76 ± 0.042
Fe, mg/100 g	65.29 ± 3.125	21.72 ± 1.131	15.85 ± 0.495	22.66 ± 1.670	14.10 ± 0.794
			A. cicer		
Ash, g/100 g	11.23 ± 0.240	8.08 ± 0.014	4.23 ± 0.021	9.81 ± 0.113	9.18 ± 0.057
K, g/100 g	3.06 ± 0.080	1.99 ± 0.071	1.59 ± 0.023	2.38 ± 0.022	3.02 ± 0.099
Ca, g/100 g	1.60 ± 0.085	1.55 ± 0.044	0.649 ± 0.023	2.09 ± 0.085	0.643 ± 0.052
Mg, g/100 g	0.506 ± 0.006	0.538 ± 0.011	0.370 ± 0.008	0.545 ± 0.010	0.328 ± 0.023
P, g/100 g	0.366 ± 0.007	0.275 ± 0.016	0.180 ± 0.007	0.288 ± 0.020	0.473 ± 0.01
Zn, mg/100 g	2.79 ± 0.127	2.97 ± 0.113	1.88 ± 0.085	2.69 ± 0.085	4.61 ± 0.170
Fe, mg/100 g	30.19 ± 2.100	14.94 ± 0.764	6.78 ± 0.368	14.52 ± 0.651	13.13 ± 0.289

2.11. Statistical Analysis. Statistical analysis of data was carried out using Statistica 7.0 software for Windows (StatSoft Inc., USA), performing basic statistics and correlation matrices. Results for mineral composition, bioactive compound, and antioxidant activity were presented as the mean of triplicate determinations ± standard deviations (SD) and expressed on a DM basis. The Pearson correlation coefficient test was performed on values obtained for isoflavones, TPC, TFC, DPPH, and FIC.

3. Results and Discussion

3.1. Mineral Composition. The mineral concentration of *Astragalus* spp. varied widely depending on the plant growth stage and morphological fraction (Table 1). In general, the mineral profile of young plants demonstrated higher content of ash and almost all elements tested than the flowering milkvetches. With respect to the growth stage, Fe concentration changed the most out of all the mineral components, decreasing as much as 3-fold in *A. glycyphyllos* and 2-fold in *A. cicer* with advancing maturity: plants at the vegetative stage contained 65.29 and 30.19 mg Fe/100 g, respectively, whereas Fe concentration in flowering plants amounted to 21.72 and 14.94 mg/100 g, respectively. Within the species, the mineral concentrations (except for Fe) differed more among plant parts (from 1.6- to 4.1-fold) than between the two growth stages (by 1- to 1.5-fold). In flowering *Astragalus* plants, the content of ash, Ca, Mg, and Fe was higher in leaves than in flowers and stems, and flowers were richer in K, P, and Zn than in the remaining two morphological fractions. The mineral profile of stems was the poorest among all samples of respective species of milkvetch; however, with regard to Fe concentration, stems of *A. glycyphyllos* were Fe-richer (15.85 mg/100 g) than its flowers (14.1 mg/100 g).

The growth stage at harvest as the most important factor governing the quality of various herbaceous plants is well documented in the scientific literature. Data on this subject for the species studied here were not available; however, research results on other perennial legumes demonstrated that harvest at an early phenological stage is preferred with regard to higher concentration of some macro- and micronutrients [38, 39]. Changes in mineral content with advancing plant maturity are related primarily not only to the increasing ratio of stem with low mineral content to leaf distinguished by higher concentration of elements, but also to a mineral dilution effect caused by accumulation of lignocellulosic components in mature plants. Results on the distribution of minerals among plant parts reported here are in agreement with earlier observations [38, 39] for other genera of Fabaceae.

With regard to the mineral profile, *Astragalus* species, in particular young milkvetch plants harvested at the vegetative stage and leaves of fully flowering plants, are a good source of important dietary elements such as Ca, Mg, and Fe. Additionally, it should be noted that Fe values for young *Astragalus* plants were higher than those in other plants widely used in foods, including spinach which is considered to be a rich source of Fe, or in many medicinal plants [26, 40]. This finding is significant as Fe deficiency is considered to be one of the top ten health challenges in modern society, being particularly prevalent in women of childbearing age [41]. Findings of this study indicate that *Astragalus* plant material could provide an important natural source of iron.

3.2. Bioactive Substances. Extracts of *A. glycyphyllos* leaves and flowers had the greatest amount of phenolic compounds (25.99 and 23.71 mg GAE/g, resp.; Figure 2(a)) and total flavonoids (21.00 and 16.71 mg RE/g, resp., Figure 2(b)). The lowest concentrations of both total phenolics (6.36 mg GAE/g) and flavonoids (1.00 mg RE/g) were measured in stems of flowering liquorice milkvetch plants. The same trend was observed with regard to TPC and TFC distribution in

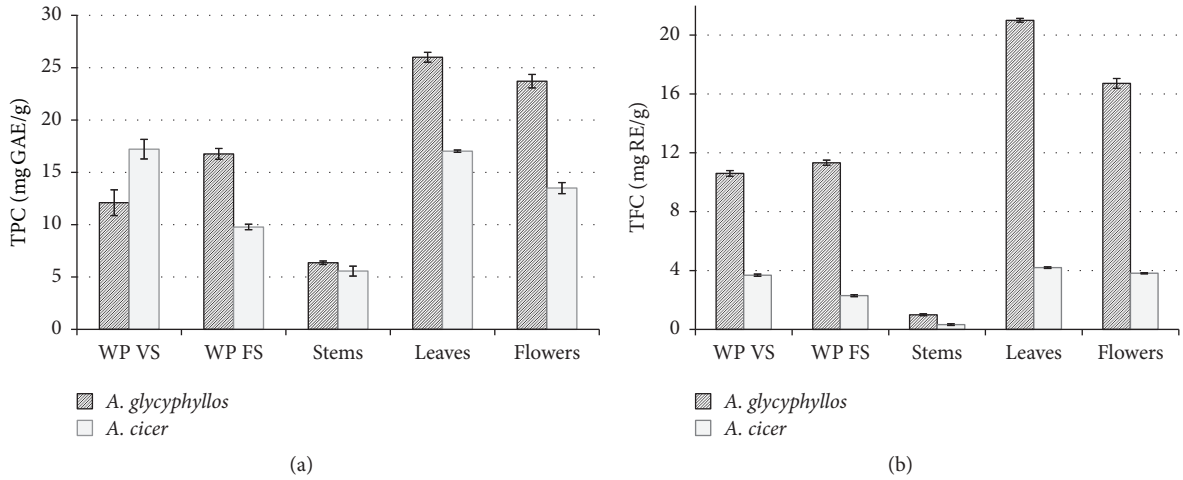

Figure 2: Total phenol (a) and total flavonoid (b) content in *A. glycyphyllos* and *A. cicer* plant material: the whole aerial parts (WP) of plants of vegetative stage (VS) and flowering stage (FS) and the separate parts of fully flowering plants (error bars indicate standard deviation).

plant parts of flowering *A. cicer*; however, concentrations of both total phenolics and flavonoids were considerably lower than those in the respective plant material of *A. glycyphyllos*. Differences in TPC and TFC concentrations in whole aerial plant parts were observed between the two stages of plant growth, with concentrations being lower in young plants of *A. glycyphyllos* than in fully flowering plants and vice versa in *A. cicer*.

Overall, results for TPC and TFC are consistent with research data presented in the literature, although published data are very sparse for the plant species studied here. Platikanov et al. [18] demonstrated that composition of volatile compounds, including those of phenolic origin, varied according to plant part of the various *Astragalus* spp. plants and within the vegetation period. Lobanova [42] similarly showed that, at the beginning of the vegetation period, *A. glycyphyllos* leaves contained fewer flavonoids (flavonols) than leaves at later stages of maturity (flowering and beginning of fruiting). Our data agree with this finding, suggesting that, among plant parts, stems accumulate the lowest concentrations of flavonoids. However, *A. glycyphyllos* collected in the mountains of Serbia and Montenegro accumulated considerably more TPC [12] than we found in herbal material from Lithuanian plants. Meanwhile, Tusevski et al. [43] reported a TPC value (15.93 mg GAE/g) in liquorice milkvetch from Macedonia similar to that found in this study in whole fully flowering plants. However, the Macedonian researchers reported lower TFC (1.62 mg catechin equivalent/g) than we determined in the whole aerial part of plants fully flowering or in plants during the vegetative phase (10.60 and 11.33 mg RE/g, resp.). Since there is a sparsity of published data on this topic, it is difficult to make a definitive statement on the discrepancies described. With regard to TPC and TFC in herbal material from *A. cicer*, published data were not found.

In the current study, we focused on the four major isoflavones (formononetin, biochanin A, genistein, and daidzein) occurring in red clover, a perennial legume species most frequently studied for isoflavones. A further reason for selecting these isoflavones was that daidzein and genistein are the primary isoflavones in soy beans, commonly used as a source of isoflavones in the production of food supplements and functional foods. Numerous differences in both the qualitative and the quantitative patterns of isoflavone composition were observed in herbal material from the two *Astragalus* species (Table 2). The dominant isoflavones in young plants of both *A. glycyphyllos* and *A. cicer* were formononetin (9.24 and 10.85 mg/100 g, resp.) and biochanin A (8.81 and 11.40 mg/100 g, resp.). Concentrations of these isoflavones were considerably lower in the whole aerial part as well as in separate morphological fractions of fully flowering plants, compared to those at the vegetative stage. In contrast, with regard to genistein concentration, the opposite trend was observed: flowering plants and their parts contained more isoflavones than plants in the vegetative phase. Among the plant parts of *A. glycyphyllos*, the highest genistein concentration was found in stems (7.29 mg/100 g). In leaves of flowering *A. cicer*, genistein concentration was of a similar level to that found in stems and the whole aerial plant. There were only trace levels of daidzein (below the limit of quantitation) in extracts of all samples from the two *Astragalus* species. According to the total amount of isoflavones quantified, young plants of *A. cicer* contained more phytoestrogens than those of *A. glycyphyllos*; however, samples of flowering *A. glycyphyllos* were richer in isoflavones than the respective plant part of cicer milkvetch. Isoflavones concentration in flowers of milkvetches was from 1.6- to 2.2-fold lower than in stems and leaves.

Comparison of the isoflavone content of milkvetches with that of more than 240 foods, reported by Kuhnle et al. [44], made it evident that only soy-based flour (124.4 mg/100 g) was richer in isoflavones than plant material of *Astragalus* accessions. Moreover, qualitative analysis of UPLC-UV chromatograms of extracts from liquorice milkvetch (Figure 3)

Table 2: Concentration of isoflavones (mg/100 g) in whole aerial part (WP) of plants harvested at the vegetative stage (VS) and fully flowering stage (FS) and the separate parts of fully flowering plants of *A. glycyphyllos* and *A. cicer*.

Isoflavone	Plant material				
	WP VS	WP FS	Stems	Leaves	Flowers
			A. glycyphyllos		
Formononetin	9.24 ± 0.7[a]	5.50 ± 0.6	6.14 ± 0.6	5.05 ± 0.5	2.20 ± 0.3
Biochanin A	8.81 ± 0.8	3.17 ± 0.4	2.50 ± 0.3	4.43 ± 0.5	2.09 ± 0.2
Daidzein	<LOQ[b]	<LOQ	<LOQ	<LOQ	<LOQ
Genistein	2.23 ± 0.3	5.71 ± 0.6	7.29 ± 0.6	5.15 ± 0.5	4.83 ± 0.5
Sum	20.28	14.38	15.93	14.63	9.11
			A. cicer		
Formononetin	10.85 ± 0.8	4.17 ± 0.4	5.55 ± 0.5	3.91 ± 0.4	1.30 ± 0.2
Biochanin A	11.40 ± 0.8	2.82 ± 0.3	3.14 ± 0.4	4.01 ± 0.5	2.49 ± 0.3
Daidzein	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Genistein	<LOQ	4.21 ± 0.4	4.08 ± 0.4	4.33 ± 0.4	1.95 ± 0.2
Sum	22.25	11.20	12.77	12.25	5.74

[a]Mean of repetitions ± standard deviation; [b]<LOQ: below the limit of quantification.

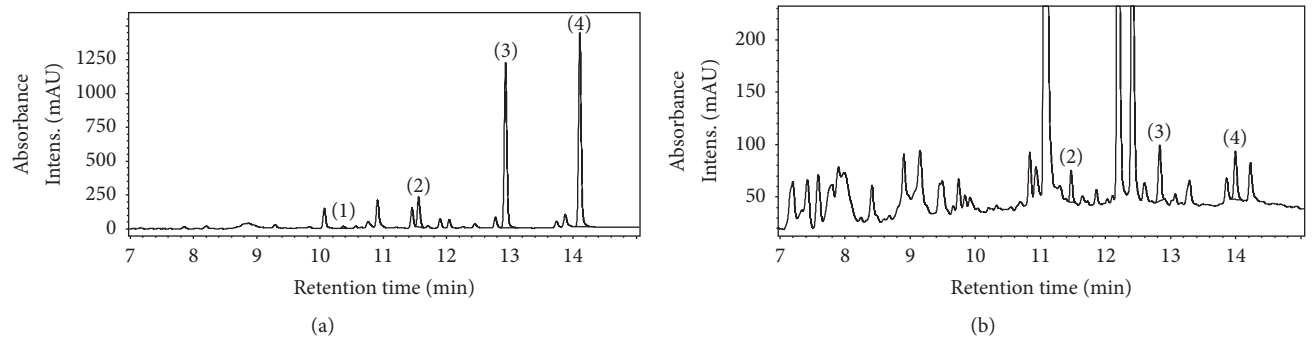

Figure 3: UPLC-UV chromatograms of isoflavones in the extracts of the whole aerial parts of flowering *Trifolium pratense* (a) and *Astragalus glycyphyllos* (b). Peaks: (1) daidzein, (2) genistein, (3) formononetin, and (4) biochanin A. Chromatographic conditions are described under Materials and Methods.

showed that the species contains compounds which eluted between daidzein and genistein as well as between genistein and formononetin.

There is evidence to suggest that *Astragalus* species have more bioactive compounds related to isoflavones than the four isoflavones selected for quantification in this study. Among the perennial legume species, isoflavones have only been quantified in some detail in red clover [30, 45, 46], and to a much lesser extent in other *Trifolium* spp. [47, 48] and *Medicago* spp. [49]. Reliable quantitative data on isoflavones in *A. cicer* and *A. glycyphyllos* are currently not available; only sporadic qualitative information can be found in the published literature [16].

The HILIC-MS/MS method was optimised and used to determine swainsonine in two samples of milkvetch species growing in Lithuania. Investigations revealed that neither of the *Astragalus* samples contained detectable amounts of the alkaloid. This finding may be attributed to swainsonine production in *Astragalus* spp. and other allied legume species being strongly correlated with the presence of a fungal endophyte, *Embellisia* spp. [50, 51]. In our study, samples were fungus-free and, consequently, swainsonine was not detected.

3.3. Antioxidant Activity. The capacity of extracts of *A. glycyphyllos* herbal material to scavenge DPPH free radicals ranged from 7.52 to 35.64 μmol/g (Figure 4(a)). The highest antioxidant activity was observed in extracts from flowers (35.64 μmol/g), followed by leaves (32.26 μmol/g) and the whole aerial part of young plants (16.65 μmol/g). The stems showed the lowest DPPH scavenging activity (7.52 μmol/g). Herbal material of *A. cicer* exhibited a higher potential to scavenge DPPH radicals than respective plant parts of *A. glycyphyllos*, with the exception of stems. This finding was despite the inverse trend in TPC and TFC distribution in the two species (Figures 2 and 4). This observation could be explained by the presence of other bioactive compounds, distinguished by strong free-radical quenching, which were not identified in the current study. It is likely that these compounds would be largely specific to *A. cicer* herbal material. At an early growth stage, *A. cicer* plants exhibited a particularly high antioxidant activity (129.0 μmol/g) similar to that in leaves (128.6 μmol/g). Extracts of flowers showed twice as low DPPH quenching capacity as leaves and young plants, but it was still fairly high (62.17 μmol/g), while stems exhibited negligible free-radical scavenging activity.

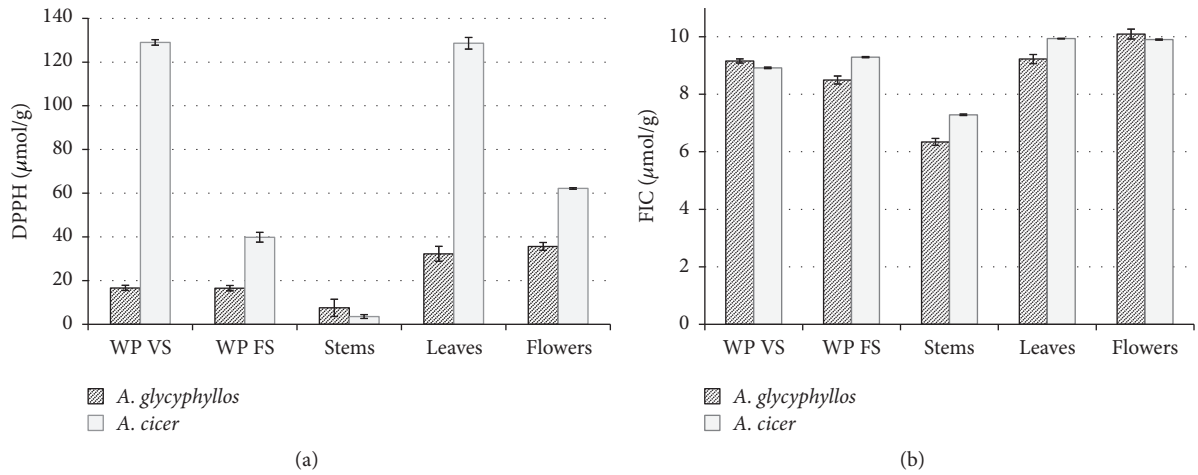

Figure 4: Antioxidant activity: DPPH radical scavenging (a) and ferrous ion-chelating (FIC) capacity (b) of extracts of *A. glycyphyllos* and *A. cicer* plant material: whole aerial parts (WP) of plants of vegetative stage (VS) and fully flowering stage (FS) and the separate parts of fully flowering plants (error bars indicate standard deviation).

In relation to the growth stage, plants harvested during the vegetative growth stage demonstrated a higher capacity to scavenge DPPH free radicals than those harvested at flowering.

Extracts from the examined herbal material of the *Astragalus* species showed a high capacity to chelate ferrous ions; Fe^{2+} chelators from 1 g of sample were shown to bind from 6.34 to 10.09 μmoles of ferrous ions (Figure 4(b)). Among plant parts of flowering *A. glycyphyllos,* flowers showed the highest FIC capacity (10.09 μmol/g) and stems had the lowest value (6.34 μmol/g). For extracts from leaves and flowers of *A. cicer*, similarly high FIC capacity (9.94 and 9.90 μmol/g, resp.) was characteristic. Regarding changes in FIC values in plants during the two growth stages, a different trend was revealed for each of the species.

Low DPPH radical-scavenging activity of *A. glycyphyllos* extract was also reported by Tusevski et al. [43]. Data on both DPPH scavenging and FIC activities of extracts from *A. cicer* plant material, as well as on FIC capacity of *A. glycyphyllos*, were not found in the published literature. The high FIC capacity of extracts from *Astragalus* species may prove to be of therapeutic importance, since synthetic compounds currently used in chelation therapy have certain side effects [52].

3.4. Relationships between Bioactive Substances and Antioxidant Properties. Analysis of linear relationships between values for FIC capacity, DPPH radical-scavenging activity, and phenolic compounds (TPC and TFC) was performed for sample sets of individual *Astragalus* species and the set which included all samples of both species (Table 3).

Generally, the results revealed significant positive correlations between TPC and TFC in all three sample sets. Significant positive linear correlations were established between TPC and DPPH in sample sets from the separate plant species only: *A. glycyphyllos* ($r = 0.952$; $p < 0.05$) and *A. cicer* ($r = 0.977$; $p < 0.01$); however, the relationship between TPC and FIC in sample sets from the separate plant species showed a weaker correlation. Correlations which ranged from weakly negative with insignificant difference ($p > 0.05$) to strongly positive were observed between TFC and antioxidant properties. The closeness of the relationship depended on the sample set; when all tested samples were included in correlation rows, poor associations were determined for both variable pairs TFC and DPPH, as well as for TFC and FIC. This may be a result of the *Astragalus* species belonging to different taxonomic units in the genus and, therefore, possessing different compositions of antioxidant agents. The different trends in correlation strength for pairs TFC versus DPPH and TFC versus FIC, which were apparent in the sample sets of the separate species, support the view that flavonoids from *A. glycyphyllos* extracts are more closely associated with scavenging of DPPH free radicals, while those from *A. cicer* have a closer association with FIC.

Isoflavone content was weakly correlated with antioxidant properties, TPC and TFC (data not shown). Statistical comparison of free-radical scavenging capabilities against FIC properties of the investigated materials showed a moderate but insignificant ($p < 0.05$) correlation. Results obtained in our study on the relationship between bioactive compounds and antioxidant properties are consistent with those of other researchers. Similarly, Tepavčević et al. [53] have documented that the DPPH scavenging activity correlated well with total polyphenolic content but did not correlate with total isoflavones in soybeans of different origin. Romani et al. [54] found no correlation between data on TPC and isoflavone content in isoflavone-based food supplements. Rau De Almeida Callou et al. [55] also reported the absence of a correlation between antioxidant capacity and isoflavone contents for soy beverages. Tusevski et al. [43] established a negative correlation between DPPH and FIC for Macedonian medicinal plants and proposed that this may be due to different reaction mechanisms involved in the two antioxidant determination methods. The antioxidant capacity of plant

Table 3: Coefficients of linear correlation between the values of bioactive properties of the *Astragalus* plant material studied.

Species	*Agly* + *Acic*[a]	*Agly*[b]	*Acic*[c]	*Agly* + *Acic*	*Agly*	*Acic*	*Agly* + *Acic*	*Agly*	*Acic*
Character	TPC			TFC			DPPH		
TFC	0.855**	0.969**	0.947*						
DPPH	0.315	0.952*	0.977**	−0.185	0.920*	0.866			
FIC	0.739*	0.872	0.861	0.450	0.873	0.973**	0.574	0.852	0.520

Correlation coefficients (r) were computed for sample sets of [a]*A. glycyphyllos* with *A. cicer*, [b]*A. glycyphyllos*, and [c]*A. cicer* separately. **,*Correlation is significant at $p < 0.01$ and $p < 0.05$ level, respectively.

extracts is considered to depend on the specific combination of bioactive compounds and their synergistic interactions [56], or their additive or antagonistic responses [57].

In summary, our findings on the distribution of minerals and phenolic compounds, as well as the antioxidant properties, in samples from two *Astragalus* species from a temperate region have demonstrated the potential pharmaceutical and nutraceutical significance of these plants.

4. Conclusion

The study assuredly demonstrates the potential of plant material from *A. glycyphyllos* and *A. cicer* as a valuable source of iron and phenolic substances, including isoflavones, free-radical scavengers, and Fe^{2+} chelators. However, mineral and phytochemical compositions, as well as antioxidant properties, were found to be species-, plant growth stage-, and plant part-dependent features. The investigated plant material may be considered as a potential source of dietary supplements and pharmaceutical and nutraceutical products, depending on species, growth stage, and plant part.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Supplementary Materials

The Supplementary Material represents a graphical abstract of the current study. It visually demonstrates the design of plant material of the two *Astragalus* species investigated and provides characters and properties tested. *(Supplementary Materials)*

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Research Article

Monoamine Oxidase-A Inhibition and Associated Antioxidant Activity in Plant Extracts with Potential Antidepressant Actions

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Monoamine oxidase (MAO) catalyzes the oxidative deamination of amines and neurotransmitters and is involved in mood disorders, depression, oxidative stress, and adverse pharmacological reactions. This work studies the inhibition of human MAO-A by *Hypericum perforatum*, *Peganum harmala,* and *Lepidium meyenii,* which are reported to improve and affect mood and mental conditions. Subsequently, the antioxidant activity associated with the inhibition of MAO is determined in plant extracts for the first time. *H. perforatum* inhibited human MAO-A, and extracts from flowers gave the highest inhibition (IC_{50} of 63.6 μg/mL). Plant extracts were analyzed by HPLC-DAD-MS and contained pseudohypericin, hypericin, hyperforin, adhyperforin, hyperfirin, and flavonoids. Hyperforin did not inhibit human MAO-A and hypericin was a poor inhibitor of this isoenzyme. Quercetin and flavonoids significantly contributed to MAO-A inhibition. *P. harmala* seed extracts highly inhibited MAO-A (IC_{50} of 49.9 μg/L), being a thousand times more potent than *H. perforatum* extracts owing to its content of β-carboline alkaloids (harmaline and harmine). *L. meyenii* root (maca) extracts did not inhibit MAO-A. These plants may exert protective actions related to antioxidant effects. Results in this work show that *P. harmala* and *H. perforatum* extracts exhibit antioxidant activity associated with the inhibition of MAO (i.e., lower production of H_2O_2).

1. Introduction

The enzyme monoamine oxidase (MAO) metabolizes xenobiotic and endogenous amines and neurotransmitters including serotonin, dopamine, norepinephrine, tyramine, tryptamine, and the neurotoxin MPTP [1, 2]. It occurs as two isoenzymes, MAO-A and MAO-B, which play an important role in the central nervous system (CNS) and peripheral organs. MAO-B is involved in neurodegenerative diseases and MAO-A in psychiatric conditions and depression. Inhibitors of MAO-B are useful as neuroprotectants, whereas inhibitors of MAO-A are effective antidepressants although their use may trigger adverse reactions (e.g., hypertensive crisis with foods containing tyramine) [1]. On the other hand, the oxidation of biogenic amines and neurotransmitters by MAO enzymes generates hydrogen peroxide (H_2O_2), oxygen radicals, and aldehydes, which are risk factors for cell oxidative injury. Therefore, the inhibition of MAO may result in protection against oxidative stress and neurotoxins [1, 3, 4]. Recent investigations have pointed out that plant and food extracts may inhibit MAO enzymes resulting in the above-mentioned biological effects [3, 5–14]. On the other hand, as a result of MAO inhibition, those products might be involved in undesirable interactions with other herbal preparations, foods, or drugs [1].

Hypericum perforatum L. (family Hypericaceae) (St. John's wort) is widely used for health purposes and their products are commercially available as herbs, nutraceuticals, teas, tinctures, juices, oily macerates, phytopharmaceuticals, and food additives and supplements [15, 16]. *H. perforatum* is popular for treatment of mild and moderate depression [17–19]. It may trigger adverse pharmacological interactions with others herbs, drugs, or foods [20–22]. Its ability to alleviate and improve mood disorders and depression is attributed to active compounds that exhibit antidepressant properties [23, 24]. The most accepted mechanism of action is monoamine reuptake inhibition but additional mechanisms including monoamine oxidase inhibition and synergistic effects can

be involved [17]. *Peganum harmala* (family Zygophyllaceae) and *Lepidium meyenii* (family Brassicaceae) (maca) are plants with CNS effects and potential antidepressant actions [14, 25, 26]. *P. harmala,* native from the Mediterranean region and Asia and extended to North America areas, is used as a multipurpose health remedy including CNS disorders. Preparations of this plant may trigger adverse pharmacological interactions [27]. *L. meyenii* is an edible plant from the central Andes whose roots are used as a food energizer and nutraceutical to improve physical and mental conditions and fertility [28]. The purpose of this work was to study the inhibition of human MAO-A by extracts of *H. perforatum, P. harmala,* and *L. meyenii* (maca) as well as by their active components that were identified and analyzed by HPLC-DAD-MS and subsequently evaluate the antioxidant activity which is specifically associated with the inhibition of MAO. This specific antioxidant activity is determined for the first time in plant extracts.

2. Materials and Methods

Hypericum perforatum L. plants collected in Ciudad Real (Spain) were dried and separated in parts: flowers; top aerial portions of the plant including branched stems and leaves but no flowers; and main stems (central and lower) and roots. They were ground and the powder used for sample preparation. Commercial herbs and herbal supplements (capsules and tablets) of *H. perforatum* were also purchased in local herbal shops. *Peganum harmala* L. plant and seeds were collected in Toledo (Spain). *Lepidium meyenii* (maca) both as powder and commercial tablets were obtained from Peru and local shops. Hypericin standard (>95% purity by HPLC) from HWI Analytik GMBH pharma solutions, hyperforin dicyclohexylammonium salt, quercetin, harmaline, harmine, catalase, clorgyline, 3,3′,5,5′-tetramethylbenzidine (TMB), and horseradish peroxidase (HRP) type II were purchased from Sigma-Aldrich.

2.1. Sample Preparation of Plant Extracts. Samples containing *H. perforatum* (i.e., plant parts, herbal preparation, capsules, or tablets) (500 mg) were homogenized in 10 mL of water/methanol (1 : 1) by using an Ultra Turrax homogenizer, centrifuged at 10000 rpm for 10 min, and the supernatant was collected. The process was repeated twice with the residue and the three supernatant fractions collected, mixed and analyzed by HPLC as mentioned below. After three consecutive extractions, the recoveries of hypericin and pseudohypericin were higher than 97%. Samples of *L. meyenii* (maca) (500 mg) and *P. harmala* seeds (500 mg) were homogenized, respectively, in 10 mL of water/methanol (1 : 1) or 10 mL of 0.6 M perchloric acid : methanol (1 : 1) by using an Ultra Turrax homogenizer, centrifuged at 10000 rpm for 10 min, and the supernatant was collected. This process was repeated twice with the residue and the collected supernatants were mixed and analyzed by HPLC as mentioned below.

2.2. RP-HPLC Analysis of Plant Extracts. The analysis of *H. perforatum* extracts was performed by RP-HPLC with UV diode array and fluorescence detection using a HPLC 1050 (Agilent) coupled with a 1100 diode array detector (DAD) (Agilent) and a 1046A-fluorescence detector. A 150 × 3.9 mm *i.d.*, 4 μm, Nova-pak C18 column (Waters) was used for separation. Chromatographic conditions were 50 mM ammonium phosphate buffer (pH 3) (buffer A) and 20% of A in acetonitrile (buffer B). The gradient was programmed from 0% (100% A) to 32% B in 8 min and 100% B at 10 min. The flow rate was 1 mL/min, the column temperature was 40°C, and the injection volume was 20 μL. Detection of hypericins was carried out by absorbance at 590 nm and fluorescence at 236 nm for excitation and 592 nm for emission. The concentration of hypericin was determined from a calibration curve of response (absorbance at 590 nm) versus concentration with solutions made in the laboratory from hypericin standard. The same response factor was applied to pseudohypericin, protohypericin, and protopseudohypericin. Flavonoids and flavonoid glycosides were analyzed at 265 nm and 355 nm and the concentration of quercetin was determined at 355 nm from a calibration curve of response versus concentration. The HPLC fraction corresponding to flavonoids and flavonoid glycosides (7 to 11 min) was collected by successive injections of *H. perforatum* extract (herbs) and, after evaporation in vacuum, dissolved in 30% methanol and used for MAO-A inhibition. The phloroglucinols (hyperforin, adhyperforin, hyperfirin, and adhyperfirin) were analyzed at 280 nm by using the same column (Nova-pak C18) and conditions but under isocratic elution with 20% of 50 mM ammonium phosphate buffer, pH 3, and 80% of acetonitrile. The concentration of these compounds was determined from a calibration curve of hyperforin standard. The analysis of β-carboline alkaloids in *P. harmala* and *L. meyenii* was carried out as previously described [14, 29].

2.3. Identification by HPLC-ESI-Mass Spectrometry. Identification of compounds in *H. perforatum* extracts was done by HPLC-MS (electrospray-negative ion mode) by using a 1200 series HPLC-DAD coupled to a 6110 quadrupole-MS (Agilent). Chromatographic separation was performed on a 150 × 2.1 mm *i.d.* Zorbax SB-C18 (5 μm) column (Agilent Technologies). The chromatographic conditions were eluent A: formic acid (0.1%); B: formic acid (0.1%) in acetonitrile; gradient: 0% to 70% B in 8 min and 100% B at 10 min, flow rate: 0.3 mL/min; T: 40°C; mass range: 50–700 u, and cone voltage: 150 V. For identification of phloroglucinols (e.g., hyperforin), separation was done using a Nova-pak C18 (4 μm) column with the same eluents and isocratic elution (eluent A, 20% and eluent B, 80%) at a flow rate of 0.7 mL/min and mass spectra recorded in negative and positive ionization. Identification of compounds was done on the basis of mass spectra, UV-vis spectra (DAD) of chromatographic peaks, and coelution with standards. β-Carbolines in *P. harmala* and *L. meyenii* were identified as previously described [14, 29].

2.4. Monoamine Oxidase (MAO-A) Inhibition Assays. MAO assays were performed as elsewhere [8, 11, 14]. Briefly, membrane protein fractions containing MAO-A (BD-Gentest) were diluted to the desired concentrations in 100 mM potassium phosphate buffer (pH 7.4). A 0.2 mL reaction mixture

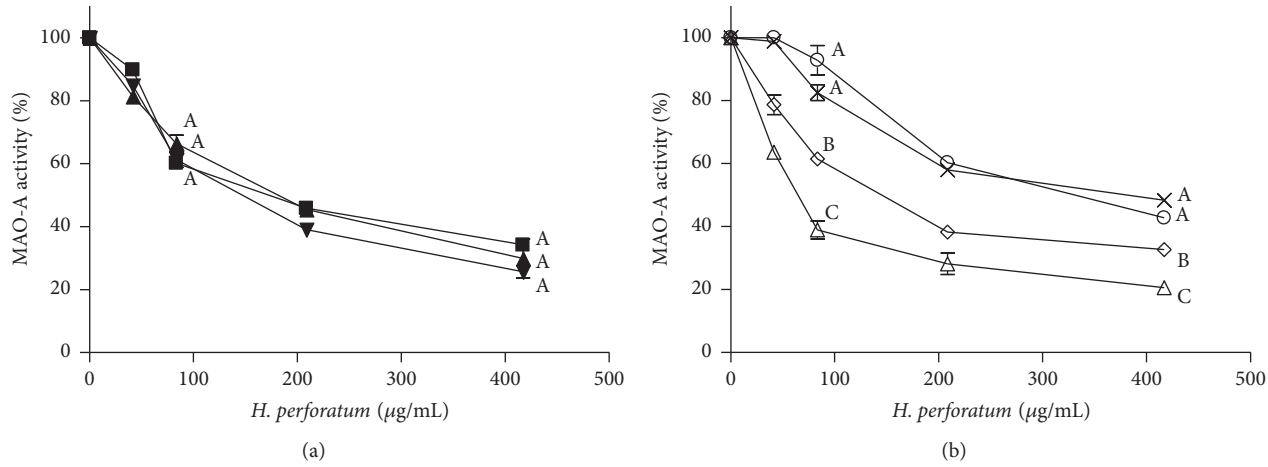

Figure 1: Inhibition of human monoamine oxidase-A (MAO-A) by extracts of commercial preparations *of H. perforatum* (a) (capsules, ■; tablets, ▲; herbs, ▼), and extracts from different parts of the plant (b) (flowers, △; top stems, ◊; main stems (central), ×; roots, ○). Significant differences ($p < 0.05$) among extracts at a selected concentration are indicated with different letters.

containing 0.01 mg/mL protein and 0.25 mM kynuramine in 100 mM potassium phosphate (pH 7.4) was incubated at 37°C for 40 min. After incubation, the reaction was stopped by the addition of 2 N NaOH (75 μL), followed by the addition of 70% $HClO_4$ (25 μL), and the sample was centrifuged (10000g) for 10 min. The supernatant (20 μL) was injected into the HPLC and the deamination product of kynuramine (i.e., 4-hydroxyquinoline) formed during enzymatic reaction determined by RP-HPLC-diode array detection at 320 nm. A response curve of area versus concentration was constructed to calculate the concentration of 4-hydroxyquinoline. In order to perform assays of MAO inhibition, aliquots of extracts from plants or commercial preparations or instead pure compounds were conveniently diluted and added to reaction mixtures containing kynuramine (0.25 mM) and MAO-A (0.01 mg/mL protein) in 100 mM potassium phosphate buffer (pH 7.4), with enzymatic reaction and analysis carried out as above, and compared with the corresponding controls containing solvent. The standard inhibitor clorgyline was used as a positive control for inhibition (>90% inhibition at 2.5 μM). Incubations were carried out at least in duplicate from different experiments and the IC_{50} values were calculated using GraphPad Prism 4.0.

2.5. Determination of Antioxidant Activity Associated with Monoamine Oxidase (MAO) Inhibition. Assays (0.2 mL) of reaction mixtures in 70 mM potassium phosphate buffer (pH 7.4), containing 0.025 mg/mL MAO-A protein and 0.25 mM kynuramine, were incubated at 37°C for 40 min in the absence (control assays) or in the presence of plant extracts. MAO assays were also performed in presence of clorgyline (25 μM), a classical inhibitor of MAO-A (positive control of inhibition), or catalase enzyme (100 μg/mL). After the incubation period, the reaction mixture was added with activated charcoal (3.5 mg), mixed, and filtered (0.45 μm). The solution was added with 20 μL of 10 mM tetramethylbenzidine (TMB) in 40% DMSO and 20 μL of horseradish peroxidase (HRP) type II (1 mg/mL), kept 5 min, and added with 0.3 mL of 0.5 M H_2SO_4 solution. The absorbance at 450 nm was measured to determine TMB diimine, a yellow product resulting from the oxidation of TMB by HRP and the H_2O_2 generated in the oxidative deamination catalyzed by MAO. The oxidation of TMB in the presence of inhibitors of MAO was compared with the corresponding controls without inhibitors and appropriate blanks showed absence of interferences.

3. Results and Discussion

Commercial preparations of *H. perforatum* inhibited human MAO-A with similar potency: IC_{50} values of 142.3 ± 30.6 μg/mL (herbal preparation), 193 ± 61 μg/mL (capsules), and 173 ± 29 μg/mL (tablets) (Figure 1(a)). Regarding plants, *H. perforatum* extracts from flowers afforded the highest inhibition (IC_{50} of 63.6 ± 9.4 μg/mL) followed by aerial stems and leaves (IC_{50} 143.6 ± 16.5 μg/mL), and the lowest in root extracts (Figure 1(b)). Extracts from the aerial parts of *H. perforatum* were analyzed by HPLC-DAD-ESI (electrospray-negative ionization). They showed the presence of two major naphthodianthrones identified as pseudohypericin and hypericin (Figure 2(a) and Table 1). Flower extracts had two additional compounds identified as protopseudohypericin and protohypericin. Phenolics and flavonoids abounded in *H. perforatum* extracts (Figure 2(b)). Chlorogenic acid and the quercetin glycosides rutin, hyperoside, isoquercitrin, miquelianin, acetyl hyperoside, and quercitrin, as well as free quercetin and biapigenin, were identified by HPLC-DAD (ESI negative ionization) and DAD (Table 1). On the other hand, flower extracts contained four phloroglucinols (Figure 2(c)) that were identified by HPLC-DAD-MS (ESI negative and positive ionization) and DAD as hyperforin, adhyperforin, hyperfirin, and adhyperfirin (Table 1). The presence of these compounds (Figure 3) in the plant agrees with other results [15, 30, 31]. The content of the main components was determined by HPLC (Table 2). Concentration of pseudohypericin was higher than hypericin, whereas

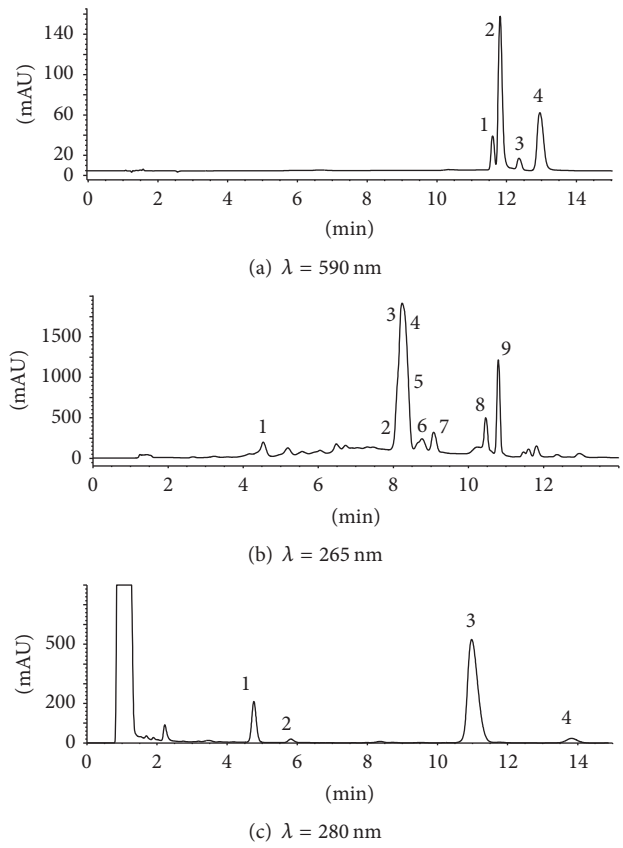

Figure 2: HPLC chromatograms of extracts from *H. perforatum* flowers. (a) Detection of hypericins at 590 nm. **1**: protopseudohypericin; **2**: pseudohypericin; **3**: protohypericin; and **4**: hypericin. (b) Detection of phenols and flavonoids at 265 nm. **1**: chlorogenic acid; **2**: rutin; **3**: hyperoside; **4**: isoquercitrin; **5**: miquelianin; **6**: acetyl hyperoside; **7**: quercitrin; **8**: quercetin; and **9**: biapigenin. (c) Detection of phloroglucinols at 280 nm. **1**: hyperfirin, **2**: adhyperfirin; **3**: hyperforin; and **4**: adhyperforin.

protopseudohypericin and protohypericin were minor compounds (0.4 μg/mg of protopseudohypericin and 0.17 μg/mg of protohypericin were detected in flowers). In the plant, the highest content of hypericins was found in flowers with significantly low levels detected in stems and absence in roots. Hyperforin was highly abundant in flowers (27.2 μg/mg), whereas the concentration in commercial preparations ranged from 0.36 to 2.4 μg/mg. In flowers, adhyperforin (1.4 ± 0.07 μg/mg), hyperfirin (4.2 ± 0.02 μg/mg), and adhyperfirin (0.46 ± 0.02 μg/mg) also appeared. Flavonoids abounded in *H. perforatum* and most of them were quercetin glycosides (Figure 2(b)) whose presence was significantly higher in flowers than in other parts of the plant. The content of free quercetin in flowers was 2.0 μg/mg, whereas a content of 6.7 μg/mg was determined in commercial preparations.

The inhibition of MAO-A by *H. perforatum* extracts indicates occurrence of inhibitors. Hypericins, hyperforin, and flavonoids are possible contributors to this inhibition and were evaluated as inhibitors (Figure 4). Hypericin inhibited MAO-A (IC_{50} of 35.5 ± 2.1 μM or 17.9 μg/mL) (Figure 4(a)). From the concentration in Table 2, hypericin is a weak contributor to MAO inhibition in *H. perforatum* extracts. Indeed, the calculated content of hypericin at IC_{50} value in assays of flower extract (i.e., 63.6 μg/mL) was 0.1 μg/mL which is low compared with IC_{50} of hypericin (17.9 μg/mL). Hyperforin did not inhibit MAO-A (Figure 4(b)). Quercetin inhibited human MAO-A (Figure 4(b)) with an IC_{50} value of 11.1 ± 0.8 μM (i.e., 3.36 μg/mL). Then, quercetin was a better inhibitor than hypericin although its potency was still low to explain entire inhibition of extracts. Thus, the calculated content of quercetin at IC_{50} in assays of flower extract was 0.13 μg/mL which is lower than the IC_{50} of quercetin (3.4 μg/mL). When the fraction corresponding to quercetin glycosides and flavonoids (7–11 min, Figure 2(b)) was collected by RP-HPLC, it inhibited MAO-A (90% inhibition at 700 μg/mL extract) indicating a contribution of these compounds to MAO inhibition in *H. perforatum*, probably by additive effects. Then, inhibition of MAO-A could arise from components such as quercetin and related flavonoids (i.e., quercetin glycosides) which are abundant in the plant. In addition, minor compounds not identified here could also contribute to MAO inhibition as major compounds in Table 2 do not explain whole inhibition.

Extracts from *P. harmala* seeds highly inhibited human MAO-A (Figure 5(a)) affording an IC_{50} value of 49.9 ± 5.6 μg/L. Chromatographic analysis indicated that inhibition was due to the presence of the β-carboline alkaloids, harmaline and harmine, that were identified by HPLC-DAD-MS (Figure 5(c)). The content of these alkaloids determined in seeds was 48.5 mg/g for harmaline and 40.0 mg/g for harmine (this means 2.4 ng/mL and 2.0 ng/mL, resp., into assays at the IC_{50}). Therefore, the inhibition potency of MAO-A by *P. harmala* seeds was 1274 times more potent than that of *H. perforatum* flowers. As shown in Figure 5(b), *Lepidium meyenii* root extracts did not inhibit human MAO-A. *L. meyenii* (maca) is a popular plant from the Andes highlands whose roots are increasingly used for its nutritional and medicinal properties as energizing and to improve mood and sexual performance [28, 32]. Previous reports have indicated that they contain alkaloids including β-carbolines [25, 26] that might inhibit MAO. Analysis of extracts for β-carboline alkaloids gave 25 μg/g (maca powder) and 11.7 μg/g (capsules) of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid as a major compound. This specific β-carboline is not an inhibitor of MAO-A [8, 11].

MAO generates hydrogen peroxide (H_2O_2) that is involved in oxidative cell damage and pathological conditions [1, 3, 4, 33–36]. Then, the inhibition of MAO may result in specific antioxidant actions [37]. In order to study the antioxidant activity associated with MAO inhibition, experiments were designed in this research which linked the activity of MAO-A with the oxidation of tetramethylbenzidine (TMB) by horseradish peroxidase (HRP) and the H_2O_2 produced during oxidative deamination catalyzed by MAO (Figure 6). *H. perforatum* and *P. harmala* extracts which inhibited MAO-A as shown above highly decreased oxidation of TMB. In contrast, *L. meyenii* root (maca) extracts that did not inhibit MAO had a low antioxidant activity in this assay. Clorgyline which is a potent inhibitor of MAO-A highly decreased the oxidation of TMB when used as a control. The same happened

Table 1: Compounds identified in *H. perforatum*.

Compounds	ESI-neg. ion $(M - H)^-$	UV max (DAD)
Naphthodianthrones		
Pseudohypericin	519	547, 590
Hypericin	503	547, 590
Protopseudohypericin	521	370, 539
Protohypericin	505	370, 539
Phenolic comp.		
Chlorogenic acid	353	324
Rutin	609	256, 355
Hyperoside	463	256, 355
Isoquercitrin	463	256, 355
Miquelianin	477	256, 355
Acetyl hyperoside	505	263, 352
Quercitrin	447	255, 348
Quercetin	301	255, 369
Biapigenin	537	268, 331
Phloroglucinols		
Hyperfirin[a]	467	274
Adhyperfirin[a]	481	274
Hyperforin[a]	535	274
Adhyperforin[a]	549	274

[a]These compounds gave also their corresponding $(M + H)^+$ and $(M + K)^+$ ions under ESI-positive ionization.

Table 2: Content (μg/mg)[1] of the main active components in *H. perforatum* samples.

H. perforatum samples	Pseudohypericin	Hypericin	Hyperforin	Quercetin
Plant				
Stems (top)	0.25 ± 0.03^a	0.11 ± 0.01^a	1.48 ± 0.3^a	0.28 ± 0.12^a
Stems (central)	0.1 ± 0.04^a	0.04 ± 0.01^a	0.59 ± 0.16^a	0.19 ± 0.01^a
Roots	-	-	0.77 ± 0.1^a	-
Flowers	2.78 ± 0.7^b	1.58 ± 0.31^b	27.2 ± 0.6^b	2.04 ± 0.08^b
Commercial prep.				
Herbs	0.51 ± 0.05^a	0.11 ± 0.01^a	1.18 ± 0.03^a	0.71 ± 0.4^a
Capsules	2.41 ± 0.2^b	0.83 ± 0.1^b	2.42 ± 0.01^b	2.4 ± 0.9^a
Tablets	2.39 ± 0.2^b	2.11 ± 0.2^c	0.36 ± 0.1^c	6.7 ± 1.7^b

Significant differences ($p < 0.05$) for a compound within a group are indicated with different letters. [1]μg of compound/mg of plant tissue for plants parts and herbs or mg of powder in capsules and tablets.

with the presence of catalase in the media that removes H_2O_2 generated by MAO-A. Therefore, these results indicate that *H. perforatum* and *P. harmala* extracts afforded specific antioxidant actions associated with a lower production of H_2O_2 by inhibition of MAO.

H. perforatum improves mood disorders and depression [17, 18, 38]. As shown here, it contains compounds such as hyperforin, hypericins, and flavonoids responsible for antidepressant effects (Figure 2 and Table 2). However, the specific mechanism for antidepressant action is not completely understood. The most accepted mechanism is inhibition of monoamine reuptake [23, 24, 39, 40]. However, some studies suggest a combination of mechanisms and synergistic effects [17, 41]. *P. harmala* exerts numerous biological and pharmacological actions. Their seeds are increasingly used for recreational purposes owing to their psychoactive and neuroactive effects [14]. The inhibition of human MAO-A is an established mechanism for antidepressant action [1]. Both irreversible and reversible inhibitors of MAO-A (*e.g.*, phenelzine and moclobemide) are successfully used as antidepressants. In this study, *H. perforatum* extracts inhibited human MAO-A. However, this inhibition was moderate. It was more than one thousand times lower than that of *P. harmala* seed extracts. Sacher et al. have reported that the occupancy of MAO-A sites into the human brain determined by PET imaging with ^{11}C-harmine binding (i.e., the same β-carboline responsible for MAO inhibition in *P. harmala*) was high for a reversible inhibitor of MAO such as moclobemide but low for *H. perforatum* extract (St. John's wort) [42]. This means that the inhibitors of MAO-A

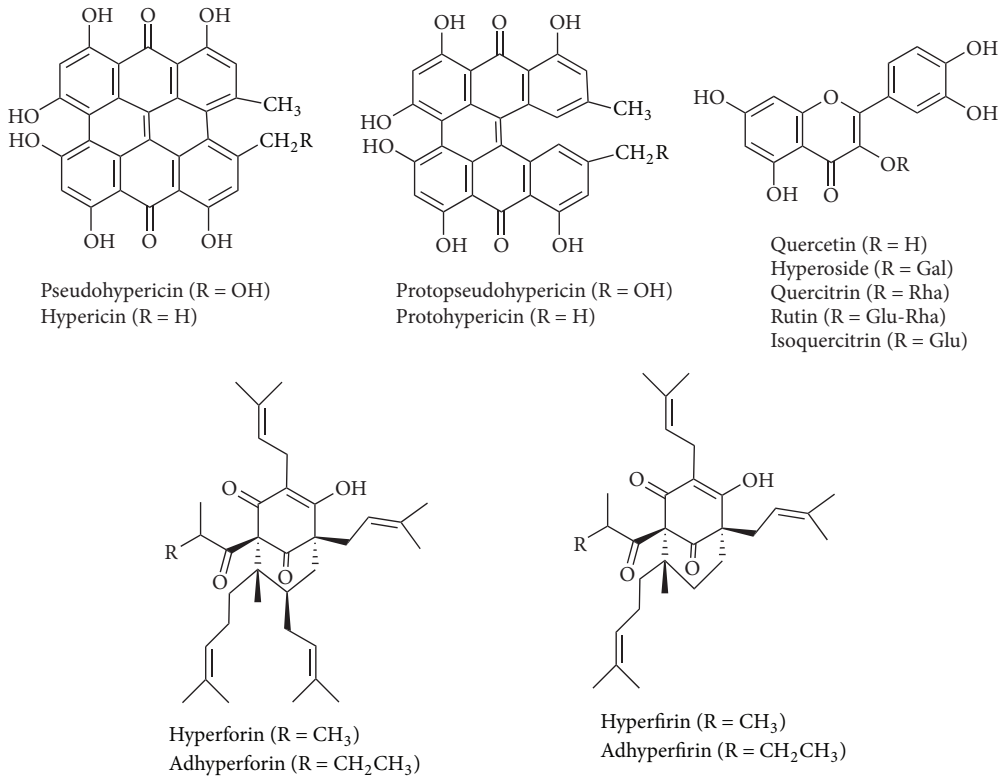

Figure 3: Structures of compounds identified in *H. perforatum*: hypericins, quercetin, and quercetin flavonoids and phloroglucinols (hyperforin, adhyperforin, hyperfirin, and adhyperfirin).

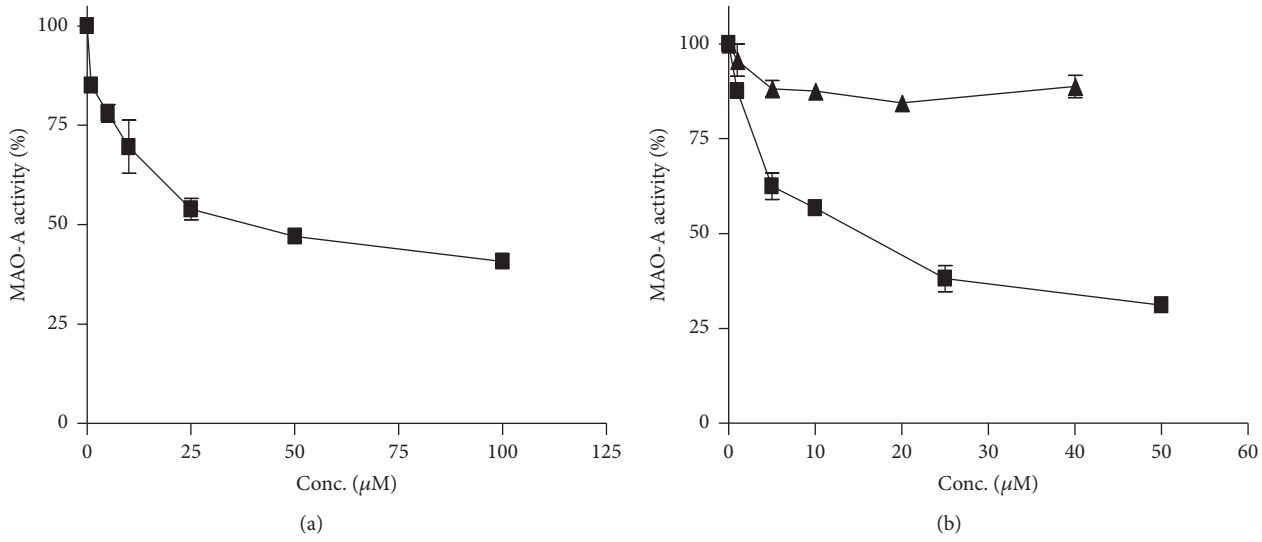

Figure 4: Inhibition of human monoamine oxidase-A (MAO-A) by active components of *H. perforatum*: hypericin (a) and quercetin (■) and hyperforin (▲) (b).

in *H. perforatum* do not bind efficiently to active sites of MAO-A in the brain in contrast to the β-carboline harmine. The inhibitors of MAO-A in *H. perforatum* are flavonoids such as quercetin and their glycosides and the levels of these compounds that reach the brain might not be enough to occupy the sites of MAO-A in the brain and inhibit the enzyme [43]. In contrast, the inhibitors of *P. harmala* are β-carboline alkaloids including harmine and harmaline which have a very good brain penetration, bind with high affinity to MAO sites, and exhibit antidepressant effects [44–46]. Therefore, *P. harmala* could afford antidepressant effects by MAO inhibition. In this regard, it could be of interest to

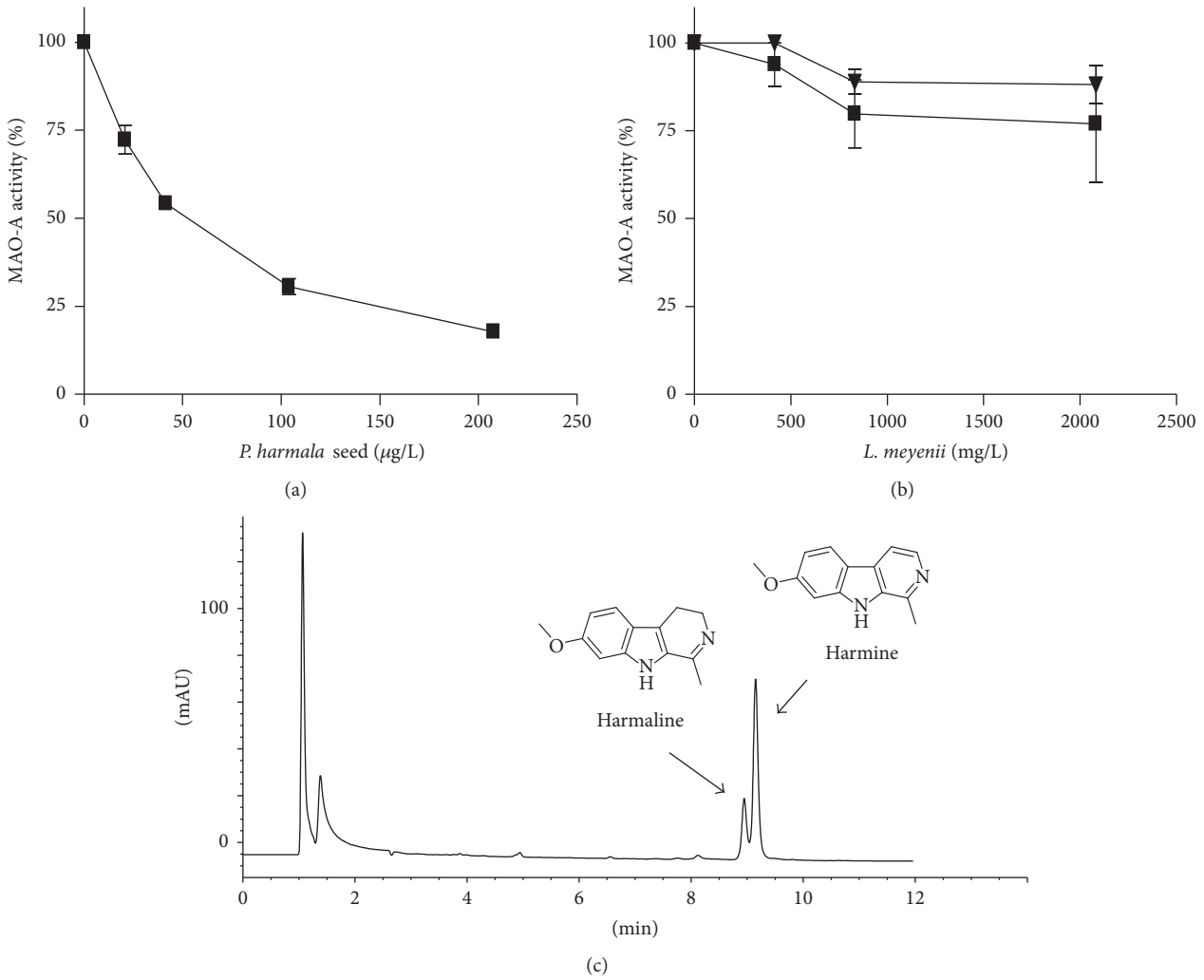

Figure 5: Inhibition of human monoamine oxidase-A (MAO-A) by *P. harmala* seed (a) and *L. meyenii* root (maca) extracts (b) (capsules, ▼ and powder, ■). (c) HPLC chromatogram of *P. harmala* seed extract which potently inhibited human MAO-A. Absorbance detection at 254 nm. Compounds identified are harmaline (*m/z* at 215 $(M + H)^+$, UV_{max} at 375 nm) and harmine (*m/z* at 213 $(M + H)^+$, UV_{max} at 245 and 322 nm).

investigate the antidepressant effects of *H. perforatum* and *P. harmala* alone and in combination as they have different mechanisms of action.

The inhibition of MAO-A by *H. perforatum* and *P. harmala* extracts may contribute to other biological effects of these plants such as antioxidant actions and adverse pharmacological reactions. Extracts of these plants exert neuroprotective and anti-inflammatory effects which have been related to antioxidant activity [6, 9, 47–50]. In this regard by using a new procedure, results in this work have evidenced that *H. perforatum* and *P. harmala* extracts show antioxidant activity associated with the inhibition of MAO (lower production of H_2O_2). On the other hand, one of the major limitations to the use of these plants is their potential for producing adverse interactions with other herbs, foods, and drugs [17, 20, 21, 27]. The inhibition of MAO-A may trigger adverse effects under certain circumstances [1, 14].

4. Conclusions

Extracts from *H. perforatum* inhibited human MAO-A, and extracts from flowers were the most potent inhibitors. They were studied by HPLC-DAD-MS and contained pseudohypericin, hypericin, hyperforin, adhyperforin, hyperfirin, and flavonoids. The highest content of these compounds appeared in flowers. Hypericin was a weak inhibitor of MAO-A; hyperforin did not inhibit the enzyme and quercetin was a moderate inhibitor. The fraction of quercetin glycosides and flavonoids contributed to MAO inhibition. *P. harmala* seed extracts highly inhibited MAO-A and its potency of inhibition was more than a thousand times higher than *H. perforatum* extracts owing to its content in harmaline and harmine alkaloids. *L. meyenii* root (maca) extracts did not inhibit MAO-A. The inhibition of MAO-A may not explain the entire CNS effects attributed to *H. perforatum* but it is expected to contribute to these actions in *P. harmala.* These plants exert antioxidant effects. By using a new method this

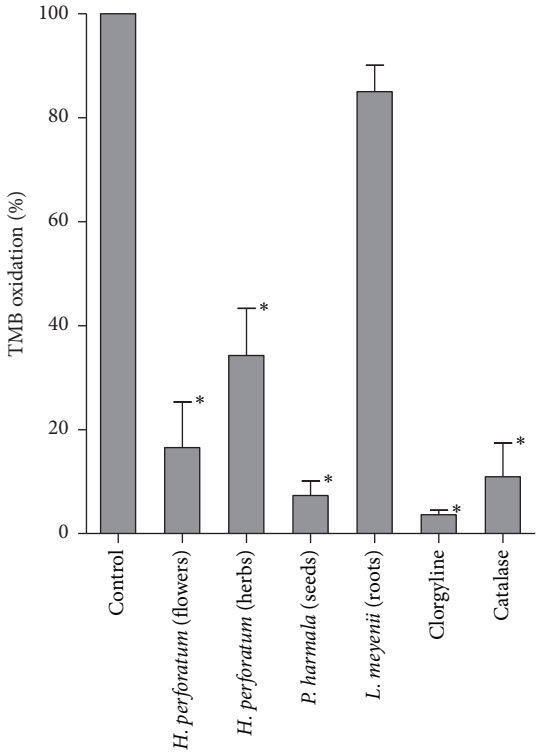

Figure 6: Antioxidant activity associated with MAO inhibition in assays coupling activity of MAO-A with the subsequent oxidation of tetramethylbenzidine (TMB) in the presence H_2O_2 generated in the reaction of MAO, and horseradish peroxidase (HRP). The graph shows the oxidation of TMB to diimine (absorbance at 450 nm) in control assays (100%), and in the presence of *H. perforatum* (herbs and flower extracts, 800 μg/mL), *P. harmala* seed extracts (0.8 μg/mL), *L. meyenii* root (maca) extracts (800 μg/mL), clorgyline (a standard inhibitor of MAO-A) (25 μM), and catalase (100 μg/mL). *Significant differences ($p < 0.01$) compared to controls.

work have evidenced that *P. harmala* and *H. perforatum* extracts exhibit antioxidant activity associated with the inhibition of MAO.

Conflicts of Interest

The authors declare no competing financial interest.

Acknowledgments

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Research Article

Effect of Dietary Chestnut or Quebracho Tannin Supplementation on Microbial Community and Fatty Acid Profile in the Rumen of Dairy Ewes

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Ruminants derived products have a prominent role in diets and economy worldwide; therefore, the capability to control the rumen microbial ecosystem, for ameliorating their quality, is of fundamental importance in the livestock sector. The aim of this study was to evaluate the effect of dietary supplementation with chestnut and quebracho tannins on microbial community and fatty acid profile, in the rumen fluid of dairy ewes. Multivariate analysis of PCR-DGGE profiles of rumen microbial communities showed a correlation among the presence of chestnut or quebracho in the diet, the specific *Butyrivibrio* group DGGE profiles, the increase in 18:3 *cis*9, *cis*12, and *cis*15; 18:2 *cis*9 and *cis*12; 18:2 *cis*9 and *trans*11; 18:2 *trans*11 and *cis*15; and 18:1 *trans*11 content, and the decrease in 18:0 concentration. Phylogenetic analysis of DGGE band sequences revealed the presence of bacteria representatives related to the genera *Hungatella*, *Ruminococcus*, and *Eubacterium* and unclassified Lachnospiraceae family members, suggesting that these taxa could be affected by tannins presence in the diets. The results of this study showed that tannins from chestnut and quebracho can reduce the biohydrogenation of unsaturated fatty acids through changes in rumen microbial communities.

1. Introduction

The manipulation of rumen microbial ecosystem is considered of primary importance in livestock sciences to improve the feed efficiency and to increase the quality of ruminant-derived products [1]. Recent findings have suggested that tannins, the second most abundant group of plant phenols after lignin, may be used as natural feed additives to modulate rumen fermentation through the inhibition of specific rumen microbial species [2, 3]. Tannins are chemically classified into two groups, hydrolysable and condensed tannins, both able to affect the growth and the metabolic activity of many species of rumen microorganisms [4]. Toxic effects of tannins have been attributed to different mechanisms, such as the inhibition of enzyme activities, the substrate or metal ion deprivation, and the detrimental action on biological membranes [1]. Moreover, tannin effects on rumen microorganisms appear to depend strongly on their chemical structure, their concentration in rumen liquor, and the microbial species involved [5]. For this reason, *in vivo* studies are requested to elucidate the effect of this class of polyphenols on rumen microbial communities and thus their actual employment in ruminant livestock.

Recently, many research studies have been attempted to understand how to increase the concentration of healthful fatty acids (FA) as rumenic acid (RA, 18:2 *cis*9, *trans*11) or vaccenic acid (VA, 18:1 *trans*11) and transient intermediate of the bacterial biohydrogenation (BH) of polyunsaturated fatty acids (PUFA), in ruminant milk and meat [6–8]. Among ruminal bacteria that appear to be involved in ruminal BH, the *Butyrivibrio* group is particularly sensitive to tannins [2, 3, 9–13]. A selective inhibition of *Butyrivibrio proteoclasticus*, involved in the last step of BH process of linoleic acid (LA, 18:2 *cis*9, *cis*12), may provide an accumulation of vaccenic acid (VA, 18:1 *trans*11) at the rumen level and, consequently, more RA in ruminant products [11, 14]. Tannins reduced *in vitro* activity and growth of *B. proteoclasticus* [11, 15, 16]. Moreover, literature [2] has shown that the inclusion of quebracho *(Schinopsis lorentzii)* tannins in ewe diet affected the *Butyrivibrio* group in a selective manner and enhanced RA content in dairy products. However, limited information is available on the *in vivo* effect of different sources of tannins on rumen microbiome and FA BH. Therefore, the aim of the present study was to investigate the effect of supplementing ewe diets with chestnut (CHT) or quebracho (QUE) tannin extracts (hydrolysable and condensed tannins, resp.) on rumen liquor FA profile, on the composition of the total rumen bacteria community, and, finally, on the composition of the *Butyrivibrio* group community, by using a polymerase chain reaction-denaturant gradient gel electrophoresis (PCR-DGGE) approach.

2. Materials and Methods

2.1. Animals and Experimental Design. The experiment was conducted at the Research Centre of the Department of Applied Biology, University of Perugia, Italy. Animals were handled according to the guidelines of the Italian law on animal welfare for experimental animals (Italian Ministry of Health, 2014) and of the University of Perugia Ethics Committee for animal use and care. Three nonlactating Bergamasca x Appenninica ewes (six years old, 60.5 ± 3.4 kg of body weight) equipped with a ruminal cannula of 10 cm internal diameter (Ankom Technology Corp., Macedon, NY, USA) were used. The animals were penned individually. The experiment was conducted as a 3 × 3 Latin square design. Each ewe was fed with the three diets in three consecutive experimental periods of 21 d, including 15 d of adaptation, before each one. At the 21st day the rumen liquor was sampled. The 3 × 3 Latin square was repeated twice with the aim of obtaining more replicates. During the whole experiment, the ewes had free access to water and hay, while the concentrates were administered twice daily (07:30 and 18:30). Orts were collected once daily.

2.2. Diets. The experimental diets were the same previously tested in an *in vivo* trial [3]. Diets were composed of chopped grass hay (particle size > 4 cm of length), administered ad libitum and by a concentrate (800 g/head/day), which contained 84.5 g kg^{-1} dry matter (DM) of soybean oil and 52.8 g kg^{-1} DM of bentonite (control, as an inert component to compensate the tannin introduction), or 52.8 g kg^{-1} DM of chestnut tannins (CHT) or 52.8 g kg^{-1} DM of quebracho tannins (QUE). The chemical composition of feeds and the ingredients of concentrates are presented in Table 1. The dose of tannins was chosen to obtain a diet tannin concentration of nearly 1.6% of expected DM intake. On the basis of results from previous studies in literature, this dose was considered as safe for the animal and practical for the farmers [7, 17, 18].

2.3. Tannin Sources. Chestnut tannins (750 g kg^{-1} DM of equivalent tannic acid) were provided by Gruppo Mauro Saviola Srl (Radicofani, Siena, Italy), while extract of QUE (456 g kg^{-1} DM of equivalent tannic acid) was provided by Guido Lapi SpA (Castel Franco di Sotto, Pisa, Italy).

Both the extracts were titrated according to Burns [19] to evaluate the equivalent tannic acid. The chemical composition and gas chromatographic profile of CHT were published by Campo et al. [20] and the characteristics of QUE were reported by Vasta et al. [2].

2.4. Feed Sampling and Analysis. Samples of feeds were collected daily and stored at −80°C until further analysis. Samples were then ground for chemical analysis by mill Cyclotec 1093 (PBI International, Milan, Italy), using a mesh size of one mm. Concentrations of crude protein (CP), ether extract (EE), and ash were determined according to the AOAC methods 976.06, 920.39, and 942.05, respectively [21]. Neutral detergent fibre (NDF), acid detergent fibre (ADF), and lignin (ADL) contents were determined according to van Soest et al. [22], using heat stable amylase and sodium sulphite, and expressed inclusive of residual ash. Metabolizable energy (ME) and net energy for lactation (NEL) were calculated according to Cannas et al. [23]. Feed FA were extracted according to Folch et al. [24], esterified according to Christie [25] with 19:0 (Sigma Chemical Co., St Louis, MO, USA) as the internal standard, and identified using the same procedure described below for FA of rumen samples.

2.5. Rumen Sample Collection and Fatty Acid Profile. Rumen content was sampled from each ewe before morning feeding from two different sites of the rumen and after 21 days of trials on each diet and immediately frozen at −80°C until further analysis.

The FA were extracted according to Folch et al. [24] and methylated according to Buccioni et al. [3]. The FA methyl ester (FAME) composition was carried out by gas-chromatography, according to Buccioni et al. [3]. Individual FAMEs were quantified using valeric acid (5:0) and nonadecanoic acid (19:0) methyl esters (cod W275204 and cod N5377, resp.; Sigma Chemical Co., St. Louis, MO, USA) as internal standards and identified by the comparison of the relative retention times of FAME peaks from samples, with those of the standard mixture 37 Component FAME Mix (C4:0-C24:0, cod 18919-1AMP, Supelco, Bellefonte, PA, USA), individual 18:1 *trans*9 and 18:1 *trans*11 (cod 46903 and v1381, resp., Sigma-Aldrich, St. Louis, MO, USA), individual 18:2 *cis*9, *trans*11 (cod 1255, Matreya Inc. Pleasant GAP, PA, USA), CLA mix standard (cod 05632; Sigma-Aldrich, St. Louis, MO, USA), and published isomeric profile [26–28]. The 18:1 isomers elution sequence was performed according

Table 1: Ingredients, chemical composition, and fatty acids profile of the experimental concentrates and of the hay and rolled barley administered to the ewes.

	Grass hay	Rolled barley	Control diet	CHT diet	QUE diet
Ingredients (g kg^{-1} of DM)					
Barley			213.8	213.8	213.8
Corn			211.3	211.3	211.3
Wheat bran			158.5	158.5	158.5
Soybean meal (44 CP)			126.8	126.8	126.8
Beet pulp			89.8	89.8	89.8
Soybean oil[1]			84.5	84.5	84.5
Bentonite			52.8	-	-
Chestnut tannin extract[2]			-	52.8	-
Quebracho tannin extract[3]			-	-	52.8
Molasses			41.3	41.3	41.3
$CaCO_3$			10.6	10.6	10.6
Sodium bicarbonate			5.3	5.3	5.3
Dicalcium phosphate			5.3	5.3	5.3
Chemical composition (g kg^{-1} of DM)					
Organic matter	847.0	859.9	816.9	858.1	869.6
Crude protein	111.2	121.0	165.6	173.7	170.3
Ether extract	12.0	16.1	109.4	105.4	102.4
NDF	636.4	134.1	174.7	181.4	172.1
ADF	501.3	54.2	77.6	72.4	74.3
ADL	105.7	14.9	10.6	13.3	8.7
Ash	69.6	21.0	84.6	39.9	39.4
ME (MJ kg^{-1} DM)	7.8	9.9	13.1	14.1	14.1
NEl (Mcal kg^{-1} DM)	0.9	1.2	2.0	2.1	2.1
Fatty acids (g/100 g of total fatty acids)					
16:0	35.5	18.2	14.0	14.4	14.9
SA, 18:0	5.8	4.6	3.6	3.4	3.4
18:1 *cis*9	9.3	21.2	23.3	22.9	22.0
LA, 18:2 *cis*9 *cis*12	28.5	45.0	51.4	51.7	51.8
LNA, 18:3 *cis*9 *cis*12 *cis*15	2.8	6.0	5.8	5.6	5.8
Other FA	18.1	4.9	1.9	2.0	2.1

[1]Fatty acid profile of soybean oil (g/100 g of total fatty acids): C16:0, 11.01; C18:0, 3.6; C18:1 *cis*9, 22.09; C18:2 *cis*9 and *cis*12, 53.7; C18:3 *cis*9, *cis*12, and *cis*15, 7.2. [2]Hydrolysable tannins extracted from chestnut wood (*Castanea sativa* Mill.) containing 750 g of equivalent tannic acid/kg DM (provided by Gruppo Mauro Saviola Srl Radicofani, Siena, Italy). [3]Condensed tannins extracted from quebracho *(Schinopsis lorentzii)* containing 456 g of equivalent tannic acid/kg DM (provided by Guido Lapi SpA, Castel Franco di Sotto, Pisa, Italy).

to Kramer et al. [29]. Moreover, standard mix of linolenic acid (LNA) isomers (cod 47792, Supelco, Chemical Co., St. Louis, MO, USA) and of LA isomers (cod 47791, Supelco, Chemical Co., St. Louis, MO, USA) and published isomeric profiles [30] were used to identify the isomers of interest (conjugated α-linolenic acid, CALNA, 18:3 *cis*9, *trans*11, *cis*15; vaccelenic acid, VLA, 18:2 *trans*11, *cis*15). Two bacterial acid methyl ester mixes (cod 47080-U Supelco, Chemical Co., St. Louis, MO; GLC110, Matreya, Pleasant Gap, PA) and individual standard for methyl ester of 14:0 *iso*, 14:0 *anteiso*, 15:0 *iso* and 17:0 *anteiso* (cods 21-1211-11, 21-1210-11, 21-1312-11, and 21-1415-11, Larodan Malmo, Sweden) were used to identify branched FA profile. Inter- and intra-assay coefficients of variation were calculated by using a reference standard butter (CRM 164, Community Bureau of Reference, Bruxelles, Belgium) and detection threshold of FA was 0.001 g kg^{-1} of FA [31]. All FA composition results are expressed as g/100 g of FA.

2.6. DNA Extraction and PCR Amplification. Total DNA was extracted from 1 mL of each frozen rumen fluid, using the Fast DNA SPIN kit for soil (MP Biomedicals, Santa Ana, CA, USA) with some modifications. Briefly, each sample was thawed and transferred to a 15 mL tube, containing 4.5 mL of a buffer consisting of 150 mM NaCl, 10 mM^{-1} Tris-HCl, pH 8.0, and 10 mM EDTA, vortexed vigorously, and centrifuged at 200*g* at 4°C for 5 min. One mL of supernatant was transferred to a 2 mL centrifuge tube and centrifuged at 14,600*g* at 4°C for 5 min. The pellet was dissolved in 978 μL of buffer sodium phosphate and 122 μL of MT buffer (both solutions are supplied by the Fast DNA SPIN kit for soil,

MP Biomedicals, Santa Ana, CA, USA) and then processed, according to the manufacturer's guidelines. DNA yield was quantified by its absorbance at 260 nm and DNA quality was verified using agarose gel electrophoresis (1% w/v).

The extracted DNA was used as a template for PCR amplification of the V6–V8 region of 16S rRNA genes of total bacteria or for the *Butyrivibrio* group. PCR amplifications were conducted using the following primer pairs: F968GC and R1401 for total bacteria (fragment size ~470 bp), according to Nübel et al. [32], and F968GC and B fib for the *Butyrivibrio* group (fragment size ~470 bp), according to Kim et al. [33]. Reactions were carried out using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hertfordshire, UK) in 25 μL volumes containing 1X PCR buffer (67 mM Tris-HCl, pH 8.8, 1.66 mM $(NH_4)_2SO_4$, 0.1% Tween-20), 1.5 mM $MgCl_2$, 250 μM deoxynucleotide triphosphates (dNTPs), 400 nM of each primer, 1U of Polytaq (Polymed, Florence, Italy), and 10 ng of DNA. PCR reactions were performed under the following conditions: initial denaturation of 94°C for 5 min, followed by 35 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 45 s, and a final extension of 72°C for 10 min. PCR products were verified by agarose gel (1.2% w/v) ecectrophoresis.

2.7. PCR-DGGE Analysis of Total Bacteria and Butyrivibrio Group Communities. The PCR amplicons were electrophoretically separated by DGGE on a 6% polyacrylamide gel (acrylamide/bis 37.5:1) in 1X TAE Buffer (40 mM Tris base; 20 mM glacial acetic acid; 1 mM EDTA) using a 50–60% denaturant gradient obtained with a 100% denaturant solution, consisting of 40% v/v deionized formamide, 7 M urea. The gels were run at 60°C and 75 V for 17 h in a Phor-U system (Ingeny International, Goes, NL) and at the end of electrophoretic runs, the gels were stained with SYBR® Gold (Molecular Probes, Eugene, OR) and gel images digitalized using the ChemiDoc XRS apparatus (Bio-Rad Laboratories, Hertfordshire, UK).

2.8. Sequence Analysis of PCR-DGGE Fragments. The middle portion of 16 bands selected from *Butyrivibrio* group DGGE profiles was aseptically excised and placed in 20 μL distilled water. The PCR products were eluted through freezing and thawing [34] and reamplified using F968/B fib primer pairs without GC clamp, as previously described. PCR products were checked by DGGE gel electrophoresis and then subjected to directly sequencing by Macrogen Service [35]. The sequence chromatograms were edited using Chromas Lite Software [36] to verify the absence of ambiguous peaks and convert them to FASTA format. The Decipher Find Chimera Web tool [37] was used to uncover chimeras hidden in the 16S rDNA sequences. The BLASTN program [38] available at the NCBI website [39] was used to find taxonomic closely related nucleotide sequences. To increase the accuracy of the assignments, different sequence similarity thresholds were used for different taxonomic levels: a similarity of ≥97% for a species level identification and 95%, 90%, 85%, 80%, and 75% for assignment at the genus, family, order, class, and phylum level, respectively [40].

A phylogenetic dendrogram was constructed to display the apparent relatedness of the partial 16S rRNA gene sequences to each other and to other sequences of equivalent length retrieved from the GenBank database using the software ClustalX 2.0.11 [41] to perform sequence alignment and the software TREECON 1.3b [42] for the construction of the phylogenetic tree using the neighbor-joining method [43]. Bootstrap analysis was performed based on 1000 resamplings.

2.9. Statistical Analysis. Statistical analysis was performed using the mixed procedure of SAS [44]. Data were analyzed with the following model:

$$Y_{ijkl} = \mu + A_i + P_j + D_k + (P_i \times D_k) + R_z + eijkz, \quad (1)$$

where Y is the dependent variable, calculated as the mean of the measurements during each sampling period, μ is the overall mean, A_i is the random animal effect (i = 1 to 3), P_j is the period effect (j = 1 to 3), D_k is the diet effect (k = 1 to 3), $D_k \times P_i$ is their interaction, R_z is the random replicates of the Latin square (z = 1 to 2), and $eijkz$ is the residual error.

Least squares means estimates are reported. For all statistical analyses, significance was declared at $P \leq 0.05$.

DGGE profiles were normalized and analyzed using GelCompar II software v 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). The number of bands (species richness) and their relative abundance were used as a proxy of richness and diversity (Shannon index, H', and Simpson index, D) of rumen microbial communities, as described by Pastorelli et al. [45]. The banding profiles of DGGEs, extracted as presence/absence matching tables, were imported into PAST software [46] for multivariate statistical analysis as previously described by Lagomarsino et al. [47]. One-way analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were performed to determine significance differences in the microbial community structure due to the different dietary regimes. In order to find potential connection between community composition and ruminal FA profile and to evidence how these connections may be influenced by the different diets two different canonical correspondence analyses (CCA) were carried out: in the first, the FA assumed to be mainly implicated in BH process (i.e., 18:0, stearic acid, SA; VA; VLA; LA; LNA; RA) were selected; in the second, the FA assumed to be markers of rumen microbial metabolism according to Fievez et al. [48] were considered (i.e., 15:0 *iso*; 15:0 *ante*; 17:0 *iso*; 17:0 *ante*). The length and the angle of vectors indicate the relative importance of that FA in discriminating the bacterial community of the different rumen liquors [49]. To identify taxa that mainly contribute to separation of microbial communities, according to the different diets, DGGE band scores were also plotted in the CCA diagrams. The *Butyrivibrio* group DGGE profiles that were mainly related to different FA profile were sequenced.

3. Results

3.1. Fatty Acids (FA) Composition of Rumen Liquor. The presence of tannins in the diets induced changes in FA profile of rumen liquor. Tannins lowered BH of PUFA leading to an accumulation of linoleic acid (LA), linolenic acid (LNA), and their BH intermediates, reducing the accumulation of

Table 2: Fatty acid profile of rumen liquor from sheep fed with 800 g/head/day of a concentrate containing 84 g kg^{-1} DM of soybean oil plus 0 (control) or 52.8 g kg^{-1} DM of a chestnut tannin extract (CHT) or 52.8 g kg^{-1} of DM of quebracho tannin extract (QUE).

FA g/100 g of total fatty acids	Control	CHT	QUE	SEM[1]	*P* value[2]
10:0	0.197 b	0.186 b	0.439 a	0.077	0.0473
12:0 *ante*	0.237 b	0.387 a	0.261 b	0.041	0.0402
12:0	0.297 b	0.318 b	0.656 a	0.087	0.0213
13:0	0.305 c	0.453 b	0.542 a	0.024	<0.0001
14:0 *iso*	0.317 b	0.613 a	0.404 b	0.068	0.0184
14:0	0.485 b	0.556 b	1.438 a	0.076	<0.0001
15:0 *iso*	0.116 c	0.185 b	0.239 a	0.020	0.0030
15:0 *ante*	0.378 b	0.376 b	0.507 a	0.039	0.0499
15:0	0.776 b	0.605 c	1.054 a	0.017	<0.0001
16:0 *iso*	0.175 b	0.178 b	0.276 a	0.029	0.0353
16:0	10,428 c	17.423 b	23.108 a	0.712	<0.0001
17:0 *iso*	7.544 a	7.418 a	6.135 b	0.288	0.0039
17:0 *ante*	0.189 b	0.154 c	0.247 a	0.010	<0.0001
17:0	0.327 b	0.342 b	0.532 a	0.037	0.0013
SA, 18:0	50.447 a	44.616 b	32.770 c	1.307	<0.0001
18:1 *trans*5	0.051 c	0.313 a	0.132 b	0.055	0.0122
18:1 *tran*6–8	0.476 c	0.682 b	1.134 a	0.131	0.0051
18:1 *trans*9	0.334 c	0.602 b	0.665 a	0.016	<0.0001
18:1 *trans*10	0.681 b	1.254 a	1.359 a	0.147	0.0071
VA, 18:1 *trans*11	1.922 c	6.304 b	7.589 a	0.244	<0.0001
18:1 *trans*12	0.535 c	0.792 b	1.299 a	0.033	<0.0001
18:1 *cis*5	0.374 b	0.697 a	0.799 a	0.069	0.0007
18:1 *cis*7	0.510 c	1.297 b	1.498 a	0.064	<0.0001
18:1 *cis*9	2.337 c	3.879 b	5.340 a	0.111	<0.0001
18:1 *cis*11	0.414 c	0.702 b	0.892 a	0.014	0.0137
18:1 *cis*12	0.258	0.315	0.363	0.042	0.1927
VLA, 18:2 *trans*11, *cis*15	0.123	0.181	0.179	0.049	0.5901
LA, 18:2 *cis*9, *cis*12	0.845 c	1.096 b	1.926 a	0.075	<0.0001
LNA, 18:3 *cis*9, *cis*12, and *cis*15	0.305 c	0.363 b	0.467 a	0.023	0.0004
RA, 18:2 *cis*9, *trans*11	0.651 c	2.137 b	2.600 a	0.042	<0.0001
CLA *trans*10, *cis*12	0.163 b	0.162 b	0.258 a	0.017	0.0008
20:0	0.230 b	0.323 b	0.637 a	0.025	<0.0001
20:4	0.738	1.016	0.959	0.110	0.1855
22:0	0.144 c	0.226 b	0.399 a	0.021	<0.0001

[1] Standard error mean; [2] probability of significant effect (a, b, and c for $P < 0.05$).

stearic acid (SA) (Table 2). In particular, vaccenic acid (VA) and rumenic acid (RA) percentage was significantly higher in rumen liquor from ewes fed with CHT and QUE than in samples from animals fed control diet. Other 18:1 isomers such as *cis*15, *cis*9, *cis*11, *trans*5, *trans*6–8, *trans*9, and *trans*10 showed a similar trend. QUE diet was also associated with an increase of 18:2 *trans*10, *cis*12 content in rumen liquor.

Considering the odd and even branched fatty acids, 14:0 *iso* content increased only in rumen liquor samples from ewes fed CHT, whereas the content of 15:0 *iso* increased in rumen liquor of both tannin-rich diets. However, the content of 15:0 *iso* was higher in rumen liquor from ewes fed with QUE. Rumen liquor samples from ewe fed with control and CHT diet had the highest concentration of 17:0 *iso* (Table 2). Considering the *ante/iso* FA, the content of 12:0 *ante* was significantly higher in CHT samples, whereas 15:0 *ante* and 17:0 *ante* content was higher in QUE samples.

3.2. Effect of Chestnut and Quebracho Tannins on Rumen Microbial Communities. The DGGE banding profiles obtained for total bacteria (Supplementary Material 1) showed a number of bands ranging from 16 to 28. The profiles generated with *Butyrivibrio* group primers were less complex, with a band number of 4–16 (Supplementary Material 2). Richness was not affected by the presence of tannins in the diet in rumen liquor bacterial ($P = 0.324$) and *Butyrivibrio* group ($P = 0.206$) communities. H' index obtained from the DGGE analysis of bacteria ($P = 0.352$) and *Butyrivibrio* group ($P = 0.117$) was similar among treatments and D index did not change significantly in relation to diet

Table 3: P values from PERMANOVA pair-wise comparison of band profiles from 16S rDNA bacterial DGGE (in boldface, upper right side) and from 16S rDNA *Butyrivibrio* group DGGE (in italics, lower left side).

Diet	Control	CHT	QUE
Control		**0.0450**[a]	**0.0272**[a]
CHT	*0.0196*[a]		**0.1310**
QUE	*0.0737*	*0.0046*[a]	

[a]Significant value ($P < 0.05$).

in bacterial ($P = 0.383$) or *Butyrivibrio* group communities ($P = 0.071$).

The ANOSIM test applied to 16S rDNA PCR-DGGE profiles showed that the different dietary regimens significantly separated the rumen bacterial communities and that bacterial banding profiles of replicates (6 animal samples × 3 diets) for each diet were more similar to each other (ANOSIM global test $R = 0.233$; $P < 0.05$) than those find when the *Butyrivibrio* group (ANOSIM global test $R = 0.4216$; $P < 0.01$) was analyzed. PERMANOVA analysis confirmed that diet significantly affected the microbial community structure (PERMANOVA global test: bacteria $F = 2.446$, $P < 0.05$; *Butyrivibrio* group $F = 4.276$, $P < 0.01$). PERMANOVA pair-wise test established that bacterial communities under CHT and QUE were significantly different from that of control diet (Table 3) and that for *Butyrivibrio* group communities under CHT were significantly different from the others, whereas the control community was not significantly different to QUE (Table 3).

3.3. Bacterial Community Composition in relation to Diet. Canonical correspondence analysis carried out between total bacteria or *Butyrivibrio* group DGGE profiles and the FA assumed to be mainly implicated in BH process (SA; VA, VLA, and LA; LNA; RA) showed that ruminal communities under tannin dietary treatments were separated from the control, Figures 1(a) and 2(a). Similarly, CCA carried out between total bacteria or *Butyrivibrio* group DGGE profiles and the FA assumed to be markers of rumen microbial metabolism (15:0 *iso*; 15:0 *ante*; 17:0 *iso*; 17:0 *ante*) according to Fievez et al. [48] indicated that ruminal community under QUE was separated from the control and CHT, Figures 1(b) and 2(b).

Both total bacterial and *Butyrivibrio* group communities under tannin extract diets were positively correlated to LA, LNA, RA, and VA production, Figures 1(a) and 2(a), whereas only those under QUE were positively correlated to C15 *ante* and C17 *ante*, Figures 1(b) and 2(b). Total bacterial and *Butyrivibrio* group communities of control samples were positively correlated to SA production, Figures 1(a) and 2(a).

3.4. Association and Identification of Butyrivibrio Group 16S rDNA PCR-DGGE Bands with Key Fatty Acids in the Biohydrogenation Pathway. Multivariate CCA analysis of data generated from *Butyrivibrio* group DGGE allowed identifying bacterial species or groups mainly correlated to a specific FA; thus, bacteria identified by sequencing DGGE bands 1, 2, 3, 4, 5, 6, and 7 (Table 4) were significantly associated with LA, LNA, RA, VA, and VLA (data not shown), whereas bacteria corresponding to bands 8, 9, 10, 11, 12, 13, 14, and 15 (Table 4) were significantly linked with SA (data not shown).

Putative taxonomic identification of DNA bands associated with LA, LNA, RA, and VA revealed that they were related to genera *Hungatella* (band 5), *Ruminococcus* (bands 2, 3, and 4), and unclassified Lachnospiraceae (bands 1, 6, and 7; Table 4; Figure 3). Moreover, putative taxonomic identification of bands associated with SA revealed that they were related to unclassified Lachnospiraceae (bands 8, 9, 10, 11, 12, 13, 14, and 15; Table 4; Figure 3).

4. Discussion

The BH process of dietary PUFA was strongly lowered by both the tannin-rich diets, regardless of the type of tannin. However, results showed that QUE tannins had a stronger ability to favor the accumulation of BH-intermediate, such as VA and RA, and to reduce the SA concentration in rumen liquor, if compared to CHT tannins. Recently, we found similar results by feeding lactating dairy ewes with diets containing soybean oil supplemented or not with chestnut and quebracho tannins [3]. In the present trial, in rumen liquor *trans*10 isomers of 18:1 and 18:2 also significantly were accumulated in response to tannin supplementation. Previously, in an *in vivo* trial on lactating dairy ewes [3] the administration of the same amount of soybean oil and tannins in the concentrate feed did not result in significant effects on *trans*10 isomers of 18:1 and 18:2, suggesting that in the present trial the rumen environment was more favorable to alternative BH pathway of LA from soybean oil.

The presence of condensed tannins in QUE diet induced the increase of 15:0 *ante* and 17:0 *ante* content and the decrease of 17:0 *iso* content in rumen liquor. Since the first two FA were associated with the growth of cellulolytic strains and the latter with the growth of amylolytic bacteria [48], this pattern or branched FA suggested a detrimental effect of condensed tannins on cellulolytic bacteria. In contrast, the pattern of branched chain FA between rumen liquor samples from CHT and control diets was quite similar, suggesting that CHT tannins did not perturb the growth of cellulolytic bacteria. Indeed, the previous *in vivo* experiment on lactating ewes [3] demonstrated that QUE tannins were more efficient than CHT in limiting cellulolytic bacteria proliferation.

As regards the composition of the whole rumen bacterial community, the diversity indices did not change. As a consequence, changes in the BH pattern could be due to rearrangements of the bacterial species. Indeed, the toxic effect of tannins on specific strains could be compensated by an increase of tannin resistant bacteria in total population. These data are in accordance with previous findings about the selective inhibition of plant extracts containing tannins on the growth and the activity of specific bacterial species representative of rumen microbial populations [2, 11, 50, 51]. Vasta et al. [2], by means of a T-RFLP analysis, demonstrated that dietary supplementation of QUE tannins affected total bacteria community structure in the rumen of lambs fed with QUE supplemented diets. However, it is worth noting that

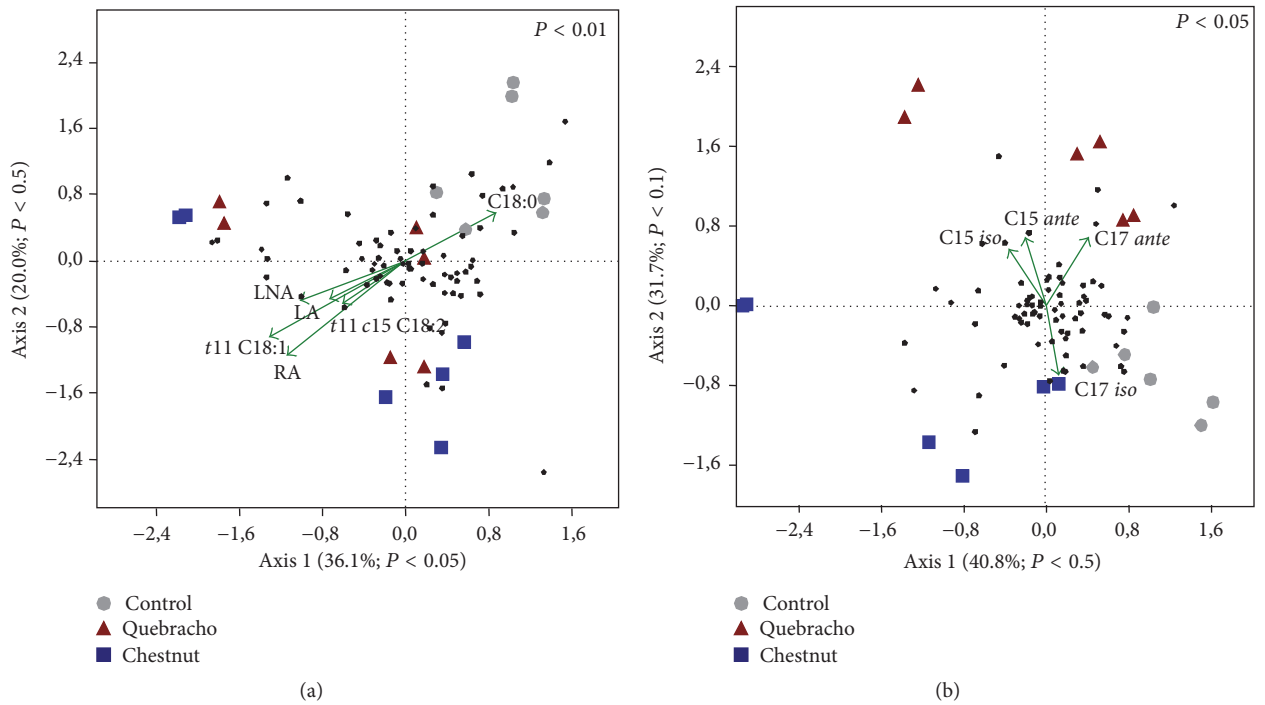

FIGURE 1: Canonical correspondence analysis (CCA) ordination diagram of ruminal bacterial communities and ruminal fatty acids variables [(a) FA assumed to be mainly implicated in BH process (SA, 18:0; VA, 18:1 *trans*11; VLA, 18:1 *trans*11, *cis*15; LA; LNA; RA); (b) FA assumed to be markers of rumen microbial metabolism (15:0 *iso*; 15:0 *ante*; 17:0 *iso*; 17:0 *ante*)] (vectors) defined by the first and second axes. DGGE band scores were also plotted (black filled circle). For each diagram significance (global test) is reported in upper right side.

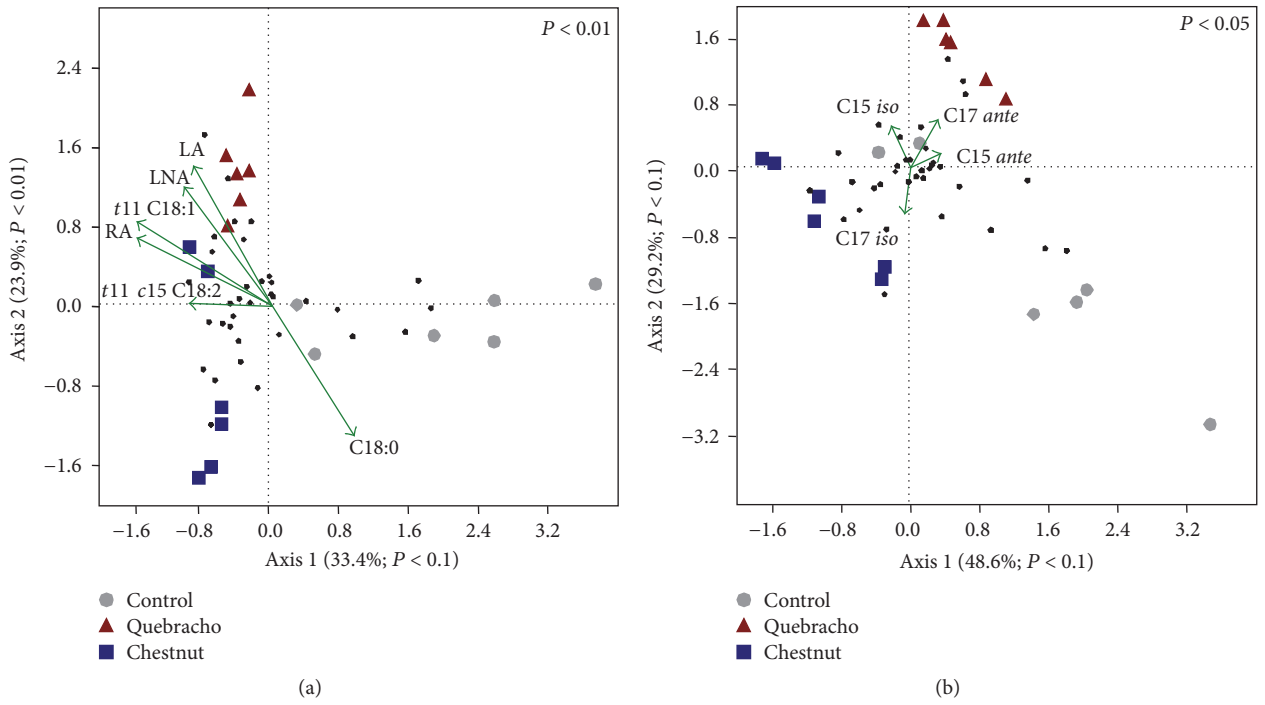

FIGURE 2: Canonical correspondence analysis (CCA) ordination diagram of ruminal *Butyrivibrio*-related communities (symbols) and ruminal fatty acids variables [(a) FA assumed to be mainly implicated in BH process (SA, 18:0; VA, 18:1 *trans*11; VLA, 18:1 *trans*11, *cis*15; LA; LNA; RA); (b) FA assumed to be markers of rumen microbial metabolism (15:0 *iso*; 15:0 *ante*; 17:0 *iso*; 17 *ante*)] (vectors) defined by the first and second axes. DGGE band scores were also plotted (black filled circle). For each diagram significance (global test) is reported in upper right side.

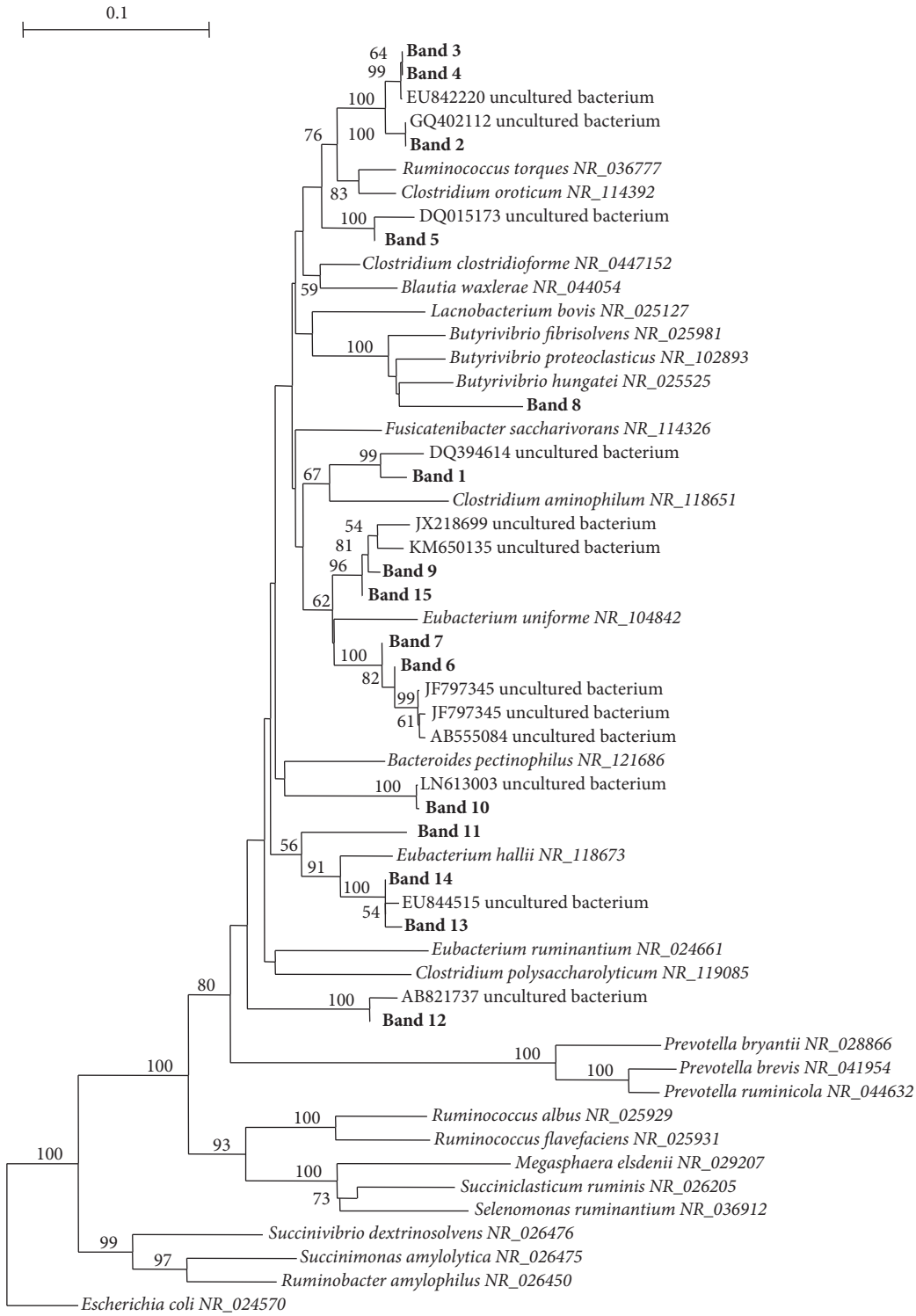

Figure 3: Phylogenetic analysis of *Butyrivibrio* partial 16S rRNA sequences obtained from PCR-DGGE bands using primers F968/B fib and identified species. Sequences obtained in this study are shown in boldface. Bootstrap values of >50% based on 1000 replications are indicated at the nodes. The 16S rRNA gene sequence of *Escherichia coli* (NR_024570) was selected as the outgroup.

Table 4: Identification of the selected polymerase chain reaction denaturing gradient gel electrophoresis (16S rDNA PCR-DGGE) fragments.

PCR-DGGE band	Nearest match (GenBank accession number; % sequence similarity)	Relative taxonomic classification
(1)	*Clostridium aminophilum* (NR_118651.1; 93%)	Unclassified Lachnospiraceae
(2)	*Ruminococcus torques* (NR_036777.1; 95%)	*Ruminococcus* spp.
(3)	*Ruminococcus torques (*NR_036777.1; 95%)	*Ruminococcus* spp.
(4)	*Ruminococcus torques (*NR_036777.1; 96%)	*Ruminococcus* spp.
(5)	*Clostridium clostridioforme* (NR_0447152; 95%)	*Hungatella* spp.
(6)	*Fusicatenibacter saccharivorans* (NR_114326.1; 93%)	Unclassified Lachnospiraceae
(7)	*Eubacterium uniforme* (NR_104842.1; 94%)	Unclassified Lachnospiraceae
(8)	*Butyrivibrio proteoclasticus* (NR_102893.1; 94%)	Unclassified Lachnospiraceae
(9)	*Clostridium oroticum* (NR_114392.1; 94%)	Unclassified Lachnospiraceae
(10)	*Blautia waxlerae* (NR_044054.1; 93%)	Unclassified Lachnospiraceae
(11)	*Roseburia intestinalis* (NR_027557.1; 92%)	Unclassified Lachnospiraceae
(12)	*Bacteroides pectinophilus* (NR_121686.1; 90%)	Unclassified Lachnospiraceae
(13)	*Eubacterium hallii* (NR_118673.1; 93%)	Unclassified Lachnospiraceae
(14)	*Eubacterium hallii* (NR_118673.1; 93%)	Unclassified Lachnospiraceae
(15)	*Clostridium oroticum* (NR_114392.1; 94%)	Unclassified Lachnospiraceae

the percentage of tannins employed in the present study was much lower (<2% DM) than that used by Vasta et al. [2]. Thus, putting together data from the present and previous *in vivo* trials on dairy ewes, the addition of practical doses of tannins in the diet is able to cause shifts in the rumen total bacterial community favoring the accumulation of LA and its BH intermediates in rumen liquor.

Bacteria involved in the BH process have been categorized traditionally into two distinct groups: those belonging to group A converting PUFA as LA or LNA into VA and those belonging to group B hydrogenating VA into SA [14]. Group A comprises many known species, among which *Butyrivibrio* spp. are the most important [52, 53], whereas *B. proteoclasticus* is the only known cultivable rumen bacterium belonging to group B [54, 55]. However, recent studies demonstrate that other microorganisms as-yet-uncultivated bacteria phylogenetically classified as *Prevotella*, Lachnospiraceae incertae sedis and unclassified *Bacteroidales*, *Clostridiales,* and Ruminococcaceae might be involved in BH processes with a relevant role [10, 12, 13, 56]. Our data from DGGE analysis showed an effect of 2% DM of QUE or CHT tannins on the composition of the *Butyrivibrio* group. *Butyrivibrio* species are particularly sensitive to condensed tannins [16]. Indeed, these phytochemicals can penetrate the cell wall of *Butyrivibrio* and other Gram positive bacteria and selectively inhibit the cell wall biosynthesis [50]. However, at the same time, remarkable differences among *Butyrivibrio* species were observed in the level of their sensitivity to tannins [11, 50]. The persistence and the appearance of some bands but not of other ones in the *Butyrivibrio* group DGGE gel are consistent with these previous findings.

Multivariate statistics allowed the selection of DGGE bands representative of *Butyrivibrio* group that are putatively involved in the BH process *in vivo*. Seven bands disappeared when ewes were fed with QUE enriched diet, and this effect was associated with reduced rumen SA concentrations. Therefore, it is possible that this group of DNA bands might be representative of other bacteria that play a role in the BH of VA into SA, confirming the findings of several studies [10, 12, 13, 56]. Indeed, the bands here identified are highly related to uncultured rumen bacteria belonging to the family Lachnospiraceae. Interestingly, no sequences were identified as *B. proteoclasticus*, which according to literature is the only known bacterial species able to efficiently biohydrogenate PUFA into SA in the rumen. However, *in vivo* studies have shown contrasting results, since a clear relationship between the reduced amount of *B. proteoclasticus* and a decreased production of SA in rumen liquor was found only in a limited number of trials [2, 3]. A possible explanation to our data may be that the concentration of tannins used in this study was not able to modify *B. proteoclasticus* growth in the rumen but only lowered its capacity to hydrogenate 18:1 *trans* FA, as previously suggested by Boeckaert et al. [10]. Otherwise, it is possible that *B. proteoclasticus* has a limited contribution to SA formation *in vivo* and that other yet not known species may have a more important role in this step of the BH pathway. Since only a limited number of rumen species is presently known, this hypothesis is likely. Moreover, it is in agreement with the opinion of other authors who evaluated the effect of marine algae [10] and fish oil [56] on rumen bacterial diversity, evidencing a possible relation between the disappearance of many uncultivated Lachnospiraceae strains, genetically distant from *B. proteoclasticus,* and a significant decrease of rumen SA concentration.

Our study evidenced also that the two types of tannins induced an increase in LA, LNA, RA, and VA and 18:2 *trans*11 and *cis*15 in rumen liquor and this was associated with a higher intensity of seven bands in the *Butyrivibrio* group DGGE profiles. Phylogenetic analysis revealed that these sequences were representative of species belonging to genera *Hungatella*, *Ruminococcus*, *Eubacterium* and to unclassified Lachnospiraceae. Once more, these data confirm that other

Butyrivibrio groups may be involved in the BH pathway and that their increased amount may promote the accumulation of 18:1 intermediates in the rumen. The employment of other and more powerful molecular techniques, such as functional metagenomics, could be useful to clear the role of these uncultured bacteria in the BH pathway.

5. Conclusions

The use of chestnut and quebracho tannins in the diet of dairy ewes at a level below 2% DM reduced the extent of ruminal BH process, lowering SA concentration and enhancing the percentage of LA, LNA, VA, RA, and other 18:1 isomers. The changes observed in the FA profile were associated with changes in total bacteria and *Butyrivibrio* group communities, even if they were more evident in presence of quebracho. Bands that disappeared or increased in presence of tannins in the *Butyrivibrio* group DGGE profiles were related to many uncultivated species of Lachnospiraceae, suggesting that these yet not known species may play a role in BH of PUFA. Our study indicates that chestnut and quebracho tannins offer an interesting possibility of modulating favorably rumen bacterial lipid metabolism toward precursors of healthful FA, which are produced in mammary tissues of lactating ewes during milk fat synthesis.

Abbreviations

ADF: Acid detergent fibre
BH: Biohydrogenation
CLNA: Conjugated linolenic acid
CHT: Chestnut tannins
DM: Dry matter
CP: Crude protein
EE: Ether extract
FA: Fatty acids
FAME: Fatty acid methyl esters
LA: Linoleic acid
LNA: Linolenic acid
ME: Metabolizable energy
NEL: Net energy for lactation
NDF: Neutral detergent fibre
PUFA: Polyunsaturated fatty acids
QUE: Quebracho tannins
RA: Rumenic acid
SA: Stearic acid
VA: Vaccenic acid
VLA: Vaccelenic acid.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Arianna Buccioni and Grazia Pallara are equal contributors.

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Supplementary Materials

Supplementary Material 1: DGGE profiles of 16S rDNA PCR products obtained from DNA extracted from rumen liquor using primer for the total bacteria (F968GC-1401R). CTR, control diet (84 g kg^{-1} DM of soybean oil); CHT, chestnut tannins diet (84 g kg^{-1} DM of soybean oil plus 52.8 g kg^{-1} DM of a chestnut tannin extract); QUE, quebracho tannins diet (84 g kg^{-1} DM of soybean oil plus 52.8 g kg^{-1} DM of a quebracho tannin extract); M, marker used for normalization of bands. Supplementary Material 2: DGGE profiles of 16S rDNA PCR products obtained from DNA extracted from rumen liquor using primer for the *Butyrivibrio* group (F968GC-B fib). CTR, control diet (84 g kg^{-1} DM of soybean oil); CHT, chestnut tannins diet (84 g kg^{-1} DM of soybean oil plus 52.8 g kg^{-1} DM of a chestnut tannin extract); QUE, quebracho tannins diet (84 g kg^{-1} DM of soybean oil plus 52.8 g kg^{-1} DM of a quebracho tannin extract); M, marker used for normalization of bands. *(Supplementary Materials)*

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Review Article

Antifungal Compounds against *Candida* Infections from Traditional Chinese Medicine

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Infections caused by *Candida albicans*, often refractory and with high morbidity and mortality, cause a heavy burden on the public health while the current antifungal drugs are limited and are associated with toxicity and resistance. Many plant-derived molecules including compounds isolated from traditional Chinese medicine (TCM) are reported to have antifungal activity through different targets such as cell membrane, cell wall, mitochondria, and virulence factors. Here, we review the recent progress in the anti-*Candida* compounds from TCM, as well as their antifungal mechanisms. Considering the diverse targets and structures, compounds from TCM might be a potential library for antifungal drug development.

1. Introduction

One severe health threat is infections caused by fungal pathogens, among which *Candida* species are the second most common fungal pathogen next to *Cryptococcus neoformans*, responsible for about 400,000 life-threatening infections per annum in the worldwide with a mortality as high as 40% [1, 2]. *Candida* spp. accounted for 98% of central venous catheter-related fungemias in patients with cancer [3]. Among the many *Candida* species, *Candida albicans* is the most common fungal pathogen of human diseases, followed by *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* [2].

As the major opportunistic fungal pathogen, *C. albicans* dwells on the skin, in the oral cavity, mucosa of gut, and urogenital tract as a symbiotic fungus under normal conditions [4]. The host could discern the commensal and pathogenic state of *C. albicans*, rendering this fungus under the surveillance of immune system, and the bacterial microbe of locales where *C. albicans* colonize also contributes to keeping this fungus in check [5, 6]. The host defense against *C. albicans* relies on a complicated network consisting of innate and adaptive immune components (e.g., epithelial cells, macrophages, neutrophils, dendritic cells, defensins, and complement). When the hosts encounter lower functions of immune system (resulting from HIV infection, organ transplant, and cancer treatment [7]) or disequilibrium of microflora due to the use of antibiotics [8], mucocutaneous and superficial infections, such as oral thrush and vaginitis, come up. This fungal pathogen could also cause life-threatening systematic infections such as candidemia. Other predisposing factors of *Candida* infections include diabetes and old age [9]. Among the nosocomial bloodstream infections, infections caused by *C. albicans* are the fourth prevalent [10].

In present, the therapeutic drugs for *Candida* infections are limited to five classes of compounds: polyenes, allylamines, azoles, fluoropyrimidines, and echinocandins [11], and amphotericin B, terbinafine, fluconazole, 5-fluorocytosine, and caspofungin are examples for them [12]. Drug resistance emerges due to pervasive application of antifungal drugs, such as fluconazole and voriconazole, for both prophylactic and therapeutic purposes [13]. Cellular and molecular

mechanisms underlying drug resistance may include reduced accumulation of intracellular drugs because of increased drug efflux (such as elevated mRNA levels of members of ABC transporter superfamily), mutations in genes of target protein (resulting in elevated levels of target protein or reduced affinity to targets), and modification of metabolism pathways (such as altered synthetic pathway of sterol which plays an important role in both structure and function of fungal cell wall) [14]. Researches indicate extensive regulation of intracellular processes in response to antifungal drugs. The fungistatic property of some drugs such as azoles and 5-flucytosine also contributes to the emergence of resistance [10], while the formation of biofilm may contribute to and elevate the resistance [15]. The paucity of antifungal drugs and the emergence of resistance make it a pressing mission to discover and identify new hits and leads from synthesized chemicals or natural products. Compared to synthesized chemicals, natural products have many advantages such as structural diversity and relatively low toxicity.

Natural products provide a potential source for antifungal drugs, either in their nascent form or as original templates for structure-optimizing for more effective and safe derivatives [16, 17]. Among the marketed antibiotics used clinically, about 80% are derived from natural products [17]. Traditional Chinese medicine is composed of mainly herbs that have been used for thousands of years. Recently, single compounds isolated from many traditional Chinese herbs have been demonstrated to have various kinds of pharmacological activities, such as antibacterial, antitumor, antiviral, and antifungal activities. Considering the present lack of antifungal drugs and the usefulness of traditional Chinese medicine, it may be a promising strategy to develop antifungal agents from traditional Chinese medicines. Here, recent antifungal compounds from traditional Chinese medicines will be briefly reviewed.

2. Compounds Targeting Cell Membrane

The plasma membrane keeps the cytoplasm from circumambient environment. The integrity and fluidity of cell membrane means being important to the survival and growth of fungal cells; one important reason is that many enzymes, channels, and transporters of drugs lie on the cell membrane. Cell membrane is the location where many metabolic processes occur and meanwhile it provides a barrier to environmental stresses.

Derived from *Sambucus williamsii*, a traditional herb broadly used for hundreds of years to treat fractures, edema, and scratches in East Asian countries, (−)-olivil-9′-*O*-*β*-D-glucopyranoside exerts its antifungal activity against *C. albicans* by depolarizing the cell membrane evidenced by influx of propidium iodide (PI) and elevated fluorescence of 3,3′-dipropylthiacarbocyanine iodide ($DiSC_3(5)$, a cyanine dye for measuring membrane potential) [18]. More important and encouraging is that this compound shows little hemolytic activity on human erythrocytes [18]. Two other components (both are lignans) from the same plant, lariciresinol [19] and (+)-pinoresinol, show similar anti-*Candida* effects by damaging the plasma membrane leading to permeabilization [19, 20]. The differential effects upon human and fungi cells imply that it may act on unique components of fungi cells, which needs further identification. Another compound isolated from *Sambucus williamsii*, glochidioboside, shows antifungal activity similar to that of (−)-olivil-9′-*O*-*β*-D-glucopyranoside against *C. albicans* by forming pores on cytoplasmic membrane with a radius range from 1.4 to 2.3 nm [21]. One of the products from the secondary metabolism of *Trachelospermum asiaticum*, dihydrodehydrodiconiferyl alcohol 9′-*O*-*β*-D-glucoside, could also depolarize the transmembrane potential via forming pores with radii ranging from 0.74 nm to 1.4 nm [22]. Changes in granularity and size revealed by the flow cytometry assays also involves alterations of the membrane properties such as osmolarity [22]. However, there are no evident causative link between disruption of membrane potential and changes of osmolarity and no conclusions about which comes first, which remain to be further investigated.

As a component of fungal cell membrane different from the mammalian parallel and a critical modulator for differentiation and pathogenicity of fungi, the glycosphingolipid glycosylceramide in the cell envelope maybe presents a better target for antifungal therapeutic treatments [23].

Ergosterol plays important roles in regulating the fluidity of the cell membrane and cell division of fungal cells, while the structural and conformational differences between ergosterol and sterol (the counterpart of ergosterol in mammalian cells) underlie the antifungal mechanism of the polyenes such as amphotericin B [12, 24]. Despite the low bioavailability and high toxicity of ergosterol-targeting drugs in humans [25, 26], ergosterol still presents a good target for antifungal drugs due to the importance of cell membrane.

Magnolol, one of the major pharmacologically active compounds from *Magnolia officinalis* which could be used to ameliorate the symptoms such as anxiety, asthma, nervous disturbance, and digestive problems [27], could reduce the content of ergosterol in the widely used *C. albicans* SC5314 [28]. Compounds from essential oil of mint, such as menthol, menthone, and carvone, suppress the growth of *C. albicans* through decreasing the contents of ergosterol in cell membrane and the hemolysis caused by them is less than that induced by fluconazole [29]. The ergosterol levels could also be decreased by carvacrol (isolated from *Origanum dictamnus L.*) and thymol, which could exert their influence on the antioxidant defense system, increase the membrane permeability, block the efflux pumps, and thus restore the antifungal susceptibility [30, 31]. Aside from *Candida* species, antifungal activities of this compound against other fungi such as *Monilinia laxa* have been identified [31–33].

Transporters such as ABC transporters on cell membrane could induce the efflux of antifungals, thus compromising the effects of drugs. Treatment with magnolol could significantly decrease the efflux of fluconazole, thus enhancing the antifungal effects of fluconazole [28].

PM-H^+ ATPase on cell membrane plays a vital role in keeping the transmembrane electrochemical proton gradient which is important for the obtaining of nutrients. The intercellular pH hemostasis modulated by PM-H^+ ATPase is

of great physiological importance. And the enzymatic activity of PM-H^+ ATPase is positively correlated with cell viability [29]. Carvone, menthol, and menthone could suppress the PM-H^+ ATPase activity, presumably the primary cause of the antifungal effects [29]. The results also indicated the existence of targets that could be easily touched by external drugs, despite the fact that more efforts need to be made.

3. Compounds Targeting Cell Wall Components

The structural integrity of cell wall is vital to the survival and growth of fungal cells, as it provides a shelter from osmotic pressure and other stresses in milieu. Recent studies showed that it likely plays an important role in the colonization and biofilm formation of *C. albicans*, as proteins associated with adhesion, such as Als1, Als3, and Hwp1, are cell wall proteins [34, 35]. Damaged cell wall leads to osmotic fragility of the fungal cell, disrupted membrane, efflux of cytoplasmic contents, and suppressed growth of fungi [13]. Cell wall is lacking in mammal cells, which makes it a preferential target for potential antifungal drugs for safety considerations. The cell wall of *Candida* species holds glycoproteins and abundant carbohydrates, among which are largely glucan, mannose, and chitin [10]. In the following part of this review we will discuss the plant-derived antifungal components acting on cell wall elements or on the synthesis of those elements.

3.1. Chitin. As one of the major components comprising fungal cell wall, chitin is a long linear homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and is synthesized by the incorporation of GlcNAc units from the precursor uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) in a reaction catalyzed by chitin synthetase (CHS) [36, 37].

Despite the small percentage in the cell wall, chitin plays important roles in maintaining the mechanical strength of the fungal cell wall, thus keeping the integrity of the fungal cell wall [38]. Damage to the cell wall may be ameliorated by the elevated quantity of chitin in cell wall due to increased synthesis and/or decreased degradation of chitin, which may increase the tolerance to antifungal drugs [38]. Since this material does not exist in human cells, this presents an attractive target for antifungal therapies [36]. The chemical structures of the classic inhibitors of CHS, namely, polyoxins and nikkomycins, make themselves be degraded easily *in vivo* and difficult to go through the cell membrane, leading to a low antifungal activity [36, 39]. This prompts us to find new CHS inhibitors.

Plagiochin E derived from liverwort *Marchantia polymorpha* L. exerts its antifungal effect through inhibiting the expression of chitin synthetase gene 1 (CHS1) and therefore suppressing the activity of CHS and subsequent synthesis of chitin both *in vivo* and in situ [13]. Interestingly, the expression of CHS2 and CHS3 gene was upregulated by this macrocyclic compound [13]. However, the same group found that plagiochin E exposure of *C. albicans* could induce accumulation of reactive oxygen species (ROS) through malfunction of mitochondria, while pretreatment with L-cysteine could contribute to the survival of *C. albicans* [40]. These studies indicate that plagiochin E may exert its antifungal activity through diverse currently unknown mechanisms.

3.2. Glucan. This carbohydrate polymer, together with chitin, is the structural component which holds the integrity and physical strength of the cell wall. The production and assembly of glucan in *C. albicans* need a series of enzymes and regulatory networks, which are fungal-specific and thus render some fascinating targets for antifungal therapies [41]. The most famous drugs of this kind are echinocandins such as caspofungin and micafungin which inhibit the synthesis of β-1,3-glucan [10]. Although they are fast-acting, less toxic, and fungicidal [10], mutations in β-1,3-glucan synthase that confer resistance to caspofungin have already emerged. Recently, a novel terpene antifungal SCY-078 demonstrated fungicidal activity against *C. albicans* through inhibiting glucan synthase [42]. Sodium houttuyfonate, a derivative from *Houttuynia cordata Thunb.,* might exert its synergistic effect with fluconazole through interfering with β-1,3-glucan synthesis and transportation [43].

4. Compounds Targeting Mitochondria

The classical respiratory chains of mitochondria are centers of energy production through oxidative phosphorylation, and meanwhile mitochondria are the organelles that produce metabolic intermediates used for amino acid and lipid biosynthesis. Both energy supply and metabolites are indispensable for the survival and growth of *C. albicans*, as well as major cellular event such as yeast-to-hyphal transition. Mitochondria are also involved in efflux-mediated resistance of *C. albicans* to fluconazole [44] while *in vitro* resistance of *C. glabrata* to azoles is associated with mitochondrial DNA deficiency [45]. Resistance to azole is also likely to be related with decreased generation of endogenous ROS that are harmful to DNA, proteins, and lipids while ROS are mainly generated by enzyme complexes (Complex I and Complex III) in classical respiratory chain as by-products of selective degradation of mitochondria [44]. Elevated levels of intracellular ROS are involved in the antifungal effects of fluconazole and miconazole and ROS also play an important role in intrinsic mitochondrial pathway of apoptosis in *C. albicans* [46, 47]. Besides the common enzymes in classical respiratory chain in *C. albicans* cells, there also exist rotenone-insensitive NAD(P)H dehydrogenase and alternative terminal oxidases constituting the cyanide-insensitive respiratory chain [48–50]. In addition, *C. albicans* and *C. parapsilosis* have additional respiratory pathway called parallel respiratory chain [51]. The differences between fungal and mammal mitochondrial enzymes also make developing drugs targeting these enzymes possible [52]. This is the case of some agrichemicals such as boscalid and carboxin that inhibit the succinate dehydrogenase in fungal cells [53]. Although there are only few studies on drugs targeting specifically mitochondria of *Candida* spp., ME1111 [2-(3,5-dimethyl-1*H*-pyrazol-1-yl)-5-methylphenol] did exert its antifungal effects upon human pathogens *Trichophyton mentagrophytes* and *Trichophyton rubrum* through inhibiting succinate dehydrogenase in mitochondria with high selectivity (the IC_{50} values

for human cells are more than thirty times higher than that for fungal cells) [53]. In a word, mitochondria might be a promising target for antifungal therapies.

As an important constituent of many herbs of Berberidaceae family such as *Berberis vulgaris*, berberine exerts its antifungal action by induction of mitochondrial dysfunction and increased ROS generation, and its effects are in synergy with fluconazole, even in fluconazole-resistant clinical isolates [54–56]. Moreover, berberine treatment could also culminate in disruption of cell wall integrity in *C. albicans* [57] and inhibit the overexpression of drug resistance gene CDR1 induced by fluphenazine [58]. Although berberine could induce apoptosis in many human cells such as HL-60 leukemia cells and thyroid carcinoma cells [59, 60], berberine could recover the mitochondrial function induced by high-fat feeding in a rat model and could decrease the triglyceride accumulation in the liver in mice [61, 62]. It also markedly decreased the ROS generation in mitochondria [62]. This makes berberine a good candidate for antifungal development although there are much more to be done.

(+)-Medioresinol from the anti-inflammatory, analgesic, and diuretic herbal plant *Sambucus williamsii*, imposed on *C. albicans,* could induce generation of ROS and cell cycle arrest and finally apoptosis [47]. Although (+)-medioresinol could inhibit *in vitro* the proliferation of mammalian cells such as A549, SK-MEL-2, SK-OV-3, and HCT-15 cells at high concentrations, the IC_{50} values for these cell lines were much higher than the MIC value against *C. albicans* cells [47, 63]. In a cohort study in Sweden, (+)-medioresinol in food did not clearly reduce the risk of esophageal and gastric cancers, but at least this compound in diet did not show bad effects [64, 65]. The safe profile of this lignin makes it more inspiring although there is no report on its effects on mammalian mitochondria.

Allyl alcohol from garlic *(Allium sativum),* which has been used as a traditional antimicrobial agent for thousands of years, exerts its antifungal effect through introducing oxidative stress such as increasing ROS production and depleting glutathione. The known targets of allyl alcohol are cytosolic alcohol dehydrogenases Adh1 and Adh2 and the mitochondrial Adh3 [66]. Although allyl alcohol could be released after ingestion of garlic, its toxicity, mediated by acrolein the production of which is catalyzed by alcohol dehydrogenase in rodents, prevents its development as antifungal agent [66, 67].

Baicalin could inhibit the activities of enzymes in mitochondria (such as Ca^{2+}-Mg^{2+}-ATPase, succinate dehydrogenase, and cytochrome oxidase) and induce cell cycle blockage and apoptosis in *C. albicans* cells [68]. However, in mammalian cells (e.g., CHO cell), baicalin could reduce ROS production [69]. There are also reports showing that baicalin induces apoptosis in human non-small lung cancer cells and osteosarcoma cells through ROS production [70, 71]. Baicalin could protect mitochondria from damage caused by streptozotocin and hepatic ischemia/reperfusion and increase the activity of citrate synthase in rats [72, 73]. Despite the discrepancy between the roles of baicalin in different cells in ROS production, the *in vivo* tests might support the use of baicalin as an antifungal candidate [69, 71, 73].

Shikonin, the major active compound isolated from *Lithospermum erythrorhizon*, could induce the endogenous ROS production, reduce the mitochondrial membrane potential, and alter mitochondrial aerobic aspiration [74]. In human gastric cancer cells and TT medullary thyroid carcinoma cells, shikonin could also induce ROS production and mitochondria-mediated apoptosis [75, 76]. The almost same cytotoxicity for fungal cells and mammalian cells makes shikonin a less attractive candidate for antifungal development.

Curcumin, the yellow pigment isolated from the turmeric (the rhizome of the plant *Curcuma longa Linn*) could also be used as an adjunct drug to treat pathogenic microorganisms such as *Helicobacter pylori*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Trypanosoma cruzi*. Antifungal activities against various kinds of fungi such as *Candida* species, *Cryptococcus neoformans*, *Aspergillus* spp., and *Sporothrix schenckii* have also been demonstrated by this compound [77, 78]. Curcumin could increase ROS production and apoptosis in *C. albicans* cells, either alone or in synergy with antifungal drugs such as azoles and polyenes [78, 79]. In mammalian cells, curcumin could protect mitochondria from damage and increase the biogenesis of mitochondria, although apoptosis-inducing effects of curcumin have also been reported in cancer cells [80, 81]. Most importantly, this compound could be safe with a maximum tolerance dose of 12,000 mg/day in Phase I clinical trials [82], which present an advantage over other antifungal compounds. However, the poor oral bioavailability and poor solubility in aqueous solutions impede its use and promote the development of methods for delivering curcumin to fight *Candida* infections [83].

Silibinin, the most famous and active compound isolated from *Silybum marianum* (milk thistle) traditionally used to protect from liver injury, could induce apoptosis related to mitochondrial Ca^{2+} influx in *C. albicans* cells [84, 85]. Silibinin could alleviate mitochondrial dysfunction in mice model of cisplatin-induced acute kidney injury through Sirt3 activation, although *in vitro* proapoptotic effects through inducing ROS production have been also reported [86, 87]. The safe profile with silibinin, evidenced by marketed health food and clinical trials, makes silibinin a very promising candidate for antifungal therapies against *Candida* infections although there is still a long way to go [88].

5. Virulence Factors

Virulence factors contributing to the *Candida* infections hold adhesins, virulence enzymes (secreted aspartyl proteinase and phospholipases functioning in host tissue invasion) [89], and morphological transition [90].

5.1. Yeast-to-Hypha Transition. Although both budding yeast type and hyphal type of *C. albicans* have been found at the loci of infections, the transition of yeast-to-hypha in *C. albicans* is considered to be a major factor involved in the colonization, invasion/penetration, virulence, immune evasion, and survival in the host tissues [91–93]. For instance, most of the hyphal growth of *C. albicans* could not be

suppressed by the macrophages after engulfment *in vitro* and lysis of macrophage caused by penetration of hypha was also observed [94]. What is worth mentioning is that, soon after phagocytosis by macrophages, the hyphal formation of *C. albicans* is required (but not sufficient) for inducing the proinflammatory pyroptosis, a kind of programmed cell death of macrophages mediated by inflammasome, before other macrophages are killed by the robust hyphal formation of *C. albicans* [95–97]. Although recently identified Candidalysin secreted by *C. albicans* hyphae plays a vital role in the mucosal pathogenesis through its cytolytic effects, no reports about the relationship between it and macrophages' damage have been published [98]. Nonetheless, Candidalysin renders a promising antifungal target for *C. albicans*. Moreover, hyphae could also support the complicated characteristic structures of mature biofilms which will be discussed later [99]. Induced by exogenous stressors (such as presence of serum and alterations in temperature, pH, levels of oxygen and glucose [91], the presence of *N*-acetyl-*D*-glucosamine (GlcNAc) [100], adherence, and starvation/nutrient limitation [92]), filamentation of *C. albicans* involves underlying alterations in protein synthesis and metabolic changes which are mainly the RAS1-Cyr1p-cAMP-PKA-EFG1 pathway and mitogen-activated protein kinase (MAPK) signaling [91, 92]. Both signaling pathways are governed by the membrane-integrated small GTPase Ras1 [101].

Magnolol and honokiol, two kinds of neolignan isolated from the root, stem, and branch bark of *Magnolia officinalis*, could inhibit the yeast-to-hypha transition of *C. albicans* under many culture conditions. Treatment of magnolol or honokiol could induce downregulation of components of the Ras1-cAMP-Efg1 pathway (such as RAS1, EFG1, TEC1, and CDC35 (the orthologue of Cyr1)), as well as reduced expression levels of the hypha-specific genes ECE1, HWP1, and ALS3, while exogenous cAMP could restore the filamentous growth in the presence of the drugs. These suggest that the transition-inhibiting effects of these two compounds may be associated with the suppression of Ras1-cAMP-EFG1 pathway [102]. Curcumin could also inhibit the yeast-to-hypha transition through targeting the transcriptional suppressor TUP1 (thymidine uptake 1) [79]. Licochalcone-A, a bioactive polyphenol from roots of licorice that has been used as a herbal remedy for hundreds of years, could inhibit the morphological transition [103]. The compound glabridin from licorice and the anthraquinone purpurin from madder root (*Rubia tinctorum* L.) could also inhibit the transition [104, 105].

Apart from the regulating role in antifungal resistance in planktonic *C. albicans*, the chaperone Hsp90 can also modulate the transition by inhibiting the filamentation via cAMP-PKA signaling [106]. Hsp90 deletion in *C. albicans* leads to virulent attenuation in a systemic candidiasis model [107]. So comes the hypothesis that inhibitors of Hsp90 may exhibit anti-*Candida* effects.

5.2. Biofilm Formation. Most infections caused by *Candida* spp. involve biofilms formed on the surfaces of biomaterials (such as intravascular catheters and prosthetic heart valves) and biotic mucosa (such as oral cavity and wound surface) [108]. Biofilm, buried in the extracellular matrix (ECM), holds a complicated three-dimensional architecture consisting mainly of yeast form cells and hyphal cells with broad heterogeneity in space [15, 109]. The spatial, structural, and metabolic heterogeneity of biofilms is considered to promote influx of nutrients, efflux of waste products, and establishment of microniches, thus facilitating the adaption of biofilms to the hypoxic environment [99, 110]. Beginning with the adherence of fungal cells to the substrate surface, the development of biofilm undergoes proliferation, maturation, and finally dissemination to finish a cycle and the cycle could repeat itself to expand the fungal population [15]. Cells in the biofilm exhibit great advantages over their free-living parallels in surviving such as increased resistance to many antimycotic drugs (e.g., *C. albicans* cells of biofilm are almost 1000 times resistant to fluconazole than free-living cells [111]) and protection offered by ECM [15]. The elevated resistance to antimycotic drugs and the ability to withstand host immune defenses, as well as the role as a reservoir for continuing infections, of *Candida* biofilms cause important clinical consequence and the presence of biofilms increases the morbidity and mortality of *C. albicans* relative to strains that could not form biofilms [99, 112]. Therefore, biofilm formation is considered as a potent virulence factor [34]. Now the *Candida* biofilms attract more and more attention, which could be reflected by the increasing number of publications on *Candida* biofilms.

Heat shock proteins play key roles in protecting cells from damage and repairing damage caused by insults, as well as in the protein synthesis, folding, transport and membrane translocation, and so on [9]. Compromising the function of Hsp90 in *C. albicans* by genetic manipulation or pharmacological means could reduce the dispersal and maturation of biofilms as well as increase the sensitivity to drugs used to abolish biofilms [106]. So inhibitors of Hsp90 may present a useful paradigm for therapy of infections caused by biofilm.

In *Candida* cells of biofilms, increased expression of many genes has been found such as genes involved in protein synthesis, drug transporting, adherence to matrix, and primary metabolism [109]. Genes encoding envelope proteins such as Hwp1, Als1, Als3, and Sun41 play critical roles in biofilm formation [92].

Although the structures of *C. albicans* biofilm can be disrupted by physical means such as mechanical removal by brushing on the surface of teeth and ultrasound (or sonication) treatment of implants [113], the clearance of *C. albicans* is primarily dependent on drugs which could prevent the formation of biofilm or abolish the matured biofilm.

Biofilm formation of *Candida* spp. and other fungi could be replicated in 96-well microtiter plates [36] as well as in animal paradigms [15], which provide us with useful tools to screen potential antifungal hits. Derived from *Cinnamomum zeylanicum*, cinnamon oil exhibits antifungal activity against *C. orthopsilosis* and *C. parapsilosis* through inhibiting the formation of biofilm as well as the growth of planktonic counterparts [114], although the exact mechanism is unknown. One of the major components cinnamaldehyde (of the oil) could also inhibit the biofilm formation of clinical isolates of *C. albicans* [115], and moreover it could suppress the

growth of *Aspergillus flavus* and *Aspergillus oryzae* which are culprits of food spoilage [116]. Berberine, an alkaloid from the medicinal plants such as *Coptis chinensis* and *Hydrastis canadensis,* also has antifungal activities against *C. albicans* biofilms, both alone and in synergy with miconazole [117]. Licochalcone-A also demonstrated *in vitro* and *in vivo* antifungal activity against *C. albicans* biofilms [103]. Purpurin also demonstrated antifungal activity against the formation and preformed biofilms of *C. albicans*, in addition to its capability of inhibiting morphological transition [104].

Aside from inhibiting the yeast-to-hypha transition, magnolol and honokiol also inhibit biofilm formation via suppressing adhesion and growth of *C. albicans* as is evidenced by XTT assay and confocal laser scanning microscopy. These two compounds could reduce the fungal burden and prolong the lifespan of *Caenorhabditis elegans* in a nematodes infection model [102]. What is more important, compounds at the concentrations used exhibit no adverse effect on the mammalian HSC-T6 cells and nematodes [102]. Curcumin could also inhibit the biofilm formation of *C. albicans* [118]. Thymol (5-methyl-2-(1-methylethyl) phenol), a major essential oil in the herb thyme (*Thymus vulgaris* L., Lamiaceae) which could be applied for treating multiple symptoms including bronchitis, whooping cough, and catarrh of the upper respiratory tract [119, 120], exhibits antifungal activity against fluconazole-sensitive and fluconazole-resistant isolates of *C. albicans* [121]. Recent study identified that thymol could inhibit the biofilm formation and development, and moreover this compound could enhance the host antimicrobial responses against *C. albicans* and increase the lifespan of *C. elegans* during the fungal infection [115, 122]. In addition, thymol has shown synergy with fluconazole against biofilms [115, 123]. Baicalein and aucubin from *Plantago major* (greater Plantain), a perennial herb used for wound healing, analgesic, anti-inflammatory, antioxidant, and infections, could inhibit the biofilm formation and decrease the cell surface hydrophobicity of *C. albicans* [124]. Eugenol, the major components of essential oils from *Syzygium aromaticum* (clove), possesses the capacity to inhibit the biofilm formation and preformed biofilms, more effective than marketed antifungal drug fluconazole. This compound could also produce synergistic effects with fluconazole [115, 123] and what is more, the structure-activity relationship of this compound is analyzed [125]. Another phenylpropanoid from clove, methyleugenol, also exhibits antifungal effect against fluconazole-resistant *Candida* isolates and synergistic effect with fluconazole [126]. Antibiofilm activity of menthol from mint, either alone or in combination with fluconazole, was also identified [123, 127]. So is the case with geraniol (3,7-dimethylocta-*trans*-2,6-dien-1-ol) [115], an acyclic monoterpene alcohol which could be isolated from many herbs such as *Pelargonium graveolens* (Geraniaceae), nutmeg, and ginger [128, 129]. Another compound from *Pelargonium graveolens,* linalool, also exhibits antifungal effect on the planktonic and biofilm cells of *C. tropicalis* [129]. Carvacrol could also sensitize the *Candida* biofilms, as well as the planktonic cells, to fluconazole [130]. Usnic acid (2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H, 9bH)-dibenzo-furandione), the major active component isolated from medicinal lichens such as *Cladonia* and *Usnea* [131], could inhibit the formation of *Candida* biofilms and other virulent traits [132, 133]. Berberine, from *Berberis aquifolium, Hydrastis canadensis, Phellodendron amurense*, has the antifungal activities against fluconazole-resistant *Candida* spp. in planktonic and biofilm form [134]. Emodin from rhizomes of *Rheum palmatum* could inhibit the formation of biofilms and hyphal development of *C. albicans* [135].

5.3. Other Factors. Virulence in mice caused by *C. albicans* mutants deficient in isocitrate lyase 1 (ICL1, a major component of the glyoxylate cycle) is evidently less than the wide type equivalents, which indicates the involvement of the glyoxylate cycle in the pathogenesis of candidiasis [136]. ICL1, as well as malate synthase, is the distinctive enzyme that has not been observed in mammalian cells; thus it may render a unique target for inhibiting the virulence of *C. albicans* to combat this fungal pathogen. Recently, inhibitors of malate synthase demonstrated antifungal effect against *Paracoccidioides species* [137], while apigenin, the active flavone compound in Chinese herbs such as thyme, could inhibit the enzymatic activity of ICL1 in *C. albicans* [138, 139]. Rosmarinic acid, the bioactive polyphenol in herbs such as basil *(Ocimum basilicum)*, oregano *(Origanum vulgare)*, sage *(Salvia officinalis),* and *Melissa officinalis*, has also been identified as an inhibitor of ICL1 in *C. albicans* [138, 140]. Recent study by Ansari et al. demonstrated that both the enzymatic activity and mRNA expression of ICL1 and malate synthase of *C. albicans* could be inhibited by the monoterpenoid perillyl alcohol, the active compound from edible and medicinal plant *Perilla frutescens* L. ex B. D. Jacks. (Lamiaceae) which has been used for treating colds, food allergy, and depression [141, 142]. Considering the absence of ICL1 in human, the well-tolerated profile in human, and the fact that Phase II trials have been conducted in patients with cancers, perillyl alcohol might serve as an interesting candidate for antifungal therapies against *C. albicans* [143, 144].

Similar to *Pseudomonas aeruginosa*, communication among fungal cells is often associated with virulence [145]. Quorum sensing means that molecules secreted by the *C. albicans* cells in response to cell density could affect the behaviors of the cells. The formation of biofilms, hyphal growth, and virulence factors of *C. albicans* could also be regulated by quorum sensing [146]. The most famous quorum sensing molecule of *C. albicans* is farnesol, one autoregulatory sesquiterpene alcohol that could prevent the filamentation (through repressing the Ras1-cAMP-PKA signaling pathway [147]), shrink the biofilm (if added before attachment or after formation but not during the initial stages of biofilm growth [148]), and block other virulence factors [149]. So comes the strategy that targeting quorum sensing molecules may contribute to the antifungal therapies [146]. Indeed, the dietary flavonoid quercetin isolated from edible and medicinal lichen *Usnea longissimi* could sensitize fluconazole-resistant isolate NBC099 to fluconazole and this kind of sensitization could be the quercetin-induced production of farnesol [146].

Another quorum sensing molecule produced by *C. albicans*, tyrosol, could also affect the development of *Candida*

biofilms [150]. This aromatic alcohol could induce the morphological transition from yeast to hyphae. At high concentrations (above 200 mM), tyrosol could cause reduction in biofilms formed by *Candida* species as well as those by *Streptococcus mutans* [151].

Extracellular hydrolytic enzymes produced by *C. albicans* are considered as virulence factors liable for the penetration into and damage to host cells caused by this pathogenic fungus [152]. These enzymes include secreted aspartic proteinases, lipases, and hemolysins [34]. Quercetin could inhibit the activities of proteinase, esterase, phospholipase, and hemolysins of fluconazole-resistant *C. albicans* strain NBC099 [146]. In addition, this compound could also synergize with fluconazole against biofilm both *in vivo* and *in vitro* [153].

6. Compounds without Identified Mechanism

Anofinic acid and fomannoxin acid isolated from *Gentiana Algida* showed weak antifungal activities against *C. albicans*, while the esterification by introducing methyl group into those compounds could enhance the anti-*Candida* activities but decrease the activities against the *Cladosporium cucumerinum*, which is a kind of plant pathogenic fungus [154]. However, no further research about the antifungal mechanism has been performed since that finding. Anofinic acid could also be isolated from another traditional Chinese medicine, *Gentiana macrophylla*, which has been used for long as therapies for constipation, pains, jaundice, and rheumatism [155]. Another dihydroflavone isolated from *Gentiana macrophylla*, kurarinone, could also inhibit the growth of *C. albicans* [155]. Nyasol ((Z)-1,3-bis(4-hydroxyphenyl)-1,4-pentadiene), isolated from the herbal plant *Anemarrhena asphodeloides Bunge* (Liliaceae) which has been used in Chinese traditional medicine as antipyretic, anti-inflammatory, antidiabetic, and antidepressant agent [156], exhibits antifungal activity against *C. albicans* alone or in synergy with azoles [157, 158]. This compound also has activity against other fungal pathogens such as *A. flavus*, *Fusarium oxysporum*, *Pythium ultimum,* and *Rhizoctonia solani*, to name a few [158, 159]. Another compound from clove, isoeugenol, also exhibited antifungal activities against *C. albicans*, as well as *Aspergillus niger* [125]. α-Terpineol (2-(4-methyl-1-cyclohex-3-enyl) propan-2-ol), from *Artemisia annua*, could inhibit a series of *Candida* species isolated from denture stomatitis patients [160]. The sesquiterpene lactone isolated from *Inula racemosa* showed good antifungal activity against *Candida* species, as well as other human fungal pathogens such as *A. flavus* and *Geotrichum candidum* [161].

7. Conclusion

In summary, many natural compounds from TCM could exert their anti-*Candida* activities through different mechanism, providing a big reservoir for developing antifungal therapies.

Combination therapies are capable of increasing the efficacy and preventing the emergence of drug resistance and many approaches have been adopted to identify effective combinations, especially the synergistic effects with marketed drugs [162–164]. An important part of the adjuvants to antibiotics might come from the previously undervalued part of chemical entities, which have been recently termed as dark chemical matter (DCM) [165]. Because these DCM showed little or no bioactivity in previous researches towards human targets, it may represent a novel and valuable repertoire for identifying hits and optimizing leads [165]. The machine learning-based synergism prediction may be a promising method to identify synergistic effects of marketed antifungal drugs and natural products isolated from traditional Chinses medicine [162]. Considering new proteins or biological processes that might be used as emerging targets such as histone deacetylase and ion homeostasis, compounds from TCM might play increasing important and diverse roles in developing antifungal therapies against *C. albicans*.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Research Article

Impact of Chestnut and Quebracho Tannins on Rumen Microbiota of Bovines

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The use of phytogenic dietary additives is being evaluated as a means to improve animal productivity. The effect of tannins seems to be the influence not only directly on the digestive process through binding of dietary proteins but also indirectly over their effects on gastrointestinal microbiota. High-throughput sequencing of 16S rRNA gene was used to analyze the impact of dietary supplementation with a blend of chestnut and quebracho tannins on the rumen microbiota of Holstein steers. Bacterial richness was lower in tannins treated animals, while the overall population structure of rumen microbiota was not significantly disturbed by tannins. The ratio of the phyla Firmicutes and Bacteroidetes, a parameter associated with energy harvesting function, was increased in tannins supplemented animals, essentially due to the selective growth of Ruminococcaceae over members of genus *Prevotella*. Fibrolytic, amylolytic, and ureolytic bacterial communities in the rumen were altered by tannins, while methanogenic archaea were reduced. Furthermore, ruminal pH was significantly higher in animals supplemented with tannins than in the control group, while urease activity exhibited the opposite pattern. Further work is necessary to assess the relation between tannins impact on rumen microbiota and alteration of rumen fermentation parameters associated with bovine performance.

1. Introduction

The use of bioactive phytochemicals as natural feed additives has been extensively studied as a strategy to manipulate rumen fermentation in pursuit of an improvement in cattle productivity by reducing methanogenesis and increasing the efficiency of nitrogen utilization [1–3]. However, the lack of sufficient understanding about the rumen microbiome is considered one of the major knowledge gaps that hinder effective enhancement of the rumen function.

Tannins are a complex group of polyphenolic compounds found in many plants species which are commonly included in ruminant diets such as forage and sorghum [4]. Tannins are classified as hydrolysable or condensed based on their chemical structure. The molecular structure of hydrolysable tannins includes a core of glucose esterified with gallic and hexahydroxydiphenic acids. Condensed tannins are usually referred to as proanthocyanidins, polymers of flavon-3-ols or flavon-3, 4-diols such as catechin and epicatechin [5]. Tannins can complex with proteins, starch, vitamins, and minerals at moderate pH, as in ruminal conditions, and dissociate at lower pH, including abomasum and the initial portion of the duodenum [6]. The increased digestibility and efficiency of feed utilization induced by addition of tannins

to ruminant diets have been attributed to their ability to precipitate proteins, allowing bypass of ruminal digestion and enhancing protein availability at small intestinal level. However, ruminal bypass cannot entirely explain the performance improvement associated with tannins addition in feed.

For a long time, tannins were thought to reduce weight gain and efficiency of nutrient utilization. However, it is now known that their effect may be either beneficial or detrimental depending on tannins origin, molecular structure, dosage, and animal species [4, 7–9]. High doses of tannins reduce voluntary feed intake and nutrient digestibility, whereas moderate concentrations can improve feed utilization [4]. A blend of tannins extracted from quebracho and chestnut tree has been used as additives to improve performance of ruminants and to reduce urinary nitrogen excretion [10]. A recent report showed that addition of moderate concentrations of chestnut and quebracho tannins to the diets of dairy cows did not affect animal performance but increased milk protein yield and decreased urinary nitrogen excretion [11]. Other authors observed that inclusion of chestnut and quebracho tannins increased dry matter intake, average daily gain, and final body weight of steers during the finishing feedlot phase [12]. These two types of tannins differ in their nutritional role and toxic effects in livestock nutrition.

Tannins modify the digestive processes of ruminants not only by binding dietary protein but also through modulation of rumen microbiota [7, 13]. The bovine rumen houses a complex and highly dense microbiota that is responsible for cattle ability to convert indigestible plant mass into energy. In recent years, a link between gut microbiota composition and energy harvesting function has been observed in humans and mice [14, 15]. Rumen microbiota composition was found to be strongly correlated with daily milk-fat yield in bovines [16, 17]. The microbial populations of the rumen and the variations associated with diet have been previously described [18]. However, although much research has been done regarding the effects of tannins on ruminants physiology and their metabolic fate [19], the impact of chestnut and quebracho tannins on rumen microbiota of bovines has not been fully described. The hypothesis under study in the present work is that tannins alter the bacterial populations of the rumen and therefore can be used as a dietary strategy to modulate rumen function. The aim of this study was to analyze the *in vivo* effects of a tannins blend derived from chestnut and quebracho on rumen bacterial populations of Holstein cattle by means of massive 16S rRNA gene sequencing, exploring the relationship between rumen microbiome composition and physiological parameters.

2. Materials and Methods

2.1. Animal Handling, Dietary Treatments, and Determination of Rumen Parameters. The study was carried out using ruminally fistulated Holstein cows of the Holando–Argentino breed ($n = 6$) with an average body weight of 584 ± 12 kg (mean ± SD). In order to emulate productive conditions, diet was gradually changed from low starch (60% alfalfa bale, 40% concentrate composed of 80% ground corn grain and 20% soybean meal) to high starch (20% alfalfa bale, 80% concentrate) over an adaptation period of 14 days (from d 1 to d 14). Animals were kept on high starch diet for 21 days (from d 15 to d 35) before the beginning of dietary treatments in order to ensure complete adaptation of rumen microbiota. On day 36, animals were randomly divided into two groups of 3 steers and each group was assigned to one of the two dietary treatments: (1) control group without additives or (2) tannins-supplemented group in which a blend of chestnut and quebracho tannins was added to diet at a concentration of 2 g per kg of feed (Table 1). Control and tannins-supplemented diets were administered to the animals until the end of the treatment period (from d 36 to d 48). The twelve-day treatment period was repeated once, after a "washout" period of 21 days during which all animals received the control diet. Ruminal samples were taken from each animal at the end of each treatment period for microbiota analysis. Diet was offered ad libitum during the whole experiment as a total mixed ration once daily at 0800 h. The average dry matter intake was 12.8 ± 0.6 kg per animal per day (mean ± SD).

Table 1: Formulation and composition of diet as percentage of dry matter.

Ingredients	% of DM
Alfalfa bale	19.0
Ground corn grain	64.0
Soybean meal	16.0
Trace mineral and vitamins	0.8
Tannins blend	0.2
Total	100.0
Composition analysis	% of DM
CP	15.0
RDP	9.3
Total calcium	0.9
Total phosphorus	0.4
Energy analysis	Mcal/kg
ME	2.97
NEm	2.01
NEg	1.35

DM: dry matter; CP: crude protein; RDP: rumen degradable protein; ME: metabolizable energy; NEm: net energy for maintenance; NEg: net energy for gain.

The tannins blend was obtained from Silvateam (Indunor SA, Argentina) and contained one-third chestnut wood *(Castanea sativa)* tannins extract and two-thirds quebracho *(Schinopsis lorentzii)* tannins extract by weight. Quebracho extract is mainly composed of flavan-3-ols condensed tannins (>84%) while chestnut extract contains mainly digalloyl glucose hydrolysable tannins (>78%). A detailed description of quebracho and chestnut extracts chemical composition has been described elsewhere [11].

Ruminal contents were collected via a dorsal fistula from the ventral sac of the rumen, taking handfuls of material from the interface between the solid material and liquid layer. Samples were collected in sterile containers (200 mL including solid and liquid material), frozen in liquid nitrogen,

and stored at −80°C until further processing. Part of the sample was used for determination of ruminal physiological parameters. The ruminal liquor pH was measured using a standard pH meter. Urease activity (UA) was measured according to the Caskey–Knapp method modified by AACC. Nonprotein nitrogen (NPN) was determined through Kjeldahl method (VELP Scientifica, Italy). Animals were cared for by trained personnel only and the experimental protocol and procedures used were conducted according to protocol 27/2011 of the Institutional Committee for the Care and Use of Experimental Animals (CICVyA-INTA).

2.2. DNA Extraction. Twenty milliliters of evenly homogenized ruminal liquor was lyophilized before DNA extraction in order to maximize microbial density (1 g of dry material per sample). DNA extraction was conducted using the QIAamp DNA stool kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer with slight modifications. Briefly, 100 mg of lyophilized rumen was weighed and lysed by incubation for 5 min at 95°C. DNA elution was done with 100 μl of Buffer AE, after incubation for 10 min at room temperature. DNA quality was assessed by agarose gel electrophoresis and DNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was kept at −20°C until further processing.

2.3. High-Throughput Sequencing of 16S rRNA Gene. The 16S rRNA gene V3-V4 regions were amplified using Illumina primers (forward: 5′ CCTACGGGNGGCWGCAG 3′, reverse: 5′ GGACTACHVGGGTATCTAATCC 3′) with standard adapter sequences attached for barcoding and multiplexing. 16S gene libraries construction and high-throughput sequencing were performed at Macrogen Inc. (Seoul, South Korea) in the Illumina MiSeq platform following manufacturer's instructions [20]. In order to reduce unbalanced and biased base compositions, 15% of PhiX control library was spiked into the amplicon pool. Due to an issue with the length of reverse reads, which were not long enough to achieve merging of paired-end sequences, only forward reads covering V3 and its flanking regions were used for further bioinformatics analysis. The datasets generated in this study are available under request.

2.4. Bioinformatics Analysis. FASTQ files were trimmed using Trimmomatic v0.33 [21], which removed all primer and adapter sequences and also removed leading and trailing bases if quality value was below 9 and 3, respectively. Sliding window trimming was also performed, as well as cutting if the average quality within a 4-base window falls below *Q*15. Demultiplexing and quality filtering were done using the script *split_libraries_fastq.py*, which is part of the QIIME v1.9.1 software package [22]. A threshold of Phred quality score ($Q > 20$) of the base was chosen for stringent quality control processing. Chimeric sequences were filtered out in QIIME using the USERCH algorithm. Open-reference operational taxonomic units (OTUs) picking was performed using UCLUST and USEARCH algorithms in QIIME. Each sequence was assigned taxonomy against Greengenes reference OTU build version 13.8, using a 97% sequence similarity threshold. OTUs with abundance below 0.005% were removed from final OTU table, in order to avoid microbial diversity overestimation [23]. Normalization of OTU counts was done by performing multiple rarefactions from 10.000 to 210.000 sequences with steps of 10.000 and with 10 repetitions at each rarefaction depth. The resulting multiple rarefied OTU tables were used for all further analysis. Principal coordinate analysis (PCoA) plots were generated in QIIME with default options using a distance matrix calculated by unweighted UniFrac metric. The significance of grouping in the PCoA plot was tested by analysis of similarity (ANOSIM) in QIIME with 999 permutations. This work used computational resources from the Bioinformatics Unit, Instituto de Biotecnología (CICVyA-INTA).

2.5. Statistical Analysis. Relative abundances of bacterial populations were statistically analyzed using STAMP v2.1.3 [24]. The relative abundances of bacterial taxa in control and tannins treated groups were compared at each level of classification (phylum, class, order, family, and genus) using White's nonparametric two-tailed t-test with 1.000 permutations. Comparisons in physiological data and diversity estimators were calculated using nonparametric two-tailed Mann–Whitney test (GraphPad Software, San Diego, CA, USA) and were considered statistically significant if $p < 0.05$.

3. Results

After high-throughput sequencing, 3,812,179 reads were obtained with an average of 346,562 ± 42,326 reads per sample. Stringent filtration of the sequences based on length and quality was performed before taxonomy assignation, resulting in 2,951,356 reads with an average length of 267 ± 22 base pairs.

The total number of OTUs detected after filtration was 2.263, but this number exhibited a high interindividual variation (Figure 1). We found that the number of OTUs tended to be lower in tannins-supplemented animals than in the control group ($p = 0.05$) (Figure 2(a)). Shannon's diversity index, which estimates the internal sample complexity, was not significantly affected by tannins ($p = 0.14$) (Figure 2(b)). PCoA based on unweighted UniFrac metric was performed in order to explore dissimilarities in microbial composition of the rumen among treated groups (Figure 3). ANOSIM detected no significant differences in bacterial diversity between control and tannins dietary treatments ($p = 0.86$).

Firmicutes and Bacteroidetes were the dominant bacterial phyla in the bovine ruminal fluid, accounting for nearly 90% of total microbiota. However, large interindividual variance was observed in the relative abundance of Bacteroidetes and Firmicutes among different animals. In the control group, bacterial populations belonging to phylum Bacteroidetes were the most abundant in all animals (52.1% on average) while Firmicutes accounted for 37.6% of total microbiota. However, this predominance was inverted in the tannins treated animals, with a significantly higher percentage of Firmicutes (46.2%, $p = 0.02$) and a reduction to 44.6% in

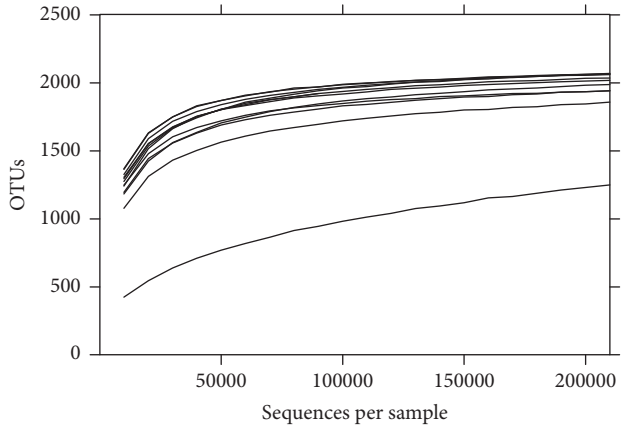

Figure 1: OTUs rarefaction curves of rumen microbiota based on 16S rRNA gene sequences. OTUs were picked using the UCLUST method with 3% dissimilarity in QIIME. Each curve corresponds to a single ruminal sample.

Bacteroidetes ($p = 0.18$). Accordingly, steers supplemented with tannins presented a trend to higher Firmicutes to Bacteroidetes ratio in comparison with the control group (1.08 versus 0.73, $p = 0.09$) (Figure 4).

Significant differences in the abundance of certain taxa were detected between control and tannins treated animals (Figure 5). Among Bacteroidetes, the most abundant genus was *Prevotella* accounting for more than 40% of this phylum. The average abundance of *Prevotella* was lower in tannins-supplemented animals than in the control group, although it exhibited a high degree of variance among animals (16.5 versus 21.9%, $p = 0.15$). *Clostridia* was the predominant class which accounted for more than 90% of total Firmicutes, and it was significantly enhanced in tannins treated animals (41.5 versus 34.6, $p < 0.001$). Among *Clostridia*, Ruminococcaceae was the most abundant family and showed a significantly higher abundance in the tannins-supplemented animals (17.8% versus 10.7%, $p = 0.009$). In the control group, most sequences corresponding to family Ruminococcaceae belonged to unclassified members (7.9%) and genus *Ruminococcus* (2.6%). Both taxa were enhanced in tannins treated steers, reaching abundances of 12.6% ($p = 0.01$) and 4.9% ($p = 0.07$), respectively. Other nonclostridial bacteria within the phylum Firmicutes were significantly altered by tannins, including members of class Erysipelotrichi. Some Erysipelotrichi were enhanced (genus *L7A-E11*, $p = 0.02$; and genus *p-75-A5*, $p = 0.06$) while others were lowered (genus *RFN20*, $p = 0.001$) in tannins-supplemented animals. Members of class Bacilli (genera *Streptococcus* and *Lactobacillus*) showed only moderate increases in their abundance. Meanwhile, genus *Fibrobacter* was significantly affected by tannins, accounting for 0.10% of total microbiota in the control group and only 0.005% in the tannins treated animals ($p = 0.01$). Other minor fibrolytic bacteria were significantly more abundant in tannins treated steers, including genus *Blautia* (0.08 versus 0.02%, $p = 0.01$) and member of family Eubacteriaceae genus *Anaerofustis* (0.06 versus 0.02%, $p = 0.03$).

Among sugar fermenting bacteria, the most abundant taxon was genus *Prevotella*, whose abundance was reduced by 5.4% in tannins treated animals, as mentioned above. Genus *Treponema* was also reduced in tannins treated steers (0.41 versus 1.21%, $p = 0.04$). Among Veillonellaceae members, genus *Succiniclasticum*, which specializes in fermenting succinate to propionate, doubled their levels in tannins treated animals (3.99 versus 1.99%, $p = 0.08$). Lipolytic genus *Anaerovibrio* was significantly enhanced by tannins (0.11 versus 0.05%, $p = 0.01$). Genus *Selenomonas* was also increased in tannins supplemented animals (0.11 versus 0.05%, $p = 0.07$). Among ureolytic bacteria, genus *Butyrivibrio* was the most abundant one and it was negatively affected by tannins treatment (1.80 versus 2.36%), as well as *Treponema* and *Succinivibrio* (0.009 versus 0.02%). On the other hand, methanogens belonging to phylum Euryarchaeota were less abundant in tannins supplemented steers (1.37 versus 2.03%) and their levels were inversely correlated with rumen pH ($r = -0.80$). Genus *Methanosphaera* was significantly reduced by tannins (0.06% versus 0.16%, $p = 0.01$).

Determination of pH, urease activity, and NPN was performed in all rumen samples along with microbiota composition analysis. Tannins treated steers had significantly higher ruminal pH than the control group (6.30 versus 5.88, $p = 0.02$) (Figure 6(a)). Urease activity exhibited the opposite pattern, showing a significant decline in the tannins treated steers (Figure 6(b)). Moreover, a strong negative correlation between pH and urease activity was detected ($r = -0.95$). NPN was not significantly altered by treatments (Figure 6(c)).

4. Discussion

Rumen microbiome diversity is a key feature of ruminants that confers cattle the ability to adapt to a wide range of dietary conditions [25]. In recent years, the concept of host microbiome individuality in ruminants is gaining support, since numerous studies found a large number of taxa whose presence or abundance in the rumen varies markedly among individuals [26–28]. Dietary tannins diminished ruminal richness but did not significantly affect the bacterial communities' complexity (i.e., balance between the relative abundances of taxa). A recent report found an increase in rumen richness but no change in Shannon's diversity index after supplementation with a blend of polyphenols essential oil in dairy heifers under a high grain diet, supporting the idea that polyphenols can affect bacterial richness without disrupting the overall rumen microbiota population structure [29]. In line with this, β-diversity analysis detected no significant differences in rumen bacterial diversity between control and tannins treated steers. Low microbial richness in the rumen has been recently found to be tightly linked to a higher feed efficiency in dairy cows [30]. The authors suggest that lower richness in the rumen of efficient animals results in a simpler metabolic network which leads to higher concentrations of specific components that are used to support the host's energy requirements. Together, diversity analyses suggest that bacterial richness was decreased, while the overall bacterial complexity of the rumen was not significantly affected by chestnut and quebracho tannins supplementation.

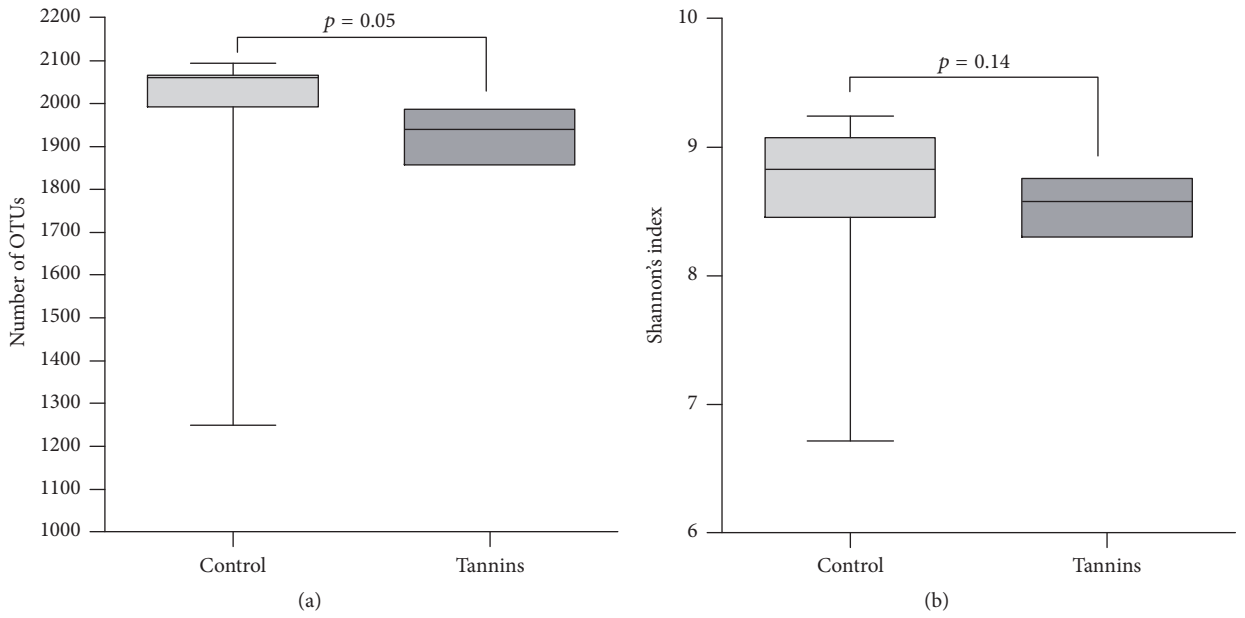

Figure 2: Effect of tannins treatment on (a) bacterial richness (number of OTUs) and (b) Shannon's diversity index of rumen microbiome. Line = median. Box = 25–75 percentiles. Bar = 5–95 percentiles.

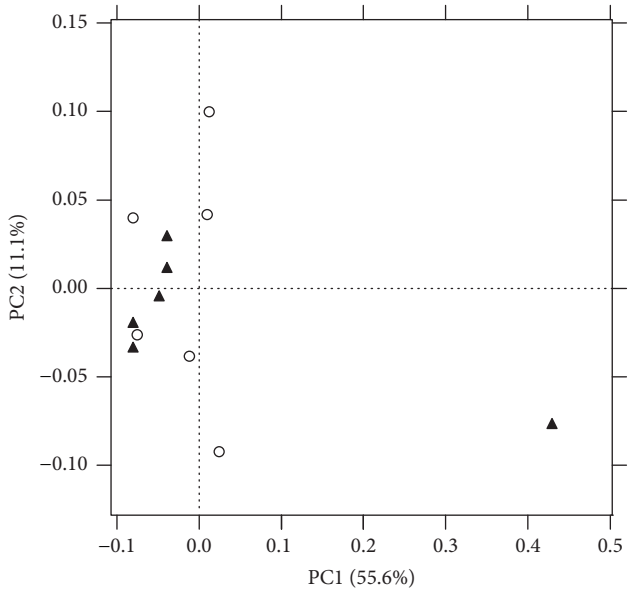

Figure 3: PCoA plot based on unweighted UniFrac metric. Items shaped with triangles and circles correspond to samples from control and tannins treated animals, respectively. Axes (PC1 = 55.6% and PC2 = 11.1%) account for 66.7% of the total variation detected.

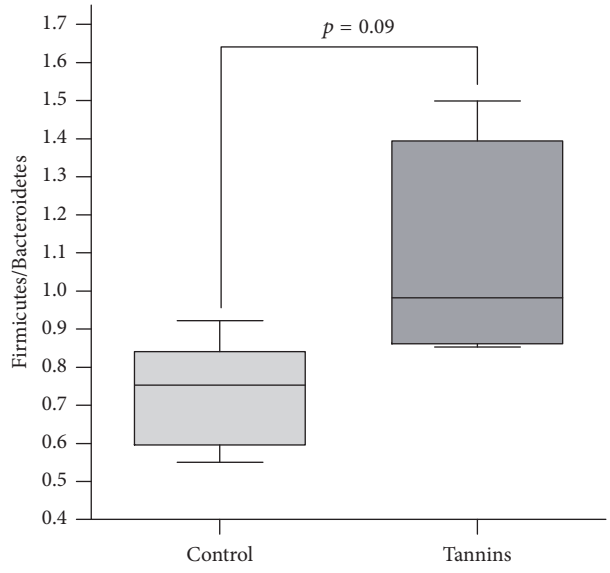

Figure 4: Effect of tannins on the ratio of phyla Firmicutes and Bacteroidetes in rumen microbiota. Line = median. Box = 25–75 percentiles. Bar = 5–95 percentiles.

The dominance of phyla Firmicutes and Bacteroidetes in the bovine ruminal fluid is a common feature in the gastrointestinal microbiome of monogastric organisms and ruminants [15, 17, 28, 31]. Henderson et al. also found that an increase in the ruminal abundance of total Firmicutes correlated with a decrease in the abundance of Bacteroidetes both in cows ($r = -0.80$) and in sheep ($r = -0.97$) [32]. Other authors found that Bacteroidetes were the most abundant phylum in ruminal samples obtained from dairy cows but some animals exhibited a higher percentage of Firmicutes compensating for a lower abundance of Bacteroidetes [17]. Our results agree with these observations, since a strong inverse correlation between the abundances of Firmicutes and Bacteroidetes was detected ($r = -0.99$). These results suggest that members of Firmicutes and Bacteroidetes compete for available resources in the rumen and tannins would tip the balance in favor of Firmicutes.

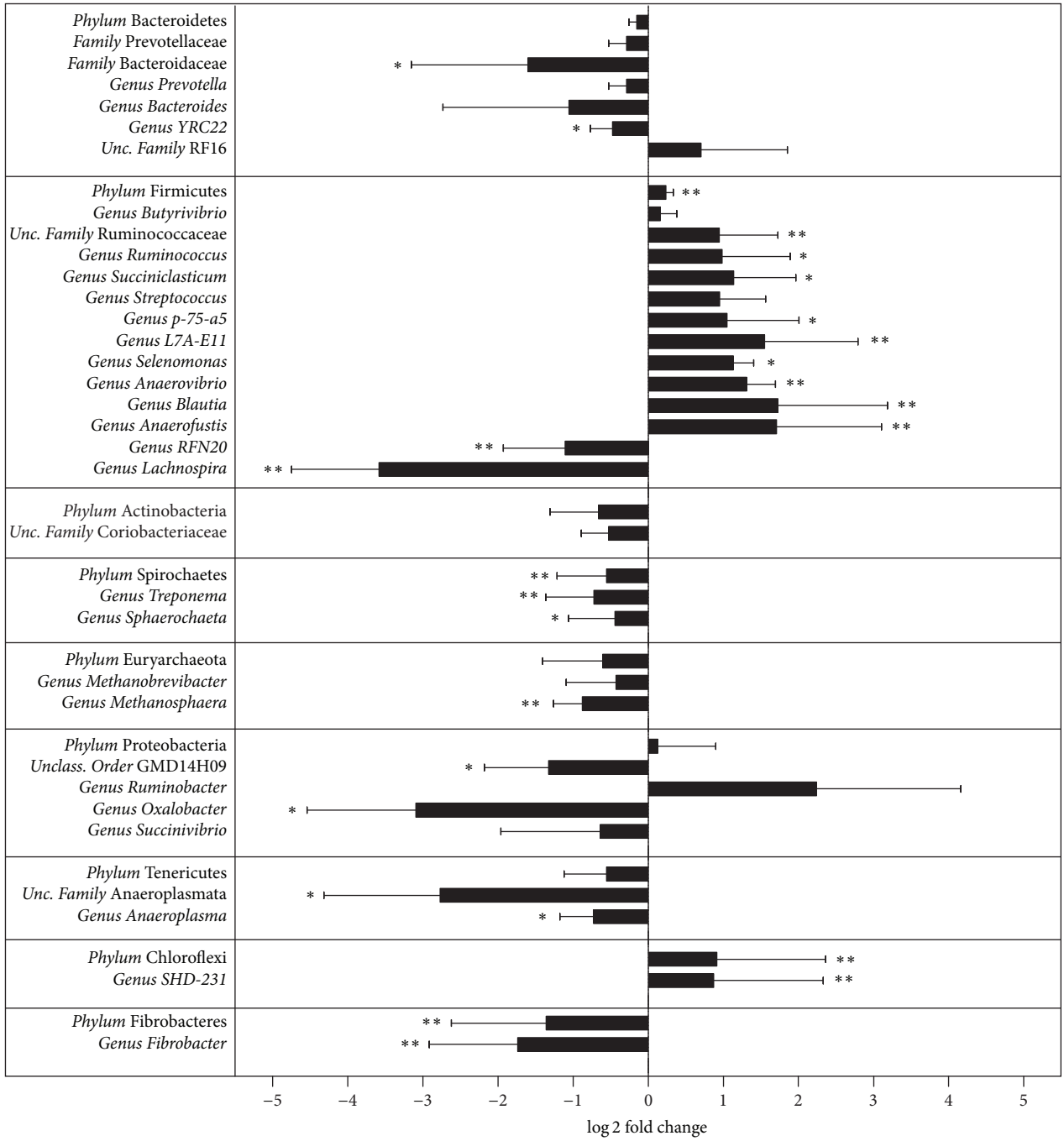

Figure 5: Relative fold changes (log 2 tannins/control) in the abundance of rumen bacterial taxa between control and tannins treated steers. $^{**}p < 0.05$. $^{*}p < 0.10$. Bar = SEM.

The ratio of Firmicutes to Bacteroidetes has been shown to affect energy harvesting and body fat accumulation in humans and mice [14, 15]. Along with increased fatty acid absorption, more energy was found to be efficiently obtained from diet in obese mice, illustrating the connection between Firmicutes and improved efficiency in energy harvesting [14]. In cows, the Firmicutes to Bacteroidetes ratio was found to be strongly correlated with daily milk-fat yield [17]. A recent study found that the abundance of Firmicutes in the rumen positively correlates with the average daily body weight gain in steers, suggesting that these bacteria play a significant role in feed efficiency of bovines [33]. Therefore, it is possible that the increase of Firmicutes to Bacteroidetes ratio induced by tannins can improve bovine performance, as previously suggested by other authors [19].

Fiber degradation is a complex process carried out by a group of microorganisms that are able to digest plant polysaccharides mainly through production of cellulolytic,

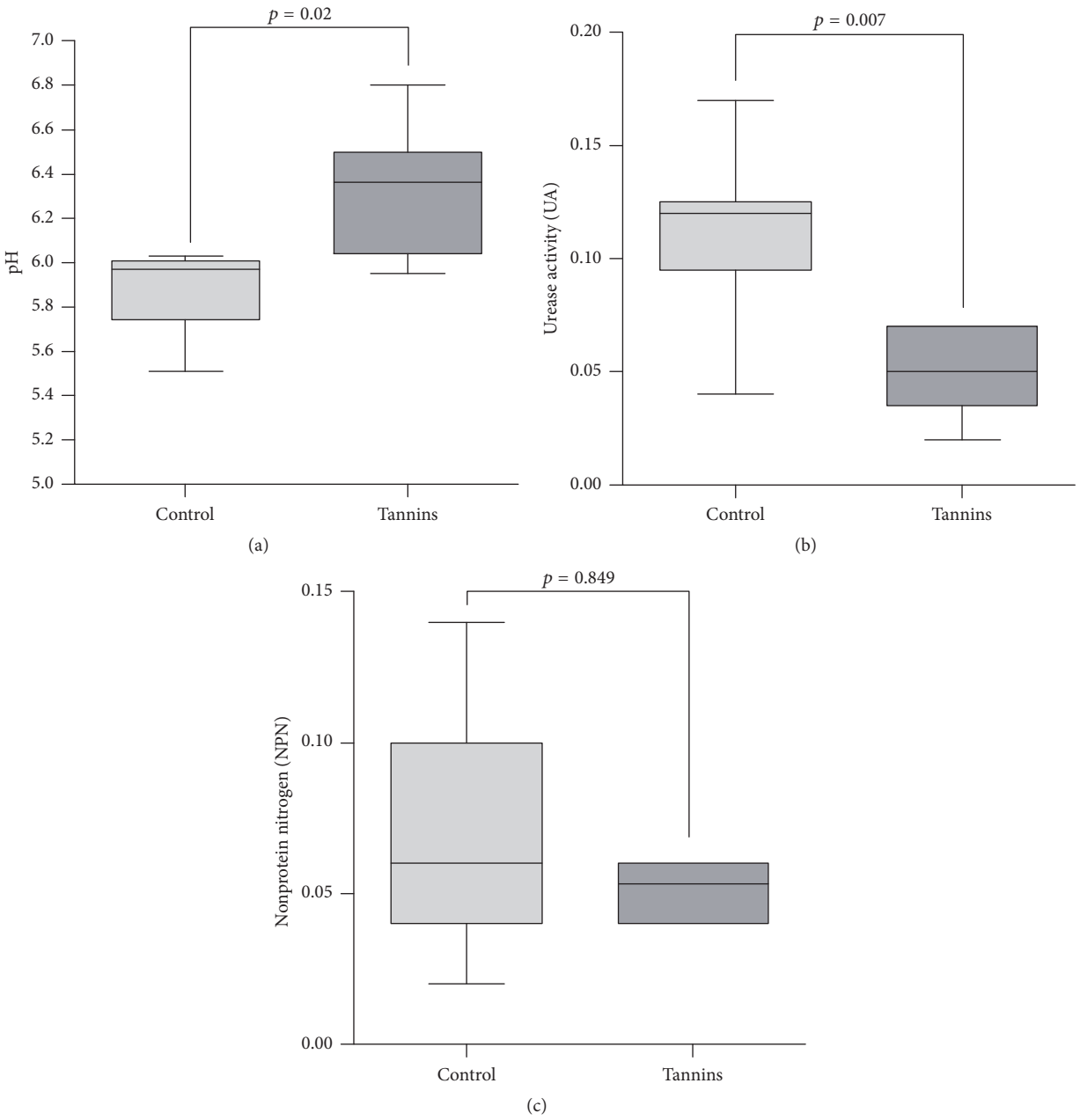

Figure 6: Effect of tannins on pH, urease activity, and NPN in the rumen liquor. Line = median. Box = 25–75 percentiles. Bar = 5–95 percentiles. Urease activity values are given in pH units proportional to urease activity. NPN levels are expressed as a percentage of soluble nitrogen in the rumen.

hemicellulolytic, and pectinolytic enzymes [34]. Cellulolytic activity in the rumen involves a diverse bacterial community whose main members belong to genera *Ruminococcus* and *Fibrobacter*, while the main hemicellulolytic bacteria belong to genera *Ruminococcus*, *Prevotella,* and *Butyrivibrio* [34–37]. The blend of tannins administrated increased Ruminococcaceae and other members of phylum Firmicutes while inhibited genera *Prevotella* and *Fibrobacter*. *Prevotella* species have a documented role in metabolism of starch, hemicellulose, pectin, and protein catabolism [25]. The high abundance of this genus might be the result of a large metabolic niche that is occupied by different species with similar metabolic capabilities or it might be associated with a high genetic variability that enables members of genus *Prevotella* to occupy different ecological niches within the rumen [38–40]. For instance, *Prevotella* abundance has been shown to be higher in the rumen of animals of beef cattle with low feed efficiency [41]. Previous studies reported a great diversity in the sensitivity of *Prevotella* species to tannins [42], which could explain the lower abundance detected for this genus in tannins-supplemented steers. Since genus *Prevotella* is characterized by a high genetic diversity and wide array of output metabolites [38–40], their replacement by *Ruminococcus* may lead to a simpler product profile specialized to

support the host's energy requirements [30]. Our results agree with previous studies that found a significant reduction in *Fibrobacter* and *Prevotella* rumen populations after supplementation with condensed and hydrolysable tannins, including chestnut and quebracho tannins [43, 44].

Rumen fibrolytic function is carried out by a largely redundant microbial community with overlapping distribution of metabolic capabilities, and this fibrolytic community has the ability to restore its structure and function after perturbation, a phenomenon known as *resilience* [27]. In this context, the observed changes in diversity of fibrolytic bacteria in tannins treated steers may result from a combination of physicochemical and biological mechanisms described in the literature, including direct interaction of tannins with fiber [45], which could alter the available surface area for microbial attack, the inhibition of certain fibrolytic taxa by means of tannins antimicrobial activity [46], and modulation of fibrolytic bacterial species driven by changes in rumen pH, since certain fibrolytic taxa of the rumen are inhibited at low pH under high-grain diets [36, 47].

Rumen amylolytic and saccharolytic bacteria were also affected by dietary treatment with tannins, mainly through the decrease of genera *Prevotella* and *Treponema*. Other amylolytic genera were moderately increased in tannins-fed steers including *Streptococcus*, *Bifidobacterium,* and *Lactobacillus*. Amylolytic activity is normally enhanced in ruminants consuming high-grain diets [18, 48, 49]. The rate of grain degradation by these microbial communities plays a key role in maintaining rumen homeostasis, since rapid starch fermentation produces large amounts of organic acids, therefore producing a drop in ruminal pH that may lead to metabolic acidosis [50]. A previous study showed that tannic acid and quebracho tannins lower the rate of microbial hydrolysis of starch-rich grains in the rumen by physical modification of the endosperm protein matrix [45]. This physical modulation of starch degradation could explain the lower abundance of sugar fermenting taxa detected in tannins treated steers, as well as the higher ruminal pH observed in this group.

Fermentation products of microbial activity in rumen, mainly short-chain volatile fatty acids, serve as a major source of energy for ruminants and have a direct impact on the physiological parameters of the animal and feed utilization efficiency [16]. Some members of family Veillonellaceae, which produce propionate as a major fermentation product and have been associated with lower methane emissions [51], were enhanced in tannins treated animals. Interestingly, some *Selenomonas* species can break tannin-protein complexes and use tannins as energy source [52, 53]. Therefore, the higher abundance of *Selenomonas* in tannins treated steers may be partly due to availability of tannins as direct energy source. Meanwhile, members of class Erysipelotrichi, which have been linked to beef cattle feed efficiency [33, 51, 54], were also modulated by tannins treatment.

Methane production during fermentation of feeds in the rumen represents a loss of 2–12% of gross energy [1], and it is performed by a group of archaea known collectively as methanogens which belong to phylum Euryarchaeota. The microorganisms produce methane, the second largest anthropogenic greenhouse gas which has a global warming potential 25 times that of carbon dioxide [55]. In the present study, a reduction of methanogenic archaea in tannins supplemented steers was detected as well as an inverse correlation between the abundances of phylum Euryarchaeota and rumen pH ($r = -0.95$). Tannins are thought to directly inhibit methanogens, as well as indirectly limit methanogenesis through reduction of hydrogen availability [1, 55]. Saminathan et al. found a significant decrease in genus *Methanobrevibacter* after treatment of rumen samples with condensed tannins *in vitro* [56]. Other authors described a linear decrease of genus *Methanobrevibacter* after dietary supplementation with condensed tannins from pine bark in goats [31].

Ruminal pH was significantly higher in tannins treated steers than in the control group, while urease activity exhibited the opposite pattern. These results agree with previous reports which found an increase in ruminal pH after supplementation with chestnut and quebracho tannins [57, 58]. Other authors observed that feeding chestnut and quebracho tannins decreased urease activity in the faeces of cows [10, 11].

Feed-grade urea is an effective source of nitrogen commonly used in beef cattle diets. Ureolytic bacteria in the rumen produce urease to hydrolyse urea to ammonia, which is subsequently used for the synthesis of amino acids and microbial protein. Normally, the rate of urea hydrolysis exceeds the rate of ammonia utilization, which leads to poor efficiency of urea utilization and increases toxic ammonia concentrations in blood [59]. Ureolytic bacteria in the rumen comprise a highly diverse group whose main species belong to genera *Succinivibrio*, *Treponema*, *Bacteroides*, *Butyrivibrio*, *Streptococcus,* and *Bifidobacterium* [60, 61]. We found that the most abundant ureolytic genera, *Butyrivibrio* and *Treponema*, were negatively affected by tannins treatment. Thus, the observed decline in ruminal urease activity may be related with the decrease of these urease-producing taxa. A previous study also observed a drop in ruminal urease activity after addition of tannins to diet but a direct interaction between tannins and urease enzyme was postulated as responsible for this inhibition [62].

5. Conclusions

The current study showed that chestnut and quebracho tannins added to the diet of Holstein steers modified rumen microbiota composition, particularly fiber and starch degrading bacteria, mainly by reducing the abundance of *Prevotella* and *Fibrobacter* while favoring Ruminococcaceae and other members of phylum Firmicutes. Tannins treatment significantly increased pH and decreased urease activity in the ruminal liquor. Further work is necessary to assess the possible relation between tannins ability to modify rumen microbiota composition and the alteration of rumen fermentation parameters associated with energy and feed efficiency of beef cattle, such as the profile of short-chain fatty acids and the emissions of ammonia and methane.

Conflicts of Interest

The authors declare that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

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Research Article

Antibacterial Activity of Ethanolic Extract of *Syzygium polyanthum* L. *(Salam)* Leaves against Foodborne Pathogens and Application as Food Sanitizer

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The aim of this study was to determine antibacterial activity of *S. polyanthum* L. *(salam)* leaves extract foodborne pathogens. All the foodborne pathogens were inhibited after treating with extract in disk diffusion test with range 6.67 ± 0.58–9.67 ± 0.58 mm of inhibition zone. The range of MIC values was between 0.63 and 1.25 mg/mL whereas MBC values were in the range 0.63 mg/mL to 2.50 mg/mL. In time-kill curve, *L. monocytogenes* and *P. aeruginosa* were found completely killed after exposing to extract in 1 h incubation at 4x MIC. Four hours had been taken to completely kill *E. coli*, *S. aureus*, *V. cholerae,* and *V. parahaemolyticus* at 4x MIC. However, the population of *K. pneumoniae*, *P. mirabilis,* and *S. typhimurium* only reduced to 3 log CFU/mL. The treated cell showed cell rupture and leakage of the cell cytoplasm in SEM observation. The significant reduction of natural microflora in grapes fruit was started at 0.50% of extract at 5 min and this concentration also was parallel to sensory attributes acceptability where application of extract was accepted by the panellists until 5%. In conclusion, *S. polyanthum* extract exhibits antimicrobial activities and thus might be developed as natural sanitizer for washing raw food materials.

1. Introduction

Food safety is a major concern for both consumers and food manufacturers alike. Despite the high degree of awareness of food preservation methods, the occurrence of disease outbreaks caused by foodborne pathogens and spoilage microorganisms in foods is still increasing [1]. Foodborne illness is also known as foodborne disease and colloquially referred to food poisoning is any illness resulting from the consumption of contaminated food, pathogenic bacteria, viruses, or parasites that contaminate food, rather than chemical or natural toxins. The symptoms for food poisoning are including diarrhea, fever, vomiting, abdominal pain, and dehydration [2]. Currently to preserve food from spoilage, some manufacturers used synthetic antimicrobial agents to prevent the growth of food spoilage and food pathogenic microorganisms include benzoates, nitrates, and nitrites [3]. However, emergence of microbial resistance to classic antimicrobial agents becomes a major health concern due to elevated use of chemical preservatives in food processing [4]. Nowadays, consumers are more aware on food safety especially on the long term effect of synthetic additives in food including toxic and carcinogenic effect. Hence, this issue has led to the increased demand for high-quality, minimally processed foods with extended shelf-life and preferably free from or with a low level of synthetic additives in food [5]. Moreover, foods need to be safe and fresh with prolonged shelf-life. Therefore, antimicrobials agent from natural plants is a good source as an alternative to synthetic preservatives in order to satisfy consumers demand for safe and healthy food [6]. Antimicrobial agents can be either synthesized or naturally occurring in plant materials [7]. The main reasons for adding antimicrobial in food are to control food spoilage and to prevent the growth of foodborne pathogens

[8]. This suggests that natural plants might be sources of antimicrobials agents that can be used to inhibit the growth of foodborne pathogens.

S. polyanthum L., which is synonym to *salam*, is a deciduous tropical tree belonging to the Myrtaceae family [9]. This plant grows wildly on lowlands and is widely distributed in the temperate, subtropical, and tropical regions in the world [10]. These leaves had several name based on the location including *S. polyanthum* in Malaysia and Indonesia it is called *serai kayu* (Malay); *meselangan* is the name that used in Sumatra, *gowok* (Sunda), *salam* (Java, Sunda, Madura), *manting* (Java), or *kastolam* (Kangean) [11]. *S. polyanthum* leaves have been used traditionally as medicine or therapeutic agents including efficiency against ulcer, hypertension, diabetes, hyperuricemia, diarrheal, gastritis, skin diseases, and inflammation [11, 12]. Furthermore, *S. polyanthum* leaves were believed to possess antibacterial activity against *Streptococcus mutans* [11] and *Staphylococcus aureus* [13]. Besides that, this plant also had antifungal activities against spoilage fungi *Euroticum* spp., *Aspergillus* spp., and *Penicillium* spp. [14]. Furthermore, according to Perumal et al. [10], *S. polyanthum* leaves are also found to be noncytotoxic to normal mammalian cell lines. Based on previous study, *S. polyanthum* leaves had antibacterial activity against *B. cereus* and *B. subtilis* [15].

Therefore the aim of this study was to determine the antimicrobial activity of *S. polyanthum* leaves extracts against a wide spectrum of foodborne pathogens.

2. Materials and Methods

2.1. Samples. Dried *S. polyanthum* leaves were purchased from Herbal Market Bandung, Indonesia, deposited, and identified in Institute of Bioscience (IBS), Universiti Putra Malaysia.

2.2. Preparation of Extract. One hundred grams of dried *S. polyanthum* leaves was ground using dry blender. Then, the samples were soaked in 400 mL absolute ethanol for seven days at room temperature as stated by Rukayadi et al. [16], with some modification. The mixture was then filtered using Whatman number 2 filter paper and concentrated by using rotary evaporator at 50°C and at speed of 150 rpm for 60 to 90 min. The extract was dissolved in 10% dimethylsulfoxide (DMSO) to obtain stock solution. The final concentration of extract was standardized at 10 mg/mL or 1%. The stock solution was kept at −4°C.

2.3. Bacteria Cultures. A total of nine strains of frequently reported as foodborne pathogens were included: *Escherichia coli* O157:H7 ATCC 43895, *Klebsiella pneumoniae* ATCC 13773, *Listeria monocytogenes* ATCC 19112, *Proteus mirabilis* ATCC 21100, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 29737, *Vibrio cholerae* (Isolate 2), and *Vibrio parahaemolyticus* ATCC 1780. All the microbial strains used in this study were maintained by subculturing them on the nutrient agar (NA) or nutrient agar mix with 3% of NaCl for *V. cholerae* and *V. parahaemolyticus* and were incubated overnight. Bacteria strains can be stored in this way for a few weeks on the agar plates before subculturing them again, while, for the stock culture preparation, 0.5 mL of overnight culture with broth media was mixed into 0.5 mL of 80% sterile glycerol. Cultures were stored at −20°C. These stock cultures were kept from 6 months to 1 year [17].

2.4. Disk Diffusion Test. *S. polyanthum* extract was tested for antimicrobial activity using the disk diffusion method as described by CLSI [18]. Bacteria species with concentration in range 10^6–10^8 CFU/mL were spread on Mueller Hinton agar (MHA) with a sterile cotton swab. Sterile filter paper discs with 6 mm diameter were placed on top of the culture and 10 μL of 10 mg/mL (w/v) of *S. polyanthum* leaves extract was loaded on the paper discs. 0.1% of commercial chlorhexidine (CHX) was used as positive control whereas 10% DMSO as negative control. The plates were incubated at 37°C for 24 hours. Evidence of clear zone indicates bacterial growth inhibition and the diameter was measured in mm.

2.5. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Determination of MIC and MBC values was performed using a method described in the CLSI [18]. MIC was conducted in 96-well U-shaped microtiter plate using twofold standard broth microdilution method with an inoculum of approximately 10^6–10^8 CFU/mL. *S. polyanthum* leaves extract with concentration 10 mg/mL was mixed and twofold diluted in the respective medium containing inoculum. Column 12 of the microtiter plate contained the highest concentration of extract (5 mg/mL) while column 3 contained the lowest concentration of extract (0.0097 mg/mL). Column 1 served as negative control (only medium, no inoculum, and no antimicrobial agent), while column 2 served as positive control for all samples (only medium and inoculum or antimicrobial agent-free well) for 24 hours. The MIC was defined as the lowest concentration of antimicrobial agent that was able to inhibit the visible growth [16] while minimal bactericidal concentration (MBC) was standing for the lowest concentration of antimicrobial agent that completely killed the growth of culture. MBC was determined by subculturing the suspension (10 μL) from each well in microtiter plate on MHA. The plates were then incubated at 37°C for 24 hours or until growth was seen at positive control.

2.6. Time-Kill Curve Assay. A time-kill curve assay was carried out with the MIC values found previously in the microplate bioassay, using a modification of the viable cells count method of de Souza et al. [19]. *S. polyanthum* leaves extract was diluted with the Muller Hinton broth (MHB) medium containing inoculum of approximately 10^6–10^8 CFU/mL to obtain final concentrations of 0x MIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC for each bacterial species. At different time intervals of exposure, (0, 0.5, 1, 2, and 4 hours), 0.1 mL of the suspension was serially diluted in 1% phosphate buffered saline (PBS) and plated onto MHA. The plates were incubated at 37°C for 24 hours. The results were expressed in log CFU/mL.

Table 1: Inhibition zone of *S. polyanthum* L. leaves extract against foodborne pathogens.

Strains	Inhibition zone (mm)		
	S. polyanthum extract	CHX	DMSO
E. coli O157:H7	7.00 ± 0.28	9.00 ± 0.00	n.a
K. pneumoniae	9.33 ± 0.50	11.50 ± 0.50	n.a
L. monocytogenes	9.67 ± 0.58	12.00 ± 0.00	n.a
P. aeruginosa	7.00 ± 0.32	10.00 ± 0.51	n.a
P. mirabilis	6.67 ± 0.40	10.00 ± 0.70	n.a
S. aureus	9.33 ± 0.52	10.00 ± 0.23	n.a
S. typhimurium	6.67 ± 0.50	8.00 ± 0.00	n.a
V. cholerae	8.33 ± 0.30	8.80 ± 0.58	n.a
V. parahaemolyticus	6.67 ± 0.50	9.00 ± 0.00	n.a

n.a: no activity; diameter of inhibition zones in mm (including disc); positive control (chlorhexidine: CHX; 0.1%); negative control (DMSO; 10%); results were expressed as means ± standard deviation (SD); $n = 3 \times 3$.

2.7. Scanning Electron Microscope (SEM). Fresh *K. pneumoniae* and *S. aureus* culture was treated with the extract and incubated at 37°C in MHB for 24 hours. The pellets were collected by centrifugation (5000 ×g for 10 min) and were fixed with 2.5% glutaraldehyde for 4–6 hours at 4°C. Then, the pellets were washed with 0.1 M sodium cacodylate buffer for 10 min and were repeated for 3 times. The pellets were then postfixed with 1% osmium tetroxide for 2 hours at 4°C, washed again with 0.1 M sodium cacodylate buffer for 10 min, and repeated for 3 times. Then the pellets were dehydrated using 35, 50, 75, and 95% acetone for 15 min each. Lastly the pellets were dehydrated using 100% acetone for 15 min and were repeated for 3 times. Cell suspensions were transferred into a specimen basket, made from aluminium foil coated with albumin, and then put in critical dryer for 0.5 hours. The specimens were mounted on a stub and the sputter was coated with gold. The morphology of the cells was observed and images were obtained using SEM instrument.

2.8. Application of S. polyanthum Extract as Food Sanitizer on Grapes. The samples of grapes fruit (approximately 10 g) were treated with tap water and natural sanitizer with concentration of 0.05%, 0.50%, 1.00%, and 5.00% of *S. polyanthum* extract according to Yusoff et al. [20] with slight modification. Grapes fruit was soaked separately at different time interval, 5, 10, and 15 min, to determine their microflora growth viability. Untreated samples remained unwashed. For bacteria growth determination, 1 mL from each treatment was diluted into 10^{-1}, 10^{-2}, and 10^{-3} dilution. Then, 0.1 mL from each dilutions series was spread on the different types of agar, Plate count agar, Eosin Methylene Blue agar (EMB), and Baird Parker agar, and incubated at 37°C for 24 hours. The presence of colonies was counted.

2.9. Evaluation of Sensory Attributes Acceptability of Treated Grapes Fruit. The sensory evaluation acceptability test was performed according to Brasil et al. [21], with slight modification. A group of 50 untrained panellists were presented with five different 3-digit coded samples placed in a random order. The evaluation was conducted based on the 9-point hedonic scale for inspection acceptance testing where panellists assessed each treated sample in terms of colour (observed with eyes), odour (smelled with nose), and the texture (touched with finger). The ratings for the each analysis of samples were given in a scale ranging from extremely disliked (scale of 1) to extremely liked (scale of 9).

3. Results

3.1. Yield of Extract. 100 g of dried weight of *S. polyanthum* leaves was extracted using ethanol solvent and yielded 8.21 g of extract, which gave the percentage value of 8.21% total yield.

3.2. Disk Diffusion Test. The inhibition zone of *S. polyanthum* leaves extract against foodborne pathogens is shown in Table 1. The inhibition zones were between 6.67 ± 0.58 and 9.67 ± 0.58 mm. Results showed the inhibition zones of *S. polyanthum* extract were 7.00 ± 0.28 mm, 9.33 ± 0.50 mm, 9.67 ± 0.58 mm, 7.00 ± 0.32 mm, 6.67 ± 0.58 mm, 9.33 ± 0.58 mm, 6.67 ± 0.50 mm, 8.33 ± 0.58 mm, and 6.67 ± 0.58 mm on *E. coli, K. pneumoniae, L. monocytogenes, P. aeruginosa, P. mirabilis, S. aureus, S. typhimurium, V. cholerae,* and *V. parahaemolyticus,* respectively. The larger inhibition zone gave the meaning of higher antibacterial activity of the extract on the tested microbial species.

3.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). From the result shown in Table 2, *S. polyanthum* leaves extract demonstrated broad-spectrum activity against all selected bacteria with the MIC values ranging from 0.63 to 1.25 mg/mL. Among them *L. monocytogenes* and *S. aureus* were found to be the most susceptible pathogens with the MIC value of 0.63 mg/mL. Results show that the MBC was in the range of 0.63 mg/mL to 2.50 mg/mL. *L. monocytogenes* gave the lower MBC value compared to other strains which was 0.63 mg/mL.

3.4. Time-Kill Curve Assay. In this study, time-killing assay was done to find the correlation between the concentrations of *S. polyanthum* leaves extract with its killing effects on

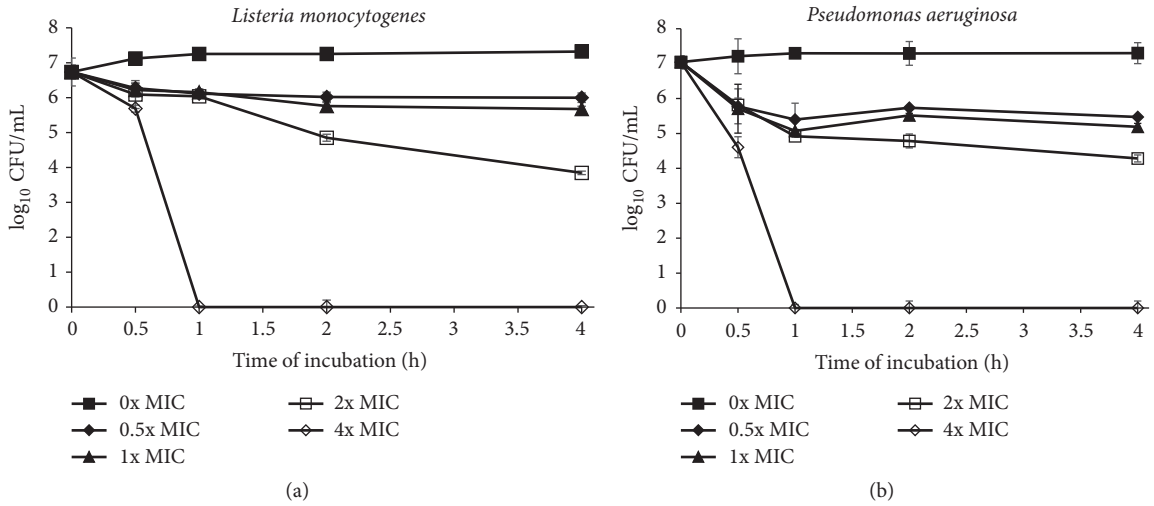


Figure 1: (a) Time-kill curve plots for *L. monocytogenes* (0, 0.315, 0.630, 1.260, and 2.520 mg/mL) following exposure to *S. polyanthum* L. extract. Values given in the brackets after species are 0x MIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC, respectively. (b) Time-kill curve plots for *P. aeruginosa* (0, 0.625, 1.250, 2.500, and 5.000 mg/mL) following exposure to *S. polyanthum* L. extract. Values given in the brackets after species are 0x MIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC, respectively.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *S. polyanthum* L. extract against foodborne pathogens.

Strains	MIC (mg/mL)	MBC (mg/mL)
E. coli O157:H7	1.25	2.50
K. pneumoniae	1.25	2.50
L. monocytogenes	0.63	0.63
P. aeruginosa	1.25	2.50
P. mirabilis	1.25	2.50
S. aureus	0.63	1.25
S. typhimurium	1.25	1.25
V. cholerae	1.25	1.25
V. parahaemolyticus	1.25	1.25

selected foodborne pathogens. Time-kill curve assay showed that *S. polyanthum* leaves extract can kill *L. monocytogenes* and *P. aeruginosa* at 4x MIC for 1 hour (Figures 1(a) and 1(b)) and *E. coli, S. aureus, V. cholerae,* and *V. parahaemolyticus* at 4x MIC for 4 hours (Figures 2(a), 2(b), 2(c), and 2(d)). The population of *K. pneumoniae*, *P. mirabilis*, and *S. typhimurium* also showed a reduction $< 3 \log_{10}$ CFU/mL when treated with the extract at 4x MIC for 4 hours as shown in Figures 3(a), 3(b), and 3(c).

3.5. Scanning Electron Microscope (SEM). Figures 4(a) and 4(b) show the treated and untreated *K. pneumoniae* cells with *S. polyanthum* extract at the concentration of 1.25 mg/mL for overnight. The untreated *K. pneumoniae* showed normal cells characteristics with rod shape and intact peptidoglycan layer. Meanwhile, after treating with *S. polyanthum* extract overnight, cells appeared to be damaged with some irregularities surfaces, whereby the rod-shaped cells shrank and deflated, and some of them were cavitated. Besides that, the effect of *S. polyanthum* extract against *S. aureus* is shown in Figures 5(a) and 5(b). The grape-like cluster morphology of *S. aureus* was altered after the treatment. Disruptions with release of intracellular material associated with *S. aureus* cells losing their cytoplasm (empty and flaccid cells) were also observed.

3.6. Application of S. polyanthum Extract as Food Sanitizer on Grapes. Table 3 shows the effect of *S. polyanthum* against natural flora in grapes. Bacterial population which was detected in grapes includes *E. coli* and *S. aureus*. This study showed that total plate count had been reduced significantly after exposure to 0.50% at 5 min soaking where the population decreased from 5.78 ± 0.05 to $5.19 \pm 0.13 \log_{10}$ CFU/mL. On the other hand, *E. coli*'s population only had been significantly reduced after treating at 1.00% for 5 min and decreased to undetected at 5% extract at 5 min treatment while *S. aureus* decreased to $\log_{10}$ 0.00 ± 0.00 CFU/mL starting at 0.50% in 5 min.

3.7. Evaluation of Sensory Attributes Acceptability of Treated Grapes Fruit. Table 4 shows the sensory acceptability of treated grapes with *S. polyanthum* extract. Based on the result, it can be concluded that most panellists accepted these grapes samples which were washed with extracts and tap water with overall acceptability of more than scale 7. There is also no significant difference between washing treatment using highest concentrations of extract (5%) and tap water. That means that panellist is not able to differentiate between using tap water and extracts. Therefore, *S. polyanthum* did not affect the physical appearances of grapes.

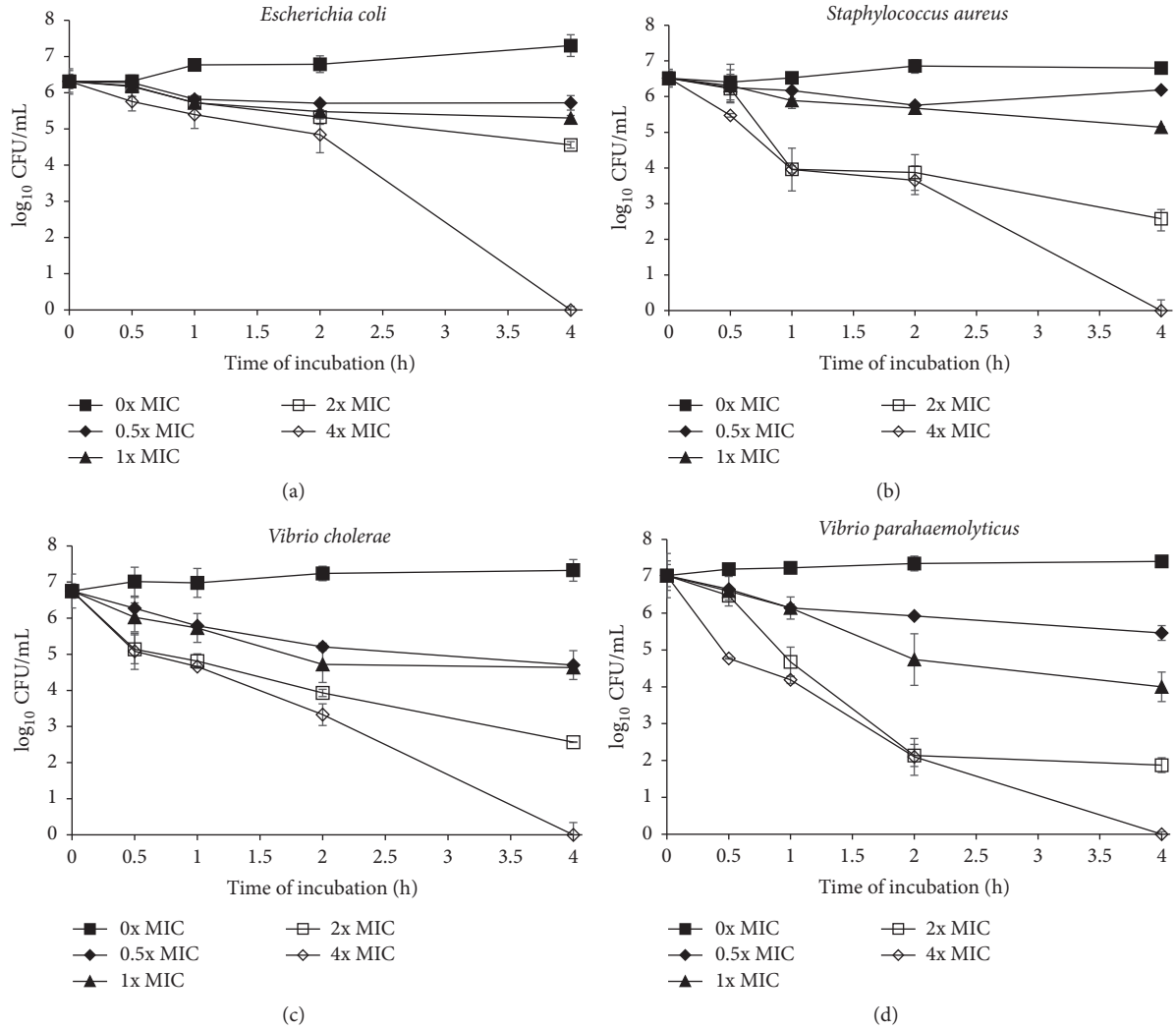


Figure 2: (a) Time-kill curve plots for *E. coli* O157:H7 (0, 0.625, 1.250, 2.500, and 5.000 mg/mL) following exposure to S. *polyanthum* L. extract. Values given in the brackets after species are 0x MIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC, respectively. (b) Time-kill curve plots for *S. aureus* (0, 0.315, 0.630, 1.260, and 2.520 mg/mL) following exposure to *S. polyanthum* L. extract. Values given in the brackets after species are 0x MIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC, respectively. (c) Time-kill curve plots for *V. cholerae* (0, 0.625, 1.250, 2.500, and 5.000 mg/mL) following exposure to *S. polyanthum* L. extract. Values given in the brackets after species are 0x MIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC, respectively. (d) Time-kill curve plots for *V. parahaemolyticus* (0, 0.625, 1.250, 2.500, and 5.000 mg/mL) following exposure to *S. polyanthum* L. extract. Values given in the brackets after species are 0x MIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC, respectively.

4. Discussion

A recent trend in food processing is to avoid the use of chemical preservatives. Thus, natural antimicrobial alternatives are required. In this research, ethanol was used as a solvent. Ethanol is also classified as a polar solvent. This means that this solvent is miscible in water and it will extract mostly the ionic compounds from *S. polyanthum* leaves. Ethanol has better dissolving capabilities compared to water because it has a slightly low dipole and is dielectric; thus it is slightly polar [22]. Moreover, according to Marriott [23], the solvents permitted for use in the preparation of food ingredients are ethanol, ethyl acetate, and acetone only.

From the disk diffusion result, *L. monocytogenes* gave the highest inhibition zone compared to others strain. On the other hand, *P. mirabilis*, *S. typhimurium,* and *V. parahaemolyticus* were observed to be more resistant against the extract. Generally, in Gram-negative bacteria, their outer membranes serve as permeability barrier which allows only small hydrophilic molecules to pass through into the cell, restricting their rate of penetration for certain antimicrobial compounds and excluding larger molecules. Besides, they also possess multidrug resistant pumps which exclude some of the antibacterial compounds across the barrier [24]. These special buildings make the Gram-negative bacteria more tolerant to any foreign compounds intake. On the other hand, disk diffusion test sometimes gave inaccurate result due to some limitations such as the ability of extract to pass through the pore discs and the inability of hydrophobic compounds to diffuse into the media agar [25]. In addition according

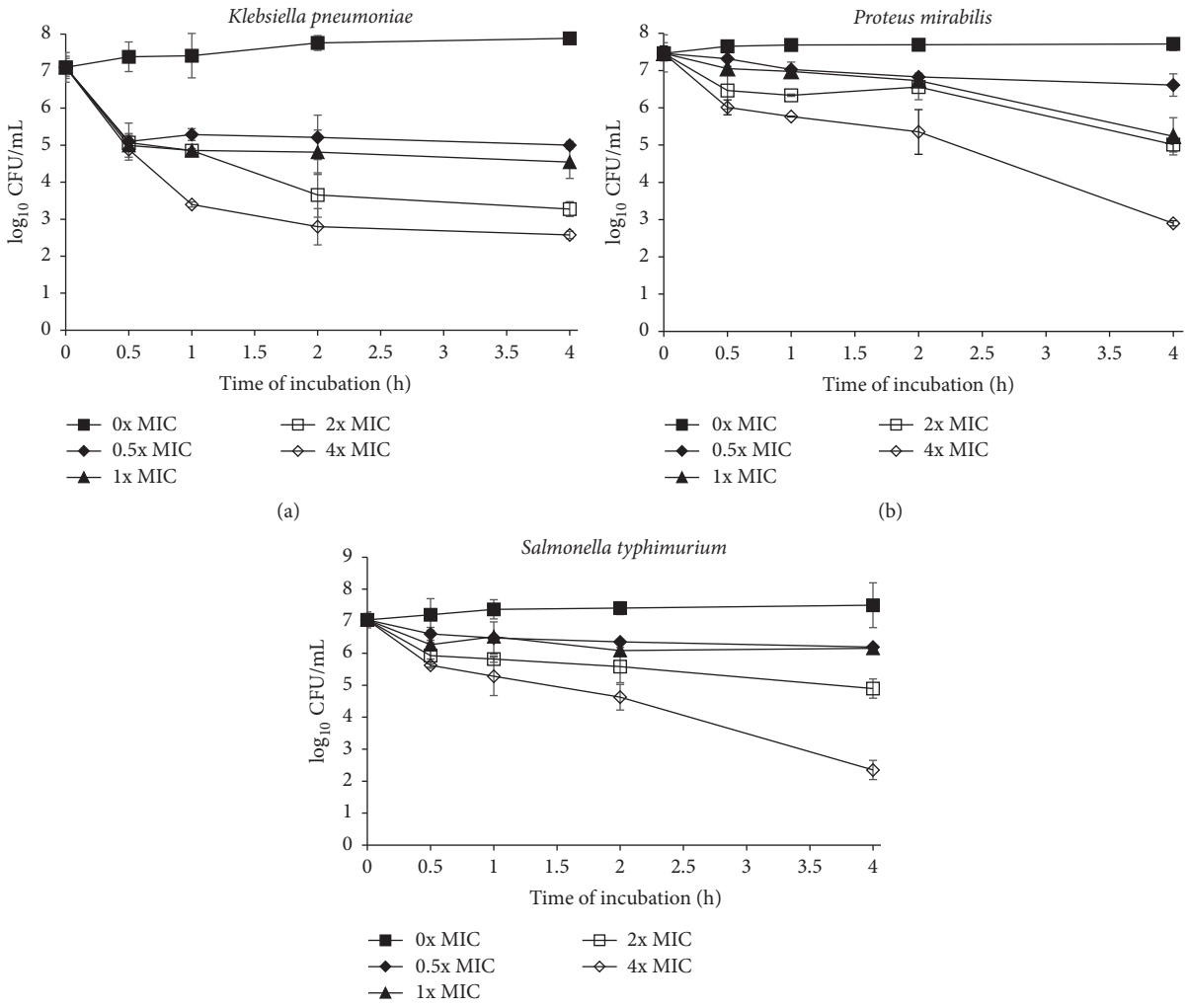
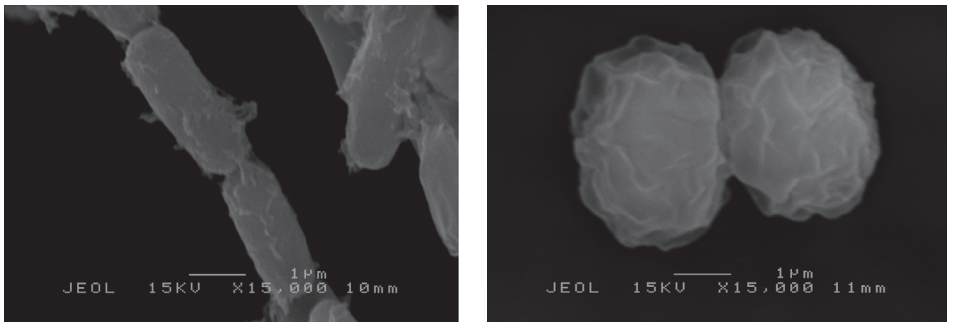
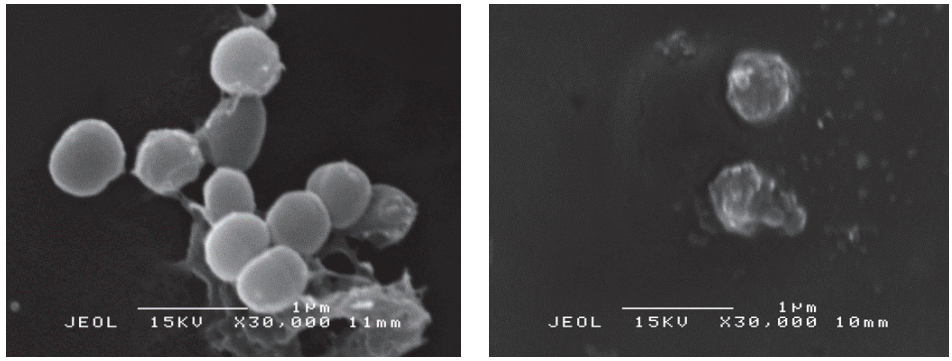


Figure 3: (a) Time-kill curve plots for *K. pneumoniae* (0, 0.625, 1.250, 2.500, and 5.000 mg/mL) following exposure to *S. polyanthum* L. extract. Values given in the brackets after species are 0x MIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC, respectively. (b) Time-kill curve plots for *P. mirabilis* (0, 0.625, 1.250, 2.500, and 5.000 mg/mL) following exposure to *S. polyanthum* L. extract. Values given in the brackets after species are 0x MIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC, respectively. (c) Time-kill curve plots for *S. typhimurium* (0, 0.625, 1.250, 2.500, and 5.000 mg/mL) following exposure to *S. polyanthum* L. extract. Values given in the brackets after species are 0x MIC, 0.5x MIC, 1x MIC, 2x MIC, and 4x MIC, respectively.



(a) (b)

Figure 4: (a) Scanning electron micrograph of untreated *K. pneumoniae*. (b) Scanning electron micrograph of *K. pneumoniae* after treating with *S. polyanthum* L. extract at MIC value for 24 hours.



(a) (b)

Figure 5: (a) Scanning electron micrograph of untreated *S. aureus*. (b) Scanning electron micrograph of *S. aureus* after treating with *S. polyanthum* L. extract at MIC value for 24 hours.

to Gangoué-Piéboji et al. [26], by using disc, some active compounds might be blocked in the disc pores and are unable to pass through the inoculated media and hence cannot express their activity. Besides that, inhibition zone of 0.1% of CHX against the pathogens was in range of 8.80 ± 0.58 to 12.00 ± 0.00 mm. This finding showed lower inhibition zone compared to the study done by Abbas et al. [27], which mentions that the inhibition zone was between 13.84 ± 0.65 and 14.87 ± 0.53 mm on *E. faecalis* by using 2% of CHX. This observation may be due to the different concentration of CHX. However, according to Gupta et al. [28], inhibition zone of CHX against *P. aeruginosa* was 10.00 mm, whereas *S. aureus* was 11.00 mm. Therefore, the finding was similar to this present study. In conclusion, the disc-diffusion test is normally used as first screening in the detection of active compounds in plant extracts before further determination was performed.

L. monocytogenes and *S. aureus* were found to be the most susceptible pathogen with the MIC value of 0.63 mg/mL while the other strains showed 1.25 mg/mL. *L. monocytogenes* also showed the lower MBC values compared to other strains with 0.63 mg/mL. Besides that, *S. typhimurium, V. cholerae,* and *V. parahaemolyticus* had the same value for MIC and MBC, meaning this bacteria can be inhibited and killed at the same concentration of plant extract. This result showed that Gram-positive bacteria were easier to inhibit compared to Gram-negative ones. Gram-negative bacteria have a hydrophilic outer membrane rich in lipopolysaccharide molecules. Therefore it serves as a penetration barrier towards macromolecules [29]. Although this description is widely accepted, and accepted for many essential oils, some researchers have stated that the Gram distinction may have little relation to growth inhibition and some herbs are equally effective against both groups of bacteria [30]. However, the outer membrane is not completely impermeable as there are porin proteins present in this layer that can create channels large enough to allow restricted passage of molecules with a molecular mass below 600 Da, such as substituted phenolics in herb extracts and essential oils, allowing their slow penetration into the periplasmic space and the cytoplasmic membrane [31]. Thus it is possible that over a longer contact time the active compounds present in leaves extract would have the same effect on Gram-negative and Gram-positive bacteria [32]. Besides that, the *Euphorbia hirta* extract showed lower antimicrobial activity on *E. coli* compared to *S. polyanthum* extract with the MIC value of 3.13 mg/mL [33]. According to Rand et al. [34], *S. polyanthum* extract demonstrated better bactericidal and bacteriostatic properties compared to *B. oleracea* extract where MIC and MBC value of *S. aureus*, *E. coli*, *P. aeruginosa,* and *K. pneumoniae* were 100 mg/mL and 400 mg/mL, 300 mg/mL and 400 mg/mL, 100 mg/mL and 200 mg/mL, and 100 mg/mL and 400 mg/mL, respectively. *Moringa oleifera* seed extract displayed weaker antibacterial activity compared to *S. polyanthum* with MIC values >4 mg/mL on *E. coli*, *P. aeruginosa,* and *S. typhimurium* [35]. Moreover, *S. polyanthum* also shows good antibacterial effect compared to garlic and ginger extract. Based on Smith-Palmer et al. [36], MIC and MBC of garlic and ginger extract on *L. monocytogenes*, *E. coli* and *S. aureus* were >1% whereas *S. polyanthum* gave bacteriostatic and bactericidal effect between 0.063% and 0.125% against the same bacteria strains. On the other hand, *S. polyanthum* and *Syzygium aromaticum* (clove) showed quite similar antibacterial activity. Inhibition zones of *S. aromaticum* against *E. coli*, *L. monocytogenes,* and *S. aureus* were 9.7, 8.4, and 8.0 mm, respectively. Meanwhile the MIC values were 0.04%, 0.03%, and 0.04% on the same pathogens. Besides that, *Syzygium cumini* showed no antimicrobial activity against *E. coli* and *K. pneumoniae;* however, it is effective against *S. aureus* with 9.00 mm shown in inhibition zone [37]. Therefore, *S. cumini* had lower antibacterial activity compared to *S. polyanthum* extract in terms of disk diffusion test. According to Chikowe et al. [38], *Syzygium forte*, *Syzygium francisii*, *Syzygium moorei*, *Syzygium puberulum,* and *S. wilsonii* illustrated weaker antibacterial activity compared to *S. polyanthum* where there was no inhibition zone against *P. mirabilis* and *S. aureus*. However, *S. francisii*, *S. moorei,* and *S. wilsonii* showed higher inhibition zone against *E. coli* compared to *S. polyanthum* extract. On the other hand, all the tested *Syzygium* spp. gave lower antibacterial activity against *E. coli* compared to *S. polyanthum* in terms of MIC value except *S. francisii* with 0.256 mg/mL. Apart from that,

Table 3: Effect of different concentrations and exposure times of *S. polyanthum* L. extract on natural microbial in grapes.

Sample	Grapes								
Bacterial species	TPC ($\log_{10}$ CFU/mL)			*E. coli* ($\log_{10}$ CFU/mL)			*S. aureus* ($\log_{10}$ CFU/mL)		
ET/Treatment	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min
Control	5.78 ± 0.05^{aA}	5.78 ± 0.05^{aA}	5.78 ± 0.05^{aA}	4.14 ± 0.05^{aA}	4.14 ± 0.05^{aA}	4.14 ± 0.05^{aA}	3.45 ± 0.03^{aA}	3.45 ± 0.03^{aA}	3.45 ± 0.03^{aA}
Tap water	5.73 ± 0.08^{aA}	5.72 ± 0.03^{aA}	5.66 ± 0.11^{aA}	4.11 ± 0.05^{aA}	4.09 ± 0.03^{aA}	4.05 ± 0.04^{aA}	3.37 ± 0.11^{aA}	3.14 ± 0.07^{aA}	3.52 ± 0.06^{aB}
0.05%	5.69 ± 0.04^{aA}	5.39 ± 0.03^{aA}	5.62 ± 0.16^{aA}	4.08 ± 0.06^{aA}	4.18 ± 0.03^{aA}	4.10 ± 0.02^{aA}	3.19 ± 0.07^{bA}	3.33 ± 0.18^{bA}	3.25 ± 0.14^{bA}
0.50%	5.19 ± 0.13^{bA}	5.16 ± 0.14^{bA}	5.07 ± 0.03^{bA}	3.88 ± 0.18^{aA}	4.01 ± 0.04^{aA}	3.95 ± 0.06^{aA}	0.00 ± 0.00^{cA}	0.00 ± 0.00^{cA}	0.00 ± 0.00^{cA}
1.00%	4.46 ± 0.22^{cA}	4.39 ± 0.12^{cAB}	3.03 ± 0.07^{cB}	3.22 ± 0.06^{bA}	3.46 ± 0.19^{bAB}	3.11 ± 0.07^{bB}	0.00 ± 0.00^{cA}	0.00 ± 0.00^{cA}	0.00 ± 0.00^{cA}
5.00%	3.74 ± 0.03^{dA}	3.49 ± 0.23^{dA}	0.00 ± 0.00^{dA}	0.00 ± 0.00^{cA}	0.00 ± 0.00^{cA}	0.00 ± 0.00^{cA}	0.00 ± 0.00^{cA}	0.00 ± 0.00^{cA}	0.00 ± 0.00^{cA}

Values with different small letters within the same columns are significantly different ($p < 0.05$). Values with different capital letters within the same rows are significantly different ($p < 0.05$). ET: Exposure Time.

Table 4: Sensory attributes acceptability of treated grapes with *S. polyanthum* L. extract.

Attributes	Tap water	0.05%	0.50%	1.00%	5.00%
Colour	8.49 ± 0.88^a	8.16 ± 1.12^a	8.10 ± 1.08^a	7.65 ± 0.80^a	7.81 ± 1.88^a
Odour	8.59 ± 1.65^a	7.11 ± 1.11^a	7.53 ± 0.82^a	7.63 ± 1.90^a	7.25 ± 0.70^a
Texture	7.04 ± 0.89^a	7.10 ± 0.94^a	6.87 ± 1.02^a	7.68 ± 0.84^a	6.84 ± 2.10^a
Overall acceptability	7.24 ± 1.40^a	7.02 ± 1.51^a	7.13 ± 0.95^a	7.71 ± 1.79^a	7.28 ± 1.84^a

Mean values ± standard deviation with different small letters in the same row have significance different ($p < 0.05$).

S. polyanthum had higher antibacterial activity against *K. pneumoniae* than *S. forte*, *S. francisii*, *S. moorei*, *S. puberulum,* and *S. wilsonii.*

Generally, different crude extracts show different antibacterial level among different microbes tested. These inconsistencies might be due to the different expression of the bioactive compounds present in the extracts. As suggested by Cowan [39], essential oils and polyphenolic compounds exhibited different bacteriostatic and bactericidal effect on bacterial strains. Therefore, minimum inhibitory concentration (MIC) is the parameter commonly used to guide the selection on the antimicrobial agent used in treatment by predicting their efficacy at a standard inoculum approximately 10^6 CFU/mL after an incubation period of 18–24 hours [18]. However, MIC only provides limited information on the kinetics of the antimicrobial action. Due to this limitation, time-killing assay was performed in order to find the correlation between the rate of bactericidal activity with the incubation time and concentration of antimicrobial agent [32].

Figures 1(a) and 1(b) showed that both *L. monocytogenes* and *P. aeruginosa* had been completely killed at 4x MIC in 1-hour incubation with 2.52 and 5.00 mg/mL of *S. polyanthum* leaves extract, respectively. These two strains were killed earlier compared to others. However finding by Penduka and Okoh [40] stated that *L. monocytogenes* can be killed completely with 0.314 mg/mL of crude *Garcinia kola* seed methanol extract in 0.5 hours of incubation. Therefore this finding suggested that *S. polyanthum* leaves extract might possess lower antibacterial activity against *L. monocytogenes*. In other case, according to Alwash et al. [41], *Melastoma malabathricum* extract had been reported to be able to kill completely *P. aeruginosa* at concentration 1.56 mg/mL within 8 hours. The comparison was hard to evaluate as both extracts are effective in completely killing *P. aeruginosa* at different concentration and incubation time. Generally, more concentrated extract will be able to kill bacteria in short period.

E. coli, *S. aureus*, *V. cholerae,* and *V. parahaemolyticus* had been killed at 4x MIC within 4 hours as shown in Figures 2(a), 2(b), 2(c), and 2(d). Five mg/mL extract had been used to kill *E. coli* completely in 4 hours. In contrast, Mamman et al. [42], had reported the bactericidal activity of *Azadirachta indica* extract on *E. coli* strain was at concentration 250 mg/mL. Therefore, results from this study revealed that *S. polyanthum* leaves extract is a good antibacterial source against *E. coli* strain. According to Witkowska et al. [29], the bactericidal effect of sage extract on *S. aureus* was at concentration > 40 mg/mL for 24 hours of incubation time. In addition, rosemary and clove extracts were able to kill *S. aureus* completely at 5 and 10 mg/mL concentration for 4 and 6 hours of incubation time, respectively. However, from this finding, *S. polyanthum* leaves extract only took 4-hour incubation to kill *S. aureus* completely at concentration 2.52 mg/mL. In comparison, *S. polyanthum* leaves have better bacterial effect against *S. aureus* compared to sage, rosemary, and clove extract. Furthermore, *S. aureus* was Gram-positive bacteria where the membrane structure was easier to disrupt compared to Gram-negative bacteria. From finding by Kwieciński et al. [43], it stated that *S. aureus* can be killed within 15 min with 1% (v/v) of tea tree oil while El-Farmawi et al. [44] showed that methicillin-resistant *S. aureus* can be killed during 2–4 hours of incubation with cinnamon and green tea extract at concentration 300 μl/mL and 200 μl/mL, respectively. In conclusion, *S. polyanthum* leaves extract had a weaker bactericidal effect as compared to tea tree oil, cinnamon, and green tea extracts. The time-kill plot obtained for *V. cholerae* and *V. parahaemolyticus* strains exhibited bactericidal end points which were at 4x MIC after 4-hour incubation. However, the population of both pathogens was reduced approximately to 3 log at 2x MIC after 4 hours. Penduka and Okoh [40] reported that 69% of *V. parahaemolyticus* was killed at 5 mg/mL after 2-hour incubation using *Garcia kola* seed methanol extract. Therefore, *S. polyanthum* leaves have a quite similar bactericidal effect with *G. kola* seed extract where >50% *V. cholerae* and *V. parahaemolyticus*' population were completely killed at same concentration and incubation time.

On the other hand, the populations of *K. pneumoniae*, *P. mirabilis,* and *S. typhimurium* were only reduced to <3 log after 4-hour incubation as shown in Figures 3(a) and 3(b). According to Supardy et al. [45], extract able to reduce bacterial cell less than 3 log was indicated as having bacteriostatic effect. Furthermore, 3 log was the minimum level of microbial population to cause infection in human. According to El-Farmawi et al. [44], *K. pneumoniae* can be killed within 6 to 8 hours of incubation with cinnamon and green tea extract at concentrations 500 μl/mL and 300 μl/mL, respectively. Its means *S. polyanthum* leaves had a quite weaker antibacterial activity compared to cinnamon. Research by Rajeh et al. [46] reported the bactericidal activity of *Euphorbia hirta* extract on *P. mirabilis* was at concentration 50 mg/mL at 24-hour incubation. Muniandy et al. [47] stated that concentration 1.08 mg/mL of *Coleus aromaticus* extract can completely kill *P. mirabilis* within 24 hours of incubation time. On the other hand, Konaté et al. [48], reported the bactericidal effect of *Sida alba* extract on *P. mirabilis* at concentration 0.05 mg/mL within 6 hours of incubation. Results revealed

that *S. polyanthum* leaves possess better antibacterial agent compared to *E. hirta* and *C. aromaticus* extracts; however this leaves extract showed lower bactericidal effect compared to *S. alba*. Foster [49] stated that *Salmonella* spp. had the ability to adapt in wide range of conditions including ability to grow in various pH and temperatures. Besides that, Mandal et al. [50] reported the reduction of *Salmonella* spp. until 2.19 log at concentration 0.512 mg/mL of *Camelia sinensis* extract within 24 hours of incubation time. Similarly, in this research, *Salmonella* spp. only reduced to 3 log and did not completely get killed after treatment with *S. polyanthum* leaves extract in 4-hour incubation. This showed that *S. polyanthum* had better antibacterial activity against *Salmonella* spp. compared to *C. sinensis* where the population reduction took only about 4 hours.

Increasing of plant extract's concentration will lead to diffusion into membrane cell thus causing membrane destruction [51]. In addition, the killing activity of *S. polyanthum* leaves extract was concentration-dependent. According to Miksusanti et al. [52], at higher concentration of extract, the membrane becomes leaky to cytoplasmic components which lead to cell death. It was also speculated that high concentrations of *S. polyanthum* leaves extract contribute to rapid killing of the microorganism because of the serious loss of membrane integrity and degenerative cell wall. In order to kill the microorganisms, leaves extract needs to bind, occupy, and remain at the target site for sufficient period of time to prevent the metabolic process and interfere with the chemical reactions of the bacteria. In addition, the increasing of plant extract can saturate the target site and cause rapid bactericidal effect [53]. The hydrophobicity of plants extract and their bioactive compounds contribute in the breaking down of the membrane cells lipid and make them more permeable for the penetration [54]. Furthermore, the bioactive compounds in extract may inhibit the synthesis of essential metabolites (folic acid) by preventing the enzymatic reaction. The protein synthesis in the microorganisms also can be inhibited if the bioactive compounds interfere and change the shape of ribosome. The interference can lead to misreading of the genetic code on the mRNA [55].

Action modes of extract against tested strains were observed as shown in Figures 4 and 5. The treated *K. pneumoniae* showed that the cell was ruptured and shrunk. This observation was supported by dos Santos et al. [56], where the electron-dense particles which stayed packed in cytoplasm before were dispersed and result in an empty hollow in cytoplasm. This indicated that cytoplasm's compartment was released acrossed the cell wall. Study conducted by Supardy et al. [45] also reported the damaged and distorted *K. pneumoniae* cell after treating with 0.5 mg/mL of *Halimeda discoidea* extract for 12, 24, and 36 hours of treatment. The same result also were obtained by Rajeshwari et al. [57] where the morphology of *K. pneumoniae* showed unusual shapes of expanding, swelling, shrinking, and other multiple disorientations that were absent in the control sets after treating with *H. discoidea* extract. In addition, the same phenomenon was reported by Derakhshan et al. [58] who treated the *K. pneumoniae* with the cumin (*Cuminum cyminum* L.) herb extract. However, not all shrunk cells after treatment represent cells death. Some of them decrease their cell surface area as the way of adaptation, in order to minimize the target site for antimicrobial compounds to attach on them [45]. However, constant exposure of plant extract with increasing of concentration and extended time treatment will eventually kill the cells [29]. Generally, the results showed the shrinkage and deformation of the cells proved that the cells were under a suppressive and stressful environment. From the results, the prompt antibacterial action on the cells was seen to specifically attack the cell membrane components. Moreover, the treated cell of *S. aureus* also showed morphology changes. The grape-like cluster morphology of *S. aureus* was altered after the treatment. Disruptions with release of intracellular material associated with *S. aureus* cells losing their cytoplasm (empty and flaccid cells) were also observed. The distortion of the physical structure of the cell could cause the expansion and destabilization of the membrane and increase membrane fluidity, which in turn increases the passive permeability and manifests itself as a leakage of various vital intracellular constituents, such as ions, ATP, nucleic acids, sugars, enzymes, and amino acids. This observation suggested that the ionic interactions between the cationic polymers and negatively charged lipopolysaccharides (as lipoteichoic acid, a component of the thick peptidoglycan layer of Gram-positive bacteria) in the outer membrane can be responsible for the growth inhibition and lysis, through blockage of important nutrients flow such as Ca^{+2} and Mg^{+2} ion entering the cell [59].

Fresh food including fruit and vegetables may harbour a variety of microbes which priory originating from the environment where they grew. The microbes will keep growing along the postharvest handling and food processing and caused spoilage to the foods if no proper decontamination methods applied [60]. The growing and survival of these microbes with prolonged time especially during storage period will spoil the foods and cause foodborne illness when consumed by people outside. As reported by Chang and Fang [61], the survivability of *E. coli* O157:H7 and *S. typhimurium* in shredded lettuce within 10–12 days imposed a potential health risk to consumers. In this study, treatment with tap water is referring to the common washing methods applied by household. There were some researchers who reported the capability of tap water to reduce the total bacterial count around 2 to 3 $\log_{10}$ CFU/mL [62, 63]. However, in this study, the treatment with tap water only showed slight reduction compared to previous study. Brackett [64] had reported that the use of tap water for washing cannot completely remove the bacterial populations on food materials. Besides, there are limitations of using tap water in washing food materials which is due to the presence of chlorine residues in treated tap water. Chlorine residues have become a concern in food safety due to their potentiality to produce carcinogenic compounds such as trihalomethanes, haloacetic acids, haloketones, and chloropicrin when reacting with organic matter [65]. As stated by Gill and Badoni [66], reusing of processing water as sanitizer will make the tap water another source of cross-contamination. In this study, the bacterial reduction in treated grapes fruit was proportional with the increasing of *S. polyanthum* extract concentration and

soaking time. Research was in the similar of Abadias et al. [67] who also reported the reductions of microbial populations were increased as the concentration of sanitizer and washing time increased. However, study conducted by Tornuk et al. [68] proved that the ability of thyme sanitizer was affected by extract concentration while different exposure time did not give significant reduction on the bacterial populations in apple fruits. Therefore, the relative influence in terms of microbial inactivation was tap water < 0.05% < 0.50% < 1.00 < 5.00%.

As stated by Vilgis [69], the ideal sanitizer is when the panellists are unable to recognize the difference between treated and nontreated samples which gives the meaning of not much change occurring before or after the treatment was applied. Study reported by Kumudavally et al. [70] reported the effectiveness of clove extract on reducing the pathogenic microflora in fresh mutton until 4-day treatment at $25 \pm 2^{\circ}C$, at the same time giving no adverse effect on physical and sensory qualities. In correlation with that, Solomon et al. [71] had reported the organoleptic and chemical evaluations of suya (boneless meat pieces) after treating with basil extract for 30, 60, 90, and 120 mins. In their sensory analysis part, authors reported that the suya soaked with basil extracts enhances eating quality as it improved the flavour of meat. However, most of the panellists were not satisfied in terms of the final colour of treated suya (brownish green colour). In this study, grapes were accepted by panellist even after treating with highest concentration of extract (5%). From this observation it can be concluded that, generally, the treated samples which had been exposed to highest concentration and longest exposure time were accepted by panellist. That means *S. polyanthum* extract did not affect or change the physical characteristics of food samples after exposure to highest concentration of extract at maximum time of exposure.

Antimicrobial activity of herbs and spices varies widely, depending on the several factors including spices type, test medium, and types of pathogens. Moreover, microorganisms differ in their resistance to different types of spices and herbs. According to Kalemba and Kunicka [72], active components of herbs at low concentrations may interact synergistically with other factors including sodium chloride, acids, and preservatives to increase preservation. However, antimicrobial activity of herb derived has been reported to diminish during food processing [73]. Therefore, further studies on the efficacy of these natural antimicrobial agents in a range of food products as well as evaluation of potential interactions of antimicrobial compounds with components of food matrices such as fats, carbohydrates, and proteins are required.

5. Conclusion

In conclusion, susceptibility test is very important step in the screening of antibacterial activity of plant material. From the result, *S. polyanthum* leaves had antibacterial activity against wide spectrum of foodborne pathogens and are able to reduce microflora count in fresh fruits. Therefore the plant might be promoted to further tests towards its evaluation as a sanitizer or preservative in wide range of foods.

Conflicts of Interest

All authors state that they have no conflicts of interest.

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Research Article

Optimization, Purification, and Starch Stain Wash Application of Two New α-Amylases Extracted from Leaves and Stems of *Pergularia tomentosa*

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A continuous research is attempted to fulfil the highest industrial demands of natural amylases presenting special properties. New α-amylases extracted from stems and leaves of *Pergularia tomentosa*, which is widespread and growing spontaneously in Tunisia, were studied by the means of their activities optimization and purification. Some similarities were recorded for the two identified enzymes: (i) the highest amylase activity showed a promoted thermal stability at 50°C; (ii) the starch substrate at 1% enhanced the enzyme activity; (iii) the two α-amylases seem to be calcium-independent; (iv) Zn^{2+}, Cu^{2+}, and Ag^{2+} were considered as important inhibitors of the enzyme activity. Following the increased gradient of elution on Mono Q-Sepharose column, an increase in the specific activity of 11.82-fold and 10.92-fold was recorded, respectively, for leaves and stems with the presence of different peaks on the purification profiles. *Pergularia* amylases activities were stable and compatible with the tested commercial detergents. The combination of plant amylase and detergent allowed us to enhance the wash performance with an increase of 35.24 and 42.56%, respectively, for stems and leaves amylases. Characterized amylases were reported to have a promoted potential for their implication notably in detergent industry as well as biotechnological sector.

1. Introduction

Maltogenic amylases are widely distributed in microorganisms, plants, and higher organisms and constitute a subfamily of amylolytic enzymes [1, 2]. Through their transglycosylation activity, they were responsible for the solubility increase, the oxidative stability, the sweetness, and the carcinogenicity decrease [3, 4]. Belonging to this enzyme family, α-amylases (1,4-a-D-glucan glucanohydrolase) catalyze the hydrolysis of α-1,4 glycosidic linkage in starch and related polysaccharides. They represent approximately 25% of industrial enzymes in the global market [5]. Hence, they have an industrial importance which is intensified by their thermal resistance and adaptation to special processes as brewing and liquefaction process [6], paper and textile sectors [7], and heavy-duty and dishwashing detergents [8]. Currently, α-amylases were implied also in chemistry, clinical, pharmaceutical, or analytical process [9].

From the worldwide enzyme manufacturing, α-amylases are included in about 30% of the global detergent industries and in 90% of the solid-liquid laundry [10, 11]. Despite the continuous need of the discovery of new enzymes suitable for new detergent formulations, microbial amylases still have the crucial interest [12–15]. A few researches focused on plants amylases quite useful for cleaning purposes, such as the case of immobilized α-amylase soybean onto chitosan enhancing

the removal of starch stains [16]. With the ignorance of their local endogenous applications [17], the need to characterize plant amylases by means of their stability, specificity, optimal activity range, and metal dependence still remains.

The fact that α-amylases are commonly extracted and purified from different plant organs, such as tubers [18], leaves [19], seeds [20, 21], and stems [22], encourage us to focus on the plant source and mainly wild plants which are not studied in the literature and which may present interesting specific and unique properties. While plants have been considered as a source of abundant enzymes which were well searched in food sectors [23], less attention has been paid to species which can be included in industrial starch processing like *Triticum aestivum, Manihot esculenta,* and *Zea mays* [24].

Asclepiadaceae family was characterized by the richness of several species in different enzymes. *Asclepias fruticosa* contains asclepain in its latex [25] and calotropain was discovered in *Calotropis procera* [26]. A cysteine protease hydrolysing the fibrinogen was found in *Pergularia extensa* and *Cynanchum puciflorum* [27]. Belonging to this family, *Pergularia tomentosa* has gained a recent scientific importance due to its proteinases, rennin, polyphenol oxidase, tyrosinase, β-amylase, lipase, L-asparaginase, and lipoxygenase widespread in the latex compared to the crude extract of the whole plant [28–30]. According to the literature, this rangeland species of *Pergularia* contains several secondary metabolites, antioxidative bioactive molecules, and potent antifungal compounds against *Fusarium oxysporum* f.sp. *lycopersici* [31, 32]. It was used as a remedy in traditional medicine in case of tuberculosis, skin diseases, and constipation [33]. It presented anti-inflammatory [34], antidermatophytic [35], molluscicidal [36], and antimicrobial activities [37, 38] and it was known as anticancer agent [39] and insect repellent [40].

In view of the above, the present study reports the purification of α-amylases extracted from leaves and stems of the wild medicinal plant, *Pergularia tomentosa*. Several conditions were also optimized such as pH, temperature, and substrate concentration. Additional ions in the reaction medium were classified as activators or inhibitors to achieve high and better enzyme activity.

We also intended to evaluate the stain remover potential of extracted enzymes view of the high detergent industry requisition of amylases using low-cost raw materials. The main purpose of the enzyme application raised in this study is to investigate the cleaning efficiency of plant amylase combined to laundry detergent and its distinctive compatibility as a highly efficient new additive.

2. Materials and Methods

2.1. Plant Material. Pergularia tomentosa L. was identified and collected from the surrounding of Bir Ben Ayed (south of Sfax, Tunisia) [31]. Stems and leaves were rinsed carefully with distilled water to be purified from soil and air environmental impurities. Plants were deposited onto filter paper until relative drying after the rinsing step. Each organ was ground separately in the minimum of distilled water. The mixture was centrifuged at 5000 ×g for 30 min and the obtained supernatant was filtered through the filter paper (150 nm, 5B, Advantec Tokyo, Japan) to obtain a clear crude extract.

2.2. Precipitation of α-Amylase and Enzyme Assay. Protein precipitation with ammonium sulphate at 80% was carried out with a continuous gentle stirring in ice bath and was stored later overnight at 4°C [41]. The solution was centrifuged at 12,000 ×g for 30 min and at 4°C. The obtained precipitate was dissolved in distilled water and dialyzed against the same solvent for 24 h at 4°C and by changing the solvent thrice. Dialysis was carried out using cellulose tubing (molecular weight cut-off 13,000 kDa, Himedia LA393-10 MT).

The protein content was determined by the Bradford method [42] referring to bovine serum albumin. α-Amylase assay was carried out following the DNS (2-OH-3.5-dinitrobenzoic acid) method of Miller [43]. Optical density was measured at 550 nm against substrate and enzyme blank. One unit of amylase was defined as the enzyme amount which releases 1 μ mole of glucose per minute.

2.3. Effect of pH and Temperature. pH optimum was determined by incubating the amylase-substrate reaction for 10 min at different pH ranging from 4.0 to 10.0. Temperature optimization of amylase was determined by carrying the reaction mixture for 10 min (40–60°C) and keeping a constant pH [44]. pH stability was studied by preincubating 0.5 mL of enzyme with 0.1 M buffer at different pH values for 3 h at 4°C [45]. The thermal stability of α-amylase was tested by incubating the enzyme for 3 h at the determined pH optimum and at 50 and 60°C. Samples were withdrawn every 15 min and residual activity was determined.

2.4. Effect of Substrate Concentration and Metal Ions. Pergularia tomentosa α-amylases activity was determined at several starch concentrations at the range of 1 to 2.5% and dissolved in 0.1 M buffer at the pH optimum. The maximum activity was taken as 100% and relative activity was plotted against different concentration values.

Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}, and Co^{2+} at 1 and 5 mM were supplemented in the reaction medium of enzyme extract and starch solution and incubated for 30 min at pH and temperature optimum of each plant organ. The enzyme activity without addition of any ions (inhibitor or activator) was considered as 100%.

2.5. Purification of α-Amylase. The dialyzed fraction was heated at 60°C in water bath for 15 min and the denatured protein precipitate was removed by centrifugation, while the supernatant was checked for activity. Obtained active fraction was loaded onto a Mono Q-Sepharose column (2.1 × 24 cm) preequilibrated with 6.5 mM sodium phosphate buffer (pH 5.0) at 4°C. The same buffer was used to wash the column. Bounded proteins were eluted by a linear gradient of NaCl (0-1 M) in the same buffer at a flow rate of 5 mL min^{-1}. Protein content was determined at 280 nm according to the method

of Bradford [42]. Amylase activity of the recovered fractions was determined following the DNS method [43].

2.6. Compatibility of Stems and Leaves α-Amylases with Commercial Detergents. The compatibility of stems and leaves α-amylases with commercial available laundry detergents, Persil, Tide, and Savex, was determined. Detergent solutions with a concentration of 7 mg/mL were boiled for 90 min to inactivate any enzyme activity included on their formulation. Cooled solutions were mixed separately with each amylase (1 : 1) and incubated at 50°C for 1 hour. The residual activity was calculated in comparison with the control (instead of the detergent solution).

2.7. Efficiency of Stems and Leaves α-Amylases in Starch Stain Wash. Wash efficiency of starch stains was studied in the presence of Savex detergent and the two *Pergularia* α-amylases [46]. White cotton cloth pieces stained with starch solution (0.5%) were placed at 80°C for 30 min to assume the firm binding of stains to the material support. Washing performance was tested by varying the cleaner, as water, water + detergent, water + enzyme, water + detergent (7 mg/mL) + enzyme. Stained cotton cloth piece was incubated in the presence of the corresponding cleaner mixture on a shaker platform (100 rpm) for 30 min at 50°C. Obtained solution was collected for each mixture to measure the concentration of reducing sugars released from starch [43]. The blank consists in distilled water instead of wash liquid. The same assay procedure was followed for stems and leaves α-amylases.

The efficiency of starch removal by the washing process was expressed as the following equation [47]:

$$\text{Efficiency\%} = \frac{100 * A * 0.9}{B}, \quad (1)$$

where A is the amount of glucose released (g/mL) during the wash procedure and B is the amount of starch (μg/mL) used for staining the cotton cloth piece.

2.8. Statistical Analysis. Data were expressed as mean ± standard deviation and comparisons were made with appropriate controls using Student's t-test. Confidence limits were set at $p < 0.05$ for all values analyzed in triplicate.

3. Results and Discussion

3.1. Optimization and Characterization of α-Amylases. The highest amylase activity of *Pergularia tomentosa* was exhibited at pH 5.5 in case of leaves and at pH 6.0 for the stems (Figure 1). Between pH 4.0 and 6.5, the relative activity of stems amylase retained more than 60% of the maximal activity. However, in case of leaves, 60% of retained activity was observed at the range of pH 5.0–7.0. Beyond pH 8.0, the amylase activity loss was 68% of the initial relative activity. It may be due to the pH effect on the ionization of the group of lateral chains maintaining the enzyme structure and its influence on the active site activities. The pH optimum of amylase extracted from germinated seeds of *Glycine max* is similar to our studied stems [48], while leaves α-amylase

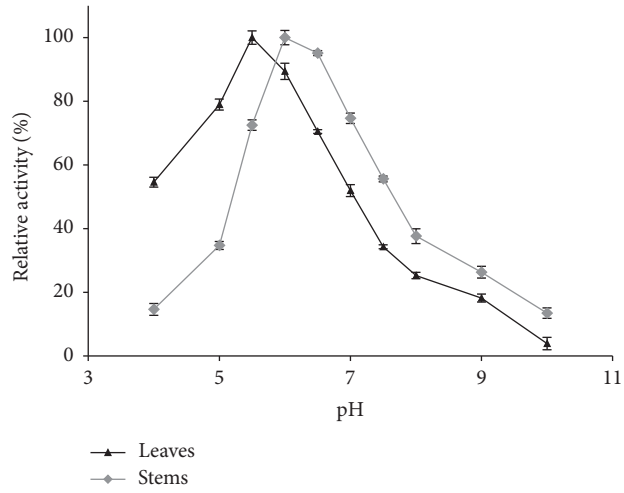

Figure 1: Effect of pH on the activity of α-amylases extracted from leaves and stems.

presented the same pH of *Carthamus tinctorius* amylase isolated from seeds [49].

Enzymes were incubated for 3 h in several buffers; more than 80% of leaves enzyme activity was retained between pH 5 and 8, suggesting that it was very stable despite the high pH (Figure 2(a)). Compared to the process of leaves amylase, a considerable loss of activity was observed in acidic pH for stems enzyme (Figure 2(b)). The latest amylase was kept stable in the pH range 6–8 and retained approximately 60% of the initial activity after 180 min of incubation.

The curves of the amylase activities as a function of the temperature looked bell-shaped with an optimum at 50°C (Figure 3). Both of stems and leaves curves coincide at the interval of 47 and 52°C. The increase of the temperature was relatively going with the α-amylases activity increase, between 40 and 50°C, as assayed at the pH optimum of each plant organ. Beyond the peak of 50°C, the activity began to decline roughly until the temperature changed from 53 to 60°C. Above 60°C, enzymes still retained more than 60% of their initial activities. The temperature optimum varies among species; furthermore, optimal activity of our identified α-amylases was slightly lower than *Vigna radiata* and *Pinus koraiensis* (65°C) [50].

Thermophilic amylases are mostly searched for starch industries applications [51]. Our leaves amylases are stable at 50°C beyond 150 min and lose just 2% of their initial activity after 90 min of incubation (Figure 4(a)). At 60°C and after 90 min of enzyme incubation, 38% of the initial activity was lost. In the case of stems, 55% of the initial activity remained at 60°C beyond 100 min (Figure 4(b)). The thermal stability of the studied enzymes greatly exceeds the results of Haifeng where the enzyme of *Aureobasidium pullulans* was completely denatured at 60°C after 50 min [52]. This higher percent of the activity retention and thermal stability further encourages the implication of *Pergularia tomentosa* L. in various practical sectors. The observed differences towards the process behavior may be due to the particular genetic heritage of each species [53]. And this thermal stability can

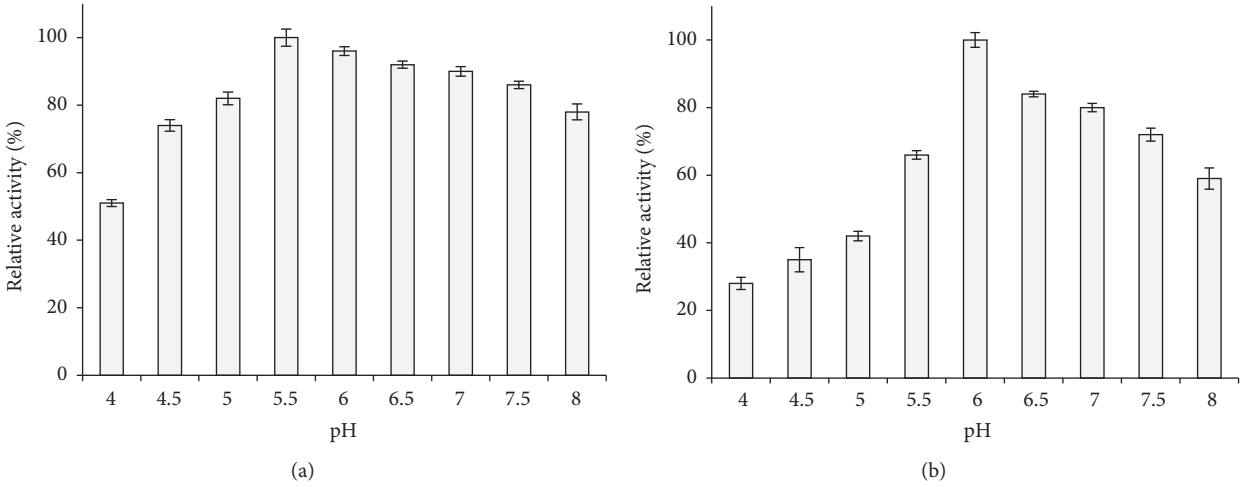

Figure 2: pH stability of α-amylases extracted from leaves (a) and stems (b).

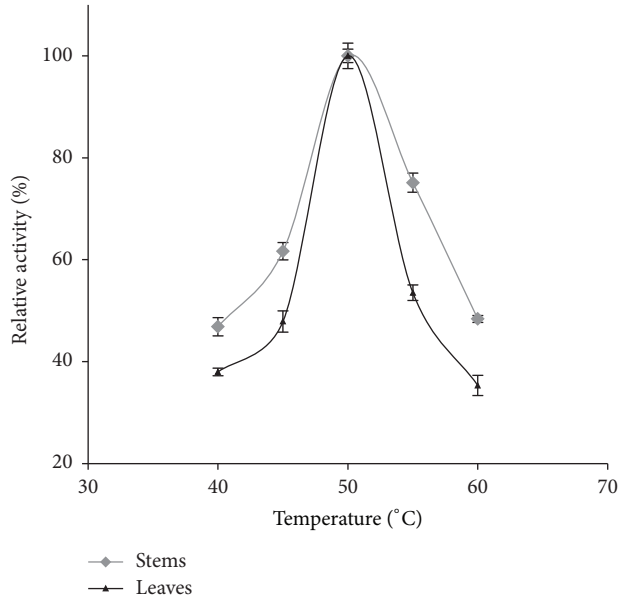

Figure 3: Effect of temperature on the activity of α-amylases extracted from leaves and stems.

be attributed to the presence of some secondary and tertiary binding of the enzymatic proteins enhancing the enzyme structure consolidation and its resistance to the thermal treatment [54].

The effect of substrate concentration was maximal at 1.0% of starch solution in case of leaves and stems (Figure 5). This concentration was also frequently used for amylase assay in previous researches [55, 56]. However, the substrate concentration starting to increase from 0.5% was significantly followed by the enzyme activity increase with enhancement of 23.81 and 31.74%, respectively, for leaves and stems. Then and at 1%, the activity declines gradually, whereas, the shape of the decrease curve for stems was wider in comparison with leaves, especially between the concentrations of range 1–1.5% where there was just 11.56% of enzyme activity lost. The lowering of amylase activity can be explained by the fact that all of the substrate binding sites were filled.

Leaves α-amylase was inhibited by all the tested ions metals, whereas stems α-amylase was activated by Co^{2+} with an increase of 35% of the relative activity and it was inhibited by all other metal ions with a variable extent. It was found that calcium has a negative effect, particularly in increasing the concentration. The same process was observed with the amylase of Fenugreek seeds [45]. This effect may be due to the metal competitions and/or to the particularity of the enzyme structure. The known inhibitors Zn^{2+} and Cu^{2+} [57] induce, respectively, a different decrease in enzyme activity at 5 mM. In case of leaves and at 5 mM, Zn^{2+} leads to 87% of activity inhibition and 72% for the stems amylase. At the same concentration, the inhibition of Cu^{2+} was more pronounced and quasi-total. Ag^{+} reported as a strong inhibitor at 2 mM [58] induces a decrease of 82 and 91%, respectively, of amylase from leaves and stems. Thus, among all the metal ions presented in Figure 6, Co^{2+}, Mg^{2+}, and Ca^{2+} seem the weaker inhibitors of the extracted amylases.

3.2. Purification of α-Amylases. The purification profiles of α-amylases were shown in Figure 7. The anion exchange chromatography of amylase extracted from leaves on Mono Q-Sepharose column eluted with a linear increased gradient of NaCl showed three peaks of activity and just two distinct peaks were revealed in case of stems.

As summarized in Table 1, amylases extracted from the two studied plant organs seemed totally different in view of their specific activities, purification fold, and yield, as well as their profiles after elution with NaCl as shown in Figure 7.

The purification procedure of leaves α-amylase leads to a 11.82-fold increase in specific activity for the elution in the range of 25–149 mM NaCl. It was considered as the highest fold in comparison with the two other peaks, while the higher yield of 38.33% was registered for the peak eluted in

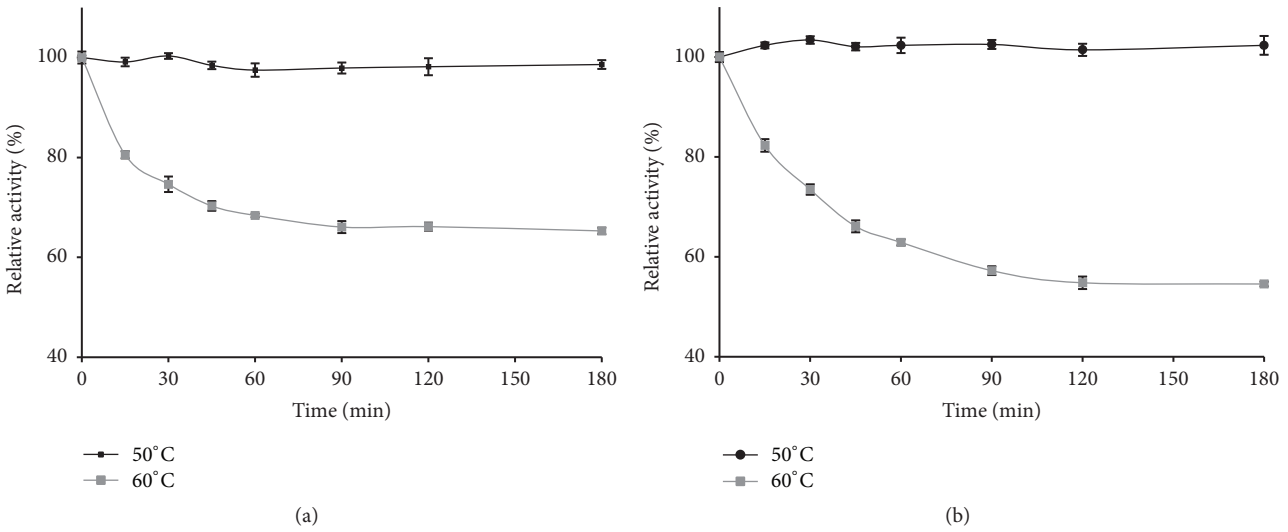

Figure 4: Thermal stability at 50 and 60°C of α-amylases extracted from leaves (a) and stems (b).

Table 1: Purification of α-amylases extracted from leaves and stems of *Pergularia tomentosa*.

Step	Specific activity (U/mg)	Purification (fold)	Yield (%)
Leaves			
Crude extract	0.547	-	-
Heat treatment	0.558	1.012	76.905
Anion exchange chromatography			
25–149 mM NaCl	6.463	11.821	17.19
225–398 mM NaCl	2.672	4.887	38.333
402–450 mM NaCl	3.787	6.927	23.81
Stems			
Crude extract	0.554	-	-
Heat treatment	0.619	1.117	84.567
Anion exchange chromatography			
290–435 mM NaCl	5.41	9.756	28.224
620–690 mM NaCl	6.048	10.924	27.272

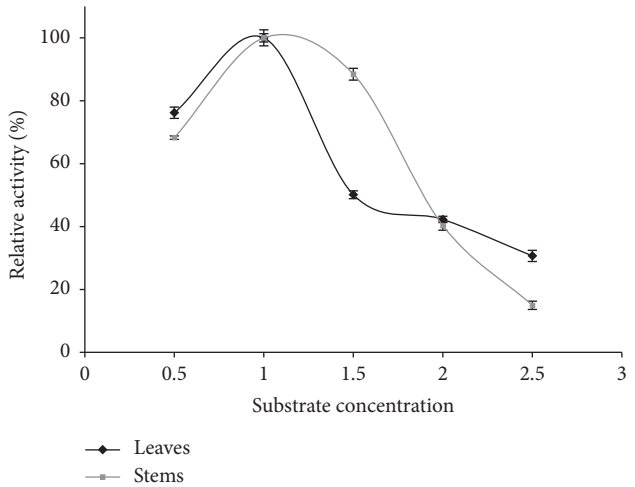

Figure 5: Effect of substrate concentration on the activity of α-amylases extracted from leaves and stems.

225–398 mM NaCl. The main purification fold for stems α-amylase was recorded for the peak eluted between 620 and 690 mM NaCl with a yield of 27.27%. Nevertheless, further steps of extract concentration and purification processes such as affinity chromatography could be used to have more pure α-amylases from *Pergularia tomentosa*.

3.3. Application of Stems and Leaves α-Amylases. The above results confirm the largest activity spectra of the two amylases extracted from stems and leaves of *Pergularia tomentosa* at a wide range of pH and temperature. The revealed interesting retained activity at alkaline pH and moderate temperature and the exhibited amylases stability were considered as important criteria during the manufacture of commercial detergents and the degradation of starchy stains residues. The negative effect of calcium on *Pergularia* enzyme activity is searched to fulfil imperfect detergents suffering from oxidants sensitives and calcium-dependent α-amylases [59].

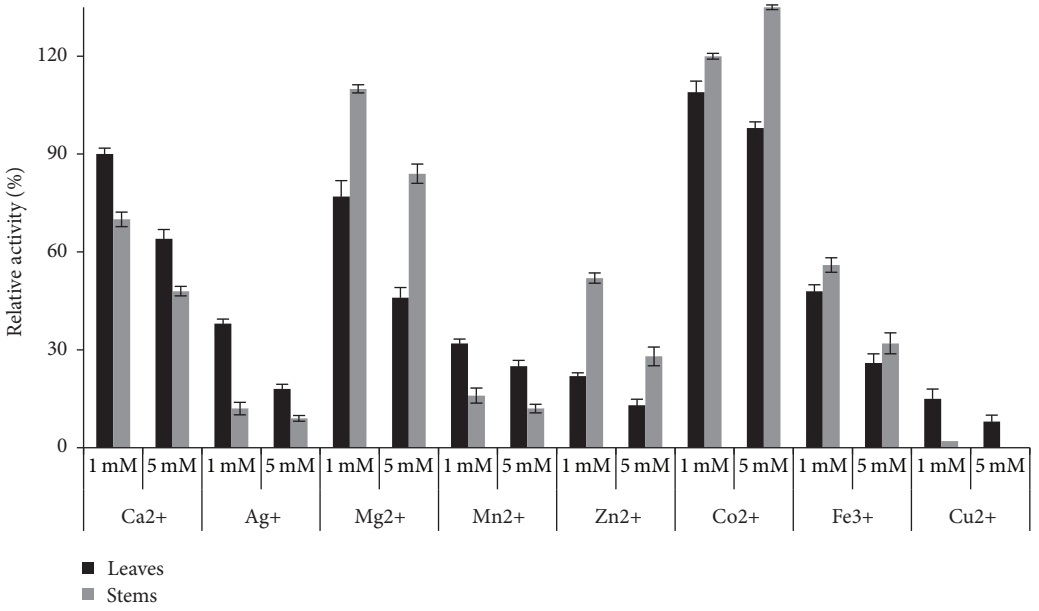

Figure 6: Effect of metal ions on α-amylase extracted from leaves and stems.

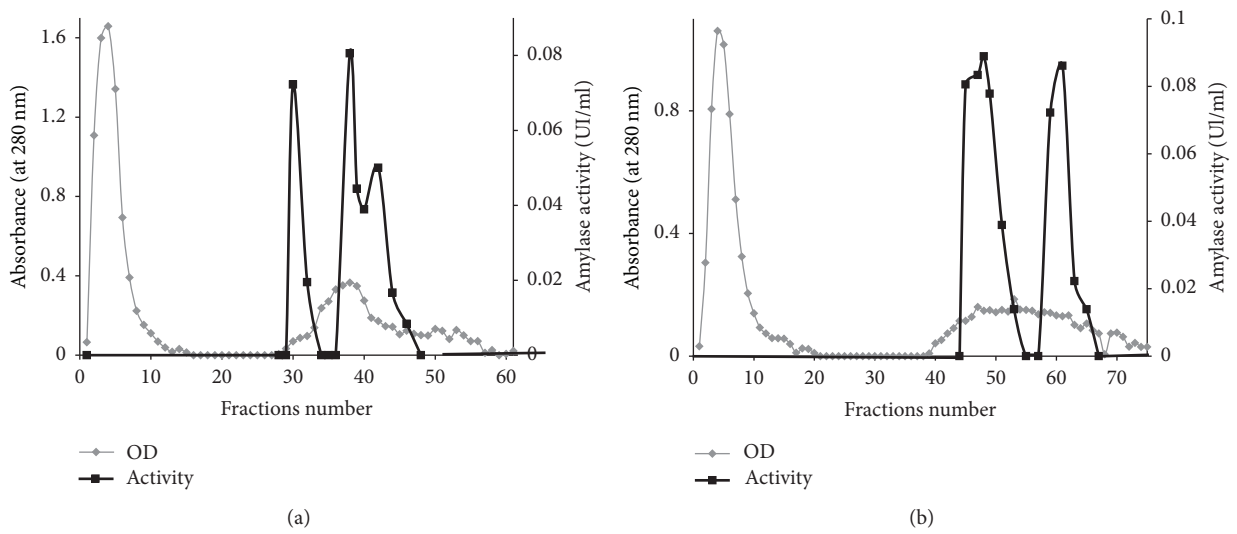

Figure 7: Anion exchange chromatography of α-amylase extracted from leaves (a) and stems (b) in Mono Q-Sepharose column eluted with an increased gradient of NaCl.

Out of the vast pool of microbial amylases enhancing whiteness effect, a similar formulation based on a wild-plant enzyme promotes widespread environmentally safe and low-cost detergents especially in rural areas [60].

However, a lucky inclusion of enzyme in the detergent formulation requires a good compatibility [61]. The data presented in Table 2 showed excellent stability and compatibility of stems and leaves α-amylases of *Pergularia tomentosa* with the tested three commercial detergents.

According to the results and compared to the two other detergents, detergent A was considered the lowest compatible with stems and leaves amylases via the obtained residual activities, respectively, 65.22 and 80.5%. Furthermore, leaves amylases seem to be more compatible with detergents A and C than stems amylase with a quasi-compatibility with

Table 2: Effect of different detergents on the residual activity of extracted α-amylases from stems and leaves of *Pergularia tomentosa*.

Additive	Residual activity of stems amylase (%)	Residual activity of leaves amylase (%)
Control	100	100
Detergent A	65.22 ± 0.08	80.5 ± 0.93
Detergent B	82.63 ± 1.14	81.80 ± 0.54
Detergent C	87.91 ± 1.5	94.05 ± 1.32

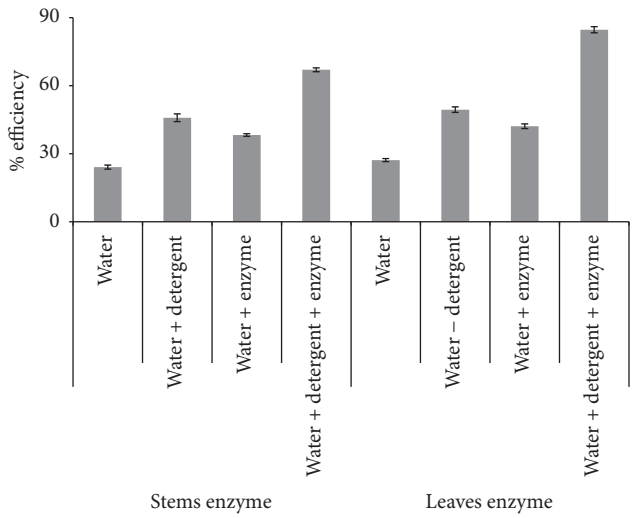

Figure 8: Efficiency of starch stain removal of stems and leaves α-amylases.

the detergent B. The detergent C was found to be more compatible with the two amylases, by retaining an enzyme activity of 87.91% and 94.05%, respectively, for stems and leaves.

Application of α-amylases is still very limited and a few studies reported the possibility of their implication in wash performance implying their compatibility with detergents, powder as well as liquid. Besides, the obtained results presented in Table 2 could be compared to previous researches yet they focused just on amylases extracted from fungi and bacteria [12, 13]. In the data described above, thermal-stable α-amylases from *Pergularia tomentosa* should be suggested as a competitive additive in detergent formulations, while the detergent effect on the residual activity of the discovered amylases may be attributed to the detergent composition [13].

Previous studies reported the efficiency of bacterial α-amylase towards several raw starch sources, for instance, soluble starch, potato curry, corn, and wheat starches [14] as well tomato sauce and egg yolk [15]. Figure 8 reveals that the combination of water, detergent, and α-amylase of stems and even leaves of *Pergularia tomentosa* greatly enhances the ability to remove starchy stains from cotton cloth pieces compared to the mere use of detergent or α-amylase. The revealed increase of washing efficiency by the supplement of amylases to detergent was in accordance with other studies [14, 47]. In fact, this combination was significantly improved when leaves α-amylase was added with an increase of 35.24% and 42.56%, respectively, with the simple enzyme wash and with the detergent wash.

The two *Pergularia tomentosa* α-amylases could be integrated in industrial sectors as catalysts of stains removal and incorporated in different formulations of detergents. It could solve the problems of human skin sensitivity and side effects of the detergents residues evacuated in the environment by decreasing the amount of industrial components like surfactants, bleach, and cobuilders through the challenge of natural α-amylases. The reputable thermal stability of *Pergularia* amylases minimising the risk of contamination and the diffusion rate warrants further investigation for further industrial and biotechnological applications with a low-cost of external cooling [62].

4. Conclusion

The described work in this paper was attempted to characterize and purify plant α-amylases which could be exploited in several fields like the hydrolysis of oil-field drilling fluids and the paper industry. The study of biochemical characteristics of amylases identified from *Pergularia tomentosa* showed a promising range of pH stability and an interesting thermal stability especially at 50°C without requirement of calcium. The simple and cheap extraction procedure of the new stems and leaves α-amylases as well as the interesting purification fold and yields raises the great potential of our studied plant in starch stain removal as a source of biological active substances. By immobilization procedure, the amylases properties may be also improved to be implied in successful and modern biotechnology sectors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Antimicrobial Natural Product Berberine Is Efficacious for the Treatment of Atrial Fibrillation

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The purpose of this study is to test the efficacy of bioactive natural product berberine in the treatment of patients with atrial fibrillation (AF). Data of 45 paroxysmal AF patients treated with berberine and 43 age, gender, New York Heart Association functional classification score, and concomitant cardiovascular disease matched patients treated with amiodarone were analyzed retrospectively to examine conversion rate, average conversion time, average heart rate, and echocardiographic parameters. There was no statistical difference between berberine and amiodarone on conversion rate or echocardiographic parameters. Berberine treatment showed a significantly longer average time to conversion and higher heart rate during sinus rhythm (SR) than amiodarone. Echocardiographic parameters showed that E/A ratio and left atrial diameter were significantly improved after 6- and 12-month berberine treatment, but only E/A ratio improved significantly at the same time points after amiodarone treatment. This is the first report to specifically compare efficacy of berberine and amiodarone in the treatment of patients with AF. We find that berberine and amiodarone are equally effective for conversion of AF and maintenance of normal SR.

1. Introduction

Bioactive natural products have been reported to exhibit antioxidant, antimicrobial, antiviral, anticancer, and anti-inflammatory activities [1]. Berberine, originally used as an antimicrobial medication for the treatment of diarrhea [2], is a natural product isoquinoline alkaloid isolated from Chinese medicinal plant *Coptis chinensis* [3]. Berberine's potential antiarrhythmic properties were first explored in a cohort of 50 patients with ventricular tachyarrhythmia conducted in Shanghai Xuhui Central Hospital by Dr. Huang et al. in 1985 [4]. Huang's team further published results of antiarrhythmic mechanisms of berberine in a canine model in 1992 [5]. Later Dr. Liu and Rao reported using berberine as an antiarrhythmic drug to treat 28 patients with ventricular premature beats in 1994 [6]. These early data showed potential for this agent as an antiarrhythmic with only minor gastrointestinal side effects but without comparing to other antiarrhythmics. The purpose of this study was to test the efficacy of berberine in the treatment of patients with atrial fibrillation (AF) in comparison with amiodarone, a frequently prescribed and widely used Vaughn-Williams class III agent efficacious in converting AF to sinus rhythm (SR) [7, 8].

2. Methods

This study was approved by the Ethics Committee of Shanghai Xuhui Central Hospital (Approval number 2013-12) with ethical standards of the Helsinki Declaration of 1964. To extend indication of berberine for AF, consecutive patients randomly received berberine or amiodarone treatment between January 2013 and March 2014 based on attending physician discretion. We conducted a retrospective cohort study in this patient database to compare the efficacy of berberine and amiodarone in the treatment of AF. Eligibility for inclusion included symptomatic paroxysmal AF (PAF) patients

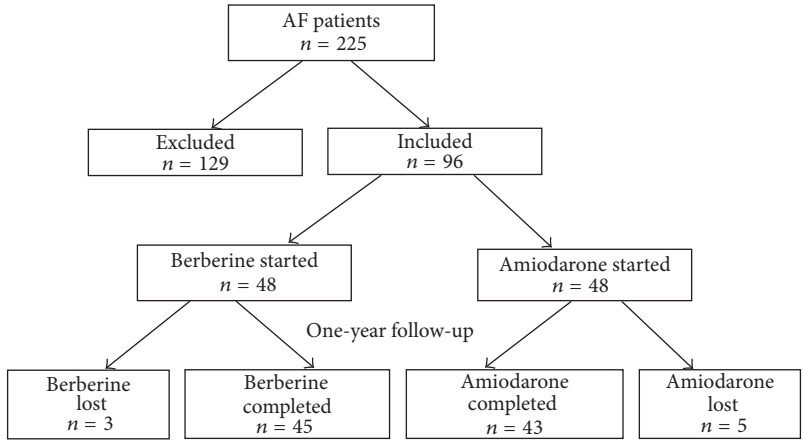

Figure 1: *Flow chart of patient selection.* From a database of 225 patients between January 2013 and March 2014, 96 met the inclusion criteria and 129 were excluded because of noneligibility. Data from 48 atrial fibrillation (AF) patients treated with berberine and 48 matched AF patients treated with amiodarone were included. Follow-up at one year was complete in 45 and 43 of berberine and amiodarone treated patients, respectively. Three patients were lost in berberine group and 5 patients were lost in amiodarone group.

diagnosed by electrocardiogram (ECG) with history of 6 months–2 years, presenting to the emergency room with symptomatic AF and onset time of 3 hours–7 days, and they were aged between 20 and 80 years old and had oral administration of either berberine or amiodarone for 12 months. Patients were excluded from the analysis if they had ECG evidence of second-degree atrioventricular block, a diagnosis of sick sinus syndrome, thyroid dysfunction, severe lung disease, severe electrolyte disorder, severe hepatic disease, renal insufficiency, and stroke or any reversible cause of AF. Berberine and amiodarone treated patients were matched by age, gender, New York Heart Association (NYHA) functional classification score, and concomitant cardiovascular disease (CVD).

2.1. Study Procedure. Flowchart of patient selection was shown in Figure 1. From a database of 225 patients, 96 met the inclusion criteria and 129 were excluded because of noneligibility. Data from 48 AF patients treated with berberine and 48 matched AF patients treated with amiodarone were included. Follow-up at one year was complete in 45 and 43 of berberine and amiodarone treated patients, respectively. Three patients were lost in berberine group and 5 patients were lost in amiodarone group. Antiarrhythmic drugs were initiated during AF. No electrical cardioversion was applied to these PAF patients. Study schedule was shown in Supplementary Figure 1. The oral administration dose of berberine was minimal at 1.2 g/day (0.3 g qid) and maximal at 2.0 g/day (0.5 g qid), and the average dose is of 1.3 g/day for 1 year. Amiodarone was taken orally with an initial dose of 0.6 g/day (0.2 g tid) in the first week and then 0.4 g/day (0.2 g bid) in the second week followed by 0.2 g/day (0.2 g qd) in the third week and lasted for 11.5 months. Surface ECG was performed for each patient every week in the first month followed by once a month in the rest of the year. Echocardiography and blood test were conducted and adverse events were recorded at the follow-up time points of 1, 3, 6, and 12 months.

2.2. Outcome Assessment. The primary outcome was presence of SR after antiarrhythmic treatment with berberine or amiodarone at 1, 3, 6, and 12 months. The secondary outcome included average time from onset of therapy to conversion of AF to SR and heart rate in SR after successful treatment. The third outcome consisted of Doppler echocardiographic parameters E/A ratio (ratio of the early to late ventricular filling velocities) and left atrial diameter (LAD) measured at the follow-up time points of 1, 3, 6, and 12 months. The fourth endpoints were adverse events.

2.3. Statistical Analysis. The primary outcome time-to-event (conversion to SR) was compared between berberine and amiodarone groups by the Kaplan-Meier method. The statistically significant difference of two groups was analyzed by Log rank test. To adjust for potential confounding, the difference between two groups was further analyzed by Cox proportional hazard model. In the model analysis, we adjusted for the effects of age, sex, AF duration, frequency, history, and smoking status. Other endpoints between groups were compared by ANCOVA analysis with baseline value of the dependent variable as covariates.

3. Results

Baseline characteristics of the 88 patients followed to one year including age, gender, PAF history, smoking status, biochemical measurements, NYHA Functional Classification, comorbidities, and concomitant therapies were summarized in Table 1; there was no significant difference between berberine and amiodarone groups.

3.1. Proportion in SR. The number of patients conversion to SR at time points of 1, 3, 6, and 12 months was summarized in Table 2, while the percent conversion to SR was plotted by Kaplan-Meier curve in Figure 2. A trend toward a larger proportion of the berberine treated group in SR at 12 months

Table 1: Characteristics of PAF patients by berberine and amiodarone groups.

Category	Berberine $N = 45$	Amiodarone $N = 43$	Significance
Age (year)	65 ± 11	63 ± 12	NS
Male	28 (62.2%)	27 (62.8%)	NS
Duration (hour)	48	46	NS
Frequency (time/month)	5.5	6.3	NS
History (month)	9.5	9.3	NS
Smoking status			
Never smoking	22 (48.9%)	20 (46.6%)	NS
Smoking < 5 year	8 (17.8%)	9 (21%)	NS
Smoking ≥ 5 year	15 (33.4%)	14 (32.6%)	NS
K (mmol/L)	4.1	4.2	NS
Mg (mmol/L)	1.0	1.1	NS
TSH (u/ml)	1.5	1.4	NS
ALT (u/ml)	17	18	NS
AST (u/ml)	16	18	
CR (μmol/L)	76	74	NS
PT (sec)	12.2	12.8	NS
INR	0.92	0.95	NS
NYHA classification			
II	31	29	NS
III	14	14	NS
Concomitant CVD			
CAD	39	36	NS
DCM	6	7	NS
HTN	41	40	NS
HF	2	3	NS
Concomitant medication			
Aspirin	21	20	NS
Nitrates	22	21	NS
ACE inhibitors	41	41	NS
Statins	21	20	NS

Notes. ACE, angiotensin-converting enzyme; ALT, alanine transaminase; AST, aspartate transaminase; CAD, coronary artery disease; CR, creatinine; CVD, cardiovascular disease; DCM, dilated cardiomyopathy; HF, heart failure; HTN, hypertension; INR, international normalized ratio; K, potassium; Mg, magnesium; NS, nonsignificant between groups; NYHA, New York Heart Association; PT, prothrombin time; TSH, thyroid-stimulating hormone.

Table 2: Reversion rates of sinus rhythm in berberine and amiodarone groups.

Group	N	Follow-up time			
		1 month	3 months	6 months	12 months
Berberine	45	42 (93.3%)	40 (88.8%)	38 (84.4%)	35 (77.7%)
Amiodarone	43	37 (86.1%)	35 (81.3%)	31 (72%)	28 (65.1%)*
Significance		NS	NS	NS	NS

Notes. * $p < 0.05$ 1 month versus 12 months; NS, nonsignificant between two groups.

was noted; however, when comparted with amiodarone, this was not statistically significant when analyzed by the Cox proportional hazard model after adjusting for the effects of age, gender, AF duration, frequency, history, and smoking status ($x^2 = 3.156$, $p = 0.0756$). There was a high proportion of SR in both groups at 1 month but noticeable attrition over time with a slight statistical difference between 1-month and 12-month time points in the amiodarone treatment group ($p = 0.04$).

3.2. Time to Conversion and Heart Rate. Patients were closely monitored during the first day of berberine or amiodarone

Table 3: Echocardiographic parameter improvement over time.

Group	Parameter	Before treatment (baseline)	After treatment			
			1 month	3 months	6 months	12 months
Berberine	E/A	0.94 ± 0.37	0.96 ± 0.36	1.05 ± 0.37	1.15 ± 0.39*	1.26 ± 0.35#
Amiodarone	E/A	0.95 ± 0.31	0.97 ± 0.30	1.04 ± 0.34	1.14 ± 0.33*	1.25 ± 0.32*
Significance		NS	NS	NS	NS	NS
Berberine	LAD (mm)	36.9 ± 13.5	35.5 ± 12.5	33.6 ± 12.5	30.8 ± 13.1*	30.5 ± 13.6*
Amiodarone	LAD (mm)	35.5 ± 13.9	34.9 ± 12.9	32.8 ± 12.1	31.4 ± 13.9	30.1 ± 13.8
Significance		NS	NS	NS	NS	NS

Notes. * $p < 0.05$ baseline versus 6 months or 12 months; # $p < 0.01$ baseline versus 12 months; E/A, ratio of the early to late ventricular filling velocities; LAD, left atrial diameter; NS, nonsignificant difference between two groups.

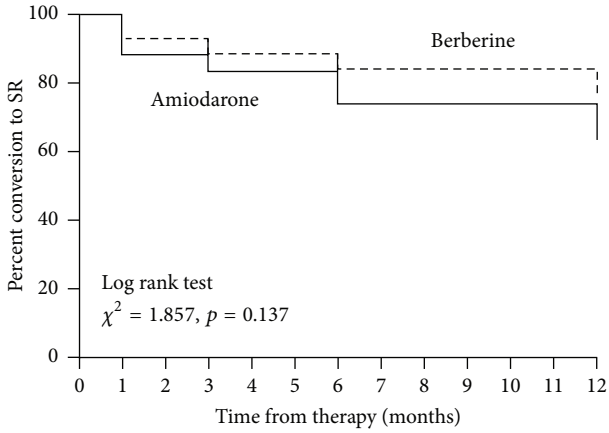

Figure 2: *Percent conversion to sinus rhythm.* Percentages of patient conversion of atrial fibrillation (AF) to sinus rhythm (SR) were plotted by Kaplan-Meier curve after 1-, 3-, 6-, and 12-month berberine or amiodarone treatment. There was no significant difference between two groups at any time points by Log rank test ($x^2 = 1.857$, $p = 0.137$). Berberine: dashed line; amiodarone: solid line.

oral therapy. The average time to achieve SR in the patients given berberine was significantly longer than that in amiodarone group (356 ± 135 versus 211 ± 126 min, $p < 0.01$). The average heart rate during SR measured at 1-month follow-up in berberine group was significantly higher than that in amiodarone group (83.5 ± 11.6 versus 69.3 ± 12.5/min, $p < 0.01$).

3.3. Echocardiographic Parameters. Measurements of echocardiographic parameters E/A ratio and LAD were summarized in Table 3. No statistical difference in the analyzed echocardiographic parameters E/A ratio and LAD was detected between berberine and amiodarone groups before treatment and 1, 3, 6, and 12 months after treatment. Nevertheless, the ANCOVA analysis did reveal a significant effect for time, $F(4, 344) = 14.32$, $p < 0.001$. In the berberine group, post hoc contrasts found that the values of E/A at 6- ($p < 0.05$) and 12-month time points ($p < 0.01$) were significantly improved than the measures observed before treatment. The LAD measures showed similar results ($p < 0.05$ at both 6- and 12-month time points). In the amiodarone group, only E/A ratio improved after 6- and 12-month ($p < 0.05$) treatment. LAD did not change statistically after amiodarone treatment at any follow-up time points.

3.4. Adverse Events. Five from the initial 48 amiodarone treated PAF patients did not complete 1-year treatment, in which 2 had dysthyroidism. Minor side effects were observed in 5 of the 43 patients in amiodarone group: 2 had transient sinus bradycardia, 2 had nausea, and 1 had dizziness. After reducing dose or withdrawing medication for several days, these reactions disappeared. Meanwhile, there were no severe adverse events recorded in the 45 patients who completed 12-month berberine treatment. But 3 patients from berberine group were lost during the study whose possible adverse events were not recorded. Only data of patients who were compliant were collected for this study.

4. Discussion

This retrospective study provides some support for an antiarrhythmic effect of berberine in patients with PAF. Although the primary outcome of proportion of SR did not show a statistical difference between groups, our data appear to demonstrate at least equivalence with the most effective antiarrhythmic medication available in clinical studies [7–9] and improved echocardiographic parameters for both E/A ratio and LAD after berberine treatment, but only E/A ratio improved in amiodarone group.

Amiodarone is not only the most commonly used antiarrhythmic medication in Chinese AF patients [10], but also the most-often-prescribed in developed countries, accounting for 34.5% of prescriptions in Europe and 32.8% in North America [8]. Nevertheless, long-term high dose administration of amiodarone is associated with both cardiac and noncardiac adverse effects, including sinus bradycardia, decreased blood pressure, pulmonary toxicity, liver toxicity, optic neuropathy, skin discoloration, and thyroid dysfunction [11]. We observed amiodarone side effects such as minor nausea and dizziness, transient sinus bradycardia, and dysthyroidism during this study. Therefore, finding alternative antiarrhythmic drugs with similar or better efficacy but less side effects is extremely desirable.

Berberine has been reported to be effective for *E. coli* diarrhea with a low dosage of 0.4 g/day but has no efficacy for *Vibrio cholera* even with a high dosage of 1.2 g/day [2].

Our data demonstrated that berberine with a minimal oral administration dose of 1.2 g/day had a nonsignificant trend toward higher proportion of SR during the one-year follow-up when compared with amiodarone (Figure 2). However, berberine needed a significantly longer time to reach SR (356 ± 135 versus 211 ± 126 min, $p < 0.01$) and a higher heart rate in SR (83.5 ± 11.6 versus 69.3 ± 12.5/min, $p < 0.01$) in comparison to amiodarone after successful treatment. These phenomena imply that berberine is efficacious at rhythm control but may not result in any rate control during AF, judged by the average heart rate in SR. The possible explanation of the slow effect of berberine is that its antiarrhythmic mechanism is via a reduction in potassium current in a concentration-dependent manner, which takes a longer time [5].

Although berberine and amiodarone were statistically equally effective in maintenance of SR after 1, 3, 6, and 12 months of treatment, evidence of reverse atrial remodelling was only statistically significant for berberine with reduction in LAD size demonstrated at 6 months ($p < 0.05$) and 12 months ($p < 0.01$) supporting a potent antiarrhythmic effect and true reduction in burden of AF in this cohort. In addition, there were no serious adverse events recorded during the 12-month berberine treatment. This result was consistent with another clinical trial with a higher berberine daily dose of 1.5 g for 16 weeks, in which minor adverse events such as anorexia, upset stomach, diarrhea, and constipation were recorded, but no serious adverse events such as heart failure, bone fractures, and liver toxicity were observed [12]. Nevertheless, our retrospective setting limited the choice of variables to record for analysis. Three lost patients from berberine group may have adverse events but were not recorded. Included patients who completed 1-year berberine treatment were less likely to have severe adverse effects.

In summary, our results are novel as no previous clinical studies have specifically compared the efficacy of berberine and amiodarone for the treatment of AF. We find that berberine and amiodarone are equally effective at maintenance of SR. The health beneficial effect of berberine is confirmed and the clinical application of berberine is promising. The knowledge of adverse effects with berberine is limited; however monitoring the safety of berberine in the high dose range is important. Therefore, further prospective studies are needed to evaluate the long-term efficacy and side effect profile of berberine in a contemporary AF population.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Hongchao Zheng and Fu Zhu contributed equally to this study.

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Supplementary Materials

Supplementary Figure 1. Study procedure. A. Berberine was administrated orally at minimal dose of 1.2 g/day (0.3 g qid) and maximal dose of 2.0 g/day (0.5 g qid) with an average dose of 1.3 g/day for 1 year. B. Amiodarone was taken orally with an initial dose of 0.6 g/day (0.2 g tid) in the first week and then 0.4 g/day (0.2 g bid) in the second week and followed by 0.2 g/day (0.2 g qd) in the third week and for the rest of the year. Follow-up time points were 1, 3, 6, and 12 months after treatment. *(Supplementary Materials)*

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Research Article

Paeoniflorin, the Main Active Ingredient of Shuyu Capsule, Inhibits $Ca_v1.2$ and Regulates Calmodulin/Calmodulin-Dependent Protein Kinase II Signalling

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The aim of this study was to explore the mechanism underlying the antidepression activity of paeoniflorin, the main active ingredient of paeony extract and Shuyu capsules, and determine its effect on the calmodulin/calmodulin-dependent protein kinase II (CaM/CaMKII) signalling pathway and on the possible target, the voltage-gated calcium channel (Ca_v). Rats at the nonacceptance stage were selected for premenstrual syndrome (PMS) depression modelling. Behavioural assays were used for model testing. Rats were given Shuyu capsules, paeony extract, and bupleurum. Western blot analysis was used to assess the expression levels of calcium voltage-gated channel subunit alpha 1 C (CACNA1C), brain-derived neurotrophic factor, and CaM/CaMKII signalling pathway proteins. Intracellular Ca^{2+} concentration in CHO cell line was measured using Fluo-4-AM and whole-cell patch clamps. The PMS depression model was successfully established and demonstrated that Shuyu can mitigate depressive behaviour in a rat PMS model. Paeony extract did not affect CACNA1C protein expression in rat hippocampi but did affect $Ca_v1.2$-mediated CaM/CaMKII signalling pathways. Paeoniflorin significantly inhibited KCl-induced increases in intracellular Ca^{2+} concentration and $Ca_v1.2$ current density. Further, it may function via the CaM/CaMKII pathway and its downstream signalling molecules by regulating $Ca_v1.2$, thus playing an important role in the treatment and alleviation of affective disorders.

1. Introduction

Premenstrual syndrome (PMS), commonly encountered in clinical gynaecology, refers to a series of mental and physical symptoms, including anxiety, breast pain, and headaches, which occur during the premenstrual period (luteal phase) in 20~50% of reproductive-aged women [1]. Symptoms lessen or disappear after menstruation but recur with each menstrual cycle [2, 3]. Epidemiological surveys conducted worldwide suggest that reproductive-aged women experience one or more PMS-related symptoms [4] that significantly influence their health and quality of life. PMS depression is characterized by symptoms including negative affect (depression, anger, aggression, crying spells, mood swings, tension, irritability, and anxiety), water retention, and pain [5, 6], and the incidences of these symptoms have recently increased, attracting increased attention from medical fields.

Shuyu, a commercially available herbal prescription of traditional Chinese medicine, comprises four herbal ingredients: Radix Bupleuri (*Bupleurum chinense* DC.), Radix Paeoniae Alba (*Paeonia lactiflora* Pall.), Rhizoma Cyperi (*Cyperus rotundus* Linn.), and Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.). Studies have shown that Shuyu can mitigate PMS depression symptoms and that its action mechanism is concentrated in specific cerebral areas [7, 8]. Following intragastric administration of Shuyu or paeony extract, paeoniflorin, one of the constituents, is absorbed into the blood [9]. Paeoniflorin, the main component of Shuyu

and paeony extract, has many biological effects, including enhancement of cognitive ability, improvements in learning disabilities, and nerve protection [10–12], suggesting that it may be the main active substance of Shuyu capsule that has psychological and, therefore, antidepressant, effects. However, the mechanism by which paeoniflorin exerts its antidepressant effect is still poorly understood.

Calcium dysregulation could be related to PMS and researches revealed a significantly decreased Ca^{2+} concentration in the blood corpuscles and serum of PMS patients [1, 13]. Voltage-gated calcium channels regulate intracellular Ca^{2+} concentration. *CACNA1C* encodes the α1C subunit of the L-type voltage-gated calcium channel (LTCC), known as $Ca_v1.2$, which is the main subunit of LTCC. $Ca_v1.2$ is a transmembrane channel protein and contains the binding sites for dihydropyridine drugs. In recent years, the relationship between *CACNA1C* and affective disorder has been widely studied. Genomewide association studies show that *CACNA1C* polymorphisms are closely related to depression, schizophrenia, and bipolar disorder and are causes of various affective disorders, making them a potential new target for treating mental and emotional disorders [14]. High levels of intracellular Ca^{2+}, regulated by Ca_v, increase the levels of Ca-bound calmodulin (CaM), which in turn activates calmodulin-dependent protein kinase II (CaMKII), which is involved in regulating the synthesis and release of proteins and neurotransmitters including brain-derived neurotrophic factor (BDNF), 5-hydroxytryptamine, dopamine, and glutamate, all of which are related to the incidence of PMS. In this study, paeoniflorin was applied to CHO cells stably expressing Ca_v *in vitro*, and a PMS depression rat model was used to explore the mechanism by which paeoniflorin exhibits an antidepressant effect. In particular, we studied the expression of key proteins in the Ca_v downstream signalling pathway under paeoniflorin treatment.

2. Materials and Methods

2.1. Laboratory Animals and Ethics Statement. Healthy female SPF Wistar rats weighing 140–160 g were selected and given free access to water and food. The feeding room temperature was $24 \pm 1^{\circ}C$ and the relative humidity was $50 \pm 10\%$. The rats were provided by the Laboratory Animal Center of Shandong Traditional Chinese Medicine University, License Number SCXK (LU) 2011-0003. Fifty rats were randomized into five groups ($n = 10$) including a control group, model group, Shuyu treatment group, paeony extract treatment group, and bupleurum extract treatment group. Rats in the control group were not stimulated while those in the model group were stimulated with leg binding (details below). In the Shuyu capsule, paeony extract, and bupleurum extract treatment groups, medicine was chronically administered to rats during the modelling period. Rats in each experimental group were weighed at consistent intervals during the study.

Hippocampal primary neurons were separated from newborn (24-h-old) Wistar mother rats (provided by Jinan Pengyue Experimental Animal Breeding Co., Ltd., License Number SCXK (Lu) 2014-0007). Laboratory animals were provided with care according to "*The Care and Use of Laboratory Animals*" by the Laboratory Animal Center of Shandong University of Traditional Chinese Medicine.

2.2. Antibodies, Drugs, and Chemicals. Primary antibodies against CaM (SAB4503194, Sigma-Aldrich, St. Louis, MO, USA), CaMKII (4436, Cell Signaling Technology, Danvers, MA, USA), p-CaMKII (Thr286) (3361, Cell Signaling Technology), CACNA1C (ab58552, Abcam, Cambridge, MA, USA), BDNF (AV41970, Sigma-Aldrich), and tubulin (M2005, Abmart, Arlington, MA, USA) were used at dilution ratios recommended in the instruction manual. The secondary antibody, peroxidase-conjugated goat anti-rabbit antibody IgG (Santa Cruz Biotechnology, Dallas, TX, USA), was diluted at a dilution ratio of 1 : 2000.

The Shuyu capsules (clinical approval number 2011L06107), paeony extract (clinical approval number 20110527), and bupleurum extract (clinical approval number 20110526) used in the experiments were intragastrically administered to animals at doses of 0.41 g/kg/d, 0.32 g/kg/d, and 36 mg/kg/d, respectively, for 5 d. During modelling, all groups were given the drug once a day at 9:00 am at a dosage equivalent to 8 times the dosage administered to humans.

Paeoniflorin (Z110736) and nifedipine (N7634-1G) standards were purchased from Shanghai Yuanye Biotech (Shanghai, China) and Sigma-Aldrich, respectively. All other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.3. Determination of the Oestrous Cycle. All rats were weighed and marked with picric acid. The oestrous cycle was determined by microscopic examination of vaginal smears [15, 16]. In a prooestrus vaginal smear, epithelial cell nuclei and keratinocytes were observed, while in an oestrus vaginal smear, anucleate keratinocytes and epithelial cells were observed. In a postoestrus vaginal smear, leukocytes, keratinocytes, and epithelial cell nuclei were observed, and in an anoestrus vaginal smear, many leukocytes and few epithelial cells and myxocytes were observed.

2.4. Generation of a PMS Depression Rat Model. Rats tend to be active at the acceptance stage (prooestrus and oestrus), and their oestrous behaviours abate or disappear at the nonacceptance stage (postoestrus and anoestrus). Rats with regular oestrus behaviours at the nonacceptance stage, with similar open-field tests and sucrose preference test scores, were selected for the trial.

The model was generated according to a previously published protocol, with some modifications [8]. The rats' legs were bound crosswise; the front and hind leg on the opposite side were bound with a sterile gauze (width: 2 cm) to prevent free movement but in a way that they could move slightly and obtain food. The same amount of sterile drinking water was provided to the experimental and control groups. Modelling lasted for 5 d.

2.5. Behavioural Assays. Locomotor activity in rats was measured using an open-field test [17, 18] with an XR-Xvideo

(including the XR-Xvideo animal behaviour analytical system). Under dim red light, experimenters placed the distal third of the rat tails at the centre of an open-field test box (size: 50 × 50 × 40 cm) with black walls and a black floor. The system recorded behavioural changes and general movement over 5 min. The model test was conducted after 5 d of modelling and simultaneous drug administration.

Furthermore, the sucrose preference experiment [19] was used to measure the reward response in the rats [20]. Depressive animals show a general decline in sucrose preference, reflecting the symptom of anhedonia in humans. In the experiment, two water bottles were provided for free selection over 24 h. The two bottles contained a 1% aqueous sucrose solution, and pure water, respectively, and were located on opposite sides of the cage. Before the experiment, the rats had free access to water and food. Consumption of each water type was measured based on bottle weight. Sucrose preference was expressed as the percentage of consumed sucrose water to the total liquid consumed. Sucrose preference rate was calculated using the following formula: sucrose water consumption (g)/[sucrose water consumed (g) + water consumption (g)] × 100%.

2.6. Western Blotting. Western blot analyses were performed according to a previously published protocol [21]. Band intensities were determined by densitometry analysis using Image J software (National Institutes of Health, Bethesda, MD, USA), and the results were expressed as a ratio of target protein to tubulin or as the ratio of phosphorylated protein to total protein amount.

2.7. Primary Culture of Hippocampal Neurons. Twenty-four-hour-old Wistar rats were decapitated and the heads were placed into iced phosphate-buffered saline (PBS). The hippocampus was removed and placed into 10 mL of PBS containing ice. Trypsin (0.25%) was added, and the specimens were incubated at 37°C for 20 min with the tubes shaken every 5 min. Trypsinized cell samples were collected and washed in Dulbecco modified Eagle's minimal essential medium (DMEM) three times. After 4 h, the medium was replaced with NBG medium (neurobasal medium : B27 : L-glutamine = 100 : 2 : 1, Gibco/Life Technologies, Carlsbad, CA, USA) for incubation. The medium was replaced after 24 h and then replaced every 3.5 d.

2.8. Changes in Intracellular Ca^{2+} Concentration. Hippocampal neurons *in vitro* were divided into four groups after being cultured for 7 d: blank control group (0 μM), high-dose paeoniflorin group (200 μM), middle-dose paeoniflorin group (100 μM), and low-dose paeoniflorin group (50 μM). The medium from each group was aspirated and the cells were rinsed three times with Hawk's Balance Salt Solution (HBSS, Biotop, Suzhou, China). Fluo-4-AM dye (1 mL, 5 μM; Molecular Probes Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) was added followed by incubation at 37°C for 45 min. The extracellular residual dyes were washed away with HBSS, and 1 mL of HBSS was added followed by incubation for 15 min. The fluorescence intensity of intracellular Ca^{2+} was measured by laser scanning confocal microscopy (LSM510, ZEISS, Germany) with an exposure time of 100 ms and excitation and emission wavelength at 488 nm and 530 nm, respectively. The images were collected every 3 s for a total duration of 240 s. At the 24th second, KCl was added to the cells of each group making the final concentration 100 mM. At the 80th second, paeoniflorin was added to the cells of each group to detect changes in the fluorescence intensity of intracellular Ca^{2+}.

2.9. Whole-Cell Patch Clamp. Chinese hamster ovary (CHO) cells stably expressing LTCCs were purchased from Ice Ion Channel Explorer (ICE-CHO- $Ca_v1.2$, Beijing, China) and cultured in a standard manner [22]. The voltage clamp mode of the Axon MultiClamp 700B (Molecular Devices, Sunnyvale, CA, USA) was used to measure intracellular voltages. The cell membrane voltage clamp was set at −60 mV when the tight-seal whole cell was formed. The clamping voltage was depolarized from −60 mV to +10 mV for 0.3 s, and the data were collected repeatedly every 20 s with EPC-10 amplifier (HEKA) and stored in the PatchMaster (HEKA) software to observe the influence of paeoniflorin on the electric current peak of L-type calcium channel.

Capillary glass tubes (BF 150-86-10, Sutter Instruments) were pulled into recording electrodes using a pipette puller (P97, Sutter, Sacramento, CA, USA), and the pipette manipulator (MP285, Sutter Instruments) was manipulated under an inverted microscope (IX71, Olympus, Tokyo, Japan) to get recording electrodes in contact with the cells to impose negative pressure suction, thus forming GΩ sealing-in. Fast capacitance compensation was then conducted, and negative pressure was applied to lyse the cell membrane, thus forming a whole-cell recording mode. Slow capacitance compensation was then conducted, and membrane capacitance and series resistance were recorded. However, electric leakage compensation was not provided.

After current stabilization of L-type calcium channel recorded by whole cells, each group (the blank control group, high-dose paeoniflorin group, middle-dose paeoniflorin group, low-dose paeoniflorin group, and nifedipine group) was given the trial drugs, exerting an effect for approximately 5 min. The cell-cover glass was placed in a recording bath under an inverted microscope, and the tested drugs and drug-free external fluid flowed through the recording chamber in the order of gravity perfusion to act on the cells. Fluid exchange was conducted in the recording using a vacuum pump exchange. The current detected in the compound-free extracellular fluid of every cell was taken as its own control group, and multiple cells were independently detected. All electrophysiological experiments were conducted at room temperature (21 ± 1°C).

The current treated by paeoniflorin at certain concentrations was first standardized [peak current of the concentration (pA)/peak current of blank control (pA)], and then the inhibition ratio was calculated [1 − (peak current of the concentration (pA)/peak current of blank control (pA))].

2.10. Statistical Analysis. One-way analysis of variance (ANOVA) was used for the weight test, open-field test, and the sucrose preference test. GraphPad Prism 6 was used for

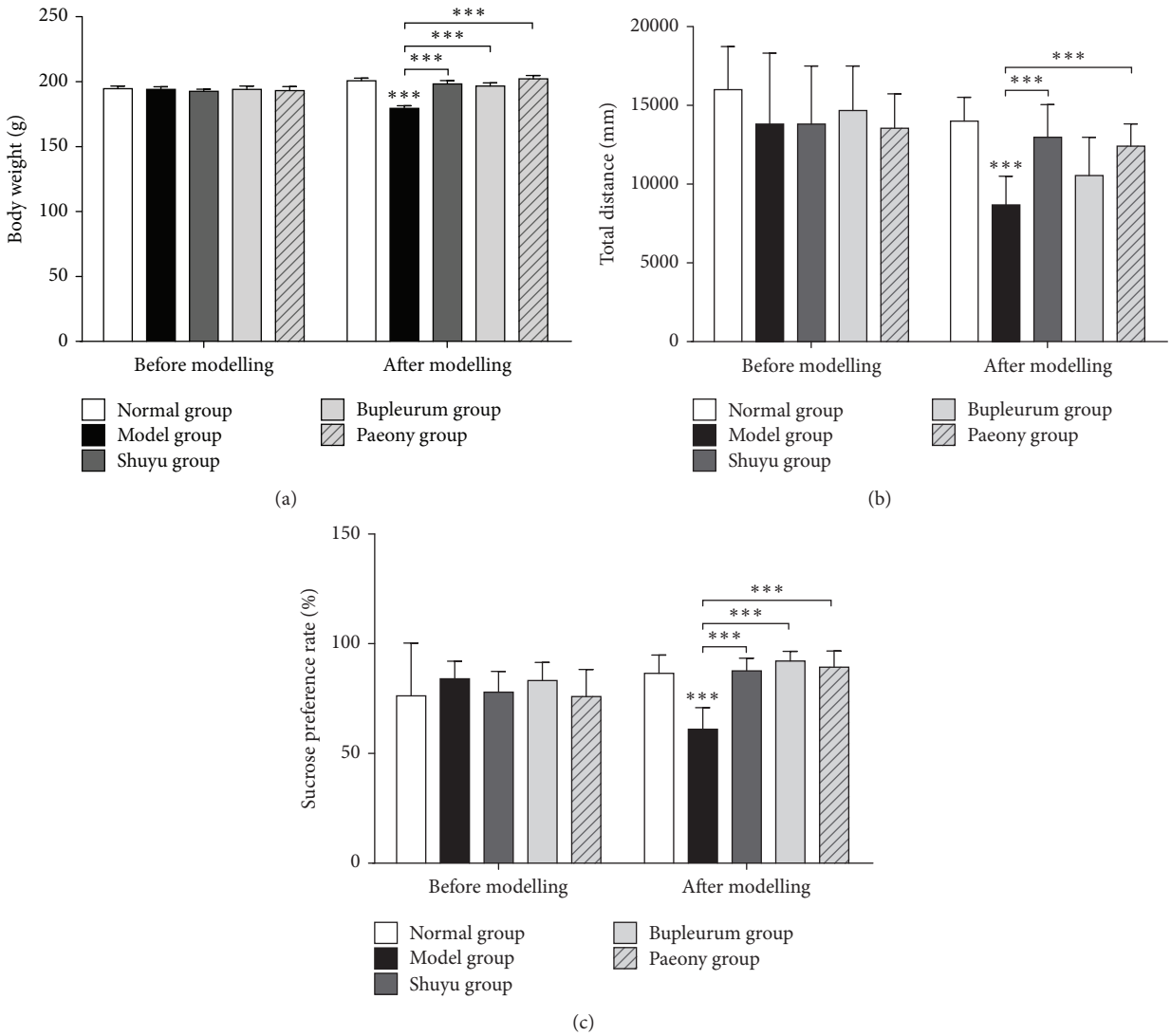

Figure 1: Behavioural assays. (a) Body weight test. (b) Open-field test. (c) Sucrose preference test. For all assays, testing was performed both before and after modelling. Moreover, the following groups were analysed: the control/normal group, model group, Shuyu capsule group, bupleurum group, and paeony group. The statistical analysis for the behaviour assays was performed by one-way ANOVA ($n = 10$, $^{***}P < 0.001$).

the calculations. Photo analyses of hippocampal neurons were performed using the default Zeiss LSM Image Browser software. PClamp 10.0 was used for the whole-cell patch clamp recording of the L-type calcium channel current. All data are shown as the mean ± SD, with the significance level set at $P < 0.05$.

3. Results

3.1. Shuyu Capsules Effectively Mitigate Depressive Behaviour in a Rat PMS Model. Rat body weights in each group before and after modelling are shown in Figure 1(a). Before modelling, no significant differences in body weight were seen, while after modelling, rats in the model group showed a significant decrease in body weight compared to that shown by the normal group rats ($P < 0.001$), but there were no significant differences between the rats in the treatment groups. Compared to the model group rats, rats in the Shuyu, bupleurum, and paeony groups showed significantly increased body weight ($P < 0.001$).

Results of the open-field test are shown in Figure 1(b). Before modelling, the open-field test scores in each group were not significantly different, while after modelling the open-field test scores of rats in the model group decreased significantly ($P < 0.001$) compared to those of the normal group. The open-field test scores of rats in the Shuyu and paeony groups increased significantly ($P < 0.001$), and those of rats in the bupleurum group showed a tendency to increase, but it was not statistically significance.

Results of the sucrose preference test are shown in Figure 1(c). Before modelling, there was no significant different in the consumption of sucrose water between the groups,

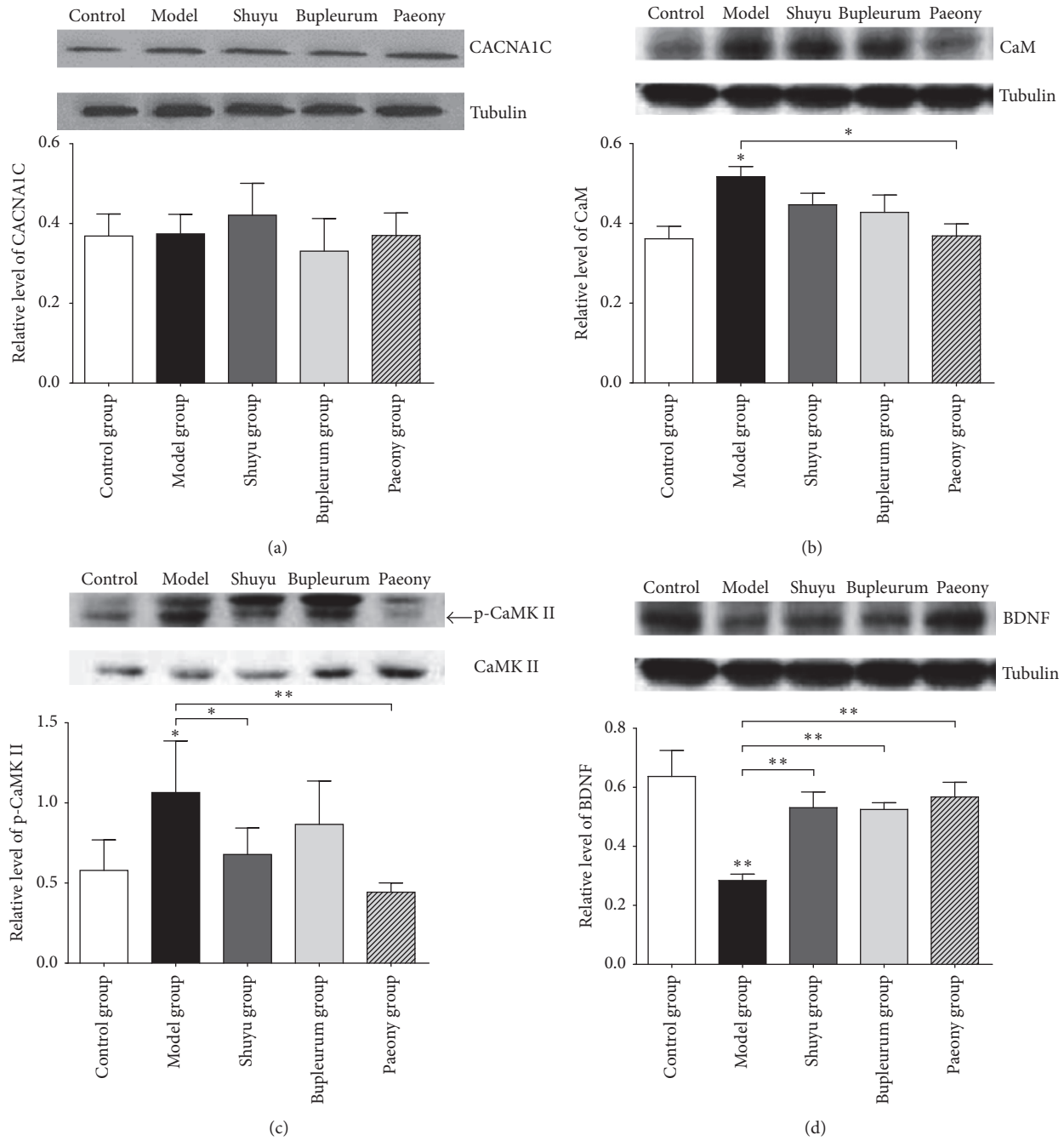

Figure 2: Western blot and analysis of hippocampus tissue samples from the control/normal group, model group, Shuyu capsule group, bupleurum group, and paeony group. (a) CACNA1C protein level analysis ($n = 6$). (b) Analysis of calmodulin (CaM) protein level ($n = 6$, $^{*}P < 0.05$). (c) Analysis of phosphorylated calmodulin-dependent protein kinase II (p-CaMKII) level ($n = 6$, $^{*}P < 0.05$ and $^{**}P < 0.01$). (d) Analysis of brain-derived neurotrophic factor (BDNF) protein expression ($n = 3$, $^{**}P < 0.01$).

while after modelling rats in the model group showed a significant decrease in sucrose preference compared to that shown by the normal group rats ($P < 0.001$). The results for the three treatment groups were not significantly different from those for the normal group. Compared with the model group, each treatment group showed a significant increase in sucrose preference ($P < 0.001$).

3.2. Paeony Extract Does Not Affect CACNA1C Protein Expression in the Rat Hippocampus but Can Affect $Ca_v1.2$-Mediated CaM/CaMKII Signalling. *CACNA1C* expression in the hippocampus is shown in Figure 2(a). Compared with the normal group, the model and treatment groups showed no significant differences in hippocampal *CACNA1C* expression.

Compared with the normal group, the model group showed increased CaM expression in the hippocampus ($P < 0.05$), and there were no significant differences in the hippocampi of treatment group rats. CaM expression in rats of the paeony group was significantly lower than that in rats of the model group ($P < 0.05$, Figure 2(b)).

Expression of phosphorylated CaMKII (p-CaMKII) in model group rats was significantly higher than that in normal group rats ($P < 0.05$). p-CaMKII expression in Shuyu and paeony group rats was significantly lower than that in model group rats ($P < 0.05$, $P < 0.01$, Figure 2(c)).

BDNF expression in the hippocampi of model group rats was significantly lower than that in normal group rats ($P < 0.01$), and there were no significant differences between the treatment groups. BDNF expression was significantly higher in the Shuyu group, bupleurum group, and paeony group rats than in model group rats ($P < 0.01$, Figure 2(d)).

3.3. Paeoniflorin Significantly Inhibits KCL-Induced Increases in Intracellular Ca^{2+} Concentration. Fluo-4-AM is an acetyl methyl ester derivative of Fluo-4 and is readily accessible from the medium. Upon entering a cell, AM is hydrolysed by intracellular esterase, and then the resultant Fluo-4 binds with Ca^{2+} and emits fluorescence. Fluo-4-AM is therefore used to probe Ca^{2+} levels. Before adding KCl to primary hippocampal neurons, the basal fluorescence intensity of each group was the same, but after KCl was added, intracellular Ca^{2+} increased due to the depolarization of the cell membrane induced by extracellular K^+, and thus the fluorescence intensity of each group increased sharply (Figures 3(a) and 3(b)). Paeoniflorin intervention inhibited intracellular Ca^{2+} overloading induced by K^+. Compared with the blank control group, the paeoniflorin treatment group showed a remarkable concentration-dependent decrease in fluorescence intensity (Figures 3(a) and 3(c)), as shown in Figure 3(b).

3.4. Paeoniflorin Inhibits $Ca_v1.2$ Calcium Channels. Compared to that of the normal group, the $Ca_v1.2$ current density of CHO cells treated with paeoniflorin decreased ($P < 0.05$) in a concentration-dependent manner; the higher the concentration of paeoniflorin, the greater the inhibition of $Ca_v1.2$ current density (Figure 4(a)). Nifedipine was used as a positive control and showed nearly 100% inhibition ($96.7 \pm 4.2\%$) of $Ca_v1.2$ current density. High-dose (200 μM), middle-dose (100 μM), and low-dose paeoniflorin (50 μM) produced $50.7 \pm 4.2\%$, $29.0 \pm 1.7\%$, and $15.3 \pm 10.0\%$ inhibition of $Ca_v1.2$ current density, respectively (Figure 4(b)).

4. Discussion

Postmenstrual symptoms are typical emotional disorders. We demonstrated that postmenstrual symptoms occur in the premenstrual phase and cease postmenstruum in a rat model. Specifically, rats showed symptoms at the nonacceptance period (premenstruum) in the oestrous cycle that disappeared or resolved at the acceptance period (postmenstruum). Other groups verified PMS or premenstrual dysphoric disorder models using similar strategies [23–25]. We selected healthy female Wistar rats through vaginal smear screening, established a PMS depression rat model by means of constraint [26], and studied the animals in a body weight gain test, open-field test, and sucrose preference test. Reduced weight gain in model rats suggested that emotional stress and chronic unpredictable mild stress have the same inhibitory effects on weight gain [27]. The open-field test score measured exploratory behaviour and excitability. The sucrose preference of rodents reflected the reward response; depressive animals commonly show a reduced sucrose preference [28]. The significance of the rat's path, sucrose preference level changes (Figure 1), and some core depression symptoms, during the PMS period were well modelled. Macroscopic behavioural experiments also showed that the administration of Shuyu capsule or paeony extract could relieve the symptoms caused by modelling (Figure 1), indicating that both the Shuyu capsule and the paeony extract contain important molecules that can effectively mitigate depressive behaviour in a rat PMS model.

Many works reported that the hippocampus was related to emotional disorders. Imaging data revealed that patients with depression had smaller hippocampus than that of healthy subjects. Further, the degree of reduction in hippocampus volume was positively correlated with the time for which depression lasted [29]. Another research revealed the relationship between hippocampal morphometry in depressed patients and that in control subjects [30]. As a result, in this study, we measured CaM, CaMKII, p-CaMKII, and BDNF protein levels in the hippocampus of rats in the model and treatment groups. The results showed that neither modelling nor treatment led to a difference in CACNA1C expression levels but that they caused an increase in key protein CaMKII phosphorylation in the $Ca_v1.2$-induced CaM/CaMKII signalling pathway and a decrease in BDNF protein expression in model group rats. When Shuyu capsule or paeony extract was administered simultaneously with modelling, the above proteins in the hippocampus of rats were restored to their original levels. Previous studies have demonstrated that exogenous BDNF has an antidepressant activity [31, 32], and this study demonstrates that antidepressant drugs (and their ingredients) can relieve the modelling-induced reduction in BDNF protein in the hippocampus (Figure 2(d)). In addition, p-CaMKII then activates cAMP response element-binding protein (CREB) [33–35], which can regulate the expression of BDNF [36]. In our study, modelling increased CaM and p-CaMKII expression, and administration of Shuyu capsule or paeony extract restored the expression of CaM and p-CaMKII to normal levels. These results indicate that modelling caused an abnormality in the CaM/CaMKII signalling pathway and further affected downstream nerve regulation, while Shuyu capsule (or its ingredients) achieved an antidepression effect by alleviating the pathway abnormality.

Specifically, western blot results suggested that bupleurum extract did not exert effects on $Ca_v1.2$-induced CaM/CaMKII signalling pathway (Figure 2). BDNF level also increased in the model group rats treated with bupleurum extract, which exhibited the same effects as paeony extract

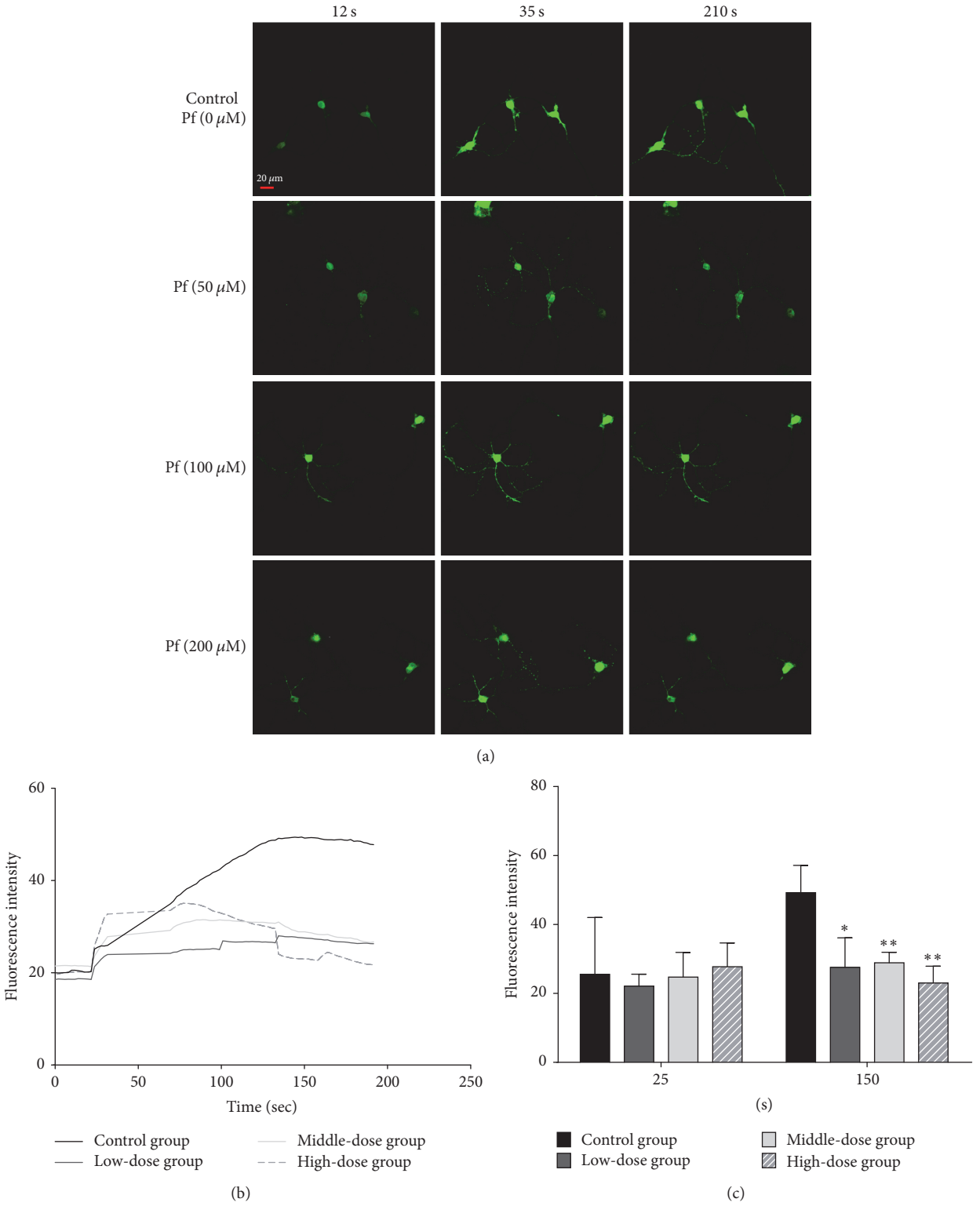

Figure 3: Intracellular calcium ion concentrations of primary culture of hippocampal neurons from groups including blank control, 50 μM paeoniflorin treatment, 100 μM paeoniflorin treatment, and 200 μM paeoniflorin treatment. (a) Morphology and fluorescence intensity of hippocampal neurons in each group before and after KCl and paeoniflorin treatment. Pf: paeoniflorin treatment. (b) Fluorescence intensity of each group over time (0–191 s). (c) Statistical comparison of fluorescence intensity at 25 s and 150 s in each group ($n = 5$, $^{*}P < 0.05$ and $^{**}P < 0.01$). Scale bar: 20 μm.

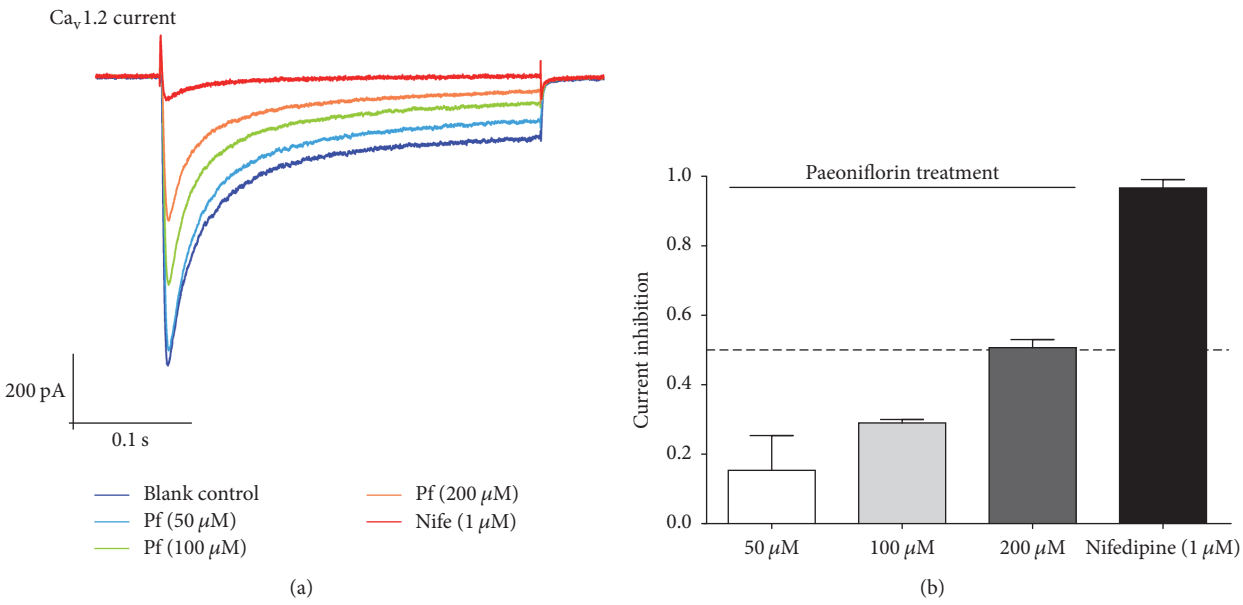

Figure 4: Whole-cell patch clamp analysis of CHO cells with LTCC constitutive expression. (a) $Ca_v1.2$ current density was recorded and analysed for the following groups: blank control, positive control (1 μM nifedipine treatment), 50 μM paeoniflorin treatment, 100 μM paeoniflorin treatment, and 200 μM paeoniflorin treatment. (b) Inhibition ratios for 1 μM nifedipine treatment, 50 μM paeoniflorin treatment, 100 μM paeoniflorin treatment, and 200 μM paeoniflorin treatment.

or Shuyu capsules did. Hence, we further studied the effect of paeoniflorin, the main active molecule in paeony, on Ca^{2+} channel. The Ca^{2+} channel is a transmembrane structure that controls the entry of Ca^{2+} into cells [14]. CACNA1C encodes the α1C subunit of the LTCC ($Ca_v1.2$ subtype), which is the main subunit of LTCC and is also the target of many antidepressant drugs. CACNA1C acts as a transmembrane channel protein; therefore, extracellular drugs can utilize it to conduct functional intervention of intracellular regulatory factors, thus influencing their physiological functions. Neither modelling nor administration resulted in a difference in CACNA1C expression level, but they did affect the downstream pathway (Figure 2), which suggests that the active molecules in Shuyu capsules may directly act on CACNA1C, although this inference has not been confirmed. Therefore, we further examined the behaviour and function of primary cultured calcium channels under drug intervention. Our previous study showed that paeoniflorin is the main substance of Shuyu capsule [37] to cause an antidepressive effect and that it passes easily through the blood-brain barrier [9]; therefore, we incubated primary hippocampal cells directly with paeoniflorin and observed changes in intracellular Ca^{2+} levels. Our results provide compelling evidence that paeoniflorin can inhibit the overloading of intracellular Ca^{2+} (Figure 3). Further, patch clamp experiments more accurately confirmed this through the constitutive expression of LTCC in CHO cell lines, where paeoniflorin has been shown to inhibit $Ca_v1.2$ current density in a concentration-dependent manner (Figure 4). Although other studies as well as ours suggest that paeoniflorin acts on $Ca_v1.2$ and influences the CaM/CaMKII pathway, there is not yet enough evidence to confirm this. The influence of paeoniflorin on $Ca_v1.2$, the CaM/CaMKII signalling pathway, and the downstream molecules may be concomitant rather than causal; therefore, more studies on this topic are needed.

5. Conclusion

This study further evaluates the mechanism of Shuyu capsule with regard to its therapeutic effect against PMS. Paeoniflorin in Shuyu capsules may influence the CaM/CaMKII signalling pathway and its downstream signalling molecules by regulating $Ca_v1.2$ current density, thus playing an important role in the treatment and alleviation of affective disorders.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Chunhong Song designed experiments, integrated data, edited images, and wrote this manuscript. Dongmei Gao and Yanhong Yu performed western blotting. Fang Li and Meiyan Wang contributed to analysis of intracellular calcium ion concentration and whole-cell patch clamp experiment. Jieqiong Wang, Sheng Wei, and Peng Sun generated rat models and performed behavioural assays. Mingqi Qiao directed this research, provided key advice and essential assistance, finished the paper, and provided funding for the project.

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Research Article

Resveratrol Inhibits Propagation of *Chlamydia trachomatis* in McCoy Cells

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Resveratrol (RESV), an antifungal compound from grapes and other plants, has a distinct ability to inhibit the *Chlamydia (C.) trachomatis* developmental cycle in McCoy cells, a classic cell line used for chlamydial research. Inoculation of *C. trachomatis* with increasing amounts of RESV (from 12.5 to 100 μM) gave a dose-dependent reduction in the number of infected McCoy cells visualized by using monoclonal antibodies against chlamydial lipopolysaccharide. A similar trend has been observed with immunoassay for major outer membrane protein (MOMP). Furthermore, there was a step-wise reduction in the number of *C. trachomatis* infective progenies caused by the increasing concentrations of RESV. The ability of RESV to arrest *C. trachomatis* growth in McCoy cells was confirmed by a nucleic acid amplification protocol which revealed dose-dependent changes in mRNAs for different genes of chlamydial developmental cycle (*euo*, *incA*, and *omcB*). Although the precise nature of the antichlamydial activity of RESV is yet to be determined and evaluated in future studies, the observed effect of RESV on *C. trachomatis* infection was not related to its potential effect on attachment/entry of the pathogen into eukaryotic cells or RESV toxicity to McCoy cells. Similar inhibitory effect was shown for *C. pneumoniae* and *C. muridarum*.

1. Introduction

Resveratrol (3,4′,5-trihydroxystilbene, $C_{14}H_{12}O_3$, RESV) belongs to a group of polyphenolic substances named stilbenes. Its fat-soluble *cis*- and *trans* isoforms bound to a glucose molecule are present in some plants (grapes, peanuts, berries, etc.) and red wine [1]. Steadily growing interest in the potential health benefits of resveratrol began to surface about 20 years ago when reduction in the risk of cardiovascular disease was linked to moderate red wine consumption [2]. Interest in the potential health benefits of RESV was also fueled by reports relating to the anti-aging properties of the compound when RESV-mediated extension of lifespan in yeast, *C. elegans* and *Drosophila,* was reported [3]. To date, RESV is acknowledged to have significant antioxidant activity and anticarcinogenic as well as cardio- and neuroprotective actions [4]. Reports on the antidiabetic activity of RSV and other plant-derived polyphenols have also emerged recently [5, 6]. The remarkable diversity of biological effects of RESV seen in mammals might be explained by multiple targets mediating its action in intermediate metabolism. Among these are molecules and pathways involved in aging and longevity, mTOR/S6K, sirtuins, AMPK, and perhaps several others [7]. Therefore, the RESV-driven health effects seen in higher organisms are likely to be secondary to the antiaging effect of RESV reported in metazoans. However, it is important to acknowledge that the beneficial effects of RESV reported in eukaryotes have little or no relevance to plant physiology. RESV is a phytoalexin, a normal constituent of the plant cell produced by plants in response to fungal/bacterial insult, stress, or elicitor treatment [7]. Therefore, antifungal and antibacterial properties seem to constitute an original biological function of RESV whose significance for medical practice has not yet been fully explored. Such an assumption is well supported by recent

studies reporting the inhibitory effect of RESV on different types of viral infections in eukaryotes [8, 9].

In the present paper we report that RESV has a significant inhibitory effect on *C. trachomatis* propagation in McCoy cells. *C. trachomatis* is the most common sexually transmitted bacterial pathogen causing a wide range of urogenital diseases (urethritis, endocervicitis, salpingitis, endometritis, and inflammatory pelvic disease) as well as trachoma and pulmonary infections [10–12]. The search for new antichlamydial agents has become especially important due to the global increase in antibiotic-resistant chlamydial infections [10, 13].

2. Material and Methods

2.1. Reagents and Organisms. All reagents were from Sigma-Aldrich unless specified otherwise. *C. trachomatis* strain L2/Bu434 and *C. pneumoniae* strain Kajaani 6 were kindly provided by Dr. P. Saikku (University of Oulu, Finland). *C. muridarum* strain Nigg (ATCC VR-123). Resveratrol (RESV) was purchased from Kaden Biochemicals (Germany), dissolved in ethanol at a concentration of 50 mM and kept at −80°C as a stock solution.

2.2. Cell Culture. *C. trachomatis* and *C. muridarum* were propagated in McCoy mouse fibroblasts grown in DMEM with 10% HyClone FCS supplemented with 2 mM glutamine, 4.0 mg/ml gentamicin, and 5.0 mg/ml amphotericin B.

C. pneumoniae was propagated in HL cells (Washington Research Foundation, Seattle, USA). *C. trachomatis* was initially propagated in McCoy cells and purified by Renografin gradient centrifugation [14]. Elementary bodies were isolated and resuspended in SPG buffer. Titers were determined by infecting cell monolayers with 10-fold dilutions of thawed stock suspension. Subconfluent McCoy cell monolayers were infected with the stock suspension of *C. trachomatis* at a multiplicity rate of 1 : 1. Plates were centrifuged for 1 hour at 1500g to synchronize the infection. A wide range of RESV additions to the culture medium were performed at the "0" time point of the postinfection period.

Nucleic acid-based assays were performed 24 hours after *C. trachomatis* inoculation of the cultured cells. Infection rate in the McCoy cells and infective progeny formation were estimated 48 hours after pathogen introduction into the incubation medium.

All experiments were supplemented with negative (additions of RESV solvent) and positive controls (addition of 0.67 μM azithromycin). This concentration of azithromycin was defined as minimum inhibitory concentration (MIC_{AZ}) under culturing conditions used in our studies.

2.3. Determining RESV Toxicity in McCoy Cells. In vitro cytotoxicity was estimated by MTT [3-(4,5-dimethylthiazol-2-y1)2,5-diphenyltetrazolium bromide] test. Briefly, McCoy cells were plated in 96-well plates. After overnight attachment in RPMI with 10% FCS (37°C, 5% CO_2) and medium replacement, addition of 10 μl of RESV was performed (final concentration was 12.5–100 μM). After 24 or 48 hours incubation, 20 μl of MTT (5 mg/ml) was added to each well. The plates were incubated for an additional 4 hours, washed in PBS, and treated with 100 μl of isopropanol. The optical density was measured at 540 nm with a reference wavelength of 630 nm using a Multiskan EX microplate photometer (Thermo Fisher Scientific, USA). RSV toxicity was also measured using the LIVE/DEAD® Viability/Cytotoxicity assay according to the manufacturer's instructions (Invitrogen, UK).

Moreover, a trypan blue exclusion test was performed routinely during all experiments. The acceptable level of cell viability was set at ≥90%.

2.4. Immunofluorescence Staining. Infected McCoy cells were grown for 48 hours on coverslips in 24-well plates. The cells were fixed with ice-cold methanol, permeabilized with 0.1% Triton X-100, and preblocked for 1 hour at 4°C with 1% bovine serum albumin in PBS. All monolayers were stained using FITC-conjugated monoclonal antibody against chlamydial lipopolysaccharide (NearMedic Plus, RF). Inclusion-containing cells were visualized using a Nikon Eclipse 50i microscope at ×1000 or lower magnification.

2.5. Assessment of Infective Progeny. Infective progeny accumulation was assessed in McCoy cell monolayers infected with *C. trachomatis* with or without additions of RESV. Infected cell monolayers were harvested 48 hours after bacterial inoculation and lysed by freezing/thawing. Serial dilutions of lysates were inoculated onto McCoy cells and plates were centrifuged for 1 hour at 1500g. The infected cells were visualized at 48 hours of the postinfection period using FITC-conjugated monoclonal antibody against chlamydial lipopolysaccharide (NearMedic Plus, RF).

2.5.1. Modified Enzyme Immunoassay (ELISA). A modified enzyme immunoassay was carried out using 96-well plates 48 hours after pathogen inoculation. In brief, the cells were fixed with ice-cold 72% ethanol, placed for 30 min at −20°C and treated with StabilZyme® HRP Conjugate Stabilizer (SurModics, USA). After washing and 1-hour incubation with monoclonal antibody against *C. trachomatis* MOMP (NearMedic Plus, RF), the cells were washed again with PBST and exposed to anti-mouse IgG conjugated with horseradish peroxidase. After additional PBS washing, the cells were incubated for 30 minutes with 0.3% 3,3,5,5-tetramethylbenzidine substrate and the reaction was stopped with H_2SO_4. The results were read in triplicate wells on a Multiskan EX at 450 nm.

2.6. Attachment and Internalization Assay. This assay was performed as described in our previous paper [15].

2.7. RNA Extraction and Reverse Transcription. RNA was isolated from *C. trachomatis*-infected McCoy cell monolayers grown on 6-well plates using TRIZol (Invitrogen) at the 24-hour time point of the postinfection period. Total mRNA was pretreated with DNase I (DNA-free™, Ambion) and quantified on a NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific, USA). 1 μg of each RNA sample was converted into cDNA using random hexamer primers

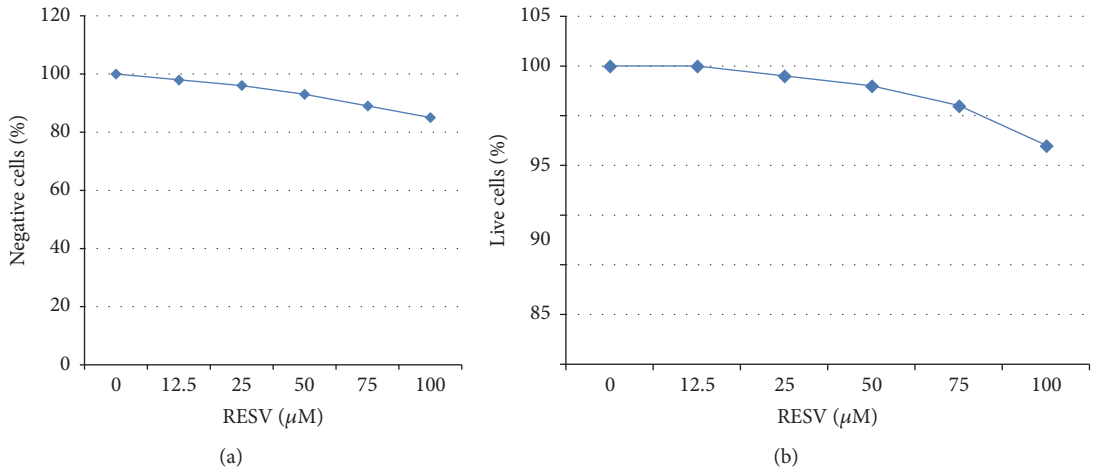

Figure 1: Assessment of cytotoxicity in McCoy cells incubated with increasing concentrations of resveratrol (RESV). McCoy cells were plated in 96-well plates. After overnight attachment and medium replacement, addition of 10 μl of RESV was performed (final concentration was 12.5–100 μM). After 24 hours incubation MTT test (a) and LIVE/DEAD Viability/Cytotoxicity assay (b) were performed as described in *Material and Methods*.

and a SuperScript III First-Strand Synthesis Kit (Invitrogen, Germany).

2.8. Quantitative Real-Time PCR. mRNA levels for three different developmental genes of *C. trachomatis* were analyzed in McCoy cells after treatment with RESV by quantitative RT-PCR using a CFX-96 thermocycler (Bio-Rad Laboratories, USA). RT-qPCR Taqman primers were designed using Primer 3 software and were validated by BLAST search as well as regression plot analysis using C_p values obtained with multiple dilutions of cDNA. Specificity of the designed primers and fluorescent probes was confirmed in different systems using cDNA from *C. trachomatis Bu-434*, *C. trachomatis UW-3/Cx, C. trachomatis UW- 31/Cx,* and *C. pneumoniae K-6*, as well as from uninfected McCoy cells and some other common bacterial pathogens.

The *C. trachomatis*-specific primers used were as follows:

(i) For *euo* gene: Pr-F 5′ TCCCCGACGCTCTCCTTTCA 3′, Pr_R 5′CTCGTCAGGCTATCTATGTTGCT 3′, Probe 5′- ROX- ATG GAC GCC ACT TGT CCC ACG GAA T- BHQ2-3′

(ii) For *incA* gene: Pr-F 5′ CTACAGAAGAAATGCGCAAACTTT, Pr-R 5′ AATGATTGCTGGTTATGCGCTAAT, Probe 5′-FAM – CGGCGAACTTCTTCTGCTAATGGGGTT BH1- 3′

(iii) For *omcB* gene: Pr-F 5′-TGTATCAGAAACTGGAACAGTCAATG 3′, Pr-F 5′TGAAAGCAGTATCAGCTGGAGATG 3′, Probe 5′-FAM CGGAAGAAAGAATCGCTTCCCCACG BH1- 3′

Primer sequences for eukaryotic beta-actin were used as published previously [15].

All primers were verified and used under thermal cycling conditions—95°C for 10 min and 50 cycles of 95°C for 15 seconds, 60°C for 1 min, and 72°C for 20 seconds. Serial dilutions of RNA, extracted from *C. trachomatis*-infected McCoy cells, were used as a standard for quantification of chlamydial gene expression. The mRNA expression levels were referenced to Ct values for chlamydial genes detected in infected McCoy cells grown at "0" concentration of RESV. This reference value was taken as 1.00. All mRNA measurements were done in triplicate.

All experiments were conducted at least three times. Statistical analysis was performed where possible using Student's *t*-test. The most representative sets of immunofluorescent images were selected and are shown above.

3. Results

3.1. Cytotoxicity Assessment. As can be seen from Figure 1, RESV showed no significant cytotoxicity in subconfluent McCoy cell monolayers when present in the incubation medium for 24 hours at a concentration of 12.5 μM to 75 μM ($P > 0.05$). However, the highest RESV concentration used (>100 μM) produced a notable increase in the number of McCoy cells positive in MTT and Live/Dead assays. An acceptable level of cell viability (~90%) was also detected at later time points during McCoy cell incubation with 12.5–75 μM RESV (up to 48 hours, results not shown) which allowed immunofluorescence studies to be carried out 48 hours after RESV additions. No significant effect of RESV on host cell proliferation was detected under the conditions used.

3.1.1. Immunofluorescence and Enzyme Immunoassay Analysis. As can be seen from Figure 2, simultaneous addition of RSV and the pathogen is accompanied by a dramatic inhibition of inclusion body formation in McCoy cells. Inclusion bodies in 48-hour McCoy cultures appeared to be large, polygonal, or round-shaped with homogeneous distribution of immunofluorescent signal. Even the lowest RESV concentration (12.5 μM; Figure 2(b)) reduced the size of the inclusion bodies and their staining intensity. The

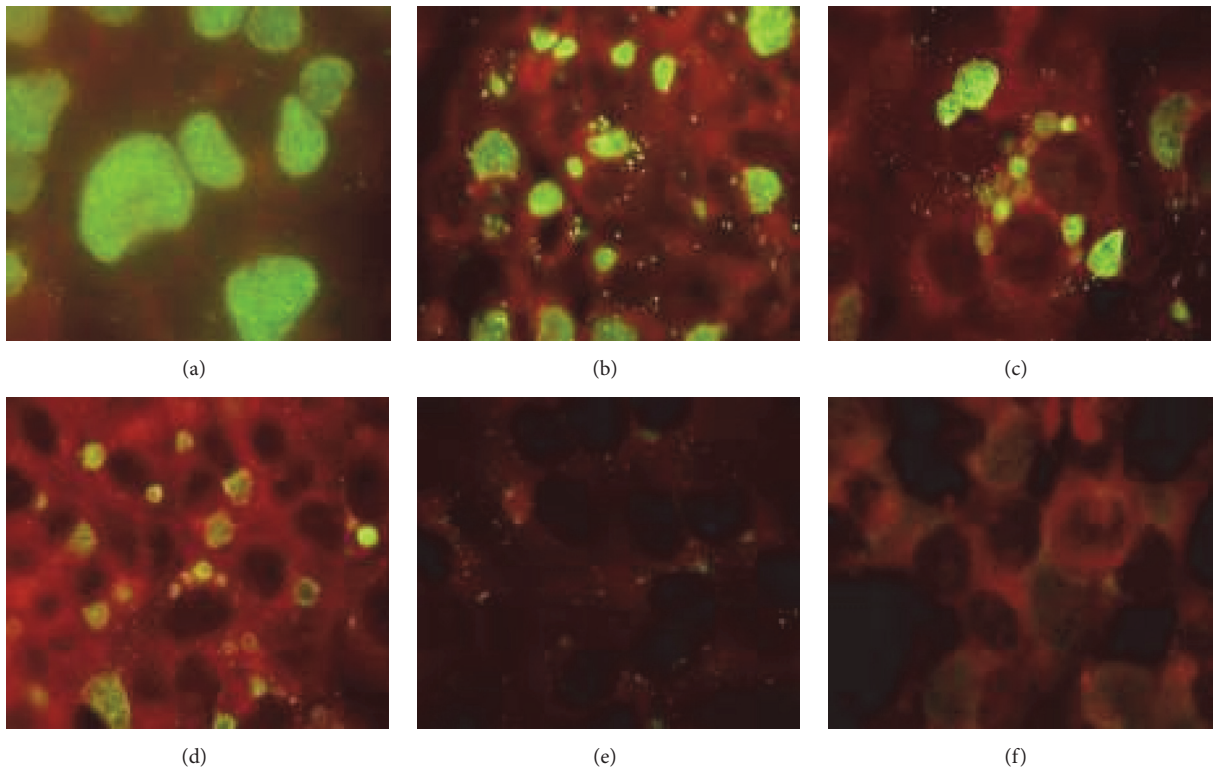

Figure 2: Resveratrol (RESV) inhibits propagation of *C. trachomatis* in McCoy cells. Immunofluorescence analysis (×1000). McCoy cells were infected with *C. trachomatis* serovar L-2 at MOI of 1. The final concentrations of RESV present in the culture medium ranged from 12,5 to 100 μM, as indicated. Cultures were fixed after 48 hours incubation at 37°C in 5% CO_2. Infected monolayers were stained using *C. trachomatis*-specific monoclonal antibodies as described in Material and Methods. (a) "0" μM RSV, (b) 12.5 μM, (c) 25 μM, (d) 50 μM, (e) 75 μM, and (f) 100 μM.

inhibitory effect of RESV was even more apparent at a concentration 25 μM when the number of inclusion bodies was reduced approximately twofold. There was an obvious "empty pocket" formation in the inclusion bodies at the 50–75 μM RESV concentration range. Drastic reduction of inclusion body numbers was seen with 75 μM RESV, while complete eradication of infection was achieved with 100 μM RESV. No inclusion body formation has been seen at 100 μM RESV. In order to quantify the RSV-induced changes in chlamydial growth in McCoy cells, immune detection of MOMP was performed. As shown in Figure 3, there was a stepwise decline in immune-detectable MOMP in McCoy monolayers infected with *C. trachomatis* in the presence of increasing concentrations of RESV. All experiments with RESV treatment were supplemented with a positive control (additions of 0.67 μM azithromycin) which consistently showed a 100% reduction in the number of chlamydial inclusions without any significant toxicity for the McCoy cell monolayer in reference strain Bu434 of *C. trachomatis* under the conditions used in our cell culture experiments.

It has to be assumed that RESV arrests the infectious cycle at the stage of pathogen attachment/entry into the host cells. Hence, we decided to infect McCoy cells with RESV present in the medium (from 0 to 75 μM) and document pathogen attachment to the cell membrane. According to our results, the presence of RESV in the medium had no noticeable impact on the attachment rate of EBs to the host cell membrane (data not shown).

3.1.2. Infective Progeny Formation. Next, we decided to verify whether dose-dependent inhibition of chlamydial growth with RESV was accompanied by reduced formation of *C. trachomatis* infective progeny. Figure 4 shows that there is a statistically significant and gradual decline in the infective progeny titer caused by additions of RESV in the concentration range of 12.5 μM to 50 μM. No infective progeny was detected at the highest RESV levels in the medium (75 μM and 100 μM).

Simultaneous addition of RESV and bacteria may arrest the infectious cycle by affecting the infectious properties of EBs. Therefore, in the following experiment we infected McCoy cells with bacterial particles pretreated with RESV and assessed *C. trachomatis* growth at 48 hpi using an IF protocol. The EBs of *C. trachomatis* were incubated for 1 hour at 37°C with increasing concentrations of RESV or vehicle alone, washed twice with SPG buffer, and inoculated into cultured cells. No statistically significant differences in percentage of infected McCoy cells were detected in these experiments (results not shown). Therefore, the RESV effect on *C. trachomatis* growth does not seem to be related to the direct effect of the compound on chlamydial progeny viability and infectivity.

Table 1: Folds and mRNA changes in McCoy cells infected with *C. trachomatis* in the presence of resveratrol (RESV).

mRNA	RESV concentrations (μM)					
	0	12.5	25	50	75	100
euo	1	$0.2 \pm .0.05^*$	$0.05 \pm 0.075^*$	$0.033 \pm 0.013^*$	$0.35 \pm 0.03^*$	$0.315 \pm 0.065^*$
incA	1	$0.175 \pm 0.075^*$	$0.09 \pm 0.01^*$	$0.055 \pm 0.015^*$	$0.07 \pm 0.01^*$	$0.064 \pm 0.005^*$
omcB	1	$0.059 \pm 0.001^*$	$0.04 \pm 0.01^*$	$0.025 \pm 0.005^*$	$0.017 \pm 0.002^*$	$0.015 \pm 0.005^*$

McCoy cells were set up, grown, and infected with *C. trachomatis* in the presence or absence of increasing concentrations of RESV (0–100 μM) as described in Material and Methods. Total RNA was extracted 24 hours later. RNA levels for the genes of interest were normalized to eukaryotic β-actin expression levels. All mRNA values were referenced to expression levels at "0" RESV concentration (1.00); $*$ indicates statistically significant values as compared to "0" RESV concentration.

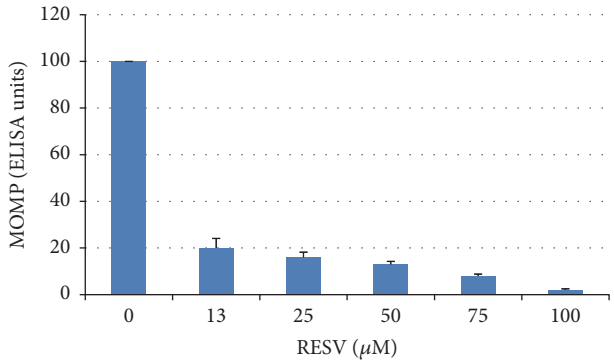

Figure 3: Relative amounts of MOMP in McCoy cells infected with *C. trachomatis* in the presence of resveratrol (RESV). McCoy cells were plated in 96-well plates, infected with *C. trachomatis* with simultaneous addition of RESV (0–100 μM). At 48 hours after infection, infected monolayers were fixed, blocked, washed, and subjected to modified enzyme immunoassay (ELISA) with *C. trachomatis*-specific monoclonal antibodies against MOMP as described in *Material and Methods*.

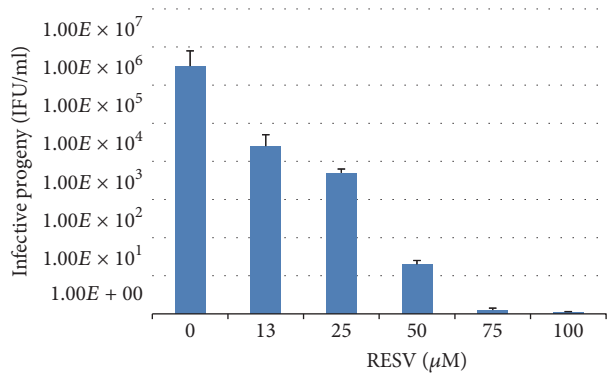

Figure 4: Infective progeny formation in McCoy cells infected with *C. trachomatis* in the presence of resveratrol (RESV). McCoy cells were plated, grown, and harvested at 48 hours after simultaneous addition of RESV (0–100 μM) and *C. trachomatis*. Infective progeny formation was measured as described in *Material and Methods*.

3.1.3. RNA Analysis. Finally, RNA analysis was employed to explore the inhibitory effect of RESV on *C. trachomatis* growth in McCoy cells. Biochemical and morphological abnormalities in the infected eukaryotic cells are secondary to transcriptional changes associated with the bacterial genome. Therefore, chlamydial mRNAs were measured at 24 hpi as opposed to the 48-hour time point used in our experiments for all other assays. As can be seen in Table 1, RESV treatment reduced significantly all mRNA values for genes representing the early, middle, and late phases of the chlamydial developmental cycle (*euo*, *incA*, and *omcB*, resp.). Such a reduction was seen even with the lowest RESV concentration used (12.5 μM) and reached a peak at 100 μM. The only exception was *euo* mRNA. Its values showed a decline at 12.5 and 25 μM RESV; however, higher RESV concentrations gave a less pronounced reduction in *euo* transcripts. Overall, the trend in chlamydial mRNA regulation in the presence of RESV was in good agreement with all of the other changes observed in *C. trachomatis*-infected McCoy cells treated with RESV.

3.1.4. RESV Effect on Chlamydiaceae. The ability of RESV to inhibit other *Chlamydiaceae* was tested. McCoy cells were infected with *C. muridarum* and *C. pneumoniae* in the presence of RESV at concentrations from 12.5 to 100 μM. Both of the *Chlamydiaceae* tested were completely inhibited by 75 μM RESV treatment.

4. Discussion

C. trachomatis is an obligate intracellular epitheliotropic bacterium with a biphasic life cycle and is responsible for a variety of human diseases. In cultured cells and the human epithelium *C. trachomatis* can exist in two distinct morphological forms: the infectious elementary body (EB) and the noninfectious metabolically active reticulate body (RB). A typical chlamydial infection cycle begins with the entry of the EB into the host cell. Upon entry, the EBs differentiate into the RBs, which multiply and differentiate back to EB within the endosome (chlamydial inclusion). Newly formed EBs are usually released from the disrupted cells to initiate a new round of infection in the adjacent host cells [12, 16].

Despite the fact that chlamydial infection can be treated with antibiotics, growing antibiotic resistance and the frequent occurrence of persistent infections dictate the necessity of the search for new non-antibiotic inhibitors of chlamydial growth [17]. Various compounds with antichlamydial activity including Toll-like receptor agonists, metalloprotease inhibitors, and inhibitors of the bacterial type III secretion system have recently been identified [18–20]. These compounds exercise their antichlamydial activity either by

direct effect on *C. trachomatis* viability and/or by modulating the host cell response. Host-directed therapeutic strategies become a new emerging reality in the treatment of chlamydial infections.

In the present paper, we report that resveratrol, an antifungal compound found in grapes and other plants, has a distinct ability to inhibit the *C. trachomatis* developmental cycle in McCoy cells, a classic cell line used for chlamydial research. This major conclusion is well documented by several lines of experimental evidence. First of all, there is a dose-dependent decline in the number of *C. trachomatis*-infected McCoy cells when treated with RESV, as revealed by IF staining with LPS-specific monoclonal antibodies. A similar tendency has been observed with another immunoassay utilizing MOMP-specific monoclonal antibody. Moreover, there was a significant stepwise reduction in the number of *C. trachomatis* infective progeny induced by increasing concentrations of RESV. Finally, the ability of RESV to arrest the infectious cycle of *C. trachomatis* in McCoy cells was confirmed by nucleic acid amplification protocol. Additionally, the inhibitory effect of RESV was reproduced for other chlamydial species including *C. pneumoniae* and *C. muridarum*.

Susceptibility assessment showed that the minimum inhibitory concentration (MIC) for RESV in *C. trachomatis*-infected McCoy monolayers is 75 μM which is approximately 100 times higher than the corresponding value for azithromycin (0.67 μM), an antibiotic widely used for treatment of chlamydial infections. However, the antichlamydial activity of RESV needs to be reassessed in further cell culture studies, animal experiments, and most importantly clinical studies. RESV is known to have low bioavailability rate in in vivo conditions owing to its high susceptibility to oxidation and the hydrophobicity of the molecule [21, 22]. Thus, it is very likely that "smart delivery" technologies (nanoparticles, nutraceutical formulations) may significantly enhance the antichlamydial effect of RESV.

Nevertheless, the newly discovered anti-infective properties of polyphenols, including RESV, mark an important development in modern pharmacotherapy of infectious diseases. Antiviral activity of RESV has recently been shown in Herpes simplex, varicella-zoster, and influenza viruses as well as human cytomegalovirus [23–25], whereas antimicrobial activity of RESV has only been reported for *Neisseria gonorrhoeae*, *Neisseria meningitidis,* and other bacterial pathogens [26].

The mechanisms behind the antiviral and antibacterial properties of RESV need to be thoroughly investigated. Although our results do not disclose the precise molecular mechanisms behind the antichlamydial activity of RESV, some conclusions can be drawn from our data. The antichlamydial activity of RESV is not related in our view to its potential toxicity to eukaryotic cells nor to RESV effect on host cell proliferation since it has been observed at a wide range of RESV concentrations proven to be nontoxic (12.5 μM–75 μM). Furthermore, according to our results, the inhibitory effect of RESV is not related to the potential effect of the compound on the attachment/entry of *C. trachomatis* to the host cells or to the direct effect of RESV on chlamydial particles. Although the exact mechanism of antichlamydial activity of RESV is yet to be determined, it is obvious that RESV affects an intracellular stage of the *C. trachomatis* infectious cycle. In our opinion, the results reported above may reveal the dependence of the chlamydial developmental cycle on the host cell sirtuin pathway, a family of NAD(+)-dependent deacetylases which are a primary target of RESV in eukaryotic cells. Therefore, therapeutic strategies targeting the sirtuin pathway of host cells exposed to chlamydial pathogens should be carefully evaluated in future studies. If our results obtained in cultured cells have some equivalence to *in vivo* systems, the addition of RESV to antibiotic regimens may hold some promise for treatment of chlamydiosis.

Moreover, there is an interesting pattern of chlamydial mRNA regulation in McCoy cells treated with RESV. An unequivocal and steep dose-dependent decline in mRNA values for *incA* and *omcB*, which are markers for the middle and late stages of the chlamydial developmental cycle, was accompanied by discordant changes in *euo* mRNA. The *euo* gene is a newly emerging informative marker of the early phase of the chlamydial developmental cycle which encodes a histone H1-specific protease [27]. Its mRNA values were significantly reduced at lower RESV concentrations (25 and 50 μM). In contrast, higher concentrations of RESV (75 and 100 μM) caused less significant euo mRNA reduction. If this pattern of euo mRNA changes is complemented by a relatively well preserved histone condensation rate, our results may suggest that the early phase of the chlamydial developmental cycle is less impacted by RESV treatment than the middle and late stages of *C. trachomatis* infection. Although such reasoning is highly speculative, further investigation focused on the middle and late stages of chlamydial infection may reveal important mechanisms behind the antichlamydial activity of RESV. Additional studies are required to verify whether RESV has some antichlamydial activity under "in vivo" conditions and whether our recent results have any relevance to clinical practice.

Conflicts of Interest

The authors declare no conflicts of interest involved.

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Research Article

Purification, Characterization, and Mode of Action of Pentocin JL-1, a Novel Bacteriocin Isolated from *Lactobacillus pentosus*, against Drug-Resistant *Staphylococcus aureus*

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Staphylococcus aureus and its drug-resistant strains, which threaten public health and food safety, are in need of effective control by biopreservatives. A novel bacteriocin, pentocin JL-1, produced by *Lactobacillus pentosus* that was isolated from the intestinal tract of *Chiloscyllium punctatum*, was purified by a four-step chromatographic process. Mass spectrometry based on MALDI-TOF indicated that pentocin JL-1 has a molecular mass of 2987.23 Da. Only six of the twenty-five amino acids could be identified by Edman degradation. This bacteriocin is thermostable and tolerates a pH range of 5–7. Also, it is sensitive to proteinase K, trypsin, pepsin, and alkaline protease. This bacteriocin has a broad inhibitory spectrum against both Gram-positive and Gram-negative strains and in particular is effective against multidrug-resistant *S. aureus*. Additionally, we showed that the cell membrane is the target of pentocin JL-1 against methicillin-resistant *S. aureus* (MRSA), causing a loss of proton motive force. Furthermore, pentocin JL-1 has a drastic impact on the structure and integrity of MRSA cells. These results suggest that pentocin JL-1 has potential as a biopreservative in the food industry.

1. Introduction

Staphylococcus aureus belongs to the Gram-positive Micrococcaceae family and is one of the most serious bacterial pathogens globally [1]. It can produce several toxins including staphylococcal enterotoxins, which are a major cause of several illnesses, especially food-borne diseases resulting from the consumption of a broad variety of contaminated food such as meats, dairy products, baked goods, and salads [1–3]. In 1961, methicillin-resistant *S. aureus* (MRSA) was first found among *S. aureus* clinical isolates [4] and carries an increased risk for morbidity and mortality. Moreover, the preferred treatment agent against MRSA, vancomycin, has been reported to have reduced efficacy [5]. In addition, many other types of multidrug-resistant *S. aureus* have been detected in the past few years [6–8]. Drug-resistant *S. aureus* strains are a potential risk to humans as they could transfer the resistance to other pathogenic bacteria of humans through the food chain, the genetic pool of bacteria, bacteriophages, or DNA fragments [9, 10]. There is thus an urgent need to discover novel and effective biopreservatives and antimicrobial drugs to inhibit *S. aureus* and its drug-resistant strains for either food preservation or prevention and control of bacterial infectious diseases [10].

Bacteriocins are prokaryotic proteins or peptides, which exhibit inhibitory activity against other prokaryotes [10, 11]. Particularly, the bacteriocins produced by lactic acid bacteria (LAB) have been the focus of much research because LAB and their metabolic products are generally regarded as safe (GRAS) [12] and have potential application as natural preservatives in the food industry [13]. Currently, bacteriocins produced by Gram-positive strain are classified into five groups [14]: class I, small (<5 kDa) and linear peptides containing posttranslationally modified amino acids, including those with thioether bridges formed between the thiol groups of Cys residues and the β-carbon of other amino acid residues; class II, small (<10 kDa), linear peptides without posttranslationally modified amino acids; class III, proteins

(>10 kDa); class IV, small (<10 kDa), circular peptides without posttranslationally modified amino acids and with an amide bond between the N- and C-termini; class V, small (<5 kDa), linear or circular peptides containing extensively posttranslationally modified amino acids with thioether bridges formed between α-carbon of other amino acid residues and the thiol groups of Cys residues. However, there is no international standard of classification, and other schemes have been proposed along with information regarding their characteristics [15, 16].

In recent years, many useful LAB bacteriocins have been identified and studied, such as lactococcin A [17], pentocin TV35b [18], amyovorin L471 [19], lacticin Q [20], plantaricin ZJ008 [11], and lactocin XN8-A [21]. So far, nisin produced by *Lactococcus lactis*, pediocin produced by *Pediococcus acidilactici*, and a combination of three bacteriocins (carnocyclin A, carnobacteriocin BM1, and piscicolin 126), all produced by *Carnobacterium maltaromaticum* UAL307, which has been commercialized in the USA and Canada, with the name of Micocin®, are used as food preservatives commercially [22, 23]. Other effective LAB bacteriocins are in the process of obtaining commercial status to be used as food preservatives [22].

Treatment with LAB bacteriocins is an effective and safe way to inhibit *S. aureus* growth in food. Many researchers have shown that LAB bacteriocins have anti-MRSA ability [11, 24, 25]. However, only a few studies have been performed to investigate the LAB bacteriocins against other drug-resistant *S. aureus* such as anticiprofloxacin, anticefoxitin, and antigentamicin [21]. In our study, we aimed to purify and characterize pentocin JL-1, which was produced by *Lactobacillus pentosus* isolated from the intestinal tract of *Chiloscyllium punctatum*, exhibiting a broad inhibitory spectrum. This bacteriocin can inhibit not only MRSA but also other multidrug-resistant *S. aureus* strains. In addition, the mode of action by which pentocin JL-1 causes cell membrane damage in MRSA was characterized.

2. Materials and Methods

2.1. Isolation and Identification of Antimicrobial Strains. The intestinal tracts of *C. punctatum* (grey carpet shark) were dissected and homogenized in 20 mL saline solution under sterile conditions and were then plated in serial dilutions in deMan, Rogosa, and Sharpe (MRS) medium. The plates were incubated aerobically at 30°C for 24 h. Several colonies were picked at random and incubated again in MRS broth. For screening for bacteriocin-producing strains, the agar-well diffusion test was used to detect antimicrobial activity in cell-free supernatants (filtered through a 0.22 μm Millipore filter) obtained by centrifugation (10,000g, 30 min, 4°C) after 18, 36, 60, and 72 h incubation [10]. The Gram-positive strain MRSA GIM 1.771 and the Gram-negative strain *Escherichia coli* O157:H7 GIM 1.707 were used as indicator strains. Before the experiments, the indicator strains were grown to 10^6 CFU/mL. Then, 1 mL of the culture was mixed with 100 mL soft agar medium and poured onto individual Petri dishes. Subsequently, 8 mm diameter wells were punched onto the plates, and each well was filled with 100 μL of the cell-free supernatants under sterile conditions. The plates were incubated overnight at their respective optimum temperatures, and the clear zones of inhibition were measured in diameter and indicated the presence of antimicrobial activity. In our study, all bacteria culture media and chemical reagents were supplied by Sigma-Aldrich (USA).

The strain with the highest antibacterial activity against MRSA GIM 1.771 and *E. coli* O157:H7 GIM 1.707 was selected and named JL-1. It was stored at −80°C in MRS broth with 25% (*v/v*) glycerol. The strain JL-1 was then identified by 16S rRNA gene sequencing with the forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and the reverse primer 5′-CTACGGCTACCTTGTTACGA-3′. Subsequently, sequence homologies were analyzed by comparing the sequence with those in the NCBI database and phylogenetic analysis was carried out using MEGA 5.0.

2.2. Purification of the Bacteriocin. The strain JL-1 was grown in MRS medium at 30°C for 72 h. The ferments were centrifuged (10,000g, 30 min, 4°C), and the cell-free supernatant was absorbed with the macroporous resin D4020 (average pore diameter 100–105 Å, specific surface area of 540–580 m^2/g, Nankai University Chemical Factory, China). The column was eluted with 20% (*v/w*) ethanol and active fractions were collected. The antimicrobial activity of each fraction was determined by measuring the diameter of the inhibition zone around the wells compared with nisin and expressed as international units (IU) per mL [11]. The active fractions were lyophilized using a freeze-dryer (Labconco, USA). The lyophilized powder was dissolved in 20 mM phosphate buffer (pH 7.0) for the next purification step. Then, cation-exchange chromatography and gel filtration were performed using the AKTA Pure 25 (GE, Uppsala, Sweden) chromatography system equipped with a full wavelength UV detector and an automatic collector. The sample was purified using an SP-Sepharose Fast Flow column (XK 16/40, GE) and elution with 0-1 M NaCl in citric acid-phosphate buffer (pH 7.0) at 1.0 mL/min. The active fraction was collected and purified by an Ultrahydrogel TM 250 gel filtration column (Waters, USA), after elution with pure water at 1.0 mL/min. Then, the active fraction was purified on a C_{18} column (10 × 250 mm, 5 μm) using the LC-6AD semipreparative High Performance Liquid Chromatography (HPLC) system (Shimadzu, Japan) at 2.5 mL/min with a gradient elution of 100% buffer A (95% water, 5% acetonitrile, and 0.1% trifluoroacetic acid) to 100% buffer B (100% acetonitrile and 0.1% trifluoroacetic acid). The active fraction was collected and repurified using an analytical C_{18} column (150 × 4.6 mm, 5 μm) with an elution with 40% acetonitrile. The active fraction was collected and lyophilized for mass spectrum (MS) detection. Purified pentocin JL-1 was lyophilized using a freeze-dryer (Labconco, USA). MRSA GIM 1.77 was used as the indicator strain for the activity test.

2.3. Mass Spectrometry and Amino Acid Sequence. The molecular mass of the purified pentocin JL-1 was detected by MALDI-TOF-MS (Shimadzu Axima Assurance, Japan), which was analyzed by GL Biochem (Shanghai, China). The N-terminal amino acid sequence of the purified pentocin JL-1

Table 1: Inhibitory spectrum of pentocin JL-1.

Indicator strains	Source	G^+/G^-	Antimicrobial activity
Lactobacillus acidophilus	ATCC314	G^+	−
Lactobacillus casei	ATCC393	G^+	+++
Bacillus subtilis	CGMCC1.1627	G^+	++
MRSA	GIM1.771	G^+	+++
Enterococcus faecalis	ATCC51575	G^+	++
Listeria monocytogenes	ATCC19112	G^+	++
Micrococcus luteus	CGMCC1.2299	G^+	++
Vibrio parahaemolyticus	GIM1.306	G^-	−
Pseudomonas aeruginosa	CGMCC1.1785	G^-	+
Shigella dysenteriae	CGMCC1.1869	G^-	+++
Escherichia coli O157:H7	GIM1.707	G^-	+++

Inhibition zone in diameter (mm): +++: 20–25; ++: 15–19; +: 10–14; −: no inhibitory activity (including the 8 mm diameter of each well). ATCC, American Type Culture Collection, Virginia, USA; CGMCC, China General Microbiological Culture Collection Center, Beijing, China. GIM, Guangdong Microbiology Culture Center, Guangdong, China.

was detected by PPSQ33A automatic sequencing system (Shimadzu, Japan), which was analyzed by Shanghai Sangon Biotech Company, China.

2.4. Bacteriocin Activity Assay. The agar-well diffusion test as described previously was used to detect the antimicrobial activity of the purified pentocin JL-1 (15 μg/mL, pH 5.5) [10]. The indicator strains are listed in Tables 1 and 2. LAB strains were grown in MRS broth at 30°C for 16 h. Other Gram-positive indicator strains were grown in Tryptone Soy Broth (TSB) medium at 37°C and the Gram-negative indicator strains were grown in Luria-Bertani broth medium at 37°C.

Additionally, the minimal inhibitory concentration (MIC) of pentocin JL-1 against MRSA GIM 1.771 was tested. Overnight culture of MRSA GIM 1.771 with the concentration of around 2×10^6 CFU/mL was collected and 50 μL of each was grown in 96 well-microtiter plates (Bio-Rad, USA) with different concentrations of pentocin JL-1, ranging from 50 ng/mL to 15 μg/mL, at 37°C for 24 h. Each concentration was done in triplicate. The MIC represents the bacteriocin concentration at which 100% of growth is inhibited measured by the absorbance at 540 nm [22].

2.5. Stability against pH, Temperatures, and Enzymes. To determine pH stability, lyophilized purified pentocin JL-1 was dissolved in 0.05% (*w/v*) acetic acid at 15 μg/mL and was adjusted with 1.0 M HCl or 1.0 M NaOH to different pH values of 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0. Then the above samples were incubated at 37°C for 1 h and were adjusted to pH 5.5 with 1.0 M HCl or 1.0 M NaOH.

To determine thermal sensitivity, lyophilized purified pentocin JL-1 dissolved in 0.05% (*w/v*) acetic acid at 15 μg/mL was evaluated at different temperatures (−20°C, 4°C, 30°C, 60°C, and 100°C) for 1 h and also at the autoclaved condition (121°C at 15 psi for 15 min).

To determine enzymatic sensitivity, lyophilized purified pentocin JL-1 dissolved in 0.05% (*w/v*) acetic acid at 15 μg/mL was treated with the following enzymes at 75 μg/mL under their respective optimum pH and temperatures: proteinase K (pH 7.5, 37°C), trypsin (pH 8.0, 37°C), pepsin (pH 2.0, 37°C), and alkaline protease (pH 8.6, 50°C) (all from Sigma-Aldrich, USA). After incubation for 1 h, the bacteriocin-enzyme mixture was boiled for 5 min to inactivate the enzymes.

The residual antibacterial activities of the above samples were calculated using the agar-well diffusion test with MRSA GIM 1.771 as the indicator strain. The area of inhibition was calculated from the diameter of the inhibition zones, and the decrease ratio was displayed as a percentage. Lyophilized pentocin JL-1 dissolved in 0.05% (*w/v*) acetic acid at 15 μg/mL with pH 5.5 was used as a control in all assays.

2.6. Mode of Action

2.6.1. Growth Curve and Time-Killing Kinetics. The bacteriostatic or bactericidal mode of action of pentocin JL-1 was tested as described previously by Zhu et al. with some modifications [11]. MRSA GIM 1.771 was cultivated to the exponential phase in 100 mL of TSB culture medium. The lyophilized purified pentocin JL-1 dissolved in 0.05% (*w/v*) acetic acid was added to the cultures at a final concentration of 1x MIC and the same volume of 0.05% (*w/v*) acetic acid was added to the aforementioned media as a control. Samples were incubated at 37°C and bacterial suspensions were taken each hour for 24 h, and the absorbance was measured at OD_{600}. In addition, the viable cell counts on TSB agar medium after the addition of 1x MIC, 2x MIC, and 3x MIC of pentocin JL-1 were also quantified every 10 min for 1 h.

2.6.2. Proton Motive Force (PMF). To test the effect of pentocin JL-1 on membrane integrity, the cell PMF was assayed. PMF includes the transmembrane electrical potential ($\Delta\Psi$) and the transmembrane pH gradient (ΔpH) [26]. $\Delta\Psi$ was monitored by the fluorescent probe 3,3′-diethylthiadicarbocyanine iodide $DisC_2(5)$ (Sigma, USA). MRSA GIM 1.771 cells were grown to the exponential phase in 50 mL TSB culture medium, harvested, and washed twice with 50 mL buffer A (250 mM glucose, 5 mM $MgSO_4$, 10 mM K_3PO_4, and 100 mM KCl, pH 7.0) at 4°C, resuspended in 5 mL of the same buffer and stored on ice for the fluorescence measurements. Then, the cells were added to a fluorescence cuvette together with 0.5 μM $DisC_2(5)$. The fluorescence emission was monitored at room temperature using an F-4600 spectrofluorometer (Hitachi, Japan) with an excitation wavelength (Ex) of 647 nm and emission wavelength (Em) of 680 nm for 400 s. When the reduction of fluorescence was stable, final concentrations of 1x, 2x, and 3x MIC of pentocin JL-1 were added to the cuvette, respectively, and 0.05% (*w/v*) acetic acid was added as a negative control. Full dissipation of the membrane potential was indicated by addition of 1% Triton X-100. To the control, an equivalent volume of 3x MIC of pentocin JL-1 was added.

ΔpH was monitored by the fluorescent pH probe 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein,

Table 2: Pentocin JL-1 activity against multidrug-resistant *S. aureus*.

Indicator strains	Isolation sources	Inhibitor zone (mm)	Resistance antibiotics[a]
Multidrug-resistant *S. aureus* 1	Pork	23.8 ± 0.8	FOX, TET
Multidrug-resistant *S. aureus* 2	Pork	22.5 ± 1.2	FOX, GEN
Multidrug-resistant *S. aureus* 3	Pork	24.3 ± 0.7	FOX, TET, GEN
Multidrug-resistant *S. aureus* 4	Pork	22.4 ± 0.6	FOX, TET, C
Multidrug-resistant *S. aureus* 5	Pork	23.9 ± 1.5	FOX, TET, GEN, C
Multidrug-resistant *S. aureus* 6	Pork	23.7 ± 0.6	CIP, FOX, C, SXT, TET, GEN

[a]FOX, cefoxitin; TET, tetracycline; GEN, gentamicin; C, chloramphenicol; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole.

acetoxymethyl ester (BCECF AM) (Beyotime, China). MRSA GIM 1.771 cells were grown to the exponential phase in 50 mL TSB culture medium, harvested, washed twice with 50 mL 5 mM HEPES buffer at 4°C, and resuspended in 5 mL of the same buffer for incubation on ice for 1 h. Subsequently, the 1 μM BCECF AM pH probe was added and the solution was incubated at 37°C for 1 h in the dark. Then 1 mL of the incubated solution was added to a fluorescence cuvette together with a final concentration of 1x, 2x, and 3x MIC of pentocin JL-1, respectively. Acetic acid (0.05%, *w/v*) was used as a negative control and 1% (*w/v*) Triton X-100 was used as a positive control. To the control, an equivalent volume of 3x MIC of pentocin JL-1 was added. The fluorescence intensity was monitored at 50 s intervals for 400 s immediately after mixing at Ex 488 nm and Em 535 nm using an F-4600 spectrofluorometer (Hitachi, Japan).

2.6.3. Scanning Electron Microscopy (SEM). MRSA GIM 1.771 cells in exponential phase were supplemented with 1x MIC of pentocin JL-1 and incubated at 37°C for 10 min. Cells without pentocin JL-1 were used as controls. Cells were collected by centrifugation at 4°C, 6000g for 5 min, and washed gently with 500 μL phosphate buffer saline (PBS, 0.1 M, pH 7.4) twice. Subsequently, the cells were fixed in 2.5% glutaraldehyde at 4°C for 16 h and washed gently with 500 μL PBS twice. Then the cells were dehydrated with gradient ethanol solutions (30%, 50%, 70%, 80%, 90%, and 100%) at 4°C and centrifuged at 6000g for 15 min. The cells were then freeze-dried using a freeze-dryer (Labconco, USA), coated with gold, and imaged using an SU8010 scanning electron microscope (Hitachi, Japan).

2.7. Statistical Analysis. All related experiments were done in triplicate and the results are expressed as mean ± standard deviation. Data analysis was performed with SPSS 19.0 and Origin 8.0. Comparison of data on stability of pentocin JL-1 against pH, temperatures, and enzymes were performed using independent sample t-test and $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Identification of the Bacteriocin-Producing Strain JL-1. The strain JL-1 was selected at 72 h incubation because of its highest antibacterial activity against MRSA GIM 1.771 and *E. coli* O157:H7 GIM 1.707 with a diameter of the inhibition zones of 24.8 ± 0.5 mm and 23.9 ± 0.2 mm, respectively. It is a Gram-positive and catalase-negative *Bacillus*. The 16S rRNA gene sequence was amplified by PCR, and a 1485 bp gene fragment was sequenced after cloning and aligned using the NCBI database. We found that the 16S rRNA sequence of strain JL-1 had 99% similarity with that of *L. pentosus* KC422317.1. Additionally, a phylogenetic tree was constructed using MEGA 5.0 (Figure 1). The sequence of the 16S rRNA gene from strain JL-1 was submitted to the GenBank database under the accession number KY777710.

It has been reported that *L. pentosus*, a *Lactobacillus* species usually isolated from fermented and pickled food and animal intestines, can produce many functional metabolites, such as exopolysaccharides [27], β-galactosidase [28], and also some bacteriocins [29–31]. *L. pentosus* is LAB, so its metabolic products are GRAS and have the potential to act as natural preservatives [12, 32].

3.2. Purification of the Bacteriocin. The bacteriocin, pentocin JL-1, is one of the secondary metabolites of *L. pentosus* JL-1 after a 72 h incubation. In order to purify this bacteriocin, macroporous resin, cation-exchange, gel filtration, and semipreparative HPLC were used. During the purification process, its antibacterial activity against MRSA GIM 1.771 was evaluated. The crude bacteriocin was collected using macroporous resin D4020 and then was purified by SP-Sepharose Fast Flow. Three fractions F1, F2, and F3 were collected, and the fraction F3 had antibacterial activity against MRSA GIM 1.771 (see Figure S1 in Supplementary Material available online at https://doi.org/10.1155/2017/7657190), with a specific activity reaching up to 432 IU/mg (Table 3). The active fraction F3 was subsequently purified with Ultrahydrogel TM 250 gel filtration chromatography (Figure S2). In this step, three fractions F3A, F3B, and F3C were collected and their antibacterial activities were tested. The highest antibacterial activity fraction of F3A was collected for further purification by semipreparative HPLC, as shown in Figure 2. The fraction F3Aa had significantly higher antibacterial activity against MRSA GIM 1.771 than F3Ab ($p < 0.05$). F3Aa was repurified by analytical HPLC (data not shown), and the active fraction was collected and lyophilized for MS detection. The purification process and antibacterial activity are listed in Table 3. After the four-step purification, pentocin JL-1 was purified 70.7-fold at a yield of 4.7%. In previous studies, pentocin SJ-65 produced by *L. pentosus* SJ65 was purified 52-fold at a yield of 8% [33], bacteriocin KU24 produced by *L. lactis* KU24 was purified 24.58-fold [25], and plantaricin ZJ5 produced by *L. plantarum* ZJ5 was purified 139.5-fold at a yield of 1.7% [13].

Table 3: Purification and activity of the bacteriocin produced by *L. pentosus* JL-1.

Samples	Total protein (mg)	Total bacteriocin activity (IU)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Supernatant	2690	100440	37	1.0	100.0
Macroporous resin D4020	460	49740	108	2.9	49.5
Cation exchange	47	20320	432	11.7	20.2
Gel chromatography	12	11090	924	25.0	11.0
C_{18} RP- HPLC	1.8	4710	2617	70.7	4.7

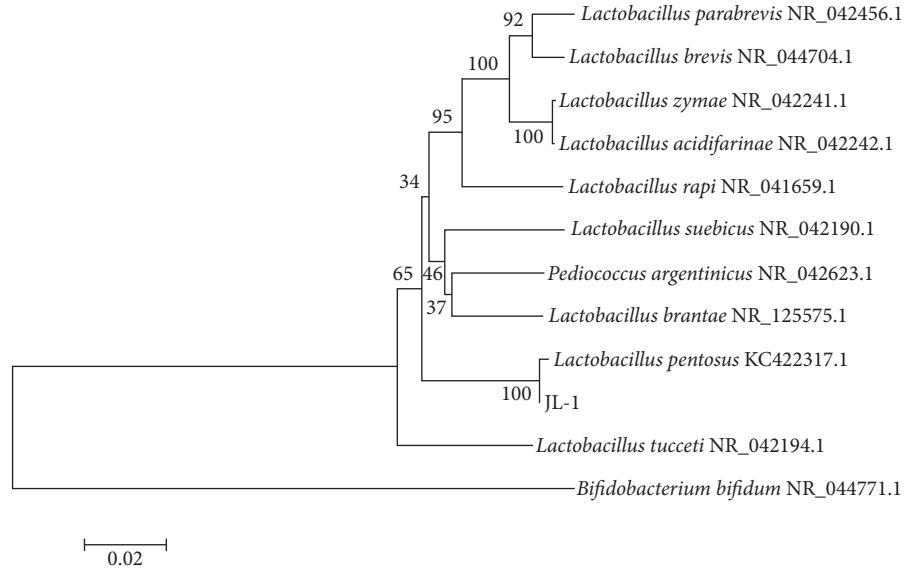


Figure 1: Phylogenetic tree of strain JL-1 based on its 16S rRNA sequence.

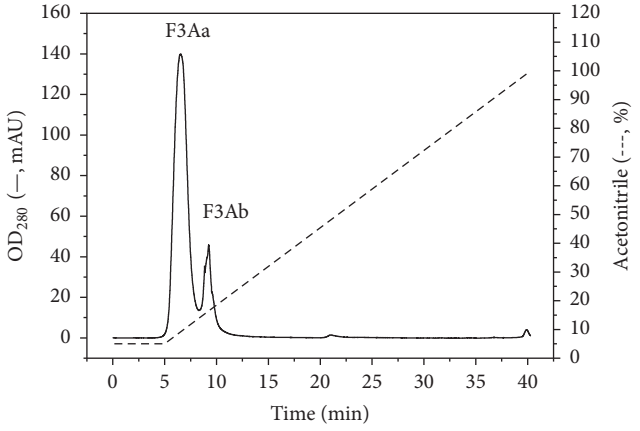


Figure 2: Purification of the bacteriocin produced by *L. pentosus* JL-1 by semipreparative HPLC.

The yield of pentocin JL-1 can be enhanced by optimizing the production conditions. In addition to incubation temperatures, initial pH values, inoculum density, loading volume, and culture medium optimization, coculture with other strains and autoinduction by a signal peptide produced by the strain itself are effective ways to improve the yield of bacteriocins. For example, at low cell densities, gassericin E was produced by *L. gasseri* EV146 after the addition of the supernatant from a previous bacteriocin-producing EV1461 culture (autoinduction) or through cocultivation with several other Gram-positive strains (inducing bacteria) [34]. In addition, genetic engineering is a strategy to enhance the production of bacteriocins. In recent years, *L. plantarum*, *L. lactis*, *L. sakei, S. thermophilus,* and various other LAB have been used as hosts for heterologous expression [32, 35, 36].

In addition, from the purification process, we observed that pentocin JL-1 is a cationic and hydrophobic peptide, which was shown by cation-exchange, hydrophobic-interactions, and C_{18} reverse-phase HPLC (C_{18} RP-HPLC). Many bacteriocins such as bacteriocin KU24 [25], bacteriocin VJ13 [37], plantaricin ZJ5 [13], and other LAB bacteriocins share similar properties. Thus, the purification strategies can be compared and referenced.

3.3. Mass Spectrometry and Amino Acid Sequence. Pentocin JL-1 was identified by MALDI-TOF-MS. The results indicated that the purified pentocin JL-1 has a molecular mass of 2987.23 Da (Figure 3), which was different from those of the bacteriocins produced by *L. pentosus* reported previously, including the bacteriocin from *L. pentosus* RL2e of around 20 kDa [29], the bacteriocin B231 produced by *L. pentosus*

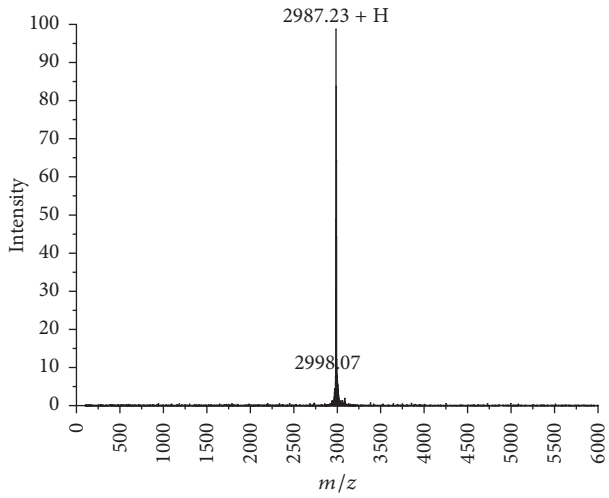


Figure 3: MALDI-TOF-MS of analytical HPLC purified pentocin JL-1.

of about 5 kDa [30], pentocin C50-6of about 2.5 kDa [31], and pentocin 31-1 of 5,592.225 Da [38]. In addition, a variety of new bacteriocins produced by LAB has been successfully purified and characterized in the past few years. The molecular masses of the majority plantaricins is >3.0 kDa, although some smaller plantaricins have also been reported, such as plantaricin DL3 (2.1 kDa) [39] and ZJ008 (1334.77 Da) [11]. However, to the best of our knowledge, the present study is the first report of a pentocin with a molecular mass of 2987.23 Da. In addition, six of the twenty-five amino acids of pentocin JL-1 could be identified by Edman degradation and the sequence is VAKVAR. Further sequencing failed probably due to the presence of a modified residue or some rare amino acids in the peptide that prevented cleavage by the Edman's reagent. The sequence showed no homology with other known bacteriocins using protein BLAST against the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Thus, pentocin JL-1 may be a novel LAB bacteriocin. To obtain more detailed information, further chemical and mass spectrometry techniques needed to be performed. In addition, the complete genome sequencing of our *L. pentosus* will be performed to gain insights into the genetic elements involved in bacteriocin production in the future.

3.4. Stability against pH, Temperatures, and Enzymes. The effects of pH, temperatures, and enzymes on the antibacterial activity of the bacteriocin were determined (Table 4). No significant differences in antimicrobial activities were found from pH 5 to pH 7 ($p > 0.05$). However, the antimicrobial activity decreased significantly when the pH decreased from 4 to 2 and increased from 8 to 10 ($p < 0.05$). Some other bacteriocins have also been reported to have a similar pH stability [21, 40]. It may be that, at extreme pH values, strong intramolecular electrostatic interactions cause a partial or total loss of activity [41]. However, even at pH 2 and pH 10, pentocin JL-1 still retained 65.69% and 52.30% of antibacterial activity, respectively, which indicates that this bacteriocin may be used in most food.

Table 4: Stability of pentocin JL-1 against pH, temperatures and enzymes.

Treatment	Residual inhibitory activity (inhibition zone diameter, mm)	Residual inhibitory activity (%)
pH value		
Control (5.5)	23.9 ± 0.7	100.00
2.0	15.7 ± 1.4	65.69*
3.0	16.2 ± 1.8	67.78*
4.0	16.8 ± 1.2	70.29*
5.0	23.9 ± 0.4	100.00
6.0	23.9 ± 0.8	100.00
7.0	23.6 ± 0.6	98.74
8.0	14.6 ± 0.3	61.09*
9.0	12.9 ± 0.9	53.97*
10.0	12.5 ± 1.6	52.30*
Temperature		
Control	23.9 ± 0.7	100.00
−20°C, 1 h	22.2 ± 1.0	93.00
4°C, 1 h	23.8 ± 1.2	99.58
30°C, 1 h	23.9 ± 0.2	100.00
60°C, 1 h	23.6 ± 0.8	98.74
100°C, 1 h	22.6 ± 0.8	94.52
121°C, 15 min	20.1 ± 0.9	84.10*
Enzyme		
Control	23.9 ± 0.7	100.00
Proteinase K (pH 7.5, 55°C)	17.3 ± 1.2	72.38*
Trypsin (pH 8.0, 37°C)	15.7 ± 1.2	65.69*
Pepsin (pH 1.8, 37°C)	0.0 ± 0.0	0.00*
Alkaline protease (pH 8.6, 50°C)	0.0 ± 0.0	0.00*

*The decrease being considered statistically significantly ($p < 0.05$).

At different temperatures, pentocin JL-1 was stable, and no significant differences were detected from −20°C to 100°C ($p > 0.05$). An antibacterial activity of 84.10% remained even after autoclavation (121°C, 15 min). This thermostable characteristic makes pentocin JL-1 suitable for use in a sterilization process.

When purified pentocin JL-1 was treated with different hydrolytic enzymes, its inhibitory action was significantly reduced by treatment with proteinase K and trypsin ($p < 0.05$). However, the inhibitory action was completely abolished by treatment with pepsin and alkaline protease. Thus, pentocin JL-1 has a proteinaceous nature like most other bacteriocins [11, 13, 19, 21].

3.5. Inhibitory Spectrum. The inhibitory spectrum of pentocin JL-1 is shown in Tables 1 and 2. As shown in Table 1, pentocin JL-1 was inhibitory against both Gram-positive and Gram-negative bacteria. Among the indicator species, the

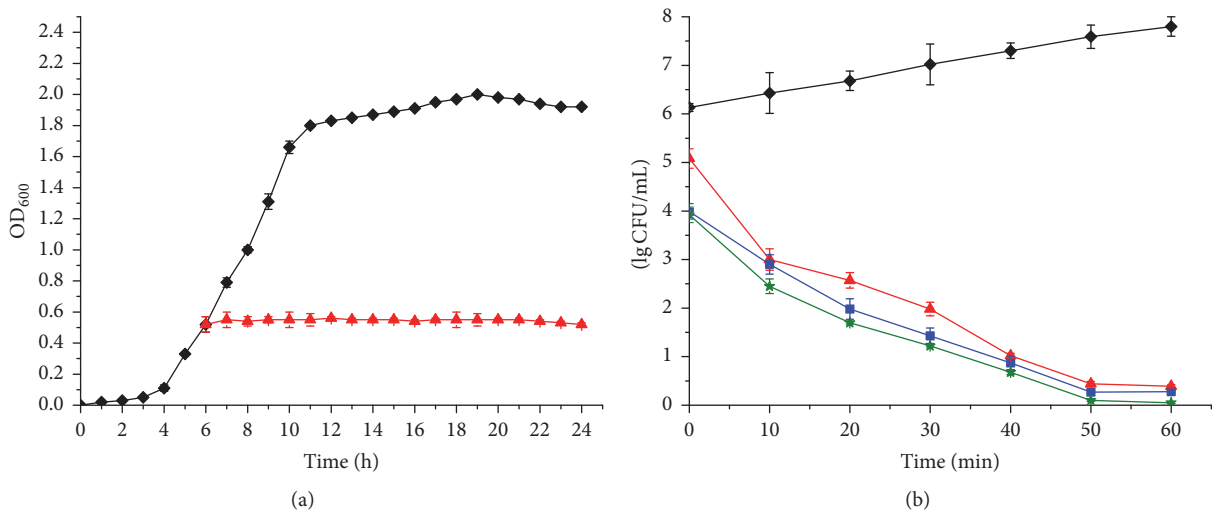


Figure 4: Effects of pentocin JL-1 on intact cells. (a) The effects of pentocin JL-1 on MRSA GIM 1.771 growth and (b) time-killing kinetics by pentocin JL-1. Control (solid diamond); 1x MIC (solid triangle); 2x MIC (solid square); 3x MIC (solid star).

bacteriocin showed the highest activity against the Gram-positive bacteria *L. casei* ATCC 393 and MRSA GIM 1.771 and the Gram-negative bacteria *Shigella dysenteriae* CGMCC 1.1869 and *E. coli* O157:H7 GIM 1.707, with a diameter of inhibition zones 20–25 mm. In addition, it inhibited *Bacillus subtilis* CGMCC 1.1627, *Enterococcus faecalis* ATCC 51575, *Listeria monocytogenes* ATCC 19112, *Micrococcus luteus* CGMCC 1.2299, and *Pseudomonas aeruginosa* CGMCC 1.1785. However, pentocin JL-1 had no inhibitory activity against *L. acidophilus* ATCC 314 and *Vibrio parahaemolyticus* GIM 1.306. Many pentocins produced from *L. pentosus* have been studied against a variety of Gram-positive and Gram-negative bacteria and the fungi *Candida albicans* [18, 29–31]. However, none have been reported to have an anti-MRSA activity, except for pentocin JL-1 in our study.

In particular, besides MRSA GIM 1.771, pentocin JL-1 could also inhibit 6 strains of multidrug-resistant *S. aureus* isolated from pork in our lab (Table 2). Not only can multidrug-resistant *S. aureus* produce toxins, but also the transfer of resistance to other pathogenic bacteria of humans is a potential threat [42]. In addition, the MIC of pentocin JL-1 against the indicator strain MRSA GIM 1.771 was 7.5 μg/mL. It has been reported that plantaricin Pln-1 inhibits MRSA with a MIC of 180 ± 20 μg/mL [43]. The MIC of lactocin XN8-A against *S. aureus* ATCC 29213 is 6.85 μg/mL, but this is not an MRSA strain [21]. Lactocin XN8-A has also antibacterial activity against pork-derived multidrug-resistant *S. aureus*, but this study did not show the MIC data [21]. In our study, pentocin JL-1 exhibited a broad inhibitory spectrum, a low MIC against MRSA, and a high antimicrobial activity against multidrug-resistant *S. aureus*, representing a potential biopreservative in the food industry. Thus, further study was needed to identify its mode of action.

3.6. Mode of Action of Pentocin JL-1

3.6.1. Growth Curve and Time-Killing Kinetics. Figure 4(a) shows that the growth curve of MRSA GIM 1.771 for 24 h is typical. However, once 1x MIC of pentocin JL-1 was added, after 6 h (the exponential phase), the OD_{600} values were nearly stable. This means that pentocin JL-1 inhibited the growth of MRSA GIM 1.771 with no clear evidence of cell lysis. However, the time-killing curve showed that when 1x MIC, 2x MIC, and 3x MIC of pentocin JL-1 were added, respectively, a significant downward tendency in the viable count was observed and was dose-dependent to some extent (Figure 4(b)). Additionally, an instantaneous killing action occurred at 0 h by addition of 1x MIC, 2x MIC, and 3x MIC of pentocin JL-1 with 1.05, 2.14, and 2.21 $\log_{10}$ reduction, respectively. These results indicate that pentocin JL-1 had a bactericidal activity against MRSA GIM 1.771. Lactocin XN8-A [21], enterocin SN11 [44], and plantaricin ZJ008 [11] have also been reported to have similar bactericidal properties.

3.6.2. PMF. As many bacteriocins are assumed to kill the target microorganism via permeabilization of the cell membrane [45], the effect of pentocin JL-1 on the membrane integrity of MRSA GIM 1.771 intact cells was determined by the membrane potential sensitive dye $DisC_2(5)$ (Figure 5) and the transmembrane pH gradient fluorescent probe BCECF (Figure 6). As shown in Figure 5, when the reduction of fluorescence was stable, the accumulated dye in the membrane interior of energized cells was quenched. After a stable signal was observed, addition of the pentocin JL-1 (indicated by the first arrow in Figure 5) caused a rapid increase in fluorescence due to the collapse of the ion gradients that generate the membrane potential [46]. After the fluorescence stabilization, 1% Triton X-100 (indicated by the second arrow in Figure 5) was subsequently added, and the results indicated the 100% dissipation of the membrane potential. As shown in Figure 5, addition of 1% Triton X-100 only caused a small further increase in fluorescence for curves (b) and (c) and was nearly flat for curve (a), showing that pentocin JL-1 causes cell membrane permeabilization. Additionally, the ability of pentocin JL-1 to disturb the membrane barrier was dose-dependent. However, the fluorescence of the control sample,

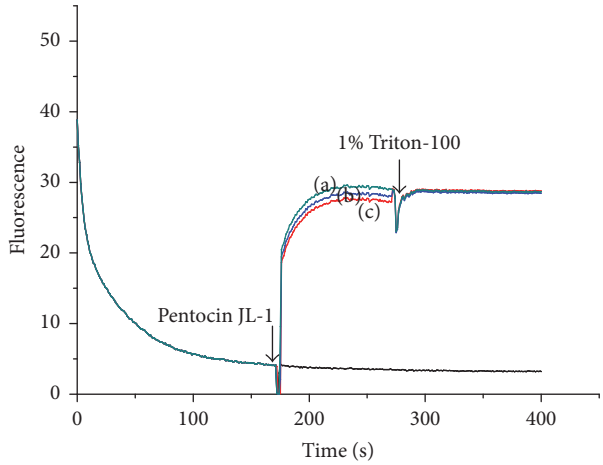


Figure 5: Analysis of ΔΨ of MRSA GIM1.771 cells. MRSA GIM 1.771 cells were treated with 3x MIC (a), 2x MIC (b), and 1x MIC (c) pentocin JL-1, respectively. 1% Triton X-100 was added as ΔΨ 100% dissipation and 0.05% (*w/v*) acetic acid was used as the negative control.

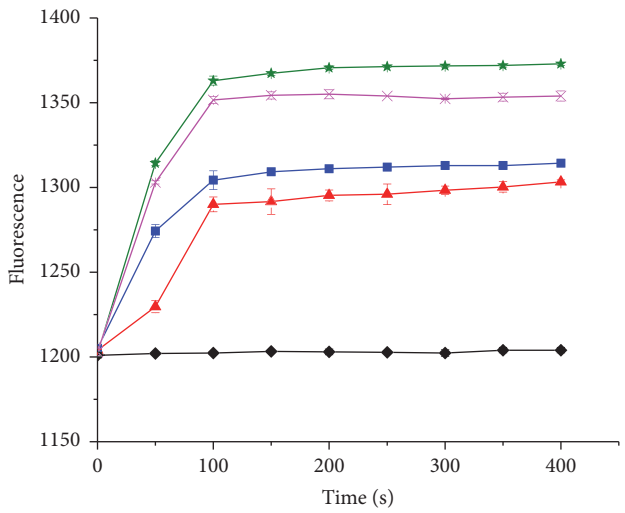


Figure 6: Analysis of ΔpH of MRSA GIM 1.771 cells. MRSA GIM 1.771 cells were treated with 3x MIC (solid star), 2x MIC (solid square), and 1x MIC (solid triangle) pentocin JL-1, respectively. 1% Triton X-100 (cross) was added as ΔpH 100% dissipation and 0.05% (*w/v*) acetic acid (solid diamond) was used as the negative control.

to which 0.05% (*w/v*) acetic acid was added, did not indicate any increase or decrease during the 400 s experiment.

Once BCECF AM is absorbed into the cell membrane, it is cleaved by an esterase into BCECF, which is a fluorescent probe that indicates a transmembrane pH gradient. As shown in Figure 6, when the samples were exposed to 1x MIC, 2x MIC, and 3x MIC of pentocin JL-1, the fluorescence of BCECF increased within 100 s and increased slightly at the later time points, indicating that ΔpH of MRSA GIM 1.771 was rapidly dissipated by pentocin JL-1. ΔpH was stable in the control sample after a 400 s incubation. However, the final fluorescence of the 1x MIC and 2x MIC treated samples was lower than that of the samples exposed to 1% Triton X-100. These results suggest that ΔpH is incompletely dissipated by 1x MIC and 2x MIC of pentocin JL-1.

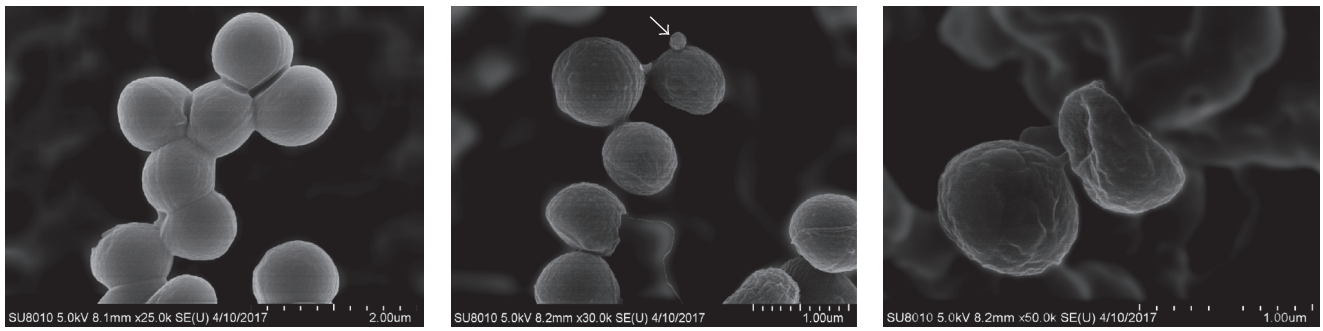
In general, cationic bacteriocins initially interact with the anionic cell membrane through electrostatic attraction [47]. Then, bacteriocins permeabilize the cell membrane to dissipate ΔΨ and ΔpH, which constitute the PMF of the cells [48]. Finally, bacteriocins have bacteriostatic or bactericidal effects. In our study, pentocin JL-1 dissipated ΔΨ and ΔpH of MRSA GIM 1.771. This result shows that the addition of pentocin JL-1 leads to the dissipation of the PMF of MRSA GIM 1.771 due to the loss of vital ion gradients and suggests that the membrane is the target of pentocin JL-1. In addition, the dissipation of the PMF was dose-dependent. Similar dose-dependent cell membrane potential dissipation results have also been shown for other bacteriocins such as aureocin A53 [49] and Pln EF [26].

ΔΨ and ΔpH dissipations were nearly complete within 100 s, which shows that the membrane permeabilization caused by pentocin JL-1 is a relatively rapid process. This is in accordance with the well-known antibiotic peptide clavanin [46], which is a membrane-targeted and dose-dependent peptide. However some other membrane-targeted bacteriocins have a gradual process of membrane potential dissipation [26, 50].

3.6.3. SEM. SEM was used to further demonstrate the membrane damage of MRSA GIM 1.771 caused by pentocin JL-1. Morphological changes of MRSA GIM 1.771 after 10 min exposure to 1x MIC of pentocin JL-1 are presented in Figure 7. Compared with the smooth surface of the control cells with integrated and plump cell structures (Figure 7(a)), cell membrane disruption and deformation with shrinking and cavities were observed on the cell surface of cells treated with pentocin JL-1 (Figures 7(b) and 7(c)). In addition, blebs (the arrow indicated in Figure 7(b)) protruded into the cell surface, which also shows that pentocin JL-1 acts on the cell surface. Blebs are a kind of vesicles, which are induced by external stimulus and might play an important role in cell-to-cell communication [26]. Cell membrane damage was clear in MRSA GIM 1.771 treated with pentocin JL-1 and is a typical characteristic caused by bacteriocins [26]. Similar membrane damage has also been reported for nisin, pediocin, and Pln EF-treated cells [26, 51, 52].

4. Conclusions

In the present study, the bacteriocin pentocin JL-1, produced by *L. pentosus* isolated from the intestinal tract of *C. punctatum*, was purified and found to have a molecular mass of 2987.23 Da. It is sensitive to proteinase K, trypsin, pepsin, and alkaline protease, indicating that it has a proteinaceous nature. Also, this bacteriocin has a broad inhibitory spectrum, is thermostable, and stable over a pH range of 5–7. Hence, pentocin JL-1 appears to have promising potential as a biopreservative in the food industry, especially for controlling multidrug-resistant *S. aureus*. Additionally, our results show that the cell membrane is the target of pentocin JL-1 against MRSA GIM 1.771, causing a loss of PMF in only a few



(a) (b) (c)

Figure 7: Scanning electron micrographs of MRSA GIM 1.771 cells. (a) Untreated control cells; (b) and (c) 1x MIC of pentocin JL-1 treated cells.

minutes, and that it has a drastic impact on the structure and integrity of the MRSA GIM 1.771 cell that finally leads to cell death, which was indicated by the growth curve and time-killing kinetics. In further studies, more detailed information on the mode of action, the exact amino acid sequence, and the structure of pentocin JL-1 will be addressed.

Disclosure

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Characterization and Identification of Cryptic Biopeptides in *Carya illinoinensis* (Wangenh K. Koch) Storage Proteins

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The objective of this research was to identify and characterize the encoded peptides present in nut storage proteins of *Carya illinoinensis*. It was found, through in silico prediction, proteomic analysis, and MS spectrometry, that bioactive peptides were mainly found in albumin and glutelin fractions. Glutelin was the major fraction with ~53% of the nut storage proteins containing at least 21 peptides with different putative biological activities, including antihypertensives, antioxidants, immunomodulators, protease inhibitors, and inhibitors of cell cycle progression in cancer cells. Data showed that using 50 μg/mL tryptic digests of enriched peptides obtained from nut glutelins is able to induce up to 19% of apoptosis in both HeLa and CasKi cervical cancer cells. To our knowledge, this is the first report that shows the potential value of the nut-encoded peptides to be considered as adjuvants in cancer therapies.

1. Introduction

Pecan is a fruit with a single stone surrounded by a husk, native of United States and México, but it has a worldwide distribution. The interest for studying Pecan has been incremented in the last years, mainly because its nut is considered as a healthy foodstuff. In this regard, it has been observed that its regular consumption decreases the risk to suffer coronary heart disease and type 2 diabetes [1]. Nut is a high-protein food as it contains from 18 to 24% on a dry weight basis [2]. When proteins from meat and plants are ingested, they elicit a wide range of nutritional and biological properties, some of which are attributed to the presence of encrypted bioactive peptides [3]. Several reports indicate that storage proteins from plants, such as soy, amaranth, and wheat, are a source of bioactive peptides [4, 5]. These peptides are inactive within the sequence of parent proteins, but they can be released during gastrointestinal digestion or foods processing [6]. Depending on the amino acid sequence, these peptides may exert a number of different activities, for example, as antihypertensive, antioxidant, antiproliferative (anticarcinogenic) activity, antithrombotic, opioid, inhibitor of enzymes, activator of proteolysis, stimulant, and metabolic regulator. Cytochemical studies have provided evidences that food-derived

bioactive peptides modulate viability, proliferation, differentiation, and apoptosis of different cell types [7], and also they are able of inhibiting cancer cells [8, 9].

In Mexico, the nut is called "the queen of dried fruits," because of its nutritional importance. It is ingested alone or mixed with other dried fruits, in desserts and ice creams, among others. Although nut has been studied in different aspects [1], to our knowledge there are no reports about either the presence of encrypted bioactive peptides in this seed, nor its role against cancer cells. In this work, we obtained different Pecan nut's protein fractions and small peptides by first time, assigned putative physiological roles, and demonstrated its effect on HeLa and CasKi cells from cervical cancer.

2. Materials and Methods

2.1. Protein Isolation of Nut. Seeds (nut) of *Carya illinoinensis* (Wangenh K. Koch), Wichita variety, were degreased for 7 h in petroleum ether, at a ratio nut/petroleum ether 1 : 14 (w/v) with the Soxhlet method reported by the AOAC [17]. The total protein isolation was developed according to the protocol established by Saravanan and Rose [18]. In brief, 5 g of nut flour was mixed with 15 mL of cold acetone containing 10% trichloroacetic acid and 0.07% β-mercaptoethanol. The mixture was homogenized by sonication for 15 min on ice and centrifuged at 2,000 ×g for 2 minutes at 4°C and proteins in the supernatant were precipitated overnight at −20°C. Proteins were concentrated by centrifugation at 10,000 ×g for 30 min at 4°C; the pellet was washed 3x in cold acetone and then dried at room temperature to remove excess of acetone and stored at −20°C [18]. For storage protein isolation, albumin fraction with nonprotein nitrogen (NPN) was obtained using distilled water as extraction agent. The suspensions of flour/solvent (1 : 10 w/v) were extracted with magnetic stirring for 1 h at 4°C and centrifuged at 10,000 ×g for 15 min at 4°C. The supernatant was collected and stored at −20°C for subsequent analysis. Pellet was resuspended in 0.1 M NaCl, 0.010 M K_2HPO_4 (pH 7.5), and 0.001 M EDTA for extracting 7S globulins. The 11S globulin fraction was obtained with 0.8 M NaCl, 0.010 K_2HPO4, and 0.001 M EDTA (pH 7.5) according to the report by De La Barba Rosa et al. [19]. Prolamin extraction was carried out using four direct extraction solvents: 70% ethanol [19]; 70% ethanol and 0.5% sodium acetate [20]; 70% ethanol, 0.5% sodium acetate, and 1% β-mercaptoethanol [21]; and 60% 2-propanol with 1% β-mercaptoethanol [22]. Finally, glutelins were obtained with a 0.1 M NaOH solution [19]. Protein fractions were quantified in triplicate using the Bradford method (Bio-Rad, Hercules, CA, USA) and then analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using β-mercaptoethanol (1% w/v) to reduce the disulfide bridges. Finally gels were stained with Coomassie Blue G250.

2.2. Prediction of Bioactive Peptides from Nut Storage Proteins. *Carya illinoinensis* nut protein sequences were obtained from the GenBank data (https://www.ncbi.nlm.nih.gov/) [23] and analyzed to obtain the profile of cryptic peptides with putative functional activity using the BIOPEP database (http://www.uwm.edu.pl/biochemia/) [24]. Nut protein sequences for analysis in silico are as follows: Glutelin (44aa, superfamily Glutelin, GenBank accession: AAC69515.1), 11S legumin (505aa, superfamily Globulin, GenBank accession: ABW86979.1), 7S vicilin (784aa, superfamily Globulin, GenBank accession: ABV49593.1), 7S vicilin (792aa, superfamily Globulin, GenBank accession: ABV49592.1), putative allergen I1 (143aa, superfamily Albumin, GenBank accession: AAO32314.1), and putative 7S vicilin (102aa, superfamily Globulin, GenBank accession: AAZ93628.1). Occurrence frequency (A) of a bioactive fragment in a protein was given by the equation: $A = a/N$, where a is number of fragments with a given activity and N is number of amino acid residues [25].

2.3. Identification of Bioactive Peptides in the Nut Protein Fractions by Mass Spectrometry (MS/MS). Nut protein fractions were digested with trypsin at a 1 : 50 ratio (trypsin : protein) for 16 h at 37°C and peptides were precipitated to obtain 200 mg of protein. Pellets were resuspended in 200 μL of urea buffer (6 M Urea, 50 mM Tris-HCl pH 8), reduced with DTT at room temperature and alkylated with iodoacetamide in the dark. Reactions were stopped adjusting pH from 3 to 4 with formic acid [26]. The analysis of liquid chromatography-mass spectrometry was performed on a SYNAP-nanoUPLC System (Waters Co., Palo Alto, CA) equipped with an ionization source unit ion spray mass spectrometry. Peptide identification was performed using MASCOT (Matrix Sciences, http://www.matrixscience.com/) [27], with information obtained in BIOPEP database.

2.4. Determination of Antiproliferative Effect of the Encrypted Peptides of Nut Glutelins in Cell Lines. HeLa and CasKi cells (immortalized cells from cervical cancer) were grown in Dulbecco's modified Eagle's media (DMEM), with high concentration in glucose, and supplemented with $NaHCO_3$ (3.5 mg/L), 10% fetal bovine serum, penicillin G (50 mg/L), streptomycin (100 mg/L), and amphotericin (1 mg/L), at 36°C and 10% of CO_2. Apoptosis assay and cell cycle distribution were done using a FACS (fluorescence activated cell sorting) in apparatus Calibur (Becton Dickinson, NJ) [28]. HeLa and CasKi cells with a confluence of 85% were treated with 50 μg/mL of trypsin-digested glutelins of nut. Cisplatin (1 μg/mL) was used as a positive control of apoptosis. Primary culture of fibroblasts was used as the control of normal cells.

3. Results

3.1. Characterization of Storage Protein in Pecan Nut. Pecan nut contained ~13.43 mg of total protein in 100 g of full-fat flour. Four soluble fractions were identified in nut total protein: albumins, globulins, prolamins, and glutelins. This last protein fraction was the most abundant representing ~53% followed by globulins, prolamins, and albumins, with percentages of ~27, 14, and 6%, respectively (Table 1). Fractions were separated by SDS-PAGE, detecting different protein patterns. For example, in the albumin fraction the proteins with apparent molecular weight of 220, 60, 55, and 52 kDa were observed. With 7S globulins the proteins of ~70, 60, 55, 50, 35, 32, 28, 18, and 17 kDa were visualized, whereas with 11S globulins the proteins with apparent molecular weight of

Table 1: Pecan nut storage proteins quantification.

Protein	Storage protein fraction	Nut flour (mg/g)	Percent (%)
Soluble	Albumin	$1.97^{a/a*} \pm 0.04$	$5.80^{a/b}$
	7S Globulin	$0.95^{b/a} \pm 0.03$	$2.80^{b/b}$
	11S Globulin	$8.09^{c/a} \pm 0.03$	$23.83^{b/b}$
	Prolamin	$4.85^{d/a} \pm 0.19$	$14.28^{c/b}$
	Glutelin	$18.08^{e/a} \pm 0.30$	$53.27^{d/b}$
Insoluble		22.00 ± 2.43	
Total		291.92 ± 11.64	

*Means with different superscript letter indicates significant difference ($p < 0.05$).

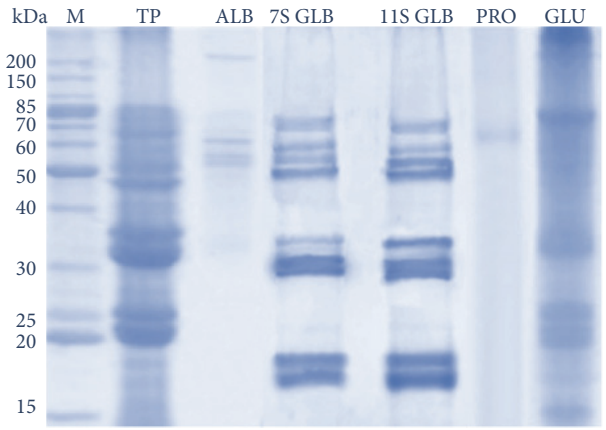

Figure 1: SDS-PAGE of nut's protein fraction. M, molecular weight marker; TP, total protein; ALB, albumins; 7S GLB, 7S globulins; 11S GLB, 11S globulins; PRO, prolamins; GLU, glutelins.

70, 60, 55, 50, 35, 30, 27, 20, 18, and 17 kDa were obtained. Under the conditions used in the assays, only one protein of ~65 kDa was resolved in the prolamin fraction, whereas in the glutelin fraction proteins of ~80, 35, 25, 22, 20, and 15 kDa were resolved (Figure 1).

3.2. Prediction of Bioactive Peptides Nut Storage Proteins. The biological activities of putative peptides associated with nut storage proteins was predicted by bioinformatic analysis. Angiotensin I-converting enzyme-inhibitory activity (antihypertensive activity) had the higher occurrence frequency, followed by the antioxidant activity. Angiotensin I-converting enzyme-inhibitory activity was mainly found in glutelin (occurrence frequency 0.2045), followed by albumin (0.175) and three 7S globulins (0.1706) (Figure 2). In addition, the highest occurrence frequency for antioxidant activity was observed in glutelin (0.04) and 11S globulin (0.017), which are the most abundant fractions of nut (Table 1). A low occurrence frequency (<0.017) was observed for other nut storage proteins, with respect to glutelin and 11S globulin.

3.3. Peptides Identified in Tryptic-Digested Nut Storage Proteins by LC-MS/MS. LC-MS/MS analysis of nut storage proteins digested with trypsin showed 29 de novo peptides.

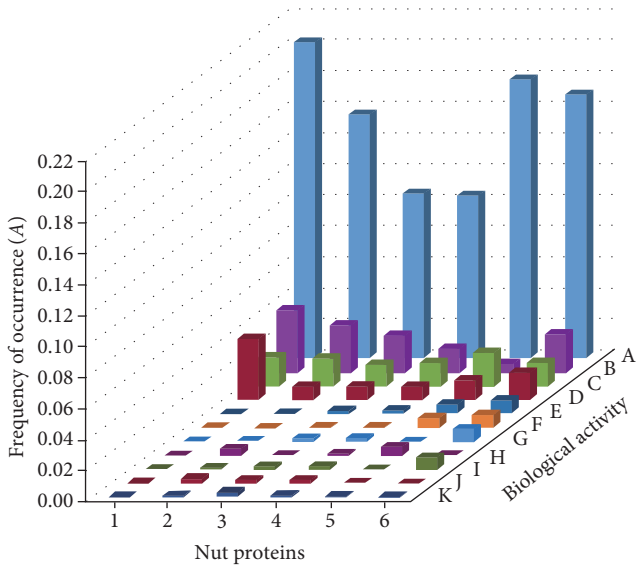

Figure 2: Prediction of peptides with potential biological activity in nut storage proteins with their occurrence frequencies. Nut proteins: (1) Glutelin (44aa, superfamily Glutelin), (2) 11S legumin (505aa, superfamily Globulin), (3) 7S vicilin (784aa, superfamily Globulin), (4) 7S vicilin (792aa, superfamily Globulin), (5) Putative allergen I1 (143aa, superfamily Albumin), and (6) Putative 7S vicilin (102aa, superfamily Globulin). Biological activity: (A) angiotensin I-converting enzyme-inhibitory activity (antihypertensive), (B) antioxidant (C) protease inhibitor, (D) metabolic stimulant, (E) metabolic regulator, (F) neuropeptide, (G) antithrombotic, (H) hypotensive, (I) antianesthetic, (J) activation of ubiquitin-mediated proteolysis (AUMP), and (K) immunomodulant.

In silico analysis showed that those peptides (Table 2) have putative biological activities, including antihypertensive, antioxidants, inhibitor of cell cycle (anticarcinogenic), antithrombotic, opioid, inhibitor of enzymes, activator of proteolysis, metabolic stimulant, and metabolic regulator. It has been reported that biologically active peptides are formed mostly by residues tyrosine, leucine, proline, glutamic acid, arginine, and alanine [3], and amino acids that were found in peptides of nut resolved by mass spectrometry analysis. As shown previously, the most abundant fraction in nut was glutelin. A total of 21 peptides with different biological activities were found: (a) in the glutelin fraction, peptides with antihypertensive, antioxidant, and anticarcinogenic activities were detected. (b) In the globulins fraction, peptide with antithrombotic activity that might inhibit the platelet aggregation was found. (c) When the prolamin fraction was analyzed, only peptides with opioid activity were observed. (d) In the albumin fraction, peptides with antihypertensive, antioxidant, and protease inhibitor activities were predicted. From all the biological activities predicted with the amino acid sequences of the peptides resolved by mass spectrometry, the antioxidant and antihypertensive activities showed the higher occurrence frequency, with values of 0.0821 and 0.063, respectively (Table 2).

3.4. Determination of Antiproliferative Effect of the Encrypted Peptides of Nut Glutelins in Cell Lines HeLa and CasKi. When

Table 2: Biopeptides identified in the tryptic digest of fractions of Pecan nut storage proteins by spectrometry MS/MS.

Activity	Occurrence frequency	Peptides with biological activity	Storage protein	Description and report	Reference
Antihypertensive	0.063	MVISR, LAASGLLLL, ALLALS, VDG, FQP, DMIPAQ, EEE, LKAWSVAR CYFQNCPR	Glutelins Albumins and Glutelins	ACE I inhibitor	[10]
Antioxidant	0.0821	GYY, Ell, IRWH, TFEEETSA, NYKQMT YYY, LKPPTY, YYG, LEGFYYY	Glutelins Albumins and Glutelins	It protects the cell from oxidation	[11]
Antiproliferative (anticarcinogenic)	0.031	AYRNRYRRQYRY EQRPRT, LPTSEAAKY	Glutelins	Inhibitor of oncogenic transcription factor (STAT3)	[12]
Antithrombotic	0.0016	DEE	11S Globulins	Antiplatelet	[13]
Opioid	0.0091	YPFPGPIP, GYK, QK	Prolamins	Powerful Painkiller	[14]
Protease inhibitor	0.002	LA	Albumins	Inhibitor of dipeptidyl aminopeptidase IV	[15]
Activators of ubiquitin-mediated proteolysis	0.00843	LA	Albumins	Activator of ubiquitin-mediated proteolysis	[16]
Other functions (metabolic stimulants and metabolic regulator)	0.001	NPHDHQ, LEANPRS, WLTTIHGS	Albumins and glutelins	—	[10]

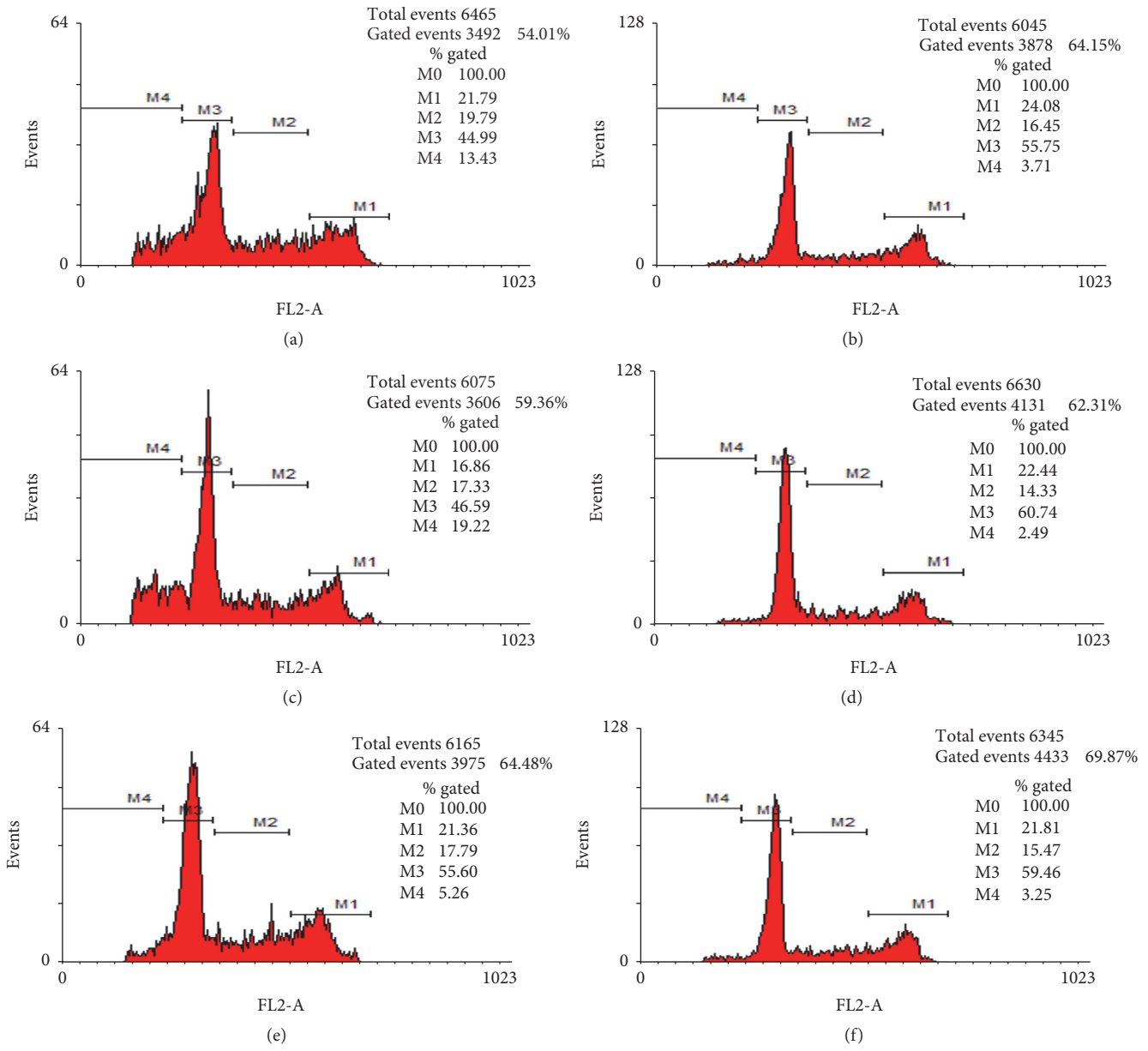

FIGURE 3: Cell cycle and antiproliferative effect of nut biopeptides on HeLa and CasKi cell lines. (a) HeLa cells treated with tryptic digest of nut, (b) untreated HeLa cells, (c) CasKi cells treated with tryptic sample, (d) untreated CasKi cells, (e) fibroblasts treated with tryptic digest of nut, and (f) untreated fibroblasts. M1, mitosis; M2, synthesis; M3, G_0 phase; M4, apoptosis.

HeLa cells were treated for 36 h with 50 μg/mL of tryptic digests of nut, it is observed that 44.99%, 19.79%, 21.79%, and 13.45% of cells were in G_0 phase, synthesis, mitosis, and apoptosis, respectively, while untreated HeLa cells showed that the 55.75% was in phase G_0, 24.08% in mitosis, and 3.71% in apoptosis. CasKi cells treated with tryptic digests showed that 46.59%, 17.33%, 16.83%, and 19.22% were in G_0 phase, synthesis, mitosis, and apoptosis, respectively, whereas untreated CasKi cells showed that 60.74% were in the G_0 phase, 14.33% in synthesis, 22.44% in mitosis, and 2.49% in apoptosis. Interestingly, when this activity was analyzed in healthy control cells (fibroblasts) treated in the same way as the cervical cancer cell, we found that 55.60% were in G_0 phase, 17.79% in synthesis, 21.86% in mitosis, and 5.26% in apoptosis, whereas in the untreated fibroblasts 59.46%, 15.47%, 21.81%, and 3.25% of the cells were G_0 phase, synthesis, mitosis, and apoptosis, respectively, which showed that antiproliferative activity can be selective of the neoplastic cells (Figure 3).

4. Discussion

There is special interest to know the nutritional or biological properties of walnut, mainly because people are using it as a common fruit in their diet, eating it alone as a dried fruit or in different food products such as desserts. To our knowledge no information has been published about encrypted peptides

from nut proteins, which can be released during the normal process of digestion and produce beneficial physiological effects.

Firstly, we determined soluble protein fraction in nut. It was found that this fraction is formed by albumins, globulins, prolamins, and glutelins, which have been also reported in plants such as amaranth. The highest soluble fraction was represented by glutelins with ~53.27%. This percentage was higher than that reported in amaranth (21–24%) but lower than those found in wheat and melon (85 and 82%, resp.) [29, 30]. The concentration of 11S globulins was slightly lower than that found in amaranth [31]. In spite of the fact that we used modified protocols for extracting prolamin (i.e., ethanol plus sodium acetate and β-mercaptoethanol) [20, 22, 26], we only were able to obtain ~14% of the total fractions.

Bioinformatic analysis predicted that angiotensin I-converting enzyme-inhibitory activity, which is related to antihypertensive, was the main biological activity found in nut storage proteins followed by the antioxidant effect. This is an important finding as the nut consumers might be protected against hypertension problems and also ingest molecules with antioxidant activity. It is known that angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) is a hypertension-responsible glycoprotein present both in biological fluids and in many tissues [32]. This enzyme can be inhibited by small encrypted peptides, for example, LKPNM and LKP, originating from fish proteins, are able to inhibit the ACE and showed activities of 66 and 91%, respectively, compared with the captopril [33]. Also, it has been confirmed that different peptides derived from protein eggs showed ACE inhibitory effect and antihypertensive effect [34]. It will be interesting in future studies to synthesize some of the peptides found in the glutelin and globulin fractions of nut (i.e., DMIPAQ, EEE, LKAWSVAR, VISR, LAASGLLLL, ALLALS, VDG, FQP, and CYFQNCPR) (Table 2) and test their potential to have an ACE inhibitory activity. On the other hand, although the antioxidant activity in nut is mainly associated with the presence of phenolic compounds [32], the occurrence frequency of the antioxidant activity owing to encrypted peptides suggest that its antioxidant effect could be not only for the presence of the phenolic compounds but also for encrypted peptides. These peptides might act as chelators of metal ions, thereby preventing cellular oxidation [35, 36].

Another activity resulting in relatively high values in the frequency of occurrence was the activity as protease inhibitor (0.002) found in albumin fraction. Peptide protease inhibitors produced by the plants are small amino acids that contribute to the defense against insects [37] and they are found primarily in grains and storage tissues of plants. Additionally and according to our in silico analysis, it seems that albumin fraction contains encrypted peptides that inhibit the dipeptidyl aminopeptidase IV (DPP-IV) (EC 3.4.14.5) and activate the ubiquitin-mediated proteolysis. This finding is important because accelerating the uptake of encrypted peptides, by activating a ubiquitin-dependent proteolytic pathway, can reduce the blood glucose level in human being [16, 38]. Additionally, we found that that globulin and prolamins fractions may have antithrombotic and opioid peptides, respectively, which can act as platelet aggregation inhibitors and powerful painkiller [14, 39].

We predicted that glutelin fractions might have an anticarcinogenic activity, and we demonstrated experimentally that protein fractions of nut digested with trypsin has an antiproliferative effect on HeLa and CasKi cell lines derivated of cervical cancer. We detected the presence of peptides AYRNRYRRQYRY, EQRPRT, and LPTSEAAKY in glutelin fraction, which might be responsible for the antiproliferative effect. Although we do not have experimental data, it is possible those last two peptides might play an important role in the inhibition of the transcription factor STAT3, which mediates the expression of various genes involved in cell proliferation and apoptosis [12]. In conclusion, we demonstrated that digested tryptic protein samples, which contain encrypted peptides, are able to exert an antiproliferative activity on neoplastic cells. Our aim in future studies will be focused in testing the different biological activities using synthetic peptides with the amino acid sequences reported here.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Research Article

Antioxidant Potential of Selected Korean Edible Plant Extracts

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This study aimed to evaluate the antioxidant activity of various plant extracts. A total of 94 kinds of edible plant extracts obtained from the Korea Plant Extract Bank were screened for cytotoxicity, following which the total phenolic content of 24 shortlisted extracts was determined. Of these, extracts from three plants, namely, *Castanea crenata* (CC) leaf, *Camellia japonica* (CJ) fruit, and *Viburnum dilatatum* (VD) leaf, were examined for antioxidant capabilities by measuring radical scavenging activity, ferric reducing/antioxidant power, and lipid peroxidation inhibitory activity. In addition, cellular antioxidant activities of the three extracts were assessed by a cell-based dichlorofluorescein assay and antioxidant response element (ARE) reporter activity assay. The results demonstrated that all three extracts concentration-dependently scavenged free radicals, inhibited lipid peroxidation, reduced the cellular level of reactive oxygen species, and increased ARE-luciferase activity, indicating antioxidant enzyme-inducing potential. In particular, CJ extract showed significantly greater antioxidative activity and antimigratory effect in a breast cancer cell line compared to CC and VD extracts. Hence, CJ extract deserves further study for its *in vivo* functionality or biologically active constituents.

1. Introduction

Oxidative stress caused by free radicals and their derivatives leads to disturbances in redox homeostasis [1]. Reactive oxygen species (ROS) are endogenously produced during intracellular metabolic processes but can also be generated by exogenous stimuli such as UV radiation, pollutants, smoke, and drugs [2, 3]. When intracellular oxidative status increases, the cell triggers its defense systems or undergoes apoptosis [2]. These responses to oxidative stress influence numerous cellular processes including core signaling pathways, which are associated with development of systematic and/or chronic disorders including aging and cancer [3, 4]. Therefore, it is critical to remove cellular oxidants and restore redox balance.

Edible plants or plant-derived compounds can be used medicinally as preventive and/or therapeutic measures against a variety of disorders [5]. A number of studies attribute health benefits of dietary plants to biologically active constituents that possess strong antioxidant activity [6]. This has resulted in extensive studies on plant extracts or constituents that are capable of scavenging free radicals and inhibiting lipid peroxidation [7, 8].

It has been well established that the antioxidant capacity of plant extracts is closely associated with their total phenolic content. Furthermore, some antioxidants are known to activate cellular antioxidant defense systems [9, 10]. The nuclear factor erythroid 2- (NFE2-) related factor 2 (Nrf2), a transcription factor, is known to be a master regulator of the cellular antioxidant response. This has given rise to the strategy of searching for substances inducing Nrf2 activation in development of functional foods or nutraceuticals [1, 9].

In the present study, we screened 94 edible plant extracts for cytotoxicity and chose 24 of these for measurement of total phenolic contents. In addition, we tested antioxidant activity, performed antioxidant response element (ARE) reporter assays for three selected plant extracts, and examined their antimigratory effects in a human breast cancer cell line, MCF-7.

2. Materials and Methods

2.1. Preparation of Plant Extracts. A total of 94 edible plant extracts were purchased from the Korea Plant Extract Bank (Ochang, Chungbuk, Korea) [11]. According to the supplier, each plant material was washed, air-dried at 70°C, ground, and extracted in 100% methanol at 30°C for 3 days. The extract solution was then filtered and vacuum-evaporated to dryness. For *in vitro* measurement of antioxidant activity, the obtained extracts were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at 20 mg/mL.

2.2. Determination of Total Phenolic Contents. The total phenolic contents of the extracts were measured as described by Ariffin and coworkers [12, 13] with minor modifications [13], using a high-performance liquid chromatography system equipped with a diode array detection module (HPLC-DAD; Waters Corp., Milford, MA).

2.3. Determination of Cytotoxicity. To test the cytotoxicity of each extract, Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was used as previously described [14]. Cytotoxicity of each extract was expressed as an IC_{50} value that indicates the concentration of the extract at which cell viability is reduced by 50% in comparison to the control treatment. The IC_{50} values were obtained by nonlinear regression using GraphPad Prism (ver. 3.0).

2.4. Determination of Free Radical Scavenging Capacity. Antioxidant activity of the extracts was assessed by the ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) or 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals as previously described [15]. Butylated hydroxytoluene (BHT), a synthetic antioxidant, or α-tocopherol was used as a positive control for both assays.

2.5. Determination of Ferric Reducing/Antioxidant Power (FRAP). The FRAP assay was performed as previously described [16, 17]. α-Tocopherol was used as a positive control at concentrations of 50, 100, 500, and 1,000 μM in comparison to the negative control (a solvent-treated condition).

2.6. Measurement of Lipid Peroxidation Inhibition. The supernatant of mouse liver homogenate was used for thiobarbituric acid reactive substances (TBARS) assay measuring the level of an end-product of lipid peroxidation, malondialdehyde (MDA) [17]. After various concentrations of samples or positive control BHT were mixed with the liver homogenate, peroxidation was induced using 20 mM ferric chloride. The absorbance of the reactant was measured at 532 nm. The lipid peroxidation inhibitory activity of a sample was calculated.

2.7. Cell Culture. The human breast cancer cell line MCF-7, used for the cytotoxicity assay, was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all from Invitrogen, Carlsbad, CA, USA).

A human hepatoma cell line HepG2, obtained from KCLB, was transfected with pGL4.37[*luc2P*/ARE/Hygro] vector (Promega, Madison, WI, USA) as previously described [18]. The transfectant carrying an ARE-luciferase construct was named HepG2-ARE and cultured in the maintenance medium including 0.4 mM hygromycin (Sigma-Aldrich). All cultures were kept in a culture incubator (37°C, 5% CO_2, humidified) for the designated period.

2.8. Quantification of Cellular Oxidative Stress: Dichlorofluorescein (DCF) Assay. The intracellular reactive oxygen species (ROS) concentration was quantified by measuring the oxidation level of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) as described by Wang and Joseph [19]. MCF-7 cells were treated with samples at designated concentrations in 0.5% FBS-containing culture medium for 24 h. ROS production was induced by 100 μM *tert*-butyl hydroperoxide (tBHP), an oxidant, for 4 h before termination of sample treatment. The cells were then treated with 50 μM DCFH-DA for 1 h at 37°C. After removal of the excess DCFH-DA, fluorescence was measured using a microplate reader at excitation and emission wavelengths of 485 and 535 nm.

2.9. Measurement of Antioxidant Response Element (ARE) Activity Assay. Luciferase reporter assay was conducted on HepG2-ARE cells as described [18, 20]. The cells were treated with samples for 12 h after serum starvation (0.5% FBS, 12 h). The luciferase activity, which corresponded to the ARE activity, was measured using a luciferase assay system (Promega) according to the manufacturer's instruction. Sulforaphane (Sigma-Aldrich), an isothiocyanate, was used as an ARE activator. Brusatol (Carbosynth Ltd., Newbury, Berkshire, UK), a quassinoid, was used as a specific inhibitor of the Nrf2 pathway [21]. The luminescence of the assay was detected and calibrated on total protein amounts. The data were then normalized against the control values.

2.10. Cell Cycle Analysis. To determine the proliferative capacity of cultured cells, 5-ethynyl-2′-deoxyuridine (EdU) uptake analysis was performed using Click-iT® EdU flow cytometry assay kit (Life Technologies). For the assay, cells were prepared as recommended by the manufacturer's instruction. Briefly, cells were cultured for 48 h and subsequently treated with 10 μM EdU for 2 h, harvested, and washed in phosphate-buffered saline (PBS; Gibco) containing 1% bovine serum albumin (BSA; Sigma-Aldrich). After fixation and permeabilization, EdU-incorporation was visualized in Click-iT reaction cocktail containing Alexa Flour® 488 azide. After being rinsed, 1×10^4 cells per condition were analyzed by the BD FACSCalibur flow cytometer (BD Biosciences).

2.11. Cell Migration Assay. For measurement of *in vitro* cell migration [22], MCF-7 cells were plated onto a 6-well plate coated with 10 μg/mL of poly-L-ornithine (Sigma-Aldrich) and 5 μg/mL of human plasma fibronectin (Life Technologies) at a density of 1×10^5 cells per well. At about 90% confluence in the growth medium (DMEM containing 10% FBS), an artificial gap was created on a cell monolayer by scraping the cells in a straight line with a P200 pipet tip.

After removing the detached cells, the growth medium was replaced with 2% FBS-containing medium for the designated period in the absence or presence of phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate; TPA), an enhancer of cell motility [23, 24]. Culture images were captured at the beginning and every 24 h for the designated period using an optimal microscope (Labomed TCM 400, Labo America, Inc., Fremont, CA, USA, photographed by Eyecam, Bimeince, Suwon, Korea). The migration rate was calculated as follows: migration rate (%) = [(width at 0 h − width at 24 h)/width at 0 h] × 100.

2.12. Statistical Analysis. The obtained data were analyzed by one-way analysis of variance and Duncan's multiple range test using the SPSS statistics 22 software (SPSS Inc., Chicago, IL, USA). Comparisons between two groups were performed by Student's unpaired *t*-test, and *p* values less than 0.05 were considered significant. Statistical differences were indicated with asterisks, hashtags, or different alphabetical letters.

3. Results and Discussion

A total of 94 plant extracts were screened for cytotoxicity (partially shown in Table 1) and a selection of them were tested for total phenolic content. Cytotoxicity was assessed based on MCF-7 cell viability at various concentrations of each extract and expressed as IC_{50} values. According to the screening program of the National Cancer Institute, USA, a plant extract is generally considered actively cytotoxic if the IC_{50} value is ≤20 μg/mL [25, 26]. As the IC_{50} values of all sample extracts tested in the study were higher than 20 μg/mL, the test samples could be considered not actively cytotoxic. To maximize the probability of antioxidant activity expression and ensure the nontoxicity of the test samples, extracts with IC_{50} values in the range of 20 to 200 μg/mL were selected (Table 1). The 24 selected extracts were then analyzed for total phenolic content by HPLC analysis. The extracts from fruits of *Camellia japonica* (CJ), leaves of *Viburnum dilatatum* (VD), and leaves of *Castanea crenata* (CC) showed the highest values for total phenolic content (Table 1) and were therefore subjected to further antioxidant assays.

The antioxidant capabilities of these extracts were evaluated by measurement of radical scavenging activity, FRAP assay, and lipid peroxidation inhibition testing (Figure 1). All three kinds of extracts induced an increase in radical scavenging activity and FRAP values in a concentration-dependent manner (Figures 1(a)–1(c)). In particular, the scavenging activity and FRAP values of CJ extract were significantly greater than those of VD or CC extracts at concentrations ≥ 25 μg/mL. In addition, lipid peroxidation inhibitory activity was determined via measurement of MDA levels using mouse liver tissue homogenate treated with various concentrations of the extracts (Figure 1(d)). The concentration-dependent decrease in MDA levels observed was consistent with radical scavenging activities of the extracts. These results demonstrated that CC, CJ, and VD extracts have relatively strong antioxidant capabilities.

To examine whether these extracts can protect mammalian cells from oxidative stress, cultures of a human mammary gland-derived epithelial cell line MCF-7 were treated with each extract prior to challenging them with tBHP. The intracellular ROS production was determined by the relative intensity of DCF fluorescence (Figure 2(a)). While intracellular ROS formation was significantly promoted by tBHP treatment, the augmented ROS level was significantly lowered by treatment with CC, CJ, or VD extracts. This finding is indicative of the antioxidative capability of the three sample extracts in a living cell model as well as in an *in vitro* system.

To elucidate the potential mechanism by which these extracts exert antioxidant activity, ARE-luciferase activity was measured in HepG2-ARE cells following extract treatment (Figure 2(b)). As expected, ARE-luciferase activity was significantly increased by sulforaphane, a known Nrf2 activator, and suppressed by simultaneous treatment with brusatol, an Nrf2 inhibitor. All three sample extracts were found to induce ARE activation, which was partially or completely abolished by brusatol. However, CC-induced ARE activation was not influenced by brusatol. These results suggest that the antioxidative function of CJ and VD extracts was, at least in part, mediated via the Nrf2 signaling pathway. Moreover, our previous data demonstrated that CC itself did not increase the levels of Nrf2 or its downstream gene transcripts in MCF-7 cells [18]. Considering that brusatol reversibly enhances ubiquitination and degradation of Nrf2 [21], CC-induced ARE activation, which is unaffected by brusatol, may be accomplished by different mechanism(s) from the other samples. For instance, CC may activate the Nrf2 signaling pathway through the modulation of PI3K and/or MAPK instead of directly interacting with Keap1 protein, which is present in the form of a heterodimer with Nrf2 in the cytoplasm [27–29]. However, the precise molecular mechanisms for the antioxidative function of CC extract must be revealed through further study.

Since Nrf2-mediated redox control is highly correlated with cancer cell proliferation [30, 31], we further examined whether those extracts influence cell cycle progression of cancer cells. MCF-7 cells that underwent G1, S, and G2-M phases were analyzed by a thymidine analogue, EdU-based cell sorting after treatment with each extract at a concentration of 50 μg/mL for 48 h (Figure 3(a)). Our data showed that 61.9 ± 0.1% of cells were analyzed at G1 phase, 27.4 ± 0.5% at S phase, and 8.7 ± 1.2% at G2-M phase in the control condition. After exposure to each extract, the cells at G1 phase were slightly decreased and those at S and G2-M phases were marginally increased (data not shown). These findings demonstrate that the three extracts may not significantly influence cell cycle progression of MCF-7 cells at a dose of treatment in this study.

Intriguingly, we found that CJ extract was capable of preventing MCF-7 cell migration *in vitro* (Figure 3(b)), whereas the other two extracts were not (data not shown). Regardless of the presence of TPA, the migration rate of MCF-7 cells was decreased by treatment with CJ extract at the concentrations of ≥50 μg/mL. Considering that Nrf2 can promote breast cancer cell migration which is associated with tumor aggressiveness *in vivo* [32], it is conceivable that CJ

Table 1: List of edible plant extracts used in the study.

Serial number	Scientific name (binomial nomenclature)	Common name	Family	Part(s) extracted from*	Total phenolic content (mg GAE/g DW)†	IC_{50} (μg/mL)#
16	*Stewartia pseudocamellia*	*Stewartia koreana*	Theaceae	L	218.71 ± 11.35	119 ± 7
18	***Camellia japonica***	*Camellia*	Theaceae	F	561.27 ± 10.47	126 ± 2
19	*Alangium platanifolium*	*Alangium*	Alangiaceae	L	46.79 ± 1.15	104 ± 15
20	*Pseudosasa japonica*	Arrow bamboo	Gramineae	L	95.63 ± 3.84	184 ± 13
25	***Viburnum dilatatum***	Linden viburnum	Caprifoliaceae	L	262.56 ± 8.32	139 ± 16
27	*Eleutherococcus divaricatus* var. *chiisanensis*	*Acanthopanax chiisanensis*	Araliaceae	S	133.71 ± 4.80	182 ± 16
29	*Artemisia annua* Linne	Sweet wormwood	Compositae	L	150.06 ± 7.50	181 ± 64
32	*Zanthoxylum piperitum*	Japanese pepper	Rutaceae	L	58.71 ± 0.88	176 ± 15
33	*Anthriscus sylvestris*	Cow parsley	Umbelliferae	L, S	5.44 ± 1.45	146 ± 29
34	*Celtis sinensis*	Chinese hackberry	Ulmaceae	L	182.56 ± 8.66	109 ± 58
39	*Toona sinensis*	Chinese cedar	Meliaceae	L	135.06 ± 4.04	188 ± 22
46	*Poncirus trifoliata*	Hardy orange	Rutaceae	F	29.29 ± 2.60	158 ± 30
48	*Euonymus hamiltonianus*	Hamilton's spindletree	Celastraceae	L	52.37 ± 5.78	53 ± 2
52	*Pinus koraiensis*	Korean nut pine	Pinaceae	L	142.37 ± 7.83	181 ± 16
59	*Michelia compressa*	*Magnolia compressa*	Magnoliaceae	L	63.90 ± 2.33	107 ± 25
61	***Castanea crenata***	Chestnut	Fagaceae	L	256.98 ± 5.69	149 ± 46
63	*Akebia quinata*	Five-leaf Akebia	Lardizabalaceae	W	35.83 ± 2.64	37 ± 11
64	*Albizia julibrissin*	Silk tree	Leguminosae	L	129.48 ± 5.36	166 ± 80
68	*Philadelphus schrenkii*	Mock orange	Saxifragaceae	L, S	85.63 ± 3.71	129 ± 57
71	*Ipomoea batatas*	Sweet potato	Convolvulaceae	L, S	62.37 ± 0.58	55 ± 11
78	*Camellia sinensis*	Tea plant	Theaceae	L, S	256.02 ± 5.84	171 ± 47
79	*Tilia amurensis*	Amur linden	Tiliaceae	L	121.02 ± 5.92	115 ± 16
86	*Ziziphus jujuba*	Jujube	Rhamnaceae	L	244.29 ± 7.17	105 ± 33
94	*Pueraria montana*	Kudzu	Leguminosae	L	54.48 ± 0.33	97 ± 19

*W, whole plant; F, fruit; L, leaf; S, stem; R, root. †Data are expressed as milligrams (mg) of gallic acid equivalents (GAE) per 1 g dry weight (DW). #IC_{50} value, the half-maximal inhibitory concentration, of cell viability was measured in a human breast cancer cell line, MCF-7; values are presented in mean ± SEM from three independent experimental sessions.

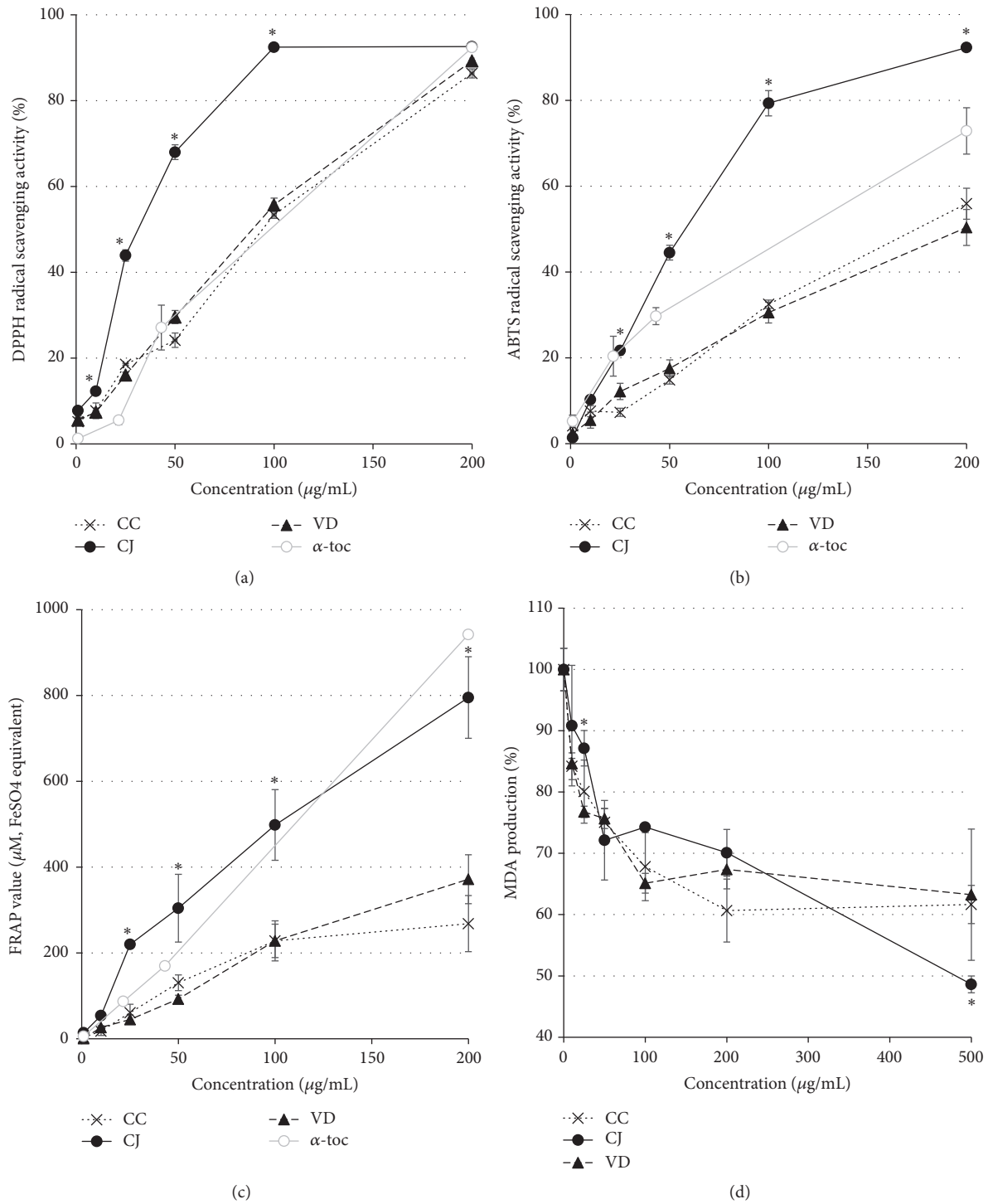


Figure 1: Antioxidant capabilities of CC, CJ, and VD extracts. The three extracts were examined for DPPH radical scavenging activity (a), ABTS radical scavenging activity (b), and FRAP (c) at various concentrations (1, 10, 25, 50, 100, and 200 μg/mL). α-Toc, α-tocopherol, is a positive control. Lipid peroxidation inhibitory activity (d) was measured at 1, 10, 25, 50, 100, 200, and 500 μg/mL. N (number of independent experimental sessions) = 3; error bars, mean ± SEM. Statistical differences were indicated with asterisks for comparisons between two groups, CJ and CC or VD, at the given concentration.

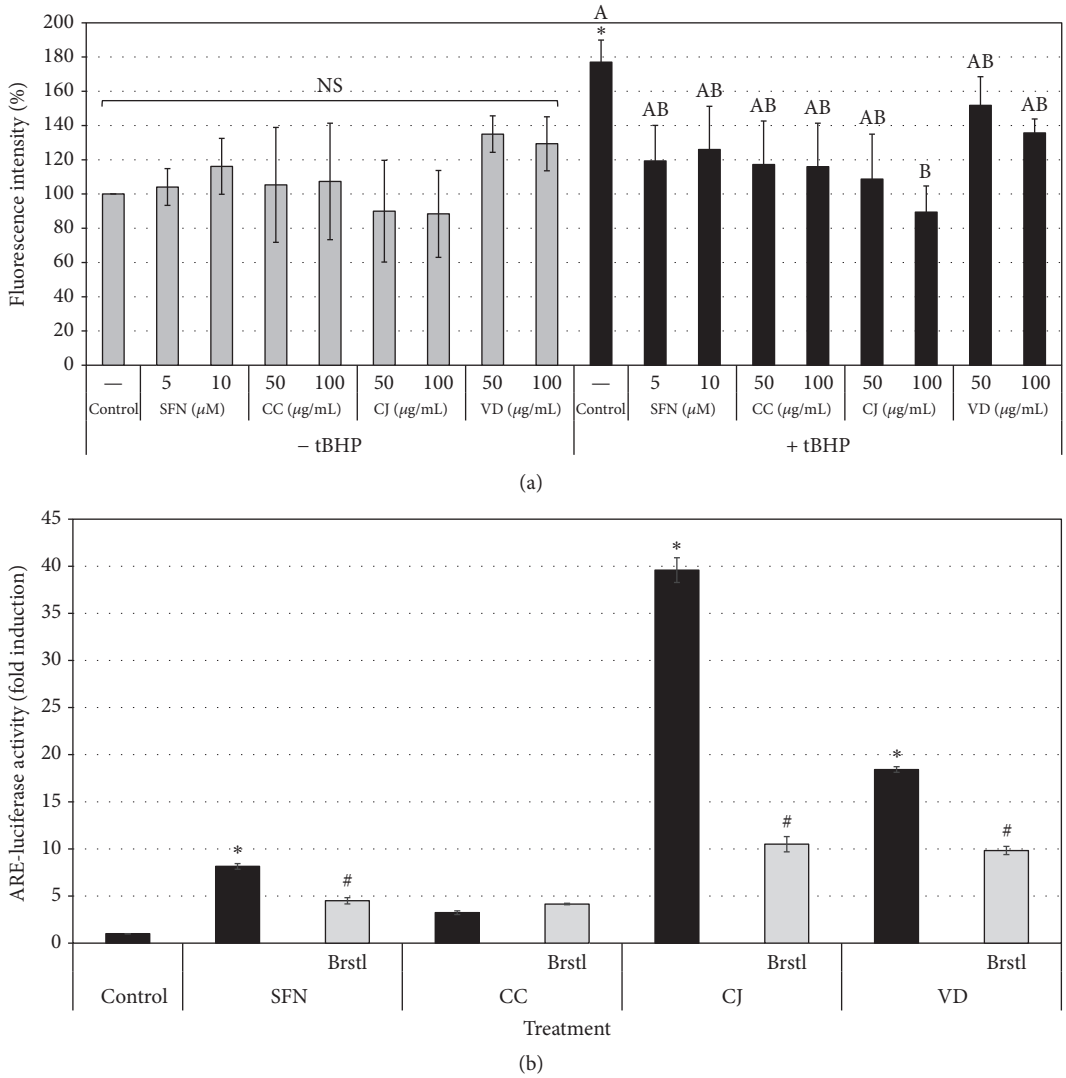
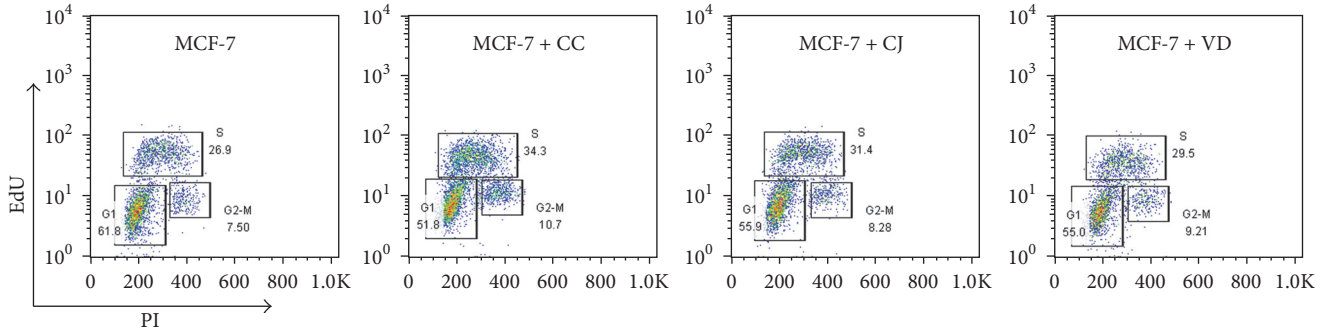


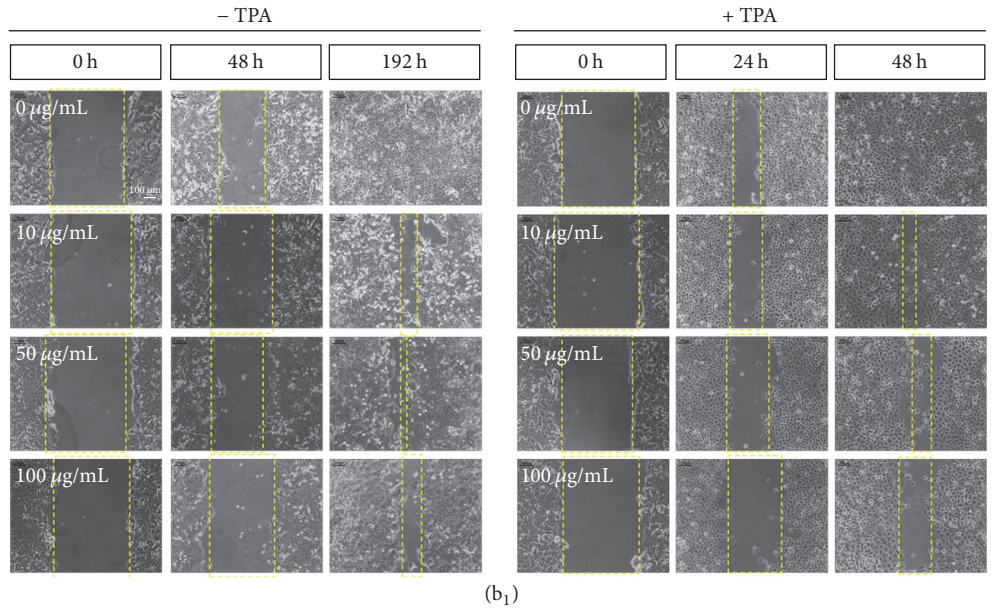
Figure 2: Cellular antioxidant effects of CC, CJ, and VD extracts. (a) MCF-7 cells were treated with the designated extracts and then exposed to tBHP. The intracellular ROS levels were determined by DCF fluorescence intensity. Treatment with CJ extract at 100 μg/mL effectively reduced the tBHP-induced ROS levels. $N = 3$; error bars, mean ± SEM. Asterisk indicates a significant difference in comparison with the control (no tBHP treatment). Different alphabetical letters indicate significant differences among the tBHP-treated conditions. (b) HepG2-ARE cells were treated with the extracts (100 μg/mL) and ARE activities were assayed. SFN, sulforaphane (5 μM), is an ARE activator. Brstl, brusatol, is an Nrf2 inhibitor. $N = 3$; error bars, mean ± SEM. Asterisks indicate significant differences in comparison with the control (no treatment). Hashtags for the brusatol-treated conditions indicate significant differences in comparison with their counteracting conditions, in which cells were treated with sample but not with brusatol.

extract may contain diverse substances that work in a combinatorial manner to enhance Nrf2-mediated antioxidation potential and to reduce cancer cell motility. Identification of bioactive substances included in CJ extract and their biological functions awaits further study.

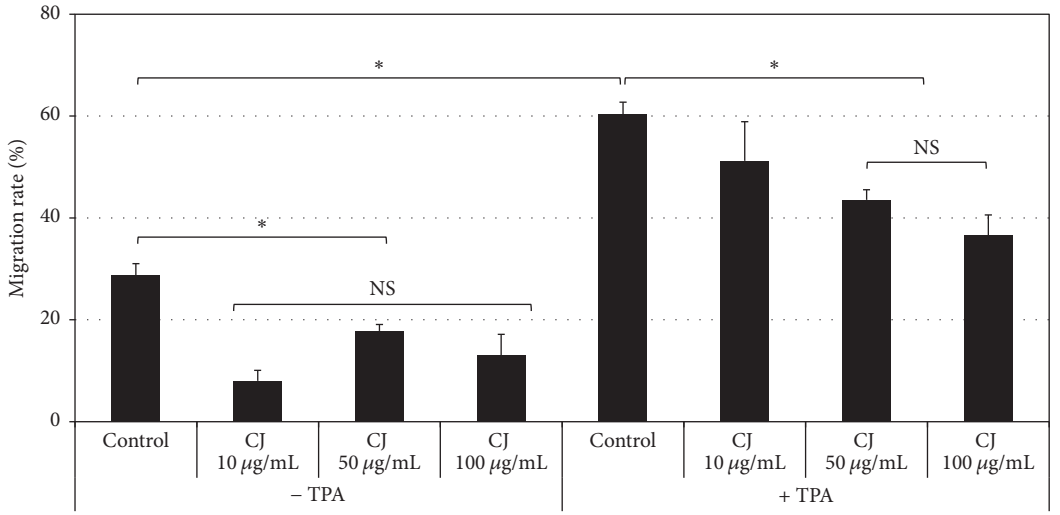
Multiple studies have demonstrated antioxidant activities of CC, CJ, and VD extracts. Several bioactive components from different parts of CJ have been reported: triterpenes in its flowers [33], flavonol glycoside in its leaves [34, 35], and saponins in its seeds [36]. Recently, it was found that the ethanol extract of CJ fruits exhibited a vascular protective effect by endothelial-dependent vasorelaxation [37] and that oleanane triterpenoids isolated from CJ fruits may be beneficial in the treatment of type 2 diabetes and obesity via PTP1B inhibitory activity [38]. Such findings suggest that CJ fruits may have biological effects through the functioning of bioavailable constituents. In addition, Noh and colleagues reported that the methanol extract of the inner shell of CC could protect hepatic cells from oxidative stress through the activation of antioxidant enzymes *in vitro* and *in vivo* and that the extract constituents scoparone and scopoletin were identified as potently active compounds [39, 40]. Kim and colleagues reported that the crude extract of VD (squeezed fruit juice) had strong antioxidant activities, reducing oxidative insults *in vitro* and *in vivo*, and that the key components involved were anthocyanins and phenolics [41, 42].



(a)



(b_1)



(b_2)

(b)

FIGURE 3: Influence of CJ extract on cell cycle progression and motility of MCF-7 cells. (a) Cells that underwent G1, S, and G2-M phases of cell cycle were analyzed by a thymidine analogue, EdU/PI-based cell sorting after treatment with each extract at a concentration of 50 μg/mL for 48 h. EdU, 5-ethynyl-2′-deoxyuridine. PI, propidium iodide. (b) Cell migration assay. (b_1) Representative images of cultured cells in the presence of CJ extract at the concentrations of 0, 10, 50, and 100 μg/mL. The scale bar in a panel of (b_1) represents 100 μm, applicable to all panels in (b_1). (b_2) Quantification of cell migration rate under the designated cultured conditions. $N = 3$; error bars, mean ± SEM. Asterisks indicate significant differences among the conditions. NS, no significant difference.

In this study, edible plant extracts were evaluated for their antioxidant potential using various *in vitro* assays. Our findings can be summarized in the form of three main contributions. Firstly, we found that CC, CJ, and VD extracts had strong free radical scavenging and lipid peroxidation inhibitory activities. Secondly, the extracts reduced tBHP-induced ROS levels, which were mediated through the activation of the Nrf2 signaling pathway. Thirdly, CJ extract among the three extracts barely affected cancer cell proliferation but decreased *in vitro* cancer cell migration. These findings demonstrated the antioxidant capability of CC, CJ, and VD extracts and potent anticancer effect of CJ, which could have implication in development of anticancer functional foods and natural source-derived nutraceuticals for cancer prevention.

Abbreviations

ABTS:	3-Ethylbenzothiazoline-6-sulfonic acid
ARE:	Antioxidant response element
BHT:	Butylated hydroxytoluene
CC:	*Castanea crenata* (leaf)
CJ:	*Camellia japonica* (fruit)
DCF:	Dichlorofluorescein
DCFH-DA:	2,7-Dichlorodihydrofluorescein diacetate
DPPH:	2,2-Diphenyl-1-picrylhydrazyl
EdU:	5-Ethynyl-2′-deoxyuridine
FBS:	Fetal bovine serum
FRAP:	Ferric reducing/antioxidant power
HPLC-DAD:	High-performance liquid chromatography-diode array detection
MDA:	Malondialdehyde
Nrf2:	Nuclear factor erythroid 2-related factor 2
PI:	Propidium iodide
ROS:	Reactive oxygen species
TBARS:	Thiobarbituric acid reactive substances
tBHP:	Tert-butyl hydroperoxide
TPA:	12-*O*-Tetradecanoylphorbol-13-acetate
VD:	*Viburnum dilatatum* (leaf).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Acknowledgments

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Review Article

An Overview of the Biological Effects of Some Mediterranean Essential Oils on Human Health

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Essential oils (EOs), extracted from aromatic plants, are interesting natural products and represent an important part of the traditional pharmacopeia. The use of some EOs as alternative antimicrobial and pharmaceutical agents has attracted considerable interest recently. Most of the EOs and their single constituents have been reported to inhibit several phytopathogens, human pathogens, and insects as well as their effective uses in food and pharmaceutical industries. The current review discussed the chemical composition and bioactivity of some important EOs extracted from some Mediterranean plants and their principal bioactive single constituents. Information has been furnished on the mechanisms, mode of actions, and factors affecting the bioactivity of some single constituents from different Mediterranean plant EOs. The current review gives an insight into some common plant EOs belonging to Lamiaceae, Apiaceae, Rutaceae, and Verbenaceae families commonly growing in Mediterranean region. Further information has been provided about the medical uses of some EOs for several human diseases covering the pharmacological effects (anti-inflammatory, antioxidant, and anticarcinogenic). The antimicrobial effects have been also considered in the current review. Although plant EOs are considered promising natural alternatives for many chemical drugs, they still need more specific research for wide application especially in food and pharmaceutical industries.

1. Introduction

Essential oils (EOs) are one of the most important natural products derived from plants for their various biological properties and medicinal uses [1, 2]. EOs have been utilized in different domestic aspects such as in perfumery, cosmetics, feed, food, and beverages. Several researchers demonstrated the possibility of utilizing EOs in cooking, where they give a pleasant taste to food and are utilized mostly in processed food. Recently, there has been great interest in the use of EOs for their curative effects in aromatherapy [3]. Plant EOs were largely utilized in pharmaceutical and other related medical uses as one of the most important and effective ingredients.

The current review intends to discuss some aspects of plant EOs and their main single constituents ranging from an overview of historical perspective, analytical techniques for chemical analysis (classical and modern methods), bioactivity of single substances (mode of action, factors affecting their bioactivity, and common families of aromatic Mediterranean plants), medical uses for human health and biological characterization including pharmacological aspects (anti-inflammatory, antioxidant, and anticarcinogenic effects), and antimicrobial effects (antibacterial and antifungal activities).

This review gives also an insight into the chemical composition of some important EOs and their principal bioactive single constituents. Detailed information focuses on the mechanism of bioactivity action of the main bioactive single constituents of some important plant EOs such as sage, oregano, thyme, marjoram, and vervain related to different families such as Lamiaceae, Apiaceae, Rutaceae, and Verbenaceae.

2. History of Plant Essential Oils

EOs have been used by many cultures around the world for centuries for different purposes according to each culture. It

is unknown exactly whether the EOs were used as healing agents or for domestic use in the beginning. However, recently great consideration has been given to the effective use of EOs in clinical procedures [4–6].

Ancient Egyptians have used aromatic oils as early as 4500 BC in cosmetics and ointments [7]. They used to make a mixture of different sources of herbal preparations such as aniseed, cedar, onion, myrrh, and grapes in perfume or medicine [7]. On the other hand, the use of aromatic oils was first recorded in traditional Chinese and Indian medicine between 3000 and 2000 BC [7]. In particular, the recorded history about China and India listed more than 700 substances including cinnamon, ginger, myrrh, and sandalwood as being effective for healing. In addition, Greek history documented the use of different EOs for the first time between 500 and 400 BC, including thyme, saffron, marjoram, cumin, and peppermint [8].

In the 18th and 19th centuries, chemists documented the active components of medicinal plants and identified many substances such as caffeine, quinine, morphine, and atropine, which were considered to play an important role in their biological effects [9].

Some EOs such as lavender, peppermint, and myrrh are still being used pharmaceutically and could be used effectively in the upcoming future as suitable alternatives for many synthetically produced medications [3].

3. Chemical Composition of Plant Essential Oils

As widely known, the chemical composition of plant EOs is principally represented by mono- and sesquiterpene hydrocarbons and their oxygenated derivatives, along with aliphatic aldehydes, alcohols, and esters [7]. It is also of great interest to highlight that the chemical profile of any EO is closely related to the extraction procedure carried out and therefore the selection of suitable extraction method is very important.

According to the characteristics of each plant material, some specific extraction techniques can be applied such as steam distillation, solvent extraction, soxhlet extraction, microwave-assisted hydrodistillation, dynamic headspace, static headspace, solvent flavor evaporation, solid-phase microextraction, and direct thermal desorption [7].

3.1. Analytical Techniques

3.1.1. Classical Analytical Techniques. Generally, the traditional classical techniques for analysis EOs were mainly focused on the quality aspects of oil, concerning mainly two properties, namely, identity and purity [10]. The following techniques are commonly applied to assess the physical properties of any EO: specific gravity, optical rotation, and determination of the refractive index [11]. Another test is for assessment of EO purity such as the presence of polar substances, like alcohols, glycols, and their esters and glycerin acetates. In addition to the solubility test of an EO in ethanol this reveals much on its quality [11]. The measurement of melting and congealing points as well as the boiling range of an EO is also of great importance for identifying its purity [11]. Another test usually performed in EO analysis is the evaporation residue, in which the percentage of the oil that is not released at 100°C is determined.

On the other hand, classical methodologies have been widely applied to assess the chemical properties of EOs [10, 11], such as the determination of halogenated hydrocarbons and heavy metals. The determination of esters derived from phthalic acid is also of great interest for the toxicity evaluation of an EO [10]. In most of the cases, the classical methods are generally focused on chemical groups and also the quantification method by titration such as the acidimetric determination of saponified terpene esters. A further test is the determination of terpene alcohols by acetylating with acetic anhydride [12]. Aldehydes and ketones could be estimated through different tests like bisulfite method which is recommended for aldehyde-rich oils such as lemongrass, bitter almond, and cassia, while the neutral sulfite test is more suitable for ketone-rich oils such as spearmint, caraway, and dill oils [12]. The chromatographic methods are considered as one of the most common and easily analytical techniques in EOs analysis. The principle of chromatography is based on the distribution of the constituents to be separated between two immiscible phases [13]. Thin Layer Chromatography (TLC) is a fast and inexpensive method for identifying substances and testing their purity [14].

The use of the above-mentioned traditional analytical techniques for the systematic study of EOs is generally applied for the assessment of pure compounds as well as some major compounds. Classical methods need to be combined with some modern analytical techniques, such as Gas Chromatography-Mass Spectrometry (GC-MS).

3.1.2. Modern Analytical Techniques. Most of the modern analytical techniques of EOs depend on chromatographic procedures. The main objective in any chromatographic separation is always the complete resolution of the compounds in the minimum time; for that, the most appropriate analytical chromatographic column with a specific dimension and stationary phase has to be used under adequate chromatographic conditions.

In particular, the GC analysis can be summarized as the evaporation of the compound and the elution by the mobile gas phase, the carrier gas, through the column. The different substances are separated on the basis of their relative vapor pressures and affinities for the stationary bed. On the other hand, the liquid chromatographic analysis depends on the elution of the compound by a liquid mobile phase consisting of a solvent or a mixture of solvents and the different substances are separated according to their affinities for the stationary bed.

Mass Spectrometry (MS) can be defined as the study of systems through the formation of gaseous ions, with or without fragmentation, which are then characterized by their mass-to-charge ratios (m/z) and relative abundances [15]. GC-MS is an analytical method that combines the features of Gas Chromatography and Mass Spectrometry to identify different substances within a test sample [16].

Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples. GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions and the phase properties. The difference of the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote their separation. Different molecules are retained by the column and then are eluted from the column at different retention times [16]. A list of some single constituents that exist in common aromatic Mediterranean plants have been reported in Table 1.

4. Bioactivity of Plant EOs and Their Single Components

The bioactivity of EOs is the sum of its constituents which act either in a synergistic or in an antagonistic way [7, 17]. The term "bioactivity" could be used for all EOs as well as their main active constituents either stable or volatile such as monoterpenes, sesquiterpenoids, benzenoids, and phenylpropanoids which demonstrate a sort of biological activity on humans, animals, and plants.

The following part of the current review covers the following points: (i) mode of action of single components; (ii) factors affecting the single components bioactivity.

4.1. Mode of Action. Most antimicrobial activities of several plant EOs depend mainly on their bioactive single components which are able to inhibit the growth of microorganisms and/or completely suppress the pathogens [17, 18]. In fact, several studies have explained that the synergetic effect between two or more chemical constituents could have a distinctive role in the biological activity of EOs [19].

The synergism between the aromatic plant components often plays an essential role in the effectiveness and reduction of the developing resistance of any pathogenic microorganism. Therefore, some constituents such as carvacrol, γ-terpinene, and p-cymene are more effective when they are combining together [20]. This synergistic action is due to p-cymene which acts as a mediator for carvacrol transportation across cell wall components and the cytoplasmic membrane of pathogenic fungi. Furthermore, the enzymatic reactions within the EOs and the lipophilic properties of the individual bioactive constituents might play a role in degrading the microbe plasma membrane and hence lead to the lyses of the hypha wall as discussed by Soylu et al. (2010) [21].

4.2. Factors Affecting the Bioactivity of EOs Composition. The composition of each EO can vary depending on certain conditions such as plant variety, plant part, growth area, climatic changes, harvesting time, storage conditions, and the chemotype of each component [8]. Therefore, the composition of EOs cannot be expected to be similar every year even if they are extracted from the same area. For example, the minimum inhibitory concentrations (MICs) of four different chemotypes of thyme oil (linalool, thuyanol, carvacrol, and thymol type) against *Staphylococcus aureus* Rosenbach ranged from 250 to 4000 μg/mL depending on the presence of thymol [22].

For improving the antimicrobial outfindings of many plant EOs, several factors either biological and experimental should be taken into consideration such as (i) appropriate and exact standardized microbiological test; (ii) available standard strains from different collections; (iii) assays including a variety of microorganisms either gram-positive and gram-negative bacteria, phytopathogens and human pathogens and yeasts; (iv) exact botanical identification of the plant EOs origin; (v) biochemical characterization of the extracted EOs as well as their production, storage conditions, and age. In addition, the distinctive water solubility and volatility of many EOs enable them to reveal a broadband spectrum of activity in various *in vitro* tests such as agar well diffusion, serial dilutions, and volatile tests [23].

Several plant EOs demonstrated different antimicrobial activities according to the tests carried out, examiners themselves, or any other factors. For example, lemon EO showed to some extent a clear inhibition effect against *Escherichia coli* Migula by using agar diffusion test as reported by Fisher and Phillips (2006) [24]. However, other researchers did not notice any inhibition activity from lemon oil against the same target organism using another nutrient media iso-sensitest agar [25].

Möse and Lukas (1957) [26] have observed a clear inhibition activity from lemon oil against *Klebsiella pneumonia* Schroeter in agar diffusion test, whereas Deans and Ritchie (1987) [25] reported that there is no antibacterial effect from lemon oil against this bacterium. The obtained results of MIC test showed diverse actions regarding the antibacterial activity of rosemary oil against *S. aureus* ranging from 20 to 400 μg/mL as reported by Panizzi et al. (1993) [27] due to differences in incubation period even at the same temperature.

On the other hand, Pellecuer et al. (1976) [28] reported the highest MIC value (1250 μg/mL) regarding rosemary oil obtained in the dilution test against *Bacillus subtilis* Ehrenberg, whereas Farag et al. (1989) [29] observed a moderate MIC value (750–800 μg/mL). However, Panizzi et al. (1993) [27] reported the lowest MIC value of the same oil (10 μg/mL) and these changes in estimated values could be due to the fluctuation of some experimental factors like temperature and incubation period.

5. Common Mediterranean Aromatic Plants

Many common Mediterranean aromatic plants are belonging to Lamiaceae, Apiaceae, Rutaceae, and Verbenaceae families. The selected discussed aromatic plants in the current review are considered as the most important Mediterranean officinal plants.

5.1. Family Lamiaceae

5.1.1. Lavender. The EO extracted from lavender (*Lavandula officinalis* Chaix.), Lamiaceae family, showed strong antibacterial and antifungal properties [30]. *L. officinalis* EO treats sinus and vaginal infections due to *Candida albicans*

Table 1: List of some single constituents existing in common aromatic Mediterranean plants.

Number	Main single constituent	Common aromatic plants
(1)	α-Thujene	Dill, balm, caraway, lavender, marjoram, oregano, sage
(2)	Camphene	Mint, hyssop, lavender, marjoram, oregano, sage, thyme
(3)	Sabinene	Dill, parsley, basil, caraway, marjoram, sage
(4)	Myrcene	Dill, parsley, mint, balm, basil, caraway, fennel, hyssop, lavender, marjoram, oregano, sage
(5)	β-Pinene	Dill, parsley, balm, basil, caraway, fennel, marjoram, oregano, sage
(6)	*cis*-3-Hexenyl acetate	Parsley, coriande, mint
(7)	α-Terpinene	Mint, balm, hyssop, marjoram, oregano, thyme
(8)	*p*-Cymene	Dill, parsley, mint, balm, caraway, fennel, lavender, marjoram, oregano, sage, thyme
(9)	β-Phellandrene d	Dill, parsley, balm, basil, caraway, fennel, hyssop, lavender, marjoram, oregano, sage, thyme, vervain
(10)	*trans*-β-Ocimene	Dill, parsley, mint, basil, caraway, hyssop, lavender, marjoram, vervain
(11)	γ-Terpinene	Parsley, mint, balm, fennel, hyssop, lavender, marjoram, oregano, sage, thyme, vervain
(12)	Terpinolene	Balm, basil, hyssop, marjoram, oregano, thyme
(13)	Linalool	Mint, balm, basil, caraway, hyssop, lavender, marjoram, oregano, sage, thyme, vervain
(14)	Nonanal	Coriander, mint
(15)	Limonene	Mint, balm, basil, caraway, hyssop, lavender, marjoram, oregano, sage, thyme, vervain
(16)	*trans*-*p*-Mentha-2.8-dien-1-ol	Mint
(17)	α-Terpineol	Parsley
(18)	Carvomenthyl acetate	Mint
(19)	Bornyl acetate	Mint, caraway, lavender, marjoram, sage
(20)	*E*-2-Undecenal	Coriander
(21)	1-Undecanol	Coriander
(22)	*cis*-Carveol	Mint, basil
(23)	α-Pinene	Dill, mint, balm, basil, marjoram, oregano, sage, thyme, vervain
(24)	*trans*-Carveol	Mint, hyssop, sage
(25)	Carvone	Dill, coriander,
(26)	α-Phellandrene	Dill, parsley, fennel, lavender, marjoram, oregano
(27)	β-Caryophyllene	Mint, balm, basil, caraway, hyssop, lavender, marjoram, oregano, sage, thyme, vervain
(28)	Dodecanal	Coriander
(29)	*trans*-β-Caryophyllene	Parsley, mint, balm, basil, caraway, hyssop, lavender, marjoram, oregano, sage, thyme, vervain
(30)	α-Humulene	Mint
(31)	*cis*-Pinane	Basil, caraway, lavender, oregano
(32)	D3-Carene	Fennel, lavender, marjoram, oregano
(33)	a-Terpinene	Balm, hyssop, marjoram, oregano
(34)	*o*-Cymene	Anise, balm, basil, caraway, hyssop, lavender, marjoram, oregano, sage, thyme, vervain
(35)	*p*-Cymene	Caraway, fennel, lavender, marjoram, oregano, sage, thyme
(36)	1,8-Cineole	Balm, basil, hyssop, marjoram, oregano, sage, vervain
(37)	*cis*-Linalool oxide	Anise, basil, fennel
(38)	(-)-Citronellal	Balm, sage, thyme
(39)	*iso*-Borneol	Balm, marjoram, thyme
(40)	Camphor	Balm, basil, lavender, marjoram, sage
(41)	*iso*-Pinocamphone	Basil, hyssop, lavender, marjoram, oregano, sage
(42)	*trans*-Pinocamphone	Balm, caraway, hyssop, sage
(43)	Terpinen-4-ol	Balm, basil, hyssop, lavender, marjoram, oregano, sage, vervain
(44)	Myrtenol	Basil, hyssop, lavender, marjoram, sage, thyme
(45)	(*E*)-Citral	Vervain, oregano

Table 1: Continued.

Number	Main single constituent	Common aromatic plants
(46)	Isobornyl acetate	Caraway, lavender, marjoram, sage
(47)	Bornyl acetate	Caraway, lavender, marjoram, sage
(48)	Thymol	Balm, marjoram, oregano, thyme
(49)	a-Copaene	Basil, hyssop, marjoram, oregano, vervain
(50)	b-Elemene	Balm, basil, caraway, vervain
(51)	b-Caryophyllene	Balm, basil, caraway, hyssop, lavender, marjoram, oregano, sage, thyme, vervain
(52)	b-Cedrene	Balm, basil, hyssop, lavender, marjoram, oregano, sage, vervain
(53)	a-Humulene	Balm, basil, hyssop, lavender, marjoram, oregano, sage, vervain
(54)	Caryophyllene oxide	Balm, lavender, oregano, sage

Berkhout and digestive disturbances including colic and helps to boost immunity [31].

Furthermore, lavender EO was well documented for the treatment of abrasions, burns, stress, headaches, skin problems, muscular pain, and boosting the immune system [32, 33]. The lavender EO is chemically composed of camphor, terpinen-4-ol, linalool, linalyl acetate, beta-ocimene, and 1,8-cineole (Table 2) [34]. Among the above-mentioned substances, linalool and linalyl acetate showed a sedative effect and marked narcotic actions, respectively. In addition, linalool and linalyl acetate have great absorbing properties for skin during massages.

5.1.2. Oregano. Oregano (*Origanum vulgare* L.), Lamiaceae family, is a perennial herb; it is considered as one of the most common culinary herbs where its leaves can enhance the flavor of food [19]. This species is used in traditional and modern medicine and in the pharmaceutical industry.

Four main groups of oregano commonly used as culinary herbs can be distinguished, that is, Greek oregano (*Origanum vulgare* L. ssp. *hirtum* (Link) Ietsw.), Spanish oregano (*Coridothymus capitatus* (L.) Hoffmanns. & Link), Turkish oregano (*O. onites* L.), and Mexican oregano (*Lippia graveolens* Kunth) [35]. *O. vulgare* L. ssp. *hirtum* is a typical eastern Mediterranean taxon, reported only for some areas in Southern Italy [36]. The main single constituents of *O. vulgare* EO have been listed in Table 2.

5.1.3. Thymus. The most common variety is *Thymus vulgaris* L. It belongs to the genus *Thymus* of the Lamiaceae family. The EO extracted from *T. vulgaris* EO showed antimicrobial activity against several phytopathogens such as *B. cinerea*, *Penicillium italicum* Wehmer, *P. citrophthora* Leonian, and *Rhizopus stolonifer* (Ehrenb.) Vuill. [37].

Thymus species are used as food plants by the larvae of some Lepidoptera (butterfly and moth) insect species, including *Chionodes distinctella* and the *Coleophora* case-bearers *C. lixella*, *C. niveicostella*, *C. serpylletorum*, and *C. struella* [38]. The main single constituents of *T. vulgaris* EO have been listed in Table 2.

5.1.4. Peppermint. The most important species are peppermint (*Mentha piperita* L.) and spearmint (*M. spicata* L.). *M. piperita* was classified in the Lamiaceae family; their oil constituents include carvacrol, menthol, carvone, methyl acetate, limonene, and menthone [6].

Peppermint EO has been intensively studied for its anti-inflammatory, anti-infectious, antimicrobial, and fungicidal effect as well as antiseptic and digestive properties. It is observed that the single constituents of peppermint EO can relieve many bacterial, fungal, and viral infections when inhaled or applied in the form of vapor balm. On the other hand, Ali et al. (2015) [3] reported that menthol, the primary constituent of peppermint EO, is responsible for pharmacological action.

5.1.5. Sage. Sage is considered to be the main genus among the Lamiaceae family, which consists of about 900 species widely distributed in the temperate, subtropical, and tropical regions all over the world but especially in the Mediterranean region, central Asia, central and South America, and southern Africa [19].

Globally, the best known species of the family used in both traditional and modern medicine are *Salvia officinalis*, *S. fruticosa,* and *S. divinorum*. Another important plant is oregano, considered to be the most valued species worldwide. About 60 plant species were listed within this common name. The main single constituents of sage EO have been listed in Table 2.

5.1.6. Marjoram. Majorana hortensis (Lamiaceae), commonly known as marjoram, is a perennial herb or undershrub with sweet pine and citrus flavors. It has a long history of medicinal and culinary use. In some Middle Eastern countries, marjoram is synonymous with oregano; therefore, some names have been used to distinguish it from other plants of the genus *Origanum* such as sweet marjoram and knotted marjoram [39]. The main single constituents of marjoram EO have been listed in Table 2.

5.2. Family Apiaceae

5.2.1. Anise. Pimpinella anisum (Apiaceae) is called aniseed. It is native to the eastern Mediterranean region and Southwest Asia [40]. Anise is an herbaceous annual plant; its leaves at the base of the plant are simple, long, and shallowly lobed, while

Table 2: Chemical composition of some common Mediterranean plant essential oils.

Some of main Single constituents	Ki[a]	Ki[b]	Percentage[c]								
			Apiaceae		Lamiaceae					Verbenaceae	Identification[d]
			Anise	Caraway	Lavender	Marjoram	Oregano	Sage	Thyme	Vervain	
α-Thujene	930	1,035	—	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	T	—	1, 2
α-Pinene	938	1,032	0.3 ± 0.0	0.5 ± 0.2	—	9.0 ± 0.1	0.4 ± 0.0	4.4 ± 0.1	2.5 ± 0.1	0.2 ± 0.0	1, 2, 3
(-)-Camphene	953	1,076	—	—	0.7 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	4.1 ± 0.0	1.0 ± 0.1	—	1, 2, 3
Sabinene	973	1,132	T	1.0 ± 0.1	T	1.1 ± 0.1	T	0.4 ± 0.0	T	0.5 ± 0.0	1, 2, 3
β-Pinene	978	1,118	—	7.4 ± 0.4	—	3.8 ± 0.9	0.2 ± 0.0	2.5 ± 0.1	—	T	1, 2, 3
cis-Pinane	980		—	0.1 ± 0.0	0.1 ± 0.0	—	0.1 ± 0.0	—	—	—	1, 2
Myrcene	993	1,174	—	0.7 ± 0.1	0.3 ± 0.0	0.7 ± 0.3	0.5 ± 0.0	0.5 ± 0.1	0.1 ± 0.0	—	1, 2, 3
α-Phellandrene	995	1,176	0.1 ± 0.0	T	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	T	T	—	1, 2, 3
o-Cymene	1,020	1,187	0.1 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	2.6 ± 0.9	41.9 ± 0.1	2.5 ± 0.2	56.2 ± 0.2	0.1 ± 0.0	1, 2, 3
p-Cymene	1,024	1,280	—	0.1 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	1.2 ± 0.1	0.1 ± 0.0	—	1, 2, 3
β-Phellandrene	1,029	1,218	T	0.6 ± 0.2	0.1 ± 0.0	9.1 ± 0.5	0.1 ± 0.0	1.0 ± 0.0	0.2 ± 0.1	0.7 ± 0.2	1, 2, 3
Limonene	1,030	1,203	—	14.3 ± 0.5	0.3 ± 0.0	6.4 ± 0.5	0.3 ± 0.0	1.4 ± 0.0	0.6 ± 0.0	2.3 ± 0.9	1, 2, 3
1,8-Cineole	1,034	1,213	—	0.1 ± 0.0	T	33.5 ± 0.3	0.6 ± 0.1	4.2 ± 0.3	T	0.4 ± 0.1	1, 2
(*Z*)-β-Ocimene	1,038	1,246	T	0.1 ± 0.0	1.7 ± 0.3	0.1 ± 0.0	T	T	T	T	1, 2, 3
(*E*)-β-Ocimene	1,049	1,280	—	0.3 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	T	T	T	0.3 ± 0.1	1, 2, 3
γ-Terpinene	1,057	1,255	T	T	T	0.8 ± 0.3	2.8 ± 0.2	0.1 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	1, 2, 3
Linalool	1,097	1,553	0.4 ± 0.1	0.5 ± 0.1	23.1 ± 0.2	9.8 ± 0.7	0.7 ± 0.3	1.1 ± 0.06	0.4 ± 0.1	0.1 ± 0.0	1, 2, 3
trans-Thujone	1,115	1,449	—	0.1 ± 0.0	—	T	—	37.9 ± 0.1	—	—	1, 2, 3
trans-Pinocarveol	1,138	1,654	—	T	T	0.1 ± 0.0	T	0.2 ± 0.0	T	T	1, 2
(-)-Citronellal	1,143	1,491	—	—	—	—	—	0.2 ± 0.0	0.5 ± 0.1	—	1, 2, 3
iso-Borneol	1,144	1,633	—	—	—	0.1 ± 0.0	—	—	0.1 ± 0.0	—	1, 2, 3
Camphor	1,145	1,532	—	T	0.9 ± 0.0	0.2 ± 0.0	T	13.9 ± 0.7	T	—	1, 2, 3
iso-Pinocamphone	1,153	1,566	—	T	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	T	0.2 ± 0.0	1, 2
trans-Pinocamphone	1,159		—	4.3 ± 0.9	—	T	T	0.3 ± 0.0	T	T	1, 2
Borneol	1,167	1,719	—	—	6.3 ± 0.9	2.0 ± 0.5	0.3 ± 0.0	7.6 ± 0.4	0.2 ± 0.0	0.1 ± 0.0	1, 2, 3
Terpinen-4-ol	1,176	1,611	—	T	0.2 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	T	0.2 ± 0.0	1, 2, 3
Dihydrocarveol	1,177		—	—	0.4 ± 0.0	0.8 ± 0.1	—	0.2 ± 0.0	0.2 ± 0.0	—	1, 2
p-Cymen-8-ol	1,185	1,864	—	—	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	T	T	1, 2
α-Terpineol	1,189	1,706	T	T	0.4 ± 0.0	0.7 ± 0.1	T	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	1, 2, 3
Myrtenal	1,193	1,648	—	0.1 ± 0.0	0.4 ± 0.1	0.7 ± 0.1	—	0.2 ± 0.0	0.3 ± 0.0	—	1, 2
Estragole	1,195	1,670	—	65.0 ± 0.9	—	0.1 ± 0.0	0.1 ± 0.0	T	—	—	1, 2, 3
Myrtenol	1,196	1,804	—	—	0.4 ± 0.0	0.2 ± 0.1	—	0.2 ± 0.0	0.3 ± 0.0	—	1, 2
Isobornyl formate	1,228		—	—	—	—	—	—	—	45.4 ± 0.9	1, 2
Linalyl acetate	1,248	1,565	—	—	44.4 ± 0.7	3.3 ± 0.6	0.1 ± 0.0	1.5 ± 0.2	—	—	1, 2, 3

Table 2: Continued.

Some of main Single constituents	Ki[a]	Ki[b]	Percentage[c]								Identification[d]
			Apiaceae		Lamiaceae					Verbenaceae	
			Anise	Caraway	Lavender	Marjoram	Oregano	Sage	Thyme	Vervain	
Geraniol	1,255	1,857	—	—	9.3 ± 0.3	0.6 ± 0.1	—	0.3 ± 0.0	—	—	1, 2
cis-Anethole	1,262		97.1 ± 0.4	T	—	—	—	—	—	0.2 ± 0.0	1, 2
(*E*)-Citral	1,270	1,727	—	—	—	—	—	—	—	44.5 ± 0.9	1, 2, 3
Isobornyl acetate	1,277		—	0.1 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	T	0.7 ± 0.0	T	T	1, 2
Bornyl acetate	1,284	1,591	—	0.1 ± 0.0	0.2 ± 0.0	1.2 ± 0.5	T	0.9 ± 0.0	T	T	1, 2
Thymol	1,290	2,198	—	—	—	0.7 ± 0.1	0.7 ± 0.0	T	8.7 ± 0.9	—	1, 2, 3
Carvacrol	1,297	2,239	—	—	—	4.1 ± 0.9	44.0 ± 0.9	0.3 ± 0.0	24.4 ± 0.9	—	1, 2, 3
Terpinyl acetate	1,333		—	—	—	0.5 ± 0.0	—	—	—	—	1, 2
α-Copaene	1,377	1,497	—	T	T	0.1 ± 0.0	0.1 ± 0.0	T	T	0.2 ± 0.1	1, 2
Geranyl acetate	1,379	1,765	—	—	—	—	—	—	—	—	1, 2
Isoledene	1,382		—	T	T	T	0.1 ± 0.0	T	T	0.1 ± 0.0	1, 2
β-Caryophyllene	1,418	1,612	T	0.1 ± 0.0	1.0 ± 0.9	0.3 ± 0.1	0.2 ± 0.1	1.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	1, 2
β-Cedrene	1,424	1,638	—	—	1.3 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	1.0 ± 0.0	—	0.4 ± 0.1	1, 2
α-Humulene	1,455	1,689	—	T	0.6 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	5.9 ± 0.9	T	0.2 ± 0.0	1, 2
α-7-*epi*-Selinene	1,518	1,740	—	T	—	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	T	0.2 ± 0.1	1, 2
Caryophyllene oxide	1,580	2,008	—	—	0.4 ± 0.0	—	0.2 ± 0.0	0.8 ± 0.0	—	—	1, 2
Total compounds			98.3	98	97.0	97	98.9	98.7	99.1	97.6	

[a]Kovats retention index on an HP-5 MS column. [b]Kovats retention index on an HP INNOWax column. [c]T, trace, less than 0.05%. A dash indicates absent. [d]1, Kovats retention index; 2, mass spectrum; 3, coinjection with authentic compound.

its higher leaves are feathery pinnate, divided into numerous small leaflets.

Anise is a food plant for the larvae of some Lepidoptera species such as butterflies and moths. Anise was first cultivated in Egypt and the Middle East and was brought to Europe for its medicinal value [41]. Anise EO can be obtained from the fruits by the steam distillation technique. The main component of anise EO is anethole (80–90%), with minor components including 4-anisaldehyde, estragole, and pseudoisoeugenyl-2-methylbutyrates [42].

5.2.2. Caraway. *Carum carvi* (Apiaceae) is called also meridian fennel. It is native to some parts of Mediterranean region like western Asia, Europe, and North Africa [43].

The plant characterized by finely divided, feathery leaves with thread-like divisions. The fruits of caraway are usually used as a whole and have a pungent, anise-like flavor and aroma that comes from its EO such as carvone, limonene, and anethole. The main single constituents of caraway EO have been listed in Table 2. Caraway fruit oil is used as a fragrance component in some cosmetic industries such as lotions and perfumes; in addition it has many uses in folk medicine [44].

5.3. Family Rutaceae

5.3.1. Lemon. Lemon, *Citrus limon* L. (Osbeck) is a species of small evergreen tree in the flowering *Rutaceae* family native to Asia. The EO extracted from *C. limon* is composed mainly of terpenes, D-limonene, and limonene. In addition, some other minor constituents are present in trace amounts such as phellandrene, pinene, and sesquiterpene [34].

Lemon EO is able to accelerate the production of white blood cells, strengthen the immune system, and help in the digestion processes [32]. The main constituents of lemon EO have demonstrated antiseptic, astringent, and detoxifying properties for blemishes associated with oily skin [45].

5.4. Family Verbenaceae

5.4.1. Vervain. *Verbena officinalis* L. classified in Verbenaceae family is commonly known as vervain. It is natively grown in Europe. Verbena has been traditionally used in herbalism and folk medicine, as herbal tea. Among other effects, it may act as a galactagogue and possibly sex steroid analogue. Verbena has been listed as one of the 38 plants used to prepare Bach flower remedies [46].

Several researches reported the antimicrobial activity of vervain EO and investigated that this activity could be related to the high amounts of monoterpenes and phenolic compounds. In fact, the major constituents of this EO were *o*-cymene, isobornyl formate, citral, carvacrol, and thymol [19]; further details of the main components of vervain EO have been reported in Table 2.

6. Medical Uses of Essential Oils in Human Diseases

Many of the plant EOs are being widely utilized in the pharmaceutical industry, aromatherapy, and other related medical uses. Many plant EOs have been used as medicine for centuries and have demonstrated several health benefits, including effects on infectious, chronic, and acute diseases [47].

The medical preparations made with plant EOs as well as their single constituents applied in the therapy of human infectious diseases are well documented. However, the selection of the suitable safe oil and the determination of the best efficient dose should be taken into consideration to avoid any side effects when they are applied for children presupposes. In particular, many EOs have been used for healing purposes and have been highly recommended especially in the treatment of some catarrhal diseases [47]. Eugenol 3, the main constituent of clove (*Syzygium aromaticum* L. Merrill & Perry) EO, can treat the systemic infections of children with fever; however, a prior study reported that it was not sufficient to prevent death among all treated patients [7]. Several investigations showed that *Ocimum gratissimum* L. EO was more effective than benzyl peroxide-based products for reducing the number of lesions (papules and pustules) [48].

A number of medical trials have investigated the effective use of some EOs in treating methicillin-resistant *S. aureus* (MRSA) *in vitro*, for example, *Lippia origanoides* Kunth, *Backhousia citriodora* F. Muell, *M. piperita*, *M. arvensis*, *M. spicata*, and *Melaleuca alternifolia* (Maiden & Betche) Cheel [17]. Further studies reported the effective use of EO extracted from *Citrus aurantium* var. *amara* L. in inhibiting *Tinea corporis* disease even at low concentrations.

The detailed pharmacological effects of many plant EOs such as anti-inflammatory, antioxidant, and anticarcinogenic were also discussed in the following section.

6.1. Pharmacological Activity. Recently, great interest was given to the curative effect of many plant EOs especially for wound treatment since the EOs have demonstrated interesting medication against some wound types, which cannot occur with pharmaceuticals [49].

For centuries, plant EOs have been used for curing many diseases such as melaleuca EO which is considered an effective factor for speeding up the healing process of wounds. Lavender EO was commonly used to heal wounds, cuts, burns, and sunburns by improving the formation of scar tissues [8]. Tea tree oil has been shown to be effective *in vitro* on several strains of methicillin-resistant *S. aureus* (MRSA) isolated from wounds [50]. The EOs extracted from frankincense and geranium can be used as antiseptic agents by burning them. They can also be applied internally to protect the wounds from developing infections [51].

6.1.1. Anti-Inflammatory. Inflammatory disorders are associated with pain, redness, and swelling, leading to loss of vital functions. EOs have been used for several decades to relieve pain and inflammation. Usually, EOs have more effective and pain-relieving properties than many pharmaceutical analgesics. The use of EOs has many benefits in the treatment of inflammation because it has fewer side effects than many synthetic and traditional drugs.

A review of the medicinal properties of chamomile documented that plants contain more flavonoids with anti-inflammatory properties than others do. These inflammation-reducing compounds can penetrate the skin easily and reduce inflammation. Tea tree oil has been shown to increase monocytic differentiation *in vitro* and reduce inflammation, therefore assisting the healing of chronic wounds. Other promising applications have been proposed for *Helichrysum italicum* (Roth) G. Don as antispastic [52], *Pelargonium roseum* L'hér. as anti-inflammatory [53], and *O. majorana* L. as antimutagenic agent [54].

The possible mechanism of the anti-inflammatory property of EOs was suggested to compete with arachidonic acid for its incorporation into the membrane. Hence, arachidonic acid generates slightly modified prostaglandins and eicosanoids, which induces a lesser extent of inflammation via reduced induction of COX-2 [55]. Further studies reported that the main component of tea tree oil, terpinen-4-ol, has been shown to suppress inflammatory mediator production by activating monocytes *in vitro* [50].

6.1.2. Antioxidant. Antioxidant activity can be defined as the molecules able to react with radicals or having a reducing power to counteract the oxidative stress caused by radicals. Antioxidant properties play an essential role in some of EOs' biological activities, which is justified by the involvement of oxidative stress in pathology. In addition, the botanical source of aromatic plants and the environmental factors such as climate may affect the actual composition of extracted EOs and thus reflect different antioxidant activities. Oregano EO is able to protect extra virgin olive oil from oxidation during storage and is able to extend the shelf life of sea bream and to reduce the formation of volatile amines and of TBAR compounds [56]. Oregano and sage EOs are able to protect the minced meat samples from autoxidation [57].

The antioxidant effect of many EOs is due to the inherent ability of some of their components, particularly phenols, to stop or delay the aerobic oxidation of organic matter [58]. On the other hand, there are phenol-free EOs that express antioxidant behaviour and this is due to the radical chemistry of some terpenoids and other volatile constituents (e.g., sulfur-containing components) [48]. In general, phenolic compounds, both natural (e.g., α-tocopherol) or synthetic (e.g., BHA), act as antioxidants due to their high reactivity with peroxyl radicals, which are disposed of by formal hydrogen atom transfer [59]. The antioxidant chemistry of sulfur-containing EOs from *Allium* and related genera is due to a direct chain-breaking activity that is expressed only upon conversion of the inactive components into thiosulfinates that ultimately yields the "active" sulfonic acid [57].

6.1.3. Anticarcinogenic. Anticarcinogenic activity is the ability of a specific substance to counteract or completely inhibit the development of carcinogen. The traditional anticancer therapy, such as multichemotherapeutic drugs, is often compromised of the development of drug resistance and the serious irreversible side effects [60]. As pointed out recently, natural products from medicinal plants represent a fertile ground for the development of novel anticancer agents. However, the utilization of plant EOs as anticancer agents is under daily diagnosis for designing the best natural alternative, which could have selectivity towards the target cells of various tumours.

In the current review, some revealing examples of plant EOs and their constituents that have been used effectively as antitumour agents have been discussed. The detailed discussion will also be reported regarding the possible mechanisms and the multiple pathways involving apoptosis, DNA repair modulation, cell cycle arrest, and antiproliferative activity through the increase of reactive oxygen and nitrogen levels (ROS/RNS) in cancer cells [61].

Some EOs showed a potential anticancer activity against liver, lung, colon, and prostate cancer such as *Artemisia lavandulaefolia* DC and its main single constituent 1,8-cineole against a subline of the ubiquitous KERATIN-forming tumour cell line HeLa which derived from an epidermal carcinoma of the mouth [62]. Jayaprakasha et al. (2013) [63] found that *C. limettioides* Tan EO inhibited colon cancer (SW480) by inducing the cells-apoptosis. Zu et al. (2010) [64] noted that the *T. vulgaris* EO exhibited the strongest cytotoxicity towards three human cancers: PC-3 from human prostate, A549 from human lung, and MCF-7 from breast adenocarcinoma.

Further studies demonstrated that some single constituents like carvacrol [65], thymol [66], limonene, and citral [67] have shown a promising cytotoxic effect against different human cancer cell lines mainly due to the induction the mitochondrial dysfunction [65–67]. Elshafie et al. [68] have pointed out the effectiveness of *O. vulgare* EO and its main single constituents (carvacrol, thymol, citral, and limonene) on tumour liver cell line (HepG2) following the MTT viability assay. The results showed that the above treatments illustrated a great effect as anticancer therapeutic agents especially thymol and carvacrol (Figure 1).

The general mechanism of cytotoxic effect of plant EOs is mostly due to the presence of phenols, aldehydes, and alcohols. In particular, the toxicity towards mammalians decreases significantly with the increase of average lipophilicity of EO components [51], while the toxicity against bacteria and fungi simultaneously increases significantly with increasing lipophilicity [51]. This mode of action refers to the extraordinary role of EOs among natural compounds, especially of their highly lipophilic constituents. The cellular mechanisms of carcinogenic prevention by EO treatments were also considered to be due to the induction of cell-apoptosis [61]. There is also a suggestion that the pathway of cancer cells is sensitive to the inhibitory actions of plant isoprenoids by reducing tumour cell-size in patients [69].

7. Antimicrobial Activity

7.1. Antibacterial. Elshafie et al. (2016) [70] evaluated the *in vitro* antibacterial activity of three EOs extracted from *Schinus terebinthifolius* Raddi (leaves and fruits) against two strains of G+ve bacteria (*Bacillus megaterium* de Bary and *Clavibacter michiganensis* Smith) and 4 strains of G−ve bacteria (*E. coli*, *Xanthomonas campestris* Pammel, *Pseudomonas*

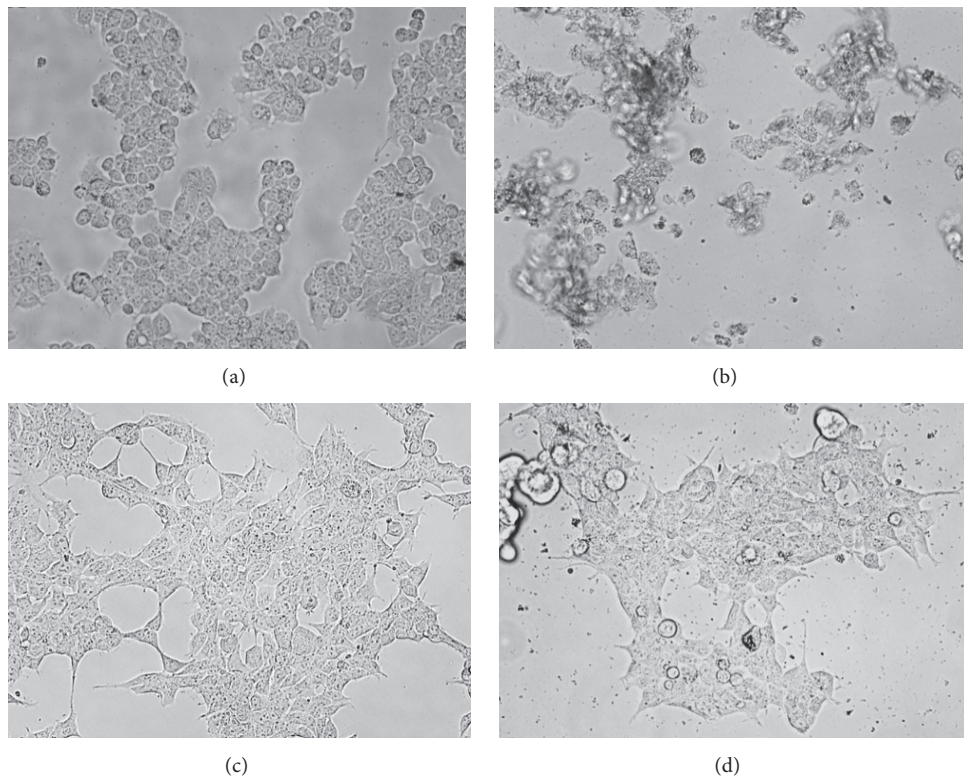

Figure 1: Effect of oregano EO on the cell morphology of hepatocarcinoma cell line (HepG2) and health renal cells (HEK293). The photographs were taken at a magnification ×40. Images are representative of three independent experiments. (a) HepG2 (control); (b) HepG2 cells treated with oregano EO (236 μg/μL); (c) HEK293 (control); and (d) HEK293 cells treated with oregano EO (236 μg/μL).

savastanoi Janse, and *P. syringae* pv. *phaseolicola* Van Hall) compared with the synthetic antibiotic tetracycline. They resulted in the fact that the above-mentioned EOs were able to significantly inhibit the growth of tested bacterial strains especially against G+ve bacteria.

El-Massrry et al. (2009) [71] studied the antibacterial activity of different crude extracts from fresh leaves of *S. terebinthifolius* cultivated in Egypt and reported that these extracts exhibited higher antibacterial activity against *S. aureus*, *P. aeruginosa*, and *E. coli*. In a recent study, the EOs extracted from *V. officinalis* L., *Majorana hortensis* L., and *Salvia officinalis* L. were able to inhibit the growth of some phytopathogenic bacteria in a dose dependent manner such as *B. megaterium*, *C. michiganensis*, and *B. mojavensis* in the case of *V. officinalis* [72], whereas the highest reduction of *C. michiganensis*, *X. campestris,* and *P. savastanoi* was observed in the case of *S. officinalis* and *M. hortensis*. The mode of action of different EOs towards the bacterial cells *in vitro* is explained by permeabilization of cell membranes, loss of ions, leakage of macromolecules, and lysis [72].

7.2. Antifungal. Numerous plant EOs and their single constituents have been reported to inhibit postharvest fungi *in vitro* and *in vivo*. Several researchers reported that some plant EOs can be potentially utilized in controlling some serious phytopathogenic fungi such as *B. cinerea* [73, 74], *Aspergillus* spp. [75], *Fusarium* spp. [76], *Penicillium* spp. [77], and *C. gloeosporioides* Penz. [78].

Elshafie et al. (2015) [2] investigated the fact that the EOs obtained from *T. vulgaris* and *V. officinalis* can be effectively utilized for controlling brown rot infection on peach fruit caused by *Monilinia laxa*, *M. fructicola*, and *M. fructigena*. Clove EO extracted from *Syzygium aromaticum* (L.) Merr. & Perry has been reported as a bioactive substance especially its active component monoterpene eugenol against *B. cinerea*, *M. fructigena*, *P. expansum* Link, and *Phlyctema vagabunda* Desm. in apples [79]. Oregano EO and its main single constituent carvacrol were reported to inhibit effectively the mycelium growth of *Neofabraea alba* (E. J. Guthrie) Verkley in apples [80].

Other studies also concluded the presence of significant potential fungicidal effects of some plant EOs such as thyme and vervain higher than chemical preparations in postharvest treatments against *M. laxa*, *M. fructigena,* and *M. fructicola* on peach fruit [1, 2]. In the same mode, EOs of fennel (*Foeniculum sativum* Mill.), marjoram, oregano, and sage exhibited a fungicidal effect against *B. cinerea* and *P. expansum* in apples [81].

Elshafie et al. (2015) [17] evaluated the antifungal effect of the single components of *O. vulgare* EO (carvacrol, thymol, linalool, and *trans*-caryophyllene) against the postharvest pathogens *M. laxa, M. fructigena,* and *M. fructicola* (Figure 2). The fungicidal activity assay of the above four single

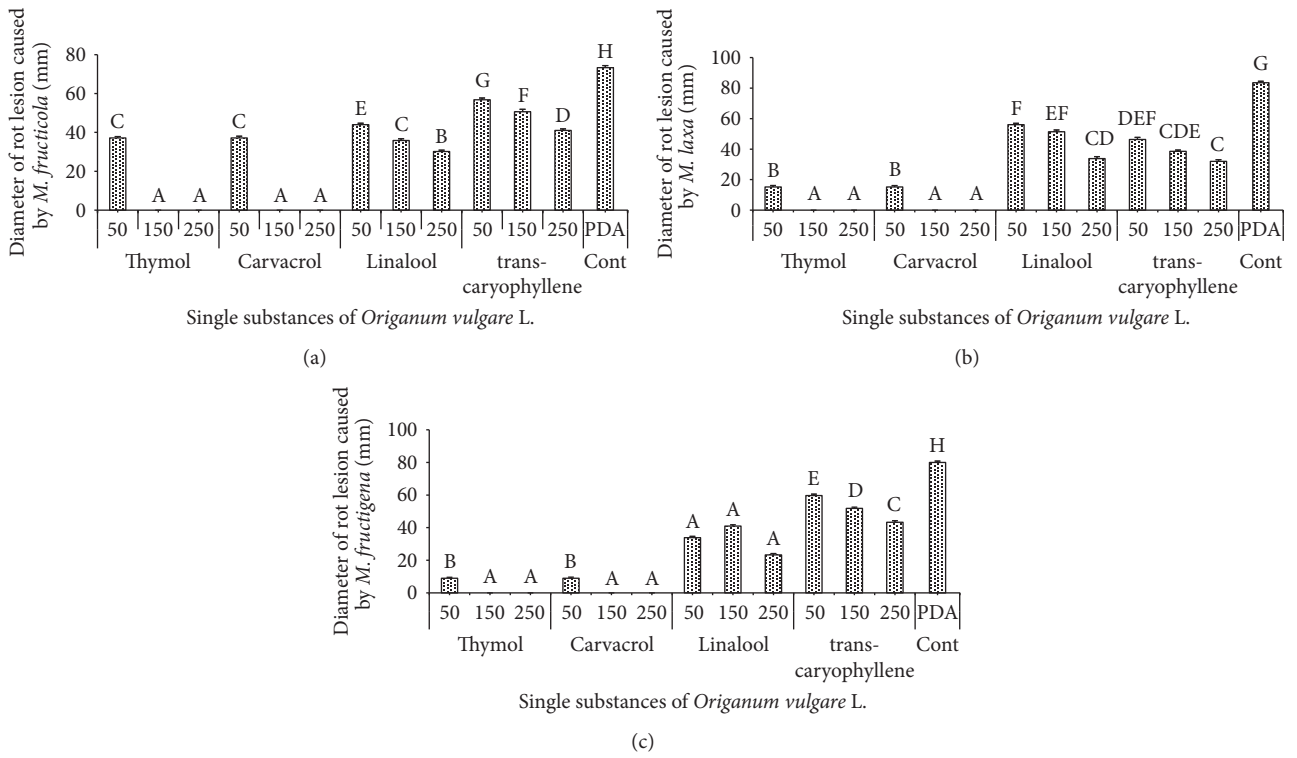

Figure 2: *In vitro* antifungal activity of the four single substances of *O. vulgare* EO against *M. laxa*, *M. fructicola*, and *M. fructigena*. Bars with different letters indicate mean values significantly different at $P < 0.05$ according to Tukey test. Data are expressed as mean of three replicates ± SE. 50, 150, and 250 are the concentrations of each single substance in ppm; PDA is potato dextrose agar.

components has been carried out *in vitro* according to the method of Soylu et al. (2010) [21] and the antifungal activity was expressed by measuring the diameter of mycelium growth [82]. They concluded that carvacrol and thymol have exhibited the highest activity in *in vitro* tests against all studied postharvest *Monilinia* pathogens. Linalool and *trans*-caryophyllene showed low antifungal activity against all studied pathogens. However, thymol showed a fungitoxic effect while carvacrol and citral showed a fungistatic effect against the previous tested fungi.

In the same study, Elshafie et al. (2015) [17] reported that the bioactive treatments which exhibited the highest *in vitro* activity were selected for further *in vivo* experiments against the same postharvest pathogenic fungi and concluded that carvacrol and thymol showed a promising inhibition of the brown rot disease of peach fruit caused by *M. laxa*, *M. fructicola,* and *M. fructigena*. The antifungal effect is mainly attributed to the inhibition of both mycelium growth and spore germination. This hypothesis suggests that impeding the initial infection and the subsequent mycelial spread beyond the infection site will restrict the expression of disease.

8. Conclusion

A great many research articles investigating the biochemical properties of plant EOs have obtained interesting results in agricultural, clinical, and pharmaceutical fields. In conclusion, the use of EOs in pharmaceutical and agrochemical industries as natural alternatives for synthetic microbicide drugs is a field of growing interest. Plant EOs mentioned in the current review are considered promising natural alternatives to conventional pharmaceutical drugs. Moreover, there is still a need for more specific and rational research that deals with the method of application of those efficient EOs and their single constituents in agriculture and food industry for manufacturing new health-oriented products as well as novel natural pharmaceutical drugs.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

The authors contributed equally to manuscript.

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Research Article

In Vitro Antibacterial Activity of Pomegranate Juice and Peel Extracts on Cariogenic Bacteria

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Aim. To evaluate the antimicrobial activity of hydroalcoholic extracts of pomegranate (*Punica granatum* L.) peel and juice, against the microorganisms considered the main etiologic agents of dental caries. *Methods*. The values of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined against *Streptococcus mutans* Clarke ATCC® 25175™ strain and *Rothia dentocariosa* clinical isolate. *Results*. Peel extracts inhibit effectively the growth and survival of *S. mutans* ATCC 25175 strain and *R. dentocariosa* clinical isolate with MIC and MBC values of 10 μg/μl and 15 μg/μl, respectively. Furthermore, the pomegranate juice extract showed high inhibitory activity against *S. mutans* ATCC 25175 strain with a MIC value of 25 μg/μl and a MBC value of 40 μg/μl, whereas, against *R. dentocariosa*, it has displayed a moderate inhibitory activity, with MIC and MBC values of 20 μg/μl and 140 μg/μl, respectively. *Conclusions*. *In vitro* microbiological tests demonstrate that the hydroalcoholic extracts of pomegranate juice and peel are able to contrast the main cariogenic bacteria involved in tooth decay. Although being preliminary data, our results suggest that pomegranate polyphenolic compounds could represent a good adjuvant for the prevention and treatment of dental caries.

1. Introduction

Even though the prevalence of dental caries has decreased through the use of preventive systems (fluoride prophylaxis, fluoride toothpastes, control of oral hygiene, and sealants) [1–4], it still remains one of the most common chronic diseases both in health and in systemic diseases affected children [5–7]. The etiology of tooth decay is multifactorial and it is induced by three main factors: host, environment, and bacteria. Today it is known that different bacterial species cause the strongest effect on the prevalence and incidence of dental caries.

Streptococcus mutans represents one of the main factors for triggering of dental caries, because it can adhere to tooth surfaces and produce large amounts of acid. The key virulence factors are the water-insoluble glucan synthesized from sucrose and involved in biofilm formation, the acidogenicity, and finally the acid tolerance [8, 9]. Many studies

have revealed that *S. mutans* represents about the 20–40% of the cultivable flora in biofilms removed from carious lesion [10]. *Rothia* spp., in particular *R. dentocariosa*, are common inhabitants of the oral cavity. Recent reports suggest that these species could be opportunistic pathogens, causing a number of diseases in addition to dental and periodontal pathologies [11]. *R. dentocariosa*, originally isolated from carious lesions of human teeth, has been found to cause endocarditis [12], pneumonia [13], and infections of the peritoneum and lung [14].

S. mutans and the other microorganisms involved in the pathogenesis of dental caries have been considered very difficult to control, because they have developed tolerance and resistance to many antimicrobial agents routinely used in the clinical practice [15].

The chlorhexidine has been studied for nearly 40 years primarily for its ability to reduce gingivitis. Classified as an antimicrobial agent, it has been proven to inhibit the formation and development of dental plaque biofilm [16]. However, it can cause a change in taste and produce yellow or brown pigments on tooth surfaces. Therefore, the use of chlorhexidine for caries prevention is controversial, especially in children [16].

The resistance of microorganisms against the antibiotics commonly used to treat oral infections, the increasing number of oral pathologies, and the lack of medications without side effects require identifying new effective strategies against oral pathogens. Since ancient times, the bioactive principles of plant origin have been used for treatment of many diseases and microbial infections. In the last decades, the use of plants with preventive and therapeutic effects contributing to health care has increased. Scientists investigated many plant products in order to find their effectiveness in the prevention of dental plaque formation [17–19].

Numerous medicinal plant extracts have been shown to inhibit the formation of dental biofilms by reducing the adhesion of microbial pathogens to the tooth surface or reducing the number of bacteria implicated in the caries pathogenesis [20, 21]. However, only few natural products have found therapeutic applications. The reasons of such limited use are due to different factors as effectiveness, stability, smell, taste, and, not last, cost [22].

Pomegranate (*Punica granatum* L.) is a common fruit of a tree belonging to the family *Punicaceae*. It is native to the region from northern India to Iran and it has been cultivated and naturalized over the entire Mediterranean region since ancient times. The ripe fruit is about five inches wide with deep red, leathery skin, grenade shape with a pointed calyx. The fruit contains many seeds separated by white membranous pericarp. Each seed is surrounded by tart and red juice [23].

Pharmacological properties of pomegranate have a long history, but, in the recent decades, the interest in evaluating therapeutic effects of pomegranate has increased noticeably. Studies show that pomegranate juice has potent antioxidant activity (capability to scavenge free radicals) due to its high polyphenols content, including ellagitannins (hydrolysable tannins) and anthocyanins (condensed tannins). There is a range of phytochemical compounds in pomegranate that have showed antimicrobial activity, but most of the researchers have found that ellagic acid and larger hydrolyzable tannins, such as punicalagin, have the most important activities. In many cases, the mixture of the pomegranate constituents offers the most advantage [24]. This fruit has also been used in traditional medicine for the treatment of dysentery, diarrhea, and respiratory pathologies [25, 26].

Many studies indicate that pomegranate extracts may be employed as natural alternative for the treatment of a wide range of bacterial and viral infections due to their antimicrobial activity. Recent study indicates that both pomegranate aril and peel extracts have an effective antimicrobial activity, as evidenced by the inhibitory effect on the bacterial growth of two important human pathogens, including *Staphylococcus aureus* and *Escherichia coli*, often involved in foodborne illness [27]. In addition, experimental data strongly support the antibacterial activity of pomegranate extracts against oral pathogen such as *S. mutans* [28]. However, little is known about the effect of pomegranate extracts on other pathogens involved in tooth decay such as *R. dentocariosa*, the first bacterium isolated from carious dentin [29].

The aim of present study was to evaluate the antimicrobial activity of hydroalcoholic extracts of pomegranate (*Punica granatum* L.) peel and juice against *S. mutans* ATCC 25175 strain and *R. dentocariosa* clinical isolates.

2. Materials and Methods

2.1. Preparation of Extracts for Microbiological Assay. Fresh fruits of pomegranate (*P. granatum* L.) were collected from trees located in the countryside of Avellino (Southern Italy) during fruit season. The fruits were handpicked, washed, and peeled, and the arils, without seeds, were hand-crushed and then squeezed in order to obtain the juice. The peel was air dried a few days and then pulverized. The samples were stored at −20°C for further analysis. The juice was defrosted at room temperature. Solution water/ethanol 25 ml 50% (v/v) was added to 5 g of juice. The same procedure was carried out for the peel powder. Each sample was mixed for 30 minutes, and then the extracts were filtered.

The analysis of phenolic compounds of the pomegranate (juice and peel) was performed by reverse phase HPLC (RP) coupled offline mass spectrometry (MS) MALDI-TOF as described in our previous study [27].

For microbiological assays, the ethanolic extracts of juice and peel were dried in Savant in order to calculate the percentage yield of total polyphenols. Each extract was reduced in volume in a rotavapor, transferred into a plastic tube, and finally lyophilized. The hydroalcoholic extracts of pomegranate peel and juice were used, as described in our previous study [27].

2.2. Microorganisms and Growth Conditions. The antimicrobial activity of the pomegranate extracts was evaluated against the strain *Streptococcus mutans* Clarke ATCC 25175 (LGC Standards, UK) isolated from carious dentine and *Rothia dentocariosa* clinical isolate Rd1, obtained from samples of dental plaque provided from the Pediatric Dentistry

Department of "Federico II" University, Naples, Italy. Permission to take dental plaque samples was acquired according to the local planning authorities. Furthermore, approval for this study was granted by the ethics committee of the "Federico II" University, Naples, Italy (Protocol number 101/14).

The identification of clinical isolates was performed, from UOC of Clinical Microbiology, AOU "Federico II" of Naples, Italy, by mass spectrometry using the Matrix Assisted Laser Desorption/Ionization (MALDI) mass spectrometer (Bruker Daltonics, MALDI Biotyper, Fremont, CA, USA), a high-throughput proteomic technique for identification of a variety of bacterial and fungal species [30, 31], and biochemical phenotyping method in an BD Phoenix™ Automated Microbiology System (Becton Dickinson, BD Franklin Lakes, NJ, USA), according to the manufacturer's instruction.

Bacteria were cultured aerobically in broth and agar media at 37°C. The media used were Brain Heart Infusion (BHI) (Oxoid, S.p.a., Rodano, Milano, Italy), Columbia CNA with 5% Sheep Blood with Colistin and Nalidixic Acid (Oxoid, S.p.a., Rodano, Milano, Italy), and Mueller-Hinton (Simad s.a.s., Naples, Italy). Microbial strains were maintained at 4°C on agar media. The isolates were stored frozen at −80°C in BHI broth supplemented with 10% glycerol (v/v) (Carlo Erba, Reagents, Milan, Italy) until use and the working cultures were activated in the respective broth at 37°C for 15–18 h.

2.3. In Vitro Antibacterial Activity Assays. The susceptibility of *S. mutans* ATCC 25175 and *R. dentocariosa* Rd1 to different concentrations of *Punica granatum* L. fruit extracts was determined by dilution tube method with 1×10^5 CFU/ml as standard inoculums [32]. The extracts were added in the series of tubes achieving final concentrations of 0, 5, 10, 15, 20, 30, 40, 60, 100, and 140 μg/μl, and tubes were incubated at 37°C for 24 h. As positive control the bacterial strains were tested with ranging concentrations of Ampicillin (Sigma-Aldrich, Milano, Italy) and with extraction buffer as negative control. After incubation, the optical density at $A_{600\,\mathrm{nm}}$ was determined; subsequently an aliquot of each sample was spread into BHI-agar plates in duplicate and then incubated for 24–48 h for the evaluation of viable counts. Minimum inhibitory concentration (MIC) was assigned to lowest concentration of pomegranate extract, which prevents bacterial growth. The minimum bactericidal concentration (MBC) was defined as the minimum extract concentration that killed 99% of bacteria in the initial inoculums.

To verify the effect of pomegranate juice and peel hydroalcoholic extracts on the fitness of *S. mutans* ATCC 25175 and *R. dentocariosa* Rd1, assays of bacterial growth and survival were performed in presence of increasing concentrations of the extracts. To evaluate the fitness of each strain, during the observation period (96 h), serial dilutions were spread on BHI-agar and incubated at 37°C for 24–48 h to evaluate viable counts. All experiments were performed in triplicate, with three independent cultures; the results obtained were analyzed and graphically reported by using "GraphPad Prism 6" software. Results are presented as mean ± SD. The statistical significance was determined by the two-way ANOVA test with a Bonferroni correction (P value ≤ 0.05).

3. Results

3.1. In Vitro Antibacterial Activity of Pomegranate Extracts. The antimicrobial activity of pomegranate extracts against *S. mutans* ATCC 25175 cariogenic strain and *R. dentocariosa* Rd1 clinical isolate was evaluated by dilution tube method, according to the CLSI (Clinical and Laboratory Standards Institute) guidelines [33].

Growth of *S. mutans* ATCC 25175 strain and *R. dentocariosa* Rd1 clinical isolate was inhibited with a concentration of pomegranate juice extract equal to 25 μg/μl and 20 μg/μl, respectively.

Pomegranate juice extracts showed a MBC value of 40 μg/μl against *S. mutans* ATCC 25175 and a MBC value of 140 μg/μl against *R. dentocariosa* Rd1 (Table 1).

The pomegranate peel extracts exhibited a MIC value of 10 μg/μl and a MBC value of 15 μg/μl against both microorganisms tested. Both the bacteria tested in this study are sensitive to ampicillin (Table 1).

3.2. Effects of Pomegranate Extracts on Bacterial Fitness. To verify the effect of pomegranate juice and peel hydroalcoholic extracts on the fitness of *S. mutans* ATCC 25175 cariogenic strain and *R. dentocariosa* Rd1 clinical isolate, the growth and survival were evaluated for 96 h, with increasing concentrations of hydroalcoholic extracts. The pomegranate juice extracts exhibited inhibitory effect on growth and survival of both strains (Figure 1). The growth evaluation was biased by the turbidity of the extracts, as clearly showed by growth curves (Figures 1(a) and 1(c)). However, the evaluation of viable counts had highlighted a strong bactericidal activity of pomegranate juice hydroalcoholic extract with a concentration of 40 μg/μl for *S. mutans* ATCC 25175 and a moderate bactericidal effect against *R. dentocariosa* Rd1 with a concentration of 140 μg/μl (Figures 1(b) and 1(d)). Interestingly, the pomegranate hydroalcoholic peel extract exhibited a strong inhibitory activity against both tested cariogenic strains (Figure 2). The hydroalcoholic peel extracts interfered with the bacterial growth, survival, and fitness in a dose dependent manner and with time-lasting effects, as previously described for other clinical isolates [27]. In addition the bactericidal activity is detectable at a very low concentration equal to 15 μg/μl for both strains. The peel extracts in ethanol were cloudy so it was impossible to test it in the bacterial growth assay.

4. Discussion

Results of the present study showed that pomegranate juice and peel extracts were effective against the main cariogenic pathogens such as *S. mutans* ATCC 25175 cariogenic strain and *R. dentocariosa*, Rd1 clinical isolate.

The present research was in line with other studies demonstrating antibacterial agents from plant were effective to prevent and contrast oral and periodontal disease and tooth decay [34–37].

In particular, among plants, *Punica granatum* L., used in traditional medicine, is known for its pharmacological properties that have been evaluated due to antiparasitic,

Table 1: Antibacterial activity of pomegranate fruit extracts against *Streptococcus mutans* and *Rothia dentocariosa.*

Cariogenic bacteria	Pomegranate juice extracts ($\mu g/\mu l$)		Pomegranate peel extracts ($\mu g/\mu l$)		Ampicillin ($\mu g/\mu l$)	
	MIC[a]	MBC[b]	MIC	MBC	MIC	MBC
S. mutans ATCC 25175	25	40	10	15	0.01	0.02
R. dentocariosa Rd1	20	140	10	15	0.04	1.2

[a]MIC: minimum inhibitory concentration. [b]MBC: minimum bactericidal concentration.

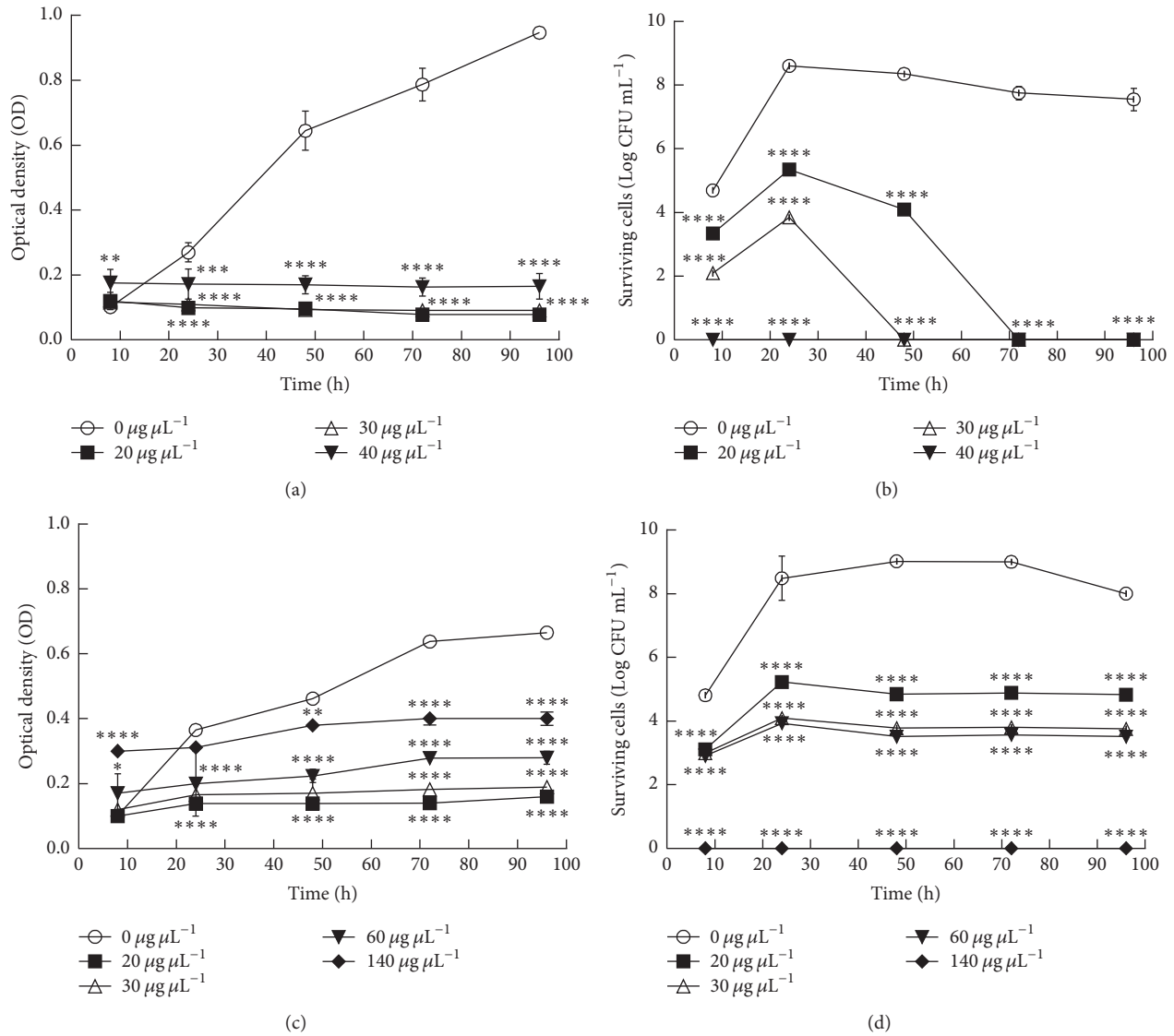

Figure 1: Effect of pomegranate juice extracts on (a) growth of *S. mutans* at different concentration (0, 20, 30, and 40 $\mu g/\mu l$); (b) survival of *S. mutans* at different concentration (0, 20, 30, and 40 $\mu g/\mu l$); (c) growth of *R. dentocariosa* at different concentration (0, 20, 30, 60, and 140 $\mu g/\mu l$); (d) survival of *R. dentocariosa* at different concentration (0, 20, 30, 60, and 140 $\mu g/\mu l$). The experiments were performed in triplicate and statistical significance was examined by the two-way ANOVA test with a Bonferroni correction. Results are indicated as means ± SDs. Asterisks indicate statistical significance ($^{*,**}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

antibacterial, antifungal, antiproliferative, apoptotic, and anticancer effects [38].

Literature data reported that extracts of *Punica granatum* L. peel in different concentrations were effective against different bacterial species such as *S. aureus*, *E. coli*, *Salmonella enterica*, *Shigella sonnei*, *Enterococcus faecalis*, and *Bacillus subtilis* [27, 39]. The amount of total polyphenols varied depending on the parts of the fruit, in particular being higher in the peel than in the juice extracts [27]. According to these findings, our data highlighted that the pomegranate peel

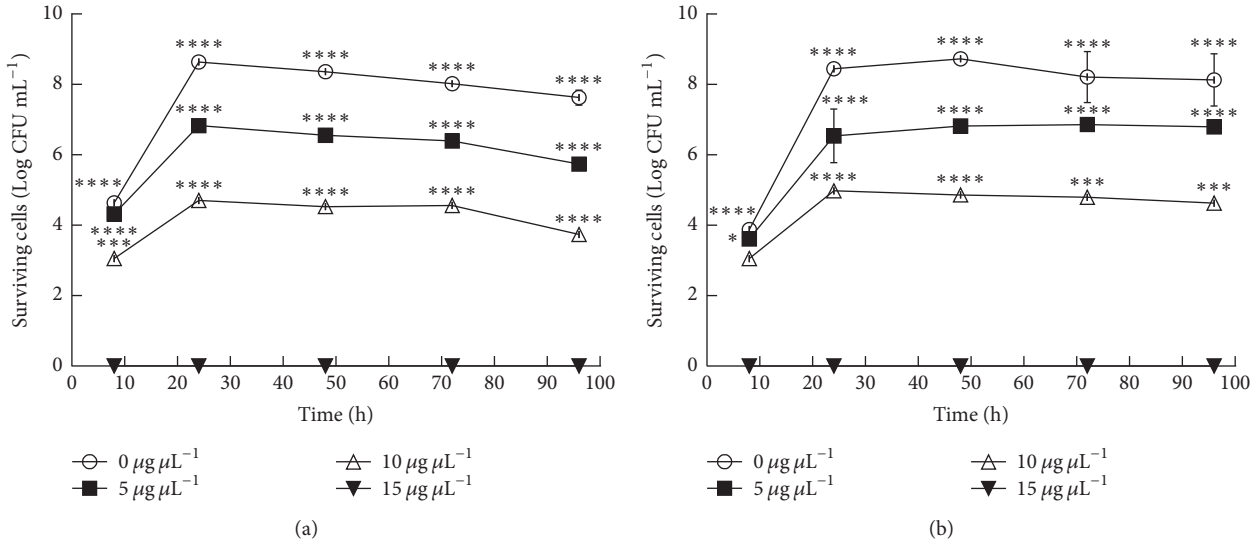

Figure 2: Effect of pomegranate peel extracts on survival of *S. mutans* (a) and *R. dentocariosa* (b) at different concentration (0, 5, 10, and 15 μg/μl). The experiments were performed in triplicate and statistical significance was examined by the two-way ANOVA test with a Bonferroni correction. Results are indicated as means ± SDs. Asterisks indicate statistical significance ($^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

extract strongly inhibits the growth and the survival of both cariogenic strains with MIC value of 10 μg/μl and MBC value of 15 μg/μl.

The MIC values of pomegranate extracts determined in different studies significantly vary. For example, the MIC against *S. aureus* isolates are reported to range from 0.62 to >250 μg/μl [27, 40–42]. This variability is not surprising, considering that it is typically recorded even with conventional antimicrobials against all clinical isolates [43], though generally within a more restricted range. Therefore, our results are in agreement with previous studies that established that pomegranate extracts could reduce the viable count of *S. mutans* to a degree equivalent to that of 1.2% CHX mouthwash [44, 45].

In 2006, also Vasconcelos et al. investigated the antimicrobial effect of *Punica granatum* L. (pomegranate) using a phytotherapeutic gel, pomegranate based, and miconazole (Daktarin® oral gel) against three standard streptococci strains (*mutans* ATCC 25175, *sanguis* ATCC 10577, and *mitis* ATCC 9811) and demonstrated the greater efficiency of pomegranate gel in inhibiting microbial adherence than miconazole [46].

While the antibacterial activity of the pomegranate peel has been the subject of numerous researches, few studies have investigated the antibacterial activity of pomegranate juice against oral pathogens, such as *S. mutans* and *R. dentocariosa*. Kote and Nagesh in 2011 conducted a clinical trial that showed the ability of pomegranate juice to reduce the microorganisms of dental plaque (streptococci and lactobacilli) [47].

This *in vitro* study demonstrates that *Punica granatum* L. peel and juice extracts are efficacious against cariogenic bacteria, supporting the hypothesis that pomegranate polyphenols could exert an anticaries effect via an antimicrobial mode-of-action.

In addition, our findings demonstrate for the first time the inhibitory effect of hydroalcoholic pomegranate extracts on *R. dentocariosa*, isolated from dental plaque, a bacterium considered as one of the main etiological agents of several oral diseases, including tooth decay [5, 48, 49].

5. Conclusions

In vitro microbiological assays demonstrated that pomegranate (*Punica granatum* L.) hydro-alcoholic peel and juice extracts are able to counteract cariogenic bacteria of dental plaque. In fact, the extracts showed inhibitory effect on the growth and survival of *S. mutans* ATCC 25175 and *R. dentocariosa* Rd1 isolate, considered among the most important etiological agents of tooth decay. The strongly bactericidal power of the pomegranate fruit extracts against oral cariogenic bacteria suggests further deep investigation.

Much of the evidence for pomegranates antibacterial and antiviral activities against foodborne pathogens and other organisms responsible of infectious disease comes from *in vitro* cell based assays, necessitating further confirmation of *in vivo* efficacy through human clinical trials. Therefore, more controlled trials using different concentrations of pomegranate fruit extracts are necessary to verify its action upon supragingival microflora *in vivo*. Within the limits of the present study, it may be concluded that the pomegranate fruit extracts are effective against dental plaque microorganisms. Further research is needed to identify the real benefits of pomegranate as a therapeutic and preventive agent for dental plaque microorganisms and to identify the specific active constituents in pomegranates that could be useful as anticaries/antiplaque agents and the safer dose that can be used in humans.

Disclosure

The present address for Gabiria Pastore is Laboratory of Molecular Microbiology and Biotechnology, Department of Medical Biotechnologies, University of Siena, Siena, Italy. The present address for Annunziata Gaetana Cicatiello is Department of Clinical Medicine and Surgery Federico II University Medical School, Naples, Italy.

Conflicts of Interest

The authors declare no conflicts of interest.

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Review Article

Cucurbitaceae Seed Protein Hydrolysates as a Potential Source of Bioactive Peptides with Functional Properties

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Seeds from Cucurbitaceae plants (squashes, pumpkins, melons, etc.) have been used both as protein-rich food ingredients and nutraceutical agents by many indigenous cultures for millennia. However, relatively little is known about the bioactive components (e.g., peptides) of the Cucurbitaceae seed proteins (CSP) and their specific effects on human health. Therefore, this paper aims to provide a comprehensive review of latest research on bioactive and functional properties of CSP isolates and hydrolysates. Enzymatic hydrolysis can introduce a series of changes to the CSP structure and improve its bioactive and functional properties, including the enhanced protein solubility over a wide range of pH values. Small-sized peptides in CSP hydrolysates seem to enhance their bioactive properties but adversely affect their functional properties. Therefore, medium degrees of hydrolysis seem to benefit the overall improvement of bioactive and functional properties of CSP hydrolysates. Among the reported bioactive properties of CSP isolates and hydrolysates, their antioxidant, antihypertensive, and antihyperglycaemic activities stand out. Therefore, they could potentially substitute synthetic antioxidants and drugs which might have adverse secondary effects on human health. CSP isolates and hydrolysates could also be implemented as functional food ingredients, thanks to their favorable amino acid composition and good emulsifying and foaming properties.

1. Introduction

The Cucurbitaceae family is formed by about 130 genera and 800 species, including squashes, pumpkins, melons, and gourds [1, 2]. Figure 1 shows the total world production of Cucurbitaceae fruits in 2014 (217,714,974 tonnes) divided into four major groups [3]. Cucurbitaceae plants are cultivated in warmer regions of the world and many of their parts are typically used as food, especially the fruit, the flowers, and the seeds [4, 5]. However, Cucurbitaceae seeds have long been used in indigenous diets as popular medicine, thanks to their bioactive and nutraceutical properties [6, 7].

Industrially, Cucurbitaceae seeds are normally used for the extraction of edible and drying oils which comprise about a half of the seed's weight [8]. The main byproduct in the oil production is the oil cake which is rich in carbohydrates and has a very high content of protein (60–65% [w/w]; [9, 10]). However, this nutritive and potentially bioactive byproduct is usually discarded as waste or only used as animal feed. Since Cucurbitaceae seeds also seem to be a valuable source of good quality proteins in terms of their amino acid composition; they could potentially be used as functional food ingredients [11–13]. Moreover, enzymatic hydrolysis can improve the bioactive and functional properties of proteins [14–16]. Therefore, Cucurbitaceae seed protein hydrolysates could be not only an interesting alternative to known food supplements but also a potential source of new bioactive peptides.

In recent years, Cucurbitaceae seed proteins and their hydrolysates have gained considerable attention from researchers and the body of evidence for their bioactive and functional properties is quickly growing. Therefore, the main objective of this paper is to provide a comprehensive review of latest research conducted in the field of Cucurbitaceae seed protein isolation and its subsequent enzymatic hydrolysis in order to obtain bioactive peptides with enhanced functional

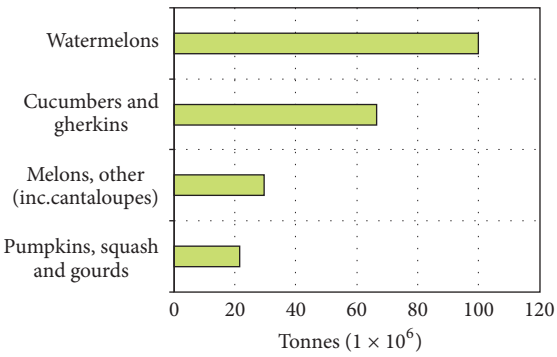

Figure 1: World production of Cucurbitaceae fruits in 2014 [3].

properties. At the same time, this review aims to put into perspective the lack of knowledge about bioactive peptides recoverable from Cucurbitaceae seed protein hydrolysates and thus, it aims to point out the many opportunities this area may provide for future research.

2. Generation of Cucurbitaceae Seed Protein Hydrolysates

This section describes the processes involved in the generation of Cucurbitaceae seed protein extracts and their enzymatic hydrolysates, starting with the extraction of the protein from the seeds themselves, then studying the main factors involved in the enzymatic hydrolysis of the Cucurbitaceae seed protein extracts and, finally, describing the steps involved in the obtainment of Cucurbitaceae seed protein hydrolysates (Figure 2).

2.1. Cucurbitaceae Seeds. First, Cucurbitaceae seeds are usually manually separated from the ripe fruit and they are dehulled if necessary and possible. In order to obtain the seed meal (sometimes referred to as seed flour), disintegrating and defatting steps are applied to the seeds. The disintegration is achieved by grinding or pulverizing the dehulled seeds. The defatting of the ground seeds is carried out with hexane and the resulting meal is dried at room temperature. The disintegration step may be repeated at this point. The resulting meal may also be passed through a 60-mesh sieve in order to obtain a fine powder [17, 18]. In the case of Cucurbitaceae seeds that are used in oil production (especially *Cucurbita pepo*, but also *Citrullus lanatus*), the residual oil cake resulting from the seed oil pressing is first ground and then defatted, following the same procedures as described above [10, 19]. The defatted Cucurbitaceae seed meal is then stored in a dry and cool place until used for the protein extraction.

2.2. Cucurbitaceae Seed Protein Extraction. At the laboratory scale, Cucurbitaceae seed protein extraction has been carried out mainly by means of fractionation, concentration, and isolation processes, resulting in potential food and pharmaceutical applications [9]. However, different factors (such as pH, temperature, ionic strength, solvent type, extraction time, and solid-liquid ratio, among others [19]) may affect the

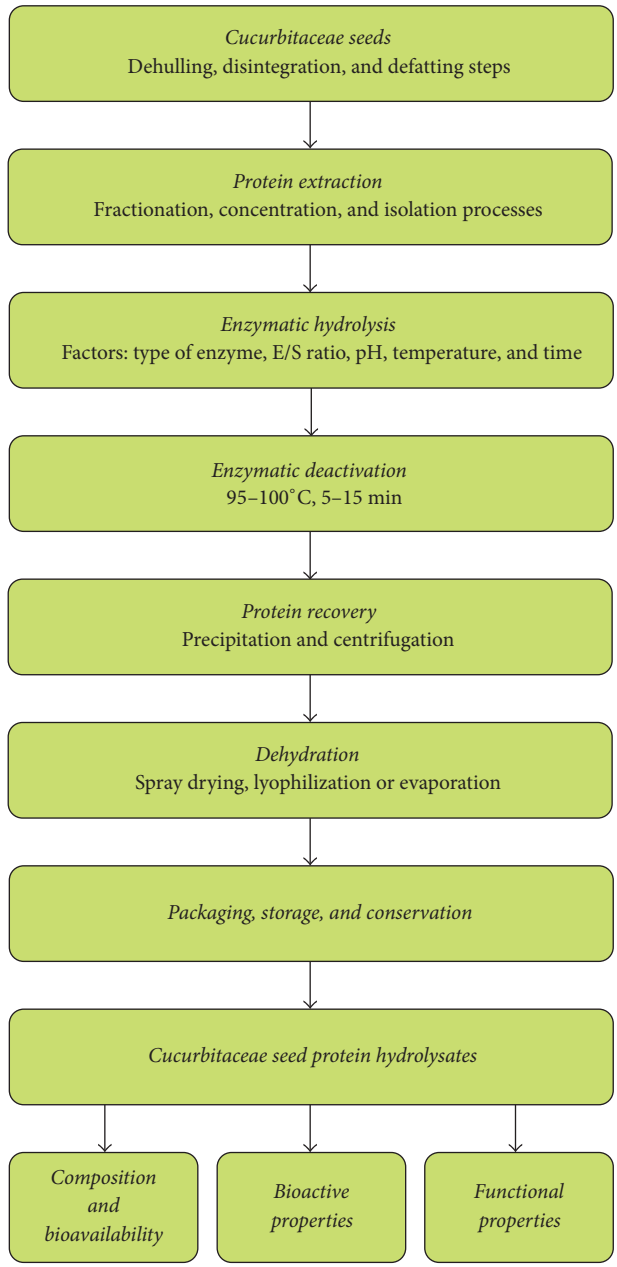

Figure 2: Generation of Cucurbitaceae seed protein hydrolysates.

protein extraction process, thus making it rather complicated and unpractical to implement at industrial level.

Traditionally, the extraction of storage proteins from seeds has been carried out following Osborne [20] who classifies proteins according to their solubility in water (albumin), salt solutions (globulin), alkali solutions (glutelin), and alcohol solutions (prolamin). Nowadays, however, many Cucurbitaceae seed protein researchers deal directly with cucurbitin (11S globulin) which is the main storage protein in Cucurbitaceae seeds [10]. Cucurbitin is a hexameric globular protein whose subunits weigh 54 kDa each. In turn, each subunit consists of an acidic and a basic subunit which are disulfide-bonded and weigh 33 kDa and 22 kDa,

respectively [21]. Peričin et al. [9] describe the standard procedure of cucurbitin extraction. First, the defatted seed meal is extracted with water and this fraction is discarded. Subsequently, the globulin fraction is extracted with NaCl solution at room temperature. The protein is then precipitated from the clarified extract by gradual addition of water. The precipitate is dissolved in standard buffer, the solution is clarified by centrifugation, and the cucurbitin is precipitated by addition of water [9, 10, 22].

On the other hand, high-yield protein isolates can be extracted from Cucurbitaceae seeds in alkaline conditions when not aiming only for cucurbitin but for all protein fractions in one [18, 19, 23–25]. First, the defatted seed meal is treated using NaOH solution (pH 8–10). Subsequently, the solution is centrifuged and an isoelectric precipitation of the protein from the supernatant is carried out, usually using dilute HCl (pH values about 4-5). The precipitate is then separated from the whey by centrifugation and the resulting protein pellet is dried in order to obtain the protein isolate. This process can be implemented to obtain protein isolates which consist of about 80–90% of protein. Moreover, Cucurbitaceae seed protein isolates have proven to have some bioactive and functional properties even before any hydrolysis takes place, so they make a very good comparison point for the Cucurbitaceae seed protein hydrolysates.

Following the extraction process, cucurbitin [10, 22], the protein isolate [23–27], or the defatted seed meal itself [17] can be hydrolyzed in order to obtain Cucurbitaceae seed protein hydrolysates. Generally, enzymatic hydrolysis is preferred for any type of protein since it can guarantee less drastic hydrolytic conditions and more specific cleavage sites in comparison to chemical hydrolysis [28, 29].

2.3. Enzymatic Hydrolysis of Cucurbitaceae Seed Protein Extracts. The main factors described to influence hydrolysis of Cucurbitaceae seed protein are the type of enzyme used, the enzyme/substrate (E/S) ratio, pH value, temperature and time of hydrolysis [14]. These factors influence not only the degree of hydrolysis (DH, defined as the proportion of cleaved peptide bonds in a protein hydrolysate [30]), but also the molecular weight distribution of the peptides conforming the hydrolysates [29, 31] and their bioactive and functional properties [32]. For the research papers revised in this section, Table 1 summarizes the main hydrolysis conditions as well as the DH, molecular weight distribution, and the bioactive and functional properties of the Cucurbitaceae seed protein hydrolysates.

One of the frequently studied factors that can influence hydrolysis is the type of enzyme used. Since different proteolytic enzymes produce compounds with different physicochemical and nutritional characteristics, it is important to identify most suitable enzymes for each substrate. Thus, enzymatic hydrolysis of the peptide linkage between amino acids can result in a mixture of peptides of various molecular sizes and free amino acids, depending on the type of enzyme used [29]. The proteases that are normally used in Cucurbitaceae seed protein hydrolysis come from animals (Pepsin and Trypsin) or fungi and bacteria (Alcalase, Neutrase, and Flavourzyme) and they can be classified as endoproteases (Alcalase, Pepsin, and Trypsin), exoproteases, and complex mixtures of endo- and exoproteases (Flavourzyme). On the one hand, endoproteases produce relatively large peptides by hydrolyzing the peptide bonds within protein molecules at random. Exoproteases, on the other hand, specialize in hydrolyzing the terminal peptide bonds. Thus, they produce free amino acids by removing them one by one from either the N- or C-terminal sites of the protein molecule [29, 40]. However, often a mixture of free amino acids, dipeptides, tripeptides, and other short peptides (<1.5 kDa) is desirable for use in special formulations (see Section 3). In these cases, a sequential use of endo- and exoproteases is applied during hydrolysis (the former facilitating the action of the latter) for a more complete and specific degradation of the protein [29].

The specificity of the enzymes used in Cucurbitaceae seed protein hydrolysis is thought to result in different DH and molecular weight distributions of the hydrolysates [17, 22, 23]. Venuste et al. [17] analyzed the influence of four different enzymes (Alcalase, Flavourzyme, Protamex, and Neutrase) on the hydrolysis process of pumpkin *(Cucurbita moschata)* seed protein meal. The DH achieved by Alcalase, Flavourzyme, Protamex, and Neutrase was 13.84%, 11.80%, 8.74%, and 4.12%, respectively. According to the authors, Protamex and Neutrase are not able to break the pumpkin protein peptide bond efficiently enough to achieve a DH comparable to those of Alcalase and Flavourzyme. As for molecular weight distribution, a larger proportion of low-molecular weight peptide fractions (1–0.18 kDa) was achieved in the hydrolysis of the protein meal by Acalase and Protamex (57.20% and 50.90%, resp.) compared to Neutrase and Flavourzyme (34.43% and 25.83%, resp.). Thus, Alcalase turned out to be the most suitable enzyme for this particular protein in order to achieve both the highest DH and the highest proportion of low-molecular weight peptides in the hydrolysate.

Vaštag et al. [22] studied the influence of the type of enzyme (Alcalase and Pepsin) on the hydrolysis process of cucurbitin obtained from pumpkin (*Cucurbita pepo* L. c. v. "Olinka") seed oil cake. Under the same E/S ratio and time conditions (0.02 g/1 g and 60 min, resp.), Alcalase achieved a higher DH (26.9 ± 1.1%) in comparison to Pepsin (18.7 ± 1.2%). The authors suggest that, due to the specificity of the two enzymes, the same protein was hydrolyzed at different peptide bonds, resulting in a different composition of the hydrolysates. Similarly, Arise et al. [23] studied the influence of enzyme type (Pepsin, Trypsin, and Alcalase) on the hydrolysis process of watermelon (*Citrullus lanatus* L.) seed proteins. In this study, Trypsin produced a higher DH (26.26 ± 0.27%) than Pepsin and Alcalase (19.38 ± 0.86 and 13.16 ± 1.82%, resp.). This trend was ascribed to the affinity of Trypsin to cleave at sites with C-terminal end, which could generate more amino acids than peptides. However, neither Vaštag et al. [22] nor Arise et al. [23] reported molecular weight distribution of the peptides in the hydrolysates. This kind of information can be very valuable for the determination of the peptide bioavailability and the functional properties of the protein hydrolysates.

Some researchers have used a sequence of different enzymes in the hydrolysis of Cucurbitaceae seed proteins

Table 1: Hydrolysis parameters of Cucurbitaceae seed protein and bioactive and functional properties of their hydrolysates.

Seed type	Substrate	Enzyme	Process parameters				Hydrolysis degree (%)	Molecular weight distribution (kDa)	Properties	Reference
			E/S ratio	pH	T (°C)	t (h)				
Watermelon (*Citrullus lanatus* L.)	Protein isolate	Pepsin	1/100	2.2	37	5	19.38	Not reported	Antioxidant and α-amylase inhibitory activity	Arise et al. [23]
		Trypsin		8	37	5	26.26			
		Alcalase		8	60	5	13.16			
Pumpkin (*Cucurbita pepo* L. c. v. "Olinka")	Protein isolate	Alcalase	1/757	8	50	1	53.23	<15	Antioxidant and ACE-inhibitory activity	Vaštag et al. [26]
		Flavourzyme	1/385			1	37.17	36–40 and <15		
		Sequential use (Alcalase + Flavourzyme)	A (1/250) F (1/385)			1 2	69.29	<15		
Pumpkin (*Cucurbita pepo* L. c. v. "Olinka")	Cucurbitin	Alcalase	2/100	8	50	1	26.94	Not reported	Antioxidant, ACE, and α-amylase inhibitory activity	Vaštag et al. [22]
		Pepsin		3	37		18.7			
Pumpkin (*Cucurbita pepo* L. c. v. "Olinka")	Cucurbitin	Alcalase	2/100	8	50	0 to 2.5	29.8	22 and <12	Antioxidant activity, emulsifying, and foaming capacity	Popović et al. [10]
		Flavourzyme		7	50		9.2	33, 22, and <12		
		Pepsin		3	37		29	<12		
Pumpkin *(Cucurbita pepo)*	Protein isolate	Alcalase	0.5 ml/g	8	50	0.5	19	36, 24, and 20–14	Emulsifying capacity	Bučko et al. [27]
		Pepsin	2/100	3	37	1.5	19	20–14		
Pumpkin (*Cucurbita pepo con.Pepo* var. *styriaca*)	Protein isolate	Alcalase	1/100 to 2/100	9	45 to 55	2 to 5	28	<6.5	Antioxidant activity	Nourmohammadi et al. [24]
		Trypsin		8	35 to 45		23	Not reported		
Pumpkin *(Cucurbita moschata)*	Protein meal	Alcalase	1/100	8	55	5	13.84	1–0.18, 5–1, and <0.18*	Antioxidant activity	Venuste et al. [17]
		Flavourzyme		7			11.8	>10, 1–0.18, and 5–1*		
		Protamex		6.5	50		8.74	1–0.18, 5–1, and <0.18*		
		Neutrase		7			4.12	5–1, 1–0.18, and <0.18*		
Bitter melon *(Momordica charantia)*	Protein pellet	Trypsin	1/50	8	37	12	Not reported	2.5–0.7	ACE-inhibitory activity	Priyanto et al. [33]
		α-Chymotrypsin			37					
		Alcalase			50					
		Pepsin		1.5	37					
		Thermolysin	1/50 to 1/800	8	40 to 70	1 to 15				
Seinat (*Cucumis melo* var. *tibish*)	Protein isolate	Sequential use (Trypsin + Pepsin)	1/100	8	37	3	28.23	0.5–0.18, 1–0.5, and 1-2*	Antioxidant activity	Siddeeg et al. [25]
				7	37	0.5 to 3				
Seinat (*Cucumis melo* var. *tibish*)	Protein fractions	Sequential use (Pepsin + Trypsin)	1/100	2	37	3	Not reported	Glutelin: 0.5–0.32 and 1–0.5*; albumin 0.5–0.18 and 1–0.5*; globulin: 1–0.5 and 0.5–0.18*; glutelin: 0.5–0.18	Antioxidant activity	Siddeeg et al. [34]
				7	37	3				

*Predominant molecular weight fractions in descending order.

[25, 26, 34]. Siddeeg et al. [34] and Siddeeg et al. [25] obtained a high proportion of low-molecular weight peptide fractions (1–0.18 kDa) in the hydrolysates of seinat (*Cucumis melo* var. *tibish*) seed proteins prepared using a sequential treatment with Pepsin-Trypsin [34] and Trypsin-Pepsin [25]. Additionally, in order to study the effect of the sequential use of different enzymes on the DH in Cucurbitaceae seed protein hydrolysates, Vaštag et al. [26] compared the use of Alcalase, Flavourzyme, and their sequential use in the hydrolysis of protein isolate from pumpkin (*Cucurbita pepo* L. c. v. "Olinka") oil cake. In addition, three different E/S ratios were used for both Alcalase and Flavourzyme (1/757, 1/385, and 1/250 [w/w]), with higher E/S ratios yielding higher DH for both enzymes. At the same E/S ratios, Alcalase produced hydrolysates with higher DH than Flavourzyme over the entire period of the hydrolysis which indicates a higher proteolytic activity of Alcalase towards this type of protein. The highest DH values were reached at the E/S ratio of 1/250 [w/w] and the hydrolysis time of 60 min, 53.23 ± 0.70% for Alcalase, and 37.17 ± 1.05% for Flavourzyme. As for the sequential use of both enzymes, this proved to be more efficient than using both enzymes separately. The maximum DH reached by using Alcalase alone did not increase with prolonged hydrolysis. However, the addition of Flavourzyme (E/S 1/385 [w/w]) at 60 min increased the maximum DH to 69.29 ± 0.9% (at 120 min). The authors suggest that Alcalase hydrolysates are a favorable substrate for the hydrolysis by Flavourzyme, probably because Alcalase increases the amount of N-terminal sites in the protein and thus facilitates hydrolysis by the exoprotease activity of Flavourzyme. Both Alcalase and the sequential use of both enzymes achieved the degradation of proteins with a molecular weight higher than 15 kDa. However, the composition of the most prominent molecular weight peptide fraction in these hydrolysates (<15 kDa) was not reported in detail.

Another factor that is inherently related to the use of different enzymes is the pH of the hydrolysis process. However, the pH value of the hydrolysis can adversely influence the solubility of the protein which, in turn, directly affects its recovery from the hydrolysate. Bučko et al. [27] obtained a higher protein recovery from pumpkin *(Cucurbita pepo)* seed protein hydrolysates at pH 8 hydrolyzed by Alcalase (19.3 ± 0.6%) than at pH 3 hydrolyzed by Pepsin (15.9 ± 1.0%). The authors attributed this difference not only to the specific ability of the enzyme to break the peptide bonds but also to the pH of the medium. However, since the solubility of the protein is directly dependent on the pH value, the recovery of the protein can be enhanced posterior to the hydrolysis by adjusting the pH and the ionic strength of the solution.

The time of hydrolysis may also significantly influence the hydrolytic breakdown of Cucurbitaceae seed protein. Typically, the DH value increases with increasing hydrolysis time. Such is the case of the study performed by Siddeeg et al. [25] who prepared hydrolysates from seinat (*Cucumis melo* var. *tibish*) seed protein isolates, using a sequential treatment by Trypsin and Pepsin (both E/S ratios 1/100 [w/w]). First, the protein isolate solutions were incubated with Trypsin for 180 min at 37°C. After the inactivation of Trypsin by adjusting the pH value from 8 to 7, Pepsin was added to the samples. The hydrolysis was stopped at different times (30, 60, 90, 120, and 180 min), obtaining a steady kinetic curve with falling reaction rate over time. Thus, the DH values increased over time and ranged from 11.27 to 28.23%. These DH values, however, are relatively low in comparison to other proteases revised in this section. The authors hypothesize that, for this particular type of protein, alkaline proteases (such as Alcalase) might have higher activity compared to neutral and acid proteases (such as Trypsin and Pepsin, used in their experiment).

As for more complex effects of the time of hydrolysis, Popović et al. [10] observed an interaction between this factor and the type of enzyme used. The authors compared three different proteases (Alcalase, Flavourzyme, and Pepsin) in the hydrolysis of cucurbitin obtained from pumpkin *(Cucurbita pepo)* seed oil cake. The final degree of hydrolysis (t = 120 min) for Alcalase and Pepsin was very similar (27.1% and 29.0%, resp.) and much higher than for Flavourzyme (8.5%). The rate of Pepsin hydrolysis was constant, resulting in a steadily increasing degree of hydrolysis during the whole of 120 min. However, when Alcalase and Flavourzyme were used, the degree of hydrolysis increased rapidly in the first 30 min and remained constant for the rest of the treatment. Thus, the authors suggest that different time of hydrolysis can result in hydrolysates with different DH, depending on the type of enzyme used.

In order to detain the hydrolysis process of Cucurbitaceae seed protein, the enzymes are deactivated and the hydrolytic reaction stops. This is most commonly achieved by heating the reaction mixture up to 95–100°C, usually by submerging the reactor in boiling water for a short period of time (5–15 min). The mixture is then cooled down to room temperature and, sometimes, the pH value is adjusted in order to precipitate the indigested protein. The reaction mixture is then centrifuged and the supernatant is used for the determination of DH [30].

After the DH analysis, the supernatant is usually freeze-dried or spray-dried and the resulting protein hydrolysate powder is refrigerated (temperatures ranging from −18°C to 4°C). If NaCl is used in the hydrolysis, the supernatant may be dialyzed against water before drying [10]. The dried protein hydrolysate powder is then analyzed for an array of chemical and physical properties of interest, including the amino acid composition, the molecular weight distribution, and the bioactive and functional properties of the peptides.

3. Composition and Bioavailability of Cucurbitaceae Seed Protein Hydrolysates

Bioactive and functional properties of proteins, their isolates, and their hydrolysates are known to depend on their composition in terms of peptides and amino acids [28]. Crucially, the peptide bond hydrolysis improves the bioactivity of protein hydrolysates compared to their parent proteins, mainly due to the generation of bioactive peptides [31, 32]. Bioactive peptides can range in size from 2 to 20 or even 50 amino acid residues [41–44] but generally do not exceed 6–12 amino acid residues (<0.8–1.5 kDa, [29, 45]). The bioavailability of these peptides in target tissues depends directly on their molecular size, which greatly affects their absorption across

the enterocytes [32]. Low-molecular weight peptides (bi- and tripeptides) can be absorbed intact and hydrolyzed later, whereas other oligopeptides (4–10 amino acid residues) and polypeptides (>10 amino acid residues) are usually hydrolyzed before being absorbed in the intestinal mucosa as free amino acids [41] which causes their bioactivity to drop dramatically [44]. Therefore, the intensity of peptide bioactivity is usually inversely correlated to the peptide length [44]. However, some of the larger peptides are able to resist degradation by digestive enzymes [41]. This depends on their amino acid composition and location within the sequence of amino acids that form the peptide [32, 34, 40].

Importantly, very few bioactive peptides from any vegetal and animal sources have been studied *in vivo* despite the years of extensive *in vitro* research. Thus, not much evidence exists in favor of bioactivity of these peptides in humans. As for *in vivo* studies of peptides from Cucurbitaceae seed protein hydrolysates in humans, none seem to exist at the moment. This is mainly due to the fact that Cucurbitaceae seed protein hydrolysates have yet to be thoroughly characterized in terms of their peptide composition and first studied *in vivo* in laboratory animals, such as rats.

Molecular weights of most abundant peptide fractions found in Cucurbitaceae seed protein hydrolysates by means of SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) can be consulted in Table 1. The existing research on Cucurbitaceae seed protein hydrolysates as a potential source of bioactive peptides has mostly focused on bioactive and functional properties of the hydrolysates as a whole, consisting of several molecular weight fractions at once (Table 1). However, a few particular polypeptides and oligopeptides with different bioactive properties *in vitro* have already been identified in Cucurbitaceae seed proteins (Table 2). As for low-molecular weight peptide fractions (<1.5 kDa) which could potentially represent a source of bioactive and bioavailable bi- and tripeptides, these were also reported by many of the revised research papers (Table 1). However, such low-molecular weight peptides have yet to be adequately extracted and isolated from the peptide mixtures and subsequently studied in terms of their bioactivity, both *in vitro* and *in vivo* [44, 46].

The amino acid profile of protein isolates and hydrolysates obtained from seeds of several Cucurbitaceae species is shown in Tables 3 and 4, respectively. It is evident that the analyzed Cucurbitaceae seed protein isolates and hydrolysates contain a majority of essential and nonessential amino acids. Thus, Cucurbitaceae seed protein isolates and hydrolysates can be considered high-quality nutritional sources since they meet most nutritional requirements for body performance in terms of amino acids [17, 24]. Moreover, there is evidence for a direct link between several amino acids (Asp, Glu, Pro, Arg, His, Met, Leu, Ile, Ala, Tyr, and Val) and the antioxidant properties of protein hydrolysates [24, 47]. However, the absorption of amino acids in form of short peptide chains (di- and tripeptides) seems to be more efficient than the absorption of an equivalent amount of free amino acids, mainly due to the presence of peptide-specific transport systems and the subsequent peptide digestion into amino acids within the enterocytes [29].

4. Bioactive Properties of Cucurbitaceae Seed Protein Isolates and Hydrolysates

4.1. Antioxidant Activity. The determination of antioxidant activity in Cucurbitaceae seed protein usually comprises different *in vitro* measures, such as radical scavenging activity (for DPPH, $ABTS^+$, and O_2^- radicals), reducing power (of Fe^{3+} to Fe^{2+}), and metal chelating activity (Fe^{2+}), among others [48, 49].

Outstanding *in vitro* antioxidant activity has been reported for the globulin fractions (cucurbitin) of watermelon *(Citrullus lanatus)* [50] and pumpkin *(Cucurbita moschata)* [51] seed protein. Also, antioxidant effects of pumpkin *(Cucurbita pepo)* seed protein isolate have been reported *in vivo* in CCl_4 induced liver injury in rats [52]. As for Cucurbitaceae seed protein hydrolysates, their antioxidant activity seems to be improved in comparison to unhydrolyzed seed protein [17]. However, it may depend on various hydrolysis-related factors, such as the type of enzyme used for the hydrolysis, the DH, and the molecular weight of the resulting peptides.

Vaštag et al. [22] studied the antioxidant activity of Alcalase and Pepsin hydrolysates of cucurbitin obtained from pumpkin (*Cucurbita pepo* L. c. v. "Olinka") seed oil cake. The use of different enzymes for the hydrolysis did not significantly affect the ABTS radical cation scavenging activity (3.3–3.4 mmol/l TEAC for both Alcalase and Pepsin hydrolysates). However, the Alcalase hydrolysate proved to have almost twofold higher reducing power than the Pepsin hydrolysate ($A_{700\,nm}$ of about 0.25 and about 0.14, resp.). The authors hypothesize this was due to the Alcalase hydrolysate containing more peptides or amino acids able to donate electrons in order to react with free radicals and form more stable products in comparison to the Pepsin hydrolysate.

Similarly, Popović et al. [10] hydrolyzed cucurbitin extracted from pumpkin *(Cucurbita pepo)* oil cake with three different enzymes (Alcalase, Pepsin, and Flavourzyme) and investigated the antioxidant activity (ABTS radical cation scavenging activity and reducing power) of the cucurbitin hydrolysates. Measured by both antioxidant tests employed, the Alcalase and Pepsin hydrolysates showed good antioxidant activity while the Flavourzyme hydrolysates did not present any significant antioxidant activity. Interestingly, Alcalase and Pepsin hydrolysates were also reported to have the highest DH and the lowest molecular weight out of the three enzymatic hydrolysates studied (Table 1).

Vaštag et al. [26] observed the radical scavenging activity and the reducing power of both the Alcalase and Flavourzyme hydrolysate of protein isolate obtained from pumpkin (*Cucurbita pepo* L. c. v. "Olinka") seed oil cake. As for the highest values of radical scavenging activity, the Alcalase hydrolysate (60 min, DH 53.23%) had significantly higher ABTS radical cation scavenging activity (7.59 ± 0.08 mM TEAC/mg) than both the Flavourzyme hydrolysate (60 min, DH 37.17%; 3.27 ± 0.19 mM TEAC/mg) and the sequential use of Alcalase and Flavourzyme (at 90 min, DH 60.94%; 4.75 ± 0.05 mM TEAC/mg and at 120 min, DH 69.29%; 4.71 ± 0.05 mM TEAC/mg). Moreover, the reducing power was not significantly affected by the sequential use

Table 2: Polypeptides and oligopeptides with different bioactive properties *in vitro* identified in Cucurbitaceae seed proteins.

Source	Sequence	Molecular weight (kDa)	Properties	Reference
Pumpkin *(Cucurbita maxima)*	KRDPDWRREQEERREQERRREQQQQRREEQQRGER	12.33	Antifungal	Vassiliou et al. [35]
	YTRGGRGGWKGGGGGKGGGGGKGGGGG	2.34		
Pumpkin (*Cucurbita moschata* cv. black pumpkin)	PQRGEGGRAGNLLREEQEI	~9	Antifungal	Wang and Ng [36]
Momordica cochinchinensis	GCEGKQCGLFRSCGGGCRCWPTVTPGVGICSSS	3.29	Anticarcinogenic	Chan et al. [37]
	GCEGKPCGLFRSCGGGCRCWPTVTPGVGICSS	3.17		
Benincasa hispida	SDYLNNNPLFPRYDIGNVELSTAYRSFANQKAPGRLNQNWALTADYTYR	5.75	Antimicrobial and antioxidant	Sharma et al. [38]
Bitter melon *(Momordica charantia)*	FREKVYNIPL	1.28	ACE-inhibitory activity	Priyanto et al. [33]
	VSGAGRY	0.71		
	ITLPYSGNYER	1.31		
	IAAGKPREKIP	1.18		
	IAAGKPREKIPIGLPA	1.63		
	LLHYDSTAAAGALLVLIQTTAEAAR	2.57		
	LHYDSTAAAGALLVLIQTTAEAAR	2.46		
	LAQGNNGIFRTPIVL	1.61		
	IFRTPIVL	0.96		
	VDNKGNR	0.80		
	FFKESPPEA	1.05		
	FFKESPPEAYN	1.33		
	IISLENQWSA	1.16		
	FRNPVDL	0.86		
	QASESLN	0.75		
	LRYDDGWM	1.05		
	IVLSSATDKGNGQQWT	1.70		

Table 3: Amino acid composition (g/100 g) of Cucurbitaceae seed protein isolates.

Amino acid		Seinat (*Cucumis melo* var. *tibish*) Siddeeg et al. [25]	Bitter melon *(Momordica charantia)* Horax et al. [18]	Watermelon *(Citrullus vulgaris)* Jyothi lakshmi and Kaul [39]	Watermelon *(Citrullus vulgaris)* cv. Mateera Wani et al. [12]	Watermelon *(Citrullus vulgaris)* cv. Sugar baby Wani et al. [12]	Pumpkin (*Cucurbita pepo con. Pepo* var. *styriaca*) Nourmohammadi et al. [24]
Essential amino acid	Histidine (His)	2.21	3.71	2.46	1.86	2.15	1.83
	Threonine (Thr)	2.87	1.6	3.47	3.49	3.17	1.88
	Valine (Val)	4.42	—	2.91	—	—	3.35
	Methionine (Met)	2.47	2.75	0.33	0.97	0.88	—
	Phenylalanine (Phe)	5.30	4.11	5.18	5.36	5.83	4.22
	Isoleucine (Ile)	3.40	3.27	1.44	5.17	5.21	2.88
	Leucine (Leu)	6.27	6.57	7.44	7.09	7.19	5.33
	Lysine (Lys)	2.76	8.49	2.18	3.21	2.92	2.65
	Tryptophan (Try)	—	—	1.07	0.96	1.17	—
Nonessential amino acid	Tyrosine (Tyr)	2.86	4.51	2.85	3.89	3.96	2.32
	Cysteine (Cys)	5.91	1.27	0.96	6.31	6.09	—
	Aspartic acid (Asp)	6.72	8.54	9.48	9.14	10.39	7.22
	Glutamic acid (Glu)	14.48	14.8	20.71	17.69	16.75	14.28
	Serine (Ser)	3.93	4.17	5.91	4.87	4.96	3.97
	Glycine (Gly)	3.86	4.13	5.82	4.86	5.04	4.23
	Arginine (Arg)	12.42	10.1	16.89	14.53	15.21	10.65
	Proline (Pro)	—	4.56	—	4.21	3.95	—
	Alanine (Al)	—	4.17	—	4.89	5.05	3.14

Table 4: Amino acid composition (g/100 g) of Cucurbitaceae seed protein hydrolysates.

Amino acid		Pumpkin *(Cucurbita moschata)*, Alcalase Venuste et al. [17]	Pumpkin *(Cucurbita moschata)*, Flavourzyme Venuste et al. [17]	Pumpkin *(Cucurbita moschata)*, Protamex Venuste et al. [17]	Pumpkin *(Cucurbita moschata)*, Neutrase Venuste et al. [17]	Pumpkin (*Cucurbita pepo con. Pepo* var. *styriaca*), Alcalase Nourmohammadi et al. [24]	Seinat (*Cucumis melo* var. *tibish*), Trypsin + Pepsin Siddeeg et al. [25]
Essential amino acid	Histidine (His)	1.37	1.43	1.17	1.6	1.67	2.06
	Threonine (Thr)	1.88	1.59	1.74	1.77	2.01	2.64
	Valine (Val)	3.09	2.55	2.55	3.57	3.45	3.63
	Methionine (Met)	2.34[x]	1.99[x]	1.76[x]	2.38[x]	—	1.96
	Phenylalanine (Phe)	6.02[y]	4.61[y]	4.82[y]	6.2[y]	4.29	4.39
	Isoleucine (Ile)	2.72	3.00	2.57	2.73	3.10	3.36
	Leucine (Leu)	4.7	3.7	4.43	4.87	5.64	5.32
	Lysine (Lys)	2.44	2.2	2.58	2.49	3.00	1.78
	Tryptophan (Try)	0.52	0.77	0.15	0.16	—	—
Nonessential amino acid	Tyrosine (Tyr)	—	—	—	—	2.88	2.25
	Cysteine (Cys)	—	—	—	—	—	7.40
	Aspartic acid (Asp)	6.47	5.47	5.72	6.44	7.56	6.79
	Glutamic acid (Glu)	15.89	15.09	16.18	16.87	17.19	12.00
	Serine (Ser)	3.74	3.39	3.72	3.79	4.04	2.98
	Glycine (Gly)	3.86	4.28	3.92	4.06	3.95	4.08
	Arginine (Arg)	11.6	11.82	11.78	13.65	—	9.68
	Proline (Pro)	2.04	1.84	1.83	2.33	13.60	—
	Alanine (Al)	3.41	2.70	2.81	3.11	3.47	—

x: methionine + cysteine; y: tyrosine + phenylalanine.

of Alcalase and Flavourzyme when compared to the use of Alcalase only (the maximum values of $A_{700\,nm}$ oscillated between 0.33 ± 0.03 and 0.38 ± 0.04). The authors attributed the results to the specificity of the enzymes used for the hydrolysis, since each enzyme hydrolyzes proteins to result in a diverse composition of the hydrolysates, the Alcalase, and the sequential hydrolysate consisting of peptides with lower molecular weight than the Flavourzyme hydrolysate (Table 1). They also concluded that using Alcalase for the hydrolysis of pumpkin *(Cucurbita pepo)* seed protein might be the best option to improve the antioxidant activity of the cucurbitin hydrolysate.

Nourmohammadi et al. [24] hydrolyzed pumpkin oil cake (*Cucurbita pepo* var. *Styriaca*) protein isolate with Alcalase and Trypsin and used response surface methodology in order to suggest optimal treatment conditions in terms of DPPH radical scavenging activity. A sample hydrolyzed with 2% Alcalase at 50°C for 3.5 h (DH 28.0 ± 0.7, peptide size < 6.5 kDa) would achieve 90% DPPH radical scavenging activity whereas, for 1% Trypsin, 35°C and 5 h of treatment (DH 23.0 ± 0.5) were suggested as the best conditions, achieving 78% DPPH radical scavenging activity. Moreover, the Alcalase hydrolysate exhibited significantly better total antioxidant properties and metal chelating activity than the Trypsin hydrolysate (exact values not reported). Thus, the authors concluded that for this specific Cucurbitaceae seed protein isolate, Alcalase hydrolysis is the optimal treatment in order to achieve best antioxidant properties. They speculated that both the enzyme specificity and the hydrolysis conditions determine the size, type, and composition of free amino acids and peptides in the final hydrolysate which, in turn, directly affects its antioxidant properties. Moreover, they suggest that the lower the molecular size of the resulting peptides in the hydrolysate, the higher its antioxidant activity.

However, seed proteins from different Cucurbitaceae families seem to work best with different enzymes. For example, Arise et al. [23] hydrolyzed watermelon (*Citrullus lanatus* L.) seed protein isolates with Pepsin, Trypsin, and Alcalase and determined the antioxidant activity of the hydrolysates (reducing power and superoxide anion radical scavenging activity). The highest ferric-reducing ability was attained by the Trypsin hydrolysate (exact values not reported), whereas the Pepsin hydrolysate showed the highest O_2^- radical scavenging activity (IC_{50} of 2.414 mg/mL, compared to 2.824 mg/mL for Trypsin and 3.205 mg/mL for Alcalase). Therefore, Alcalase does not seem to be the most suitable enzyme for the hydrolysis of this particular protein.

The antioxidant activity also seems to depend on the DH of the hydrolysate and on the molecular weight distribution of the peptides in the hydrolysate. In general, the higher the DH and the smaller the size of the peptides, the better the antioxidant activity of Cucurbitaceae seed protein hydrolysates. In Popović et al. [10], both the Pepsin and Alcalase hydrolysates had better ABTS radical cation scavenging activity and reducing power at higher DH values and lower molecular weights. Similarly, Vaštag et al. [26] observed that the radical scavenging activity and the reducing power of both the Alcalase and Flavourzyme hydrolysate were dependent on their DH and the size of the resulting peptides. However, while the radical scavenging activity of both hydrolysates increased with increasing DH, the reducing power decreased with increasing DH, except for the Alcalase hydrolysate whose reducing power increased with the DH but only up to DH of 40%.

Moreover, Siddeeg et al. [25] reported a similar trend when studying the effects of the hydrolysis time on the antioxidant activity of hydrolysates from seinat (*Cucumis melo* var. *tibish*) seed protein isolates, using a sequential treatment by Trypsin and Pepsin (both E/S ratios 1/100 [w/w]). At various hydrolysate concentrations (1–5 mg/mL), the authors observed that the DPPH radical scavenging activity, reducing power, ABTS radical scavenging activity, and Fe^{2+} chelating activity all gradually increased with increasing DH (11.27% at 30 min to 28.23% at 180 min). As for the best values, DPPH radical scavenging activity (5 mg/mL of hydrolysate) increased from 58.83 to 78.0% with the increase of DH, as well as the ABTS radical cation scavenging activity which significantly improved from IC_{50} of 3.09 mg/mL to 2.25 mg/mL. Also, the highest percentage of ferrous chelating activity (71.13%) was found at the highest DH (28.23%) at the concentration 2.0 mg/mL. Interestingly, molecular weight distribution of the hydrolysates did not change dramatically over the course of the hydrolysis (30–180 min), although the authors mention slight changes towards lower molecular weights.

Since none of the *in vitro* methods revised in this section measure antioxidant activity using living cells, it would be desirable to carry out experiments of such nature before studying the antioxidant activity of Cucurbitaceae seed protein hydrolysates *in vivo*.

4.2. Antihypertensive (ACE Inhibitory) Activity. Treating hypertension with synthetic drugs with angiotensin-I converting enzyme (ACE) inhibitory activity can have undesirable side effects, so food-derived peptides with ACE inhibitory activity are considered to be a better alternative [42, 53]. Food-derived peptides are encrypted in proteins and are usually released during food ripening or fermentation but can also be prepared by means of *in vitro* enzymatic hydrolysis [54]. While Cucurbitaceae seed protein isolates do not seem to have a significant ACE inhibitory activity [26], Cucurbitaceae seed protein hydrolysates could be a valuable source of peptides with ACE inhibitory activity [22, 26, 33].

The ACE inhibitory activity of peptides depends on various factors. As for the peptides derived from Cucurbitaceae seed proteins, their ACE inhibitory activity seems to directly depend on their concentration: the higher the concentration of the peptide, the higher its ACE inhibitory activity [33]. Moreover, the ACE inhibitory activity of Cucurbitaceae seed protein hydrolysates which contain the peptides seem to be dependent on the type of enzyme used for the hydrolysis, on the DH, and on their molecular weight distribution [22, 26]. However, the results are inconclusive and more research is needed in this area.

Priyanto et al. [33] identified peptides with ACE inhibitory activity in bitter melon *(Momordica charantia)* seed protein enzymatic (thermolysin) hydrolysates (Table 2). The hydrolysates were fractionated by HPLC and an ACE inhibitory assay was carried out on the different fractions.

Crucially, a novel ACE inhibitory peptide (VY-7: VSGAGRY; molecular weight: 0.7 kDa) was found in momordin A proteic fraction (GI:157,832,029), having the best IC_{50} value of all identified peptides in the hydrolysate (8.64 ± 0.60 μM). *In vivo* (studied in spontaneously hypertensive rats), the bitter melon *(Momordica charantia)* seed protein hydrolysate (at 10 mg/kg of body weight) showed moderate antihypertensive effects which became very pronounced for the VY-7 peptide alone (at 2 mg/kg of body weight).

As for the type of enzyme used for the hydrolysis, Vaštag et al. [22] hydrolyzed pumpkin (*Cucurbita pepo* L. c. v. "Olinka") oil cake cucurbitin and found that both Alcalase and Pepsin hydrolysates proved to have ACE inhibitory activity (apparent IC_{50} values of 0.0244 mg and 0.0445 mg, resp.). However, Vaštag et al. [26] observed different ACE inhibitory activities for pumpkin oil cake (*Cucurbita pepo* L. c. v. "Olinka") protein isolate hydrolysates by Alcalase, Flavourzyme, and the sequential use of both enzymes. While both the unhydrolyzed pumpkin seed protein isolate and the Flavourzyme hydrolysate showed no ACE inhibitory activity, for the Alcalase and the sequential hydrolysate, the ACE inhibitory activity depended on the DH. The highest ACE inhibitory activity (71.05 ± 7.50%; EC_{50} 0.442 mg/mL) was observed for the Alcalase hydrolysate with DH of 53.23 ± 0.70%. Below this DH, the ACE inhibitory activity of the Alcalase hydrolysate was lower. However, the sequential use of both enzymes (Alcalase and Flavourzyme) also showed a lower ACE inhibitory activity (about 55%), despite a higher DH (69.29 ± 0.90). The authors speculate that the ACE inhibitory peptides that were liberated when the Alcalase hydrolysate reached molecular weights below 15 kDa were probably broken down by the subsequent use of Flavourzyme which, in turn, caused the drop in the ACE inhibitory activity.

4.3. Antihyperglycaemic (α-Amylase Inhibitory) Activity. In the management of type 2 diabetes, α-amylase inhibitors can delay the absorption of glucose since α-amylase is responsible for the breakage of starch into products with low-molecular weight, such as glucose and maltose [55]. Therefore, substances with α-amylase inhibitory activity can be found in many commercially available antihyperglycaemic drugs. However, Cucurbitaceae seed protein isolates and hydrolysates may also be an interesting source of compounds with such properties [56]. In Cucurbitaceae seed protein isolates, α-amylase inhibitory activity has been reported for *in vivo* studies [57]. However, Cucurbitaceae seed protein isolates do not seem to possess α-amylase inhibitory activity *in vitro* and for Cucurbitaceae seed protein hydrolysates there are only a few *in vitro* studies of α-amylase inhibitory activity [22, 23].

Vaštag et al. [22] hydrolyzed pumpkin (*Cucurbita pepo* L. c. v. "Olinka") oil cake cucurbitin and found that both Alcalase and Pepsin hydrolysates showed modest α-amylase inhibitory activity (<30%), the Pepsin hydrolysate showing slightly higher α-amylase inhibition than the Alcalase hydrolysate at all studied protein concentrations (0.5-2.0 mg/mL). However, Arise et al. [23] reported potent α-amylase inhibition (beyond 50%) for Pepsin, Trypsin, and Alcalase hydrolysates of watermelon (*Citrullus lanatus* L.) seed protein isolates at all studied protein concentrations (0.5–2.0 mg/mL). The Alcalase hydrolysate showed the strongest α-amylase inhibition, followed by the Trypsin and the Pepsin hydrolysate. The authors speculate that the strong α-amylase inhibition of Alcalase and Trypsin hydrolysates may be due to the enzymes creating specific (cationic and/or branched) amino acid residues. However, both Alcalase and Pepsin hydrolysates achieved a significantly lower IC_{50} in comparison to the Trypsin hydrolysate (0.149, 0.165, and 0.234 mg/mL, resp.).

As mentioned before, neither Vaštag et al. [22] nor Arise et al. [23] reported molecular weight distribution of peptides in the hydrolysates. This kind of information could be very valuable for linking the α-amylase inhibitory activity of the hydrolysate to peptides of particular size and structure.

5. Functional Properties of Cucurbitaceae Seed Protein Isolates and Hydrolysates

5.1. Protein Solubility. Solubility can influence other functional and bioactive properties of protein isolates and their hydrolysates and thus, it is considered one of the most important characteristics of protein [43, 58].

Protein solubility depends on the surface hydrophobic-hydrophilic balance of the protein and on the protein-protein and protein-solvent interactions [59]. These interactions are caused by charged, polar, and nonpolar groups of amino acid residues present on the surface of the protein [60]. Therefore, the solubility of Cucurbitaceae seed protein isolates and hydrolysates is highly dependent on the pH value of the solution. As a result of this, a pH solubility profile of Cucurbitaceae seed protein isolates and hydrolysates is often carried out by determining the protein solubility at different pH values. The Cucurbitaceae seed protein isolates and hydrolysates are dissolved in an aqueous solution, being occasionally stirred. After a certain amount of time (60 min), the mixture is centrifuged and the amount of protein that has dissolved is determined in the supernatant. The protein solubility is then expressed as a percentage of the dissolved protein to the total protein [w/w].

A typical pH solubility profile of Cucurbitaceae seed protein isolates is a U-shaped curve [10] on which the solubility of the protein reaches minimum values around its isoelectric point (pI). In Cucurbitaceae seed protein isolates, the pI usually corresponds to slightly acidic pH values (4-5) and a relatively low solubility of the protein isolate (0–15%). However, below pH 3 and above pH 5, the solubility of the protein isolates drastically improves, reaching the highest values at extreme pH values [18, 19, 61].

Wani et al. [19] carried out a pH solubility profile of watermelon *(Citrullus lanatus)* seed protein isolate (pH 1–12). The authors reported minimum solubility at the pH value of 4.0 (10.45–15.21%) and the highest solubility values (above 95%) were achieved at the pH values of 11 and 12. According to the authors, improved solubility of the watermelon seed protein isolate in the alkali region might be due to the higher amounts of aspartic and glutamic acid in the protein isolates. Similarly, Horax et al. [18] studied a pH solubility profile of bitter melon *(Momordica charantia)* seed protein isolate

(pH 2–10). They observed the lowest protein solubility at pH values of 4.5–5.0 and the maximum protein solubility was reached at pH 2 (>80%) and in the alkali region of pH 7–10 (62.0–67.5%). The authors suggested that lower solubility of bitter melon *(Momordica charantia)* seed protein isolate in the alkali region may be due to a relatively low content of charged residues, such as aspartic or glutamic acid.

Additionally, Bučko et al. [61] investigated the effect of pH, ionic strength, and suspension concentration of pumpkin *(Cucurbita pepo)* seed protein isolate on its solubility. As for the influence of pH in the studied range of values (pH 3–8), the lowest solubility was observed at pH 5 (11% [w/v]) and the highest solubility at pH 8 (68% [w/v]). Furthermore, the influence of suspension concentration of protein isolate on its solubility was studied at different pH values (3, 5, and 8). Regardless of the suspension pH value, the authors observed an increase in the concentration of dissolved proteins with increasing suspension concentration. However, the solubility yield (the concentration of the dissolved protein to the suspension concentration) actually decreased with increasing suspension concentration but only at pH values of 3 and 8. At pH 5, suspension concentration had no effect on the solubility yield of the dissolved protein. As for the influence of the ionic strength on the solubility of the protein, slight salting-in effects (an increase in solubility) were observed at pH 5 and 8 while also moderate salting-out effects (a decrease in solubility) occurred at pH 3.

Importantly, enzymatic hydrolysis introduces a series of changes to the solubility of Cucurbitaceae seed protein isolates. During hydrolysis, the protein is degraded into smaller peptides and, in general, its solubility increases, which can be especially evident near the pI of the original protein [31]. However, this might not be the case at extreme pH values (especially above pH 9) where the solubility of the unhydrolyzed protein may maintain higher values than the solubility of the enzymatic hydrolysates [10].

The differences in solubility of the Cucurbitaceae seed protein isolates and hydrolysates can be attributed to the reduction of secondary structure of the original protein during the hydrolysis, which exposes ionizable amino and carboxyl groups and, in turn, increases the hydrophilicity of the peptides present in the hydrolysates [10, 27]. As a result of enzymatic hydrolysis, the overall influence of the pH on the solubility of the protein is attenuated and both the characteristic U-shaped pH profile and the pI can be lost for the hydrolysates [10, 27].

Bučko et al. [27] studied the influence of enzymatic (Alcalase and Pepsin) hydrolysis of pumpkin *(Cucurbita pepo)* seed protein isolate on protein solubility at different pH values, ionic strengths, and suspension concentrations. Both hydrolysates had the same DH (19%) and significantly higher solubility in comparison to the unhydrolyzed protein isolate, previously characterized in Bučko et al. [61]. This was particularly obvious near the isoelectric point of the pumpkin seed protein isolate (pI = 5) where the solubility of the protein isolate, the Alcalase hydrolysate, and the Pepsin hydrolysate was 12%, 68%, and >90%, respectively. Over the whole pH range (pH 3–8), the solubility of the Alcalase hydrolysate followed a slightly increasing trend (in the range of 60–76%). On the contrary, the solubility of the Pepsin hydrolysate decreased over the whole pH range but stayed above 90% at all pH values. The effects of the ionic strength on the protein solubility were also modulated by the hydrolysis. The Alcalase hydrolysate was the least influenced by ionic strength when compared to the protein isolate and the Pepsin hydrolysate. However, the authors claim that the overall effect of ionic strength seems to be a result of various factors, such as electrostatic interactions, ion specific effects, and hydrophobic effects, among others.

Similarly, Popović et al. [10] studied the pH profile of cucurbitin from pumpkin (*Cucurbita pepo* L. c. v. "Olinka") oil cake and its enzymatic hydrolysates (Alcalase, Flavourzyme, and Pepsin). The solubility profile of cucurbitin was typically U-shaped, with high solubility at acidic and basic pH values and extremely low solubility in the pH range of 5–7 (0.05 mg/mL). Pepsin produced hydrolysates without a pI and with better solubility over the whole pH range, comparably to the results obtained by Bučko et al. [27]. However, Popović et al. [10] observed that Alcalase and Flavourzyme maintained the U-shaped pH solubility profile of the cucurbitin but reduced the pI to pH 5 (Alcalase) and pH 5-6 (Flavourzyme) in comparison to the unhydrolyzed protein (pH 5–7).

In addition to the aforementioned factors (pH, suspension concentration, and ionic strength), the solubility of the Cucurbitaceae seed protein hydrolysates also seems to depend on the DH, which in turn depends on the particular enzyme used for the hydrolysis. In general, the higher the DH of hydrolysis, the higher the solubility of the hydrolysate, as observed by Popović et al. [10]. The authors carried out the pH profile of cucurbitin and its enzymatic hydrolysates (Alcalase, Flavourzyme, and Pepsin) at different DH (14.6% and 29.8% for Alcalase, 5.6% and 9.2% for Flavourzyme, and 15% and 29% for Pepsin). They observed better solubility for the higher values of DH for all three enzymes in comparison to the lower values of DH and over the whole spectrum of pH values (2–11).

The solubility of protein hydrolysates may also directly depend on the time of hydrolysis. In theory, hydrolysis time should have the same effect as the DH, since longer hydrolysis times lead to higher DH. This was observed by Siddeeg et al. [25] who prepared Trypsin and Pepsin hydrolysates from seinat (*Cucumis melo* var. *tibish*) seed protein isolate at different times (30, 60, 90, 120, and 180 min). However, the authors also observed a decrease in solubility of the hydrolysates over hydrolysis time (instead of the expected increase). Moreover, they noted similar effects of the hydrolysis time on other functional properties, such as water and oil absorption capacities, emulsifying activity, and foaming capacity of the hydrolysates. This may point to possible adverse effects of long hydrolysis times on the quality of the final product. In fact, excessive hydrolysis of protein has been suggested to diminish the functional properties of the hydrolysate despite its increased solubility [28].

5.2. Emulsifying and Foaming Properties. Cucurbitaceae seed protein isolates and hydrolysates could be potentially used as functional food ingredients thanks to their balanced amino

acid composition and their bioactive properties. However, the incorporation of protein isolates and hydrolysates into foods requires certain functional properties. In addition to enhanced solubility, it is crucial to find out whether Cucurbitaceae seed protein isolates and hydrolysates possess favorable emulsifying and foaming properties. These functional properties seem to be closely related to the size of the peptides conforming the hydrolysate, with excessively small peptides affecting adversely the functionality of the hydrolysate [25].

In order to investigate the emulsifying properties of Cucurbitaceae seed protein isolates and hydrolysates, emulsions of a vegetable oil (sunflower, soy, or corn, at 20–25%) with the isolates or hydrolysates are prepared at a concentration of dissolved protein, then homogenized, and analyzed. The analysis can include measuring the absorbance at 500 nm, where the absorbance at 0 min time is the emulsifying capacity (EC) and the decrease in absorbance in time is the emulsion stability (ES), or measuring of the diameter of the emulsion droplets. As for the foaming properties of Cucurbitaceae seed protein isolates and hydrolysates, air is introduced to the protein solution in phosphate buffer. The foam volume at 0 min represents the foaming capacity (FC) and the foam stability (FS) is expressed as the decrease in the foam volume over time.

Emulsifying and foaming properties of Cucurbitaceae seed protein isolates have been studied in bitter melon *(Momordica charantia)* seed protein isolate [18] and in watermelon *(Citrullus lanatus)* seed protein isolate [19]. In these Cucurbitaceae seed protein isolates, emulsifying and foaming properties are normally assessed at pH values of about 7 [18, 19]. This is because these functional properties depend on protein solubility which, in turn, is pH dependent. However, Bučko et al. [61] investigated emulsifying properties of pumpkin seed *(Cucurbita pepo)* protein isolate at different pH values (3, 5, and 8) and ionic strengths (0 and 0.5 mol/dm^3 NaCl) of the emulsions. The emulsifying capacity and emulsion stability correlated with the results of protein solubility at the different pH values. This suggests that the principal limitation of Cucurbitaceae seed protein isolates is their low solubility at their pI and, thus, low functional properties at these pH values. Moreover, FC and FS have been reported to be relatively low in cucurbitin extracted from *Cucurbita maxima* pumpkin seed [62].

In comparison, functional properties of Cucurbitaceae seed protein hydrolysates seem to be improved over a range of pH values and ionic strengths, especially at the pI of the protein they are prepared from. Bučko et al. [27] investigated emulsifying properties of pumpkin *(Cucurbita pepo)* seed protein isolate and its hydrolysates (Alcalase and Pepsin) at different pH values (3, 5, and 8) and ionic strengths (0 and 0.5 mol/dm^3 NaCl) of the emulsions. While pumpkin seed *(Cucurbita pepo)* protein isolate failed to form emulsions at pH 5 at both ionic strengths studied and at pH 3 and ionic strength of 0.5 mol/dm^3 NaCl, both of its hydrolysates (Alcalase and Pepsin) showed improved emulsifying properties regardless of pH and ionic strength of the emulsion. These results suggest that enzymatic hydrolysis of Cucurbitaceae seed protein isolates can improve their functional properties over a wide range of pH values and ionic strength conditions. Importantly, Bučko et al. [27] reported the presence of relatively large peptides in all hydrolysates (14–20, 24, and 36 kDa) which could be responsible for the excellent emulsifying properties of the hydrolysates. On the other hand, small-sized peptides resulting from long hydrolysis times are less effective in creating stable emulsions than peptides with higher molecular size resulting from shorter hydrolysis times [31]. Such is the case of a study performed by Siddeeg et al. [25], who observed a decrease in both EC and FC of seinat (*Cucumis melo* var. *tibish*) seed protein hydrolysates over hydrolysis time (30, 60, 90, 120, and 180 min).

Another factor that influences the emulsifying and foaming properties of *Cucurbitaceae* protein hydrolysates is the DH. Popović et al. [10] reported an improved EC and ES for cucurbitin from pumpkin (*Cucurbita pepo* L. c. v. "Olinka") oil cake and its enzymatic hydrolysates (Alcalase, Flavourzyme and Pepsin) at pH 7 for DH up to 15%. However, DH of 29-30% negatively affected these emulsifying properties. These findings are in line with the previously mentioned effects of peptide molecular size on the functional properties of the hydrolysates. Popović et al. [10] observed more peptides with higher molecular weight (33 and 22 kDa) in hydrolysates with DH up to 15%, whereas these were practically absent in hydrolysates with DH over 15%. On the other hand, the foaming properties of the protein hydrolysates were improved independently of the DH. It is therefore possible that foaming properties of *Cucurbitaceae* protein hydrolysates are not as sensitive to excessive hydrolysis as emulsifying properties are.

6. Future Trends and Conclusions

For millennia, Cucurbitaceae seeds have been widely used both as a protein-rich food ingredient and a nutraceutical agent by many indigenous cultures. However, relatively little is known about the bioactive components of the Cucurbitaceae seed proteins and their specific effects on human health. Technological advances have made it possible to extract different protein fractions from Cucurbitaceae seeds in order for them to be further analyzed. Enzymatic hydrolysis *in vitro* has been implemented to simulate the breakdown of these proteins during digestion. Nevertheless, to evaluate the real effect that Cucurbitaceae seed protein isolates and hydrolysates might have on living organisms, it is necessary to validate the findings of *in vitro* studies by means of *in vivo* studies, both in laboratory animals and in humans. However, only a few *in vivo* studies in rats have been carried out with Cucurbitaceae seed protein isolates [52, 57]. As for Cucurbitaceae seed protein hydrolysates, there is a need for *in vivo* studies since none seem to exist at the moment.

Not many bioactive properties of Cucurbitaceae seed protein hydrolysates have been investigated beyond their antioxidant activity and ACE/α-amylase inhibitory activities. For example, some proteins and peptides isolated directly from the seeds have shown anticarcinogenic, antifungal, and antimicrobial properties [35–38, 63] (Table 2). However, these properties have not yet been studied in the Cucurbitaceae seed protein hydrolysates. As for the functional properties of Cucurbitaceae seed protein isolates and hydrolysates, their solubility has been investigated

thoroughly since all the other functional properties depend on it. However, information on their emulsifying and foaming properties is still very scarce. It would be particularly interesting to explore the direct relationship between the distribution of peptide molecular weight in the Cucurbitaceae seed protein hydrolysates and their bioactive and functional properties. The body of research reviewed in this paper seems to suggest that bioactive properties could be attributed to low-molecular weight peptides (<6.5–15 kDa) while enhanced functional properties could depend on the presence of larger peptides (>12–15 kDa) in the hydrolysates. However, there also seems to be only a small range of molecular weights that would guarantee the bioactive and functional properties of the hydrolysates. Therefore, future research should focus on purifying and characterizing the individual bioactive peptides within Cucurbitaceae seed protein hydrolysates. The obvious next step would be determining the bioavailability of these peptides, both in animals and in humans.

In order to benefit from oil industry byproducts, such as Cucurbitaceae seed oil cakes, finding cost-effective methods for Cucurbitaceae seed protein isolation and hydrolysis is a crucial step for the industrial implementation of such processes. Future research in this area should focus on applying hydrolysis pretreatments to Cucurbitaceae seed proteins and on the use of green technologies with the objective of reducing both the costs and the impact they may have on the environment. Moreover, the balanced amino acid composition and the bioactive properties of Cucurbitaceae seed protein isolates, hydrolysates, and peptides call for their use as functional food ingredients. However, studying such complex food systems is not the same as studying protein isolates, hydrolysates, or peptides on their own. Therefore, a lot of future research is needed in these areas.

Conflicts of Interest

César Ozuna and Ma. Fabiola León-Galván (the authors) declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

"Gold" Pressed Essential Oil: An Essay on the Volatile Fragment from *Citrus* Juice Industry By-Products Chemistry and Bioactivity

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Present essay explores the potentials of *Citrus* juice industry's by-products as alternative bioactive natural products resources. Four crude Cold Pressed Essential Oils (CPEOs), derived from orange, lemon, grapefruit, and mandarin, were studied. All CPEOs were subjected to water distillation, in order to obtain the volatile fragment, which was further fractionated with respect to distillation period in two parts, concluding to eight samples. These samples along with the four original CPEOs were assessed in relation to their phytochemical content and their repellent and larvicidal properties against Asian Tiger Mosquito. The volatiles recovery rates ranged from 74% to 88% of the CPEO. Limonene presented a significant increase in all samples ranging from 8% to 52% of the respective CPEO's content and peaked in mandarin's 2nd volatile fragment which comprised 97% of the essential oil. The refinement process presented clear impacts on both bioassays: a significant increase in larvicidal potency was observed, annotated best by the improvement by 1100% and 1300% of the grapefruit volatile fractions; repellence testing provided only one significant result, the decrease of landings by 50% as a response to mandarin's second volatile fraction. The applied methodology thus may be considered for the improvement of *Citrus* juice industry's by-products chemistry and bioactivity.

1. Introduction

Among agricultural commodities and the consequent industries, *Citrus* fruits hold a significant position. According to Food and Agricultural Organization [1], global *Citrus* fruit production in the year 2014 reached approximately 140 Mt, 60% of which were oranges. Worldwide, it is estimated that annually over 30% of *Citrus* fruits produced (40% of oranges) are being processed by the food industry to produce mainly juice based products. This endeavor is generating a considerably high amount of by-products that can be potentially used as a biorefinery raw material [2].

The residues of citrus juice production consisted of peel, pulp seeds, and whole citrus fruits that do not meet the quality requirements, while only 50% of the fresh fruit's mass is transformed into juice [3]. In general, these citrus juicing by-products have an insignificant economic value, even though they are rich in soluble sugars, cellulose, hemicellulose, pectin, flavonoids, and essential oils [2, 4]. Among them the Cold Pressed Essential Oil (CPEO) is widely used by the food, beverage, cosmetic, and pharmaceutical industries as flavoring and fragrance agent due to its characteristic aroma profile [5]. This utilization as industrial raw materials has created a market for these CPEOs in which their price escalation is 6 to 7€ per kg for grapefruit; 8 to 12€ per kg for orange; 12 to 15€ per kg for mandarin; 30 to 35€ per kg for lemon [6]. CPEOs consisted of volatile and nonvolatile fractions that are composed of more than 200 compounds [7]. The volatile fraction, which represents an 85% to 99% of the CPEO, is well characterized in the literature

Table 1: The four *Citrus* fruits samples *taxon* attribution, definition, and recovery yield (CPEO = Cold Pressed Essential Oil; F1 = fragment 1 (first 15 min); F2 = fragment 2 (consequent 2 h and 45 min). *Volume of the respective CPEO. **In the case of CPEO, the industrial figure corresponds to % W/W of produced juice [6]).

Taxon	Code	Sample definition	Volume distilled*, recovered (mL)	Yield (% V/V)**
C. paradisi	C 01	CPEO	50,0	0,4–0,6%
	C 02	F1	23,2	46,4%
	C 03	F2	18,5	37,0%
		Total vol. fr.	41,7	83,4%
C. limon	C 04	CPEO	20,0	0,4–0,6%
	C 05	CPEO vol. fr. 1	8,3	41,5%
	C 06	CPEO vol. fr. 2	6,5	32,5%
		Total vol. fr.	14,8	74,0%
C. reticulata	C 07	CPEO	25,0	0,4–0,6%
	C 08	CPEO vol. fr. 1	10,8	43,2%
	C 09	CPEO vol. fr. 2	10,1	40,4%
		Total vol. fr.	20,9	83,6%
C. sinensis	C 10	CPEO	25,0	0,4–0,6%
	C 11	CPEO vol. fr. 1	12,0	48,0%
	C 12	CPEO vol. fr. 2	10,0	40,0%
		Total vol. fr.	22,0	88,0%

[8]. Phytochemicals commonly found in *Citrus* CPEOS are monoterpene and sesquiterpene hydrocarbons, their oxygenated derivatives and aliphatic aldehydes, alcohols, and esters. The main volatile compound of *Citrus* essential oils is D-limonene, a nonoxygenated monoterpene derived by the combination of two isoprene units [6]. The CPEOs obtained as by-products from the *Citrus* juice industry are recovered in yields ranging from 0,4 to 0,6 mL per kg [6]. However, CPEOs are characterized by high percentages of nonvolatile residues, which contain hydrocarbons, sterols, fatty acids, waxes, carotenoids, coumarins, psoralens, and flavonoids [8].

D-Limonene is one of the world's most widespread terpenes constituting up to 90–95% of the orange peel oil and 75% of lemon peel oil. The worldwide annual production of D-limonene is over 70 million kg and rising fast [9]. In addition to its flavor and fragrance properties, this phytochemical is used in a broad variety of consumer products due to its physicochemical properties. For example, D-limonene is being used as a nontoxic solvent in oleochemical, wax, resin, paint, and glue industrial preparations or as a valuable renewable biosolvent, an alternative to hazardous petroleum solvents [10]. Another major application of D-limonene is its application as a cleaning agent replacing various environmentally unfriendly cleaning agents such as toluene, hexane, and chlorinated organic solvents [11]. Due to its low price, D-limonene is an attractive starting compound for the biotechnological production of industrially relevant fine chemicals and flavor compounds with identical carbon skeletons, such as carveol, carvone, and perillyl alcohol [12].

Herein we present the outcome of our study concerning the sustainable valorization of the *Citrus* juice industry CPEO by-products incorporating a binary approach. This biorefinery method focuses on the retrieval and simultaneous fragmentation of the CPEOs volatile fraction aiming at the increase of the (a) content of D-limonene, a fine chemical with distinct exploitation potentials and (b) bioactivity of the processed CPEO volatile fragments. As a target organism for the bioactivity assessment, the Asian Tiger Mosquito was chosen, *Aedes (Stegomyia) albopictus* (Skuse 1894) (Diptera: Culicidae), which is currently considered as the most invasive mosquito species in the world [13] with great public health importance since it is a confirmed vector of Yellow Fever [14], dengue fever [15], Chikungunya fever [16], and Zika [17] viruses.

2. Materials and Methods

2.1. Materials. The original material of the study consisted of the CPEOs derived from the industrial processing [18] of the following four different *Citrus* species, (a) Orange, *Citrus sinensis* (L.) Osbeck; (b) Lemon, *C. limon* (L.) Osbeck; (c) Grapefruit, *C. paradisi* Macfad.; (d) Mandarin, *C. reticulata* Blanco, which were kindly provided by the industry of fruit juices Christodoulou Bros SA. All sampling details are included in Table 1.

2.2. Methods

2.2.1. Isolation and Fragmentation of the CPEO Volatile Fraction. The four-industrial CPEOs were subjected to conventional hydrodistillation. All distillations were performed with 3 L of H_2O in a modified Clevenger apparatus for 3 hours. Four EOs were obtained as the initial fragments (first 20 min) of the hydrodistillation of these industrial by-products and another four were isolated from the remaining fragments (consequent 2 h and 40 min). The essential oil yields are included in Table 1.

2.2.2. Gas Chromatography-Mass Spectrometry (GC/MS). The GC/MS analyses were performed on a Agilent Technologies 7890A Gas Chromatograph, equipped with a HP 5MS 30 m × 0.25 mm × 0.25 μm film thickness capillary column, connected with an Agilent 5957C, VL MS Detector with Triple-Axis Detector system operating in EI mode, and He as the carrier gas (1 mL/min). The initial column temperature was 60°C and heated gradually to 280°C with a 3°C/min rate. The identification of the compounds was based on comparison of their Retention Indices (RI) obtained, using various n-alkanes (C_9–C_{24}) and their EI-mass spectra were compared with the NIST/NBS, Wiley libraries spectra, and literature [19, 20]. Additionally, the identity of the indicated phytochemicals was confirmed by comparison with available authentic samples. All authentic samples utilized for the identification of EOs compounds were obtained from Sigma-Aldrich, except for germacrene D and α-thujene, which had been isolated in the context of previous studies.

2.2.3. Rearing Ae. albopictus in the Laboratory. Larvae and adults of *Ae. albopictus* were obtained from a laboratory colony which was maintained at 25 ± 2°C, 80% relative humidity and photoperiod of 16 : 8-h light/dark (L/D), in the laboratory of the Benaki Phytopathological Institute, Kifissia, Greece. The adults were kept in wooden frame cages (33 × 33 × 33 cm) with a 32 × 32 mesh, with easy access to 10% sucrose solution on a cotton stick. Females were fed with fresh chicken blood with Hemotek© blood feeding system. The larvae were reared in tap water-filled cylindrical enamel pans with diameter of 35 and 10 cm deep covered by fine muslin. Approximately 400 larvae were fed in excess with powdered fish food (JBL Novo Tom 10% Artemia) in each pan until the emergence of adults. Adult mosquitoes were often collected with a mouth aspirator and transferred to the rearing cage. Plastic beakers with 100 ml water and strips of moistened filter paper were provided in the cage for oviposition. The eggs were kept damp for a few days and then placed in the pans for hatching [21].

2.2.4. Larvicidal Bioassays. The larval mortality bioassays were carried out according to the test method of larval susceptibility as recommended by the World Health Organization [22] with modifications. Sufficient amounts of each compound were transferred to a vial and the residual solvent was removed under high vacuum. Stock solutions of 10% (w/v) in dimethyl sulfoxide (DMSO) were prepared for each testing material. Twenty late third-to early fourth-instar mosquito larvae were placed in 2% (v/v) aqueous solution of DMSO (98 ml of tap water plus 2 ml of DMSO), followed by the addition of 29 μl of the tested material solution. The aqueous solutions were then gently shaken for homogenization [21]. Five replicates per dose were tested and a treatment with 98 ml of tap water and 2 ml of DMSO was included in each bioassay as the control. The mortality rates of the essential oils tested were arbitrary classified to "low," "moderate," and "very good" if the mortality rates ranged between 0–50%, 50–80%, and 80–100%, respectively.

2.2.5. Repellent Activity Bioassay. For the repellent activity of the essential oils, the assessment was based on the human landing counts [23, 24]. The study was conducted into a cage (33 × 33 × 33 cm) with a 32 × 32 mesh and with a 20 cm diameter circular opening fitted with cloth sleeve. Each cage contained 100 adult mosquitoes (sex ratio, 1 : 1), 5 to 10 days old, starved for 12 h at 25 ± 2°C, and 70–80% relative humidity. A plastic glove with an opening measuring of 5 × 5 cm was employed for all the bioassays. Different doses (from 0.05 to 1 μlcm^{-2}) for DEET were applied and it was found that the lowest dose, where zero landings were counted, was ≈0.2 μlcm^{-2}. All testing materials were applied on paper (Whatman chromatography paper) of 24 cm^2 total area and tested at two doses: 50 μl ("low," ≈0.2 μlcm^{-2} of testing material) and 100 μl ("high," ≈0.4 μlcm^{-2} of testing material) of 100 μgμl^{-1} stock solution. Control treatments without the components and with DEET were also included for the repellency tests as standards (control and positive control, resp.). Each treatment was repeated eight times and four human volunteers were used [21, 25].

2.2.6. Data Analysis. Larvicidal effect was recorded 24 h after treatment. Data obtained from each dose-larvicidal bioassay (total mortality, mgl^{-1} concentration in water) were subjected to probit analysis in which probit-transformed mortality was regressed against $\log_{10}$-transformed dose; LC_{50}, LC_{90} values and slopes were calculated (SPSS 11.0).

Data concerning the repellency of the samples (mosquito landings) were analyzed using Kruskal-Wallis test. When significant differences were detected, Mann-Whitney U tests were carried out for pairwise comparison. Bonferroni correction was applied to correct for 66 pairwise comparisons leading to an adjusted $a = 0.0006$ [25].

3. Results

The volatile fractions of the four *Citrus taxa* presented essential oil yields and recovery rates as percent of the relative industrial CPEO, which are included in Table 1. From the four CPEOs studied orange exhibited the highest volatile recovery rate (0,880), whereas lemon exhibited the lowest (0,740). In all cases, the essential oil yields were higher during the first 15 min of the hydrodistillation (fragment 1) of the CPEOs.

3.1. Phytochemical Assessment. The detailed qualitative and quantitative analytical data of the main volatile constituents of the CPEOs have been summarized in Table 2. It must be noted that 30 different phytochemicals, representing 73.89% to 99.98% of the respective samples were identified by combined GC and GC/MS analyses as constituents of the samples studied. The investigated samples were found to contain mainly monoterpenes, mostly cyclic, and only occasionally aliphatic. More specifically, particularly, in the case of orange, grapefruit, and mandarin, D-limonene was by far the most abundant component (up to 97.79%), while compounds like myrcene and α-pinene where found in lower percentages. On the contrary, in the case of lemon EOs, D-limonene was found in lower percentages (up to 56.50%)

Table 2: *Citrus* samples compounds in percentage of total composition.

Compounds	RI	C 01	C 02	C 03	C 04	C 05	C 06	C 07	C 08	C 09	C 10	C 11	C 12	Identification
α-Thujene	930				0,6	1,3								a, b, c
α-Pinene	939	0,8	0,9		2,3	5,1	2,1	0,8	1,7		0,9	1,3	0,9	a, b, c
Sabinene	975	0,9	1,2						1,2				0,8	a, b, c
β-Pinene	976				10,5	19,2	13,7							a, b
Myrcene	991	2,6	2,9	1,8	2,0	3,7	1,9	2,7	4,3	2,2	3,1	3,6	4,1	a, b, c
D-Limonene	1029	72,4	89,6	79,6	37,2	52,7	56,5	80,1	92,8	97,8	83,9	95,0	90,8	a, b, c
trans-β-ocimene	1050		0,3		0,2	0,3								a, b
γ-Terpinene	1060				10,4	13,9	13,4							a, b, c
α-Terpinolene	1089		0,3	0,6	0,7	0,9	0,7						1,1	a, b
Nonanal	1101				0,2									a, b
cis-limonene oxide	1137												0,4	a, b
Citronellal	1153		0,3	0,5	0,3	0,3								a, b
α-Terpineol	1179	0,8	1,0	3,2	0,3		0,5							a, b
Decanal	1202	0,4	0,7	1,9									0,9	a, b
Neral	1238				1,2	0,6	1,5							a, b
Carvone	1243			0,6									0,5	a, b
Lavandulyl acetate	1290				0,9	0,2	1,2							a, b
Citral	1320				2,0	0,9	2,6							a, b
Neryl acetate	1362			0,7	1,2	0,4	1,5							a, b
α-Copaene	1377		0,4	1,6										a, b
β-Cubebene	1388		0,4	1,7										a, b
β-Caryophyllene	1419	0,9	0,9	4,3	0,6		0,8							a, b, c
α-Bergamotene	1435				1,0	0,2	1,5							a, b
α-Humulene	1456			0,7										a, b
Germacrene D	1485			0,9										a, b, c
Valencene	1496				0,2								0,5	a, b
Bicyclogermacrene	1500				0,1									a, b
β-Bisabolene	1506				1,5	0,2	2,2							a, b, c
δ-Cadinene	1523			1,4										a, b
Nootkatone	1807	1,1		0,5										a, b
Total		79,8	98,9	100,0	73,4	99,8	100,0	83,5	100,0	99,9	87,9	99,9	99,9	

Sample names according to Table 1; RI = Retention Index; identification method: a = MS, b = RI, and c = comparison with authentic standard.

followed by β-pinene, γ-terpinene, myrcene, α-pinene, neral, and citral, among others.

Similar findings of a previous study on the CPEOs derived from industrial cold-pressing of fresh fruit peels of orange, mandarin, and lemon are in accordance with the above-mentioned results, where D-limonene was the most abundant compound in all fruits with concentrations reaching 85.5% in orange, 74.38% in mandarine, and 59.1% in lemon [26]. Ahmad et al. [27] investigated the chemical composition of citrus essential oils collected by cold-pressing of shredded fruit peels. According to their findings, the abundance of D-limonene in two sweet orange varieties was 61.08% and 76.28%, in grapefruit 86.27%, and in lemon 53.61%. According to previous results on the chemical composition of lemon CPEO, D-limonene exhibited the highest percent (75.68%) followed by β-pinene (8.7%) and γ-terpinene (7.19%) [28]. Concerning mandarine, Sawamura et al. [29] studied the CPEO retrieved by a hand-pressing method of whole fruits and peels and resulted in a high percent of D-limonene (80.3%). Thus, few studies investigate the chemistry of citrus CPEOs, while no previous study exists concerning the CPEOs produced as by-products by the citrus juice producing industry. It is important to notice that the quantitative results of the previous studies mentioned above refer to uncorrected percentages of chemical constituents due to the presence of nonvolatile compounds in the CPEOs that are not detected by the analytical instruments used.

The applied herein process consisted of the simultaneous tasks of isolation and fractionation of the CPEOs volatile content, which was evaluated for first time presenting intriguing results in respect to the produced essential oils major compounds (above 3% of the essential oil). More specifically, the D-limonene concentration was increased in all cases in both hydrodistillation fragments (1 and 2) in comparison with its original occurrence in the respective CPEOs as indicated in Figure 1. In grapefruit, the percent of D-limonene in the CPEO (72.35%) reached 89.63% in fragment 1 and 79.56% in fragment 2 of the hydrodistillation, whereas other main

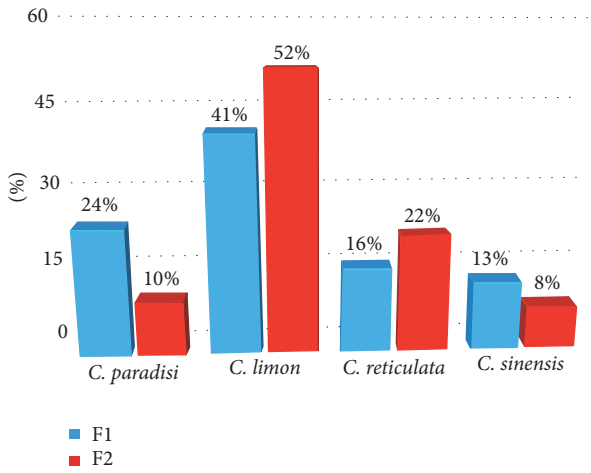

Figure 1: D-Limonene increase in the refined samples, expressed as percentage of the relevant CPEO limonene content.

compounds with increased abundancy in fragment 2 were α-terpineol (3,19%) and β-caryophyllene (4,26%). For lemon, the abundance of D-limonene was increased from 37.22% in the CPEO to 52.66% in fragment 1 and 56.50% in fragment 2, while a noticeable increase of β-pinene (19.16%), α-pinene (5.08%), and γ-terpinene (13.86%) content is also observed in fragment 1. A sharp increase of D-limonene content is observed after the hydrodistillation of the mandarin CPEO. Specifically, the presence of D-limonene reached 92.75% and 97.79% in fragments 1 and 2 of the hydrodistillation, respectively, as compared to its initial abundance in the CPEO which was determined as 80.06%. Other constituents with increased abundance in the first fragment of the hydrodistillation are myrcene (4.28%), a-pinene (1.74%), and sabinene. Finally, for oranges, the D-limonene content was increased in the first fragment of the hydrodistillation, reaching the 95.01%. In the second fragment, an increase of the content of D-limonene, myrcene, and a-terpinolene was observed.

3.2. Bioactivity Assessment

3.2.1. Larvicidal Bioassays. *Citrus* based essential oils (EOs) have already been pinned as potent population control agents against *Aedes* sp., by Khan and Akram [30], who suggested the relevant consideration of EOs from *C. grandis, C. sinensis, C. paradisi, C. reticulata, C. limon*, and *C. aurantium* but also identified the necessity for the design and implementation of relevant field trials. Four years earlier, in 2009 Michaelakis et al. [31] reported the increased larvicidal activity of the EOs derived from the dried peels of *C. sinensis, C. limon,* and *C. aurantium* against *Culex pipiens* (Diptera: Culicidae). Since then the toxicity of *Citrus* EOs has been confirmed against two *Culex* sp. *taxa, Cx. pipiens* [31] and *Cx. quinquefasciatus* [32]; two *Anopheles* sp. *taxa, An. labranchiae* [33] and *An. stephensi* [34]; two *Aedes* sp. *taxa, Ae. albopictus* [21, 35] and *Ae. aegypti* [36, 37]. Adaptation of these results as a starting point for scientific discussion was implemented after the performance of an insect toxicity sketch, which included as complementary targets the *larvae* of *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) investigated by Bilal et al. [38] and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) performed by Villafañe et al. [39].

Table 3: Mean percentage (±s.e.) of dead larvae of *Aedes albopictus* in larvicidal bioassays by the 12 *Citrus* samples.

Code	Mean percentage (±s.e.) of dead larvae
C 01	2.0 ± 1.2
C 02	28.0 ± 6.0
C 03	24.0 ± 6.0
C 04	23.0 ± 8.8
C 05	74.0 ± 6.0
C 06	31.0 ± 2.9
C 07	2.0 ± 1.2
C 08	14.0 ± 4.3
C 09	0.0 ± 0.0
C 10	10.0 ± 8.8
C 11	51.0 ± 6.2
C 12	94.0 ± 3.7
DMSO*	0.0 ± 0.0

*Control.

Since the larvicidal properties of *Citrus* EOs are solidly established, the serious question phrased by Khan and Akram [30] concerning the necessity for field trials implementation remains. The latter require the availability of a large volume of a consistent quality EO. An intriguing answer to this question was suggested in 2011 by Did et al. [35] that employed the EOs isolated from the *Citrus* juice industry solid wastes, which though potent as a source required additional infrastructure development and additional production stages to become broadly available. Our approach herein refers to the exploitation of readily available by-products of the *Citrus* juice industry that are defined as the CPEOs, all four of which are assessed on their toxicity per se, and after refinement, for first time herein.

The larvicidal bioassay results are cumulated in Table 3, where the mean percentage (±s.e.) of dead larvae is presenting and indicates results 24 h after the implementation of the testing material. The last consisted of the 4 CPEO and 8 EO samples of Table 1. From the four CPEOs tested those from grapefruit and mandarin were found to be of insignificant toxicity, the one from orange exhibited a mild toxicity, and this from lemon presented a moderate toxicity, justifying thus the depreciated value of the respective by-products. The applied process though proved significant by improving the larvicidal properties in seven of the eight in total, processed samples, as indicated in Figure 2. Even the percentages of Figure 2 indicate an impressive increase of toxicity for the two grapefruit EOs, as most potent proved the lemon first volatile fraction (C05) and the second volatile fraction from orange (C12), exhibiting, respectively, 74% and 94% larvae mortality. Among the eight volatile fractions, only the second volatile fraction of mandarin presented no toxicity (C9). This sample presented a unique phytochemical profile consisting of the highest percentage of D-limonene (97,8%).

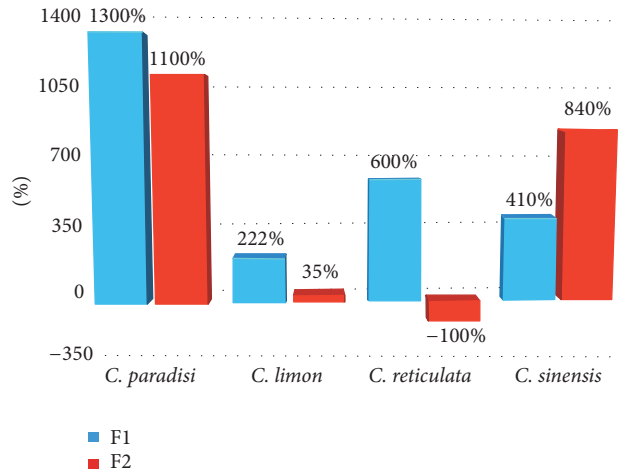

Figure 2: Larvicidal toxicity of the refined samples, expressed as percentage of the relevant CPEO toxicity.

These preliminary results from the 12 samples tested did not provide any relation between the phytochemical content or the fluctuation of the major compounds and the larvicidal pattern.

The combined study of larvicidal results in conjunction with the low toxicity of mandarin second volatile fraction is indicative that the accompanying the limonene phytochemicals could be attributed to the increase of toxicity. Previous studies [21] indicated that some commonly found substances in EOs with significant larvicidal activity are *a*-pinene, 3-carene, (*R*)-(+)-limonene, myrcene, and terpinen-4-ol. The increased toxicity that is recorded in all samples containing amounts of myrcene exceeding the limit of 3% may partly explain this toxicity pattern. In either case, the obtained results could justify the application of the simultaneous volatile fraction isolation and fragmentation.

3.2.2. Repellence Bioassays. The repellent properties of *Citrus* derived EOs have been studied and confirmed in a band wider than the larvicidal band of target organisms. In these, the following is included: six Coleoptera, *Periplaneta americana* [40, 41], *P. fuliginosa* [41], *Blattella germanica* [40, 41], *Neostylopyga rhombifolia* [40], *Tribolium castaneum* [38, 42], and *Sitophilus zeamais* [43]; the aphid *Tetranychus urticae* [44]; seven Diptera, *Loutzomyia youngi* [45], *Simulium damnosum* [46], *Aedes albopictus* [21, 47], *Ae. aegypti* [48], *Anopheles dirus* (ibid.), *An. stephensi* [34], and *Culex quinquefasciatus* [49]. Within this rough background and based upon these significant research efforts, the present study focused on the investigation of the four-industrial origin CPEO and the eight derived volatile fragments, which is a novelty of the study.

The results summarized in Table 4 present the mean number of landings for five minutes. All four CPEOs presented a low to moderate repellent activity. The applied process of CPEOs refinement did not provide any significant results on the respective repellence activity. Specifically, the orange volatile fractions (C11, C12) exhibited almost the same figures with the respective CPEO (C10) landing averaging

Table 4: Mean number (±s.e.) of landings of *Aedes albopictus* on the uncovered area of the glove per 5 minutes and comparison with the positive control (DEET) and the control (DCM) by using the 12 *Citrus* essential oils (d.f. = 1. $\alpha = 0.05$).

Code	Mean number (±s.e.) of landings/5 min	P_{DEET}	P_{DCM}
C 01	32.9 ± 3.9	0.0003*	0.0011*
C 02	50.6 ± 3.6	0.0003*	0.1712
C 03	38.0 ± 1.0	0.0003*	0.0008*
C 04	23.0 ± 5.3	0.0003*	0.0011*
C 05	39.0 ± 5.2	0.0003*	0.0117*
C 06	39.5 ± 7.7	0.0003*	0.1275
C 07	36.0 ± 2.7	0.0003*	0.0008*
C 08	32.6 ± 0.9	0.0003*	0.0008*
C 09	18.0 ± 3.0	0.0003*	0.0008*
C 10	49.6 ± 1.9	0.0003*	0.0237*
C 11	51.0 ± 4.3	0.0003*	0.1412
C 12	52.1 ± 5.1	0.0003*	0.1267
DEET	0.0 ± 0.0	—	—
DCM	56.0 ± 4.0	—	—

*Significant difference.

to around 50; the application of grape fruit (C02, C03) and lemon (C05, C06) volatile fractions presented an increase of landings compared with the relevant CPEOs (C01, C04), therefore presenting a significant decrease in correlation with their repellent properties; on the other hand, mandarin volatile fragments presented a differentiated performance since both the first and second fragments improved the CPEO's (C07) repellence exhibiting less landings, by 50% for the second fragment (C09) and by 22% for the first fragment (C08). As potential factor for the observed differentiation, the phytochemicals contained in the colorant fragment of the CPEOs may be considered, the lack of which is the major difference between the CPEO and the relative EOs. Those results agree with previous studies indicating that most hydrocarbons (e.g., *a*-pinene) had a lower repellent efficacy against adult mosquitoes compared to aldehydes, oxides, or alcohols [50].

4. Conclusions

In conclusion, the current work exploiting invaluable industrial by-products demonstrated a protocol that can provide D-limonene of analytical grade (97%), revealing thus in the form of the mandarin second volatile fragment an alternative source for the retrieval of this valuable molecule.

Industrial by-products as natural insecticides could be a promising tool especially for targeting mosquito larvae. The lack of any significant difference in the phytochemical content of the mandarin three samples, with the simultaneous differentiation of bioactivity in both larvicidal and repellency bioassays, indicates that the root cause may not be detected in the volatile fragment components and there are some factors that need more investigation to extract useful conclusions such as enantioselectivity of major and minor ingredients [21, 51, 52].

Disclosure

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Mycochemical Characterization of *Agaricus subrufescens* considering Their Morphological and Physiological Stage of Maturity on the Traceability Process

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Agaricus subrufescens Peck is a basidiomycete with immunomodulatory compounds and antitumor activities. This research evaluated the mycochemical composition of *A. subrufescens*, considering their morphological and physiological stage of maturity, with a particular focus on the development of a traceability process for the formulation of new nutritional products based on fungal foods. The stipes contained a high amount of dry matter (10.33%), total carbohydrate (69.56%), available carbohydrate (63.89%), and energy value (363.97 kcal 100 g^{-1} DM). The pilei contained a high amount of moisture (90.66%), nitrogen (7.75%), protein (33.96%), ash (8.24), crude fat (2.44%), acid detergent fiber (16.75 g kg^{-1}), neutral detergent fiber (41.82 g kg^{-1}), hemicellulose (25.07 g kg^{-1}), and lignin (9.77 g kg^{-1}). Stipes with mature physiological stage had higher values of dry matter (10.50%), crude fiber (5.94%), total carbohydrate (72.82%), AC (66.88%), and energy value (364.91 kcal 100 g^{-1} DM). Pilei of the mushrooms in the immature physiological stage had higher values of P (36.83%), N (8.41%), and A (8.44%). Due to the differences between the mycochemical compositions of the morphological parts of mushrooms linked to their physiological stage of maturity, such characteristics have immense potential to be considered for a traceability process. This study can be used for the purpose of providing the consumer with more product diversity, optimizing bioactivities of composts, and allowing farmers an efficient and profitable use of the mushroom biomass.

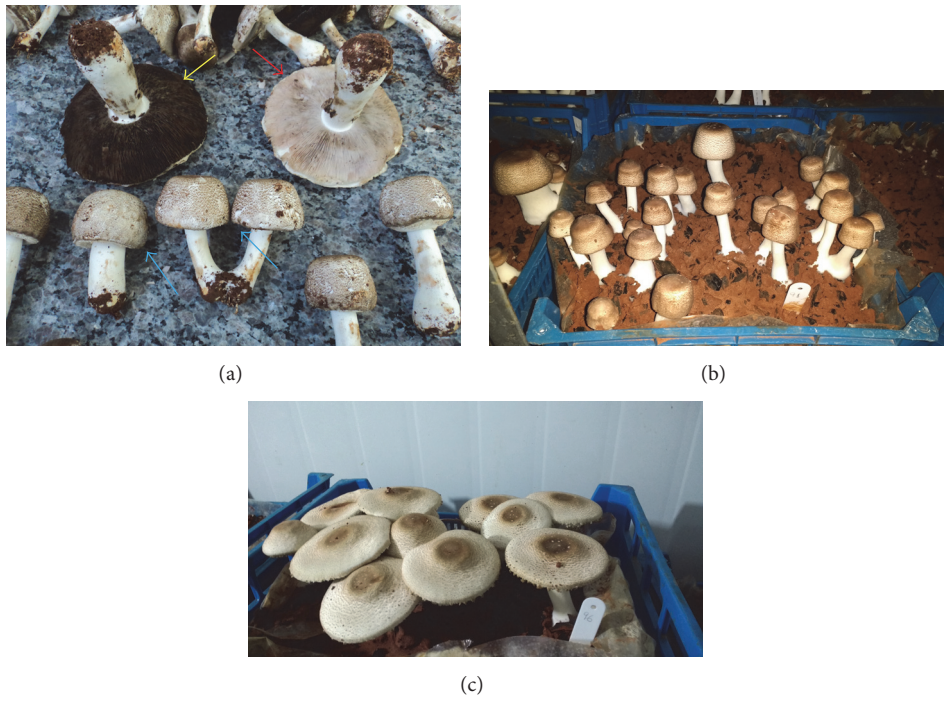
1. Introduction

Agaricus subrufescens Peck, which is synonymous with *Agaricus brasiliensis* (Wasser et al.) and *Agaricus blazei* (Murrill) *sensu* Heinemann [1], is a basidiomycete fungus commonly referred to as "almond mushroom," "medicinal mushroom," or "sun mushroom." This mushroom has gained interest in the international scientific community because of its immunomodulatory compounds and antitumor activities [2–4].

Particular attention is required in the process of mushrooms cultivation designated for medicinal purposes [origin of the spawn (quality of the inoculum for production), compost and casing used, and production environment] accompanied with a detailed knowledge of mycochemical characteristics, based on morphological and physiological development of mushrooms, to improve their nutritional and pharmacological effect in humans consumption.

It has been shown that productivity of *A. subrufescens* is related to the quality of the compost, among others factors [5].

According to Wisitrassameewong et al. [6], various studies have attempted to optimize the composition of compost



(a) (b)

(c)

Figure 1: (a) Mushroom in different physiological stages (yellow arrow refers to mature mushroom with mature spore "dark brown spores"; red arrow refers to mature mushroom with immature spores; and blue arrow refers to immature mushrooms with closed pilei without lamella break). (b and c) Immature and mature mushrooms with immature spores used in the manuscript.

for mushroom cultivation. However, the association between the compost medium and nutritional quality of the harvested mushrooms has not been fully investigated. In this regard, Zied et al. [7] presented a detailed study on which process of mushroom cultivation influenced the amount of β-glucan in the mushrooms. The study aimed to create a cultivation protocol to standardize mushroom; however, the morphological and physiological development of the mushrooms were not emphasized in this research. These mushroom cultivation processes are important because they allow the grower to adopt practices that enhance the medicinal characteristics of the fungus.

Morphologically, a mushroom can be divided into two main parts: pilei (superior part of the fruit body where the lamella and spores are located) and stipe (inferior part of the fruit body, consisting of a bulk sterile hyphal tissue) [8, 9]. Physiologically, *A. subrufescens* can be classified according to the following stages of maturity: immature (pilei closed), mature (pilei opened) with immature spores, and mature (pilei widely opened) with mature spores (Figure 1(a)) [10, 11].

We suggest that a systematic program of development could be devised, considering the morphological and physiological characteristics of mushrooms in traceability process. The definition of traceability process varies according to the industry sector, the current legislation, the country concerned, and the objectives of the process. For the agro-based food chain, food traceability can be defined as the information necessary to describe the production history of a food crop and any subsequent transformations or processes that the crop might be subject to in its distribution process from the grower to the consumer's plate, with detailed explanations of each stage [12, 13].

A targeted and more rigorous definition of food supply chain not specific to food commodities was provided by the International Organization for Standardization in 1994 [14] and supported by EC regulation 178/2002 [15]. This defines "traceability as the ability to trace and follow a food, feed, food producing animal or ingredients, through all stages of production and distribution."

The production system and traceability of *A. subrufescens* cultivation involve three steps: the process of mushroom cultivation (studied by Zied et al. [7]), the morphological and physiological development of the mushroom (intended aim of this manuscript), and the postharvest method used for mushroom commercialization (dehydration, freeze-drying, extraction process of metabolic fractions, or bioactive compounds, etc.), a subject for future research.

Therefore, this study aimed to evaluate the mycochemical composition of *A. subrufescens*, considering their morphological and physiological development, with a particular focus on the development of a traceability process for the formulation of new nutritional products based on fungal foods.

2. Materials and Methods

The traceability system for mushrooms in Europe (Spain) was evaluated. The same strain, casing layer, and cultivation environment (chamber with controlled climate) were used, varying only the compost at each mushroom farm.

Table 1: Analytical characteristics of the substrates at the end of phase II. CM corresponds to Compost Manchego and CV to Compost Villacasa.

Parameter	CM	CV
pH (1 : 5, w/v)	7.63 ± 0.02	7.44 ± 0.02
Moisture (g kg^{-1})	664.7 ± 3.8	654 ± 3.3
Total nitrogen (g kg^{-1} d.m.)	19.7 ± 0.9	21.3 ± 0.7
Protein (g kg^{-1} d.m.)	123.1 ± 5.4	132.9 ± 4.1
Ash (g kg^{-1} d.m.)	237.2 ± 2.4	224.6 ± 6.5
Organic matter (g kg^{-1} d.m.)	762.8 ± 2.4	775.4 ± 6.5
Carbone/Nitrogen	22.5 ± 1.0	21.2 ± 0.6
Crude fiber (g kg^{-1} d.m.)	330 ± 17.8	292.8 ± 9.6
Crude fat (g kg^{-1} d.m.)	2.5 ± 0.8	3.3 ± 0.7
N-free extracts (g kg^{-1} d.m.)	307.2 ± 15.5	346.4 ± 10.3
Total carbohydrates (g kg^{-1} d.m.)	637.2 ± 6.7	639.2 ± 7.1
Hemicellulose (g kg^{-1} d.m.)	123.4 ± 1.3	106.8 ± 5.7
Cellulose (g kg^{-1} d.m.)	204.8 ± 15.8	190.3 ± 5.7
Lignin (g kg^{-1} d.m.)	208.8 ± 11.1	226.7 ± 3.8
Neutral-detergent sol. fiber (g kg^{-1} d.m.)	225.8 ± 12.4	251.6 ± 13.0

Each value is expressed as mean ± standard deviation ($n = 4$); d.m.: dry matter.

Table 2: Physical and chemical characteristics of Dutch commercial casing.

Parameter	Value
pH (1 : 5, v/v)	8.26 ± 0.05
Electrical conductivity (1 : 5, v/v; μS cm^{-1})	231 ± 2
Particle real density (g mL^{-1})	1.759 ± 0.009
Ash (g kg^{-1})	286.7 ± 9.4
Bulk density, wet (g mL^{-1})	0.613 ± 0.009
Moisture (g kg^{-1})	831 ± 0.8
Bulk density, dry (g mL^{-1})	0.104 ± 0.001
Total pore space (mL L^{-1})	941 ± 1
Water-holding capacity (kg kg^{-1})	6.97 ± 0.31
Organic matter (g kg^{-1})	713.3 ± 9.4
Nitrogen (g kg^{-1})	8.6 ± 0.3
Carbone/nitrogen	48.1 ± 0.8
Total carbonates (g kg^{-1})	137 ± 7
Active lime (g kg^{-1})	55 ± 2

Each value is expressed as mean ± standard deviation ($n = 4$).

2.1. Traceability Process of Mushroom Cultivation. The *A. subrufescens* strain, ABL 99/30, was collected in 1999, from Piedade city, São Paulo State, Brazil, as described by Zied et al. [7]. Spawn was prepared according to the following steps: selection of mushroom, production of subculture, production of mother spawn, and production of grain spawn [16].

The *Agaricus bisporus* growing substrates, based on wheat straw and chicken manure, were provided by Compost Manchego (CM) SL (Villanueva de la Jara, Spain) and Compost Villacasa (CV) SL (Casasimarro, Spain). Table 1 presents the analytical characteristics of the two substrates used, at the end of phase II of composting (pasteurization and aerobic thermophilic conditioning procedure). For each characteristic, four repetitions were performed.

After phase II of the composting process, the substrate was inoculated with *A. subrufescens* at 12 g kg^{-1} fresh compost. Euroveen® (BVB Substrates, Grubbenvorst, Netherlands), a Dutch commercial mixture, was used as casing material (Table 2). A 4 cm thick casing layer was applied 20 days after spawning.

Analyses of substrates and casing were carried out with four repetitions for each parameter in the Laboratory of the Centro de Investigación, Experimentación y Servicios del Champiñón (Quintanar del Rey, Spain). Competitor molds, mites, and nematodes were absent in both the composts and the Dutch commercial casing layer.

The crop cycle was conducted in an experimental chamber of the Centro de Investigación, Experimentación y Servicios del Champiñón (Quintanar del Rey, Spain), equipped with humidification systems, heating/cooling, and recirculating/outside air ventilation, which allowed automatic control of temperature, relative humidity (RH), and carbon dioxide concentration. After spawning, the compost was incubated at 28 ± 1°C, 95 ± 2% RH for 20 d, without external ventilation or lighting.

Ruffling was done eight days after casing application when the mycelia appeared on the surface. A day later, the environmental temperature (19 ± 1°C), RH (88 ± 2%), and carbon dioxide level (<800 ppm) were decreased, with illumination (150 lux, 12 h per d) provided to induce fruiting. After three days, the environmental temperature was increased to 24 ± 1°C until first flush cessation. To induce the second and third flush, the environmental temperature (19 ± 1°C) was decreased again for 3 d. The total duration of the crop cycle was 82 d.

2.2. Morphological and Physiological Development of Mushroom. Mushrooms, in two different stages of maturity (immature and mature) in the second flush, were harvested manually, totalizing 50 mushrooms in each stage. The immature mushrooms had the pilei with approximately 30 to 55 mm of diameter and the mature mushroom had the pilei with approximately 60 to 100 mm of diameter (Figures 1(b) and 1(c)). The mature mushrooms used were with immature spores. The bottoms of the stipes were scraped to remove casing layer residues. The immature and mature mushrooms were divided into three groups, according to morphological parts (pilei, stipes, and whole mushrooms); thus, a total of six parameters (pilei immature, stipes immature, whole mushrooms immature, pilei mature, stipes mature, and whole mushrooms mature) were analyzed, according to their mycochemical composition. Samples were dried at 50°C for 48 h and triturated for analysis. For each characteristic, six repetitions were performed.

2.3. Analyses in the Mushrooms, Composts, and Casing. Physical, chemical, and biological characteristics of mushrooms, composts, and casing layer were determined according to Pardo-Giménez et al. [17, 18]. The following measurements were taken:

(i) Moisture and dry matter contents (gravimetric method, drying at 103–105°C to a constant weight)

(ii) pH (potentiometric method, extracting a test portion of sample with water at 22 ± 3°C; extraction ratios of 1 + 5 (W/V) and 1 + 5 (V/V) were used)

(iii) Electrical conductivity (conductimetric method; a test portion is extracted with water at 22 ± 3°C in an extraction ratio of 1 + 5 (V/V) to dissolve the electrolytes)

(iv) Total N content (Kjeldahl method, based on digestion with sulphuric acid/potassium sulphate)

(v) Protein (calculation by multiplying the total nitrogen content, obtained by the Kjeldahl method by a conversion factor of 6.25 for substrates and 4.38 for mushrooms)

(vi) Organic matter and ash (thermogravimetric method, measuring the loss of weight after calcination at 540°C)

(vii) C/N ratio (calculation from organic matter and nitrogen contents; the conversion of organic matter into its carbon content is made based on the assumption that organic matter contains 58% organic carbon)

(viii) Crude fiber (Weende technique adapted to the Ankom filter bag technique; this method determines the organic residue remaining after digestion with solutions of sulphuric acid and sodium hydroxide)

(ix) Crude fat (gravimetric method adapted to the Ankom filter bag technique, extracting the sample with petroleum ether)

(x) Total carbohydrates (calculation by subtracting the sum of the crude protein, total fat, water, and ash from the total weight of the material)

(xi) Nitrogen-free extracts/available carbohydrate content (calculation by subtracting the crude fiber from the total carbohydrate content)

(xii) Energy value (calculation from the relative content of protein, fat, and carbohydrates using modified Atwater factors)

(xiii) Cellulose, hemicellulose, lignin, and acid and neutral detergent fiber (Van Soest fiber detergent methods: acid and neutral detergent fiber method by Ankom filter bag technique and Ankom acid detergent lignin in beakers method were used)

(xiv) Particle real density (calculation from ash content considering that the density of the organic matter is 1.55 kg m^{-3} and that of the ashes 2.65 kg m^{-3})

(xv) Bulk density, fresh (weight of certain volume of material after compaction, determined in laboratory using a cylinder of 1L of capacity by compacting the sample under defined conditions)

(xvi) Bulk density, dry (calculation from fresh bulk density and moisture content)

(xvii) Total of pore space (calculation from dry bulk density and real density)

(xviii) Water holding capacity (saturation and drainage method, determined from the moisture content of the sample after being subjected to three cycles of saturation and drainage at atmospheric condition)

(xix) Active lime (ammonium oxalate method, calculated by means of a gasometric dosage of the CO_2 of the ammonium carbonate, formed by reacting the calcium carbonate of the sample with a solution of ammonium oxalate with the aid of a Bernard calcimeter)

(xx) Total carbonates (hydrochloric acid method, based on the measurement of the volume of CO_2 released when treating the carbonates with hydrochloric acid)

2.4. Statistical Analysis. ANOVA was used to analyze the data, and the Fisher's LSD test was employed to establish significant differences between means ($p \leq 0.05$). All calculations were performed using the Statgraphics Plus software, v. 4.1 (Statistical Graphics Corp., Princeton, NJ, USA). PCA (Principal Component Analysis) was performed with R software [19], to reduce the dimensionality of the data and to identify components which allow discriminating the material.

3. Results and Discussion

Table 3 shows the ANOVA results for the 16 mycochemical characteristics, considering the type of compost (a), morphological mushroom parts (b), and physiological stage of mushroom maturity (c). Table 4 shows the respective mean tests for the variables that were significant by ANOVA, to describe the traceability process linking the cultivation information (using the two composts) and its response to the harvested mushrooms composition.

The compost is an important factor in the quality (physical, chemical, and biological) of mushroom [17]. However, in this study, a comparison of the two composts (CM and CV) indicated significance for only five mushroom characteristics: CFa (crude fat), EV (energy value), ADF (acid detergent fiber), CE (cellulose), and LI (lignin), as shown in Table 3(a). The reasons that only those characteristics showed differences are unclear, particularly regarding the percentage of CFa and CE, that composts with a higher percentage of these elements result in harvested mushrooms with lower value of these elements, which makes us understand that there is no direct correlation between CFa and CE content in the compost and harvested mushroom.

It should be noted that the characteristics analyzed in the substrates do not differ drastically (Table 1), the composts being quite similar, meeting the compost quality index for mushroom cultivation [20]. Pardo et al. [21] introduce a HACCP system in the compost elaboration line, which allow the composting companies to design and establish a self-control system to ensure the quality of their productions, but the authors did not verify the mycochemical characteristics of the harvested mushrooms. Zied and Minhoni [22] obtained the highest protein content in mushrooms cultivated on

Table 3: Results of variance analyses for chemical characteristics of mushrooms considering (a) type of compost; (b) morphological parts of mushrooms; (c) physiological stage of maturity. DM: dry matter (%); M: moisture (%); N: nitrogen (%); P: protein (%); A: ash (%); CFi: crude fiber (%); CFa: crude fat (%); TC: total carbohydrate (%); AC: available carbohydrate (%); EV: energy value (kcal 100 g^{-1} DM); ADF: acid detergent fiber (g kg^{-1}); NDF: neutral detergent fiber (g kg^{-1}); HE: hemicellulose (g kg^{-1}); CE: cellulose (g kg^{-1}); LI: lignin (g kg^{-1}).

(a)

		Mean Square[1]														
	df	DM	M	N	P	A	CFi	CFa	TC	AC	EV	ADF	NDF	HE	CE	LI
Treatments	1	0.27^{ns}	0.34^{ns}	0.02^{ns}	0.32^{ns}	0.39^{ns}	0.09^{ns}	4.86^{***}	1.07^{ns}	1.74^{ns}	223.05^{***}	111.84^{**}	10.63^{ns}	53.46^{ns}	1.39^{*}	94.29^{**}
Mean		9.76	90.13	6.49	28.44	7.67	5.60	2.13	61.65	56.05	359.55	14.22	35.30	21.08	7.00	7.33
CV (%)		5.73	0.65	18.18	18.19	6.98	6.64	25.34	9.30	10.01	1.20	22.14	16.49	18.39	7.88	41.58

(b)

		Mean Square[1]														
	df	DM	M	N	P	A	CFi	CFa	TC	AC	EV	ADF	NDF	HE	CE	LI
Treatments	2	6.16^{***}	5.94^{***}	47.66^{***}	914.30^{***}	9.55^{***}	0.08^{ns}	3.24^{***}	1241.90^{***}	1222.10^{***}	355.40^{***}	193.04^{***}	1301.40^{***}	491.90^{***}	0.15^{ns}	180.11^{***}
Mean		9.76	90.13	6.49	28.44	7.67	5.60	2.13	61.65	56.05	359.55	14.22	35.30	21.08	7.00	7.33
CV (%)		4.43	0.53	9.54	9.55	3.87	6.66	24.71	4.15	4.22	1.03	18.70	7.15	10.81	8.07	34.84

(c)

		Mean Square[1]														
	df	DM	M	N	P	A	CFi	CFa	TC	AC	EV	ADF	NDF	HE	CE	LI
Treatments	5	4.96^{***}	3.98^{***}	25.91^{***}	497.10^{***}	4.87^{***}	0.85^{***}	4.98^{***}	612.10^{***}	585.60^{***}	265.42^{***}	87.12^{***}	553.60^{***}	216.21^{***}	1.17^{**}	76.62^{***}
Mean		9.76	90.13	6.49	28.44	7.67	5.60	2.13	61.65	56.05	359.55	14.22	35.30	21.08	7.00	7.33
CV (%)		2.40	0.42	2.01	2.01	2.37	5.59	13.44	0.99	1.13	0.75	18.28	6.17	9.82	7.40	34.73

[1]Significance codes: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; $^{ns}p > 0.05$. CV: coefficient of variation; df: degree of freedom.

Table 4: Results of mean comparison tests for characteristics which showed a significant result according to ANOVA. Mean values followed by the same letter do not differ according to the F test ($p < 0.05$) in (a) or according to Tukey's test ($p < 0.05$) in (b) and (c). Treatment levels are related to (a) type of compost; (b) morphological mushroom parts; (c) physiological stage of maturity. DM: dry matter (%); M: moisture (%); N: nitrogen (%); P: protein (%); A: ash (%); CFi: crude fiber (%); CFa: crude fat (%); TC: total carbohydrate (%); AC: available carbohydrate (%); EV: energy value (kcal $100\,g^{-1}$ DM); ADF: acid detergent fiber ($g\,kg^{-1}$); NDF: neutral detergent fiber ($g\,kg^{-1}$); HE: hemicellulose ($g\,kg^{-1}$); CE: cellulose ($g\,kg^{-1}$); LI: lignin ($g\,kg^{-1}$).

(a)

	DM	M	N	P	A	CFi	CFa	TC	AC	EV	ADF	NDF	HE	CE	LI
MC	—	—	—	—	—	—	2.35^{a}	—	—	361.07^{a}	13.14^{b}	—	—	6.88^{b}	6.34^{b}
VC	—	—	—	—	—	—	1.90^{b}	—	—	359.02^{b}	15.30^{a}	—	—	7.12^{a}	8.31^{a}

Note. MC: Manchego Compost; VC: Villacasa Compost.

(b)

	DM	M	N	P	A	CFi	CFa	TC	AC	EV	ADF	NDF	HE	CE	LI
Stipes	10.33^{a}	89.67^{c}	4.96^{c}	21.73^{c}	6.99^{c}	5.67	1.72^{b}	69.56^{a}	63.89^{a}	363.97^{a}	11.13^{c}	27.25^{c}	16.12^{c}	—	4.34^{c}
Wh. Mush.	9.69^{b}	90.09^{b}	6.63^{b}	29.03^{b}	7.73^{b}	5.58	2.18^{a}	60.83^{b}	55.25^{b}	358.83^{b}	14.50^{b}	36.07^{b}	21.57^{b}	—	7.60^{b}
Pilei	9.34^{c}	90.66^{a}	7.75^{a}	33.96^{a}	8.24^{a}	5.56	2.44^{a}	55.36^{c}	49.80^{c}	356.54^{c}	16.75^{a}	41.82^{a}	25.07^{a}	—	9.77^{a}

Note. Wh. Mush: whole mushroom.

(c)

	DM	M	N	P	A	CFi	CFa	TC	AC	EV	ADF	NDF	HE	CE	LI
Mat. Stipes	10.50^{a}	89.50^{c}	4.32^{e}	18.91^{e}	6.68^{e}	5.94^{a}	1.59^{c}	72.82^{a}	66.88^{a}	364.91^{a}	10.10^{d}	24.69^{d}	14.58^{e}	6.54^{b}	3.71^{d}
Imm. Stipes	10.17^{b}	89.83^{bc}	5.61^{d}	24.55^{d}	7.29^{d}	5.41^{bc}	1.85^{c}	66.31^{b}	60.90^{b}	363.03^{ab}	12.16^{cd}	29.82^{c}	17.66^{d}	7.29^{a}	4.98^{cd}
Mat. Pilei	8.81^{e}	91.19^{a}	7.10^{b}	31.10^{b}	8.04^{b}	5.73^{ab}	3.12^{a}	57.75^{e}	52.02^{e}	360.82^{b}	16.13^{ab}	42.35^{a}	26.22^{a}	6.99^{ab}	9.22^{ab}
Imm. Pilei	9.88^{c}	90.12^{b}	8.41^{a}	36.83^{a}	8.44^{a}	5.39^{c}	1.76^{c}	52.97^{f}	47.58^{f}	352.27^{d}	17.37^{a}	41.29^{a}	23.92^{ab}	7.15^{ab}	10.32^{a}
Mat. Wh. Mush.	9.37^{d}	90.18^{b}	6.09^{c}	26.67^{c}	7.52^{c}	5.78^{a}	2.56^{b}	62.80^{c}	57.02^{c}	360.64^{b}	13.94^{bc}	35.94^{b}	22.00^{bc}	6.80^{ab}	7.25^{bc}
Imm. Wh. Mush.	10.01^{bc}	89.99^{b}	7.17^{b}	31.40^{b}	7.93^{b}	5.40^{c}	1.80^{c}	58.87^{d}	53.47^{d}	357.03^{c}	15.06^{ab}	36.21^{b}	21.15^{c}	7.21^{a}	7.95^{ab}

Note. Mat: mature; Imm.: immature; Wh. Mush: whole mushroom.

substrate with high protein content, a result not verified in the present manuscript.

Food traceability systems are therefore becoming critical for the food industry and the public sector, as well as for consumers. The increased requirements for documentation and reporting systems are taking a toll on developing countries that are hoping to expand their trade in food or break into new markets [23].

Concerning the morphological characteristics of the mushrooms (stipes, pilei, and whole mushrooms), the results (Table 3(b)) indicated that only CFi (crude fiber) and CE were not significantly different from each other, by the F test ($p < 0.05$). Regarding the physiological characteristics, all variables were significantly different, depending on the stage of mushroom maturation (Table 3(c)). The means test results of the characteristics that were significant by ANOVA are presented in Table 4, associated, respectively, with the morphological parts of the mushroom (b) and physiological stage of maturity (c). The results show the importance of this study in characterizing the distribution of mycochemicals within the mushroom.

Notably, the stipes contained a high amount of dry matter (DM), total carbohydrate (TC) and available carbohydrate (AC), and energy value (EV). Furthermore, the pilei had a high amount of moisture (M), nitrogen (N), protein (P), ash (A), CFa, ADF, neutral detergent liquid (NDF), hemicellulose (HE), and LI (Table 4(b)). These results highlight the wide variation of particular characteristics based on the morphological parts of the mushroom, such as N (36%), P (36%), CFi (29%), ADF (33%), NDF (35%), HE (36%), and LI (55%).

In contrast to the results verified in this study, Mol and Wessels [24] studied the differences in wall structure between substrate hyphae and hyphae of fruit-body stipes in *Agaricus bisporus* and concluded that there was no significant difference in gross chemical wall composition of the two hyphal types. However, Jasinghe and Perera [25] found a varying amount of ergosterol depending on the morphological part of shiitake mushrooms, accumulating more in the gills than in the stipe.

According to the review of Manning [26], on the chemical composition and nutritional value of cultivated mushrooms, carbohydrates are the main component of mushrooms apart from water and account for an average of 4.2% of the fresh weight. Glycogen and HE are the main polysaccharides found in mushrooms; contents of 8.18% (dry weight) of HE have been recorded in *A. campestris* [26], markedly lower than those obtained in this research with *A. subrufescens* (between 14.58 and 26.22%) (Table 4(c)). Pardo et al. [27] studied different strains and composts of *A. subrufescens* and found HE values between 17.5 and 22.7%.

To better understand the composition of the mushrooms, we also examined their physiological characteristics to provide a complete mycochemical profile of *A. subrufescens* (Table 4(c)). We observed that the stipes of the mushrooms with opened pilei (mature stipes) had higher values of DM, CFi, TC, and AC compared to equivalent stipes of the mushrooms with closed pilei (immature stipes). The values increased in proportion to the physiological fungus maturity.

Pilei of the mushrooms in the immature physiological stage had higher values of DM, P, N, and A compared with equivalent pilei in mushrooms at the mature physiological stage. The values decreased in proportion to the physiological fungus maturity.

The results of the whole mushrooms represent intermediate values between the stipes and pilei for all mycochemical characteristics analyzed. The physiological characteristics were more influenced than the morphological characteristics, according to the maturity of the mushroom, with the following values obtained: N (49%), P (49%), CFa (49%), AC (29%), NDF (42%), ADF (42%), HE (44%), and LI (64%).

A limited number of studies have reported the tissue distribution of bioactive metabolites in mushrooms during development, despite increasing evidence that maturation affects the concentration of natural compounds in mushrooms [28]. Camelini et al. [10] emphasized that the yield and structural diversity of glucans in *Agaricus brasiliensis* increased in the fruiting bodies matured. The amount of (1→3)-β-glucans in the mature stage was higher than in the immature stage [10]. According to Chang and Miles [29], the amount of (1→4)-β-glucan in the mushroom can also be associated with the amount of cellulose, with the highest amount being found in the stipes of immature mushrooms (7.29%) and in the immature of whole mushroom (7.21%), which may justify the market of this mushroom with closed pilei.

As this study aimed to develop a traceability process for *A. subrufescens* and assuming that the variables evaluated were necessary and sufficient, a PCA was performed to obtain components to discriminate the evaluated material. The first and the second estimated principal components (PC1 and PC2) retained 74.56% of the variance contained in the original variables (Figure 2). It is important to note that the dispersion is not due to a specific characteristic, but that the characteristics are combined, requiring full evaluation to attain traceability of the material.

In estimating the principal components, the variables with the highest weighting in the smallest eigenvalue are considered of minor importance in explaining the variability of the studied material. Thus, minor variables, in decreasing order, are AC, NDF, HE, and EV. The scatter plot analysis of the first two principal components showed three major regions (Figure 2, morphological parts), associated with the morphological parts of mushrooms: the left region, which had the lowest value for PC1, was associated with the pilei of the mushrooms; the opposite region, which had the highest value for PC1, was associated with the stipes; and the central region was associated with the whole mushrooms.

Figure 2 (physiological state of maturity) shows a subdivision of the evaluated characteristics with regard to the physiological stage of mushroom maturity. In this instance, there was a subdivision of the materials related to the morphological parts, stipes, and pilei, respectively. The pilei could be separated, by means of PC2, into mature (positive values) and immature (negative values). For the region associated with the stipes, there was also a subdivision but, in this instance, it was associated with PC1, in which highest values were correlated to mature stipes.

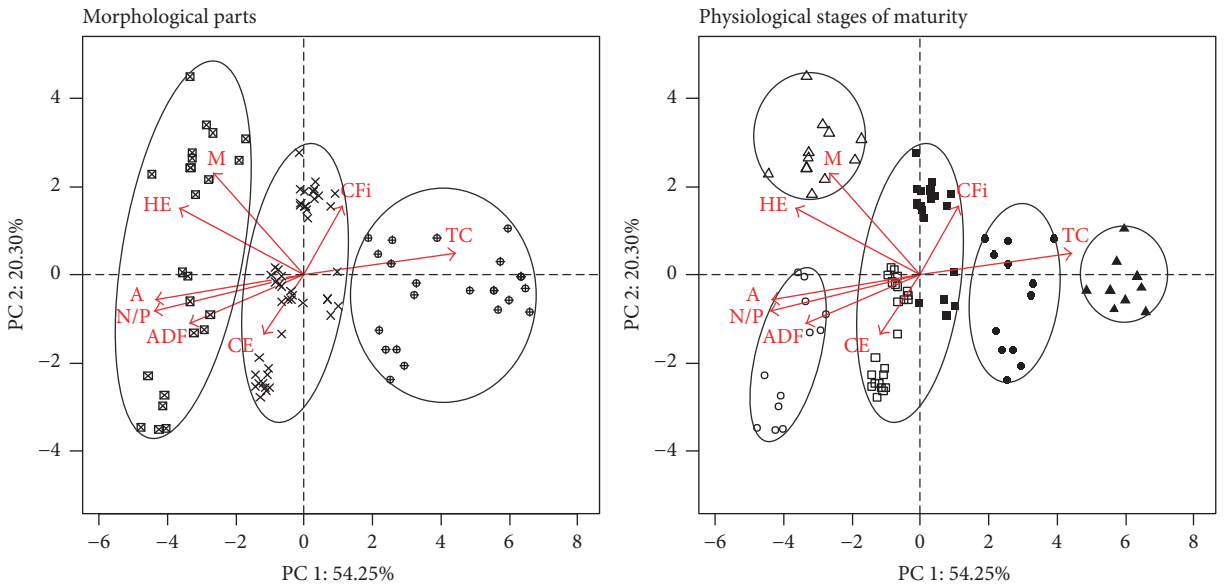


Figure 2: Score plot obtained from PCA that allowed the distinction of the evaluated material with respect to its morphological parts, ⊠: pilei; ×: whole mushrooms; ⊕: stipes, and with respect to its physiological stages of maturity, ∘: immature pilei; •: immature stipes; △: mature pilei; ▲: mature stipes; □: immature whole mushrooms; ■: mature whole mushrooms. In the second scatter plot, the evaluated characteristics do not allow the complete distinction between samples of immature and mature whole mushrooms.

From interpretation of the scatter plot, the results suggested that the pilei had a higher content of EV, M, HE, A, N, P, and ADF, compared to the stipes and whole mushrooms. Still, higher EV, M, and HE values were present when the pilei were opened and higher amounts of A, P, N, and ADF were present when the pilei were closed. CFi and CE values were typically associated with the whole mushrooms, with a slight slope of CFi in the opened mushrooms and CE in the closed pilei (understood in this situation that the closed pilei had a greater amount of β-glucan). The highest amount of TC was found in the stipes with pilei opened.

Previous literature has shown that the antioxidant activity was highest during early developmental stages in button mushrooms, *Agaricus bisporus* [30], but no explanation was provided. In our study, we propose that the concentration of some components in specific morphological parts (i.e., pilei) of the mushroom is related to biochemical processes involved in the reproduction of fungi, mainly formation and maturation of the spores (events of karyogamy, meiosis, and sporogenesis). Karyogamy is a fusion of two haploid nuclei. Meiosis is a reduction division where chromosome number in the two nuclei is reduced to half and variability is generated due to random distribution of chromosomes from the parents. Sporogenesis is like mitotic division in which chromatids separate and variability generated through crossing over of chromatids at chromosome pairing stage is released. As a result, each of the four nuclei produced after meiosis contains different combination of alleles. It would be particularly important to conduct a detailed study focused on the mycochemical characteristics in pilei, separating the gills from the remainder of the pileus, to improve the traceability process.

The traceability process presented in this manuscript allowed the identification of differences between morphological and physiological characteristics of mushroom with respect to their mycochemical compositions and distribution. This methodology is extremely important for the formulation of new nutritional products to be applied in specific diets based on fungal foods, such as diets with high protein and mineral "ash" content (use of pilei) or diets with high carbohydrates and energy value content (use of stipes). However, more research is needed in these topics of study.

4. Conclusions

Due to the differences between the mycochemical compositions of the morphological parts of mushrooms linked to their physiological stage of maturity and the type of compost (with minor significance), such characteristics have immense potential to be considered for a traceability process. This study can be used for the purpose of providing the consumer with more diversity and news products, optimizing bioactivities of composts, and allowing farmers an efficient and profitable use of the mushroom biomass.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

The authors contributed equally to this article.

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Research Article

Thiocarbamates from *Moringa oleifera* Seeds Bioactive against Virulent and Multidrug-Resistant *Vibrio* Species

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Prospect of antibacterial agents may provide an alternative therapy for diseases caused by multidrug-resistant bacteria. This study aimed to evaluate the in vitro bioactivity of *Moringa oleifera* seed extracts against 100 vibrios isolated from the marine shrimp *Litopenaeus vannamei*. Ethanol extracts at low (MOS-E) and hot (MOS-ES) temperature are shown to be bioactive against 92% and 90% of the strains, respectively. The most efficient Minimum Inhibitory Concentration (MIC) levels of MOS-E and MOS-ES against a high percentage of strains were 32 μg mL^{-1}. Bioguided screening of bioactive compounds showed that the ethyl acetate fraction from both extracts was the only one that showed antibacterial activity. Vibriocidal substances, niazirine and niazimicine, were isolated from the aforementioned fraction through chromatographic fractionation.

1. Introduction

Bacteria of *Vibrio* genus are ubiquitous in the marine environment and are part of the indigenous microbiota of marine invertebrates. Some species are recognized as human pathogens, often associated with diseases such as cholera and acute gastroenteritis [1, 2]. Vibrios are also seen as opportunistic pathogens of cultured aquatic organisms, which is one of the reasons for observing the use of antibiotics in shrimp cultivation.

Furthermore, inappropriate use of antimicrobial drugs in aquaculture has been associated with negative environmental impacts: selection of bacterial populations resistant to drugs [3, 4] and contamination of adjacent ecosystems to culture ponds.

Thus, detection of antibacterial activity in higher plants against vibrios with virulent, antimicrobial-resistant profiles is of utmost importance. In the present research, due to its high medicinal activity [5], the vibriocidal capacity of the angiosperm *Moringa oleifera* was investigated. The antimicrobial effect of *Moringa* has been researched since the 1980s and seems to be related to some specific components including pterygospermin and *Moringa* glycosides, as well as 4-(α-L-rhamnosyloxy)-benzyl isothiocyanate and 4-(α-L-rhamnosyloxy)-phenyl-acetonitrile, which act especially against *Bacillus subtilis*, *Mycobacterium phlei*, *Serratia marcescens*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella* sp., and *Streptococcus* sp. [6].

Despite the extensive scientific evidence of the bioactivity of *Moringa* against bacteria [7–10], studies on its effects against vibrios are still incipient. Thus, this research aimed to evaluate the bioactive potential from extracts of *Moringa* seeds against vibrios with virulent and multidrug antimicrobial profile.

2. Material and Methods

2.1. Origin of the Strains. One hundred *Vibrio* sp. strains isolated from the hemolymph of *Litopenaeus vannamei* shrimp were used, all of which belonged to the bacteriological

collection of the Laboratory of Environmental and Fishery Microbiology at Sea Sciences Institute (LABOMAR-UFC-Brazil). The 100 strains were phenotypically identified as *V. navarrensis* ($n = 53$), *V. brasiliensis* ($n = 15$), *V. parahaemolyticus* ($n = 10$), *V. xuii* ($n = 8$), *V. coralliilyticus* ($n = 5$), *V. cholerae* ($n = 4$), *V. neptunis* ($n = 2$), *V. alginolyticus* ($n = 1$), *V. diazotrophicus* ($n = 1$), and *V. vulnificus* B3 ($n = 1$) [11]. The enzymatic profile and antimicrobial resistance were used as a criterion for selection [12].

2.2. Botanical Material. *Moringa oleifera* seeds were collected from two specimens grown in a *campus* of the Federal University of Ceará (Pici, Fortaleza, Ceará). Separation from the fruit (pod), removal of husks, and posterior packing in plastic polyethylene bags followed the material collection.

2.3. Moringa oleifera Extracts. All extraction procedures were performed in the Department of Organic and Inorganic Chemistry at Federal University of Ceará (UFC). Part of the crushed seeds of *M. oleifera* (110 g) was subjected to three extractions with 300 mL cold hexane (PA) at 24 h intervals. After filtration and evaporation of the solvent under reduced pressure in a rotary evaporator, 15.36 g of an extract of fluid and yellowish appearance called MOS-H was obtained. The resulting cake was subjected to three cold extractions with 300 mL ethanol (PA) in 24 h intervals. After filtration and evaporation of the solvent under reduced pressure in a rotary evaporator, 11.64 g of an extract of fluid and dark appearance called MOS-E was obtained. 139 g of crushed *M. oleifera* seeds was used for hot extraction in a Soxhlet apparatus with 800 ml of hexane (PA) for 48 h. After filtration and evaporation of the solvent under reduced pressure in a rotary evaporator, 28.29 g of an extract of fluid and yellowish aspect called MOS-HS was obtained. Another extraction was carried out with 800 ml of ethanol (PA) for 48 h. After filtration and evaporation of the solvent under reduced pressure in a rotary evaporator, 13.66 g of a pasty and dark-colored appearance called MOS-ES was obtained.

2.4. In Vitro Susceptibility Testing of Moringa oleifera Extracts. Susceptibility of *Vibrio* sp. strains to the four extracts types (MOS-H, MOS-E, MOS-HS, and MOS-ES) was assessed using the disk diffusion method (DDM) and by Minimum Inhibitory Concentration (MIC) [13]. In order to proceed with the DDM, paper discs (6 mm) containing 100 μL of each extract were applied in triplicate on Mueller-Hinton plates previously seeded with bacterial cultures (10^8 UFC mL^{-1}). As negative and positive Gram control, strains of *V. parahaemolyticus* IOC and *Staphylococcus aureus* ATCC 25923, respectively, were used. For MIC determination, macrodilution technique in Mueller-Hinton broth containing 1% NaCl was used. Concentrations of 4, 8, 16, 32, and 64 μg mL^{-1} were tested, using the MOS-E oils (cold extraction with ethanol) and MOS-ES (hot extraction with ethanol) in comparison to isolates susceptible to crude extracts in the DDM test.

2.5. Chromatographic Fractionation of MOS-E and ES-MOS and Obtaining Active Fraction Ethyl Acetate (MOS-ESA). Part of the MOS-E extract (3.1 g) was adsorbed onto 7.3 g of silica gel and chromatographed on 52.5 g of silica gel in open column (Ø 5,0 cm). Elution was done in order of increasing polarity with dichloromethane (1200 ml) (MOS-ED), ethyl acetate (800 mL) (MOS-EA), and methanol (600 mL) (MOS-EM). Solvents were evaporated under reduced pressure in a rotary evaporator, yielding the following mass and yields: MOS-ED 1,920.8 mg, 61.96%; MOS-EA 231.6 mg, 7.47%; MOS 660.8 mg, 21.31%. MOS-ES extract (2.6 g) was adsorbed onto 3.9 g of silica gel and chromatographed on 48.3 g of silica gel in open column (Ø 5,0 cm). Elution was done in order of increasing polarity with dichloromethane (500 ml) (MOS ESD), ethyl acetate (700 ml) (MOS-ESA), and methanol (600 ml) (MOS-ESM). Solvents were evaporated under reduced pressure in a rotary evaporator, yielding the following mass and yields: MOS-ED 34.8 mg, 1.33%; MOS-EA 335.3 mg, 12.89%; MOS 1,978 mg, 76.07%. All fractions were subjected to antimicrobial activity test by disk diffusion method. The bioactive fraction was subjected to chromatographic fractionation by High-Performance Liquid Chromatography (HPLC) in order to isolate its active principles.

2.6. Chromatographic Fractionation of the Ethyl Acetate Fraction (MOS-ESA) by High-Performance Liquid Chromatography (HPLC) and Isolation of Active Substances. Part of the MOE-ESA active fraction (285 mg) was analyzed by HPLC in a chromatograph Shimadzu® (UFLC model) equipped with a UV-Vis detector with diode array (model SPD-M20A). Separation was performed in reverse phase conditions in semi-preparative column (C-18.5 μm), with isocratic elution using MeOH/H_2O (1 : 1) with a 4.72 mL min^{-1} flow. Chromatographic fractionation of the ethyl acetate's fraction from the MOS-ES fixed oil resulted in the detection (at 284 nm) and isolation of three main substances (Figure 1), which were obtained as whitish, amorphous solids: the compound related to the peak 1 (23.3 m; $t_r = 4.99$ min) was called MOS-ES-1, related to peak 2 (4.0 mg; $t_r = 7.06$ min) was called MOS-ES-2, and related to peak 3 (65.1 mg, $t_r = 17.45$ min) was called MOS-ES-3. Isolated substances (S1 and S3) had their structures determined by the analysis of Nuclear Magnetic Resonance (NMR) and Infrared (IR) spectral data and also by comparison to other findings described in the literature [14, 15].

3. Results

3.1. Disk Diffusion Test. From all the 100 strains tested, only five were resistant to MOS-E fixed oil; on the other hand, 36 had its growth inhibited, with average inhibition zones ranging from 13 to 15 mm (Table 1). When comparing the oils, MOS-ES tests demonstrated inhibitory bacterial rate somewhat lower, since most strains ($n = 37$) had inhibitions indicated by halos in the range 10 to 12 mm (Table 2). In addition, seven strains were resistant to the MOS-ES. The larger inhibition halo (22 to 24 mm) was observed on MOS-E test against a *V. navarrensis* strain (Table 1). Both extracts showed antibacterial effect against the Gram-positive *(Staphylococcus aureus)* and Gram-negative *(Vibrio parahaemolyticus)* controls (Tables 1 and 2). MOS-H and MOS-HS extracts showed no bioactivity against any of the isolates ($n = 100$).

Table 1: Distribution, according to the average inhibition zone (mm), of the number of strains inhibited by cold ethanol extract of *Moringa oleifera* seeds (MOS-E).

Vibrio species	*n*	Average inhibition zone (mm)						
		22–24	19–21	16–18	13–15	10–12	7–9	0
V. navarrensis	53	1	2	5	27	15	3	—
V. brasiliensis	15	—	1	2	4	2	5	1
V. parahaemolyticus	10	—	—	—	—	—	9	1
V. xuii	8	—	—	—	1	—	5	2
V. coralliilyticus	5	—	—	2	1	1	1	—
V. cholerae	4	—	—	—	2	1	1	—
V. neptunis	2	—	—	1	—	1	—	—
V. alginolyticus	1	—	—	—	1	—	—	—
V. diazotrophicus	1	—	1	—	—	—	—	—
V. vulnificus B3	1	—	—	—	—	—	—	1
V. parahaemolyticus IOC[a]	1	—	—	1	—	—	—	—
S. aureus ATCC25923[b]	1	—	1	—	—	—	—	—
Total	102	1	5	11	36	20	24	5

n: number of isolates. a: standard strain used as Gram-negative control. b: standard strain used as Gram-positive control.

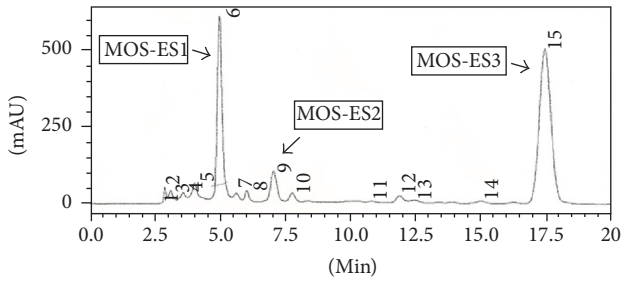

Figure 1: HPLC chromatogram analysis of an active fraction of MOS-ESA and isolation of MOS-ES-1, MOS-ES-2, and MOS-ES-3.

3.2. Minimum Inhibitory Concentration (MIC). MIC levels of the MOS-E show that 83 (90.2%) of the strains were inhibited in the presence of a 32 μg mL^{-1} concentration. Levels of 8, 16, and 64 μg mL^{-1} were able to inhibit 1, 6, and 2 strains, respectively. Also, MIC levels of the MOS-ES able to inhibit the highest percentage of strains (n = 88; 97.8%) were that of 32 μg mL^{-1}. On the other hand, only 2 (2.2%) of the strains were inhibited by a MOS-ES MIC of 16 μg mL.

3.3. Bioactivity of the Fractions. Study of the bioactivity of dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and methanol (MeOH) fractions of MOS-E and MOS-ES revealed that only EA fractions from both extracts showed antimicrobial activity (Table 3). Ethyl acetate fractions were selected and subjected to chromatographic fractionation by HPLC in order to isolate bioactive substances.

3.4. Identification of Individual Substances. From the MOS-EA fraction, three substances were identified S1 (MOS-ES-1), S2 (MOS-ES-2), and S3 (MOS-ES-3). Due to the small amount obtained from MOS-ES-2 substance (S2), it was impossible to obtain spectroscopic data enough for a structural characterization. Those two substances derived from MOE-EA fraction were also obtained in small amounts and identified based only by Thin Layer Chromatography (TLC). Furthermore, the drain of this fraction yielded 23 mg of unidentified less polar substances.

3.5. Structural Characterization of MOS-ES-1 (S1) and MOS-ES-3 (S3). S1 was isolated as a white amorphous solid and presented 129–132°C, $[M + Na]^+$ = 302,1012. RMN ^{1}H (500 MHz, CD_3OD): δ 7,28 (2H, d, J = 8,6 Hz); 7,07 (2H, d, J = 8,6 Hz); 5,43 (1H, d, J = 1,4 Hz); 3,84 (1H, dd, J = 3,4 e 9,5 Hz); 3,82 (2H, s); 3,82 (2H, m); 3,62 (1H, m); 3,46 (1H, t, J = 9,5 Hz); 1,21 (3H, d, J = 6,2 Hz). RMN ^{13}C (125 MHz, CD_3OD): δ 157,4 (C-1); 118,1 (C-2 e C-6); 130,2 (C-3 e C-5); 125,8 (C-4); 22,7 (C-7); 119,8 (C-8); 99,9 (C-1′); 72,0 (C-2′); 72,0 (C-3′); 73,8 (C-4′); 70,7 (C-5′); 18,0 (C-6′). IV ν_{max}/cm^{-1} (KBr) 3411 ($\nu_{O\text{-}H}$), 2948 ($\nu_{\text{-}C\text{-}H}$), 2250 ($\nu_{C\equiv N}$), 1611–1513 ($\nu_{C=C}$), 1227 ($\nu_{C\text{-}O}$).

Analysis of spectral data and the comparison with information in the literature [15] were used to characterize S1 as the 4-[(α-L-rhamnosyloxy)benzyl] nitrile or niazirine.

S3 was also isolated as a white amorphous solid and presented 130–133°C, $[M + Cl]^-$ = 392,0951, RMN ^{1}H (500 MHz, CD_3OD): δ 7,25 (2H, d, J = 8,7 Hz); 7,01 (2H, d, J = 8,7 Hz); 5,40 (1H, d, J = 1,8 Hz); 4,63 (2H, s); 4,46 (2H, q, J = 7,1 Hz); 3,99 (1H, dd, J = 1,8 e 3,4 Hz); 3,83 (1H, dd, J = 3,4 e 9,5 Hz); 3,62 (1H, m); 3,45 (1H, t, J = 9,5 Hz); 1,29 (3H, t, J = 7,1 Hz); 1,21 (3H, d, J = 6,2 Hz), RMN ^{13}C (125 MHz, CD_3OD): δ 157,1 (C-1); 117,6 (C-2 e C-6); 130,1 (C-3 e C-5); 133,1 (C-4); 49,0 (C-7); 192,3 (C-8); 66,9 (C-9); 14,7 (C-10); 100,2 (C-1′); 72,1 (C-2′); 72,3 (C-3′); 73,9 (C-4′); 70,6 (C-5′); 18,0 (C-6′). IV ν_{max}/cm^{-1} (KBr) 3405 ($\nu_{O\text{-}H}$), 2948 ($\nu_{\text{-}C\text{-}H}$), 1611–1513 ($\nu_{C=C}$), 1233 ($\nu_{C=S}$).

Analysis of spectral data and the comparison with information in the literature [14] were used to characterize S3 as *O*-ethyl-4-[(α-L-rhamnosyloxy)benzyl] thiocarbamate or niazimicine.

3.6. Evaluation of Antibacterial Activity of the Isolated Substances. Bioactivity results of S1 and S3 substances against ten

Table 2: Distribution, according to the average inhibition zone (mm), of the number of strains inhibited by hot ethanol extract of *Moringa oleifera* (MOS-ES).

Vibrio species	*n*	Average inhibition zone (mm)					
		19–21	16–18	13–15	10–12	7–9	0
V. navarrensis	53	1	6	14	26	5	1
V. brasiliensis	15	1	2	2	4	3	3
V. parahaemolyticus	10	—	—	—	—	9	1
V. xuii	8	—	—	—	1	7	—
V. coralliilyticus	5	—	—	1	3	1	—
V. cholerae	4	—	—	1	1	1	1
V. neptunis	2	—	—	—	2	—	—
V. alginolyticus	1	—	—	1	—	—	—
V. diazotrophicus	1	—	1	—	—	—	—
V. vulnificus B3	1	—	—	—	—	—	1
V. parahaemolyticus IOC[a]	1	—	1	—	—	—	—
S. aureus ATCC25923[b]	1	—	1	—	—	—	—
Total	102	2	11	19	37	26	7

n: number of isolates. a: standard strain used as Gram-negative control. b: standard strain used as Gram-positive control.

Table 3: Average inhibition halos of ethyl acetate (EtOAc) fractions of ethanolic extracts of *Moringa oleifera* seeds extracted cold (MOS-E) and hot (MOS-ES) with ethanol against ten *Vibrio* sp. strains isolated from the hemolymph of *Litopenaeus vannamei*.

Strain	*Vibrio* species	EtOAc (MOS-E)	EtOAc (MOS-ES)
1	*V. coralliilyticus*	14.51 ± 0.07	12.10 ± 0.08
7	*V. alginolyticus*	12.65 ± 0.06	10.52 ± 0.45
13	*V. navarrensis*	15.71 ± 0.21	14.03 ± 0.07
35	*V. diazotrophicus*	17.14 ± 0.04	16.58 ± 0.42
40	*V. navarrensis*	15.75 ± 0.35	11.82 ± 0.31
42	*V. xuii*	12.39 ± 0.24	11.80 ± 0.25
46	*V. parahaemolyticus*	8.53 ± 0.15	7.66 ± 0.12
89	*V. neptunis*	18.54 ± 0.42	11.18 ± 0.09
97	*V. cholerae*	8.46 ± 0.09	7.32 ± 0.10
98	*V. brasiliensis*	15.01 ± 0.05	14.61 ± 0.15

Vibrio sp. strains are summarized in Table 4. It is possible to attest, in a comparison between substances 1 and 3 (S1 and S3) using the size of the inhibition zone as a criterion, a greater antibacterial efficiency of S3 against all strains.

4. Discussion

Studies on the bioactive properties of *Moringa* seeds highlight multiple uses of this phanerogam, for example, turbidity removal of contaminated water by coagulation [16], biosorption of heavy metals in effluents [17], anti-inflammatory [18], and antibacterial activity against *S. aureus* and *E. coli* [19]. However, bioactivity against vibrios has not been widely researched. Thus, the high inhibition level of both extracts (95% for MOS-E; 93% for MOS-ES) (Tables 1 and 2) against antimicrobial-resistant vibrios with virulence factors must be stressed. This vibriocidal activity is congruent with the findings of Vieira et al. (2010), who investigated antibacterial activity of ethanol extracts of *Moringa* seeds and found inhibition zones ranging from 26 to 29.5 mm against classical *V. cholerae* 569B.

Vibriocidal activity of aerial parts of *M. oleifera* was reported by Peixoto et al. [20]. The authors tested the bioactivity of its extracts against standard *V. parahaemolyticus* strain and found average inhibition halos of 21.9 and 20.7 mm for ethanol and aqueous extracts, respectively.

Moringa seed extracts have also been used in tests against standard strain of *V. cholerae*. Atieno et al. [21] observed the bioactivity of hexane and methanolic extracts of *M. oleifera* and *M. stenopetala* seeds against *Salmonella* ser. Typhi, *E. coli*, and *V. cholerae*. For the species of *Vibrio*, the authors reported inhibition halo sizes of 22.2 and 13.8 mm for hexane and methanolic extracts of *M. oleifera*, respectively. The aforementioned data support the assertion that *Moringa* seeds have vibriocidal potential; however, it cannot be compared to the ones presented in the present study, since antibacterial activity in leaf extracts was not detected (MOS-H and MOS-HS).

Despite the occurrence of compounds with antibacterial activity in different parts of *Moringa* is being reported in the scientific literature since the early 1980s [22], their use for epizootic purposes has been little explored. The data obtained by the disk diffusion test and the results of the Minimum Inhibitory Concentration (MIC) serve as evidence of the high antibacterial potential of ethanol extracts of MOS-E and MOS-ES against vibrios.

Satisfactory results in disk diffusion tests and the definition of MIC levels were pivotal for the decision of carrying out complementary studies, starting with the bioguided screening of bioactive compounds. The selective antimicrobial effect of *Moringa*'s crude extract fractions was also verified by Nantachit [23]. The author noted that the dichloromethane fraction was active against *E. coli*.

Guevara et al. [24] reported similar structures described in this study, namely, *O*-ethyl-4-(α-L-rhamnosyloxy) benzyl carbamates, 4(α-L-rhamnosyloxy)-benzyl isothiocyanate,

Table 4: Average size of inhibition halos from substances 1 (S1) and 3 (S3) isolated from fractions of ethyl acetate (EtOAc) of *Moringa* seed in cold extraction with ethanol (MOS-E) and in hot extraction with ethanol (MOS-ES), against ten *Vibrio* sp. strains isolated from the hemolymph of *Litopenaeus vannamei*.

Strains	Species	Fraction of EtOAc MOS-ES		
		S1	S2	S3
1	*V. coralliilyticus*	8.11 ± 0.09	nt	14.04 ± 0.02
7	*V. alginolyticus*	8.32 ± 0.02	nt	15.98 ± 0.09
13	*V. navarrensis*	9.76 ± 0.43	nt	19.89 ± 0.11
35	*V. diazotrophicus*	7.42 ± 0.24	nt	21.21 ± 0.24
40	*V. navarrensis*	9.77 ± 0.15	nt	17.89 ± 0.12
42	*V. xuii*	8.76 ± 0.29	nt	20.33 ± 0.06
46	*V. parahaemolyticus*	7.66 ± 0.45	nt	9.90 ± 0.07
89	*V. neptunis*	7.14 ± 0.11	nt	20.11 ± 0.03
97	*V. cholerae*	7.30 ± 0.27	nt	10.15 ± 0.18
98	*V. brasiliensis*	7.99 ± 0.02	nt	18.75 ± 0.06

nt: not tested.

niazimicine, 3-*O*-(6′-*O*-oleoyl-*β*-D-glucopyranosyl)-*β*-sitosterol, *β*-sitosterol-3-*O*-*β*-D-glucopyranoside, niazirine, *β*-sitosterol, and glycerol-1-(9-octadecanoate) by studying the ethanol extracts of *Moringa* seeds. The authors mentioned the bioactivity potential of niazimicine and niazirine. Furthermore, Gilani et al. [25] isolated four bioactive compounds out of the bioguided fractionation of ethanolic extracts of *M. oleifera* leaves and also found compatible substances with those described in the present study: niazinine A, niazinine B niazinine, niazimicine, and niazinine A + B.

In this context, Padla et al. [10] isolated 4-(alpha-L-Rhamnosyloxy)benzyl isothiocyanate and 4-(4′-O-acetyl-alpha-L-rhamnosyloxy)-benzyl isothiocyanate from *Moringa oleifera* seeds and demonstrated that both substances were bioactive against Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis*. In the present study, we isolated from *Moringa* seeds the substances (1) 4-[(*α*-L-rhamnosyloxy)benzyl]nitrile (niazirine) and (2) *O*-ethyl-4-[(*α*-L-rhamnosyloxy)benzyl] thiocarbamate (niazimicine).

The compounds *O*-ethyl-*p*-hydroxybenzene carbamate and *O*-methyl-4-[(2′,3′,4′-tri-*O*-acetyl-*α*-L-rhamnosyloxy)benzyl] thiocarbamate must also be added to the previously mentioned substances as isolates from leaf extracts of *M. oleifera* [26, 27]. The addition of methanol or ethanol to isothiocyanate is considered a pathway to the synthesis of thiocarbamates glycosides in *Moringa* [26].

Since the 1990s, biological activities of pharmacological interest concerning nitriles isolated from *Moringa* have been described, and its antitumor [28] and antihypertensive [14] functions are noteworthy, along its use in the prevention of carcinogenesis [24].

The presence of niazimicine in *Moringa* seeds is often cited as a strong antitumor factor. It can be used as a prophylactic or therapeutic measure while treating HSV-1 infections [29].

Although this is not the first description of carbamate glycosides in constituent parts of the *M. oleifera* species, its unexploited vibriocidal potential must be highlighted. In the present study, niazirine and niazimicine showed high antibacterial efficiency against vibrios with phenotypic profiles compatible to the presence of virulence factors (exoenzymes and *β*-hemolysis producers), cross-resistance to *β*-lactams, and mono/multiresistance to antibiotics. These findings suggest a new class vibriocidal compounds. Moreover, the results are consistent with the demand for new alternatives to antibacterial drugs in order to mitigate the impact caused by the indiscriminate use of antimicrobials in aquaculture.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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