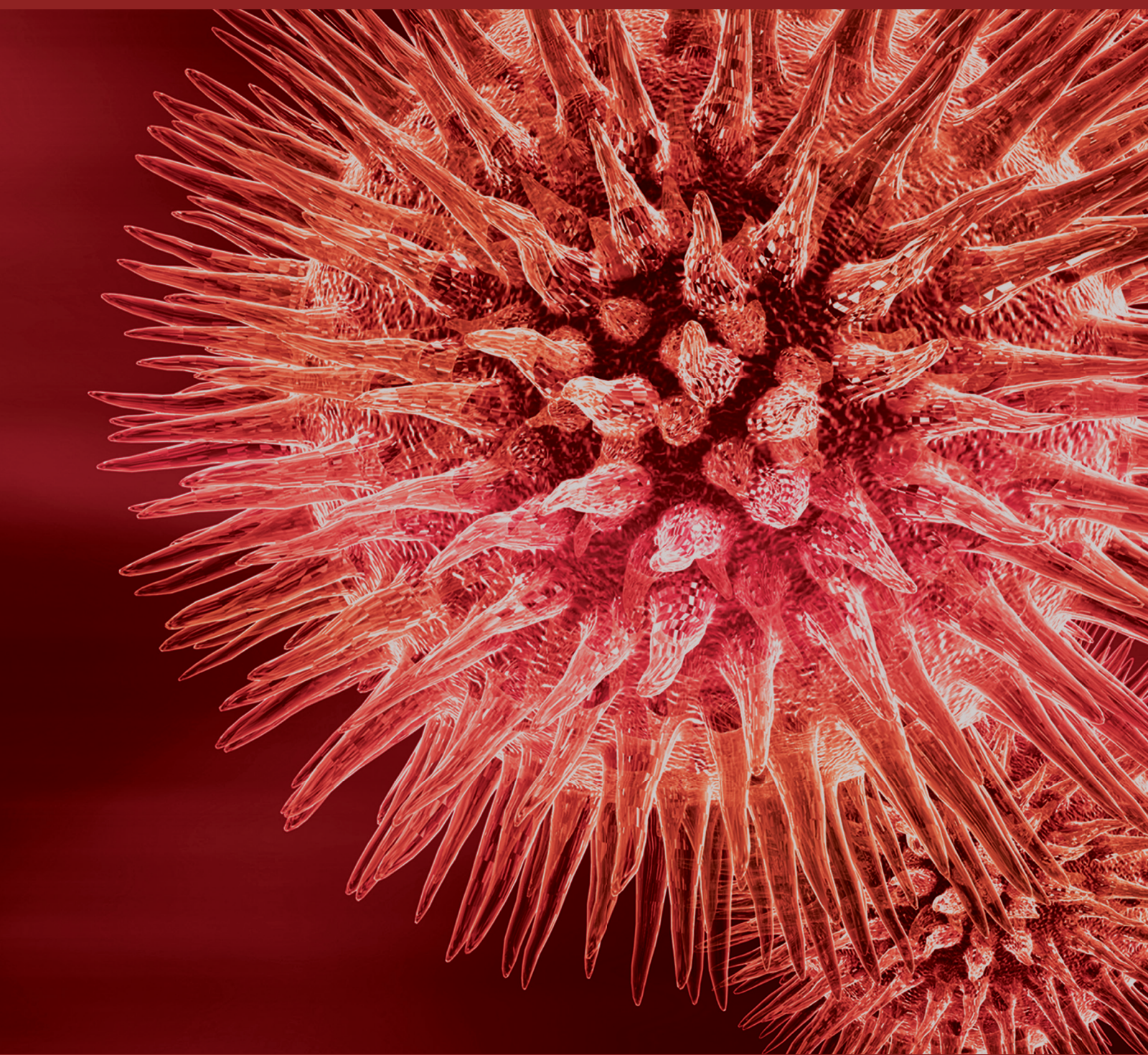
BioMed Research International

Bioactive Natural Products 2016

Guest Editors: Yiannis Kourkoutas, Nikos Chorianopoulos, Kimon A. G. Karatzas, and Ibrahim M. Banat



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Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 9749305, 2 pages
http://dx.doi.org/10.1155/2016/9749305

Editorial

Bioactive Natural Products 2016

Yiannis Kourkoutas,[1] Nikos Chorianopoulos,[2] Kimon A. G. Karatzas,[3] and Ibrahim M. Banat[4]

[1] *Laboratory of Applied Microbiology and Biotechnology, Department of Molecular Biology and Genetics, Democritus University of Thrace, 68100 Alexandroupolis, Greece*
[2] *Institute of Technology of Agricultural Products, Hellenic Agricultural Organization-DEMETER, 15310 Athens, Greece*
[3] *Department of Food and Nutritional Sciences, University of Reading, Reading RG6 6AD, UK*
[4] *School of Biomedical Sciences, Pharmaceutical Science and Practice Research Group, Ulster University, Cromore Road, Coleraine, County Londonderry, UK*

Correspondence should be addressed to Yiannis Kourkoutas; ikourkou@mbg.duth.gr

Received 25 August 2016; Accepted 25 August 2016

Various preservatives are being used to ensure that manufactured foods remain safe and unspoiled. The excessive use of chemical preservatives, many of which are believed to exert potential carcinogenic activities, as well as residual toxicity, has resulted in mistrust among European consumers. Since consumers need to feel reassured that they consume safe foods, an increasing pressure on food manufacturers and authorities is applied with respect to the elimination of harmful chemical preservatives from food preparations, strengthening the research activity towards the discovery of alternative agents [1, 2]. In this context, the use of natural products presents an intriguing case. They exhibit a wide range of biological and pharmacological activities and are considered to have beneficial effects in human nutrition [1, 2]. Currently, natural products are used in food preparations mainly as flavouring agents [3] and by cosmetic and pharmaceutical industries as fragrances and functional additives [4]. These natural substances have been suggested for use in foodstuffs [5] because they were found to display significant antimicrobial properties against bacteria and fungi [1, 2, 6–8].

The main objective of this special issue was to provide a number of documents focused on the facts, applications, and challenges of bioactive natural products and present the methodologies in use to evaluate the effectiveness of such products. Moreover, the challenges that the industry faces with respect to the use of bioactive natural products as antimicrobial agents in terms of safety and microbial growth prevention are discussed. Hence, the antioxidant, antimicrobial, anti-HIV, and cholinesterase inhibitory activities of aqueous and alcoholic extracts from leaves, stems, and flowers of *Euphorbia characias* were evaluated. It was suggested that *Euphorbia characias* extracts represent a good source of natural bioactive compounds which could be useful for pharmaceutical application, as well as in food systems for the prevention of the growth of food-borne bacteria and to extend the shelf-life of processed foods. Furthermore, it was evident from the findings of another study that *Pterospartum tridentatum* and *Mentha pulegium* may constitute an important reservoir of phytochemicals with antiradical activity and antibacterial capacity and thus they might be used in a preventive way or in a combined pharmaceutical and antibiotic therapy against pathogenic bacteria.

Sulfated polysaccharides extracted from five seaweed samples collected or cultivated in Mexico were tested to evaluate their effect on measles virus *in vitro*. The synergistic effect would allow reduction of the treatment dose and toxicity and minimization of the induction of antiviral resistance. Sulfated polysaccharides of the tested seaweed species appeared as promising candidates for the development of natural antiviral agents.

The main types of natural products that have been characterized as splicing inhibitors, the recent advances regarding molecular and cellular effects related to these

molecules, and the applications reported so far in cancer therapeutics were also summarized in the current special issue. Likewise, hyperoside, an active compound found in plants of the genera *Hypericum* and *Crataegus*, is reported to exhibit antioxidant, anticancer, and anti-inflammatory activities. The results presented suggested that hyperoside may have potential as a therapeutic agent for the treatment of liver fibrosis.

Polyphenols from diverse sources have shown anti-inflammatory activity [9, 10]. In atherosclerosis, macrophages play important roles including matrix metalloproteinases synthesis involved in degradation of matrix extracellular components affecting the atherosclerotic plaque stability. These data suggested a potential role of polyphenols from Chilean propolis in the control of extracellular matrix degradation in atherosclerotic plaques. Besides, the results of another investigation indicated that Chilean propolis has a dose-dependent effect on the inhibition of genes involved in *Streptococcus mutans* virulence and adherence through the inhibition of glucosyltransferases, showing an anticariogenic potential of polyphenols from propolis beyond *S. mutans* growth inhibition.

S. mutans, with the ability of high-rate acid production and strong biofilm formation, is considered the predominant bacterial species in the pathogenesis of human dental caries [11, 12]. In this vein, LongZhang Gargle, completely made from Chinese herbs, was investigated for its effects on acid production and biofilm formation by *S. mutans*. The findings suggested that LongZhang Gargle may be a promising natural anticariogenic agent as it suppressed planktonic growth, acid production, and biofilm formation against *S. mutans*.

Moreover, the production and incorporation of conjugated linoleic acids (CLA) in biomass were reported for the first time. *B. breve* WC 0421 stored CLA in the form of free fatty acids, without changing the composition of the esterified fatty acids, which mainly occurred in the plasmatic membrane. Finally, 19 *Streptococcus thermophilus* strains with high exopolysaccharide production abilities were isolated from traditional Chinese fermented dairy products. The exopolysaccharide and viscosity of milk fermented by these 19 isolates were investigated. It was demonstrated that the selected higher exopolysaccharide producing cultures could be used as yogurt starter cultures reducing the amount of added stabilizer, which can compare favorably with the imported commercial one.

Yiannis Kourkoutas
Nikos Chorianopoulos
Kimon A. G. Karatzas
Ibrahim M. Banat

References

[1] S. Burt, "Essential oils: their antibacterial properties and potential applications in foods—a review," *International Journal of Food Microbiology*, vol. 94, no. 3, pp. 223–253, 2004.

[2] C. C. Tassou, P. N. Skandamis, N. G. Chorianopoulos, and G.-J. E. Nychas, "Herbs, spices and their active components as natural preservatives in food preparation," in *Handbook of Herbs and Spices*, K. V. Peter, Ed., vol. 2, chapter 2, pp. 17–50, CRC Press Woodhead, 2nd edition, 2012.

[3] K. Oosterhaven, B. Poolman, and E. J. Smid, "S-carvone as a natural potato sprout inhibiting, fungistatic and bacteristatic compound," *Industrial Crops and Products*, vol. 4, no. 1, pp. 23–31, 1995.

[4] K. Bauer and D. Garbe, *Common Fragrance and Flavor Materials: Preparation, Properties and Uses*, VCH Verlagsgesellschaft, Weinheim, Germany, 1985.

[5] R. S. Farag, Z. Y. Daw, F. M. Hewedi, and G. S. A. El-Baroty, "Antimicrobial activity of some Egyptian spice essential oils," *Journal of Food Protection*, vol. 52, no. 9, pp. 665–667, 1989.

[6] D. E. Conner and L. R. Beuchat, "Sensitivity of heat-stressed yeasts to essential oils of plants," *Applied and Environmental Microbiology*, vol. 47, no. 2, pp. 229–233, 1984.

[7] D. E. Conner and L. R. Beuchat, "Effects of essential oils from plants on growth of food spoilage yeasts," *Journal of Food Science*, vol. 49, no. 2, pp. 429–434, 1984.

[8] A. Galli, L. Franzetti, and D. Briguglio, "Antimicrobial properties *in vitro* of essential oils and extract of spices used for food," *Industrial Alimentaries*, vol. 24, pp. 463–466, 1985.

[9] H. Pratsinis, D. Kletsas, E. Melliou, and I. Chinou, "Antiproliferative activity of Greek propolis," *Journal of Medicinal Food*, vol. 13, no. 2, pp. 286–290, 2010.

[10] M. Viuda-Martos, Y. Ruiz-Navajas, J. Fernández-López, and J. A. Pérez-Álvarez, "Functional properties of honey, propolis, and royal jelly," *Journal of Food Science*, vol. 73, no. 9, pp. R117–R124, 2008.

[11] W. H. Bowen and H. Koo, "Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms," *Caries Research*, vol. 45, no. 1, pp. 69–86, 2011.

[12] R. Ma, M. Sun, S. Wang et al., "Effect of high-fructose corn syrup on the acidogenicity, adherence and biofilm formation of *Streptococcus mutans*," *Australian Dental Journal*, vol. 58, no. 2, pp. 213–218, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 3278359, 15 pages
http://dx.doi.org/10.1155/2016/3278359

Review Article

Use of Plant Extracts as an Effective Manner to Control *Clostridium perfringens* Induced Necrotic Enteritis in Poultry

J. M. Diaz Carrasco,[1,2] L. M. Redondo,[1,2] E. A. Redondo,[1,2] J. E. Dominguez,[1,2] A. P. Chacana,[1] and M. E. Fernandez Miyakawa[1,2]

[1]*Instituto de Patobiología, Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria, Calle Las Cabañas y Los Reseros s/n, Casilla de Correo 25, 1712 Castelar, Buenos Aires, Argentina*
[2]*Consejo Nacional de Investigaciones Científicas y Técnicas, Rivadavia 1917, 1033 Ciudad Autónoma de Buenos Aires, Argentina*

Correspondence should be addressed to M. E. Fernandez Miyakawa; fernandezmiyakawa.m@inta.gob.ar

Received 19 March 2016; Revised 3 August 2016; Accepted 14 August 2016

Academic Editor: Yiannis Kourkoutas

Necrotic enteritis (NE) is an important concern in poultry industry since it causes economic losses, increased mortality, reduction of bird welfare, and contamination of chicken products for human consumption. For decades, the use of in-feed antimicrobial growth promoters (AGPs) has been the main strategy to control intestinal pathogens including *Clostridium perfringens* (CP), the causative agent of NE. However, the use of AGPs in animal diet has been linked to the emergence and transmission of antimicrobial resistance through food-borne microorganisms, which has led to the ban of AGPs in many countries. This scenario has challenged the poultry industry to search for safer alternative products in order to prevent NE. In this context, the utilization of natural plant extracts with antimicrobial properties appears as a promising and feasible tool to control NE in chicken. In this paper, we review the scientific studies analyzing the potential of plant extracts as alternative feed additives to reduce NE in poultry, with focus on two types of plant products that arise as promising candidates: tannins and essential oils. Some of these products showed antimicrobial activity against CP and coccidia *in vitro* and *in vivo* and are able to increase productive performance, emulating the bioactive properties of AGPs.

1. Necrotic Enteritis in Chickens and *Clostridium perfringens*

Necrotic enteritis (NE) is a worldwide extended disease caused by *Clostridium perfringens* (CP). The disease was first reported in 1961 and from that moment onwards many outbreaks have been documented in all countries where intensive poultry breeding is carried out [1–3]. NE has different presentations: sudden, clinical, and subclinical; among them, subclinical NE is one of the main causes of economic loss for the poultry industry. The estimated prevention cost of NE is U$S 0.05 per chicken with a total global loss of nearly U$S 2 billion per annum [4]. CP is a ubiquitous Gram-positive, spore forming, toxigenic, anaerobic bacterium, generally classified according to the production of five major toxins [5]. In poultry industry, CP type A is the most significant, since it is capable of producing many toxins responsible for the disease [6].

CP can be found in the environment in soil, feces, feed, and poultry litter and in the intestines of animals as part of the normal gut microbiota [7]; thus, the presence of CP by itself does not necessarily imply the occurrence of the disease. NE reports showed that the disease is mostly found in 2- to 5-week-old chickens and the incidence of the disease can be low as well as high, as most CP strains are relatively innocuous. Clinical presentation of NE in outbreaks depends on a complex interaction of the microorganism with other predisposing factors such as diet, the presence of other microorganisms, and the immunological status of the birds [2, 4, 8, 9]. The ingredients included in diet, and even changes in it, may affect both physical and chemical properties of intestinal contents. Presence of *Eimeria* spp.

and viral infections are important NE-predisposing factors as they lead to the destruction of enterocytes and increase the mucosal secretion. Stress, immunosuppression, or medical treatment can also induce changes in the composition of the microbiota. All of these factors contribute to facilitating the mucosal colonization for pathogenic CP strains, which are able to degrade the mucus and colonize the gut. When this happens, the bacteria begin to synthesize enzymes, acting in the basement membrane and lateral part of the enterocytes, spread through the lamina propria, and induce damage to endothelial cells [6, 10–13].

Clinical signs of NE include decreased appetite, diarrhea, weight loss, and several nonspecific signs that can be found even without any gut lesion [1, 2]. Gross lesions are diverse, usually affecting the small intestine and liver; jejunum and ileum are the most affected portions of the gut. Intestines are visualized with gas as well as bleeding and blood clots can be found in their contents. The mucosa can be either thickened by edema or thinned by epithelial erosion [1, 2, 6, 8] and sometimes a yellow or green pseudomembrane adhered to mucosa can be found. It is likely to find in the same animal both changes in different parts of the intestine [1–3, 6]. In the liver, necrotic foci and cholecystitis can appear dispersed throughout the parenchyma. These injuries are commonly associated with a subclinical presentation of the disease [2, 14]. Microscopic lesions comprise shortening villous, epithelium detachment in the apical portion, and also intense mucosal necrosis extending to the crypts or submucosa. Bacilli can sometimes be seen in the mucosa or in lamina propria. The inflammatory cell infiltration in lamina propria is a mixed type and more evident in some cases than others [1–3, 8].

The treatment of NE outbreaks are based on antimicrobial therapy with the aim of diminishing economic losses. Bacitracin, lincomycin, virginiamycin, penicillin, and tylosin have been the antibiotics of choice worldwide. However, the most important losses are associated with subclinical presentation of NE, which has been controlled by the use of subtherapeutic doses of antimicrobials in feed [15]. As it happens with several microorganisms, CP susceptibility to antibiotics has declined over the years.

2. Antimicrobial Control of NE and Alternatives

Control of NE and predisposing factors in poultry often becomes a really complex labor. For many years, antimicrobial therapy was the first, and most of the times the only, strategy to control CP-induced NE. Therapeutic antimicrobials administered at high doses over a short period of time are generally used to control acute outbreaks [16]. To control subclinical NE presentations, antimicrobial growth promoters (AGPs) are generally used. Although these compounds were first included into feed to improve growth rate and feed conversion efficiency in poultry [17], they are now used mostly to control CP and other Gram-positive pathogens [18]. Bacitracin (a polypeptide antimicrobial) and virginiamycin (a streptogramin) are nowadays two commonly used AGPs in poultry production to improve feed conversion ratios, body weight gain, and well-being of animals [16]. Despite the longtime use of AGPs, mechanisms involved in the improvement caused by the administration of subtherapeutic doses of the antimicrobials in broilers flocks are far from being fully understood. Proposed potential mechanisms include regulation of digestive functions and gut immunological responses [19]. The most accepted mechanism is that AGPs modulate gut microbiota, which plays a critical role in maintaining the host health [20].

The use of AGPs at subinhibitory doses for long periods of time is particularly favorable to select antimicrobial-resistant microorganisms. In countries where AGPs are still used, reduced susceptibility of poultry CP strains was reported [21]. Continuous administration of AGPs may lead to changes in the bacterial environment by eliminating susceptible strains and allowing antimicrobial-resistant bacteria (i.e., those with lower susceptibility to AGPs) to survive and predominate [22]. Furthermore, continuous administration of APGs in the feed may cause cross-resistance to therapeutic antimicrobials [21]. Antimicrobial resistance together with a gradual decrease in sensitivity to anticoccidials by some strains of *Eimeria* spp. (an important predisposing factor to NE) has exacerbated the presence of such CP strains.

Establishment of resistant and pathogenic CP strains in poultry farms also may lead to the transfer of resistant bacteria and their resistant factors from animals to humans. Studies considering *Campylobacter* spp., *E. coli,* and *Enterococcus* spp. suggest that the use of nontherapeutic antimicrobial is linked to the propagation of multidrug resistance, including resistance against drugs that were never used in the farm [23].

The impact of AGPs on the appearance and transmission of antimicrobial-resistant bacteria has been the aim of several investigations and has led to their ban in the European Union in 2006 [24]. After these measures were taken in Europe [25], the consequence was the increase in NE incidence together with an increase in the use of therapeutic antimicrobials to control diseases [26–28]. The European experience and recent moves toward reduction or termination of AGPs in North America [25] have pressured the poultry industry to search for suitable alternatives in order to control NE outbreaks, reduce productive consequences of subclinical presentation under conditions of average management of the farms [26], and diminish resistance to antimicrobials. Natural substances with antimicrobial properties can be an essential part of this control strategy.

In this context, an increasing number of antimicrobial-free tools and strategies have been developed for prevention and control of CP-induced NE in poultry [27, 29]. Any alternative to AGPs is expected to be safe to the public health, cost-effective, and friendly to the environment together with antimicrobial activity to be considered as a viable option [30]. Proposed alternatives include vaccines, immunomodulatory agents, bacteriophages and their lysins, antimicrobial peptides, pro-, pre-, and synbiotics, plant extracts, inhibitors for bacterial quorum sensing, biofilm, and virulence and feed enzymes [27, 31]. Vaccination against the pathogen and the use of probiotic and prebiotic products have been suggested but at the present time are not yet available for practical use in

the farms. One of the most promising alternatives to AGPs is the use of plant extracts added to the diet to improve nutrition and health in farm animals and to control enteric clostridial diseases; these additives have been used for many years in poultry and their efficiency has been demonstrated [21, 32].

3. Plant Extracts

Plant materials are used widely in traditional systems of medicine [55]. Plant extracts, also known as phytobiotics, have been exploited in animal nutrition, particularly for their antimicrobial, anti-inflammatory, antioxidant, and antiparasitic activities [56–58].

Many plants have beneficial multifunctional properties derived from their specific bioactive components. Biologically active components of plants are mostly secondary metabolites, such as terpenoids, phenolics, glycosides, and alkaloids, present as alcohols, aldehydes, ketones, esters, ethers, and lactones [17]. These secondary metabolites may have a protective function in vegetal tissues. Final effect on animals will depend on both the combination and concentration of these bioactive molecules and minor changes in these aspects will explain why some of these compounds can have either beneficial or detrimental effects in animals [59–61].

Plant extracts are generally considered safe and effective against certain bacteria. They are extensively used in feed as growth promoters and health protectants [62, 63], particularly in Asian, African, and South American countries, and in recent years are gradually being used in developed countries. Effects of phytogenic feed additives on the production performance of poultry are also reported [57]. It is considered that plant extracts possess antibacterial activities when their minimum inhibitory concentrations range from 100 to 1000 μg/mL according to *in vitro* bacterial susceptibility tests [64].

In the last years, several studies reported that the use of raw plant extracts and derived phytogenic compounds as poultry feed supplements [65] may have a positive effect on birds health and productivity. NE gross lesions and intestinal CP burden is a parameter commonly used to evaluate the effects of including plant extract in chicken diets [35, 47]. Different plant extracts may have direct inhibitory effect either on CP vegetative cells or in some of the toxins involved in NE pathogenesis [32].

Useful antimicrobial phytochemicals can be divided into several categories, such as polyphenols/tannins, essential oils (EOs), alkaloids, and lectins/polypeptides [66]. Phytochemicals exert their antimicrobial activity through different mechanisms. For example, (1) tannins act by iron deprivation and interactions with vital proteins such as enzymes [67]; (2) cryptolepine, the main indoloquinoline alkaloid, is a DNA intercalator and inhibitor of topoisomerase [68]; and (3) saponins form complexes with sterols from the membrane of microorganisms causing damage and consequent collapse of the cells [69]. EOs have long been recognized for their antimicrobial properties [70], but their precise antimicrobial mechanism is poorly understood. In fact, the antimicrobial activities of many plant extracts have not been clearly elucidated yet [71]. *In vivo* observations support the assumption that general antimicrobial potential of phytogenic feed additives is due to a substantial reduction of intestinal pathogen pressure [66].

In the global context to reduce or avoid the use of antimicrobials in animal production, not only biological activity of alternatives to AGPs but also the suitability of the active principles to be produced and applied at the industrial level should be considered. In the last years, two types of plant-derived extracts emerged as promising candidates to be used in poultry industry to control NE: tannins and essential oils.

4. Tannins

Tannins are polyphenolic secondary metabolites found in almost all the parts of the plants and therefore present in most animal diets. Tannins are generally classified into two groups based on their chemical structure: hydrolyzable tannins (HT) which are present in plants as gallotannins or ellagitannins [72] and condensed tannins (CT), the most common type of tannins found in forage, which are polymers of flavonol units [73]. However, tannins have an enormous structural diversity, with molar masses ranging from 300 to 20,000 Da [74]. Multiple biological properties including anticancer [75, 76] and antimicrobial [67, 77, 78] activities have been attributed to tannins [79], mainly due to their ion-complexation, protein-binding, and antioxidant capabilities [80–82].

Scientific evidence suggests significant potential for the use of tannins to enhance nutrition and animal health in both cattle and poultry [30, 62, 65, 83]. Although tannins have been generally considered as antinutritional factors for monogastric animals [59, 84, 85], it is now known that their beneficial or detrimental properties depend on both tannin nature (i.e., plant source, chemical structure, and astringency) and animal factors (i.e., animal species, physiological state, and diet composition) [39, 57, 61, 66, 86, 87] as well as administration factors such as dosage and formulation. The antinutritional effects attributed to tannins are mostly based on assays performed with elevated concentrations of CT or plant parts with elevated tannin content, as may be the case of tannic acid in sorghum.

In recent years, many reports showed that moderate tannin concentrations from diverse vegetal sources can improve not only nutrition but also health status in monogastric farm animals, including poultry. Furthermore, inclusion of polyphenol-rich plant extracts has been found to improve weight gain/feed ratio in growing pigs [88]. In poultry, Schiavone et al. (2008) [34] showed that the use of a chestnut extract has a positive influence on growth performance if included in the diet up to 2 g/kg of dry matter and also a significant decrease in total nitrogen in the litter was observed. This supports the observation that administration of chestnut tannins often results in firmer droppings, which positively affects the litter status thus improving the overall health status and welfare of chickens in intensive production

systems. Similarly, other authors have observed that the inclusion of phenolic compounds in diet enhanced growth performance, decreased lipid oxidation, decreased cholesterol value, and increased beneficial fatty acids content in broiler chickens [89]. However, other tannin formulations are unable to enhance growth performance but produce different beneficial effects in productive aspects of chicken physiology, including delay of meat lipid oxidation [37, 41, 90–92], increase of protein digestibility and feed conversion [38, 42], enhancement of gut health and microbiota biodiversity [40, 93, 94], and higher capacity to overcome deleterious effects of persistent heat stress [95, 96].

Numerous *in vitro* and *in vivo* studies have verified the activity of tannins against several types of intestinal pathogens including helminthes [97–100], coccidia [33, 36, 101], viruses [45, 77], and bacteria [102–104] with particular interest in *Salmonella* Typhimurium [44, 105, 106], *Campylobacter jejuni* [43], and CP [32, 35], which are major disease-causing or food-borne bacteria in poultry [107].

Incidence of CP-associated NE in poultry has considerably increased in countries that stopped the use of AGP [26, 108]. Elizondo et al. (2010) [32] showed that two of the most common sources of tannins, chestnut (*Castanea sativa*, HT) and quebracho (*Schinopsis lorentzii*, CT), extracts have *in vitro* antibacterial and antitoxin activities against CP and its toxins. Similarly, other authors have observed *in vitro* antimicrobial activity against CP using tannins derived from chestnut and grape products [40, 109].

These findings are consistent with recent *in vivo* studies that tested the effect of tannins added to diet of chickens on *Eimeria* spp. and CP. Tosi et al. (2013) [35] found that the addition of a chestnut tannin extract significantly reduces the counts of CP and macroscopic gut lesions in broiler chickens challenged with coccidia and CP. Subsequent results confirm the effects of chestnut and quebracho tannins in a broiler NE model reducing the incidence and severity of gross lesions and improving the productive performance of the chicken [110]. Although chestnut tannins show strong bactericidal activity against CP, most ingested HT are degraded in the intestinal tract and do not remain in the feces. In contrast, quebracho-derived CT have lower antibacterial activity but most of the administered tannins remain in the feces and therefore in the litter. Combination of CT and HT may be used to readily diminish the intestinal CP load and also to avoid the reinfection by controlling the environmental contamination (i.e., feces and bedding). In agreement with this, Cejas et al. (2011) [36] found that quebracho tannins also decreased oocyst excretion in *Eimeria* spp. challenged broiler chicks. Consistent results were also obtained with other tannin-rich plant extracts. McDougald et al. (2008) [33] showed that inclusion of muscadine pomace in the diet significantly reduced intestinal lesion scores and mortality rates using a similar NE model of broilers challenged with *Eimeria* spp. and CP. Dietary supplementation of chicken diet with a polyphenol extract of *Curcuma longa* enhanced coccidiosis resistance as demonstrated by increased body weight gains, reduced fecal oocyst shedding, and decreased gut lesions, and it was also shown to attenuate coccidia-induced inflammation-mediated gut damage [101]. *Artemisia annua* leaves, which contain both EOs and tannins [111], showed antimicrobial activity against CP proliferation *in vitro* and were able to reduce intestinal load and severity of NE-related small intestinal lesions *in vivo* [47].

A recent work reported that chestnut extracts improve lactobacilli tolerance to gastric transit and tolerance to low pH values and bile juice salts, indicating that tannins may also be used in combination with probiotics for synergist enhancement of gut health [112]. An additional benefit of the use of tannins as alternative AGPs in poultry is the difficulty of CP to multiply and develop resistance in the presence of such diverse range of molecules these plant compounds contain [21].

Although tannins can have beneficial effects on poultry performance and gut health, still little is known about the mechanisms involved in their final *in vivo* antimicrobial and growth promoter effects. Some authors suggest that low concentration of tannins can improve palatability of feed thus increasing performance of monogastric animals by stimulating feed intake [66]. Nevertheless, antimicrobial activity has been linked to their biochemical properties including metabolism inhibition by enzyme complexation and iron deprivation [67, 80, 113, 114]. Iron is essential for most pathogenic bacteria and tannic acid has been shown to function like a siderophore that chelates iron from the medium, making it unavailable for some microorganisms but without affecting lactic acid bacteria [102]. Regarding the growth promotion effect, some of the explained modes of action for antimicrobials may help to define tannin mechanisms. How antimicrobials increase performance is not clear, but possible mechanisms include reduction in total bacterial load, suppression of pathogens, thinning of the mucosal layer, and direct modulation of the immune system [115]. In general terms, like AGPs, tannins may be involved in the modulation of gut microbiota and its highly complex interactions. As reported by several authors, Gram-positive bacteria seem to be more sensitive to tannin-rich plant extracts [104, 116]. Regardless of the mode of action, chemical characteristics of the tannins are highly variable and different types of tannins can be found in a single plant extract. The origin of the plant extract added to the feed will be determinant in the final impact on microbiota and consequently in growth performance. Table 1 summarizes the effects of different tannin-rich plant extracts on performance and health of poultry *in vivo* and their antimicrobial activities *in vitro*.

The use of tannins appears as an attractive alternative to control NE since these natural products do not leave residues in poultry-derived products and given the complexity of their structures and bioactive principles it is more difficult for tannins to induce selection of resistant microorganisms in comparison with AGPs. Among the wide range of tannin-rich plant extracts with beneficial effects in poultry nutrition and health, chestnut and quebracho tannins are probably the most readily available commercial products that are being used and there are a significant number of publications that demonstrate their properties. Further research needs to be done in order to elucidate the mechanisms associated with

Table 1: *In vivo* and *in vitro* effects of tannin-rich plant extracts on performance and health of poultry.

Tannin source and type	Major findings		Doses (g/kg)	References
	In vivo effect of tannin-rich plant extracts on performance and health of poultry			
	Performance effects	Health effects		
Muscadine pomace (HT and flavonoid; *Vitis rotundifolia*)	Birds given 50 g/kg had poorer average live weight. Extracts at 5 and 20 g/kg improved BWG after challenge with CP in a NE model.	Treated birds showed increased resistance to coccidia infection and lower lesion scores after challenge with coccidia (*Eimeria* spp.). Extracts at 5 and 20 g/kg reduced mortality and lesion scores using an established model of NE.	5, 20, and 50	[33]
Chestnut (HT; *Castanea sativa*)	Chestnut extract did not influence feed digestibility, carcass quality, or N balance and showed a positive influence on growth performance up to 2 g/kg.	Carcass analysis showed no gross lesions in organs and no significant differences in thigh and breast composition among groups.	1.5, 2, and 2.5	[34]
Chestnut (HT)	—	Chestnut extract reduced the counts of CP and macroscopic gut lesions in a NE model challenged with coccidia and CP. Results were more pronounced at higher tannins doses.	1.5, 3, 5, and 12	[35]
Quebracho (CT; *Schinopsis lorentzii*)	Quebracho supplementation significantly increased BWG and intestinal V : C ratio.	Birds challenged with coccidia (*Eimeria* spp.) and treated with quebracho extract showed decreased oocyst excretion. No differences in mortality or intestinal lesion were observed.	100	[36]
Grape pomace (CT)	No negative effects on growth performance, digestive organ sizes, or protein digestibility were detected at levels up to 60 g/kg to 42 d of age.	Antioxidant activities in diet, excreta, ileal content, and breast muscle were increased by grape pomace concentrate.	15, 30, and 60	[37]
Grapeseed (CT; *Vitis vinifera*)	Inclusion of grapeseed extract up to 3.6 g/kg did not affect growth performance and increased protein and polyphenol digestibility.	Grapeseed extract caused a significant increase of antioxidant activity in diet and excreta.	0.6, 1.8, and 3.6	[38]
Grapeseed, mimosa (CT), and cranberry (CT + HT)	Mimosa extract reduced BWG and FCR while cranberry reduced feed digestibility.	Mimosa and cranberry supplements decreased minor VFA concentration, which is probably associated with inhibition of microbiota activity.	373, 258, and 24, respectively	[39]
Grape pomace and grapeseed (CT)	Grapeseed diet showed decreased BWG. Both extracts reduced intestinal crypt depth and increased muscular thickness.	Both extracts decreased the counts of *Clostridium* in ileum but these were increased in cecum. Only grapeseed extract showed antimicrobial activity against CP.	60 and 7.2, respectively	[40]
Grape pomace (CT)	No differences in BWG or FCR were observed. Birds fed with grape pomace diets had a higher content of meat polyunsaturated fatty acids.	Grape pomace added up to 100 g/kg prevented meat lipid oxidation similar to vitamin E.	50 and 100	[41]
Quercetin (flavonoid)	FCR decreased as quercetin level increased. Laying rate was maximized by supplementation of quercetin at 0.2 g/kg. No significant effect on egg quality was observed.	Microbial population of aerobes and coliforms decreased at higher levels of quercetin, while bifidobacteria increased. Antioxidant activity in liver was increased by quercetin.	0.2 to 0.6	[42]

Table 1: Continued.

Tannin source and type	*Major findings*	*Doses (mg/mL)*	*References*
	In vitro effect of tannin-rich plant extracts on CP and other poultry pathogens		
Chestnut (HT) and quebracho (CT)	CP types A, B, C, D, and E growth was inhibited in a dose-dependent manner in the presence of all tannins extracts. Quebracho tannins showed partial bactericidal activity, whereas chestnut tannin activity was stronger. Both tannins reduced alpha toxin lecithinase activity and epsilon toxin cytotoxicity.	0.03 to 8	[32]
Blackberry, cranberry (CT + HT), chestnut (HT), mimosa, quebracho, and sorghum (CT)	Tannins exhibited a variable degree of inhibition of *Campylobacter jejuni* depending on source and medium composition. Inhibitory activity of CT extracts but not HT was mitigated by casamino acid supplementation, likely because the added amino acids saturated the binding potential of CT.	100	[43]
Chestnut, tara, sumach (HT), quebracho, *Calliandra* (CT), green tea, and acacia (flavonoid)	Despite their structural diversity, all tested tannins showed antimicrobial effects against *Salmonella* Typhimurium either in liquid culture or in the diffusion assay. Tara extracts effectively reduced the counts of *Salmonella* Typhimurium at low doses (1 mg/mL).	1 to 6	[44]
Chestnut (HT) and quebracho (CT)	All compounds showed extracellular antiviral effect against both avian reovirus and avian metapneumovirus at concentrations ranging from 25 to 66 μg/mL. Quebracho extract also had intracellular activity.	0.01 to 0.15	[45]

CP: *C. perfringens*; BWG: body weight gain; HT: hydrolyzable tannins; CT: condensed tannins; FI: feed intake; FCR: food conversion rate; NE: necrotic enteritis.

antimicrobial activity of tannins as well as their impact on the development of a healthy gut microbiota in poultry.

5. Essential Oils

Essential oils (EOs) are considered to be secondary metabolites in plants which are organic compounds that are not directly involved in the normal growth, development, or reproduction of the plant [117]. These compounds are assumed to be involved in plant defense and most of them may possess antimicrobial properties [117, 118].

The composition and the percentage of different components of EOs vary amongst species and parts of the plants; most of these components are chemically derived from terpenes and their oxygenated derivatives, terpenoids, which are aromatic and aliphatic acid esters and phenolic compounds. EOs can be extracted from plant tissues by extraction or fermentation, but steam distillation is the most commonly used method in industry. EOs have been historically included in the formulation of perfumes and cosmetic products as well as herbs and spices for foods. These oily components are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) of the United States and have been used as artificial flavorings and preservatives [119]. Also, herbs and spices and their EOs have been used as pharmaceuticals in alternative or complementary medicine for many years [120].

Recently, there was a renewed interest on the antimicrobial activity of EOs since many reports demonstrated the potential to control bacterial pathogens [121–123]. The first scientific test of their bactericidal properties had been carried out by de la Croix in 1881 [123]. In more recent years, many EOs or their components have been shown to possess broad-range antibacterial properties [124, 125].

Antimicrobial activities of EOs are related to chemical characteristics such as their hydrophobicity which enables them to interact with the lipids of the bacterial cell membrane thus disturbing bacteria metabolism and cell wall and membrane permeability, leading to extensive leakage of critical molecules and ions from bacterial cells. Phenolic groups present in EOs molecules target bacterial cell membrane by changing its structure and function [126]; microscopy studies demonstrate that low concentrations of some oils may generate holes on the cell wall of sensitive bacteria including CP, being vegetative forms particularly lysed [127].

Evidence about inhibitory spectrum of EOs is contradictory. Some studies concluded that Gram-positive bacteria are more resistant than Gram-negative bacteria [128]. However, most works reported that Gram-positive bacteria are more susceptible to EOs than Gram-negative bacteria [123, 129]. The weaker antimicrobial activity against Gram-negative can be explained considering the structure of their cellular walls, mainly with regard to the presence of lipoproteins and lipopolysaccharides in the external membrane that form a barrier to hydrophobic compounds [129, 130].

Unlike common antibiotics that are often composed of only a single molecular entity, EOs are multicomponent substances and the antibacterial efficacy is related to the overall composition and relative concentrations of active components. For example, thymol and carvacrol, two common terpenoids present in many EOs, have similar antimicrobial properties but act differently against Gram-positive or Gram-negative bacteria based on the locations of one or more functional groups in these two molecules [30, 131]. The mechanism underlying antibacterial activity against CP and other Gram-positive bacterial pathogens is unclear at present and therefore further studies are needed.

The use of EOs to control the proliferation of CP and reduce NE impact on poultry production has been explored [48, 49, 53, 54]. There are numerous reports about the antibacterial effects of *Origanum vulgare*, *Piper nigrum*, *Syzygium aromaticum*, and *Thymus vulgaris*, and their components, thymol, carvacrol, and eugenol, against *Clostridium* species [132, 133] including CP [46, 134]. EOs effects on CP-induced NE may be related to a direct antimicrobial effect on bacterial cells and an indirect effect by modulating gut microbiota and digestive functions. *In vitro* CP inhibition was described for many plant extracts and their EOs [127]. Antimicrobial activity was found in 50% of the tested plant species. Great differences in the inhibitory effect and potency are found among scientific studies on the activity of EOs that can be partially explained by the variety of protocols used to obtain EOs solution and to measure antimicrobial activity [127, 135]. For example, one report [135] used disc diffusion methods and reports high antimicrobial activity (inhibition > 95%) against CP for thyme (*T. vulgaris*) and oregano (*O. vulgare*), while Si et al. (2009) [127] used broth microdilution methods and reported similar results for thyme but low antimicrobial activity (inhibition between 50 and 80%) for oregano. Since antimicrobial activity of EOs is related to the combined effects of several molecules, most reported results choose one of the main components as indicator of biological activity. Carvacrol and thymol are two of the most common single molecules used to determine spices/EOs antimicrobial activity [50, 123], and differences in presence and concentration of these molecules will contribute to explain the contradiction of published results. The aforementioned molecules are main components of several EOs with antimicrobial activity such as oregano, rosemary, and thyme oils [123, 136].

Differences in activity may also be related to vegetal growth conditions and storage conditions after harvest [137]. These authors compared several commercial stocks of spices Angelica (*Angelica archangelica*) and Japanese mint (*Mentha arvensis* var. *piperascens*) and found clear differences in antimicrobial activity [137]. Some works also reported variations in thymol and carvacrol concentrations within thyme and oregano [137]. Moreover, while some works mention that carvacrol is the main active molecule in thyme, Nevas et al. (2004) [137] described inhibitory effect against CP in Finnish thyme extract without detection of carvacrol.

In poultry, many works report that the inclusion of blends of EOs as dietary supplements has improved productive performance [52, 138] including weight gain and body mass; however, none of these works reported changes in intestinal microbiota, apparent metabolizable energy, or the calculated coefficients of digestibility. According to Jamroz et al. (2003)

[139], the inclusion of blended supplements containing carvacrol, capsaicin, and cinnamaldehyde has improved body weight and feed conversion rate in broilers even to a greater extent than avilamycin in 21-day-old chickens.

The inclusion of EOs supplementation in poultry feed alleviated intestinal gross lesions compatible with NE in a dose-dependent manner on days 21 and 28 [50, 140]. Reduction of CP-induced intestinal damage can be achieved after reducing the intestinal burden of the microorganism. Si et al. (2009) [127] reported reductions of 2 or 3 log units of CP counts in chicken ileal content by carvacrol or citronellol; these results agreed with previous *in vivo* studies which showed that EOs containing thymol and/or carvacrol were able to decrease CP counts in both small and large intestines [51].

One important criterion that may be considered to select good candidates for the substitution of AGPs to control CP-induced NE and other poultry bacterial pathogens is their stability at low pH, as all compounds need to pass through the stomach with a pH as low as 2. Some EOs like carvacrol, chamomile roman oil, or citronellal resist acid and retained their inhibitory activity toward CP after the *in vitro* preacidic treatment [127]. These results suggest that some EOs can be added to feed and have intact effect against CP vegetative cells located in the intestinal lumen. *In vivo* trials support this since they showed that birds fed with EOs supplemented had lower concentrations of CP in jejunum, cecum, cloaca, and feces on day 14, in jejunum, cecum, and feces on day 21, and feces on day 30. Chickens fed with EOs showed significantly lower CP counts in all portions of the intestine and in the feces, while the proportion of CP positive birds was also reduced [46]. Unlike tannins [32], no antitoxic activity against CP toxins was demonstrated for EOs.

Together with direct antimicrobial effects of EOs against CP, changes in intestinal microbiota also might be related to alleviation of development of NE intestinal lesions. Several studies have reported that changes in intestinal microbiota induced by essential oil dietary supplementation are to the same extent as avilamycin [139]. Once again, evidence is contradictory and needs to consider variations on EOs origins as well as feed supplement presentation. Cross et al. (2007) [52] reported that the inclusion of rosemary (*R. officinalis*), yarrow (*A. millefolium* var. *alba*), and thyme (*T. vulgaris*) in poultry diets reduced CP counts in cecum and increased coliforms counts in the same intestinal portion in chickens given any of the mentioned herbal treatments. Other works mention that blends of EOs reduce the growth of *E. coli* and CP in broilers [141, 142]. EOs exhibited a minor or no inhibition on *Lactobacillus* spp. [52] and some works report an increased number of lactobacilli counts [142]. Thus, EOs may act in a different way compared to AGPs, which tend to depress bacterial numbers in all the species. While some works report higher susceptibility to EOs in Gram-positive bacteria, other studies demonstrated the selectivity of EOs against CP over lactobacilli, both groups of Gram-positive bacteria. Undoubtedly, further studies are required to understand the mechanism underlying the group selectivity.

To control CP-induced NE and other infectious diseases, it is important to reduce intestinal and environmental bacteria burden. Some EOs formulations also reduce bacterial populations when applied directly on the soil and can be used to reduce potential contamination of fresh organic products, including poultry feed. Previous works with different bacterial pathogens on food products intended for human alimentation, including products of plant or animal origin, suggest a promising scene [47, 143, 144]. In the actual poultry productive context where synthetic antimicrobials are limited or banned, EOs could play an important role in the innovation of preventive or therapeutic strategies. It is likely that it will be more difficult for bacteria to develop resistance to the multicomponent EOs than to common antibiotics that are often composed of only a single molecular entity. Previous works with tannins [21], another multicomponent natural antimicrobial substance, may reinforce this idea. Nevertheless, the lack of studies to determine the safety and toxicity evaluation of potential changes in flavor, odor, and other organoleptic characteristics of poultry-derived food products may limit the use of EOs in poultry. Available information regarding safety in relation to oral administration of EOs in human and poultry is scanty, so determinations upon the potential toxicity of EOs administered by this route are required. The ways in which EOs are applied and the concentrations at which they are used are important factors related to their effectiveness. Inhibition studies showed that some pathogenic bacteria can be inhibited by direct application of EOs components without affecting the flavor of the food products [145]. Table 2 summarizes the available EOs additives for NE prevention in poultry as well as their performance and intestinal and antimicrobial effects.

6. Conclusions and Perspectives

The European ban of AGPs in poultry products and recent restrictions on the use of these compounds in other countries, including Australia and USA, present several challenges to the poultry industry. Reports from the EU have shown that the key problem of in-feed antibiotic withdrawal from poultry diets is the control of NE. Therefore, the cost-benefit in replacing AGPs with natural alternatives is critical for ensuring the long-term sustainable poultry production. Plant extracts have a large variety of bioactive ingredients and thus represent one of the most promising alternatives to replace AGPs, particularly tannins and essential oils. However, their application in poultry production has been largely avoided due to inconsistent evaluation of their efficacy and lack of full understanding of the modes of action behind them. In order to support the use of natural plant products to maintain the productivity rates achieved by AGPs and become acceptable by the mainstream poultry industry market, different research groups provided solid scientific evidence addressing the issue of inconsistency across many studies in literature. In this sense, the development and utilization of a standardized methodology for production of phytobased feed additives and evaluation of their biological activity is urgently needed in order to support the use of

Table 2: EOs additives for NE prevention in poultry.

Feed additive	Inclusion rate	Performance effects	Intestinal effects	Antimicrobial effects	Reference
EOs from *Thymus vulgaris* (30% thymol)	100 ppm	—	—	Decreased CP counts in the gut and feces.	[46]
EOs mix from *Thymus vulgaris* and *Origanum vulgare* (15% thymol and 15% carvacrol)	100 ppm	—	—	Decreased CP counts in the gut and feces.	[46]
Artemisia annua dry leaves	10 g/kg	Decreased BWG. Decreased FI. Improved FCR.	Reduced NE-related gross intestinal lesion score.	*In vitro* antibacterial activity against CP. Decreased CP counts in ileum and ceca.	[47]
Artemisia annua n-hexane extract	250 mg/kg	Decreased BWG. Decreased FI. Improved FCR.	Reduced NE-related gross intestinal lesion score.	*In vitro* antibacterial activity against CP. Decreased CP counts in ileum and ceca.	[47]
Capsicum/*Curcuma longa* oleoresin mix	4 mg/kg	Increased body weight after CP challenge.	Reduced NE-related gross intestinal lesion score. Reduced inflammatory response against NE.	—	[48]
Citral (mix of *cis*/*trans*-isomers, 95% purity)	250–650 μg/g	—	Reduced NE-related gross intestinal lesion score.	*In vitro* antibacterial activity against CP. Decreased CP counts in ileum and ceca. No effects in lactic acid bacteria.	[49]
Commercial EOs product (25% thymol and 25% carvacrol)	60, 120, or 240 mg/kg	No influence in growth performance.	Reduced NE-related gross intestinal lesion score.	*In vitro* antibacterial activity against CP.	[50]
Commercial herbal mix (thyme and star anise 17.0% and 17.0%)	250 mg/kg	Improved FCR. Improved digestibility of dry matter.	Reduced NE-related gross intestinal lesion score.	Decreased CP counts in large and small intestines.	[51]
EOs from *A. millefolium*	1 g/kg	Decreased BWG. Decreased FI. Improved FCR.	—	—	[52]
EOs from *O. majorana*	1 g/kg	Improved BWG and FI under suboptimal conditions for growth.	—	—	[52]
EOs from *O. vulgare* subsp. *hirtum*	1 g/kg	Decreased BWG. Decreased FI.	—	—	[52]
EOs from *R. officinalis*	1 g/kg	Decreased BWG. Decreased FI.	—	—	[52]
EOs from *T. vulgaris*	1 g/kg	Improved BWG under suboptimal conditions for growth.	—	—	[52]
EOs mix (thymol, cinnamaldehyde, and essential oil of eucalyptus)	150 g/ton	—	Reduced NE-related gross intestinal lesion score.	*In vitro* antibacterial activity against CP.	[53]
Marjoram (*O. majorana*)	10 g/kg	Improved BWG and feed conversion rate under suboptimal conditions for growth.	—	—	[52]
Oregano (*O. vulgare* subsp. *hirtum*)	10 g/kg	Decreased BWG. Decreased FI. Improved FCR.	—	—	[52]

Table 2: Continued.

Protected blend of EOs (ginger oil and carvacrol 1%)	1.5 g/kg	Improved BWG.	Reduced NE-related gross and histopathological intestinal lesion score. Increase in intestinal villus lengths and V : C ratio.	—	[54]
Rosemary (*R. officinalis*)	10 g/kg	—	—	Decreased CP counts in ceca and feces. No effect in lactic acid bacteria.	[52]
Thyme (*T. vulgaris*)	10 g/kg	Reduced BWG and FI under suboptimal conditions for growth.	—	Decreased CP counts in ceca. No effect in lactic acid bacteria.	[52]
Yarrow (*A. millefolium* var. *alba*)	10 g/kg	Improved BWG and feed conversion rate under suboptimal conditions for growth.	—	Decreased CP counts in ceca and feces. No effect in lactic acid bacteria.	[52]

CP: *C. perfringens*; BWG: body weight gain; FI: feed intake; FCR: food conversion rate; NE: necrotic enteritis.

different additives. Furthermore, a better understanding on the impact of phytogenic compounds on gut microbiota, physiology, and immunology will allow a better use of these products for economically effective and sustainable poultry production.

Besides plant extracts, there are other suitable strategies to control NE in poultry in order to fill the gap left by the ban of AGPs, including competitive exclusion products, probiotics, prebiotics, organic acids, enzymes, hen egg antibodies, bacteriophages, and vaccination. However, to date, no single preventive therapy that can effectively substitute AGPs and control NE has been found. Therefore, the combination of different in-feed additives and limiting exposure to CP and other NE-predisposing microorganisms through biosecurity and vaccination might be a tool to reduce the incidence of NE and improve gut health in the absence of AGPs. Effective nonantibiotic prevention of CP-associated health and performance problems will only be achieved by means of multidisciplinary research efforts, covering both *in vitro* molecular functionality approaches together with *in vivo* feeding experiments. Plant extracts exert specific effects on gut microbiota which influence both the emergence of intestinal pathogens and growth performance of chickens. It has been shown that tannins and essential oils possess activities in the digestive tract that cover many of the requirements to control NE. The ability of some tannins to remain active even in poultry bedding after fecal excretion appears as an interesting feature to control CP reinfection. Moreover, it has been proved that resistance of CP against tannins is not easily generated, allowing a continuous use of these compounds over time. Therefore, these products may play a key role as a viable, cost-efficient, and safe alternative to AGPs that could be used to enhance chicken performance and health as well.

Competing Interests

Some of the authors provide consulting services to companies related to poultry nutrition.

Authors' Contributions

J. M. Diaz Carrasco and L. M. Redondo contributed equally to this work.

References

[1] K. K. Cooper and J. G. Songer, "Virulence of *Clostridium perfringens* in an experimental model of poultry necrotic enteritis," *Veterinary Microbiology*, vol. 142, no. 3-4, pp. 323–328, 2010.

[2] K. K. Cooper, J. G. Songer, and F. A. Uzal, "Diagnosing clostridial enteric disease in poultry," *Journal of Veterinary Diagnostic Investigation*, vol. 25, no. 3, pp. 314–327, 2013.

[3] A. L. Keyburn, S. A. Sheedy, M. E. Ford et al., "Alpha-toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens," *Infection and Immunity*, vol. 74, no. 11, pp. 6496–6500, 2006.

[4] W. Van der Sluis, "Clostridial enteritis is an often underestimated problem," *World Poultry*, no. 16, pp. 42–43, 2000.

[5] J. G. Songer, "Clostridial enteric diseases of domestic animals," *Clinical Microbiology Reviews*, vol. 9, no. 2, pp. 216–234, 1996.

[6] K. K. Cooper and J. G. Songer, "Necrotic enteritis in chickens: a paradigm of enteric infection by *Clostridium perfringens* type A," *Anaerobe*, vol. 15, no. 1-2, pp. 55–60, 2009.

[7] J. Vierheilig, C. Frick, R. E. Mayer et al., "*Clostridium perfringens* is not suitable for the indication of fecal pollution from ruminant wildlife but is associated with excreta from nonherbivorous animals and human sewage," *Applied and Environmental Microbiology*, vol. 79, no. 16, pp. 5089–5092, 2013.

[8] A. R. Gholamiandehkordi, L. Timbermont, A. Lanckriet et al., "Quantification of gut lesions in a subclinical necrotic enteritis model," *Avian Pathology*, vol. 36, no. 5, pp. 375–382, 2007.

[9] T. Fukata, Y. Hadate, E. Baba, and A. Arakawa, "Influence of bacteria on *Clostridium perfringens* infections in young chickens," *Avian Diseases*, vol. 35, no. 1, pp. 224–227, 1991.

[10] N. J. Rodgers, R. A. Swick, M. S. Geier, R. J. Moore, M. Choct, and S.-B. Wu, "A multifactorial analysis of the extent to which eimeria and fishmeal predispose broiler chickens to necrotic enteritis," *Avian Diseases*, vol. 59, no. 1, pp. 38–45, 2015.

[11] J. F. Prescott, V. R. Parreira, I. Mehdizadeh Gohari, D. Lepp, and J. Gong, "The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know: a review," *Avian Pathology*, vol. 45, no. 3, pp. 288–294, 2016.

[12] F. Al-Sheikhly and A. Al-Saieg, "Role of Coccidia in the occurrence of necrotic enteritis of chickens," *Avian Diseases*, vol. 24, no. 2, pp. 324–333, 1980.

[13] C. T. Collier, C. L. Hofacre, A. M. Payne et al., "Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth," *Veterinary Immunology and Immunopathology*, vol. 122, no. 1-2, pp. 104–115, 2008.

[14] D. K. Onderka, C. C. Langevin, and J. A. Hanson, "Fibrosing cholehepatitis in broiler chickens induced by bile duct ligations or inoculation of *Clostridium perfringens*," *Canadian Journal of Veterinary Research*, vol. 54, no. 2, pp. 285–290, 1990.

[15] E. Olazabal, R. Gonzalez, S. Flores, and L. Alcina, "Efectividad de diferentes combinaciones de tratamientos con antibioticos en la mortalidad por Clostridium Perfringens en una empresa avicola," *Revista Elecrtonica de Veterinaria*, vol. 6, no. 2, pp. 1–9, 2005.

[16] M. S. Diarra and F. Malouin, "Antibiotics in Canadian poultry productions and anticipated alternatives," *Frontiers in Microbiology*, vol. 5, article 282, 2014.

[17] G. Huyghebaert, R. Ducatelle, and F. Van Immerseel, "An update on alternatives to antimicrobial growth promoters for broilers," *Veterinary Journal*, vol. 187, no. 2, pp. 182–188, 2011.

[18] R. M. Engberg and J. S. Petersen, "Poultry production with and without questionable feed additives and ingredients," in *Proceedings of the 13th European Symposium on Poultry Nutrition*, pp. 118–124, Blankenberge, Belgium, October 2001.

[19] S. W. Page, "Current use of antimicrobial growth promoters in food animals: the benefits," in *Antimicrobial Growth Promoters: Where Do We Go from Here?* pp. 19–51, 2006.

[20] K. M. Tuohy, G. C. M. Rouzaud, W. M. Brück, and G. R. Gibson, "Modulation of the human gut microflora towards improved health using prebiotics—assessment of efficacy," *Current Pharmaceutical Design*, vol. 11, no. 1, pp. 75–90, 2005.

[21] L. M. Redondo, J. E. Dominguez, B. C. Rabinovitz, E. A. Redondo, and M. E. Fernández Miyakawa, "Hydrolyzable and condensed tannins resistance in *Clostridium perfringens*," *Anaerobe*, vol. 34, pp. 139–145, 2015.

[22] T. F. O'Brien, "Emergence, spread, and environmental effect of antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else," *Clinical Infectious Diseases*, vol. 34, supplement 3, pp. S78–S84, 2002.

[23] B. M. Marshall and S. B. Levy, "Food animals and antimicrobials: impacts on human health," *Clinical Microbiology Reviews*, vol. 24, no. 4, pp. 718–733, 2011.

[24] J. I. R. Castanon, "History of the use of antibiotic as growth promoters in European poultry feeds," *Poultry Science*, vol. 86, no. 11, pp. 2466–2471, 2007.

[25] M. Casewell, C. Friis, E. Marco, P. McMullin, and I. Phillips, "The European ban on growth-promoting antibiotics and emerging consequences for human and animal health," *Journal of Antimicrobial Chemotherapy*, vol. 52, no. 2, pp. 159–161, 2003.

[26] F. Van Immerseel, J. De Buck, F. Pasmans, G. Huyghebaert, F. Haesebrouck, and R. Ducatelle, "*Clostridium perfringens* in poultry: an emerging threat for animal and public health," *Avian Pathology*, vol. 33, no. 6, pp. 537–549, 2004.

[27] J. P. Dahiya, D. C. Wilkie, A. G. Van Kessel, and M. D. Drew, "Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era," *Animal Feed Science and Technology*, vol. 129, no. 1-2, pp. 60–88, 2006.

[28] K. Grave, M. Kaldhusdal, H. Kruse, L. M. Fevang Harr, and K. Flatlandsmo, "What has happened in Norway after the ban of avoparcin? Consumption of antimicrobials by poultry," *Preventive Veterinary Medicine*, vol. 62, no. 1, pp. 59–72, 2004.

[29] D. L. Caly, R. D'Inca, E. Auclair, and D. Drider, "Alternatives to antibiotics to prevent necrotic enteritis in broiler chickens: a microbiologist's perspective," *Frontiers in Microbiology*, vol. 6, article 1336, Article ID 01336, 2015.

[30] C. Yang, M. A. K. Chowdhury, Y. Huo, and J. Gong, "Phytogenic compounds as alternatives to in-feed antibiotics: potentials and challenges in application," *Pathogens*, vol. 4, no. 1, pp. 137–156, 2015.

[31] S. Millet and L. Maertens, "The European ban on antibiotic growth promoters in animal feed: from challenges to opportunities," *The Veterinary Journal*, vol. 187, no. 2, pp. 143–144, 2011.

[32] A. M. Elizondo, E. C. Mercado, B. C. Rabinovitz, and M. E. Fernandez-Miyakawa, "Effect of tannins on the *in vitro* growth of *Clostridium perfringens*," *Veterinary Microbiology*, vol. 145, no. 3-4, pp. 308–314, 2010.

[33] L. R. McDougald, E. C. Hofacre, G. Mathis et al., "Enhancement of resistance to coccidiosis and necrotic enteritis in broiler chickens by dietary muscadine pomace," *Avian Diseases*, vol. 52, no. 4, pp. 646–651, 2008.

[34] A. Schiavone, K. Guo, S. Tassone et al., "Effects of a natural extract of chestnut wood on digestibility, performance traits, and nitrogen balance of broiler chicks," *Poultry Science*, vol. 87, no. 3, pp. 521–527, 2008.

[35] G. Tosi, P. Massi, M. Antongiovanni et al., "Efficacy test of a hydrolysable tannin extract against necrotic enteritis in challenged broiler chickens," *Italian Journal of Animal Science*, vol. 12, no. 3, pp. 386–389, 2013.

[36] E. Cejas, S. Pinto, F. Prosdócimo et al., "Evaluation of quebracho red wood (*Schinopsis lorentzii*) polyphenols vegetable extract for the reduction of coccidiosis in broiler chicks," *International Journal of Poultry Science*, vol. 10, no. 5, pp. 344–349, 2011.

[37] A. Brenes, A. Viveros, I. Goñi et al., "Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens," *Poultry Science*, vol. 87, no. 2, pp. 307–316, 2008.

[38] A. Brenes, A. Viveros, I. Goñi, C. Centeno, F. Saura-Calixto, and I. Arija, "Effect of grape seed extract on growth performance, protein and polyphenol digestibilities, and antioxidant activity in chickens," *Spanish Journal of Agricultural Research*, vol. 8, no. 2, pp. 326–333, 2010.

[39] D. E. Cross, R. M. McDevitt, and T. Acamovic, "Herbs, thyme essential oil and condensed tannin extracts as dietary supplements for broilers, and their effects on performance, digestibility, volatile fatty acids and organoleptic properties," *British Poultry Science*, vol. 52, no. 2, pp. 227–237, 2011.

[40] A. Viveros, S. Chamorro, M. Pizarro, I. Arija, C. Centeno, and A. Brenes, "Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks," *Poultry Science*, vol. 90, no. 3, pp. 566–578, 2011.

[41] S. Chamorro, A. Viveros, A. Rebolé, B. D. Rica, I. Arija, and A. Brenes, "Influence of dietary enzyme addition on polyphenol utilization and meat lipid oxidation of chicks fed grape pomace," *Food Research International*, vol. 73, pp. 197–203, 2015.

[42] H. N. Liu, Y. Liu, L. L. Hu et al., "Effects of dietary supplementation of quercetin on performance, egg quality, cecal microflora populations, and antioxidant status in laying hens," *Poultry Science*, vol. 93, no. 2, pp. 347–353, 2014.

[43] R. C. Anderson, M. Vodovnik, B. R. Min et al., "Bactericidal effect of hydrolysable and condensed tannin extracts on *Campylobacter jejuni* in vitro," *Folia Microbiologica*, vol. 57, no. 4, pp. 253–258, 2012.

[44] A. Costabile, S. Sanghi, S. Martín-Pelaez et al., "Inhibition of *Salmonella Typhimurium* by tannins in vitro," *Journal of Food, Agriculture and Environment*, vol. 9, no. 1, pp. 119–124, 2011.

[45] C. Lupini, M. Cecchinato, A. Scagliarini, R. Graziani, and E. Catelli, "In vitro antiviral activity of chestnut and quebracho woods extracts against avian reovirus and metapneumovirus," *Research in Veterinary Science*, vol. 87, no. 3, pp. 482–487, 2009.

[46] P. Mitsch, K. Zitterl-Eglseer, B. Köhler, C. Gabler, R. Losa, and I. Zimpernik, "The effect of two different blends of essential oil components on the proliferation of *Clostridium perfringens* in the intestines of broiler chickens," *Poultry Science*, vol. 83, no. 4, pp. 669–675, 2004.

[47] R. M. Engberg, K. Grevsen, E. Ivarsen et al., "The effect of Artemisia annua on broiler performance, on intestinal microbiota and on the course of a Clostridium perfringens infection applying a necrotic enteritis disease model," *Avian Pathology*, vol. 41, no. 4, pp. 369–376, 2012.

[48] S. H. Lee, H. S. Lillehoj, S. I. Jang, E. P. Lillehoj, W. Min, and D. M. Bravo, "Dietary supplementation of young broiler chickens with *Capsicum* and turmeric oleoresins increases resistance to necrotic enteritis," *British Journal of Nutrition*, vol. 110, no. 5, pp. 840–847, 2013.

[49] Y. Yang, Q. Wang, M. S. Diarra, H. Yu, Y. Hua, and J. Gong, "Functional assessment of encapsulated citral for controlling necrotic enteritis in broiler chickens," *Poultry Science*, vol. 95, no. 4, pp. 780–789, 2016.

[50] E. Du, L. Gan, Z. Li, W. Wang, D. Liu, and Y. Guo, "In vitro antibacterial activity of thymol and carvacrol and their effects on broiler chickens challenged with *Clostridium perfringens*," *Journal of Animal Science and Biotechnology*, vol. 6, no. 1, article 58, 2015.

[51] J. H. Cho, H. J. Kim, and I. H. Kim, "Effects of phytogenic feed additive on growth performance, digestibility, blood metabolites, intestinal microbiota, meat color and relative organ weight after oral challenge with *Clostridium perfringens* in broilers," *Livestock Science*, vol. 160, no. 1, pp. 82–88, 2014.

[52] D. E. Cross, R. M. McDevitt, K. Hillman, and T. Acamovic, "The effect of herbs and their associated essential oils on performance, dietary digestibility and gut microflora in chickens from 7 to 28 days of age," *British Poultry Science*, vol. 48, no. 4, pp. 496–506, 2007.

[53] L. Timbermont, A. Lanckriet, J. Dewulf et al., "Control of *Clostridium perfringens*-induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils," *Avian Pathology*, vol. 39, no. 2, pp. 117–121, 2010.

[54] A. Jerzsele, K. Szeker, R. Csizinszky et al., "Efficacy of protected sodium butyrate, a protected blend of essential oils, their combination, and *Bacillus amyloliquefaciens* spore suspension against artificially induced necrotic enteritis in broilers," *Poultry Science*, vol. 91, no. 4, pp. 837–843, 2012.

[55] D. Savoia, "Plant-derived antimicrobial compounds: alternatives to antibiotics," *Future Microbiology*, vol. 7, no. 8, pp. 979–990, 2012.

[56] H. Vondruskova, R. Slamova, M. Trckova, Z. Zraly, and I. Pavlik, "Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: a review," *Veterinarni Medicina*, vol. 55, no. 5, pp. 199–224, 2010.

[57] S. R. Hashemi and H. Davoodi, "Phytogenies as new class of feed additive in poultry industry," *Journal of Animal and Veterinary Advances*, vol. 9, no. 17, pp. 2295–2304, 2010.

[58] T. Steiner, *Phytogenics in Animal Nutrition: Natural Concepts to Optimize Gut Health and Performance*, Nottingham University Press, 2009.

[59] S. Smulikowska, B. Pastuszewska, E. Święch et al., "Tannin content affects negatively nutritive value of pea for monogastrics," *Journal of Animal and Feed Sciences*, vol. 10, no. 3, pp. 511–523, 2001.

[60] M. Longstaff and J. M. McNab, "The inhibitory effects of hull polysaccharides and tannins of field beans (*Vicia faba* L.) on the digestion of amino acids, starch and lipid and on digestive enzyme activities in young chicks," *British Journal of Nutrition*, vol. 65, no. 2, pp. 199–216, 1991.

[61] M. A. Longstaff and J. M. McNab, "The effect of concentration of tannin-rich bean hulls (Vicia faba L.) on activities of lipase (EC 3.1.1.3) and α-amylase (EC 3.2.1.1) in digesta and pancreas and on the digestion of lipid and starch by young chicks," *British Journal of Nutrition*, vol. 66, no. 1, pp. 139–147, 1991.

[62] S. R. Hashemi and H. Davoodi, "Herbal plants and their derivatives as growth and health promoters in animal nutrition," *Veterinary Research Communications*, vol. 35, no. 3, pp. 169–180, 2011.

[63] A. C. Abreu, A. J. McBain, and M. Simões, "Plants as sources of new antimicrobials and resistance-modifying agents," *Natural Product Reports*, vol. 29, no. 9, pp. 1007–1021, 2012.

[64] M. Simões, R. N. Bennett, and E. A. S. Rosa, "Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms," *Natural Product Reports*, vol. 26, no. 6, pp. 746–757, 2009.

[65] L. M. Redondo, P. A. Chacana, J. E. Dominguez, and M. E. Fernandez Miyakawa, "Perspectives in the use of tannins as alternative to antimicrobial growth promoter factors in poultry," *Frontiers in Microbiology*, vol. 5, article 118, 2014.

[66] W. Windisch, K. Schedle, C. Plitzner, and A. Kroismayr, "Use of phytogenic products as feed additives for swine and poultry," *Journal of Animal Science*, vol. 86, no. 14, supplement, pp. E140–E148, 2008.

[67] A. Scalbert, "Antimicrobial properties of tannins," *Phytochemistry*, vol. 30, no. 12, pp. 3875–3883, 1991.

[68] D. Karou, A. Savadogo, A. Canini et al., "Antibacterial activity of alkaloids from Sida acuta," *African Journal of Biotechnology*, vol. 4, no. 12, pp. 1452–1457, 2005.

[69] J. P. Morrissey and A. E. Osbourn, "Fungal resistance to plant antibiotics as a mechanism of pathogenesis," *Microbiology and Molecular Biology Reviews*, vol. 63, no. 3, pp. 708–724, 1999.

[70] K.-W. Lee, H. Everts, H. J. Kappert, H. Wouterse, M. Frehner, and A. C. Beynen, "Cinnamaldehyde, but not thymol, counteracts the carboxymethyl cellulose-induced growth depression in female broiler chickens," *International Journal of Poultry Science*, vol. 3, no. 9, pp. 608–612, 2004.

[71] M. Stavri, L. J. V. Piddock, and S. Gibbons, "Bacterial efflux pump inhibitors from natural sources," *Journal of Antimicrobial Chemotherapy*, vol. 59, no. 6, pp. 1247–1260, 2007.

[72] I. Mueller-Harvey, "Analysis of hydrolysable tannins," *Animal Feed Science and Technology*, vol. 91, no. 1-2, pp. 3–20, 2001.

[73] P. Schofield, D. M. Mbugua, and A. N. Pell, "Analysis of condensed tannins: a review," *Animal Feed Science and Technology*, vol. 91, no. 1-2, pp. 21–40, 2001.

[74] K. Khanbabaee and T. van Ree, "Tannins: classification and definition," *Natural Product Reports*, vol. 18, no. 6, pp. 641–649, 2001.

[75] W.-Y. Huang, Y.-Z. Cai, and Y. Zhang, "Natural phenolic compounds from medicinal herbs and dietary plants: potential use for cancer prevention," *Nutrition and Cancer*, vol. 62, no. 1, pp. 1–20, 2010.

[76] E. Szliszka and W. Krol, "The role of dietary polyphenols in tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis for cancer chemoprevention," *European Journal of Cancer Prevention*, vol. 20, no. 1, pp. 63–69, 2011.

[77] K. Ueda, R. Kawabata, T. Irie, Y. Nakai, Y. Tohya, and T. Sakaguchi, "Inactivation of pathogenic viruses by plant-derived tannins: strong effects of extracts from persimmon (*Diospyros kaki*) on a broad range of viruses," *PLoS ONE*, vol. 8, no. 1, Article ID e55343, 2013.

[78] L. Marín, E. M. Miguélez, C. J. Villar, and F. Lombó, "Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties," *BioMed Research International*, vol. 2015, Article ID 905215, 18 pages, 2015.

[79] J. Serrano, R. Puupponen-Pimiä, A. Dauer, A.-M. Aura, and F. Saura-Calixto, "Tannins: current knowledge of food sources, intake, bioavailability and biological effects," *Molecular Nutrition and Food Research*, vol. 53, no. 2, pp. S310–S329, 2009.

[80] E. Haslam, "Natural polyphenols (vegetable tannins) as drugs: possible modes of action," *Journal of Natural Products*, vol. 59, no. 2, pp. 205–215, 1996.

[81] C. Santos-Buelga and A. Scalbert, "Proanthocyanidins and tannin-like compounds—nature, occurrence, dietary intake and effects on nutrition and health," *Journal of the Science of Food and Agriculture*, vol. 80, no. 7, pp. 1094–1117, 2000.

[82] T. Okuda, "Systematics and health effects of chemically distinct tannins in medicinal plants," *Phytochemistry*, vol. 66, no. 17, pp. 2012–2031, 2005.

[83] P. Frutos, G. Hervás, F. J. Giráldez, and A. R. Mantecón, "Review. Tannins and ruminant nutrition," *Spanish Journal of Agricultural Research*, vol. 2, no. 2, p. 191, 2004.

[84] L. G. Butler, "Antinutritional effects of condensed and hydrolyzable tannins," *Basic Life Sciences*, vol. 59, pp. 693–698, 1992.

[85] F. Khajali and B. A. Slominski, "Factors that affect the nutritive value of canola meal for poultry," *Poultry Science*, vol. 91, no. 10, pp. 2564–2575, 2012.

[86] T. Hofmann, A. Glabasnia, B. Schwarz, K. N. Wisman, K. A. Gangwer, and A. E. Hagerman, "Protein binding and astringent taste of a polymeric procyanidin, 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose, castalagin, and grandinin," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 25, pp. 9503–9509, 2006.

[87] P. F. Surai, "Polyphenol compounds in the chicken/animal diet: from the past to the future," *Journal of Animal Physiology and Animal Nutrition*, vol. 98, no. 1, pp. 19–31, 2014.

[88] A. Fiesel, D. K. Gessner, E. Most, and K. Eder, "Effects of dietary polyphenol-rich plant products from grape or hop on pro-inflammatory gene expression in the intestine, nutrient digestibility and faecal microbiota of weaned pigs," *BMC Veterinary Research*, vol. 10, no. 1, article 196, 2014.

[89] K. Starčević, L. Krstulović, D. Brozić et al., "Production performance, meat composition and oxidative susceptibility in broiler chicken fed with different phenolic compounds," *Journal of the Science of Food and Agriculture*, vol. 95, no. 6, pp. 1172–1178, 2015.

[90] I. Goñi, A. Brenes, C. Centeno et al., "Effect of dietary grape pomace and vitamin E on growth performance, nutrient digestibility, and susceptibility to meat lipid oxidation in chickens," *Poultry Science*, vol. 86, no. 3, pp. 508–516, 2007.

[91] M. A. Fellenberg, A. Espinoza, I. Peña, and J. Alarcon, "Antioxidant and bacteriostatic effects of the addition of extarct of quillay polyphenols (*Quillaja saponaria*) in the marinade of broiler chicken," *Brazilian Journal of Poultry Science*, vol. 13, no. 1, pp. 71–79, 2011.

[92] M. Goliomytis, D. Tsoureki, P. E. Simitzis, M. A. Charismiadou, A. L. Hager-Theodorides, and S. G. Deligeorgis, "The effects of quercetin dietary supplementation on broiler growth performance, meat quality, and oxidative stability," *Poultry Science*, vol. 93, no. 8, pp. 1957–1962, 2014.

[93] A. A. Kamboh and W.-Y. Zhu, "Individual and combined effects of genistein and hesperidin on immunity and intestinal morphometry in lipopolysacharide-challenged broiler chickens," *Poultry Science*, vol. 93, no. 9, pp. 2175–2183, 2014.

[94] B. Mansoori, A. Rogiewicz, and B. A. Slominski, "The effect of canola meal tannins on the intestinal absorption capacity of broilers using a D-xylose test," *Journal of Animal Physiology and Animal Nutrition*, vol. 99, no. 6, pp. 1084–1093, 2015.

[95] A. A. Kamboh, S. Q. Hang, M. Bakhetgul, and W.-Y. Zhu, "Effects of genistein and hesperidin on biomarkers of heat stress in broilers under persistent summer stress," *Poultry Science*, vol. 92, no. 9, pp. 2411–2418, 2013.

[96] L. L. Liu, J. H. He, H. B. Xie, Y. S. Yang, J. C. Li, and Y. Zou, "Resveratrol induces antioxidant and heat shock protein mRNA expression in response to heat stress in black-boned chickens," *Poultry Science*, vol. 93, no. 1, pp. 54–62, 2014.

[97] S. Athanasiadou, I. Kyriazakis, F. Jackson, and R. L. Coop, "Effects of short-term exposure to condensed tannins on adult *Trichostrongylus colubriformis*," *Veterinary Record*, vol. 146, no. 25, pp. 728–732, 2000.

[98] N. L. Butter, J. M. Dawson, D. Wakelin, and P. J. Buttery, "Effect of dietary condensed tannins on gastrointestinal nematodes," *Journal of Agricultural Science*, vol. 137, no. 4, pp. 461–469, 2001.

[99] B. R. Min and S. P. Hart, "Tannins for suppression of internal parasites," *Journal of Animal Science*, vol. 81, no. 2, pp. 102–109, 2003.

[100] M. Marzoni, A. Castillo, and I. Romboli, "Dietary inclusion of Quebracho (*Schinopsis lorentzii*) tannins on productive performances of growing pheasant females," *Italian Journal of Animal Science*, vol. 4, no. 2, pp. 507–509, 2005.

[101] D. K. Kim, H. S. Lillehoj, S. H. Lee, S. I. Jang, E. P. Lillehoj, and D. Bravo, "Dietary *Curcuma longa* enhances resistance against *Eimeria maxima* and *Eimeria tenella* infections in chickens," *Poultry Science*, vol. 92, no. 10, pp. 2635–2643, 2013.

[102] K.-T. Chung, Z. Lu, and M. W. Chou, "Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria," *Food and Chemical Toxicology*, vol. 36, no. 12, pp. 1053–1060, 1998.

[103] S. Sakanaka, L. R. Juneja, and M. Taniguchi, "Antimicrobial effects of green tea polyphenols on thermophilic spore-forming bacteria," *Journal of Bioscience and Bioengineering*, vol. 90, no. 1, pp. 81–85, 2000.

[104] C. Engels, A. Schieber, and M. G. Gänzle, "Inhibitory spectra and modes of antimicrobial action of gallotannins from mango

kernels (*Mangifera indica* L.)," *Applied and Environmental Microbiology*, vol. 77, no. 7, pp. 2215–2223, 2011.

[105] L. F. Kubena, J. A. Byrd, C. R. Young, and D. E. Corrier, "Effects of tannic acid on cecal volatile fatty acids and susceptibility to *Salmonella typhimurium* colonization in broiler chicks," *Poultry Science*, vol. 80, no. 9, pp. 1293–1298, 2001.

[106] A. Van Parys, F. Boyen, J. Dewulf, F. Haesebrouck, and F. Pasmans, "The use of tannins to control *Salmonella* typhimurium infections in pigs," *Zoonoses and Public Health*, vol. 57, no. 6, pp. 423–428, 2010.

[107] Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, *Diseases of Poultry*, Blackwell, 2008.

[108] S. A. M'Sadeq, S. Wu, R. A. Swick, and M. Choct, "Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide," *Animal Nutrition*, vol. 1, no. 1, pp. 1–11, 2015.

[109] R. Graziani, G. Tosi, and R. Denti, "In vitro antimicrobial activity of SILVA FEED ENC® on bacterial strains of poultry origin," in *Proceedings of the 12th European Poultry Conference (EPC '06)*, Verona, Italy, September 2006.

[110] L. M. Redondo, E. A. Redondo, F. Delgado et al., "Control of Clostridium perfringens necrotic enteritis by tannins added to the diet," in *Proceedings of the 8th International Conference on the Molecular Biology and Pathogenesis of the Clostridia (ClostPath 8)*, Palm Cove, Australia, 2013.

[111] Y. Song, K. T. Desta, G. Kim et al., "Polyphenolic profile and antioxidant effects of various parts of *Artemisia annua* L," *Biomedical Chromatography*, vol. 30, no. 4, pp. 588–595, 2016.

[112] G. Blaiotta, B. La Gatta, M. Di Capua, A. Di Luccia, R. Coppola, and M. Aponte, "Effect of chestnut extract and chestnut fiber on viability of potential probiotic *Lactobacillus* strains under gastrointestinal tract conditions," *Food Microbiology*, vol. 36, no. 2, pp. 161–169, 2013.

[113] Hee Dong Bae, T. A. McAllister, J. Yanke, K.-J. Cheng, and A. D. Muir, "Effects of condensed tannins on endoglucanase activity and filter paper digestion by *Fibrobacter succinogenes* S85," *Applied and Environmental Microbiology*, vol. 59, no. 7, pp. 2132–2138, 1993.

[114] I. Mila, A. Scalbert, and D. Expert, "Iron withholding by plant polyphenols and resistance to pathogens and rots," *Phytochemistry*, vol. 42, no. 6, pp. 1551–1555, 1996.

[115] H. K. Allen, U. Y. Levine, T. Looft, M. Bandrick, and T. A. Casey, "Treatment, promotion, commotion: antibiotic alternatives in food-producing animals," *Trends in Microbiology*, vol. 21, no. 3, pp. 114–119, 2013.

[116] L. J. Nohynek, H.-L. Alakomi, M. P. Kähkönen et al., "Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens," *Nutrition and Cancer*, vol. 54, no. 1, pp. 18–32, 2006.

[117] G. S. Fraenkel, "The raison d'**ê**tre of secondary plant substances; these odd chemicals arose as a means of protecting plants from insects and now guide insects to food," *Science*, vol. 129, no. 3361, pp. 1466–1470, 1959.

[118] M. M. Tajkarimi, S. A. Ibrahim, and D. O. Cliver, "Antimicrobial herb and spice compounds in food," *Food Control*, vol. 21, no. 9, pp. 1199–1218, 2010.

[119] M. Hyldgaard, T. Mygind, and R. L. Meyer, "Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components," *Frontiers in Microbiology*, vol. 3, 12 pages, 2012.

[120] L. A. Mitscher, S. Drake, S. R. Gollapudi, and S. K. Okwute, "A modern look at folkloric use of anti-infective agents," *Journal of Natural Products*, vol. 50, no. 6, pp. 1025–1040, 1987.

[121] M. T. Baratta, H. J. D. Dorman, S. G. Deans, A. C. Figueiredo, J. G. Barroso, and G. Ruberto, "Antimicrobial and antioxidant properties of some commercial essential oils," *Flavour and Fragrance Journal*, vol. 13, no. 4, pp. 235–244, 1998.

[122] D. Kalemba and A. Kunicka, "Antibacterial and antifungal properties of essential oils," *Current Medicinal Chemistry*, vol. 10, no. 10, pp. 813–829, 2003.

[123] S. Burt, "Essential oils: their antibacterial properties and potencial applications in foods—a review," *International Journal of Food Microbiology*, vol. 22, no. 3, pp. 201–206, 2004.

[124] S. G. Deans and G. Ritchie, "Antibacterial properties of plant essential oils," *International Journal of Food Microbiology*, vol. 5, no. 2, pp. 165–180, 1987.

[125] M. Oussalah, S. Caillet, L. Saucier, and M. Lacroix, "Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella Typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*," *Food Control*, vol. 18, no. 5, pp. 414–420, 2007.

[126] F. Solórzano-Santos and M. G. Miranda-Novales, "Essential oils from aromatic herbs as antimicrobial agents," *Current Opinion in Biotechnology*, vol. 23, no. 2, pp. 136–141, 2012.

[127] W. Si, X. Ni, J. Gong et al., "Antimicrobial activity of essential oils and structurally related synthetic food additives towards *Clostridium perfringens*," *Journal of Applied Microbiology*, vol. 106, no. 1, pp. 213–220, 2009.

[128] C. M. Lin, J. F. Preston, and C. I. Wei, "Antibacterial machanism of allyl isothiocyanate," *Journal of Food Protection*, vol. 63, no. 6, pp. 727–734, 2000.

[129] S. Inouye, T. Takizawa, and H. Yamaguchi, "Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact," *Journal of Antimicrobial Chemotherapy*, vol. 47, no. 5, pp. 565–573, 2001.

[130] C. M. Mann, S. D. Cox, and J. L. Markham, "The outer membrane of *Pseudomonas aeruginosa* NCTC 6749 contributes to its tolerance to the essential oil of *Melaleuca alternifolia* (tea tree oil)," *Letters in Applied Microbiology*, vol. 30, no. 4, pp. 294–297, 2000.

[131] G. P. Kamatou, I. Vermaak, and A. M. Viljoen, "Eugenol—from the remote Maluku Islands to the international market place: A review of a remarkable and versatile molecule," *Molecules*, vol. 17, no. 6, pp. 6953–6981, 2012.

[132] N. Paster and R. Nitzan, "Inhibitory effect of oregano and thyme essential oils," *Thymus*, pp. 33–37, 1990.

[133] H. J. D. Dorman and S. G. Deans, "Antimicrobial agents from plants: antibacterial activity of plant volatile oils," *Journal of Applied Microbiology*, vol. 88, no. 2, pp. 308–316, 2000.

[134] J. Briozzo, L. Núñez, J. Chirife, L. Herszage, and M. D'Aquino, "Antimicrobial activity of clove oil dispersed in a concentrated sugar solution," *Journal of Applied Bacteriology*, vol. 66, no. 1, pp. 69–75, 1989.

[135] N. Silva, S. Alves, A. Gonçalves, J. S. Amaral, and P. Poeta, "Antimicrobial activity of essential oils from mediterranean aromatic plants against several foodborne and spoilage bacteria," *Food Science and Technology International*, vol. 19, no. 6, pp. 503–510, 2013.

[136] C. A. O'Bryan, S. J. Pendleton, P. G. Crandall, and S. C. Ricke, "Potential of plant essential oils and their components in animal agriculture—*in vitro* studies on antibacterial mode of action," *Frontiers in Veterinary Science*, vol. 2, article 35, 8 pages, 2015.

[137] M. Nevas, A.-R. Korhonen, M. Lindström, P. Turkki, and H. Korkeala, "Antibacterial efficiency of finnish spice essential oils against pathogenic and spoilage bacteria," *Journal of Food Protection*, vol. 67, no. 1, pp. 199–202, 2004.

[138] F. Hernández, J. Madrid, V. García, J. Orengo, and M. D. Megías, "Influence of two plant extracts on broilers performance, digestibility, and digestive organ size," *Poultry Science*, vol. 83, no. 2, pp. 169–174, 2004.

[139] D. Jamroz, J. Orda, C. Kamel, A. Wilczkiewicz, T. Wertelecki, and J. Skorupinska, "The influence of phytogenic extracts on performance nutrient digestibility, carcass characteristics, and gut microbial status in broiler chickens," *Journal of Animal and Feed Sciences*, vol. 12, no. 3, pp. 583–596, 2003.

[140] N. Mathlouthi, T. Bouzaienne, I. Oueslati et al., "Use of rosemary, oregano, and a commercial blend of essential oils in broiler chickens: in vitro antimicrobial activities and effects on growth performance," *Journal of Animal Science*, vol. 90, no. 3, pp. 813–823, 2012.

[141] R. Losa and B. Kohler, "Prevention of colonisation of Clostridium perfringens in broilers intestine by essential oils," in *Proceedings of the 13th European Symposium on Poultry Nutrition*, pp. 133–134, Blankenberge, Belgium, 2001.

[142] L. Tucker, "Maintaining poultry performance in antibiotic free diets by supplementation with commercial botanical feed ingredients," in *Proceedings of the 7th WPSA Asian Pacific Federation Conference*, pp. 227–230, Gold Coast, Australia, 2002.

[143] N. Yossa, J. Patel, P. Millner, and M. Lo, "Inactivation of *Salmonella* in organic soil by cinnamaldehyde, eugenol, ecotrol, and sporan," *Foodborne Pathogens and Disease*, vol. 8, no. 2, pp. 311–317, 2011.

[144] Y. Lu and C. Wu, "Reduction of *Salmonella enterica* contamination on grape tomatoes by washing with thyme oil, thymol, and carvacrol as compared with chlorine treatment," *Journal of Food Protection*, vol. 73, no. 12, pp. 2270–2275, 2010.

[145] W.-X. Du, C. W. Olsen, R. J. Avena-Bustillos, M. Friedman, and T. H. McHugh, "Physical and antibacterial properties of edible films formulated with apple skin polyphenols," *Journal of Food Science*, vol. 76, no. 2, pp. 149–155, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 3681094, 14 pages
http://dx.doi.org/10.1155/2016/3681094

Review Article

Microbial and Natural Metabolites That Inhibit Splicing: A Powerful Alternative for Cancer Treatment

Nancy Martínez-Montiel,[1] Nora Hilda Rosas-Murrieta,[2,3] Mónica Martínez-Montiel,[1] Mayra Patricia Gaspariano-Cholula,[1] and Rebeca D. Martínez-Contreras[1]

[1]*Laboratorio de Ecología Molecular Microbiana, Centro de Investigaciones en Ciencias Microbiológicas, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, Edificio IC11, Ciudad Universitaria, 72570 Colonia San Manuel, PUE, Mexico*
[2]*Laboratorio de Bioquímica y Biología Molecular, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, Edificio 103H, Ciudad Universitaria, 72550 Colonia San Manuel, PUE, Mexico*
[3]*Posgrado en Ciencias Químicas, Benemérita Universidad Autónoma de Puebla, Edificio 105 I, Ciudad Universitaria, 72570 Colonia San Manuel, PUE, Mexico*

Correspondence should be addressed to Rebeca D. Martínez-Contreras; rebeca.martinez@correo.buap.mx

Received 19 March 2016; Revised 27 June 2016; Accepted 3 July 2016

Academic Editor: Yiannis Kourkoutas

In eukaryotes, genes are frequently interrupted with noncoding sequences named introns. Alternative splicing is a nuclear mechanism by which these introns are removed and flanking coding regions named exons are joined together to generate a message that will be translated in the cytoplasm. This mechanism is catalyzed by a complex machinery known as the spliceosome, which is conformed by more than 300 proteins and ribonucleoproteins that activate and regulate the precision of gene expression when assembled. It has been proposed that several genetic diseases are related to defects in the splicing process, including cancer. For this reason, natural products that show the ability to regulate splicing have attracted enormous attention due to its potential use for cancer treatment. Some microbial metabolites have shown the ability to inhibit gene splicing and the molecular mechanism responsible for this inhibition is being studied for future applications. Here, we summarize the main types of natural products that have been characterized as splicing inhibitors, the recent advances regarding molecular and cellular effects related to these molecules, and the applications reported so far in cancer therapeutics.

1. Introduction

In eukaryotes, coding regions of the genome called exons are interrupted by noncoding sequences known as introns. During transcription, exons are identified while introns are removed from the immature mRNA (or pre-mRNA) to generate a mature and functional mRNA molecule. The mechanism responsible for this process corresponds to splicing and the machinery that performs this highly regulated event is the spliceosome, which is integrated by five small nuclear ribonucleoproteic particles (snRNPs) and more than 200 proteins that include auxiliary regulatory factors and components of other co- and posttranscriptional machineries [1]. During splicing, a series of RNA-RNA, RNA-protein, and protein-protein interactions are responsible for the decisions that determine which sequences will be included in the mature transcript [2]. Moreover, some sequences can be incorporated differentially into separated splicing events, leading to an increase in the coding potential of the genome by a process called alternative splicing.

2. Alternative Splicing and the Spliceosome

The general splicing mechanism involves the recognition of exon/intron boundaries in a sequence-dependent manner. In mammals, the 5′ end of the intron (5′ splice site or 5′ss) contains a characteristic TG, which recruits snRNP U1. On the opposite side, the 3′ end of the intron (3′ss) shows an invariant region called the branch point sequence (BPS), followed by a polypyrimidine-rich tract (pY-tract) and a

conserved AG dinucleotide that indicates the end of the intron [3]. The recognition of the 3′ss involves the binding of SF1 to the BPS and the recruitment of the snRNP U2 auxiliary factor (U2AF) to the pY-tract and the AG dinucleotide. After the recognition of both exon/intron boundaries, an early complex is formed that commits pre-mRNA to undergoing splicing, where U2 snRNP is also recruited to the 3′ss. U2 snRNP recruitment to the pre-mRNA is one of the key steps that triggers additional interactions, leading to the formation of catalytic spliceosome complexes due to the incorporation of the tri-snRNP U4/U5/U6 within which numerous RNA rearrangements and modifications in protein composition contribute to complete a splicing cycle [2, 3].

Like most of the snRNPs, U2 is a ribonucleoproteic complex formed by 7 Sm proteins (which are common for spliceosomal snRNPs) and 17 specific proteins, being the largest snRNP [3]. Among the specific snRNP U2 components, two protein subcomplexes are found: SF3a and SF3b [3–5]. SF3a includes 3 subunits of 60, 66, and 120 kDa [6] while SF3b shows at least 8 specific subunits of 10, 14a, 14b, 49, 125, 130, 140, and 155 kDa [7]. Components of the SF3a and SF3b subcomplexes bind to sequences in the pre-mRNA tethering U2 snRNP to the BPS and the 3′ss. SF3b 155 is one of the most conserved subunits of U2 snRNP and it has shown the ability to bind splicing factors U2AF65 and p14 [3, 8]. Interestingly, this subunit has been related to the antiproliferative effect observed for some natural products that regulate the splicing mechanism and it results clear in the fact that targeting the spliceosome and modulating splice-site recognition could be relevant for the development of new therapeutic approaches, as will be further discussed.

3. The Role of Alternative Splicing in Human Disease

Over the past 10 years, the role of alternative splicing in human disease has been growing. When the human genome project was completed, *in silico* analysis predicted that 75% of the human genes underwent splicing [26] and that 15 to 50% of the genetic diseases were related to aberrant splicing events [27]. From this initial observation, several studies have linked splicing defects with specific genetic disorders. However, the full significance of the role in alternative splicing in human disease remains to be elucidated. Some diseases that have been linked to defects on splicing include dilated cardiomyopathy, autism spectrum disorder, spinal muscular atrophy, schizophrenia, cardiac hypertrophy, amyotrophic lateral sclerosis, and frontotemporal dementia [28]. In all these cases, the molecular insights related to the splicing defect that originates the disease have been dissected. The precise regulation of the splicing event varies for each pre-mRNA and for this reason it is time consuming to demonstrate the molecular mechanism that regulates the alternative splicing for each gene. Moreover, this regulation also depends on the cellular context, complicating the scene. In this regard, future efforts need to be developed in order to dissect the alternative splicing event that is related to each disease and the possible therapeutic tools that could be applied.

One specific group of diseases that have been related to splicing corresponds to different types of cancer and only recently the determinant role of splicing in cancer has been acknowledged [29, 30]. Several features of splicing events related to tumor progression have been reported and it is well documented that the alternative splicing of different pre-mRNAs is altered during oncogenic progression with the concomitant development of cancer features, like an increase in vascularization, cell proliferation, and invasion [31, 32]. The molecular hallmarks documented for several types of cancer have been recapitulated in an attempt to orientate future efforts towards cancer treatment through alternative splicing modulation [33–35]. Considering all this evidences, several studies have been oriented to modulate alternative splicing in order to treat cancer.

4. Microbial Metabolites That Regulate Splicing

Natural products have been traditionally sought from actinomycetes, filamentous fungi, and medicinal plants. In this regard, several derivatives of bacterial fermentation as well as their synthetic equivalents possess the ability to interact with components of the spliceosome. In some cases, the effect on splicing associated with these drugs is achieved through the direct regulation of the expression of genes that are relevant for cancer progression [36]. Dozens of small molecule effectors targeting the alternative splicing process have been identified and evaluated as drug candidates, including a natural product of *Pseudomonas* sp. number 2663 called FR901464 [9], natural products from *Streptomyces platensis* Mer-11107 that originated the group of Pladienolides [37], Herboxidiene [15], and Isoginkgetin [38]. These molecules and their derivatives have shown activity as splicing inhibitors and many of them demonstrated potent antiproliferative properties in human cancer cell lines, being in general less toxic to normal human cells [39].

4.1. FR901464 and Derivatives. Spliceostatins are a group of compounds derived from the natural product FR901464, which was identified initially as an antitumor compound. In the original study, FR901463, FR901464, and FR901465 were isolated from the fermentation broth of *Pseudomonas* sp. number 2663 [9]. These 3 compounds are soluble in acetonitrile, chloroform, and ethyl acetate and poorly soluble in water and insoluble in hexane. They all show strong UV absorption at 235 nm distinctive of a conjugated diene, while the IR spectra indicated the presence of hydroxyl, ester, and a conjugated amide carbonyl (Figure 1). Initially, the three compounds from the FR9014 series mentioned before were tested for their biological activity. As a result, they all enhanced the transcriptional activity of SV40 in a CAT assay. Besides, they were all cytotoxic according to the MTT method in the following human adenocarcinomas: A549 lung cells, MCF-7 mammary cells, or HCT116 colon cells [9]. Moreover, the 3 compounds extended the life of mice bearing ascetic tumors, FR901464 being the one showing the most potent effect on the tumor systems assayed [9]. Furthermore, FR901464 induced

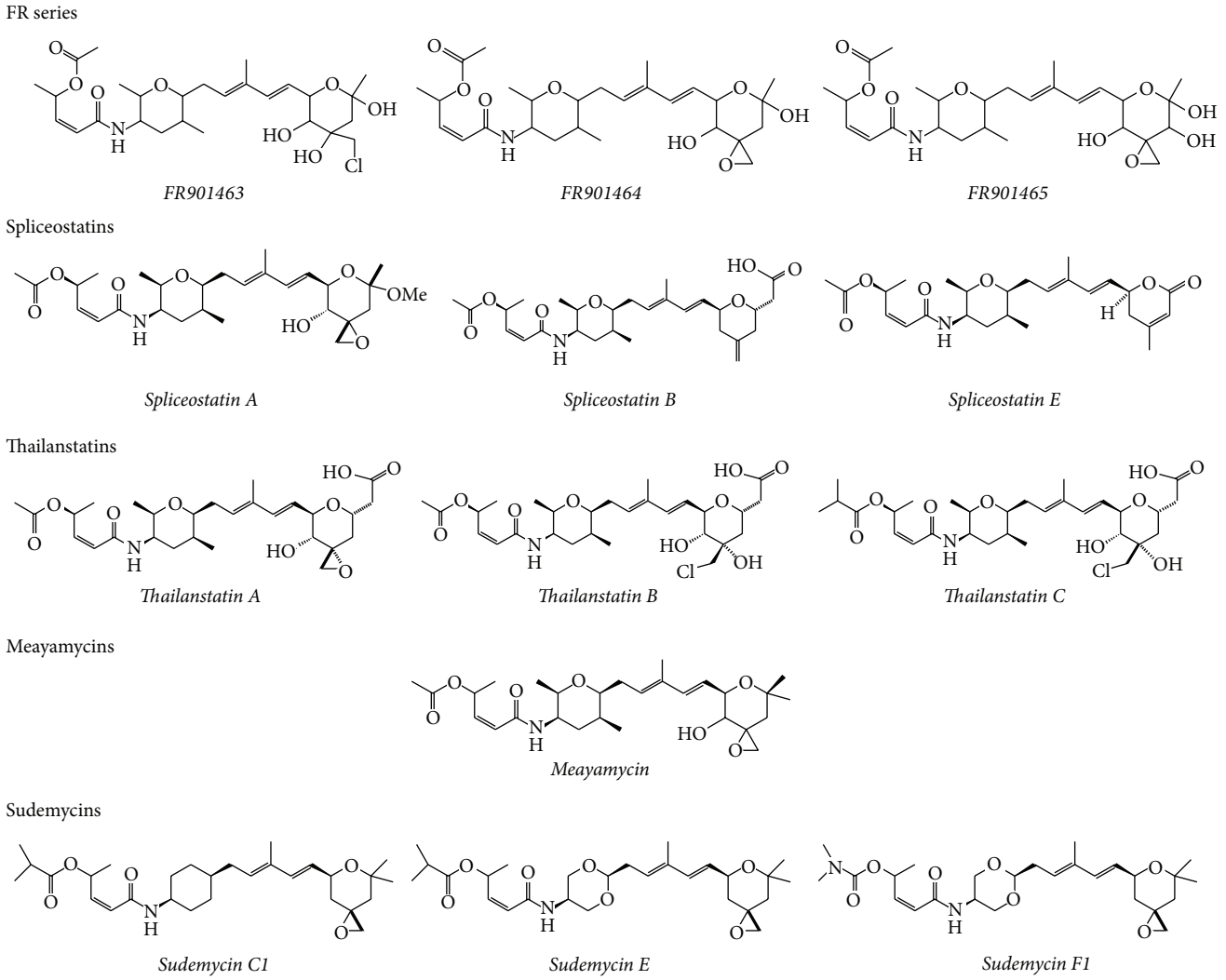


Figure 1: FR901464 and derivatives. The FR9014 series were isolated from *Pseudomonas* sp. number 2663 and constitute the first antiproliferative molecules associated with splicing inhibition. Spliceostatin A is a methylated derivative of FR901464. Spliceostatin B was also isolated from *Pseudomonas* sp. number 2663. Spliceostatin E was isolated from *Burkholderia* sp. FERM BP3421. Thailanstatins were recovered from *Burkholderia thailandensis* MSMB43. Meayamycin and Sudemycins are synthetic derivatives from the natural products depicted.

characteristic G1 and G2/M phase arrest in the cell cycle and suppressed the transcription of some inducible endogenous but not housekeeping genes in M-8 cells. In this same cell line, internucleosomal degradation of genomic DNA showed the same kinetics corresponding to the activation of SV40 promoter-dependent cellular transcription, suggesting that a chromatin rearrangement occurs upon the treatment with the drug. Despite this effect in inducing viral gene promoters, it was observed that FR901464 reduces the mRNA levels of several endogenous genes, including c-Myc [9].

Spliceostatin A is a methylated and more stable derivative of FR901464 (Figure 1) and they both show similar activity [37, 40]; the synthesis and activity for both molecules have also been reported [41, 42]. Even when there are few studies on the molecular interactions that mediate the effect of Spliceostatin and related molecules, the antiproliferative effect of Spliceostatin has been associated with splicing and seems to be equivalent to the one registered after knocking down SF3b155 [10]. Using immunoprecipitation assays, further studies demonstrated that FR901464 and its methylated derivative Spliceostatin A inhibit pre-mRNA splicing both *in vivo* and *in vitro* by binding noncovalently to the SF3b subcomplex in the U2 snRNP. In the same study, the treatment with Spliceostatin A allowed the identification of immature forms of p27 by RT-PCR, suggesting that pre-mRNA molecules that have not been fully spliced are transported to the cytoplasm, inducing the translation of aberrant mRNAs [11].

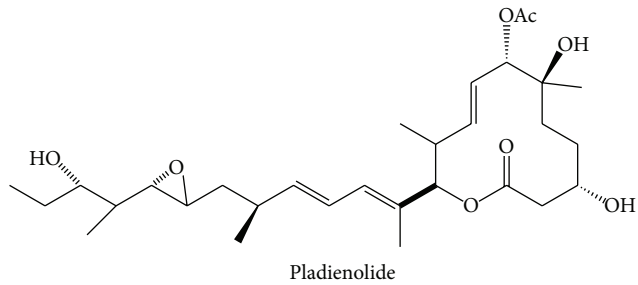
Another analogous compound of FR901464 named Spliceostatin B was purified from the fermentation broth of *Pseudomonas* sp. number 2663 [43]. Spliceostatin B is soluble in DMSO, acetonitrile, acetone, water, chloroform, and dichloromethane. The structure of Spliceostatin B was determined using UV, IR, HR-MS, and NMR spectroscopic

analyses, showing that it differs structurally from FR901464 at four points: the substitution of an epoxide group at C3 position with a terminal methylene moiety, the presence of a carboxyl moiety at C17 position, and the absence of two hydroxyl groups at C1 and C4 positions, respectively. These structural features are relevant for the biological function given the fact that it has been reported that loosing the C4 hydrogen bond donor decreases the cytotoxicity and that the C3 epoxide moiety is necessary for bioactivity [44]. The functional analog Spliceostatin B showed cytotoxic effect in three human cancer cell lines: HCT-116, MDA-MB-235, and H232A using the MTT method [45], but its activity was weaker than the one observed for FR901464 according to the IC values obtained [43] and in good correlation with the structural features just mentioned.

Other natural products considered Spliceostatin analogs were isolated from the fermentation broth of *Burkholderia* sp. strain FERM BP3421 [46]. Among these new molecules, Spliceostatin E exhibited good potency against multiple human cancer cell lines with IC_{50} values ranging from 1.5 to 4.1 nM. The structure of Spliceostatin E was elucidated by extensive spectroscopic studies and resulted structurally in less complex than Spliceostatins A and B [46]. Even when Spliceostatin E maintains the cytotoxic activity, the synthetic molecule showed no inhibition of splicing and it did not alter the structure of nuclear speckles [42].

It has been determined that the fr9 gene cluster is responsible for the biosynthesis of FR901464 in *Pseudomonas* sp. number 2663. The biosynthetic fr9 gene cluster spans a DNA region of approximately 81 kb and includes 20 genes (fr9A through fr9T). Using this information, a bioinformatic approach was conducted in order to identify other strains that could produce Spliceostatin-like metabolites. Using this comparative analysis while mining the genome of *Burkholderia thailandensis* MSMB43 elicited the identification of a biosynthetic gene cluster similar to fr9 that was named tst, referring to the Thailanstatin compounds it produces, which are functional analogs of Spliceostatins. The tst gene cluster spans a DNA region of 78 kb, which contains 15 ORFs designated tstA through tstR. The putative functions for the tst gene products were deduced by sequence comparisons with the FR9 proteins and with other bacterial homologs, where the most striking difference is the absence of the equivalent fr9S and fr9T genes from the tst gene cluster. A detailed analysis of this cluster suggested a possible biosynthetic route for Thailanstatins, which is similar to the one demonstrated for FR901464 and corresponds to a hybrid pathway involving a polyketide synthase and a nonribosomal peptide synthetase [47].

Consistent with the bioinformatic approach, Thailanstatins A, B, and C were isolated from the culture broth of *Burkholderia thailandensis* MSMB43 and they proved to be significantly more stable natural analogs of FR901464 [47]. These molecules are more stable because they lack a hydroxyl group found in FR901464 and they show an extra carboxyl moiety instead as revealed by the HR-MS, NMR, UV, and IR spectrometry. Thailanstatins possess the same linear polyketide-peptide framework observed in FR901464, but they lack a hydroxyl group at the C1 position while showing an extra carboxyl moiety at the C17 position. Thailanstatins B and C have a chloride substituent at the C3 position instead of the epoxide functionality observed for both Thailanstatin A and FR901464. Finally, Thailanstatin B possesses a dimethyl acetyl group at the distal end while Thailanstatin C shows an acetyl group instead [47].



Pladienolide

Figure 2: Pladienolide structure. Pladienolide is a 12-membered macrolide that possesses a long side chain at the carbon bearing lactone oxygen.

During the biological tests performed, all Thailanstatins exhibited strong antiproliferative activities when tested in the following human cancer cell lines: DU-145 (prostate cancer), NCI-H232A (non-small-cell lung cancer), MDA-MB-231 (triple-negative breast cancer), and SKOV-3 (ovarian cancer), Thailanstatin A being the one showing the strongest effect. Moreover, the three compounds showed the ability to inhibit *in vitro* splicing and again Thailanstatin A showed the best result being as strong as FR941464 in inhibiting splicing [47]. A summary of the molecular effects depicted for FR9414 series and other splicing inhibitors is presented in Table 1.

Considering that the molecules just presented are structurally quite complex, this results in difficulty to accomplish their structural modification. However, synthetic derivatives have been generated (Figure 1), including Meayamycin and Sudemycins [48].

4.2. Pladienolides. Pladienolide B is a macrocyclic lactone originally obtained from *Streptomyces platensis* Mer-11107, strain isolated from a soil sample collected in Kanagawa, Japan (accession number FERM P-18144, Bioconsortia Program Laboratory National Institute of Advanced Industrial Science and Technology, Japan). The compound deposited in the Open Chemistry Database (PubChem ID 52946850) has a molecular weight of 538.7132 g/mol (Figure 2).

Pladienolide B was initially identified in 2004, as part of a work that reported the isolation and structural and functional characterization of seven 12-membered macrocyclic compounds named Pladienolides A to G [37]. All these compounds displayed antiproliferative and tumor suppressive activities when assayed in cell culture and xenograft models, particularly Pladienolides B and D.

The initial extraction to isolate Pladienolides was performed with n-butanol from the fermentation broth of *Streptomyces platensis*. Further chromatography steps over Sephadex $LH\text{-}_{20}$ and silica gel column were accomplished.

Table 1: Molecular effects of different splicing inhibitors.

Splicing inhibitor	Cell line	Effect	Reference
FR9014 series	MCF-7	Induces G1 and G2/M arrest of the cell cycle	[9]
	HeLa	Inhibits the recognition of the branch point sequence	[10]
		Binding affinity to SAP145	[11]
		Arrest of SF3b	[12]
	MDA-MB-468	Interacts with SF3b subunit SAP145	[13]
Pladienolide	WiDr	Interacts with SF3b subunit SAP130	[13]
	HeLa	Interacts with SF3b. Remodeling of U2 snRNP to expose the branch point-binding region	[14]
Herboxidiene	Normal human fibroblast cell line WI-38.2	Induces G1 and G2/M arrest of the cell cycle	[15]
	HeLa	Causes arrest in G1 and G2/M phases and interacts with SF3b1 subunit SAP145	[16]
Trichostatin	WiDr	Interacts with SF3B subunit SAP130	[13]
Isoginkgetin	HT1080	Inhibition of Cathepsin K and MMP9	[17]
		Inhibits metalloproteinase MMP9 production and increases the synthesis of metalloproteinase inhibitor TIMP-1	[18]
	HEK293	Stimulates IL-8 expression	[19]
	Thyroid cancer	Increases expression of specific IL-32 isoforms and stimulates the expression of IL-8 and CXCR1	[19]

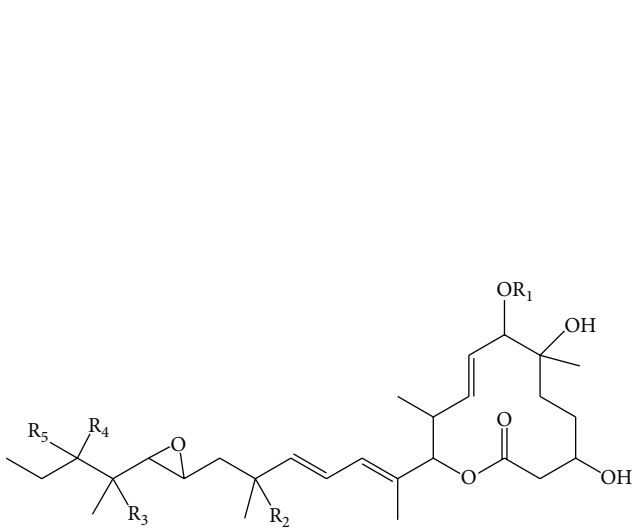
The bioactive fractions recovered were subjected to preparative HPLC and each fraction containing pure Pladienolides was freeze-dried.

The chemical properties of Pladienolides were determined using spectroscopic methods. According to the physicochemical characterization of Pladienolides (A–G), they are soluble in methanol, acetone, n-butanol, ethyl acetate, and DMSO, but not in n-hexane, or poorly soluble in water. In all compounds there is a diene system evidenced by the UV absorption at 240 nm. The chemical structure of Pladienolides A (1), B (2), C (3), D (4), E (5), F (6), and G (7) (Figure 3) was determined by the analyses of NMR, MS, IR, and 2D NMR spectra. The carbonyl and hydroxyl groups were detected in the IR spectra. All Pladienolides are 12-membered macrolides possessing a diene unit and one epoxide moiety with a long side chain at the carbon that bears lactone oxygen [37]. Regarding their biological activity, Pladienolides have highly potent *in vitro* and *in vivo* antitumor activities with potential for use in anticancer therapy [20].

Pladienolide B has shown strong *in vitro* and *in vivo* antitumor activity and growth inhibitory effect against various cell lines, some of them being resistant to chemotherapeutic agents routinely used. Pladienolide B and some for their analogs induce cell cycle arrest at both G1 and G2/M [49].

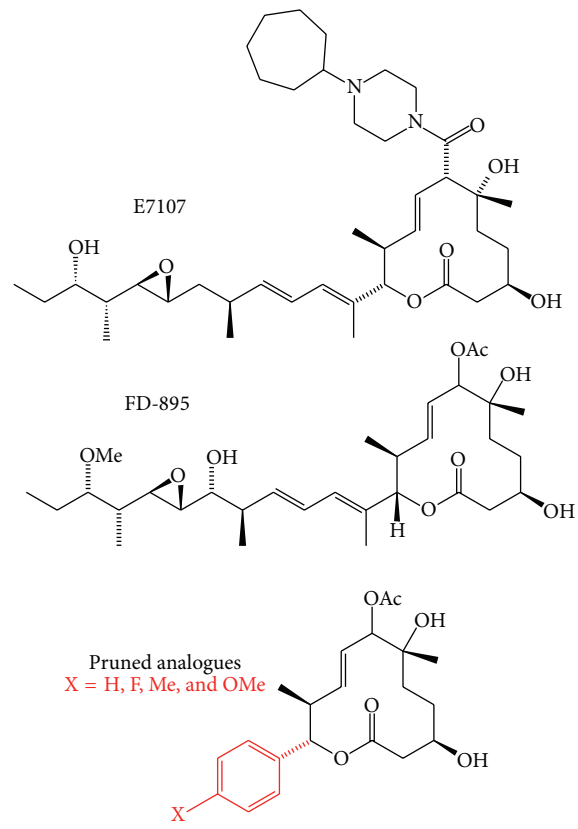
A different study using Pladienolides was oriented to identify compounds that contribute of the adaptation of cancer cells to hypoxia using HIF-1, an HLH transcription factor involved in hypoxia adaptation in cancer cells. This approach consisted in searching inhibitors of hypoxia adaptation involved in the regulation of angiogenesis and anaerobic metabolism, considering that hypoxia-inducible genes are relevant for growth of cancer cells. The screening system consisted of the placental alkaline phosphatase (PLAP) gene reporter under the control of the human VEGF promoter containing the hypoxia-responsive element (HRE) that binds HIF-1. The reporter construction was transfected and the hypoxia-induced PLAP expression was analyzed in the U251 human glioma cells. Using a high throughput screening, Pladienolides were identified as inhibitors of hypoxia-induced PLAP expression when the cells were exposed to hypoxic conditions [50, 51]. The pure compounds were probed in their anti-VEGF-PLAP and antiproliferative activity, but only Pladienolides B and D showed a strong activity in both tests, with IC_{50} of 1.8 and 3.5 for Pladienolide B and of 5.1 and 6 nM for Pladienolide D. In other studies, Pladienolide B showed a potent tumor regression and inhibition of mouse xenograft acting at low-nanomolar concentrations (Table 2). The cell growth inhibition properties of Pladienolide B were identified in a study using a 39-cell line drug-screening panel and additional cell cycle analysis indicated that Pladienolide B blocks cell growth in both the G1 and the G2/M phase [13].

Pladienolide B is the most potent metabolite of *S. platensis* with antitumor activity, but the chemical synthesis of Pladienolide is complicated [52] and only three approaches have been reported for the synthesis of these unique macrolides [53–55]. On the other hand, the great amount of compound required for *in vivo* studies remains a significant challenge, due to the synthetic complexity inherent to this class of compounds. In an attempt to generate simple molecules that retain the biological activity of Pladienolides, several analogs have been developed. The more effective Pladienolide analogs for antitumor or anticancer application reported to date are Pladienolide D, E7107, and truncated-Pladienolide versions. Pladienolide D (16-hydroxylated pladienolide) is produced



Pladienolide	R_1	R_2	R_3	R_4	R_5
A (1)	H	H	H	H	OH
B (2)	Ac	H	H	H	OH
C (3)	Ac	H	H	=O	=O
D (4)	Ac	OH	H	H	OH
E (5)	Ac	H	OH	H	OH
F (6)	H	OH	H	H	OH
G (7)	H	H	OH	H	OH

(a)



(b)

Figure 3: Pladienolide analogs. (a) General structure of Pladienolides A–G, which was determined by ^{1}H, ^{13}C NMR, MS, IR, and 2D NMR analyses. Radicals for each isoform are summarized in the table. (b) Different functional analogs have also been reported.

to a lesser extent than Pladienolide B on *S. platensis* Mer-11107. In order to facilitate the production of Pladienolide D, a biotransformation step of Pladienolide B into Pladienolide D was developed. In this alternative approach, the production of Pladienolide D was increased by 15-fold in the A-1544 strain of *S. bungoensis* by overexpressing the psmA gene, which encodes the Pladienolide B 16-hydroxylase (PsmA), responsible for the production of Pladienolide D [56]. Using a similar approach, the modified strain *S. platensis* Mer-11107 expressing the psmA gene from *S. bungoensis* A-1544 was obtained and in this case the production level of Pladienolide D was 10-fold higher [57]. Pladienolides B and D are promising candidates for further drug development because of their high efficacy and low toxicity; besides, their highly complex structure has been directed to the analog synthesis on a production scale [58].

E-7107 is a synthetic urethane derivative of Pladienolide D with activity against tumor cell lines and human xenografts [13]. E-7107 has a selective and potent antitumor activity in human tumor xenograft models such as human lung cancer LC-6-JCK, where E7107 caused complete tumor remission with poor toxicity. Moreover, E-7107 shows strong cell growth inhibitory activity against a large variety of human cancer cell lines (IC_{50} values range from 0.2 nM to 21.1 nM). Using an *in vivo* approach, E7107 produced significant tumor regression in a range of xenograft models. In this regard, animals with BSY-1 (breast), MDA-MB-468 (breast), LC-6-JCK (lung), NIH:OVCAR-3 (ovary) PC-3 (prostate), and WiDr (colon) xenografts were cured [25]. For this reason, E7107 rapidly advanced to Phase I clinical trials and the tests are currently in progress in Europe and the US (https://clinicaltrials.gov/ct2/show/NCT00459823?term=E-7107&rank=1). E7107 was tested in a Phase 1 clinical trial with patients with different types of solid tumors refractory to standard therapies, such as colorectal, esophageal, pancreatic, gastric, renal, and uterine, and was found to stabilize tumor growth [24, 59]. 40 patients received E7107 at doses from 0.6 to 4.5 mg/m^2 as a 30-minute intravenous infusion on days 1 and 8 every 21 days. The MTD for E7107 using this schedule is 4.0 mg/m^2 [24].

Finally, a different Pladienolide analog called FD-895 was isolated from *Streptomyces hygroscopicus* strain A-9561. FD-895 is a 12-membered macrolide antibiotic with a planar structure similar to Pladienolide D, but FD-895 has a hydroxyl group at the C-17 position and a methoxy group substituted for the hydroxy group at the C-21 position. FD-895 showed a cytotoxic activity against several types of cancer cells such as Adriamycin-resistant HL-60 [60]. In patients with chronic lymphocytic leukemia, FD-895 and Pladienolide B induced intron retention and spliceosome modulation.

Table 2: Antitumor activity of pladienolides.

Molecule	Cancer type or cell line	Effect	Reference
Pladienolide B	Breast (BSY-1, MCF-7) Central nervous system (SF-539) Colon (HCT-116) Lung (NCI-H522, NCI-H460, A549, DMS273, and DMS114) Melanoma (OVCAR-3) Stomach (MKN74) Prostate (DU-145)	*Growth inhibition* Cell viability was evaluated with MTT and alamarBlue assay. The growth inhibitory activity corresponded to the concentration at which cell growth was inhibited to 50% of control growth (IC_{50}). The strongest effect was observed for lung and breast cancer cell lines.	[20]
Pladienolide B	Anticancer drug-resistant cell lines: P388/CPT, P388/ETP, P388/CDDP, P388/VCR, HCT-116/5-FU, and MES-SA/Dx5	*Growth inhibition* Cell viability was evaluated with MTT and alamarBlue reagent and IC_{50} was determined. Pladienolide B showed differential strength depending on the cell line.	[20]
Pladienolide B	Human tumor xenografts: BSY-1, PC-3, OVCAR-3, DU-145, WiDr, and HCT116	*Antitumor* Cell suspensions of various human cancer cells were implanted subcutaneously into female or male BALB/c nu/nu mice. Tumor volume (TV) and relative body weight (RBW) were measured for 3 months after the treatment. Pladienolide B showed strong inhibitory or regressive activities against these xenografts.	[20]
Pladienolide B	WiDr and DLD1 human colorectal cancer cell lines	*Antiproliferative* Cells were incubated with 10 nM pladienolide B, 5-fluorouracil, taxol, or vincristine and then stained with propidium iodide. Pladienolide B caused a cell cycle arrest in both G1 and G2/M phases in a time-dependent manner according to FACS analysis.	[21]
Pladienolide B	Gastric cancer cell lines and primary cultured cancer cells from carcinomatous ascites of gastric cancer patients	*Antitumor* Using an MTT assay, the mean IC_{50} value was 1.2–1.1 nM for gastric, lung, and breast cancer cell lines. The mean IC_{50} value for primary cultured cells from the 12 cases studied was 4.9–4.7 nM. In xenograft models, the tumors completely disappeared within 2 weeks.	[22]
FD-895	Chronic lymphocytic leukemia	*Apoptosis* Peripheral blood mononuclear cells from CLL patients were exposed to 100 nM FD-895. Apoptosis was induced after 2 h exposure in an irreversible manner as measured by flow cytometry using a $PI/DiOC_6$ assay.	[23]
E7107	Lung, breast, and colon tumors	*Antineoplastic* Tumor regression in different xenograft models: BSY-1 (breast), MDA-MB-468 (breast), LC-6-JCK (NSCLC), and NIH:OVCAR-3 (ovary). The efficacy was limited in 26 patients enrolled for a trial. Cell-cycle arrest at G1 and G2/M phases was observed by flow cytometry. Highest efficiency against tumors was accompanied by functional loss of Rb and an increase of the expression of p16 and cyclin E.	[24, 25]

The cytotoxic effect of FD-895 involved the apoptosis induction in a caspase-dependent pathway [23].

4.3. Herboxidiene. Herboxidiene (GEXA1) is a polyketide [61] with the structure of a tetrahydrofuran with a residue of acetic acid and a conjugated diene [62]. These structural characteristics and its biological properties contributed to its name [63]. This compound was initially identified as a secondary metabolite from *Streptomyces chromofuscus* A7841. This compound was extracted with butanol and purified using HPLC. Further structural analysis was completed using HRFAB-MS and spectroscopic studies (RMN-^{1}H y ^{13}C).

Initial applications for Herboxidiene included its herbicide activity [36]. In 2002, six molecules sharing similar structures were isolated from *Streptomyces* sp. and were called GEX1 (Figure 4). These compounds showed antibiotic and antitumor activities, GEX 1A being the molecule with the strongest antiproliferative effect, which was later identified as Herboxidiene [15, 64]. This cytotoxic activity seems to be related to cell cycle arrest in G1 and G2/M according to some

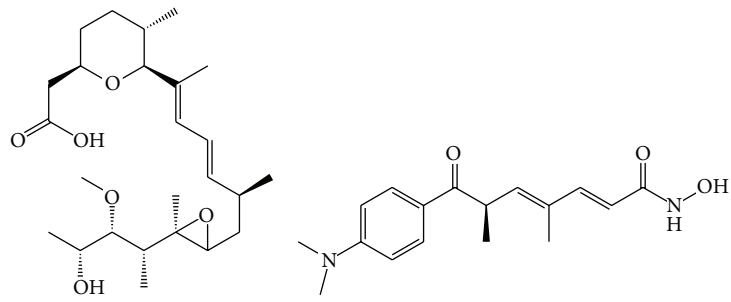


Figure 4: Herboxidiene structure. The characteristic structure of GEXA1 consisting of a tetrahydrofuran with a residue of acetic acid and a conjugated diene is shown in the left. This natural product was isolated from *Streptomyces* sp. The derivative Trichostatin is shown at right.

in vitro experiments [65]. Besides the biological activities mentioned before, Herboxidiene has also shown activity as anticholesterol agent and as a potent splicing inhibitor.

Due to the multiple biological effects demonstrated for Herboxidiene, several groups have attempted the chemical synthesis of the compound. The first total synthesis was accomplished in 1999, where the relative and absolute configurations of Herboxidiene were confirmed [66]. Later attempts used several routes, including a stereochemical synthesis in 18 steps [67], an enantioselective synthesis in 16 steps [68], or an alternative synthesis in 16 steps with a global yield of 3.4% [69]. An additional chemical synthesis reported was performed starting from two chiral ketones derived from lactate in 14 steps with a global yield of 8% [70]. A total enantioselective synthesis of Herboxidiene was reported in 2014 and the obtained product showed a mild inhibitory activity on the spliceosome [71]. In that same year, the alternative synthesis of Herboxidiene and some other analogs like a Pladienolide-Herboxidiene hybrid was reported, where alternative splicing was efficiently modulated [72]. These observations supported the potential role of Herboxidiene analogs as drug candidates for cancer treatment.

Some molecules with pharmacological activities similar to those reported for Herboxidiene are Trichostatin and TMC-49A (Figure 4). These compounds have also shown anticholesterol activity. However, Herboxidiene has shown a stronger effect on lowering the amount of cholesterol in plasma by regulating the LDL (Low Density Lipoproteins) receptor [73].

It has been shown that Herboxidiene inhibits splicing due to its ability to bind SAP155, a component of the SF3b complex of the spliceosome, altering its functionality [65]. This was accomplished using tagged molecules of Herboxidiene [73].

The effect of Herboxidiene on the splicing of cancer-related genes has also been demonstrated for a couple of cases. For example, Herboxidiene inhibits the splicing of p27, generating an isoform that is unable to bind the E3 ligase, inducing the accumulation of p27, which is in turn free to recognize and block the E-Cdk2 complex responsible for the E3-mediated degradation of p27. Some of the evidence supporting the role of Herboxidiene in cancer regulation is summarized in Table 1.

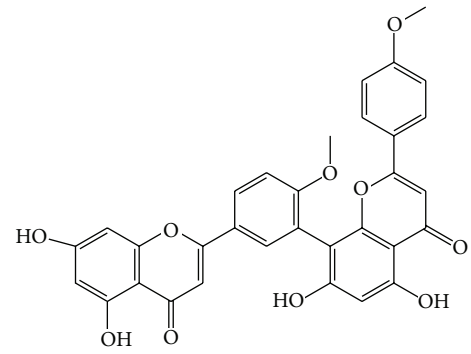


Figure 5: Structure of Isoginkgetin. The structure of the 7-*O*-*β*-D-glucopyranoside isolated from dried leaves of *Gingko biloba* is depicted.

4.4. Isoginkgetin. Isogingketin (7-*O*-*β*-D-glucopyranoside) is a glycosylated biflavonoid (Figure 5) initially isolated from dried leaves of *Gingko biloba*, a medicinal plant long utilized in traditional eastern medicine. General extraction uses methanol and isolation was performed by column chromatography [38] while further characterization was completed using spectroscopic approaches including IR, UV, HR-FAB-MS, and NMR [74].

Baker and Ollis started to work on the isolation of a compound known as Ginkgetin in 1957; however, they were not able to succeed until later when the molecule was separated using a potassium salt using an approach developed in collaboration with Nakasawa [75]. When applying this technique, the recovered compound was an isomer of Ginkgetin and for this reason the molecule was called Isoginkgetin. From that moment, several studies have been performed in order to analyze the properties and applications of this natural compound.

After the initial isolation, Isoginkgetin has been obtained from other plants including *Dysoxylum lenticellare Gillespie* [76], *Chamaecyparis obtusa* [77], *Cephalotaxus koreana* [78], and *Cycas circinalis* [79] as well as from the fruits of *Capparis spinosa* [80], *Cyperus rotundus* [81], *Selaginella* [82], and *Podocarpus henkelii* [83].

The biological activity of several biflavonoid compounds has been analyzed in various studies due to its natural origin and its abundance in plants, especially in ferns. Demonstrated activities for the extract of *Ginkgo biloba* are miscellaneous, according to the following evidence. The anti-inflammatory activity has been related to the inhibition of arachidonic acid [84], the inhibition of COX-2 [85], SOD [86], cAMP phosphodiesterases [87], and the suppression of lymphocyte proliferation [88]. Other activities include the neuroprotector and cytoprotector effects when cells are exposed either to external or intrinsic factors like UV radiation [89] or the accumulation of β-amyloid in neurons [90, 91]. This extract has also been shown to increase adiponectin secretion [92] and the activity of AMP-kinases [93]. Considering these biological activities, Isoginkgetin has been a candidate compound to treat disorders like diabetes, Alzheimer's disease, and other neurodegenerative diseases [94].

In relation to the effect of Isoginkgetin in splicing, it has been shown that the molecule has the ability to inhibit splicing both *in vitro* and *in vivo* at similar concentrations (30–33 μM). Isoginkgetin was identified as a splicing inhibitor using a cell-based reporter assay in HEK293 cells [95]. In the same study, it was suggested that the inhibitory effect possibly occurs due to the prevention of the stable recruitment of the U4/U5/U6 trismall nuclear ribonucleoprotein. Using HeLa cells, Isoginkgetin was applied to study *in vivo* mRNA dynamics, where an accumulation of intron-containing mRNAs was observed upon the treatment [96]. In this same cell line, Isoginkgetin mimics the effect of RNA exosome inhibition and causes accumulation of long human Telomerase RNA transcripts [97]. The effect of Isoginkgetin on splicing was also evaluated by studying the expression of interleukin 32 (IL-32) alternative isoforms, where IL-32γ isoform is overexpressed upon the treatment (Table 1), correlating with cell death in cell lines derived from thyroid cancer [19].

As observed for other splicing inhibitors, Isoginkgetin also shows antitumor activity, which has been related to the inhibition of metalloprotease MMP-9 production and to the increase of the inhibitors of metalloproteinase TIMP-1, resulting in a decrease of tumor invasion [18].

Further applications involving the use of splicing inhibitors can also expand our knowledge concerning the global regulation of gene expression. In a recent study, Isoginkgetin was coupled to a modified RNA-Seq method in order to provide a genome-wide insight into gene expression and to detect specific defects on splicing and transcription [96].

5. Mechanistic Insights

It has been demonstrated that Spliceostatin A, Pladienolide B, and Herboxidiene show the ability to interact directly with the spliceosome and that the molecular target for this molecule is the SF3b spliceosome subunit (Figure 6), a subcomplex of U2 snRNP [13]. The particular details observed for Pladienolide B are presented here.

Some studies have demonstrated that drug-treated human tumor xenografts can result in complete loss of the full-length mRNA for certain genes such as MDM2 in rhabdomyosarcoma cells. Pladienolide treatment causes an accumulation of unspliced or incompletely spliced pre-mRNAs and gives rise to fewer and larger nuclear speckles, the intranuclear sites where splice factors are stored [98]. Recently, it was demonstrated that splicing inhibition by Pladienolide B decreased phospho-Ser2 level [99] suggesting that the alteration of gene splicing may represent a suitable target for those drugs.

To identify the interaction between Pladienolides and a splicing protein, Pladienolide-tagged probes were used by modification of the acetoxy group at position 7 of Pladienolide B. Chemical tags included ^{3}H-labeled, fluorescence-tagged, and photoaffinity/biotin- (PB-) tagged "chemical probes" (BODIPY-FL). The chemical probes were used in the VEGF-reporter gene expression and cell growth inhibition assays at low-nanomolar to submicromolar IC_{50}. Pladienolide B blocks splicing and prompts nuclear export of intron-containing transcripts as observed by fluorescence microscopy, where the BODIPY-FL probe was concentrated in the nuclei of HeLa cells used as expression system overlapping with the signal obtained of splicing factor SC-35, a marker of nuclear speckles, where splicing factors are located [100]. Using immunoprecipitation experiments, the splicing proteins that interact with Pladienolide B were identified, such as 2,2,7-trimethylguanosine (TMG), Sm BB$'$&D1 protein, the U2 snRNP-specific protein U2B$''$, spliceosome-associated protein 120 (SAP120, SF3a subunit 1), spliceosome-associated protein 155 (SAP155, SF3b subunit 1), and cyclin E. Therefore, U2 snRNP that functions at the 3$'$ splicing site (Figure 6) seemed to correspond to the target for Pladienolide B [21]. Further immunoprecipitation assays allowed the identification of SAP145 (SF3b subunit 2) and SAP130 (SF3b subunit 3) as Pladienolide B partners. In addition, it was reported that there is a direct interaction between the Pladienolide B probe and SAP130. The mechanism by which Pladienolide impairs *in vivo* splicing involves SF3b modulation by interacting with SAP130 in the SF3b complex, but it is also possible that Pladienolide B shows a partial interaction with SAP155 or SAP145. Besides, time- and dose-dependent disturbance of *in vivo* splicing with Pladienolide B results in the formation of enlarged "megaspeckles," as observed when RNPS1 is overexpressed [101]. In a different approach using the CRISPR/Cas9 genome engineering system, it was demonstrated that the spliceosomal target of Pladienolide B is the SF3b1 subunit [102]. In the search of the mechanism by which Pladienolide B or E-7107 promote the formation of a defective spliceosome, it was found that E7 blocks ATP-dependent remodeling of U2 snRNP that exposes the branch point-binding region. Under this scenario, U2 snRNP fails to bind tightly to the pre-mRNA without disrupting the U2 particle or its association with SF3b [49, 103].

The use of the CRISPRCas9 system in HEK293T cells allowed the observation that some mutations in the subunits of the SF3b complex I promote higher levels of resistance to Pladienolide B. It was also found that R1074H mutation in SF3b1 could have a role in the resistance to Pladienolide [104]. In some reports, it has been proposed that mutations in SF3b1 are associated with numerous types of cancers such as acute myeloid leukemia, primary myelofibrosis, chronic

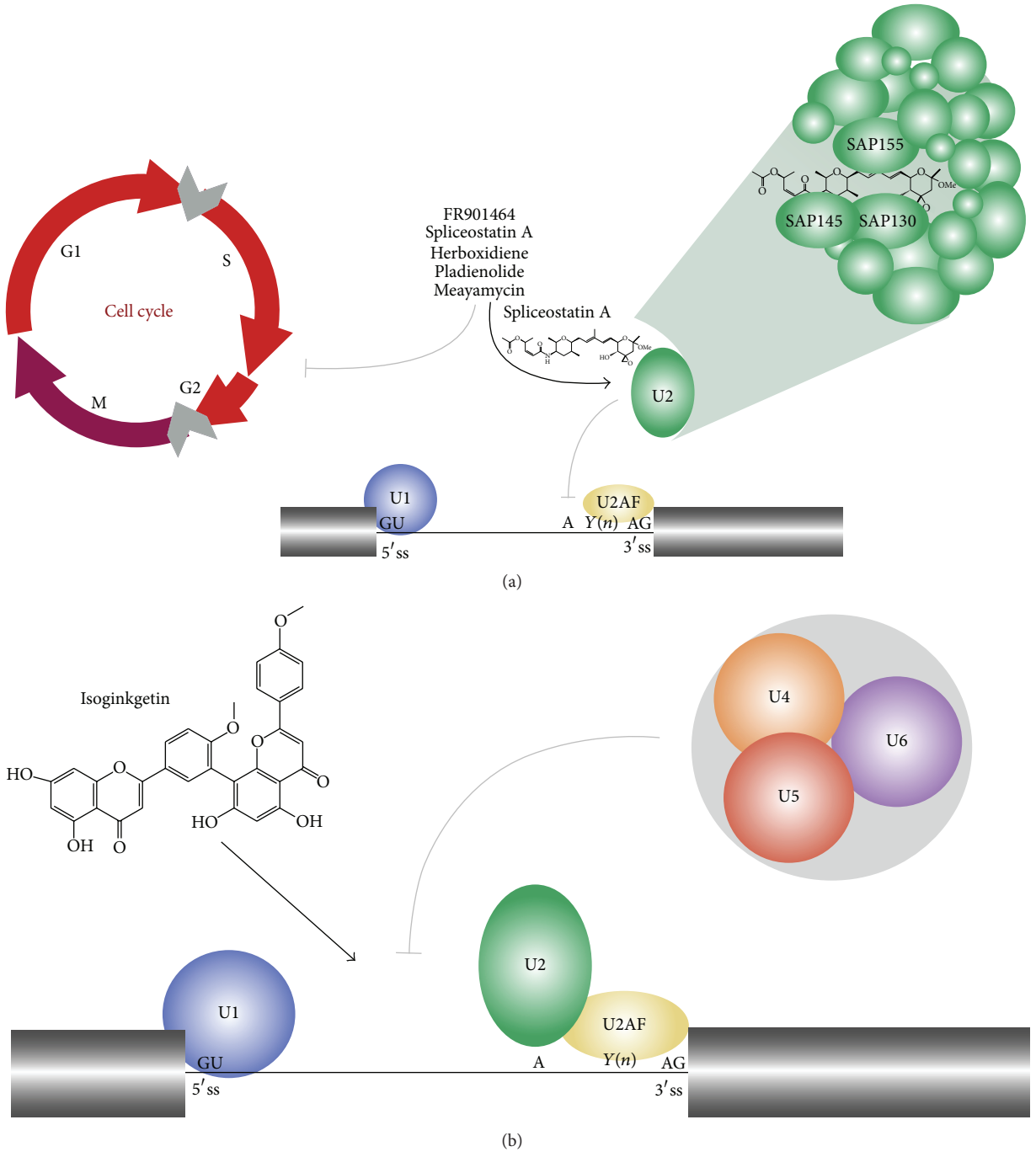


Figure 6: Molecular mechanism depicted for the natural products that inhibit splicing. (a) It has been demonstrated that FR901464, Spliceostatin A, Pladienolide B, Herboxidiene, and Meayamycin have the ability to block splicing by binding SAP130, SAP145, and SAP155 subunits of snRNP U2 (green). Besides, these natural products block cell cycle in G1 and G2/M transitions (gray arrows). (b) On the other hand, Isoginkgetin blocks splicing by inhibiting the incorporation of the tri-snRNP U4/U5/U6 complex to the spliceosome.

myelomonocytic leukemia, breast cancer, chronic lymphocytic leukemia, and multiple myeloma [105, 106]. The sequence of the SF3b1 gene of 2087 patients with myelodysplastic syndromes (MDS) showed mutations in 20% of all of them, where the K700E mutation was the most frequently found [105]. Finally, the interaction of spliceosome modulators as Pladienolides with the SF3b complex leads to an imbalance in the splicing program in susceptible cells, which may induce apoptosis by changing the levels and/or ratios of essential (and aberrant) proteins in tumor cells [106].

6. Concluding Remarks

Alternative splicing is responsible for increasing the coding potential of the human genome and the implication of this mechanism in human health is starting to be elucidated. Future studies could analyze particular splicing events related to a specific genetic disease making the discovery of new drugs for the treatment of particular disorders possible. In the case of the treatment of cancer, available molecules that target the spliceosome have effectively reverted proliferation of different types of tumors with very low toxicity, suggesting that splicing modulation is an attractive target for cancer treatment. Moreover, considering that some of the splicing inhibitors recently discovered are natural microbial metabolites, it is possible to assume that there are several molecules with antitumor activity that remain to be discovered. Considering that the molecular mechanism related to particular types of cancer is being explored, it could be possible to develop new drugs that could be oriented to modulate a precise splicing event in order to treat a special genetic disease.

Competing Interests

The authors declare that they have no competing interests.

References

[1] M. S. Jurica and M. J. Moore, "Pre-mRNA splicing: awash in a sea of proteins," *Molecular Cell*, vol. 12, no. 1, pp. 5–14, 2003.

[2] A. J. Matlin, F. Clark, and C. W. J. Smith, "Understanding alternative splicing: towards a cellular code," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 5, pp. 386–398, 2005.

[3] C. L. Will and R. Lührmann, "Spliceosomal UsnRNP biogenesis, structure and function," *Current Opinion in Cell Biology*, vol. 13, no. 3, pp. 290–301, 2001.

[4] B. K. Das, L. Xia, L. Palandjian, O. Gozani, Y. Chyung, and R. Reed, "Characterization of a protein complex containing spliceosomal proteins SAPs 49, 130, 145, and 155," *Molecular and Cellular Biology*, vol. 19, no. 10, pp. 6796–6802, 1999.

[5] A. Krämer, P. Grüter, K. Gröning, and B. Kastner, "Combined biochemical and electron microscopic analyses reveal the architecture of the mammalian U2 snRNP," *Journal of Cell Biology*, vol. 145, no. 7, pp. 1355–1368, 1999.

[6] R. Brosi, H.-P. Hauri, and A. Kramer, "Separation of splicing factor SF3 into two components and purification of SF3a activity," *The Journal of Biological Chemistry*, vol. 268, no. 23, pp. 17640–17646, 1993.

[7] M. C. Wahl, C. L. Will, and R. Lührmann, "The spliceosome: design principles of a dynamic RNP machine," *Cell*, vol. 136, no. 4, pp. 701–718, 2009.

[8] K. R. Thickman, M. C. Swenson, J. M. Kabogo, Z. Gryczynski, and C. L. Kielkopf, "Multiple U2AF65 binding sites within SF3b155: thermodynamic and spectroscopic characterization of protein-protein interactions among pre-mRNA splicing factors," *Journal of Molecular Biology*, vol. 356, no. 3, pp. 664–683, 2006.

[9] H. Nakajima, Y. Hori, H. Terano et al., "New antitumor substances, FR901463, FR901464 and FR901465: II. Activities against experimental tumors in mice and mechanism of action," *Journal of Antibiotics*, vol. 49, no. 12, pp. 1204–1211, 1996.

[10] A. Corrionero, B. Miñana, and J. Valcárcel, "Reduced fidelity of branch point recognition and alternative splicing induced by the anti-tumor drug spliceostatin A," *Genes and Development*, vol. 25, no. 5, pp. 445–459, 2011.

[11] D. Kaida, H. Motoyoshi, E. Tashiro et al., "Spliceostatin A targets SF3b and inhibits both splicing and nuclear retention of pre-mRNA," *Nature Chemical Biology*, vol. 3, no. 9, pp. 576–583, 2007.

[12] G. A. Roybal and M. S. Jurica, "Spliceostatin A inhibits spliceosome assembly subsequent to prespliceosome formation," *Nucleic Acids Research*, vol. 38, no. 19, Article ID gkq494, pp. 6664–6672, 2010.

[13] Y. Kotake, K. Sagane, T. Owa et al., "Splicing factor SF3b as a target of the antitumor natural product pladienolide," *Nature Chemical Biology*, vol. 3, no. 9, pp. 570–575, 2007.

[14] E. G. Folco, K. E. Coil, and R. Reed, "The anti-tumor drug E7107 reveals an essential role for SF3b in remodeling U2 snRNP to expose the branch point-binding region," *Genes & Development*, vol. 25, no. 5, pp. 440–444, 2011.

[15] Y. Sakai, T. Tsujita, T. Akiyama et al., "GEX1 compounds, novel antitumor antibiotics related to herboxidene, produced by Streptomyces sp. II. The effects on cell cycle progression and gene expression," *Journal of Antibiotics*, vol. 55, no. 10, pp. 863–872, 2002.

[16] M. Hasegawa, T. Miura, K. Kuzuya et al., "Identification of SAP155 as the target of GEX1A (Herboxidiene), an antitumor natural product," *ACS Chemical Biology*, vol. 6, no. 3, pp. 229–233, 2011.

[17] G.-Z. Zeng, N.-H. Tan, X.-J. Hao, Q.-Z. Mu, and R.-T. Li, "Natural inhibitors targeting osteoclast-mediated bone resorption," *Bioorganic and Medicinal Chemistry Letters*, vol. 16, no. 24, pp. 6178–6180, 2006.

[18] S.-O. Yoon, S. Shin, H.-J. Lee, H.-K. Chun, and A.-S. Chung, "Isoginkgetin inhibits tumor cell invasion by regulating phosphatidylinositol 3-kinase/Akt-dependent matrix metalloproteinase-9 expression," *Molecular Cancer Therapeutics*, vol. 5, no. 11, pp. 2666–2675, 2006.

[19] B. Heinhuis, T. S. Plantinga, G. Semango et al., "Alternatively spliced isoforms of IL-32 differentially influence cell death pathways in cancer cell lines," *Carcinogenesis*, vol. 37, no. 2, pp. 197–205, 2016.

[20] Y. Mizui, T. Sakai, M. Iwata et al., "Pladienolides, new substances from culture of Streptomyces platensis Mer-11107. III. In vitro and in vivo antitumor activities," *Journal of Antibiotics*, vol. 57, no. 3, pp. 188–196, 2004.

[21] A. Yokoi, Y. Kotake, K. Takahashi et al., "Biological validation that SF3b is a target of the antitumor macrolide pladienolide," *FEBS Journal*, vol. 278, no. 24, pp. 4870–4880, 2011.

[22] M. Sato, N. Muguruma, T. Nakagawa et al., "High antitumor activity of pladienolide B and its derivative in gastric cancer," *Cancer Science*, vol. 105, no. 1, pp. 110–116, 2014.

[23] M. K. Kashyap, D. Kumar, R. Villa et al., "Targeting the spliceosome in chronic lymphocytic leukemia with the macrolides FD-895 and pladienolide-B," *Haematologica*, vol. 100, no. 7, pp. 945–954, 2015.

[24] F. A. L. M. Eskens, F. J. Ramos, H. Burger et al., "Phase I pharmacokinetic and pharmacodynamic study of the first-in-class spliceosome inhibitor E7107 in patients with advanced solid tumors," *Clinical Cancer Research*, vol. 19, no. 22, pp. 6296–6304, 2013.

[25] M. Iwata, I. Ozawa, T. Uenaka et al., "E7107, a new 7-urethane derivative of pladienolide D, displays curative effect against

several human tumor xenografts," *Proceedings of the American Association for Cancer Research*, vol. 64, no. 7, supplement, p. 691, 2014.

[26] J. M. Johnson, J. Castle, P. Garrett-Engele et al., "Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays," *Science*, vol. 302, no. 5653, pp. 2141–2144, 2003.

[27] J. Tazi, N. Bakkour, and S. Stamm, "Alternative splicing and disease," *Biochimica et Biophysica Acta (BBA)—Molecular Basis of Disease*, vol. 1792, no. 1, pp. 14–26, 2009.

[28] B. Chabot and L. Shkreta, "Defective control of pre–messenger RNA splicing in human disease," *The Journal of Cell Biology*, vol. 212, no. 1, pp. 13–27, 2016.

[29] S. Bonomi, S. Gallo, M. Catillo, D. Pignataro, G. Biamonti, and C. Ghigna, "Oncogenic alternative splicing switches: role in cancer progression and prospects for therapy," *International Journal of Cell Biology*, vol. 2013, Article ID 962038, 17 pages, 2013.

[30] M. Ladomery, "Aberrant alternative splicing is another hallmark of cancer," *International Journal of Cell Biology*, vol. 2013, Article ID 463786, 6 pages, 2013.

[31] R. M. Hagen and M. R. Ladomery, "Role of splice variants in the metastatic progression of prostate cancer," *Biochemical Society Transactions*, vol. 40, no. 4, pp. 870–874, 2012.

[32] S. Oltean and D. O. Bates, "Hallmarks of alternative splicing in cancer," *Oncogene*, vol. 33, pp. 5311–5318, 2013.

[33] N. Martínez-Montiel, N. Rosas-Murrieta, and R. Martínez-Contreras, "Alternative splicing regulation: implications in cancer diagnosis and treatment," *Medicina Clinica*, vol. 144, no. 7, pp. 317–323, 2015.

[34] M. Contreras Rebeca and M. Montiel Nancy, "Alternative splicing modification as a treatment for genetic disorders," *Gene Technology*, vol. 4, article 126, pp. 1–4, 2015.

[35] R. D. Martínez-Contreras and N. Martínez-Montiel, "The role of splicing factors in cancer prognosis and treatment," in *Alternative Splicing and Disease*, E. Massey, Ed., Nova Science, New York, NY, USA, 2016.

[36] S. Bonnal, L. Vigevani, and J. Valcárcel, "The spliceosome as a target of novel antitumour drugs," *Nature Reviews Drug Discovery*, vol. 11, no. 11, pp. 847–859, 2012.

[37] T. Sakai, N. Asai, A. Okuda, N. Kawamura, and Y. Mizui, "Pladienolides, new substances from culture of *Streptomyces platensis* Mer-11107. II. Physico-chemical properties and structure elucidation," *The Journal of Antibiotics*, vol. 57, no. 3, pp. 180–187, 2004.

[38] S. S. Kang, J. S. Kim, and W. J. Kawk, "Flavonoids from Ginkgo biloba leaves," *Korean Journal of Pharmacognosy*, vol. 21, pp. 111–120, 1990.

[39] L. Fan, C. Lagisetti, C. C. Edwards, T. R. Webb, and P. M. Potter, "Sudemycins, novel small molecule analogues of FR901464, induce alternative gene splicing," *ACS Chemical Biology*, vol. 6, no. 6, pp. 582–589, 2011.

[40] H. Motoyoshi, M. Horigome, K. Ishigami et al., "Structure-activity relationship for FR901464: a versatile method for the conversion and preparation of biologically active biotinylated probes," *Bioscience, Biotechnology and Biochemistry*, vol. 68, no. 10, pp. 2178–2182, 2004.

[41] A. K. Ghosh and Z.-H. Chen, "Enantioselective syntheses of FR901464 and spliceostatin A: potent inhibitors of spliceosome," *Organic Letters*, vol. 15, no. 19, pp. 5088–5091, 2013.

[42] A. K. Ghosh, Z.-H. Chen, K. A. Effenberger, and M. S. Jurica, "Enantioselective total syntheses of fr901464 and spliceostatin A and evaluation of splicing activity of key derivatives," *Journal of Organic Chemistry*, vol. 79, no. 12, pp. 5697–5709, 2014.

[43] X. Liu, S. Biswas, G.-L. Tang, and Y.-Q. Cheng, "Isolation and characterization of spliceostatin B, a new analogue of FR901464, from Pseudomonas sp. No. 2663," *Journal of Antibiotics*, vol. 66, no. 9, pp. 555–558, 2013.

[44] S. Osman, B. J. Albert, Y. Wang, M. Li, N. L. Czaicki, and K. Koide, "Structural requirements for the antiproliferative activity of pre-mRNA splicing inhibitor FR901464," *Chemistry*, vol. 17, no. 3, pp. 895–904, 2011.

[45] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," *Journal of Immunological Methods*, vol. 65, no. 1-2, pp. 55–63, 1983.

[46] H. He, A. S. Ratnayake, J. E. Janso et al., "Cytotoxic spliceostatins from Burkholderia sp. and their semisynthetic analogues," *Journal of Natural Products*, vol. 77, no. 8, pp. 1864–1870, 2014.

[47] X. Liu, S. Biswas, M. G. Berg et al., "Genomics-guided discovery of thailanstatins A, B, and C as pre-mRNA splicing inhibitors and antiproliferative agents from *Burkholderia thailandensis* MSMB43," *Journal of Natural Products*, vol. 76, no. 4, pp. 685–693, 2013.

[48] T. Schneider-Poetsch, T. Usui, D. Kaida, and M. Yoshida, "Garbled messages and corrupted translations," *Nature Chemical Biology*, vol. 6, no. 3, pp. 189–198, 2010.

[49] D. Pham and K. Koide, "Discoveries, target identifications, and biological applications of natural products that inhibit splicing factor 3B subunit 1," *Natural Product Reports*, vol. 33, no. 5, pp. 637–647, 2016.

[50] C. Blancher and A. L. Harris, "The molecular basis of the hypoxia response pathway: tumour hypoxia as a therapy target," *Cancer and Metastasis Reviews*, vol. 17, no. 2, pp. 187–194, 1998.

[51] B. Burke, A. Giannoudis, K. P. Corke et al., "Hypoxia-induced gene expression in human macrophages: implications for ischemic tissues and hypoxia-regulated gene therapy," *The American Journal of Pathology*, vol. 163, no. 4, pp. 1233–1243, 2003.

[52] A. L. Mandel, B. D. Jones, J. J. La Clair, and M. D. Burkart, "A synthetic entry to pladienolide B and FD-895," *Bioorganic and Medicinal Chemistry Letters*, vol. 17, no. 18, pp. 5159–5164, 2007.

[53] A. K. Ghosh and D. D. Anderson, "Enantioselective total synthesis of pladienolide B: a potent spliceosome inhibitor," *Organic Letters*, vol. 14, no. 18, pp. 4730–4733, 2012.

[54] R. M. Kanada, D. Itoh, M. Nagai et al., "Total synthesis of the potent antitumor macrolides pladienolide B and D," *Angewandte Chemie-International Edition*, vol. 46, no. 23, pp. 4350–4355, 2007.

[55] V. P. Kumar and S. Chandrasekhar, "Enantioselective synthesis of pladienolide B and truncated analogues as new anticancer agents," *Organic Letters*, vol. 15, no. 14, pp. 3610–3613, 2013.

[56] K. Machida, Y. Aritoku, T. Nakashima, A. Arisawa, and T. Tsuchida, "Increase in pladienolide D production rate using a Streptomyces strain overexpressing a cytochrome P450 gene," *Journal of Bioscience and Bioengineering*, vol. 105, no. 6, pp. 649–654, 2008.

[57] K. Machida, Y. Aritoku, and T. Tsuchida, "One-pot fermentation of pladienolide D by *Streptomyces platensis* expressing a heterologous cytochrome P450 gene," *Journal of Bioscience and Bioengineering*, vol. 107, no. 6, pp. 596–598, 2009.

[58] S. R. Bathula, S. M. Akondi, P. S. Mainkar, and S. Chandrasekhar, "'Pruning of biomolecules and natural products (PBNP)': an innovative paradigm in drug discovery," *Organic and Biomolecular Chemistry*, vol. 13, no. 23, pp. 6432–6448, 2015.

[59] M. Salton and T. Misteli, "Small molecule modulators of pre-mRNA splicing in cancer therapy," *Trends in Molecular Medicine*, vol. 22, no. 1, pp. 28–37, 2016.

[60] M. Seki-Asano, T. Okazaki, M. Yamagishi et al., "Isolation and characterization of a new 12-membered macrolide FD-895," *Journal of Antibiotics*, vol. 47, no. 12, pp. 1395–1401, 1994.

[61] B. G. Isaac, S. W. Ayer, R. C. Elliott, and R. J. Stonard, "Herboxidiene: a potent phytotoxic polyketide from *Streptomyces* sp. A7847," *The Journal of Organic Chemistry*, vol. 57, no. 26, pp. 7220–7226, 1992.

[62] A. J. F. Edmunds, W. Trueb, W. Oppolzer, and P. Cowley, "Herboxidiene: determination of absolute configuration by degradation and synthetic studies," *Tetrahedron*, vol. 53, no. 8, pp. 2785–2802, 1997.

[63] A. R. Pokhrel, D. Dhakal, A. K. Jha, and J. K. Sohng, "Herboxidiene biosynthesis, production, and structural modifications: prospect for hybrids with related polyketide," *Applied Microbiology and Biotechnology*, vol. 99, no. 20, pp. 8351–8362, 2015.

[64] Y. Sakai, T. Yoshida, K. Ochiai et al., "GEX1 compounds, novel antitumor antibiotics related to herboxidiene, produced by *Streptomyces* sp. I. Taxonomy, production, isolation, physicochemical properties and biological activities," *The Journal of Antibiotics*, vol. 55, no. 10, pp. 855–862, 2002.

[65] Y. Gao, A. Vogt, C. J. Forsyth, and K. Koide, "Comparison of splicing factor 3b inhibitors in human cells," *ChemBioChem*, vol. 14, no. 1, pp. 49–52, 2013.

[66] P. R. Blakemore, P. J. Kocieński, A. Morley, and K. Muir, "A synthesis of herboxidiene," *Journal of the Chemical Society, Perkin Transactions 1*, vol. 8, pp. 955–968, 1999.

[67] M. Banwell, M. McLeod, R. Premraj, and G. Simpson, "Total synthesis of herboxidiene, a complex polyketide from Streptomyces species A7847," *Pure and Applied Chemistry*, vol. 72, no. 9, pp. 1631–1634, 2000.

[68] Z. Yun and J. S. Panek, "Total synthesis of herboxidiene/GEX 1A," *Organic Letters*, vol. 9, no. 16, pp. 3141–3143, 2007.

[69] T. J. Murray and C. J. Forsyth, "Total synthesis of GEX1A," *Organic Letters*, vol. 10, no. 16, pp. 3429–3431, 2008.

[70] M. Pellicena, K. Krämer, P. Romea, and F. Urpí, "Total synthesis of (+)-Herboxidiene from two chiral lactate-derived ketones," *Organic Letters*, vol. 13, no. 19, pp. 5350–5353, 2011.

[71] A. K. Ghosh, N. Ma, K. A. Effenberger, and M. S. Jurica, "Total synthesis of GEX1Q1, assignment of C-5 stereoconfiguration and evaluation of spliceosome inhibitory activity," *Organic Letters*, vol. 16, no. 11, pp. 3154–3157, 2014.

[72] C. Lagisetti, M. V. Yermolina, L. K. Sharma, G. Palacios, B. J. Prigaro, and T. R. Webb, "Pre-mRNA splicing-modulatory pharmacophores: the total synthesis of herboxidiene, a pladienolide-herboxidiene hybrid analog and related derivatives," *ACS Chemical Biology*, vol. 9, no. 3, pp. 643–648, 2014.

[73] Y. Koguchi, M. Nishio, J. Kotera, K. Omori, T. Ohnuki, and S. Komatsubara, "Trichostatin A and herboxidiene up-regulate the gene expression of low density lipoprotein receptor," *Journal of Antibiotics*, vol. 50, no. 11, pp. 970–971, 1997.

[74] S. K. Hyun, S. S. Kang, K. H. Son, H. Y. Chung, and J. S. Choi, "Biflavone glucosides from *Ginkgo biloba* yellow leaves," *Chemical and Pharmaceutical Bulletin*, vol. 53, no. 9, pp. 1200–1201, 2005.

[75] W. Baker and W. D. Ollis, *Recent Developments in the Chemistry of Natural Phenolic Compounds*, Pergamon Press, London, UK, 1961.

[76] K. He, B. N. Timmermann, A. J. Aladesanmi, and L. Zeng, "A biflavonoid from *Dysoxylum lenticellare* gillespie," *Phytochemistry*, vol. 42, no. 4, pp. 1199–1201, 1996.

[77] M. Krauze-Baranowska, L. Pobłocka, and A. A. El-Hela, "Biflavones from *Chamaecyparis obtusa*," *Zeitschrift für Naturforschung C*, vol. 60, no. 9-10, pp. 679–685, 2005.

[78] M. K. Lee, S. W. Lim, H. Yang et al., "Osteoblast differentiation stimulating activity of biflavonoids from *Cephalotaxus koreana*," *Bioorganic and Medicinal Chemistry Letters*, vol. 16, no. 11, pp. 2850–2854, 2006.

[79] A. Moawad, M. Hetta, J. K. Zjawiony et al., "Phytochemical investigation of *Cycas circinalis* and *Cycas revoluta* leaflets: moderately active antibacterial biflavonoids," *Planta Medica*, vol. 76, no. 8, pp. 796–802, 2010.

[80] H.-F. Zhou, C. Xie, R. Jian et al., "Biflavonoids from caper (*Capparis spinosa* L.) Fruits and their effects in inhibiting NF-kappa B activation," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 7, pp. 3060–3065, 2011.

[81] Z. Zhou and C. Fu, "A new flavanone and other constituents from the rhizomes of Cyperus rotundus and their antioxidant activities," *Chemistry of Natural Compounds*, vol. 48, no. 6, pp. 963–965, 2013.

[82] J.-K. Weng and J. P. Noel, "Chemodiversity in Selaginella: a reference system for parallel and convergent metabolic evolution in terrestrial plants," *Frontiers in Plant Science*, vol. 4, article 119, 2013.

[83] V. P. Bagla, L. J. McGaw, E. E. Elgorashi, and J. N. Eloff, "Antimicrobial activity, toxicity and selectivity index of two biflavonoids and a flavone isolated from Podocarpus henkelii (Podocarpaceae) leaves," *BMC Complementary and Alternative Medicine*, vol. 14, no. 1, article 383, 2014.

[84] S. J. Lee, R. H. Son, H. W. Chang, S. S. Kang, and H. P. Kim, "Inhibition of arachidonate release from rat peritoneal macrophage by biflavonoids," *Archives of Pharmacal Research*, vol. 20, no. 6, pp. 533–538, 1997.

[85] J. K. Son, M. J. Son, E. Lee et al., "Ginkgetin, a biflavone from *Ginko biloba* leaves, inhibits cyclooxygenases-2 and 5-lipoxygenase in mouse bone marrow-derived mast cells," *Biological and Pharmaceutical Bulletin*, vol. 28, no. 12, pp. 2181–2184, 2005.

[86] P. S. S. Yuan, "Effect of isoginkgetin on scavenge of oxygen free radical in anoxic rats," *Traditional Chinese Drug Research & Clinical Pharmacology*, vol. 2, pp. 1–4, 1993.

[87] R. Saponara and E. Bosisio, "Inhibition of cAMP-phosphodiesterase by biflavones of *Ginkgo biloba* in rat adipose tissue," *Journal of Natural Products*, vol. 61, no. 11, pp. 1386–1387, 1998.

[88] S. J. Lee, J. H. Choi, K. H. Son, H. W. Chang, S. S. kang, and H. P. Kim, "Suppression of mouse lymphocyte proliferation in vitro by naturally-occurring biflavonoids," *Life Sciences*, vol. 57, no. 6, pp. 551–558, 1995.

[89] S.-J. Kim, "Effect of biflavones of Ginkgo biloba against UVB-induced cytotoxicity in vitro," *Journal of Dermatology*, vol. 28, no. 4, pp. 193–199, 2001.

[90] S. K. Sam, J. Y. Lee, Y. K. Choi et al., "Neuroprotective effects of naturally occurring biflavonoids," *Bioorganic and Medicinal Chemistry Letters*, vol. 15, no. 15, pp. 3588–3591, 2005.

[91] H. Sasaki, Y. Kitoh, M. Tsukada et al., "Inhibitory activities of biflavonoids against amyloid-β peptide 42 cytotoxicity in PC-12 cells," *Bioorganic and Medicinal Chemistry Letters*, vol. 25, no. 14, pp. 2831–2833, 2015.

[92] G. Liu, M. Grifman, J. Macdonald, P. Moller, F. Wong-Staal, and Q.-X. Li, "Isoginkgetin enhances adiponectin secretion from differentiated adiposarcoma cells via a novel pathway involving AMP-activated protein kinase," *Journal of Endocrinology*, vol. 194, no. 3, pp. 569–578, 2007.

[93] C. R. Cederroth, M. Vinciguerra, A. Gjinovci et al., "Dietary phytoestrogens activate AMP-Activated protein kinase with improvement in lipid and glucose metabolism," *Diabetes*, vol. 57, no. 5, pp. 1176–1185, 2008.

[94] S. Choudhary, P. Kumar, and J. Malik, "Plants and phytochemicals for Huntington's disease," *Pharmacognosy Reviews*, vol. 7, no. 14, pp. 81–91, 2013.

[95] K. O'Brien, A. J. Matlin, A. M. Lowell, and M. J. Moore, "The biflavonoid isoginkgetin is a general inhibitor of pre-mRNA splicing," *Journal of Biological Chemistry*, vol. 283, no. 48, pp. 33147–33154, 2008.

[96] J. M. Gray, D. A. Harmin, S. A. Boswell et al., "SnapShot-Seq: a method for extracting genome-wide, in Vivo mRNA dynamics from a single total RNA sample," *PLoS ONE*, vol. 9, no. 2, Article ID e89673, 2014.

[97] C.-K. Tseng, H.-F. Wang, A. M. Burns, M. R. Schroeder, M. Gaspari, and P. Baumann, "Human telomerase RNA processing and quality control," *Cell Reports*, vol. 13, pp. 2232–2243, 2015.

[98] R. J. van Alphen, E. A. C. Wiemer, H. Burger, and F. A. L. M. Eskens, "The spliceosome as target for anticancer treatment," *British Journal of Cancer*, vol. 100, no. 2, pp. 228–232, 2009.

[99] M. Koga, M. Hayashi, and D. Kaida, "Splicing inhibition decreases phosphorylation level of Ser2 in Pol II CTD," *Nucleic Acids Research*, vol. 43, no. 17, pp. 8258–8267, 2015.

[100] L. L. Hall, K. P. Smith, M. Byron, and J. B. Lawrence, "Molecular anatomy of a speckle," *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology*, vol. 288, no. 7, pp. 664–675, 2006.

[101] P. Loyer, J. H. Trembley, J. M. Lahti, and V. J. Kidd, "The RNP protein, RNPS1, associates with specific isoforms of the p34(cdc2)-related PITSLRE protein kinase in vivo," *Journal of Cell Science*, vol. 111, no. 11, pp. 1495–1506, 1998.

[102] M. Aouida, A. Eid, and M. M. Mahfouz, "CRISPR/Cas9-mediated target validation of the splicing inhibitor Pladienolide B," *Biochimie Open*, 2016.

[103] M. Iwata, Y. Ozawa, T. Uenaka et al., "E7107, a new urethane derivative of pladienolide D, displays curative effect against several human tumor xenografts," *Proceedings of American Association for Cancer Research*, vol. 45, p. 691, 2004.

[104] E. Papaemmanuil, M. Cazzola, J. Boultwood et al., "Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts," *The New England Journal of Medicine*, vol. 365, no. 15, pp. 1384–1395, 2011.

[105] F. Damm, F. Thol, O. Kosmider et al., "*SF3B1* mutations in myelodysplastic syndromes: clinical associations and prognostic implications," *Leukemia*, vol. 26, no. 5, pp. 1137–1140, 2012.

[106] T. R. Webb, A. S. Joyner, and P. M. Potter, "The development and application of small molecule modulators of SF3b as therapeutic agents for cancer," *Drug Discovery Today*, vol. 18, no. 1-2, pp. 43–49, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 8654317, 8 pages
http://dx.doi.org/10.1155/2016/8654317

Research Article

Conjugated Linoleic Acid Production by Bifidobacteria: Screening, Kinetic, and Composition

Stefano Raimondi, Alberto Amaretti, Alan Leonardi, Andrea Quartieri, Caterina Gozzoli, and Maddalena Rossi

Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 103, 41125 Modena, Italy

Correspondence should be addressed to Stefano Raimondi; stefano.raimondi@unimore.it

Received 18 March 2016; Accepted 29 May 2016

Academic Editor: Ibrahim M. Banat

Conjugated linoleic acids (CLA) are positional and geometric isomers of linoleic acid involved in a number of health aspects. In humans, CLA production is performed by gut microbiota, including some species of potential probiotic bifidobacteria. 128 strains of 31 *Bifidobacterium* species were screened with a spectrophotometric assay to identify novel CLA producers. Most species were nonproducers, while producers belonged to *B. breve* and *B. pseudocatenulatum*. GC-MS revealed that CLA producer strains yielded 9*cis*,11*trans*-CLA and 9*trans*,11*trans*-CLA, without any production of other isomers. Hydroxylated forms of LA were absent in producer strains, suggesting that the myosin-cross-reactive antigen (MCRA) protein that exerts hydratase activity is not involved in LA isomerization. Moreover, both CLA producer and nonproducer species bear a MCRA homologue. The strain *B. breve* WC 0421 was the best CLA producer, converting LA into 68.8% 9*cis*,11*trans*-CLA and 25.1% 9*trans*,11*trans*-CLA. Production occurred mostly during the lag and the exponential phase. For the first time, production and incorporation of CLA in biomass were assessed. *B. breve* WC 0421 stored CLA in the form of free fatty acids, without changing the composition of the esterified fatty acids, which mainly occurred in the plasmatic membrane.

1. Introduction

Conjugated linoleic acids (CLA) are a group of positional and geometric isomers of linoleic acid (LA, *cis9*, *cis12*-C18:2) that have conjugated double bonds. Diverse CLA isomers have been identified with different position (ranging from Δ7, Δ9 to Δ12, Δ14) and cis/trans geometry. 9*cis*,11*trans*-CLA and 10*trans*,12*cis*-CLA are the major isomers occurring in human diet, the former being contained mostly in ruminant meat and dairy products and the latter in hydrogenated oils. Considerable research has been directed toward understanding the production and the physiological effects of CLA and there is increasing evidence of their involvement in a number of health aspects. Potential health-promoting properties of CLA include anticarcinogenesis, antiatherosclerosis, antidiabetes, antiobesity, antiallergy activities, and enhancement of immune functions [1].

CLA are intermediates of the biohydrogenation of linoleic acid (LA) and unsaturated fatty acids, a microbial process by which the fatty acids released by lipolytic enzymes undergo the progressive saturation. Microbial isomerization of LA into CLA was firstly described in the gut of ruminants, but diverse isolates from human feces have been demonstrated to take part in LA biohydrogenation, indicating that production of CLA and 11*trans*-C18:1 (vaccenic acid) occurs in the human gut as well [2–5]. In fact, humans excrete some linoleic acid (LA) with feces [6], suggesting that this substrate is available for microbial production of CLA within the colon. Several colonic species belonging to Cluster IV, Cluster XIVa, and *Bifidobacterium* genus are involved in LA transformation toward CLA, including 9*cis*,11*trans*-CLA and vaccenic acid.

Some species of bifidobacteria, natural colonizers of the gut, are capable to transform LA into 9*cis*,11*trans*-CLA and into lower amounts of 9*trans*,11*trans*-CLA and 10*trans*,12*cis*-CLA. Previous studies suggested that the MCRA protein of bifidobacteria may be involved in isomerization of LA to CLA, through hydration of unsaturated fatty acids, for example, converting palmitoleic, oleic, and linoleic acids to

the corresponding 10-hydroxy fatty acids [7, 8]. However, more recent evidences excluded the role of bifidobacterial MCRA in CLA production [9].

The ability of various bifidobacteria to produce 9*cis*,11*trans*-CLA raised the question as to whether CLA production may be regarded as one of the mechanisms by which bifidobacteria exert some of their beneficial properties [10]. In fact, it is known that commensal bifidobacteria exert a number of beneficial health effects through a variety of different mechanisms.

For this reason, they are increasingly being used in functional foods and pharmaceutical products and are generally regarded as probiotics [11–13]. The present study aimed to identify novel CLA producers among a wide range of *Bifidobacterium* species and strains and to explore unedited aspects of CLA accumulation, such as the effect of LA and CLA in microbial lipid composition and the accumulation of hydroxylated forms of linoleic acid, ascribable to MCRA activity.

2. Materials and Methods

2.1. Chemicals, Strains, and Culture Conditions. All chemicals were purchased from Sigma Aldrich (Steinheim Germany) unless otherwise stated. 128 strains of *Bifidobacterium* (Table 1) were obtained from ATCC or from our culture collection (WC-labeled strains). Bifidobacteria were cultured in lactobacilli deMan-Rogosa-Sharpe broth (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 0.5 g/L L-cysteine·HCl (hereinafter called MRS), at 37°C in an anaerobic cabinet (Forma Scientific, Marietta, OH, USA) under N_2 85%, CO_2 10%, and H_2 5% atmosphere.

The analysis of the biotransformation of linoleic acid (LA) into conjugated linoleic acid (CLA) was carried out in MRS broth supplemented with 0.5 g/L LA (hereinafter called MRS-LA). A solution of 40 mg/mL LA in 20 g/L Tween 80 was prepared, filter sterilized (cellulose acetate, 0.22 μm; Millipore, Billerica, MA, USA), and added to the sterile MRS in proper amount to obtain 0.5 g/L LA. Bifidobacteria from 16 h MRS cultures were inoculated (10% v/v) into MRS-LA and incubated at 37°C for 48 h.

2.2. Kinetic of CLA Production and Bioreactor Operation. Controlled-pH batch cultures were carried out in laboratory-scale fermenters (Sixfors, Infors, Bottmingen, Switzerland) with 500 mL of MRS or MRS-LA medium. The bioreactor was inoculated with 50 mL of a 24 h seed culture grown in MRS or MRS-LA medium, respectively. To investigate CLA production during stationary phase, LA was added to MRS cultures after 24 h of growth. Temperature was kept at 37°C, stirring at 200 rpm, and pH was maintained constant at 5.5 through the automatic addition of 4 M NaOH. The bioreactor was kept under nitrogen overpressure to maintain anaerobiosis conditions. The culture was periodically sampled to monitor bacterial growth, glucose consumption, and the concentration of LA and CLA in the broth. The biomass/substrate yield ($Y_{X/S}$) was expressed as g of dry biomass g^{-1} consumed glucose. During the mid-exponential growth phase and at the entrance of the stationary phase,

Table 1: *Bifidobacterium* strains screened for CLA production, by measuring the absorbance of conjugate diene (A_{233}) after 24 h growth in MRS-LA. ∗ indicates species bearing a gene homologue to MCRA from *B. breve* NCIMB 702258. The 34 strains that were selected for GC quantification of CLA and PCR amplification are underscored.

Species	Strains
B. adolescentis∗	ATCC 15703, WC 9806
B. angulatum∗	ATCC 27535
B. animalis subsp. *animalis*∗	ATCC 25527, WC 0409, WC 9343 WC 0410
B. animalis subsp. *lactis*∗	ATCC 27536, WC 0411, WC 0412, WC 0413, WC 0414, WC 0432, WC 0455, WC 0459, WC 0460, WC 0469, WC 0471
B. asteroides	ATCC 25910
B. bifidum∗	WC 0415, WC 0417, WC 0418
B. boum	ATCC 27917
B. breve∗	WC 0420, WC 0421, WC 0422, WC 0423, WC 0424, WC 9436, WC 9437, WC 9445, WC 9445, WC 9446, WC 9449, WC 9491, WC 9492, WC 9493, WC 9495, WC 9498, WC 9499, WC 9500, WC 9501, WC 9504, WC 9505, WC 9508
B. catenulatum∗	ATCC 27539, WC 0458, WC 0467
B. choerinum	ATCC 27686
B. coryneforme	ATCC 25911
B. cuniculi	ATCC 27916
B. dentium∗	ATCC 27534
B. gallicum	ATCC 49850
B. gallinarum	ATCC 33777
B. indicum	ATCC 25912
B. longum subsp. *infantis*∗	ATCC 15697, WC 0433, WC 0434
B. longum subsp. *longum*∗	ATCC 15707, WC 0435, WC 0436, WC 0437, WC 0438, WC 0439, WC 0440, WC 0441, WC 0442, WC 0443, WC 0444, WC 0473, WC 9711, WC 9712, WC 9717, WC 9718, WC 9721, WC 9722, WC 9724, WC 9741, WC 9742, WC 9743, WC 9745, WC 9746, WC 9747, WC 9748, WC 9749, WC 9751, WC 9752, WC 9753, WC 9754, WC 9756, WC 9757, WC 9758, WC 9759, WC 9760, WC 9761, WC 9762, WC 9763, WC 9765, WC 9766, WC 9767, WC 9768, WC 9769, WC 9770,

Table 1: Continued.

Species	Strains
	WC 9772, WC 9773
B. longum subsp. *suis*	ATCC 27533
B. magnum	ATCC 27540
B. merycicum	ATCC 49391
B. minimum	ATCC 27538
B. pullorum	ATCC 27685
B. pseudocatenulatum[*]	ATCC 27919, WC 0400, WC 0401, WC 0402, WC 0403, WC 0404, WC 0405, WC 0407, WC 0408, WC 9359
B. pseudolongum[*]	ATCC 25526
B. psychraerophilum	NCIMB 13940
B. ruminantium	ATCC 49390
B. saeculare	ATCC 49392
B. scardovii[*]	ATCC BAA-773
B. subtile	ATCC 27683
B. thermophilum[*]	ATCC 25525

200 mL of the culture were collected in order to separate the biomass and perform on it the analysis of lipid fraction.

2.3. Spectrophotometric Analysis of CLA in the Supernatant. In order to screen all the bifidobacteria for the capability to produce CLA, a rapid spectrophotometric method was used, based on UV absorption of conjugated double bonds [14]. The culture sample was centrifuged (13,000 ×g for 5 min at 4°C) and 1 mL of supernatant was mixed with 2 mL of isopropanol. After the addition of 1.5 mL of hexane, the sample was thoroughly vortexed in order to extract the lipids and then allowed to stand 5 min. The hexane layer was collected and the absorbance was measured at 233 nm (A_{233}).

2.4. Extraction of Lipids and Preparation of Fatty Acyl-Methyl-Esters. To extract cell-associated lipids, the biomass of 200 mL of culture was harvested by centrifugation (9,000 ×g for 10 min at 4°C), washed with water, frozen at −80°C, and lyophilized (Heto Lyolab 3000, Allerød, Denmark). Lyophilized biomass was mixed (2% w/v) with a 2 : 1 (v/v) chloroform : methanol solution and shaken at r.t. for 16 h. The extract was filtered through celite and anhydrous Na_2SO_4, solvents were removed using a rotavapor apparatus, and lipids were weighed. To determine the amount of LA and CLA in the supernatant, 3 mL was thoroughly vortexed with 3 mL of ethyl acetate. The organic phase was separated and anhydrified; then the solvent was removed.

The free fatty acids (FFAs) and the esterified fatty acids (EFAs), such as glycerides and phospholipids, were transformed into the corresponding methyl-esters (FAMEs) to be analyzed by GC-MS. The FFAs contained in the lipid extract of the biomass or the supernatant were methylated through diazomethane reaction [15]. The extract was dissolved in the ethyl ether solution of diazomethane, freshly prepared from Diazald, and incubated at r.t. for 10 min. Once the solvent had evaporated, FAMEs were dissolved in 1 mL of ethyl acetate containing 0.5 mg/mL methyl undecanoate as internal standard and analyzed.

The EFAs contained in the lipid extract of the biomass were transesterified through alkali-catalyzed reaction [16]. The lipid extract was dissolved in 2 mL of ethyl ether containing 1 mg/mL triundecanoin as internal standard. 50 μL of 3.3 M sodium methoxide (methanol solution) was added to the reaction mixture and the sample was homogenized. After 10 min incubation at r.t., the reaction was stopped with 30 μL of acetic acid.

2.5. GC-MS Analysis. The organic phases containing FAMEs were injected (0.5 μL) into a GC apparatus (HP5890 Series II, Agilent, Waldbronn, Germany) equipped with the column (100 m × 0.25 mm, 0.25 μm) CP-Select FAME (Varian, Palo Alto, CA, USA). The injector was kept at 270°C. To determine the whole profile of FAs, the oven temperature ramped from 135°C to 250°C (2.5°C/min) and was maintained at 250°C for 23 min. The same program was followed, starting from 160°C, when only LA and CLA had to be determined. Elution was performed with high-purity helium, with constant column head pressure of 200 kPa. Qualitative analyses of FAMEs were performed with a MS quadrupole detector (HP5972, Agilent). Analytes were identified by comparison with standards (O5632, Sigma Aldrich) and by analysis of their fragmentation patterns (EI, 70 eV) with the mass spectrum library NIST 2005 (Gatesburg, USA). Quantitative analyses were performed with a flame ionization detector held at 300°C.

2.6. Alignment Analysis of Putative Bifidobacterium MCRA Genes and PCR Amplification. In order to verify the presence of homologues of MCRA from *B. breve* NCIMB 702258 (Protein ID ADY18551.1) a search was performed among all the species of *Bifidobacterium*, using the tblastn program provided by the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The available sequences of putative MCRA gene of *Bifidobacterium* spp. were aligned using the Clustal omega program (http://www.ebi.ac.uk/). To amplify the conserved region of MCRA genes, the primers MCRAf (5′-GCGGSCGCGARATGGACAA-3′) and MCRAr (5′-CCGCCGTTGGTGATGAACAC-3′) were designed. These primers anneal with the gene sequence of *B. breve* NCIMB 702258 (GenBank accession HQ593838H) at positions 236–255 and 958–978 and generate amplicons of 742 bp. Amplification was performed with the following program: 4 min at 94°C; 30 cycles of 30 s at 94°C; 30 s at 58°C; 1 min at 72°C; 1 min at 72°C.

2.7. Statistical Analysis. All values are described by the means and the standard deviation of three separate experiments. Comparisons were performed with one-way ANOVA followed by Tukey *post hoc* comparisons. Differences were considered statistically significant for $P < 0.05$. Statistical analysis was done using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Spectrophotometric Screening of Conjugated Linoleic Acid Production. 127 *Bifidobacterium* strains belonging to 31 different species or subspecies (Table 1) were cultured in MRS-LA medium, containing 0.5 g/L of free LA. With the exception of 3 strains belonging to the species *B. coryneforme*, *B. gallinarum*, and *B. saeculare*, unable to grow in presence of LA, all bifidobacteria grew in MRS-LA achieving OD_{600} ranging between 1.1 and 5.7 after 24 h (data not shown).

To obtain a preliminary estimation of CLA production, the supernatant of 24 h cultures in MRS-LA was extracted, and the UV absorbance by conjugated double bonds was measured at $\lambda = 233$ nm (A_{233}). A_{233} ranged between 0 and 2.37 a.u., with the vast majority of the strains (>75%) yielding an extract with absorbance lower than 0.5 a.u. With the aim to validate the spectrophotometric assay, total CLA were quantified by GC-MS in the supernatant of 34 strains belonging to the species *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, and *B. pseudocatenulatum*, covering the whole A_{233} range. The dot dispersion of A_{233} against CLA production revealed that CLA production can be excluded for $A_{233} < 0.5$ (Figure 1(a)). Above this value, higher absorbance roughly corresponded to higher CLA concentration, although a direct relationship could not be established ($R^2 = 0.87$).

All the strains belonging to the species *B. adolescentis, B. angulatum, B. bifidum, B. boum, B. choerinum, B. cuniculi, B. dentium, B. gallicum, B. indicum, B. magnum, B. merycicum, B. minimum, B. pullorum, B. pseudolongum, B. psychraerophylum, B. ruminantium, B. scardovii, B. subtile*, and *B. thermophilum* and most of the strains belonging to *B. animalis* subsp. *animalis, B. animalis* subsp. *lactis, B. longum* subsp. *infantis, B. longum* subsp. *longum,* and *B. longum* subsp. *suis* fell into the group of CLA producers (Figure 1(b)).

The species *B. breve* and the group of *B. catenulatum* and *B. pseudocatenulatum* were the highest CLA producers, even though wide variability was observed. The extracts of *B. breve* presented the highest A_{233} (mean = 1.08, median = 0.73), followed by those of *B. catenulatum* and *B. pseudocatenulatum* (mean = 0.62, median = 0.45). All the strains with A_{233} higher than the 90th percentile belonged to the species *B. breve* and *B. pseudocatenulatum* (Figure 1(b)). In particular, one strain of *B. pseudocatenulatum* and five of *B. breve* presented A_{233} higher than 2.0 a.u. and were selected for deeper investigation of CLA production.

3.2. Search of MCRA Gene in Bifidobacterium Species. A tblastn search was performed for all the species of *Bifidobacterium* reported in Table 1, in order to verify the presence of a homologue of MCRA from *B. breve* NCIMB 702258 (Protein ID ADY18551.1). Of the 31 species, 14 bear a gene encoding a protein with sequence identity from 55 to 94%: *B. adolescentis*, *B. angulatum*, *B. animalis* subsp. *animalis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, *B. pseudocatenulatum*, *B. pseudolongum*, *B. scardovii*, and *B. thermophilum*. All the 34 strains that were selected for the GC quantification of CLA were positive to MCRA-targeted PCR, yielding an amplicon of approx. 750 bp (Table 1).

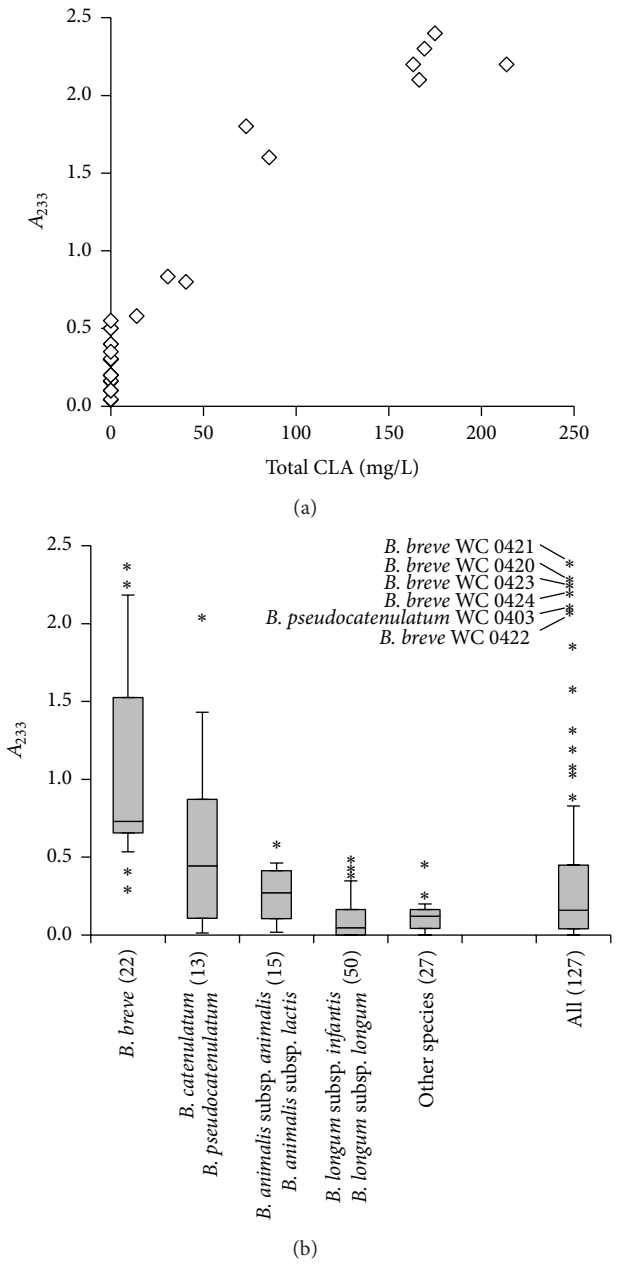


Figure 1: UV absorbance (A_{233}) of the lipophilic extracts that were obtained from the supernatants of 24 h MRS-LA cultures of bifidobacteria. (a) Relationship between A_{233} and total CLA concentration in the supernatant in a selection of 34 strains. (b) Screening of 127 *Bifidobacterium* strains. Boxes represent the range between 25th and 75th percentiles and the line inside represents the median. Whiskers denote the range between 10th and 90th percentiles and asterisks represent outlier values. Strains numbers are in brackets.

3.3. CLA Production by Selected Strains. Yields and the composition of CLA were determined in the supernatants of *B. breve* WC 0420, *B. pseudocatenulatum* WC 0403, *B.*

Table 2: Residual LA (%) and conversion yield into 9*cis*,11*trans*-CLA, 9*trans*,11*trans*-CLA, and total CLA (%) by bifidobacteria cultured for 48 h in MRS-LA medium.

	LA	9*cis*,11*trans*-CLA	9*trans*,11*trans*-CLA	Total CLA
B. breve WC 0420	86.8[a]	13.2[a]	0.0[a]	13.2[a]
B. pseudocatenulatum WC 0403	81.5[a]	14.3[a]	4.2[b]	18.5[b]
B. breve WC 0422	58.2[b]	37.2[b]	4.7[b]	41.8[c]
B. breve WC 0424	22.7[c]	70.2[c]	7.0[c]	77.3[d]
B. breve WC 0423	12.4[d]	80.3[d]	7.3[c]	87.6[e]
B. breve WC 0421	11.9[d]	81.1[d]	7.0[c]	88.1[e]

Values are means, $n = 3$; SD always < 5%. Means in a column with different superscripts significantly differ ($P < 0.05$).

breve WC 0422, *B. breve* WC 0424, *B. breve* WC 0423, and *B. breve* WC 0421, after 48 h of growth in MRS-LA (Table 2). Conversion of LA into CLA ranged between 13.2 and 88.1%. *B. breve* WC 0421 and *B. breve* WC 0423 were the most efficient CLA producers, with yields of 88.1 and 87.6%, respectively. 9*cis*,11*trans*-CLA and 9*trans*,11*trans*-CLA were the sole CLA isomers occurring in supernatants. The former was the major product of LA isomerization, accounting for 13.2 to 81.1% of LA conversion and for 77 to 100% of total CLA isomers. The latter was generally found in lower amount, never exceeding the 7.3% of LA conversion and the 23% of total CLA. The ratio 9*cis*,11*trans*-CLA : 9*trans*,11*trans*-CLA ranged between 3.4 and 11.6 and increased augmenting the total amount of CLA produced. None of these strains produced hydroxylated forms of LA.

3.4. Kinetic of LA Isomerization into CLA by B. breve WC 0421. Constant-pH batch cultures of *B. breve* WC 0421 were carried out in MRS and MRS-LA. The presence of 0.5 g/L LA caused an extension of the lag-phase of approx. 6 h, slowed down the specific growth rate (0.33 and 0.11 h^{-1} in MRS and MRS-LA, resp.), and delayed the entrance into stationary phase of 2 h. The biomass/glucose yield was lower in MRS-LA than in MRS (8 and 13%, resp.), whereas the percentage lipid/biomass was higher in the medium containing LA (7.5% and 2.5%, resp.). The isomerization of LA into CLA occurred mostly during the lag and the exponential phases (Figure 2). After 24 h of fermentation, during the exponential phase, the transformation of LA resulted in the production of 9*cis*,11*trans*-CLA (83.6%) and, at a minor extent, of *trans*9,*trans*11-CLA (4.5%). During the late exponential and stationary phases, LA isomerization proceeded slowly and 9*trans*,11*trans*-CLA progressively accumulated at the expenses of 9*cis*,11*trans*-CLA. Altogether, the conversion yield of LA into CLA was 94.3% after 52 h. In particular, conversion yields into 9*cis*,11*trans*-CLA and 9*trans*,11*trans*-CLA were 68.8 and 25.1%, respectively. All other CLA isomers were always negligible throughout the fermentation.

When 0.5 g/L LA was added to MRS cultures at the entrance into the stationary phase, the isomerization occurred at minor extent, resulting in production of the sole 9*cis*,11*trans*-CLA isomer with a conversion of 6.1% after 48 h incubation.

3.5. Lipid Composition of the Biomass. The profile of free and esterified fatty acids (FFA and EFA) within the lipid fraction

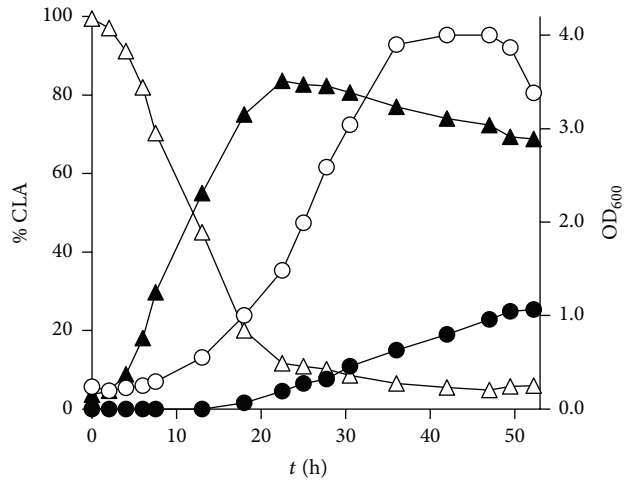


Figure 2: Time-course of growth and CLA production by *B. breve* WC 0421 in MRS medium containing 0.5 g/L LA. ○: turbidity (OD_{600}), △: LA (%), ▲: 9*cis*,11*trans*-CLA (%), and ●: *trans*9,*trans*11-CLA (%). The results are representative of the three independent experiments.

of *B. breve* WC 0421 biomass was investigated (Table 3). In MRS, lipids were dominated by EFA (*ca* 93% of total lipids), while FFA were found in low amounts (≤8%). At the exponential and stationary phases, oleic acid was the most abundant among both EFA and FFA, occurring for more than 40%. Palmitic and myristic acids and palmitic and stearic acids represented the other major fatty acids among EFA and FFA, respectively.

During the cultivation in MRS-LA, *B. breve* WC 0421 accumulated a high amount of FFA, which accounted for *ca* 75% of total lipids at the stationary phase. LA occurred in low amount (<4%) among both EFA and FFA. 9*cis*,11*trans*-CLA dominated the profile of FFA, always accounting for more than 56%. The composition of EFA was similar in MRS and MRS-LA.

The supplementation of 0.5 g/L LA at the entrance into the stationary phase did not cause accumulation of relevant amounts of CLA as free or esterified fatty acid in the microbial biomass. 24 h after LA addition, EFA composition remained unchanged, while FFA increased up to 70% of the lipid extract. In this case, FFA were mostly composed of LA (77%), whereas CLA altogether accounted for *ca* 4%.

Table 3: Relative composition (%) of esterified and free fatty acids (EFAs and FFAs, resp.) in the biomass of *B. breve* WC 0421 at the exponential and the stationary phase in MRS and MRS-LA.

			MRS		MRS-LA	
			Exponential	Stationary	Exponential	Stationary
EFA	EFA/total FA		93[a]	92[a]	26[b]	25[b]
	C10		0.8[a]	1.7[b]	1.2[a]	1.7[b]
	C12		2.8[a]	4.2[b]	3.9[b]	4.2[b]
	C14		8.6[a]	16.6[b]	13.7[b]	15.7[b]
	C16		17.4	21.5	18.9	22
	C16:1		1.1	1.9	1.9	2
	C17		0.9	0.5	0	0.4
	C18		4.2[a]	3.1[a]	0.0[b]	1.7[b]
	C18:1		55.8[a]	41.3[b]	50.9[a]	43.9[b]
	C18:2	LA	2.1	3.2	3.7	3.1
	C18:2	9*cis*,11*trans*-CLA	1.6	1.2	0.9	1
	C18:2	9*trans*,11*trans*-CLA	3.6	3.9	5	4
	C20		0.3	0.3	0	0.1
	C22		0.8	0.5	0	0.4
FFA	FFA/total FA		7[a]	8[a]	74[b]	75[b]
	C10		0	0	0	0
	C12		0	0	0	0
	C14		3.2[a]	12.7[b]	1.2[a]	0.6[a]
	C16		23.6[a]	22.0[a]	5.7[b]	2.4[b]
	C16:1		0	0	0	0
	C17		3.1[a]	1.6[a]	0.6[b]	0.3[b]
	C18		10.4[a]	6.8[a]	2.5[b]	1.2[b]
	C18:1		47.0[a]	46.2[a]	13.9[b]	8.3[b]
	C18:2	LA	0.0[a]	0.0[a]	1.7[b]	2.8[b]
	C18:2	9*cis*,11*trans*-CLA	2.1[a]	2.7[a]	58.6[b]	56.6[b]
	C18:2	9*trans*,11*trans*-CLA	3.3[a]	7.1[a]	15.2[b]	27.0[c]
	C20		4	0.9	0.2	0.4
	C22		3.2	0	0.5	0.3

Values are means, $n = 3$; SD always < 5%. Means in a row with different superscripts significantly differ ($P < 0.05$).

4. Discussion

The present study aims to gain further insight into CLA production by bifidobacteria, since the capability of certain strains to convert LA into CLA could find application in the development of specific probiotic strains able to accelerate or improve *in vivo* conversion of LA into CLA.

In vivo studies supporting biological activity of CLA were initially performed using a mixture of CLA isomers, while purified isomers have been used more recently, supporting the evidence that different isomers have distinct effect on the major health targets, such as tumorigenesis and lipid metabolism [1]. However, most of commercial formulations of CLA still contain both major isomers, whose biological activity is mediated by diverse signaling pathways. 9*cis*,11*trans*-CLA isomer exerts an enhancing effect on the nuclear transmission of PPARγ, a master regulator of adipocyte differentiation, acting as a stimulator of adiponectin secretion [17]. This mechanism can to some extent explain the antihypertensive, antihyperlipidemic, antiarteriosclerotic, anticarcinogenic, and antidiabetic effects mediated by this CLA [18]. Conversely, *trans*10,*cis*12-CLA increases lipolysis and has the function of diminishing the synthesis of fatty acids [19] but also presents a linkage with proatherogenic effects, insulin resistance, and inflammation [20].

The perspective to exploit specifically selected probiotic bifidobacteria to produce a definite CLA isomer, such as the 9*cis*,11*trans*-CLA, is quite attractive. The data herein presented demonstrate that selected strains of bifidobacteria specifically convert linoleic acid (LA) into 9*cis*,11*trans*-CLA isomer, whereas 10*trans*,12*cis*-CLA, for which potential harmful biological activities are still debated, is not produced by the tested strains.

The screening of CLA production among the major species of bifidobacteria demonstrated that the most efficient CLA producers belong to the species *B. breve* and *B. pseudocatenulatum*. Consistently, *B. breve* strains have been

previously identified among the best CLA producers, whereas low or negligible activity was found in the strains belonging to *B. adolescentis*, *B. angulatum*, *B. bifidum,* and *B. infantis* [2, 9, 21]. Unlike previous studies that indicated *B. pseudocatenulatum* as almost incapable of LA transformation [2], our data revealed that some strains of *B. pseudocatenulatum* can efficiently accomplish the isomerization of LA.

The data herein presented are in agreement with observations by O'Connell et al. [9], which excluded that the product of MCRA gene is involved in LA isomerization to CLA. In the present study, all the best producers of CLA, belonging to the species *B. breve* and *B. pseudocatenulatum*, were positive to this gene, yet no hydroxylated forms of LA were detected in their supernatants or in the biomass of *B. breve* WC 0421. A further clue hinting that MCRA is not involved in CLA production is the fact that both MCRA-targeted PCR and BLAST search for the gene revealed the presence of a homologue in CLA producer and nonproducer species.

B. breve WC 0421 emerged as the most promising strain for production of 9*cis*,11*trans*-CLA isomer. Our results associate the production of *cis*9,*trans*11-CLA to the lag and early growth phases, whereas supplementation of LA into stationary phases resulted in negligible production of CLA. This result supports the hypothesis that transformation of LA in CLA can be a strategy of bacteria to detoxify unsaturated fatty acid that interfere with membrane lipids. These *in vitro* experiments have been carried out in batch, with a pure culture, on a rich medium, with condition very different from the intestinal ecosystem, where bifidobacteria represent only a few percent of a complex mixed population. However, bioproduction of CLA by supplemental probiotic bifidobacteria has been previously demonstrated [10]. For this reason, *B. breve* WC 0421 may represent a promising candidate for *in vivo* production of the specific health-promoting CLA isomer.

The attention of the scientific community has been focused on CLA production and absorption in the gastrointestinal tract. The analysis of fatty acid profile of biomass associated lipids revealed that when *B. breve* WC 0421 was cultured in presence of LA it accumulated CLA in the form of free fatty acids, without changing the composition of its esterified fatty acids, mainly occurring in the plasmatic membrane.

5. Conclusions

Bifidobacteria are one of the most relevant health-promoting indigenous species of the human gut microbiota, exerting beneficial positive effects through a variety of different mechanisms. Our study strengthened the perspectives on a more specific use of probiotics, where the intrinsic health-promoting properties of bifidobacteria can be associated with peculiar features, such as producing *in vivo* health-promoting CLA. Based on the current knowledge [2, 9, 21] and on the outcome of this study, selected strains of *Bifidobacterium*, mostly belonging to the species *B. breve*, can be exploited as probiotics for *in vivo* production of 9*cis*,11*trans*-CLA.

The assessment of production and incorporation of CLA in bifidobacterial biomass is a relevant result of our study. Since the host-bacteria interaction is mediated by several surface compounds, it is necessary to investigate the location of 9*cis*,11*trans*-CLA within bifidobacterial cells and to examine whether CLA enriched bifidobacteria exert a diverse biological effect than the corresponding CLA-free cells.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Stefano Raimondi and Alberto Amaretti contributed equally to this work.

References

[1] J. H. Kim, Y. Kim, Y. J. Kim, and Y. Park, "Conjugated linoleic acid: potential health benefits as a functional food ingredient," *Annual Review of Food Science and Technology*, vol. 7, no. 1, pp. 221–244, 2016.

[2] M. Coakley, R. P. Ross, M. Nordgren, G. Fitzgerald, R. Devery, and C. Stanton, "Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species," *Journal of Applied Microbiology*, vol. 94, no. 1, pp. 138–145, 2003.

[3] E. Devillard, F. M. McIntosh, S. H. Duncan, and R. J. Wallace, "Metabolism of linoleic acid by human gut bacteria: different routes for biosynthesis of conjugated linoleic acid," *Journal of Bacteriology*, vol. 189, no. 6, pp. 2566–2570, 2007.

[4] F. M. McIntosh, K. J. Shingfield, E. Devillard, W. R. Russell, and R. J. Wallace, "Mechanism of conjugated linoleic acid and vaccenic acid formation in human faecal suspensions and pure cultures of intestinal bacteria," *Microbiology*, vol. 155, no. 1, pp. 285–294, 2009.

[5] H. C. Soo, H. K. In, G. P. Hui et al., "Synthesis of conjugated linoleic acid by human-derived Bifidobacterium breve LMC 017: utilization as a functional starter culture for milk fermentation," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 9, pp. 3311–3316, 2008.

[6] A. O. Edionwe and C. Kies, "Comparison of palm and mixtures of refined palm and soybean oils on serum lipids and fecal fat and fatty acid excretions of adult humans," *Plant Foods for Human Nutrition*, vol. 56, no. 2, pp. 157–165, 2001.

[7] A. Volkov, A. Liavonchanka, O. Kamneva et al., "Myosin cross-reactive antigen of *Streptococcus pyogenes* M49 encodes a fatty acid double bond hydratase that plays a role in oleic acid detoxification and bacterial virulence," *The Journal of Biological Chemistry*, vol. 285, no. 14, pp. 10353–10361, 2010.

[8] E. Rosberg-Cody, A. Liavonchanka, C. Göbel et al., "Myosin-cross-reactive antigen (MCRA) protein from *Bifidobacterium breve* is a FAD-dependent fatty acid hydratase which has a function in stress protection," *BMC Biochemistry*, vol. 12, article 9, 2011.

[9] K. J. O'Connell, M. O. Motherway, A. A. Hennessey et al., "Identification and characterization of an oleate hydratase-encoding gene from *Bifidobacterium breve*," *Bioengineered*, vol. 4, no. 5, pp. 313–321, 2013.

[10] J. B. Ewaschuk, J. W. Walker, H. Diaz, and K. L. Madsen, "Bioproduction of conjugated linoleic acid by probiotic bacteria occurs in vitro and in vivo in mice," *Journal of Nutrition*, vol. 136, no. 6, pp. 1483–1487, 2006.

[11] M. Rossi, A. Amaretti, A. Leonardi, S. Raimondi, M. Simone, and A. Quartieri, "Potential impact of probiotic consumption on the bioactivity of dietary phytochemicals," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 40, pp. 9551–9558, 2013.

[12] M. Rossi and A. Amaretti, "Probiotic properties of bifidobacteria," in *Bifidobacteria-Genomics and Molecular Aspects*, B. Mayo and D. van Sinderen, Eds., pp. 97–123, Caiser Academic Press, Norfolk, UK, 2010.

[13] N. T. Williams, "Probiotics," *American Journal of Health-System Pharmacy*, vol. 67, no. 6, pp. 449–458, 2010.

[14] E. Barrett, R. P. Ross, G. F. Fitzgerald, and C. Stanton, "Rapid screening method for analyzing the conjugated linoleic acid production capabilities of bacterial cultures," *Applied and Environmental Microbiology*, vol. 73, no. 7, pp. 2333–2337, 2007.

[15] W. W. Christie, "Preparation of ester derivatives of fatty acids for chromatographic analysis," in *Advances in Lipid Methodology—Two*, W. W. Christie, Ed., pp. 69–111, Oily Press, Dundee, UK, 1993.

[16] S. Raimondi, M. Rossi, A. Leonardi, M. M. Bianchi, T. Rinaldi, and A. Amaretti, "Getting lipids from glycerol: new perspectives on biotechnological exploitation of *Candida freyschussii*," *Microbial Cell Factories*, vol. 13, no. 1, article 83, 2014.

[17] G. Yuan, X. Chen, and D. Li, "Modulation of peroxisome proliferator-activated receptor gamma (PPAR γ) by conjugated fatty acid in obesity and inflammatory bowel disease," *Journal of Agricultural and Food Chemistry*, vol. 63, no. 7, pp. 1883–1895, 2015.

[18] G.-F. Yuan, X.-E. Chen, and D. Li, "Conjugated linolenic acids and their bioactivities: a review," *Food and Function*, vol. 5, no. 7, pp. 1360–1368, 2014.

[19] S. V. Martins, A. Madeira, P. A. Lopes et al., "Adipocyte membrane glycerol permeability is involved in the anti-adipogenic effect of conjugated linoleic acid," *Biochemical and Biophysical Research Communications*, vol. 458, no. 2, pp. 356–361, 2015.

[20] T. E. Lehnen, M. R. da Silva, A. Camacho, A. Marcadenti, and A. M. Lehnen, "A review on effects of conjugated linoleic fatty acid (CLA) upon body composition and energetic metabolism," *Journal of the International Society of Sports Nutrition*, vol. 12, no. 1, article 36, 2015.

[21] S. H. Chung, I. H. Kim, H. G. Park et al., "Synthesis of conjugated linoleic acid by human-derived *Bifidobacterium breve* LMC 017: utilization as a functional starter culture for milk fermentation," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 9, pp. 3311–3316, 2008.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 8502123, 11 pages
http://dx.doi.org/10.1155/2016/8502123

Research Article

Synergistic Effects of Sulfated Polysaccharides from Mexican Seaweeds against Measles Virus

Karla Morán-Santibañez,[1] Lucia Elizabeth Cruz-Suárez,[2] Denis Ricque-Marie,[2] Daniel Robledo,[3] Yolanda Freile-Pelegrín,[3] Mario A. Peña-Hernández,[1] Cristina Rodríguez-Padilla,[1] and Laura M. Trejo-Avila[1]

[1]*Laboratorio de Inmunología y Virología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Ciudad Universitaria, CP 66455, San Nicolás de los Garza, NL, Mexico*
[2]*Programa Maricultura, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Ciudad Universitaria, CP 66455, San Nicolás de los Garza, NL, Mexico*
[3]*Cinvestav Unidad Mérida, Km 6 Carretera Antigua a Progreso, Cordemex, AP 73, 97310 Mérida, YUC, Mexico*

Correspondence should be addressed to Laura M. Trejo-Avila; laura.trejoav@uanl.edu.mx

Received 18 March 2016; Revised 11 May 2016; Accepted 16 May 2016

Academic Editor: Ibrahim M. Banat

Sulfated polysaccharides (SPs) extracted from five seaweed samples collected or cultivated in Mexico (*Macrocystis pyrifera*, *Eisenia arborea*, *Pelvetia compressa*, *Ulva intestinalis*, and *Solieria filiformis*) were tested in this study in order to evaluate their effect on measles virus *in vitro*. All polysaccharides showed antiviral activity (as measured by the reduction of syncytia formation) and low cytotoxicity (MTT assay) at inhibitory concentrations. SPs from *Eisenia arborea* and *Solieria filiformis* showed the highest antiviral activities (confirmed by qPCR) and were selected to determine their combined effect. Their synergistic effect was observed at low concentrations (0.0274 μg/mL and 0.011 μg/mL of *E. arborea* and *S. filiformis* SPs, resp.), which exhibited by far a higher inhibitory effect (96% syncytia reduction) in comparison to the individual SP effects (50% inhibition with 0.275 μg/mL and 0.985 μg/mL of *E. arborea* and *S. filiformis*, resp.). Time of addition experiments and viral penetration assays suggest that best activities of these SPs occur at different stages of infection. The synergistic effect would allow reducing the treatment dose and toxicity and minimizing or delaying the induction of antiviral resistance; sulfated polysaccharides of the tested seaweed species thus appear as promising candidates for the development of natural antiviral agents.

1. Introduction

Latin America has an important and diverse group of seaweed species [1]. Recent data on seaweed management in this region have described the main harvest and aquaculture taking place in Argentina, Brazil, Chile, Peru, and Mexico [2]. One of the goals of seaweeds exploitation is to diversify their application by screening their diverse bioactive compounds, which remain unexplored in nutraceutical and pharmaceutical areas [3]. Different chemical compounds have been isolated from algae, including polysaccharides, which have been subjected to a variety of studies due to their extensive bioactivities and applications [4].

An increasing number of biological activities of seaweed polysaccharides have been reported in the last decades, where sulfated polysaccharides (SPs) are among the most studied compounds [5]. SPs include a complex group of macromolecules with numerous activities such as antioxidant [6, 7], antitumor [8, 9], anticoagulant [6], anti-inflammatory [6, 10], and antiviral [11, 12].

Antiviral activity of SPs was first reported in 1958 [13] and over the years a substantial research has been focused on this field. SP can be obtained from each of the three main classes of seaweed: fucoidans and alginates from brown algal species, agaroids and carrageenans from red macroalgae, and ulvans from green seaweeds [14].

Fucoidans have shown a potent antiviral activity against numerous enveloped viruses including herpes simplex virus type 1 (HSV-1) [15], human immunodeficiency virus [16],

influenza A virus [17], and different kind of paramyxoviruses such as Newcastle disease virus (NDV) and canine distemper virus (CDV) [18, 19]. *In vitro* and *in vivo* antiretroviral effects of alginates preventing syncytium formation and reducing the P24 core antigen level have been demonstrated [20]. Antiviral activity of carrageenans has been demonstrated *in vitro* against human papillomavirus (HPV), acting mainly on the inhibition of HPV virions binding to cells, and also *in vivo* by preventing infection by different HPV genotypes [21, 22]. Recently, antiviral activity against NDV of ulvan from *Ulva clathrata* cultivated in Mexico has been reported [23].

Nowadays, combining multiple drugs is a primary approach for improving antiviral effects within the antiviral drug therapy field. The advantages of multidrugs combination are the reduction of individual drugs doses, a decrease in the side effects of antiviral agents, and the prevention of drug-resistant viruses emergence. Drug combination theories provide an ideal tool for this purpose to understand the benefits of multidrugs combinations therapy [24].

Measles virus (MeV) belongs to the Paramyxoviridae family of Mononegavirales, is a nonsegmented negative-strand RNA virus, and causes a highly contagious disease [25]. Although preventable by vaccination, measles still remains one of the causes of death among young children worldwide [26]. Many new antiviral drugs have been licensed in recent years, most of which are used for the treatment of HIV infections [27]. The investigation of natural antivirals isolated from marine sources is an interesting approach in the development of new antiviral agents. In the present study, we tested the antiviral activity of SPs isolated from five Mexican seaweeds against MeV. The aim of this research was to develop new candidates of antiviral drugs that could help to control viral infection diseases.

2. Materials and Methods

2.1. Antiviral Agents

2.1.1. Collection of Seaweed. Five species of macroalgae were collected from the Mexican coasts and tested for this study: three brown seaweeds from Baja California (*Macrocystis pyrifera*, *Eisenia arborea*, and *Pelvetia compressa*), one green seaweed from Southern Baja California (*Ulva intestinalis*), and one red seaweed from Yucatan (*Solieria filiformis*).

Macrocystis pyrifera (Linnaeus) C. Agardh was collected in Bahía de Ensenada (Manto Jantay) in front of the Salsipuedes beach (31.983–116.815), in January 2013. *Eisenia arborea* J. E. Areschoug and *Pelvetia compressa* (J. Agardh) De Toni were collected in the Escalera Zone, North of Punta China (31.520–116.650) in December 2014-January 2015. The green alga *Ulva intestinalis* (Linnaeus) was collected from the water drainage channel of the Gran Mar shrimp farm, on the Baja California West coast (24.434–111.584) in August 2014.

Solieria filiformis (Kützing) P. W. Gabrielson, a red seaweed considered as a potential source of ι-carrageenan [28], was obtained from an aquaculture facility at the Telchac Marine station-CINVESTAV, Yucatan (Mexico), where it is periodically cultivated in bimonthly cycles in semiopen tanks as part of an Integrated Multitrophic aquaculture system. The sample used came from a batch cultured from April to May 2014.

Once harvested, the brown and green algae samples were washed in seawater to eliminate sand, shells, and epibionts and dried under shade, while the cultivated red algae was washed with fresh water and dried in an oven at 60°C. Prior to extraction, the samples were cut into small 2-3 cm pieces and ground to pass through a 0.5 mm sieve (Turbomolino Pulvex 200 mill).

2.1.2. Extraction and Purification of Sulfated Polysaccharides. Polysaccharides extraction was performed after extraction of polyphenols [29]. Briefly 10 g of alga powder was washed with distillated water and dried at room temperature overnight. The washed powder was extracted with 200 mL 50% v/v ethanol and sonicated for 30 min at room temperature, followed with an extraction period in a bath shaker at 70°C during 2 hours. The samples were centrifuged for 15 min (2500 rpm). The pellet was used for the polysaccharide extraction according to the procedure described by Tako et al. 2000 and Ale et al. 2012 [30, 31]. Briefly, 200 mL of 0.1 M HCl was added to the algae pellet and heated for 1 hour at boiling temperature and centrifuged at 3500 rpm for 10 minutes. The supernatant was recovered and absolute ethanol was added (4 : 1) for polysaccharides precipitation. Once precipitated, the polysaccharides were separated from the aqueous medium by centrifugation at 3500 rpm for 10 minutes; the supernatant was discarded and the pellet was washed three times with 96% ethanol to remove residual pigments and finally resuspended in a minimum amount of distilled water for a 72-hour dialysis with stirring. The dialyzed product was precipitated with absolute ethanol (4 : 1). Polysaccharide extracts were lyophilized and weighed to calculate their yield.

2.1.3. Characterization of Selected Polysaccharide Extracts

(1) FT-IR Spectra Analysis. IR spectra of aqueous extracted polysaccharides from *Solieria filiformis* and *Eisenia arborea* were obtained using diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS). Scans were performed at room temperature in the infrared region between 4000 and 400 cm^{-1} on a Thermo Nicolet Nexus 670 FT-IR spectrometer. The infrared spectra of commercial available carrageenan (ι-carrageenan C1138, κ-carrageenan C1013), fucoidan from *Fucus vesiculosus* (F5631), alginic acid (A7003) from Sigma-Aldrich (St. Louis, MO, USA), and λ-carrageenan from Celtic Colloids Inc. (B. Blakemore) were included for comparison.

(2) NMR Spectra Analysis. ^{13}C-NMR spectra were acquired on a Varian 600 spectrometer. The extracts were exchanged twice with 99.8% deuterium oxide (D2O) with intermediate lyophilization and dissolved at 10 mg mL^{-1} in D2O. Sodium [3-trimethylsilyl 2,2′,3,3′-2-H4] propionate (TSP-d4) was used as an internal reference to 0.00 ppm.

(3) Carbohydrate Determination. For determination of total sugars in the samples acid hydrolysis of the extracts was performed. A solution with 25 mg of polysaccharide extract in 100 mL of 1 M H_2SO_4 was prepared and boiled for 3 hours;

subsequently, an aliquot of 1 mL of each extract was taken. Anthrone reagent (5 mL) was added to the aliquot, placed in a water bath for 12 minutes, and cooled down at room temperature. Absorbance was read at 630 nm. Quantification was performed against a calibration curve of a stock solution of fucose.

(4) Sulfate Content Determination. The analysis was performed using the turbidimetric method of Jackson and McCandless, 1978 [32]. Briefly, this quantification of sulfates was determined by measuring turbidity as barium sulfate when adding 1.2 mL of TCA 8% and 0.6 mL of 0.01% reaction reagent (agarose/barium chloride) to the sample; reaction was homogenized by stirring for 35 minutes. The turbidity was determined at 500 nm in a Shimadzu UV-Vis spectrophotometer 1601. The calibration curve was performed with potassium sulfate (K_2SO_4) with a concentration of 0 to 100 μg of $SO_4{}^{-2}$/mL. SP extracts of *Solieria filiformis* and *Eisenia arborea* were weighed (7 mg), and 1 mL of 1 N HCl was added and heated at 105°C for 12 hours in a thermoblock (Lab line). A dilution was performed with 10 mL of deionized water; samples were then filtered using a microfilter with Whatman paper of 1.2 μm and an aliquot of 1.1 mL of the samples was taken for quantification. Analysis was performed by triplicate.

2.2. Cells and Virus. Vero cells were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12, Gibco Invitrogen, USA) supplemented with 5% fetal bovine serum (FBS, Gibco Invitrogen, USA) and 1% antibiotic (Gibco Invitrogen, USA).

Measles virus (Edmonston strain) was purchased from ATCC (ATCC® VR-24™). Virus was propagated on Vero cells and viral titers were determined by cytopathogenic effect and expressed as 50% tissue culture infectious dose (TCID50)/mL. Aliquots of viral stock were stored at −80°C until use.

2.3. Cytotoxicity Assays. The effect of SPs on cell viability of Vero cells was determined by MTT assay. The cells were cultured in 96-well plates at a density of 1.5×10^4 cells/well at 37°C in an atmosphere of CO_2. After 1 day of incubation, increasing concentrations of SPs diluted in DMEM were added; after 48 h of incubation the media were replaced with 22 μL of 2.5 mg/mL MTT dissolved in phosphate-buffered saline (PBS). After 1 h 30 min 150 μL of DMSO was added and incubated at room temperature for 15 min. The optical density (OD450 nm) was measured using a microplate reader (Multiskan FC, Thermo, USA). Cell viability was expressed by percentage as the mean value of three independent experiments considering control cells absorbance as 100% viable. CC_{50} was the concentration of the test substances that inhibited the Vero cells growth by 50% compared with the growth of the untreated cells.

2.4. Syncytia Reduction Assays. The antiviral activity of the SPs was evaluated by syncytia reduction assays. Vero cells seeded in 12-well plates were treated with different concentrations of SPs (0.01–5 μg/mL) and infected with MeV ($1 \times 10^{3.5}$ TCID50 of Edmonston strain) at the same time. After virus adsorption for 1 h at 37°C the medium was removed and monolayers were washed with PBS, after which the corresponding concentrations of SPs were added again. Each concentration was tested using three culture wells per PS concentration per experiment; the experiments were performed by triplicate. After incubation of 48 or 72 h at 37°C in a 5% CO_2 incubator monolayers were fixed with methanol : acetone (1 : 1) and stained with 1% crystal violet. Syncytia were counted and the result was expressed as a percentage of the number of syncytia observed in viral control monolayers (untreated cultures); IC_{50} was determined from dose-response curves. The selectivity index (SI) values were calculated as CC_{50}/IC_{50}. SPs showing the best SI were selected for the subsequent experiments.

2.5. Quantitative Real-Time PCR. Total RNA was isolated from treated Vero cells using RNAzol® RT (MRC Inc., USA). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and the viral genome was amplified with specific primers (MeVF: 5′ GAGGGTCAAACAGAGTCGAG 3′, MeVR: 5′ CGGTTGGAAGATGGGCAG 3′) that amplified a 95 nt fragment. The real-time PCR was carried out using SensiFAST™ SYBR® No-ROX Kit (BIOLINE, USA) and the Chromo4™ Real-Time PCR Detector (Bio-Rad, USA) with the following procedures: 95°C for 2 min, followed by 50 cycles of 95°C for 2 s, 60°C for 10 s, and 72°C for 20 s. The number of viral copies was calculated by using a standard curve. Serial 10-fold dilutions of a synthetic oligonucleotide encompassing the target measles gene were used to establish the standard curves.

2.6. Evaluation of SPs Synergy. Potential synergistic effects of selected SPs on MeV infection were evaluated using syncytia reduction assays. Each combination was tested on its corresponding IC_{75}, IC_{50}, and IC_{25} values. The synergistic effect of SPs was calculated by using a combination index (CI) described previously by Chou [33] and CompuSyn software. CI was calculated from the data as a measure of the interaction among drugs. CI values lower than 0.9 indicate synergy, CI values from 0.9 to 1.1 indicate an additive effect, and CI values higher than 1.1 indicate antagonism. Combinations with synergistic antiviral effect were selected and qPCR assays were performed in order to confirm the inhibitory effect as described above.

2.7. Time of Addition Assay. Vero cell monolayers were infected with MeV. SPs were added at a concentration of 5 μg/mL at different times of infection: 60 min before infection and 0, 15, 30, 60, and 120 min after infection. Thereafter, for each treatment, cells were incubated with SP for 1 h and then washed three times with PBS. Monolayers were fixed with methanol : acetone after incubation for 48 or 72 h at 37°C and 5% CO_2 and stained with 1% crystal violet; syncytia were counted subsequently.

2.8. Viral Penetration Assay. Virus penetration into Vero cells was evaluated according to the method reported by Huang and Wagner [34] with some modifications [18]. Vero cell

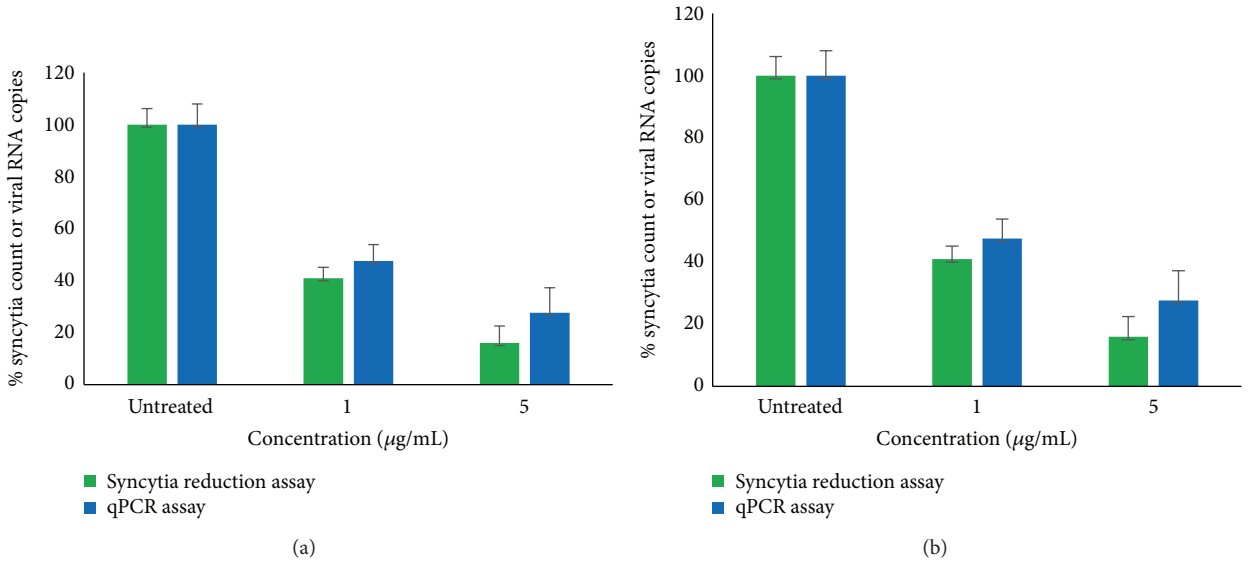


Figure 1: Confirmation of antiviral activity of *Eisenia arborea* (a) and *Solieria filiformis* (b) SPs at their best inhibitory concentrations by syncytia reduction and qPCR assays. Syncytia count and viral RNA copies number are given in % of the untreated control values.

monolayers precooled at 4°C for 3 h were infected with MeV at 4°C for 1 h in the absence of SP. After washing three times with ice-cold PBS, different concentrations of SP were added to the monolayers, and the temperature was shifted to 37°C. After 1 h of incubation at 37°C, the cells were treated with 40 mM citrate buffer (pH 3.0) to inactivate unpenetrated viruses. Buffer was replaced by culture medium and the cells were incubated for 48 or 72 h at 37°C and 5% CO_2 and stained with 1% crystal violet; syncytia were counted subsequently.

2.9. Statistical Analysis. The variables (tested by triplicate in each experiment that were in turn repeated at least three times) were submitted to a one-way analysis of variance followed by Dunnett's test (SPSS software, $\alpha = 0.05$). CC_{50} and IC_{50} values were determined by probit regression analysis.

3. Results

3.1. Cytotoxicity and Antiviral Activity of SPs. The MTT assay indicated no cytotoxicity for any of the SPs at concentrations from 0.1 to 1500 μg/mL up to 2 days (data not shown).

Antiviral activity of SPs against MeV was evaluated by syncytia reduction inhibition assays at concentrations of 0.01, 0.1, 1, and 5 μg/mL of each compound (data not shown). All tested compounds showed significant antiviral activity, but only compounds with the best SI values were selected for the subsequent experiments. As shown in Table 1, SPs of *Eisenia arborea* and *Solieria filiformis* exhibited antiviral activity at the lowest concentrations (IC_{50} 0.275 μg/mL and 0.985 μg/mL, resp.) without cytotoxic effect at concentrations of 0.1 to 1500 μg/mL. Therefore, SPs of *Eisenia arborea* and *Solieria filiformis* were selected based on their SI and antiviral activity for the combination experiments.

Table 1: Cytotoxic effect, antiviral activity, and selectivity index of SPs.

Algae[a]	CC_{50} (μg/mL)[b]	IC_{50} (μg/mL)[c]	SI[d]
Macrocystis pyrifera	>1500	1.00	>1500
Eisenia arborea	>1500	0.275	>5454.54
Pelvetia compressa	>1500	1.00	>1500
Ulva intestinalis	>1500	3.6	>416.7
Solieria filiformis	>1500	0.985	>1522.84

[a]Algal sulfated polysaccharide extract. [b]Concentration of test compound (μg/mL) that reduced Vero cell viability by 50%. [c]Concentration of a test compound that reduced the number of MeV syncytia in Vero cells by 50%. [d]Selectivity index value.

Antiviral effect of selected SPs was confirmed by qPCR assays, as shown in Figure 1. Inhibitory effect of *Eisenia arborea* and *Solieria filiformis* SP was tested at the best inhibitory concentrations (1 μg/mL and 5 μg/mL for both SPs). Results of qPCR assays were consistent with the results observed by syncytia reduction inhibition assays.

3.2. Characterization of SPs. Infrared spectroscopy has been used for the qualitative characterization of carrageenans and has proven to be a valuable tool for the characterization of sulfated oligosaccharides [35]. FT-IR and NMR spectra analyses of selected SPs extracts were performed. The FT-IR spectrum of *Solieria filiformis* extract (Figure 2(a)) indicates the presence of a typical ι-carrageenan type. Characteristics signal bands are indicated: 3,6 anhydrogalactose-2-sulfate (804 cm^{-1}) characteristic of ι-carrageenan; galactose-4-sulfate (846 cm^{-1}) signal present in κ- and ι-carrageenan. The signal between 1210 and 1260 cm^{-1} is common to all types of compounds containing sulfate.

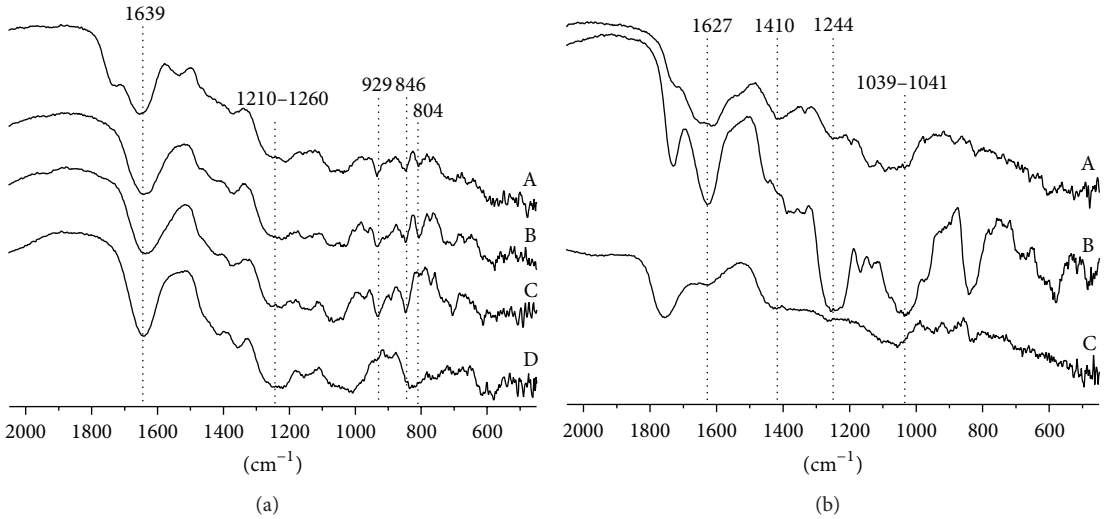


Figure 2: (a) Infrared spectra of (A) *Solieria filiformis* aqueous extract, (B) ι-carrageenan, (C) κ-carrageenan, and (D) λ-carrageenan. (b) Infrared spectra of (A) *Eisenia arborea* aqueous extract, (B) fucoidan, and (C) alginic acid.

^{13}C-NMR spectroscopy has been highly recommended for distinguishing the polysaccharides of the agar and carrageenan group [36]. Spectrum and expansion ^{13}C-NMR of the *S. filiformis* extract (Figure 3(a)) showed signals at 20 and 60 ppm typical of residual ethanol. Carbohydrates signals (63.79–104.66 ppm) observed, particularly two upfield-shifted signals (94.51 and 104.66 ppm), suggest that the molecule has two anomeric carbons. Overall its spectrum showed the presence of the ι-carrageenan. The next assignment is the mapping of the carbon signals of the molecule. Carbons of 2-sulfate-3,6-anhydrogalactose are 94.51 (C1), 77.44 (C2), 80.25 (C3), 80.84 (C4), 79.49 (C5), and 72.33 (C6) ppm [37]. Carbons of 4-sulfate-galactose are 104.66 (C1), 71.68 (C2), 79.27 (C3), 74.51 (C4), 77.27 (C5), and 63.79 (C6) ppm [37]. Sulfate content of *S. filiformis* showed 21.14% (±0.056) of total sulfate and total polysaccharide determination resulted in 91% of polysaccharide.

The FT-IR spectrum of *Eisenia arborea* extract (Figure 2(b)) indicates the presence of a mixture of fucoidan and alginic acid. Characteristics signal bands are indicated: carboxylate vibrations (1627 and 1410 cm^{-1}) can be attributed to uronic acids. Stretching vibrations at 1039–1041 cm^{-1} can be assigned to pyranose ring from guluronic and mannuronic acid residues. The broad band at 1244 cm^{-1} indicates the presence of sulfated ester groups, which are characteristic in fucoidans. ^{13}C-NMR spectrum of *E. arborea* extract (Figure 3(b)) showed typical signals of alginate ranging from 66.04 to 177.68 ppm. The signal at 66.04 ppm is characteristic of carbon-2 of guluronic acid (G) [38]. The signals at 72.51, 72.79, 74.07, 78.90, 80.82, 102.81, 102.93, and 177.68 ppm correspond to repeating blocks of mannuronic (M) and guluronic acid [39]. The signals at 102.81 and 102.93 ppm may indicate the presence of two repeating units, one of MMM and another of GMM [39]. Sulfate content of *E. arborea* showed 12.85% (±0.346) of total sulfate.

3.3. Combined Antiviral Effect of SPs. The combined effect of SPs of *Eisenia arborea* and *Solieria filiformis* on MeV infections was examined: each SP was tested at different concentrations combining its corresponding IC_{25}, IC_{50}, and IC_{75} values. E_{25}, E_{50}, and E_{75} correspond to IC_{25}, IC_{50}, and IC_{75} values of *Eisenia arborea* SPs and S_{25}, S_{50}, and S_{75} correspond to the respective values of *Solieria filiformis* SP (Table 2). Syncytia reduction assay results were expressed in relative syncytia percentage according to the number of syncytia in viral control. Best inhibitory effect was observed for E_{50}-S_{25} combination.

The evaluation of drug synergism based on a median-effect equation has been extensively used in the literature. CI values of SPs combinations were calculated as described previously using the CompuSyn software and are given in Table 2. Median-effect and the normalized isobologram generated with the software determined the presence of three synergistic combinations, represented by points below the lines at normalized isobologram (Figure 4).

Results showed strong synergistic effects at low concentrations combinations (E_{50}-S_{25}, E_{25}-S_{50}, and E_{25}-S_{25}) and antagonism at high concentrations combinations (E_{25}-S_{75}, E_{50}-S_{50}, E_{50}-S_{75}, E_{75}-S_{25}, E_{70}-S_{50}, and E_{75}-S_{75}). Combinations with synergistic effect were selected and qPCR assays were performed. As shown in Figure 5 the inhibitory effect of the synergistic combinations was confirmed. These data were consistent with results observed by syncytia reduction inhibition assays.

3.4. Effect of SPs on Viral Infection at Different Times of Addition. In order to determine which step of the MeV cycle was targeted by SPs, "time of addition" experiments were performed in Vero cells infected with MeV and exposed to PS at different times of infection. The most efficient inhibition by *S. filiformis* was observed in early phases of infection, 0 and

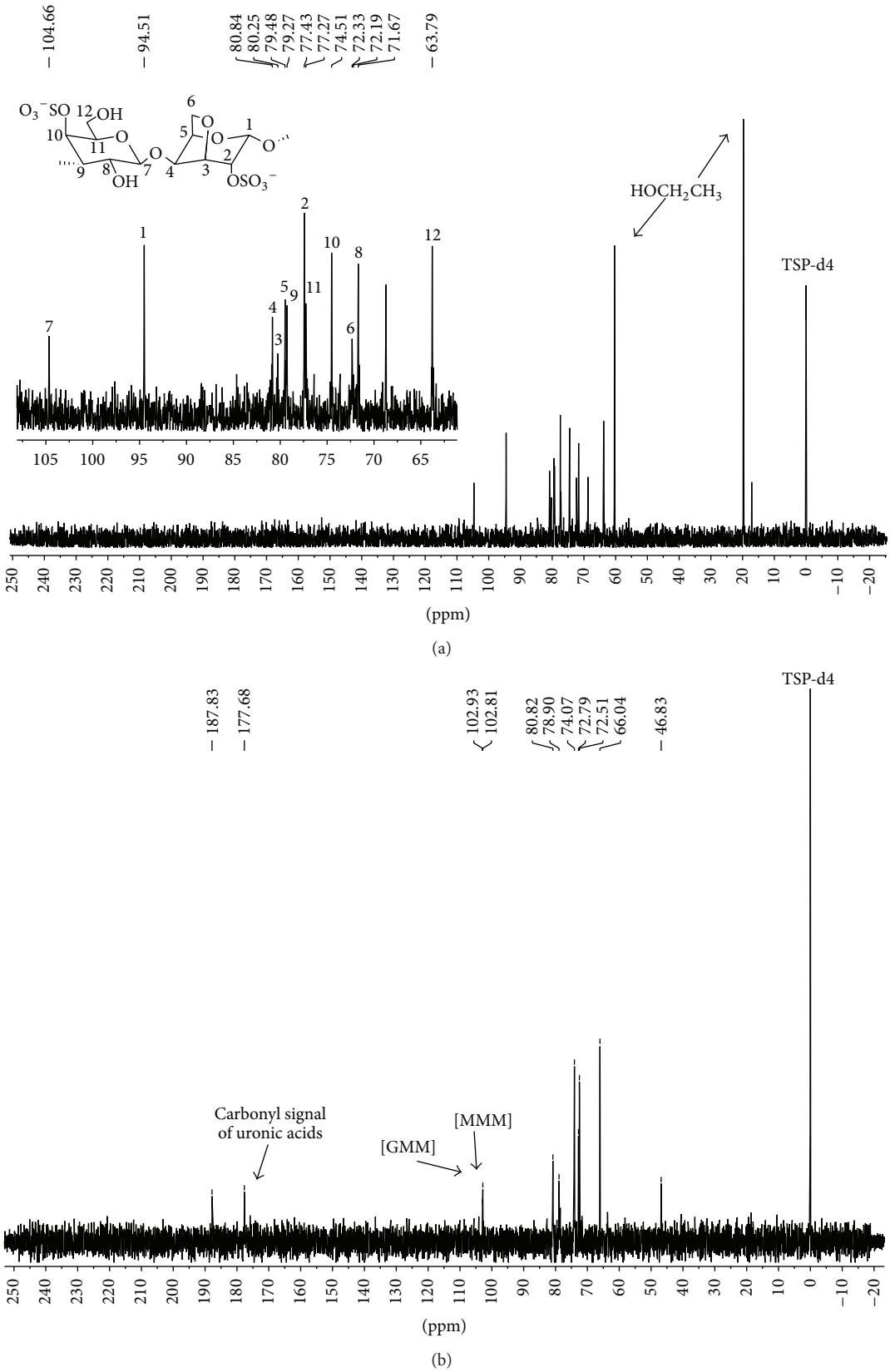


Figure 3: (a) Spectrum and expansion ^{13}C-NMR of the aqueous extract of *S. filiformis*. (b) ^{13}C-NMR spectrum of the aqueous extract of *E. arborea*.

Table 2: Synergistic effects of SPs on MeV infection.

Compounds combination	Compound concentration (μg/mL)		% relative syncytia formation in presence of the different SPs combinations	SD	CI	Description
	Eisenia arborea	*Solieria filiformis*				
IC_{75}-IC_{75}	2.98	3.027	34.5	4.4	10.59	Antagonism
IC_{75}-IC_{50}	2.98	0.985	26.4	5.6	3.08	Antagonism
IC_{75}-IC_{25}	2.98	0.011	33	7.3	1.47	Antagonism
IC_{50}-IC_{75}	0.275	3.027	28.4	4.1	3.71	Antagonism
IC_{50}-IC_{50}	0.275	0.985	32	5.9	1.88	Antagonism
IC_{50}-IC_{25}	0.275	0.011	4	3.2	0.001	*Synergism*
IC_{25}-IC_{75}	0.01	3.027	22.5	6.6	1.85	Antagonism
IC_{25}-IC_{50}	0.01	0.985	16.7	7.1	0.31	*Synergism*
IC_{25}-IC_{25}	0.01	0.011	39.4	2.5	0.05	*Synergism*

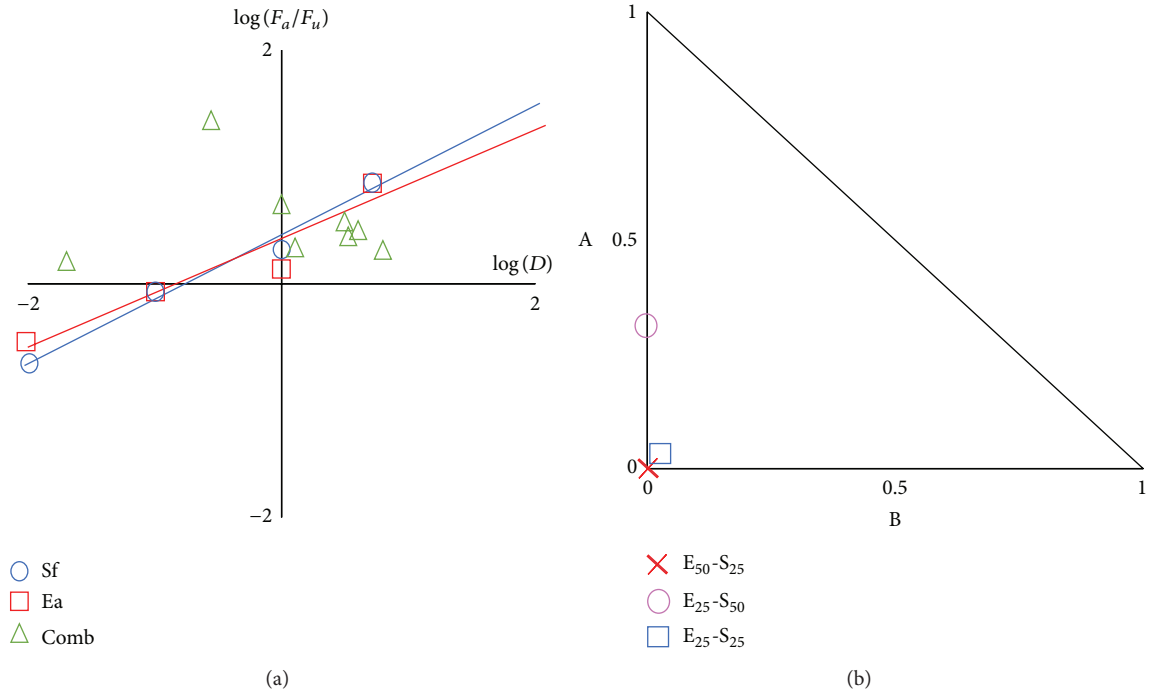


Figure 4: Analysis of *Eisenia arborea* and *Solieria filiformis* combinations. (a) Median-effect plot for combinations of *Eisenia arborea* and *Solieria filiformis* was generated with the CompuSyn software (F_a, affected fraction; F_u, unaffected fraction; D, concentration of SP used; Sf *Solieria filiformis* SP; Ea, *Eisenia arborea* SP; Comb, *Eisenia arborea* and *Solieria filiformis* combinations). (b) Normalized isobologram plots for Sf and Ea at nonconstant combination ratios. For each SP different combinations of various concentrations based on IC_{25}, IC_{50} values were tested and combination index (CI) values were determined using the CompuSyn software. CI values, represented by points below the lines, indicate synergy.

15 min after infection (Figure 6); syncytia inhibition before infection and 30 min after infection was not significant. *E. arborea* showed the most efficient inhibition 1 hour before infection and 0 and 15 min after infection. At 30, 60, and 120 min after infection, a minimal syncytia inhibition by *E. arborea* was still observed.

3.5. Effect of Fucoidan on Viral Penetration into Host Cells. Viral penetration assays were performed to determine whether entry events downstream of virus binding were inhibited by SPs. Vero cells were plated and incubated with MeV at 4°C for 1 h to allow virus binding but prevent viral internalization. Unbound virus was inactivated and SPs (1 μg/mL or 5 μg/mL) were added to the cells and incubated at 37°C. Figure 7 shows that SP from *S. filiformis* (5 μg/mL) significantly decreased viral infection by 58%, while SPs from *E. arborea* (5 μg/mL) decreased viral infection only by 24%, when compared with the findings in infected cells in the absence of treatment.

4. Discussion

Since the first studies by Gerber in 1958 showing the inhibition of mumps and influenza B virus by marine algae

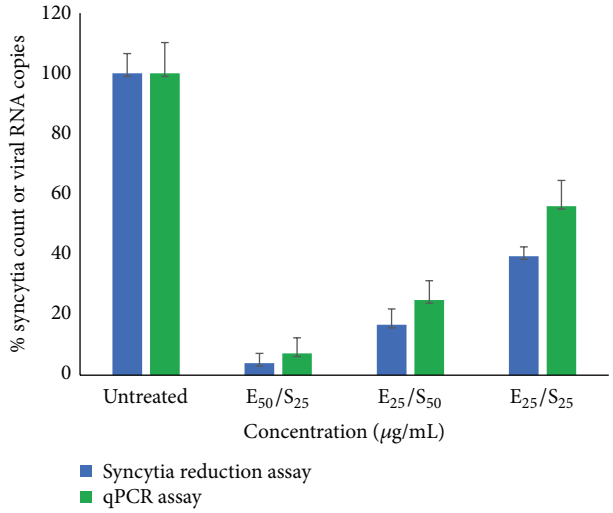


Figure 5: Antiviral activity confirmation by qPCR of the RNA extracted from Vero cells infected with MeV and cultivated in presence of synergistic SPs combinations. E_{25} and E_{50} are the SPs concentrations corresponding to IC_{25} and IC_{50} values of *Eisenia arborea* SPs. S_{25} and S_{50} concentrations correspond to the respective IC values of *Solieria filiformis* SP.

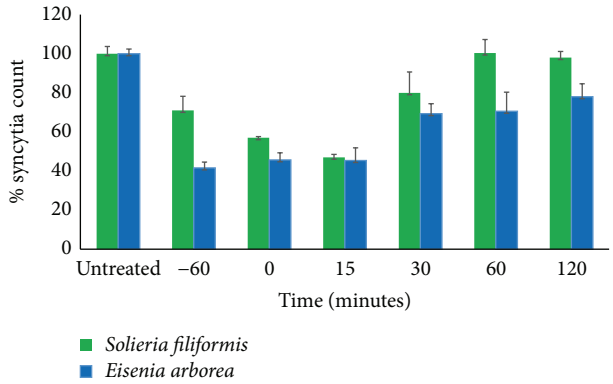


Figure 6: Time of addition experiments. Antiviral activity of SP was tested at different times of infection and analyzed by syncytia inhibition assays. SPs were added at 60 min before infection and 0, 15, 30, 60, and 120 min after infection. The data are expressed as relative syncytia count (%) compared to that of untreated virus-infected control cells, which was defined as 100%. The data shown are the mean ± SD of triplicate experiments.

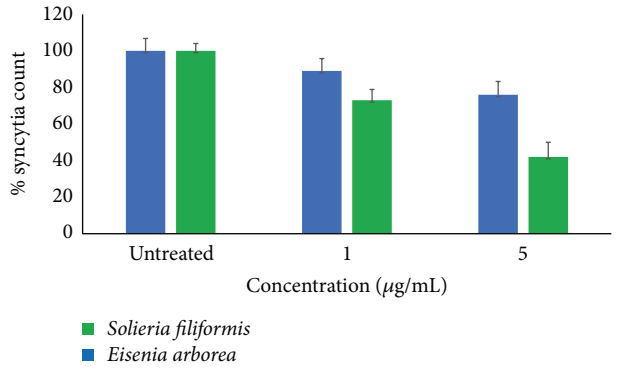


Figure 7: Effect of SPs on viral penetration. Vero cells were infected with MeV at 4°C in the absence of SPs and then shifted to 37°C to permit penetration of the adsorbed virus in the presence of SPs. Antiviral effect of SPs was evaluated using syncytia inhibition assays. The data shown are the mean ± SD of triplicate experiments.

polysaccharides, increased efforts and research have been carried out in this field [13]. Previous studies have also demonstrated no cytotoxicity of SPs isolated from certain seaweed species [40]. The absence of cytotoxicity to the host cells is one of the principal challenges in the development of new antivirals.

Eisenia arborea, an edible brown alga used in folk medicine in Japan, is the kelp species with the largest and most southerly latitudinal distribution on the North Pacific East Coast [41, 42]. Researches on *Eisenia* biological activities have been focused on the evaluation of their polyphenolic compounds [43]. To our knowledge, the antiviral effects of *Eisenia arborea* extracts have never been tested before. In this study, the extract of *Eisenia arborea* is rich in fucoidans and alginates and also showed the best SI of the five seaweed extracts (Table 1). Previous chemical characterization of Mexican *E. arborea* extracts also reported the presence of alginates, with higher yields than the one reported in this study [44]. Alginates with antiviral effects have been previously tested against HIV, IAV, and HBV, showing a potent antiviral activity [4]. Antiviral activity of fucoidan has been also reported *in vitro* and *in vivo* against many RNA and DNA viruses such as HIV, HSV1-2, dengue virus, and influenza virus [39, 45–47].

Macrocystis pyrifera has been harvested since 1956 along the Pacific coast of Baja California and exported to the United States for the production of alginates [48]. SPs extracts of Mexican *Macrocystis pyrifera* showed a significant antiviral effect but were not selected for subsequent assays because of their IC_{50} value (Table 1). Previous studies with crude dialyzed extracts of *Macrocystis pyrifera* have shown antiviral effects against VSV, with the fucoidan being responsible for these results [49].

In this study, antiviral effects of the extract from *Solieria filiformis* display the second lowest IC_{50} among the analyzed extracts. *In vitro* studies have reported antiviral properties of carrageenans against DNA and RNA viruses [21, 50]. Recently it has been shown that carrageenan (Rong Yuan F.F.I. Co., Ltd.) can inhibit influenza virus A/Swine/Shandong/731/2009 H1N1 (SW731) responsible for the influenza pandemic of 2009. Carrageenans can significantly inhibit SW731 replication by interfering with different steps of viral replication, including adsorption, transcription, and expression of the viral proteins; however, they act especially by inhibiting the interactions between the viral receptor (HA) and the target cell [51]. Sulfate content analysis and total polysaccharide determination of *S. filiformis* extract resulted in 21.14% (±0.056) total sulfate and 91% polysaccharide; these data are consistent with previous reports [52]. Degree of sulfation has a major impact on the antiviral activity of polysaccharides, including carrageenans [53].

In relation to the combination therapy approach used in this study, results showed a strong synergistic effect at low concentrations combinations of SPs and antagonism at high concentrations combinations. Our results determined that low concentrations combinations (0.0274 μg/mL and 0.011 μg/mL of *E. arborea* and *S. filiformis*, resp.) exhibited the higher inhibitory effect (96%) in comparison to the individual effect of SP (50% of inhibition with 0.275 μg/mL and 0.985 μg/mL of *E. arborea* and *S. filiformis*, resp.). Synergistic effect observed in this study has been also reported for the sulfated polysaccharides from *Fucus vesiculosus* in combination with AZT against HIV [54]. Furthermore, this effect has been also observed with acyclovir in combination with 3, 19-isopropylideneandrographolide against herpes simplex virus (wild type) and drug-resistant strains. Low concentrations of these compounds were required for a complete inhibition of DNA replication and late protein synthesis of HSV-1 wild type and drug-resistant HSV-1 [55]. The combined effect of nitazoxanide with neuraminidase inhibitors against influenza A viruses tested *in vitro* suggests that regimens that combine neuraminidase inhibitors and nitazoxanide exert synergistic anti-influenza effects [56]. In contrast, antagonistic effects at high concentrations were observed in our study; this antagonism of SPs was previously observed in a combination of ulvan and fucoidan against NDV infection [23]. Particular chemical features of SPs like chain ramifications could explain antagonism effects of SPs. Moreover, carbohydrate to carbohydrate interactions could be responsible to adhesion events; these aggregates have been previously observed in marine sponges [57].

To understand if a synergistic effect was related to different modes of action of the tested SPs, viral penetration and time of addition assays were performed. Results suggested the possibility that SP from *S. filiformis* inhibits postbinding events, because best inhibition effect was observed at 0 and 15 minutes after viral infection (Figure 6). To support this idea, a viral penetration assay was performed (Figure 7), and results show the best antiviral effect after viral adsorption. Our results are in agreement with those observed by Elizondo-Gonzalez et al. [18], who demonstrated the ability of fucoidan from *C. okamuranus* to be responsible for the antiviral activity against Newcastle disease virus, suggesting that fucoidan inhibits viral penetration into host cells, must probably by blocking the F protein.

Similar results were also observed by Bouhlal et al. [58], who suggested that carrageenans can inhibit DENV replication by interfering viral entrance, but they also suggested that SPs could avoid viral adsorption into the cell as a second mode of action. This mode of action could be similar to the mechanism observed with SPs of *E. arborea*. Alginates and fucoidan of *E. arborea* were able to show the best antiviral effect 1 hour before infection and this effect lasted up to 0–15 minutes after infection. Although both SPs, from *S. filiformis* and *E. arborea*, exhibited antiviral activity at 0 and 15 min after infection, only *E. arborea* showed inhibitory effect at 60 min. This result suggests the capability of these SPs to avoid viral adsorption to the cell; these data were confirmed by viral penetration assays where we observed less antiviral activity after viral attachment to the cell. More recent studies have demonstrated that fucoidans exhibit their antiviral activity when the compound is present during the virus adsorption period by blocking the interaction of viruses to the cells [59].

SPs tested in this study exhibit the best antiviral effect at different stages of infection: viral penetration and viral adsorption (*S. filiformis* and *E. arborea*, resp.). Multiple-drug antiviral therapy with two or more drugs that target different proteins or act in different stages of infection may decrease drug resistance and may enhance clinical outcomes by allowing a reduction of individual drug doses, thus decreasing dose-related drug toxicity [60].

5. Conclusions

In this study sulfated polysaccharides from Mexican seaweed showed antiviral activity against measles virus. Due to the lack of cytotoxicity at inhibitory concentrations, as indicated by the selectivity index, potential application can be found for these SPs. *Eisenia arborea* and *Solieria filiformis* extracts showed the higher antiviral activity and were selected to determine their combined effect. Synergistic effect was observed at the lowest concentrations tested for each SP of these species. Results suggest that SPs combined in this study are acting at different level of first stages in viral infection. Synergistic therapeutic effect allows dose and toxicity reduction and would minimize or delay the induction of antiviral resistance. Sulfated polysaccharides of Mexican seaweed are potential candidates for the development of new antiviral drugs that can help to control viral infection diseases.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The authors thank E. Hernández, E. Caamal, and C. Chavéz for chemical analytical support; M. Maldonado for polysaccharide extraction support; I. Beamonte, R. Marcos, and R. Ojeda for seaweed collection support; S. Salcedo for the confirmation of seaweeds species identification; and K. Ledezma for cytotoxicity assays support. This work was supported by Consejo Nacional de Ciencia y Tecnología (CONACYT) Mexico (Project no. 10002-255075).

References

[1] R. O'Dor, P. Miloslavich, and K. Yarincik, "Marine biodiversity and biogeography—regional comparisons of global issues, an introduction," *PLoS ONE*, vol. 5, no. 8, Article ID e11871, 2010.

[2] C. Rebours, E. Marinho-Soriano, J. A. Zertuche-González et al., "Seaweeds: an opportunity for wealth and sustainable livelihood for coastal communities," *Journal of Applied Phycology*, vol. 26, no. 5, pp. 1939–1951, 2014.

[3] L. Wang, X. Wang, H. Wu, and R. Liu, "Overview on biological activities and molecular characteristics of sulfated polysaccharides from marine green algae in recent years," *Marine Drugs*, vol. 12, no. 9, pp. 4984–5020, 2014.

[4] A. Ahmadi, S. Zorofchian Moghadamtousi, S. Abubakar, and K. Zandi, "Antiviral potential of algae polysaccharides isolated

from marine sources: a review," *BioMed Research International*, vol. 2015, Article ID 825203, 10 pages, 2015.

[5] S. Kraan, "Algal polysaccharides, novel applications and outlook," in *Carbohydrates—Comprehensive Studies on Glycobiology and Glycotechnology*, InTech, 2012.

[6] C. M. P. G. Dore, M. G. D. C. Faustino Alves, L. S. E. Pofírio Will et al., "A sulfated polysaccharide, fucans, isolated from brown algae *Sargassum vulgare* with anticoagulant, antithrombotic, antioxidant and anti-inflammatory effects," *Carbohydrate Polymers*, vol. 91, no. 1, pp. 467–475, 2013.

[7] B. W. S. Souza, M. A. Cerqueira, A. I. Bourbon et al., "Chemical characterization and antioxidant activity of sulfated polysaccharide from the red seaweed *Gracilaria birdiae*," *Food Hydrocolloids*, vol. 27, no. 2, pp. 287–292, 2012.

[8] V. Suresh, N. Senthilkumar, R. Thangam et al., "Separation, purification and preliminary characterization of sulfated polysaccharides from *Sargassum plagiophyllum* and its in vitro anticancer and antioxidant activity," *Process Biochemistry*, vol. 48, no. 2, pp. 364–373, 2013.

[9] P. Shao, X. Chen, and P. Sun, "Chemical characterization, antioxidant and antitumor activity of sulfated polysaccharide from *Sargassum horneri*," *Carbohydrate Polymers*, vol. 105, no. 1, pp. 260–269, 2014.

[10] C. O. Coura, I. W. F. de Araújo, E. S. O. Vanderlei et al., "Antinociceptive and anti-inflammatory activities of sulphated polysaccharides from the red seaweed *Gracilaria cornea*," *Basic and Clinical Pharmacology and Toxicology*, vol. 110, no. 4, pp. 335–341, 2012.

[11] C. A. Pujol, S. Ray, B. Ray, and E. B. Damonte, "Antiviral activity against dengue virus of diverse classes of algal sulfated polysaccharides," *International Journal of Biological Macromolecules*, vol. 51, no. 4, pp. 412–416, 2012.

[12] H. H. A. Gomaa and G. A. Elshoubaky, "Antiviral activity of sulfated polysaccharides carrageenan from some marine seaweeds," *International Journal of Current Pharmaceutical Review and Research*, vol. 7, no. 1, pp. 34–42, 2016.

[13] P. Gerber, J. D. Dutcher, E. V. Adams, and J. H. Sherman, "Protective effect of seaweed extracts for chicken embryos infected with influenza B or mumps virus," *Experimental Biology and Medicine*, vol. 99, no. 3, pp. 590–593, 1958.

[14] M. F. De Jesus Raposo, A. M. B. De Morais, and R. M. S. C. De Morais, "Marine polysaccharides from algae with potential biomedical applications," *Marine Drugs*, vol. 13, no. 5, pp. 2967–3028, 2015.

[15] S. F. Mohamed and F. A. Agili, "Antiviral sulphated polysaccharide from brown algae *Padina pavonia* characterization and structure elucidation," *International Journal of ChemTech Research*, vol. 5, no. 4, pp. 1469–1476, 2013.

[16] T. T. T. Thuy, B. M. Ly, T. T. T. Van et al., "Anti-HIV activity of fucoidans from three brown seaweed species," *Carbohydrate Polymers*, vol. 115, pp. 122–128, 2015.

[17] M. Kim, J. H. Yim, S.-Y. Kim et al., "In vitro inhibition of influenza A virus infection by marine microalga-derived sulfated polysaccharide p-KG03," *Antiviral Research*, vol. 93, no. 2, pp. 253–259, 2012.

[18] R. Elizondo-Gonzalez, L. E. Cruz-Suarez, D. Ricque-Marie, E. Mendoza-Gamboa, C. Rodriguez-Padilla, and L. M. Trejo-Avila, "In vitro characterization of the antiviral activity of fucoidan from *Cladosiphon okamuranus* against Newcastle Disease Virus," *Virology Journal*, vol. 9, no. 1, article 307, 2012.

[19] L. M. Trejo-Avila, M. E. Morales-Martínez, D. Ricque-Marie et al., "In vitro anti-canine distemper virus activity of fucoidan extracted from the brown alga *Cladosiphon okamuranus*," *Virus-Disease*, vol. 25, no. 4, pp. 474–480, 2014.

[20] G. Meiyu, L. Fuchuan, X. Xianliang, L. Jing, Y. Zuowei, and G. Huashi, "The potential molecular targets of marine sulfated polymannuroguluronate interfering with HIV-1 entry: interaction between SPMG and HIV-1 rgp120 and CD4 molecule," *Antiviral Research*, vol. 59, no. 2, pp. 127–135, 2003.

[21] C. B. Buck, C. D. Thompson, J. N. Roberts, M. Müller, D. R. Lowy, and J. T. Schiller, "Carrageenan is a potent inhibitor of papillomavirus infection," *PLoS Pathogens*, vol. 2, no. 7, pp. 0671–0680, 2006.

[22] A. Rodríguez, K. Kleinbeck, O. Mizenina et al., "In vitro and in vivo evaluation of two carrageenan-based formulations to prevent HPV acquisition," *Antiviral Research*, vol. 108, no. 1, pp. 88–93, 2014.

[23] J. A. Aguilar-Briseño, L. E. Cruz-Suarez, J.-F. Sassi et al., "Sulphated polysaccharides from *Ulva clathrata* and *Cladosiphon okamuranus* seaweeds both inhibit viral attachment/entry and cell-cell fusion, in NDV infection," *Marine Drugs*, vol. 13, no. 2, pp. 697–712, 2015.

[24] Y. Koizumi and S. Iwami, "Mathematical modeling of multi-drugs therapy: a challenge for determining the optimal combinations of antiviral drugs," *Theoretical Biology & Medical Modelling*, vol. 11, p. 41, 2014.

[25] W. J. Moss and D. E. Griffin, "Measles," *The Lancet*, vol. 379, no. 9811, pp. 153–164, 2012.

[26] W. J. Moss and D. E. Griffin, "Global measles elimination," *Nature Reviews Microbiology*, vol. 4, no. 12, pp. 900–908, 2006.

[27] G. Antonelli and O. Turriziani, "Antiviral therapy: old and current issues," *International Journal of Antimicrobial Agents*, vol. 40, no. 2, pp. 95–102, 2012.

[28] D. Robledo and Y. Freile-Pelegrín, "Prospects for the cultivation of economically important carrageenophytes in Southeast Mexico," *Journal of Applied Phycology*, vol. 23, no. 3, pp. 415–419, 2011.

[29] J. Xi, D. Shen, S. Zhao, B. Lu, Y. Li, and R. Zhang, "Characterization of polyphenols from green tea leaves using a high hydrostatic pressure extraction," *International Journal of Pharmaceutics*, vol. 382, no. 1-2, pp. 139–143, 2009.

[30] M. Tako, E. Yoza, and S. Tohma, "Chemical characterization of acetyl fucoidan and alginate from commercially cultured *Cladosiphon okamuranus*," *Botanica Marina*, vol. 43, no. 4, pp. 393–398, 2000.

[31] M. T. Ale, J. D. Mikkelsen, and A. S. Meyer, "Designed optimization of a single-step extraction of fucose-containing sulfated polysaccharides from *Sargassum* sp.," *Journal of Applied Phycology*, vol. 24, no. 4, pp. 715–723, 2012.

[32] S. G. Jackson and E. L. McCandless, "Simple, rapid, turbidometric determination of inorganic sulfate and/or protein," *Analytical Biochemistry*, vol. 90, no. 2, pp. 802–808, 1978.

[33] T.-C. Chou, "Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies," *Pharmacological Reviews*, vol. 58, no. 3, pp. 621–681, 2006.

[34] A. S. Huang and R. R. Wagner, "Penetration of herpes simplex virus into human epidermoid cells," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 116, no. 4, pp. 863–869, 1964.

[35] P. Volery, R. Besson, and C. Schaffer-Lequart, "Characterization of commercial carrageenans by Fourier transform infrared spectroscopy using single-reflection attenuated total reflection,"

Journal of Agricultural and Food Chemistry, vol. 52, no. 25, pp. 7457–7463, 2004.

[36] V. L. Campo, D. F. Kawano, D. B. da Silva Jr., and I. Carvalho, "Carrageenans: biological properties, chemical modifications and structural analysis—a review," *Carbohydrate Polymers*, vol. 77, no. 2, pp. 167–180, 2009.

[37] F. Van De Velde, L. Pereira, and H. S. Rollema, "The revised NMR chemical shift data of carrageenans," *Carbohydrate Research*, vol. 339, no. 13, pp. 2309–2313, 2004.

[38] D. D. McIntyre, H. Ceri, and H. J. Vogel, "Nuclear magnetic resonance studies of the heteropolysaccharides alginate, *Gum arabic* and gum xanthan," *Starch*, vol. 48, no. 7-8, pp. 285–291, 1996.

[39] H. Grasdalen, B. Larsen, and O. Smisrod, " ^{13}C-n.m.r. studies of monomeric composition and sequence in alginate," *Carbohydrate Research*, vol. 89, no. 2, pp. 179–191, 1981.

[40] S. Dinesh, T. Menon, L. E. Hanna, V. Suresh, M. Sathuvan, and M. Manikannan, "In vitro anti-HIV-1 activity of fucoidan from *Sargassum swartzii*," *International Journal of Biological Macromolecules*, vol. 82, pp. 83–88, 2016.

[41] Y. Sugiura, K. Matsuda, Y. Yamada et al., "Anti-allergic phlorotannins from the edible brown alga, *Eisenia arborea*," *Food Science and Technology Research*, vol. 13, no. 1, pp. 54–60, 2007.

[42] J. A. Zertuche-González, M. Sánchez-Barredo, J. M. Guzmán-Calderón, and Z. Altamirano-Gómez, "*Eisenia arborea* J.E. Areschoug as abalone diet on an IMTA farm in Baja California, México," *Journal of Applied Phycology*, vol. 26, no. 2, pp. 957–960, 2014.

[43] Q.-T. Le, Y. Li, Z.-J. Qian, M.-M. Kim, and S.-K. Kim, "Inhibitory effects of polyphenols isolated from marine alga *Ecklonia cava* on histamine release," *Process Biochemistry*, vol. 44, no. 2, pp. 168–176, 2009.

[44] D. L. Arvizu, Y. E. Rodríguez, G. Hernández, and J. I. Murillo, "Chemical constituents of *Eisenia arbórea* Areschoug from Baja California Sur, México," *Investigaciones Marinas*, vol. 35, no. 2, pp. 63–69, 2007.

[45] K. I. P. J. Hidari, N. Takahashi, M. Arihara, M. Nagaoka, K. Morita, and T. Suzuki, "Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga," *Biochemical and Biophysical Research Communications*, vol. 376, no. 1, pp. 91–95, 2008.

[46] K. Hayashi, T. Nakano, M. Hashimoto, K. Kanekiyo, and T. Hayashi, "Defensive effects of a fucoidan from brown alga *Undaria pinnatifida* against herpes simplex virus infection," *International Immunopharmacology*, vol. 8, no. 1, pp. 109–116, 2008.

[47] K. Hayashi, J.-B. Lee, T. Nakano, and T. Hayashi, "Anti-influenza A virus characteristics of a fucoidan from sporophyll of *Undaria pinnatifida* in mice with normal and compromised immunity," *Microbes and Infection*, vol. 15, no. 4, pp. 302–309, 2013.

[48] M. C. Valdez, E. S. Zaragoza, D. L. Belda, R. Marcos, and R. A. Ramírez, "Effect of climatic change on the harvest of the kelp *Macrocystis Pyrifera* on the Mexican Pacific coast," *Bulletin of Marine Science*, vol. 73, no. 3, pp. 545–556, 2003.

[49] A. M. S. Mayer, A. Diaz, A. Pesce, M. Criscuolo, J. F. Groisman, and R. M. de Lederkremer, "Biological activity in *Macrocystis pyrifera* from Argentina: sodium alginate, fucoidan and laminaran. III. Antiviral activity," *Hydrobiologia*, vol. 151-152, no. 1, pp. 497–500, 1987.

[50] Z. Luo, D. Tian, M. Zhou et al., "λ-Carrageenan P32 is a potent inhibitor of rabies virus infection," *PLoS ONE*, vol. 10, no. 10, Article ID e0140586, 2015.

[51] Q. Shao, Q. Guo, W. P. Xu, Z. Li, and T. T. Zhao, "Specific inhibitory effect of κ-carrageenan polysaccharide on swine pandemic 2009 H1N1 influenza virus," *PLoS ONE*, vol. 10, no. 5, Article ID e0126577, 2015.

[52] I. W. F. De Araújo, E. D. S. O. Vanderlei, J. A. G. Rodrigues et al., "Effects of a sulfated polysaccharide isolated from the red seaweed Solieria filiformis on models of nociception and inflammation," *Carbohydrate Polymers*, vol. 86, no. 3, pp. 1207–1215, 2011.

[53] T. Ghosh, K. Chattopadhyay, M. Marschall, P. Karmakar, P. Mandal, and B. Ray, "Focus on antivirally active sulfated polysaccharides: from structure-activity analysis to clinical evaluation," *Glycobiology*, vol. 19, no. 1, pp. 2–15, 2009.

[54] I. Sugawara, W. Itoh, S. Kimura, S. Mori, and K. Shimada, "Further characterization of sulfated homopolysaccharides as anti-HIV agents," *Experientia*, vol. 45, no. 10, pp. 996–998, 1989.

[55] T. Priengprom, T. Ekalaksananan, B. Kongyingyoes, S. Suebsasana, C. Aromdee, and C. Pientong, "Synergistic effects of acyclovir and 3, 19- isopropylideneandrographolide on herpes simplex virus wild types and drug-resistant strains," *BMC Complementary and Alternative Medicine*, vol. 15, no. 1, article 56, 2015.

[56] G. Belardo, O. Cenciarelli, S. La Frazia, J. F. Rossignol, and M. G. Santoroa, "Synergistic effect of nitazoxanide with neuraminidase inhibitors against influenza A viruses in vitro," *Antimicrobial Agents and Chemotherapy*, vol. 59, no. 2, pp. 1061–1069, 2015.

[57] E. Vilanova, C. C. Coutinho, and P. A. S. Mourão, "Sulfated polysaccharides from marine sponges (Porifera): an ancestor cell-cell adhesion event based on the carbohydrate-carbohydrate interaction," *Glycobiology*, vol. 19, no. 8, pp. 860–867, 2009.

[58] R. Bouhlal, C. Haslin, J.-C. Chermann et al., "Antiviral activities of sulfated polysaccharides isolated from *Sphaerococcus coronopifolius* (*Rhodophytha, Gigartinales*) and *Boergeseniella thuyoides* (*Rhodophyta, Ceramiales*)," *Marine Drugs*, vol. 9, no. 7, pp. 1187–1209, 2011.

[59] N. N. Besednova, I. D. Makarenkova, T. N. Zvyagintseva, T. I. Imbs, L. M. Somova, and T. S. Zaporozhets, "Antiviral activity and pathogenetic targets for seaweed sulfated polysaccharides in herpesvirus infections," *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry*, vol. 10, no. 1, pp. 31–42, 2016.

[60] E. A. Govorkova and R. G. Webster, "Combination chemotherapy for influenza," *Viruses*, vol. 2, no. 8, pp. 1510–1529, 2010.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 5829823, 8 pages
http://dx.doi.org/10.1155/2016/5829823

Research Article

Effect of LongZhang Gargle on Biofilm Formation and Acidogenicity of *Streptococcus mutans In Vitro*

Yutao Yang,[1,2] Shiyu Liu,[1,2] Yuanli He,[1,2] Zhu Chen,[3,4] and Mingyun Li[1,2]

[1] *State Key Laboratory of Oral Diseases, Sichuan University, Chengdu 610041, China*
[2] *Department of Operative Dentistry and Endodontics, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China*
[3] *Wuhan First Hospital of Stomatology, Jianghan University, Wuhan 430065, China*
[4] *Guiyang Hospital of Stomatology, Guiyang 550002, China*

Correspondence should be addressed to Zhu Chen; 1605894600@qq.com and Mingyun Li; limingyun@scu.edu.cn

Received 14 March 2016; Revised 26 April 2016; Accepted 5 May 2016

Academic Editor: Nikos Chorianopoulos

Streptococcus mutans, with the ability of high-rate acid production and strong biofilm formation, is considered the predominant bacterial species in the pathogenesis of human dental caries. Natural products which may be bioactive against *S. mutans* have become a hot spot to researches to control dental caries. LongZhang Gargle, completely made from Chinese herbs, was investigated for its effects on acid production and biofilm formation by *S. mutans* in this study. The results showed an antimicrobial activity of LongZhang Gargle against *S. mutans* planktonic growth at the minimum inhibitory concentration (MIC) of 16% and minimum bactericidal concentration (MBC) of 32%. Acid production was significantly inhibited at sub-MIC concentrations. Biofilm formation was also significantly disrupted, and 8% was the minimum concentration that resulted in at least 50% inhibition of biofilm formation ($MBIC_{50}$). A scanning electron microscopy (SEM) showed an effective disruption of LongZhang Gargle on *S. mutans* biofilm integrity. In addition, a confocal laser scanning microscopy (CLSM) suggested that the extracellular polysaccharides (EPS) synthesis could be inhibited by LongZhang Gargle at a relatively low concentration. These findings suggest that LongZhang Gargle may be a promising natural anticariogenic agent in that it suppresses planktonic growth, acid production, and biofilm formation against *S. mutans*.

1. Introduction

Dental caries is a chronic, progressive, and infectious disease which happens to the hard tissue of the teeth [1]. It is one of the most prevalent chronic human infectious diseases worldwide [2, 3]. The progress begins with extensive destruction of the enamel and dentin, followed by cavitation, inflammation of the pulp, and periapical tissue, even ending with tooth loss [4]. Unlike most infectious diseases exhibiting classic virulence factors such as endotoxin (lipopolysaccharide, LPS), the etiologic factors of dental caries are initiated with the bacterial adherence to the tooth surface and dental plague biofilm formation. After that, cariogenic bacteria within dental plague biofilm produce acid by metabolizing carbohydrates ingested from the host, leading to demineralization of enamel surfaces and the development of caries [5, 6].

Streptococcus mutans (*S. mutans*) has been identified as the major pathogen of human dental caries [5, 7], although additional microorganisms may be involved [8, 9]. Reasons are as follows: *S. mutans* can easily adhere to enamel surfaces, it can synthesize extracellular polysaccharides (EPS) in the presence of carbohydrate, which mediates the irreversible adhesive interaction between bacterial cells and forms a high-cell-density biofilm [10], and it exhibits a high-rate acid production and is highly aciduric, which allows it to survive and continue to produce acids in low pH microenvironments [11, 12].

Many products are being developed for caries control. Among them, the most effective and widely used cariostatic agent is fluoride. It prevents dental caries by inhibiting demineralization and enhancing remineralization on the enamel surface. However, side effects such as fluorosis limited

its use for public health [13, 14]. The antimicrobials including chlorhexidine and antibiotics have also been used to prevent dental caries. However, they may cause tooth and tongue discoloration, host cytotoxicity, pathogen drug resistance, and disturbance of oral flora [15–17]. Therefore, alternative agents for caries control with minimal adverse effects are promising.

In recent years, natural products which may be bioactive against *S. mutans* have been of great interest to the researchers in the field of caries control, mainly due to their few side effects. Currently, many cariostatic natural products have been identified, typical natural products such as ginkgoneolic acid [4], epigallocatechin gallate [18, 19], and cranberry polyphenols [20]. LongZhang Gargle, completely made from Chinese folk herbs, was created and used by Miao people, who are the ethnic minorities mainly living in Southwest China. Industrially, it has been manufactured by Guiyang Xintian Pharmaceutical Company, Guizhou, China, for nearly 20 years. The components of the Gargle include the root and leaves of *Toddalia asiatica* (L.) Lam., Cortex Lycii, and *Cimicifuga foetida*. *Toddalia asiatica* (L.) Lam. (*T. asiatica*), as the main component of LongZhang Gargle, belongs to the familyRutaceae and is a woody liana that grows in tropical and subtropical areas of the world. It is widely recognized as a medicinal plant in China, India, Japan, and Africa. All parts of the plant are believed to have medicinal properties, and its root and leaves have been processed to treat many diseases, such as rheumatism, influenza, malaria, indigestion, stomachache, and toothache [21, 22]. Currently, the pharmacological effects of *T. asiatica* considered mainly include antipyretic, antinociceptive, anti-inflammatory, and antimicroorganism. For example, a study showed the antinociceptive and anti-inflammatory effects of its root extract on Swiss albino mice [23]. Some studies also demonstrated the antibacterial and antifungal activities of *T. asiatica* extracts by measuring the planktonic growth of various kinds of microorganism, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida albicans* [22, 24–26]. At present, LongZhang Gargle is mainly applied for treating gingivitis, periodontitis, and oral ulcer on clinic [27, 28]. It can also significantly reduce bacteria in root canals and act as an effective root canal disinfectant [29]. Although there are many clinical researches confirming the value of LongZhang Gargle for curing oral diseases, studies on pharmacological mechanism of it are still scarce. Moreover, the studies of *T. asiatica* on microorganism have all been performed in planktonic cultures and the existing researches completely ignore the effect of *T. asiatica* on oral biofilms. In addition, cariogenic bacteria such as *S. mutans* have never been studied either.

Considering the antimicrobial effect of *T. asiatica* and LongZhang Gargle as previous studies showed, we suppose that the gargle can inhibit the growth of *S. mutans* in planktonic culture. Our research also aims to explore the effects of LongZhang Gargle on virulence factors of *S. mutans* including biofilm formation and acid production. The study contributes to the finding of a new natural medication treatment of dental caries without any significant side effects, as well as the finding of the multiple uses of LongZhang Gargle, so as to promote LongZhang Gargle at a wider clinical application.

2. Materials and Methods

2.1. Chemicals, Bacterial Strains, and Growth Conditions. The agent of LongZhang Gargle was provided by Guiyang Xintian Pharmaceutical Co. Ltd., Guizhou, China. *S. mutans* UA159 was provided by State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, China. Brain-heart infusion (BHI) broth was used for investigating the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and acidogenicity. For the biofilm formation, 1% (wt/vol) sucrose was added to BHI (BHIS). Alexa Fluor 647-labeled dextran conjugate, as a red fluorescent stain for EPS, was bought from Invitrogen, Carlsbad, CA, USA. SYTO 9, as a green fluorescent nucleic acid stain, was bought from Molecular Probes, Eugene, OR, USA. The bacterium was grown at 37°C under anaerobic conditions (5% CO_2).

2.2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MIC and MBC of LongZhang Gargle against *S. mutans* were determined by liquid medium double dilution method modified from that of Lombardo Bedran et al. [30]. The wells of a 96-well tissue culture plate, each containing 190 μL of serially diluted LongZhang Gargle (1%, 2%, 4%, 8%, 16%, 32%, and 64%) in BHI culture media, were inoculated with 10 μL of an overnight culture of *S. mutans* diluted in fresh BHI broth to obtain an OD_{600} of 0.2 (about 10^8 CFU/mL). Each concentration contained three parallel samples and BHI broth without gargle was used as a control. The MIC was the lowest concentration of the gargle that no bacteria were grown in the broth. To determine the MBC, the 200 μL cultures with 10 μL of *S. mutans* and 190 μL of BHI at a drug concentration above MIC were aspirated into BHI agar plates and incubated at 37°C for 24 h. The MBC was the lowest concentration of the gargle that no bacteria could be observed on the agar plate.

2.3. Effect of LongZhang Gargle on Biofilm Formation. The effect of LongZhang Gargle on *S. mutans* biofilm formation was measured by the method modified from that of Li et al. [31] and Assaf et al. [32]. Overnight-grown *S. mutans* was diluted in BHI broth to obtain an OD_{600} of 0.2 (about 10^8 CFU/mL). The wells of a sterile 96-well tissue culture plate, each contained 10 μL of such cell suspension and 190 μL of BHIS broth with different LongZhang Gargle concentrations below the MIC (1%, 2%, 4%, and 8%), were incubated for a biofilm formation. Each concentration contained three parallel samples and BHIS broth without gargle was used as a control. After 24 h, supernatant from each well was aspirated, and the biofilm in each well was fixed with methanol for 15 min, stained with crystal violet (0.5%) for 30 min, and washed three times with distilled deionized water to remove the unbound crystal violet. After that, 200 μL of 100% ethanol was added to each well to dissolve the crystal violet on the biofilm. The plate was rocked at room temperature

for 20 min, and the absorbance was read at 600 nm by a spectrophotometer.

2.4. Effect of LongZhang Gargle on Biofilm Morphology. The structure of *S. mutans* biofilms formed in the presence of LongZhang Gargle was observed by scanning electron microscopy modified from that of Bitoun et al. [33] and Jongsma et al. [34]. Briefly, 100 μL of overnight-grown *S. mutans* suspension diluted in fresh BHI at an initial OD_{600} of 0.2 and 1900 μL BHIS broth with different concentrations of LongZhang Gargle below the MIC (1%, 2%, 4%, and 8%) was added to the wells of a 24-well tissue culture plate. Each concentration contained three parallel samples and BHIS broth without gargle was used as a control. Glass coverslips (5 mm in diameter) were prefixed in each well. After incubation for 24 h, the biofilm-coated glass coverslips were immersed in 2.5% glutaraldehyde at 4°C overnight, washed three times with distilled deionized water, dehydrated using ascending graded serious of ethanol (30%, 50%, 70%, 80%, 85%, 90%, 95%, and 100%), and coated with gold. The samples were then examined by SEM.

2.5. Effect of LongZhang Gargle on Extracellular Polysaccharide (EPS) Synthesis and Bacterial Viability. The *S. mutans* biofilms were observed for the volume of their major components (EPS and bacteria) by confocal laser scanning microscopy modified from Xiao and Koo [35] and Qiu et al. [36] as described previously. 100 μL overnight-grown *S. mutans* diluted in fresh BHI at an initial OD_{600} of 0.2 was added to the wells of a 24-well tissue culture plate and grown in 1900 μL BHIS with 0, 1%, 2%, and 4% of LongZhang Gargle, respectively. Sterile slides were prefixed in each well. 1 μL of 2.5 μM Alexa Fluor 647-labeled dextran conjugate was added to each well during the formation of biofilms. After incubation for 24 h, the biofilms were washed three times with PBS and stained with 50 μL of 2.5 μM SYTO 9 green fluorescent nucleic acid stain at 4°C for 30 min. Then the samples were examined by a confocal laser scanning microscope (CLSM) under 63x oil immersion objectives (Figure 3). Two samples and three fields per sample were observed for each group.

2.6. Effect of LongZhang Gargle on Acidogenicity. The effect of LongZhang Gargle on acidogenicity of *S. mutans* was examined by a standard pH drop. We modified the measure from that of Xu et al. [19]. Bacteria were harvested at mid-logarithmic phase, washed with phosophate-buffered saline (PBS), and grown in potassium phosphate buffer with glucose (contained 0.5 mM K_2HPO_4, 0.5 mM KH_2PO_4, 37.5 mM KCl, 1.25 mM $MgCl_2$, and 1% wt/vol glucose, initial pH was 6.50). The initial optical density (OD) of the mixture at 600 nm was 0.5. LongZhang Gargle was then added to reach different concentrations below the MIC (1%, 2%, 4%, and 8%), as well as a nontreated control. The decrease in pH, as a result of glycolytic activity of *S. mutans* UA159, was monitored at 15 min intervals over a period of 120 min.

2.7. Statistical Analysis. All the experiments were repeated at least three times. Differences between the experiment group and the untreated control group were statistically analyzed by SPSS (version 20.0 for Windows). One-way ANOVA and post hoc Tukey's multiple-comparison test were applied for the comparison of multiple means. The chosen level of significance was set at $P < 0.05$.

3. Results

3.1. LongZhang Gargle Exhibits Antimicrobial Activity against S. mutans. We determined the antimicrobial effect of LongZhang Gargle by measuring MIC and MBC against *S. mutans*. The results showed that LongZhang Gargle inhibited the growth of planktonic *S. mutans* UA159 at a MIC of 16% and MBC of 32%.

3.2. S. mutans Biofilm Is Susceptible to LongZhang Gargle. The effect of LongZhang Gargle on biofilm formation by *S. mutans* was determined by measuring the absorbance at 600 nm in crystal violet assay. As is shown in Figure 1, the results were 0.860, 0.798, 0.648, 0.529, and 0.189 relative light units at the concentrations of 0, 1% (1/16 MIC), 2% (1/8 MIC), 4% (1/4 MIC), and 8% (1/2 MIC), respectively. Statistical analysis indicated that LongZhang Gargle significantly inhibited *S. mutans* biofilm formation at 2% ($P < 0.05$), 4% ($P < 0.05$), and 8% ($P < 0.01$). No significant difference was found between control (0%) and 1%. Furthermore, differences are significant between 1% and 2% ($P < 0.05$), 2% and 4% ($P < 0.05$), and 4% and 8% ($P < 0.001$). This demonstrated that the inhibitory effect was dosage dependent. The percentage of inhibition was calculated using the following equation: $(1 - A_{600}$ of the test group/A_{600} of blank control) $\times 100\%$. And the inhibition percentages are 38.5% for concentration of 4% and 78.0% for 8%. So 8% was considered as $MBIC_{50}$ which meant the minimum concentration that resulted in at least 50% inhibition of biofilm formation compared with that in control.

The morphology alteration of *S. mutans* biofilm was observed by SEM. As is shown in Figure 2, thick and relatively thick biofilm was formed in control (without the gargle). However, biofilm integrity was disrupted when treated with different concentrations of LongZhang Gargle. With a concentration of 8% ($MBIC_{50}$), only scattered bacteria could be seen.

The major components of *S. mutans* biofilms are EPS and bacteria. However, crystal violet assay and scanning electron microscopy cannot accurately differentiate one from the other. Therefore, we conducted the CLSM study to investigate how these two components were affected by LongZhang Gargle. As is shown in Figure 4, the control group (without the gargle) witnessed a high density of both EPS and bacteria cells. The intensity of fluorescence, red for EPS and green for bacteria cells, was both significantly decreased when LongZhang Gargle added. A higher concentration seemed to contribute a sharper decrease for the volume of both EPS and bacteria. To conclude, the results of crystal violet assay, SEM, and CLSM all suggest that LongZhang Gargle could inhibit the formation of *S. mutans* biofilm.

3.3. LongZhang Gargle Reduces Acid Production of S. mutans. We determined the effect of LongZhang Gargle on

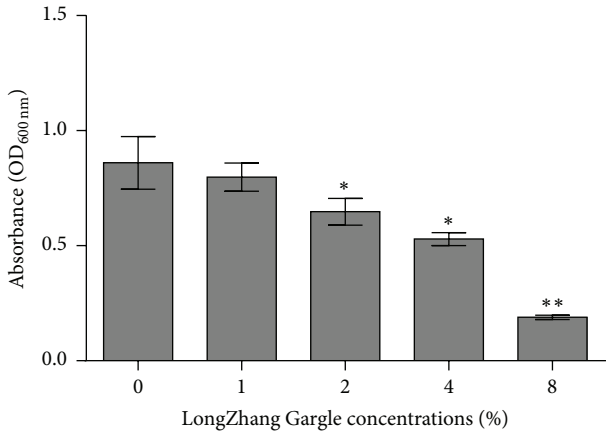


Figure 1: Effect of LongZhang Gargle on biofilm formation. *S. mutans* was incubated in BHIS broth for 24 h with LongZhang Gargle at concentrations of 0, 1% (1/16 MIC), 2% (1/8 MIC), 4% (1/4 MIC), and 8% (1/2 MIC). $^{*}P < 0.05$, $^{**}P < 0.01$.

acidogenicity of *S. mutans* by monitoring the glycolytic pH drop in planktonic culture. As is shown in Figure 4, the acid production of *S. mutans* UA159 was inhibited by LongZhang Gargle at sub-MIC levels. Higher concentration (1/2 MIC, 8%) contributed a more obvious inhibitory effect on pH drop than other concentrations. Also, the initial 30 min showed the highest pH drop.

4. Discussion

Natural products are a rich source for discovery of new antimicrobial agents [37]. Yet we know very little about an antimicrobial effect of LongZhang Gargle, a traditional Chinese herbal compound. In this study, we found that LongZhang Gargle showed an antimicrobial effect on *S. mutans* in planktonic culture. Diluted LongZhang Gargle at concentration of 16% could effectively and completely inhibit the growth of *S. mutans* and at the concentration of 32% showed an obvious bactericidal activity. Considering that high concentration of the drug may cause side effects, the MIC and MBC may help to determine the appropriate concentration for applying it. However, the bacteria in human mouth exist in biofilms rather than planktonic form, and other oral bacterial species in biofilms probably also contribute to oral diseases. Therefore, we conducted the crystal violet assay, SEM and CLSM, to explore the effects of LongZhang Gargle on *S. mutans* biofilm formation as described above. For further study, other common cariogenic bacteria and biofilms formation could also be similarly investigated.

Dental caries is caused by oral microorganisms through metabolizing carbohydrate, producing acid, and demineralizing the tooth surface. Thus, the reduction of acid production is a logical approach to prevent dental caries. However, previous studies completely ignore the effects of LongZhang Gargle on acid production of oral bacteria. In our research, *S. mutans* was examined at mid-logarithmic phase to guarantee a best state of acidogenicity. The results showed that concentration of 1% (1/16 MIC) of LongZhang Gargle could inhibit acid production of *S. mutans*. This finding suggested that acidogenicity of *S. mutans* in biofilms could probably also be inhibited. Therefore, we may conduct further experiment to measure the influence of LongZhang Gargle on the change of pH of the *S. mutans* biofilm model. Moreover, complicated dental plaque naturally formed will also be the research object to be tested. Furthermore, since the acidogenicity inhibition concentration was very low (1/16 MIC), we hypothesize that the mechanism of the suppression may be attributed to the inhibition of enzymes that related to acid production, rather than bactericidal activity of the drug.

S. mutans is a bacterium primarily adhering to dental surface in dental plaque in the mouth. Biofilms provide protection against the action of antibiotics and supply a barrier to prevent or reduce the penetration of antimicrobial agents through the matrix [38] and are also considered as the initiating factor for dental diseases [39]. Therefore, the reduction of stable biofilm formation is an effective way to prevent dental caries. To the best of our knowledge, the effect of LongZhang Gargle on the *S. mutans* biofilm has not yet been documented. In this study, we identified that the biofilm of *S. mutans* was susceptible to LongZhang Gargle. In crystal violet assay, we found that biofilm formation was significantly suppressed at the concentration of 2% LongZhang Gargle, and this effect was dosage dependent. Moreover, we also found that the $MBIC_{50}$ (8%) was lower than MIC (16%), and this result indicated that the gargle was more effective to inhibit aggregation of cells and reduce biofilm formation, compared with its bacteriostatic activity. In addition, under scanning electron microscopy, we observed that the biofilm was relatively thick and homogeneous without the gargle compared with those samples treated with gargle at different concentrations. As the concentration of the gargle increases, the biofilm integrity and structure is gradually being disrupted. Moreover, this disruption to biofilm integrity was also dosage dependent. At a concentration of 8%, *S. mutans* biofilm became very sparse and could not cover the surface of the slips, and the bacteria cluster became much smaller compared with the control group. Furthermore, for the confocal laser scanning microscopy, we found that the volume of EPS and bacterial cells became much less when adding the gargle, even though the concentration was relatively low. As we described above, the synthesis of EPS is a vital process for dental caries as it mediates irreversible adherence between bacteria and tooth surface, as well as aggregation between bacterial cells. Therefore, LongZhang Gargle can reduce the adherence and aggregation of the bacteria, followed by the inhibition of local pH drop on tooth enamel, which leads to dental caries.

Recently, anticariogenic-active natural products are gradually being studied. It has become a hot spot for researchers to explore traditional Chinese herbs that can prevent dental caries [40, 41]. LongZhang Gargle, completely made from traditional Chinese herbs, has been widely used since being manufactured 20 years ago, mainly applied for treating gingivitis, periodontal disease, and oral ulcer. Our study revealed the prospect of LongZhang Gargle as an anticariogenic

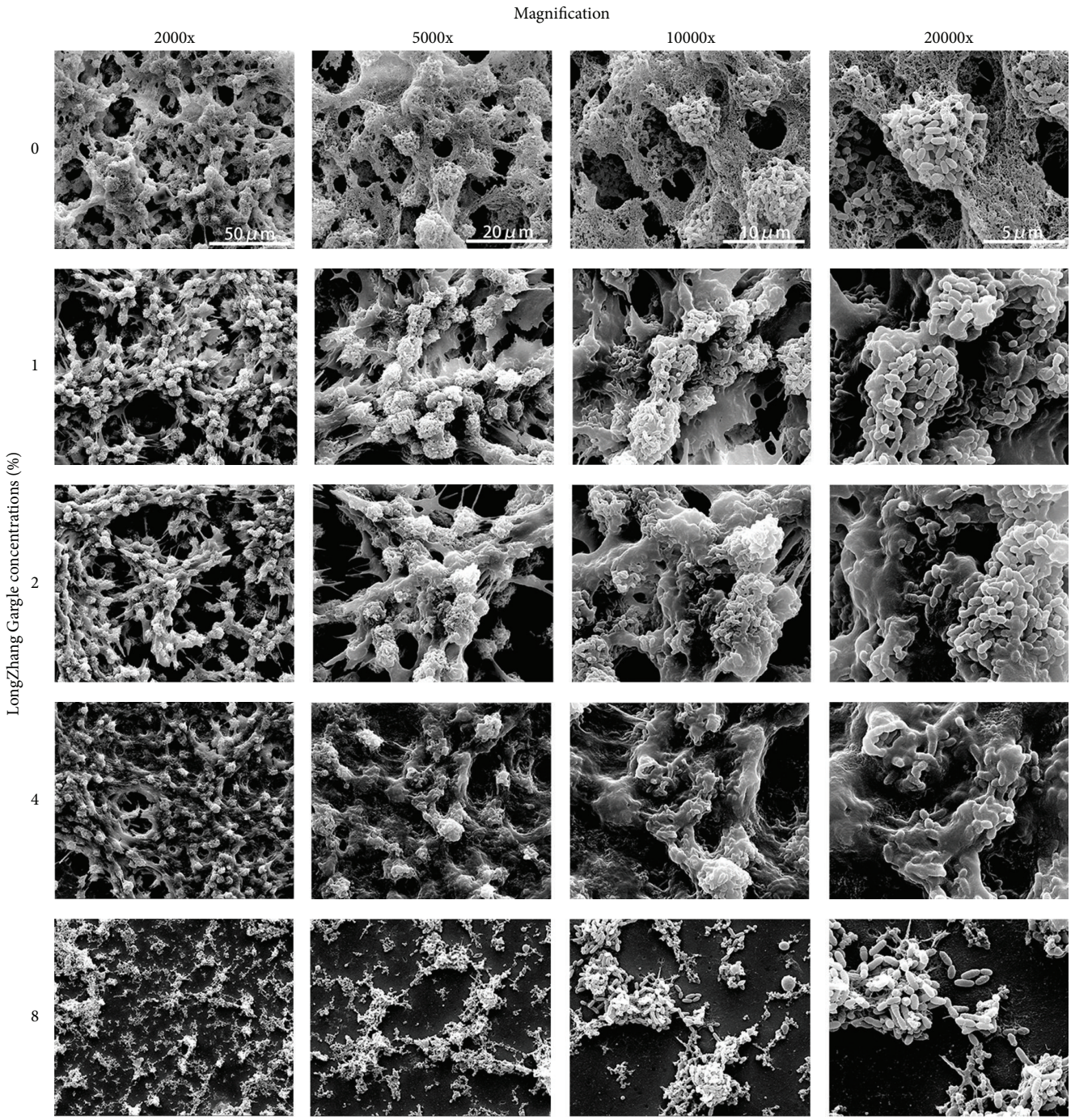


Figure 2: Effect of LongZhang Gargle on biofilm morphology. *S. mutans* was incubated for 24 h in BHIS broth, with LongZhang Gargle at concentrations of 2%, 4%, and 8%. The control group without drug was also conducted. Magnification was 2000x, 5000x, 10000x, and 20000x, respectively, for each concentration.

treatment *in vitro*. Therefore, we suppose it can logically reduce caries incidence rate for susceptible population. If this speculation is correct, LongZhang Gargle will be an integrated drug to prevent and cure the common oral diseases, including dental caries, gingivitis, periodontal disease, and oral ulcer. To confirm our speculation, more clinical studies of LongZhang Gargle need to be conducted.

5. Conclusion

This study has identified that LongZhang Gargle owns an antimicrobial effect against *S. mutans*, and it is the first to identify the inhibitory effects on acid production and biofilm formation. This indicates that LongZhang Gargle might act as a promising way to control dental caries. However, further

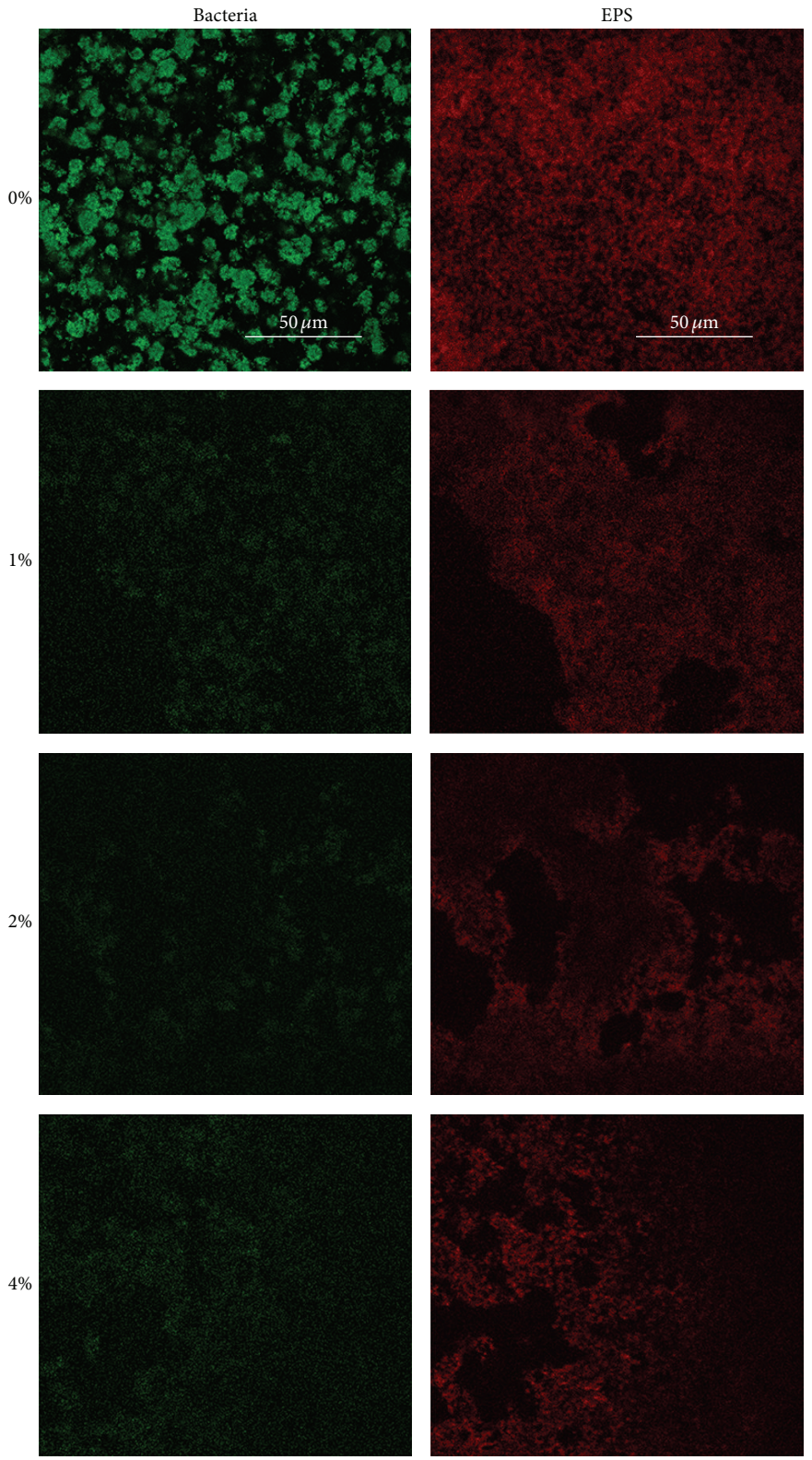


Figure 3: Effect of LongZhang Gargle on Extracellular Polysaccharide (EPS) Synthesis of *Streptococcus mutans*. The cells were treated with LongZhang Gargle at concentrations of 0, 1% (1/16 MIC), 2% (1/8 MIC), and 4% (1/4 MIC) for 24 h. EPS was labeled red (Alexa Fluor 647) and bacterial cells were labeled green (SYTO 9) under a confocal laser scanning microscope. Magnification was 63x for oil immersion objective.

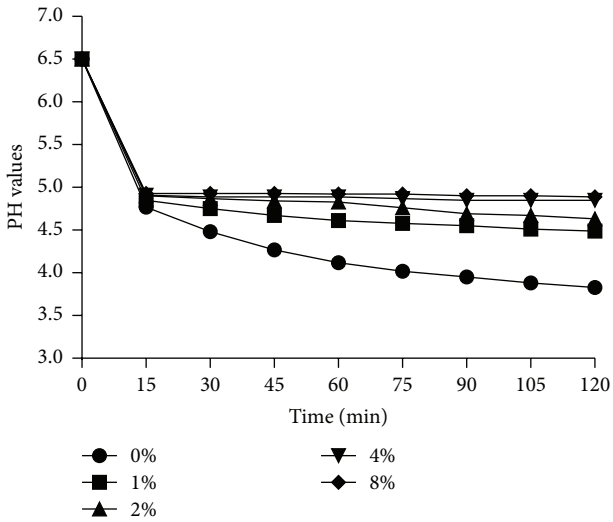


Figure 4: Effect of LongZhang Gargle on the acid production of *S. mutans*. *S. mutans* was incubated in BHI broth with LongZhang Gargle at concentrations of 0, 1% (1/16 MIC), 2% (1/8 MIC), 4% (1/4 MIC), and 8% (1/2 MIC). Glycolytic acid production was determined by monitoring the pH drop in glucose solution (1% wt/vol) at 15 min intervals over a period of 120 min.

and more studies should be considered in future, for example, the effects on other cariogenic bacteria, such as *Actinomyces viscosus*, and the clinical study on dosage, efficacy, and side effects of LongZhang Gargle.

Competing Interests

The authors declare no potential competing interests with respect to the authorship and/or publication of this paper.

Authors' Contributions

Yutao Yang and Shiyu Liu contributed equally to this study.

Acknowledgments

The authors are thankful to Chaoliang Zhang for the technical support of SEM. This study was supported by the National Natural Science Foundation of China (81400501 to Mingyun Li) and the Special Fund of State Key Laboratory of Oral Diseases, Sichuan University (SKLOD201525 to Mingyun Li), 2012 Innovative Talents Program (Zhu Chen), and Sichuan University Students' Innovative Training Program (201510611917 to Yutao Yang).

References

[1] R. H. Selwitz, A. I. Ismail, and N. B. Pitts, "Dental caries," *The Lancet*, vol. 369, no. 9555, pp. 51–59, 2007.

[2] P. E. Petersen, "The world oral health report 2003: continuous improvement of oral health in the 21st century—the approach of the WHO global oral health programme," *Community Dentistry and Oral Epidemiology*, vol. 31, no. 1, pp. 3–24, 2003.

[3] B. A. Dye, S. Tan, V. Smith et al., "Trends in oral health status: United States, 1988–1994 and 1999–2004," *Vital and Health Statistics*, vol. 248, pp. 1–92, 2007.

[4] J. Z. He, S. D. Wang, T. X. Wu, Y. P. Cao, X. Xu, and X. D. Zhou, "Effects of ginkgoneolic acid on the growth, acidogenicity, adherence, and biofilm of *Streptococcus mutans* in vitro," *Folia Microbiologica*, vol. 58, no. 2, pp. 147–153, 2013.

[5] W. H. Bowen and H. Koo, "Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms," *Caries Research*, vol. 45, no. 1, pp. 69–86, 2011.

[6] R. Ma, M. Sun, S. Wang et al., "Effect of high-fructose corn syrup on the acidogenicity, adherence and biofilm formation of *Streptococcus mutans*," *Australian Dental Journal*, vol. 58, no. 2, pp. 213–218, 2013.

[7] E. G. Smith and G. A. Spatafora, "Gene regulation in *S. mutans*: complex control in a complex environment," *Journal of Dental Research*, vol. 91, no. 2, pp. 133–141, 2012.

[8] N. Takahashi and B. Nyvad, "Caries ecology revisited: microbial dynamics and the caries process," *Caries Research*, vol. 42, no. 6, pp. 409–418, 2008.

[9] J. M. Tanzer, J. Livingston, and A. M. Thompson, "The microbiology of primary dental caries in humans," *Journal of Dental Education*, vol. 65, no. 10, pp. 1028–1037, 2001.

[10] S. Hamada, T. Koga, and T. Ooshima, "Virulence factors of *Streptococcus mutans* and dental caries prevention," *Journal of Dental Research*, vol. 63, no. 3, pp. 407–411, 1984.

[11] J. A. Banas, "Virulence properties of *Streptococcus mutans*," *Frontiers in Bioscience*, vol. 9, no. 5, pp. 1267–1277, 2004.

[12] R. Matsui and D. Cvitkovitch, "Acid tolerance mechanisms utilized by *Streptococcus mutans*," *Future Microbiology*, vol. 5, no. 3, pp. 403–417, 2010.

[13] J. D. B. Featherstone, "Prevention and reversal of dental caries: role of low level fluoride," *Community Dentistry & Oral Epidemiology*, vol. 27, no. 1, pp. 31–40, 1999.

[14] J. M. B. ten Cate, "The need for antibacterial approaches to improve caries control," *Advances in Dental Research*, vol. 21, no. 1, pp. 8–12, 2009.

[15] V. S. Da Silva Pierro, R. Barcelos, L. C. Maia, and A. N. Da Silva, "Pediatricians' perception about the use of antibiotics and dental caries—a preliminary study," *Journal of Public Health Dentistry*, vol. 64, no. 4, pp. 244–248, 2004.

[16] N. B. Arweiler, N. Boehnke, A. Sculean, E. Hellwig, and T. M. Auschill, "Differences in efficacy of two commercial 0.2% chlorhexidine mouthrinse solutions: a 4-day plaque re-growth study," *Journal of Clinical Periodontology*, vol. 33, no. 5, pp. 334–339, 2006.

[17] A. A. Scheie and F. C. Petersen, "Antimicrobials in caries control," in *Dental Caries, the Disease and Its Clinical Management*, O. Fejerskov and E. Kidd, Eds., pp. 265–277, Blackwell Munksgaard, Oxford, UK, 2nd edition, 2008.

[18] Y. Koyama, S. Kuriyama, J. Aida et al., "Association between green tea consumption and tooth loss: cross-sectional results from the Ohsaki Cohort 2006 Study," *Preventive Medicine*, vol. 50, no. 4, pp. 173–179, 2010.

[19] X. Xu, X. D. Zhou, and C. D. Wu, "The tea catechin epigallocatechin gallate suppresses cariogenic vrrulence factors of *Streptococcus mutans*," *American Society for Microbiology*, vol. 55, no. 3, pp. 1229–1236, 2011.

[20] S. Duarte, S. Gregoire, A. P. Singh et al., "Inhibitory effects of cranberry polyphenols on formation and acidogenicity of

Streptococcus mutans biofilms," *FEMS Microbiology Letters*, vol. 257, no. 1, pp. 50–56, 2006.

[21] A. Watanabe, T. Kato, Y. Ito et al., "Aculeatin, a coumarin derived from *Toddalia asiatica* (L.) Lam., enhances differentiation and lipolysis of 3T3-L1 adipocytes," *Biochemical and Biophysical Research Communications*, vol. 453, no. 4, pp. 787–792, 2014.

[22] V. Duraipandiyan, M. Ayyanar, and S. Ignacimuthu, "Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India," *BMC Complementary and Alternative Medicine*, vol. 6, article 35, 2006.

[23] H. N. Kariuki, T. I. Kanui, A. Yenesew, N. Patel, and P. M. Mbugua, "Antinocieptive and anti-inflammatory effects of *Toddalia asiatica* (L) Lam. (Rutaceae) root extract in Swiss albino mice," *The Pan African Medical Journal*, vol. 14, no. 133, 2013.

[24] J. Hu, X. Shi, J. Chen et al., "Alkaloids from Toddalia asiatica and their cytotoxic, antimicrobial and antifungal activities," *Food Chemistry*, vol. 148, pp. 437–444, 2014.

[25] M. Karunai Raj, C. Balachandran, V. Duraipandiyan, P. Agastian, and S. Ignacimuthu, "Antimicrobial activity of Ulopterol isolated from Toddalia asiatica (L.) Lam.: a traditional medicinal plant," *Journal of Ethnopharmacology*, vol. 140, no. 1, pp. 161–165, 2012.

[26] V. Duraipandiyan and S. Ignacimuthu, "Antibacterial and antifungal activity of Flindersine isolated from the traditional medicinal plant, *Toddalia asiatica* (L.) Lam," *Journal of Ethnopharmacology*, vol. 123, no. 3, pp. 494–498, 2009.

[27] A. Y. Hu, J. Xie, J. Yiao, and Y. Wei, "Curative observation of applying LongZhang Gargle to 100 cases of ROU patients," *Journal of Guiyang College of Traditional Chinese Medicine*, vol. 24, no. 4, pp. 26–27, 2002.

[28] Y. J. Zhang, B. Z. Liu, F. Jing, and Y. Z. Peng, "Clinical study of Miao medicine gargle on preventing the fixed orthodontic gingivitis," *Journal of Guiyang College of Traditional Chinese Medicine*, vol. 36, no. 5, pp. 53–54, 2014 (Chinese).

[29] M. L. Tian, L. M. Chen, T. Y. Zeng, and H. B. Wu, "Clinical study on Longzhang Gargle for root canal disinfection," *Guizhou Medical Journal*, vol. 25, no. 9, pp. 828–829, 2001 (Chinese).

[30] T. B. Lombardo Bedran, J. Azelmat, D. Palomari Spolidorio, and D. Grenier, "Fibrinogen-induced *Streptococcus mutans* biofilm formation and adherence to endothelial cells," *BioMed Research International*, vol. 2013, Article ID 431465, 8 pages, 2013.

[31] M.-Y. Li, R.-J. Huang, X.-D. Zhou, and R. L. Gregory, "Role of sortase in Streptococcus mutans under the effect of nicotine," *International Journal of Oral Science*, vol. 5, no. 4, pp. 206–211, 2013.

[32] D. Assaf, D. Steinberg, and M. Shemesh, "Lactose triggers biofilm formation by *Streptococcus mutans*," *International Dairy Journal*, vol. 42, pp. 51–57, 2015.

[33] J. P. Bitoun, S. Liao, G. G. Xie, W. L. Beatty, and Z. T. Wen, "Deficiency of BrpB causes major defects in cell division, stress responses and biofilm formation by *Streptococcus mutans*," *Microbiology*, vol. 160, no. 1, pp. 67–78, 2014.

[34] M. A. Jongsma, H. C. van der Mei, J. Atema-Smit, H. J. Busscher, and Y. J. Ren, "In vivo biofilm formation on stainless steel bonded retainers during different oral health-care regimens," *International Journal of Oral Science*, vol. 7, no. 1, pp. 42–48, 2015.

[35] J. Xiao and H. Koo, "Structural organization and dynamics of exopolysaccharide matrix and microcolonies formation by *Streptococcus mutans* in biofilms," *Journal of Applied Microbiology*, vol. 108, no. 6, pp. 2103–2113, 2010.

[36] W. Qiu, X. Zheng, Y. Wei et al., "D-alanine metabolism is essential for growth and biofilm formation of *Streptococcus mutans*," *Molecular Oral Microbiology*, 2015.

[37] J. W.-H. Li and J. C. Vederas, "Drug discovery and natural products: end of an era or an endless frontier?" *Science*, vol. 325, no. 5937, pp. 161–165, 2009.

[38] W. Costerton, R. Veeh, M. Shirtliff, M. Pasmore, C. Post, and G. Ehrlich, "The application of biofilm science to the study and control of chronic bacterial infections," *The Journal of Clinical Investigation*, vol. 112, no. 10, pp. 1466–1477, 2003.

[39] N. Takahashi and B. Nyvad, "The role of bacteria in the caries process: ecological perspectives," *Journal of Dental Research*, vol. 90, no. 3, pp. 294–303, 2011.

[40] R. W. K. Wong, U. Hägg, L. Samaranayake, M. K. Z. Yuen, C. J. Seneviratne, and R. Kao, "Antimicrobial activity of Chinese medicine herbs against common bacteria in oral biofilm. A pilot study," *International Journal of Oral & Maxillofacial Surgery*, vol. 39, no. 6, pp. 599–605, 2010.

[41] C. H. Hu, J. He, R. Eckert et al., "Development and evaluation of a safe and effective sugar-free herbal lollipop that kills cavity-causing bacteria," *International Journal of Oral Science*, vol. 3, no. 1, pp. 13–20, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 1538703, 11 pages
http://dx.doi.org/10.1155/2016/1538703

Research Article

Biological Activities of Aerial Parts Extracts of *Euphorbia characias*

Maria Barbara Pisano,[1] Sofia Cosentino,[1] Silvia Viale,[1] Delia Spanò,[2] Angela Corona,[2] Francesca Esposito,[2] Enzo Tramontano,[2] Paola Montoro,[3] Carlo Ignazio Giovanni Tuberoso,[2] Rosaria Medda,[2] and Francesca Pintus[2]

[1]*Department of Public Health, Clinical and Molecular Medicine, University of Cagliari, Cittadella Universitaria, 09042 Monserrato, Italy*
[2]*Department of Sciences of Life and Environment, University of Cagliari, Cittadella Universitaria, 09042 Monserrato, Italy*
[3]*Department of Pharmacy, University of Salerno, 84084 Fisciano, Italy*

Correspondence should be addressed to Francesca Pintus; fpintus@unica.it

Received 22 January 2016; Revised 27 April 2016; Accepted 3 May 2016

Academic Editor: Ibrahim M. Banat

The aim of the present study was to evaluate antioxidant, antimicrobial, anti-HIV, and cholinesterase inhibitory activities of aqueous and alcoholic extracts from leaves, stems, and flowers of *Euphorbia characias*. The extracts showed a high antioxidant activity and were a good source of total polyphenols and flavonoids. Ethanolic extracts from leaves and flowers displayed the highest inhibitory activity against acetylcholinesterase and butyrylcholinesterase, showing potential properties against Alzheimer's disease. Antimicrobial assay showed that leaves and flowers extracts were active against all Gram-positive bacteria tested. The ethanolic leaves extract appeared to have the strongest antibacterial activity against *Bacillus cereus* with MIC value of 312.5 μg/mL followed by *Listeria monocytogenes* and *Staphylococcus aureus* that also exhibited good sensitivity with MIC values of 1250 μg/mL. Moreover, all the extracts possessed anti-HIV activity. The ethanolic flower extract was the most potent inhibitor of HIV-1 RT DNA polymerase RNA-dependent and Ribonuclease H with IC_{50} values of 0.26 and 0.33 μg/mL, respectively. The LC-DAD metabolic profile showed that ethanolic leaves extract contains high levels of quercetin derivatives. This study suggests that *Euphorbia characias* extracts represent a good source of natural bioactive compounds which could be useful for pharmaceutical application as well as in food system for the prevention of the growth of food-borne bacteria and to extend the shelf-life of processed foods.

1. Introduction

There has been a remarkable increment in scientific articles dealing with research of antioxidant molecules because of their protective action from the damage induced by oxidative stress. It causes serious cell and tissue damage leading it to be the major cause of the pathogenesis of several disease processes like cancer, diabetes, aging, and cardiovascular and neurodegenerative diseases. Plant materials represent a great source of antioxidant and bioactive compounds which are different in their composition and physical and chemical properties [1]. Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants. The identification and development of phenolic compounds or extracts from different plants has become a major area of health- and medical-related research. Among these compounds, flavonoids have been especially highlighted because they have been shown to have protective roles against many human diseases, due to their antioxidant capacity, which depends mainly on the number and position of hydroxyl groups within their structure, and their anti-inflammatory, anticancer, and antiviral activities [2]. Their activity as inhibitors of cholinesterase has also been demonstrated and correlated with their structure and could be useful for treatment of Alzheimer's disease (AD) [3]. AD results from a deficit of cholinergic functions in the brain. Hence, one of the most promising approaches for treating this disease is to restore the acetylcholine level

by inhibiting cholinesterase activity. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are two cholinesterase enzymes that metabolize acetylcholine but differ in substrate specificity, enzyme kinetics, and activity in different brain regions. In a healthy brain, AChE is the most responsible enzyme in regulating acetylcholine levels but, in patients with AD, AChE activity gradually decreases with the concomitant increase of BChE. Thus, both AChE and BChE are legitimate therapeutic targets for treatment of cholinergic deficit characteristic of AD. Effective therapeutic options for AD are limited up to now; thus there is a demand for new natural drugs without side effects.

Moreover, the demand for bioactive compounds from natural sources as an alternative to synthetic molecules is continuously increasing also because of the emerging problem of the multidrug resistance of microorganisms related to antibiotics and their extensive use. Thus, several studies related to plant antimicrobials have demonstrated their efficacy towards a large number of pathogens and food-borne agents causing disease [4–6] as well as viral infections [7–10]. Although many plant-derived compounds are currently being used for the treatment of infectious diseases and for the preservation and extension of the shelf-life of foods, several medicinal and aromatic plants present worldwide remain still unexplored.

Euphorbiaceae is a large flowering plant family (300 genera and 8,000 species) widely distributed all around the world and composed of all sorts of plants (large woody trees, climbing lianas, or simple weeds) with a wide variety of chemical substances, many of them with a medicinal application. Some extracts from Euphorbiaceae plants have been characterized and patented as modern drugs [11]. Among Euphorbiaceae, the species *Euphorbia characias*, a nonsucculent shrub commonly occurring in vast areas of the Mediterranean region, has been analyzed and several biological active compounds were identified [12]. The plant latex has been the object of several researches and its screening has revealed the presence of natural rubber and numerous enzymes some of which interact in a common metabolism [13–22]. On the other hand, only little attention has been paid to the other parts of the plant [23–25].

Thus, the objective of this research was to evaluate antioxidant, antimicrobial, and anticholinesterase properties of the aqueous and ethanolic extracts of leaves, stems, and flowers from *E. characias* in order to find a novel potential source of bioactive molecules. Moreover, the antiviral efficacy of *E. characias* extracts was also evaluated on the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase-(RT-) associated RNA-dependent DNA polymerase (RDDP) and Ribonuclease H (RNase H) activities. Both RT-associated functions are essential for viral replication and are validated drug targets for which new drugs are still needed [26, 27].

2. Materials and Methods

All chemicals were obtained as pure commercial products and used without further purification. Acetylcholinesterase (AChE) from *Electrophorus electricus*, acetylthiocholine iodide, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), aluminum nitrate, butyrylcholinesterase (BChE) from *equine serum*, *S*-butyrylthiocholine chloride, 3-*O*-caffeoylquinic acid (chlorogenic acid), catechin, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), ellagic acid, Folin-Ciocalteu phenol reagent, ferric chloride, galantamine hydrobromide, gallic acid, 4-hydroxybenzyl alcohol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), myricetin, 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), and quercetin were purchased from Sigma Aldrich (Milan, Italy). LC-MS grade acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). Standards of myricetin-3-*O*-glucoside, quercetin-3-*O*-glucoside, and acacetin were purchased from Extrasynthese (Genay, France). HPLC grade water (18 MΩ·cm) was prepared by using a Millipore (Bedford, MA, USA) Milli-Q purification system.

Spectrophotometric determinations were obtained with an Ultrospec 2100 spectrophotometer (Biochrom Ltd., Cambridge, England) using cells with a 1 cm path length.

Figure 1: The Mediterranean shrub *Euphorbia characias* subsp. *characias*.

2.1. Plant Material. *E. characias* subsp. *characias* (Figure 1) was identified by Professor Annalena Cogoni and a voucher specimen has been deposited in the Department of Sciences of Life and Environment, University of Cagliari, Italy (number 1216/16 Herbarium CAG). The different parts of *E. characias* were collected from February to June, in southern Sardinia (Dolianova, CA, Italy). The GPS coordinates were 39°24′19.0″N and 9°12′57.6″E.

Leaves, stems, and flowers were immediately frozen at −80°C and then lyophilized in intact condition. The lyophilized plant materials (1 g) were reduced in powder and 10 mL of water (aqueous extract) or ethanol (ethanol extract) was added to the dried samples. The extraction was carried out in the dark at room temperature for 24 h under continuous stirring. Ethanol extracts were diluted 10-fold with water in order to freeze and then lyophilize the samples [24]. Before use, 1 mg of dried powders was dissolved in water or 10% ethanol (1 mL), for aqueous and ethanol extracts, respectively. For antimicrobial and antiviral activity dried

powders were dissolved in DMSO (100%) as solvent. The yield (%, w/w) from all the dried extracts was calculated as follows: yield (%) = $(A1 \times 100)/A2$, where $A1$ is the weight of the dried extract (after lyophilization) and $A2$ is the weight of the plant powder.

2.2. Antioxidant Assays. In every extract total free radical scavenging molecules were determined by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ methods using Trolox as antioxidant standard, as previously reported [28, 29]. For both free radical methods, antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC; mmol/g dw). The FRAP (ferric reducing antioxidant power) assay was performed as previously described [30]. Quantitative analysis was performed according to the external standard method ($FeSO_4$, 0.1–2 mmol/L, $r = 0.9997$) and results were expressed as mmol Fe^{2+}/g dw.

2.3. Determination of the Total Polyphenols and Flavonoids. Total content of polyphenols and flavonoids in the extracts was determined as previously reported [29]. Polyphenol concentration was calculated using gallic acid as a referred standard and was expressed as mg of gallic acid (GAE) per 1 g of dry weight (dw). Flavonoid concentration was expressed as mg of quercetin equivalent (QE) per 1 g of dry extract.

2.4. Acetylcholinesterase and Butyrylcholinesterase Activity. Acetylcholinesterase from *Electrophorus electricus* and *equine serum* butyrylcholinesterase were used for the inhibitory assays. AChE activity was measured using Ellman's reagent according to the method previously reported [31]. Briefly, the reaction mixture contained 0.1 M phosphate buffer (pH 8.0), 1.5 mM 5,5′-dithiobis-2-nitrobenzoate (DTNB), acetylthiocholine iodide (1.5 mM), and extract at the desired concentrations or solvent alone (control) in a final volume of 1 mL. Finally, enzyme was added to the reaction mixture and the absorbance immediately monitored at 405 nm. For butyrylcholinesterase assay, the same procedure was followed except for the use of enzyme and substrate, which were BChE and *S*-butyrylthiocholine, respectively. Galantamine was used as the standard cholinesterase inhibitor. Results were expressed as IC_{50} values calculated as concentration of extracts that produces 50% cholinesterase activity inhibition.

2.5. Microbial Strains, Culture Conditions, and Antimicrobial Activity. *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 11178, *Listeria monocytogenes* ATCC 19115, *Escherichia coli* ATCC 35150 (serotype O157:H7), *Salmonella typhimurium* ATCC 14028, *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 2601, *Aspergillus flavus* ATCC 46283 (aflatoxin producer), and *Penicillium chrysogenum* ATCC 10135 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and used as indicators strains. All bacterial strains were stored on nutrient broth (NB, Microbiol, Cagliari, Italy) plus 20% (v/v) glycerol at −20°C except yeasts and molds strains which were maintained in potato dextrose broth (Microbiol) with 15% (v/v) glycerol. Before use, they were subcultured twice in appropriate medium.

Minimum inhibitory concentrations (MICs) and minimum bactericidal/fungicidal concentrations (MBCs/MFCs) of the *E. characias* extracts were determined by a broth microdilution method [5]. All tests were performed with NB for bacteria and RPMI 1640 (Sigma, Milan, Italy) buffered to pH 7.0 with morpholinepropanesulfonic acid (MOPS, Sigma) for yeasts and molds. The extracts were dissolved in DMSO (5% v/v). Serial doubling dilutions of each extract were performed in a 96-well microtiter plate ranging from 19.5 to 5000 μg/mL. Overnight broth cultures were prepared in NB or RPMI and adjusted so that the final concentration in each well following inoculation was approximately 5.0×10^5 cfu/mL. The concentration of each inoculum was confirmed using viable counts on Tryptic Soy Agar (TSA, Microbiol) plates for bacteria, Sabouraud Dextrose Agar (SDA, Microbiol) for yeasts, and potato dextrose agar (PDA, Microbiol) for molds. The controls included sterility of NB and RPMI broths, sterility of the extracts, control culture (inoculum), and control DMSO to check the effect of solvent on the growth of microorganisms. Furthermore, gentamicin, ketoconazole, and amphotericin B were used as positive controls for bacteria, yeasts, and molds, respectively.

The MICs and MBCs were determined after 24 h incubation of the plates at 37°C for bacteria and 30°C for fungi. Microbial growth was indicated by the presence of turbidity and a "pellet" on the well bottom. MICs were determined presumptively as the first well, in ascending order, which did not produce a pellet. To confirm MICs and to establish MBCs, 10 μL of broth was removed from each well and inoculated on TSA, SDA, or PDA plates. After incubation under the conditions described above, the number of surviving microorganisms was determined. The MIC was the lowest concentration which resulted in a significant decrease in inoculum viability (>90%) while the MBC/MFC was the concentration where 99.9% or more of the initial inoculum was killed.

All tests were conducted in triplicate and with three replications, and the modal MIC and MFC values were selected.

2.6. HIV-1 RT-Associated Functions Biochemical Assays. HIV-1 RT gene subcloned into the p6HRT_prot plasmid was kindly provided by Stuart Le Grice (National Cancer Institute, Frederick, USA). Protein expression and purification was performed in *E. coli* M15 strain as described [32]. The HIV-1 RT-associated RDDP and RNase H activity were measured as previously described [33, 34].

2.7. LC Detection and Quantitative Analysis of Phenolic Compounds

2.7.1. (HR) LC-ESI-Orbitrap-MS and (HR) LC-ESI-Orbitrap-MS/MS. The electrospray ionisation (ESI) source of a Thermo Scientific LTQ-Orbitrap XL (Thermo Scientific, Germany) mass spectrometer was tuned in negative ion mode with a standard solution of kaempferol-3-*O*-glucoside

Table 1: Yield and antioxidant and antiradical properties of *E. characias* extracts. DPPH and ABTS values are expressed as mmol TEAC/g dw; FRAP value is expressed as mmol Fe^{2+}/g dw.

Extracts		Yield (w/w%)	ABTS	DPPH	FRAP
Leaves	Aqueous	20.4 ± 1.5	2.52 ± 0.18^{b}	1.8 ± 0.21^{b}	2.70 ± 0.17^{c}
	Ethanolic	24.9 ± 2.6	4.68 ± 0.49^{a}	6.73 ± 0.70^{a}	4.57 ± 0.12^{a}
Stems	Aqueous	12.3 ± 1.4	0.10 ± 0.01^{d}	0.17 ± 0.02^{c}	0.89 ± 0.04^{f}
	Ethanolic	15.2 ± 1.8	0.85 ± 0.10^{c}	0.88 ± 0.09^{c}	1.36 ± 0.07^{e}
Flowers	Aqueous	17.6 ± 2.3	0.62 ± 0.05^{cd}	0.53 ± 0.05^{c}	1.95 ± 0.02^{d}
	Ethanolic	20.6 ± 1.9	0.91 ± 0.10^{c}	0.58 ± 0.05^{c}	3.49 ± 0.09^{b}

Results are expressed as mean ± standard deviation of three independent experiments. Means followed by distinct letters in the same column are significantly different ($p < 0.05$).

(1 μg/mL) infused at a flow rate of 5 μL/min with a syringe pump. In the FT experiment, resolution of the Orbitrap mass analyzer was set at 30000. The mass spectrometric spectra were acquired by full range acquisition covering m/z 120–1200 in LC-MS. The data recorded were processed with Xcalibur 2.0 software (Thermo Fisher Scientific). Initial calibration of the instrument was performed using the standard LTQ calibration mixture with caffeine and the peptide MRFA, dissolved in 50 : 50 (v/v) water/acetonitrile solution. LC/ESI/LIT-Orbitrap-MS was performed using a Finnigan Surveyor HPLC (Thermo Finnigan, San Jose, CA, USA) equipped with a Waters (Milford, MA, USA) Xselect CSH C18 3.5 μm column (150 mm × 2.1 mm i.d.) and coupled to a hybrid Linear Ion Trap- (LIT-) Orbitrap mass spectrometer (Thermo Scientific). Linear gradient elution with a mobile phase comprising water acidified with 0.1% formic acid (solvent A) and acetonitrile acidified with 0.1% formic acid (solvent B) starting from 95% A was converted in 65% A in 45 min, from 65% to 0% (A) in 1 min, remaining 0% A for 4 minutes, and then from 0% to 95% (A) followed by 10 min of maintenance. The mobile phase was supplied at a flow rate of 200 μL/min keeping the column at room temperature, and the effluent was injected directly into the ESI source. The mass spectrometer was operated in negative ion mode. ESI source parameters were as follows: capillary voltage −12 V; tube lens voltage −121.47 V; capillary temperature 280°C; sheath and auxiliary gas flow (N_2) 30 and 5; sweep gas 0; and spray voltage 5 V. MS spectra were acquired by full range acquisition covering m/z 120–1600. LC-ESI-LIT-MS/MS data were obtained by applying a data dependent scan experiment, by directing to fragmentation the two highest peaks obtained in LC-ESI-Orbitrap-MS trace. Each parent ion was submitted to fragmentation with energy of 30% to produce an MS/MS spectrum in the MS range specific relative to its mass. 1 mg of dried extract obtained from ethanolic extract from *E. characias* leaves was dissolved in 10 mL of a mixture of water in acetonitrile and 10 μL was injected in the LC-MS system.

2.7.2. LC-DAD. Detection and quantitative analysis of phenolic compounds were carried out using an HPLC-DAD method [30]. Chromatograms and spectra were elaborated with a ChromQuest V. 2.51 data system (ThermoQuest, Rodano, Milan, Italy). Flavonols were detected and quantified at 360 nm and all the other compounds at 280 nm. Stock solutions were prepared at 1 mg/mL dissolving pure standards in methanol: water (50 : 50, v/v). The calibration curves for each compound were calculated by regression analysis, by plotting the peak area obtained after standards injection (3 replicates at each concentration) against the known standard concentrations. The stock solutions were diluted with methanol in order to obtain work solutions and the correlation values were 0.9992–0.9998. *E. characias* leaves extract was dissolved and injected in the LC-DAD system with the same condition of the LC-MS analysis.

2.8. Statistical Analysis. Data are reported as mean ± standard deviation of three independent experiments. The data were analyzed using one-way analysis of variance (one-way ANOVA) and Tukey's posttest. Statistical analysis was performed with GraphPad Prism 7 software (GraphPad Software, San Diego, California, USA). A difference was considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Antioxidant Activity, Polyphenol and Flavonoids Content, and Cholinesterase Activity Inhibition. Total free radical scavenging capacities determined with ABTS and DPPH assays are reported in Table 1. Comparable TEAC values of each extract were detected using the two methods. Leaves extracts exhibited significantly higher free radical scavenging activity ($p < 0.05$) than other extracts, with ethanolic extract showing the highest activity. Antioxidant activity was also examined with FRAP assay, confirming that ethanolic extract of leaves possessed the significantly highest antioxidant activity ($p < 0.05$), followed by flower ethanolic extract.

The polyphenol and flavonoid content of the extracts, expressed as mg of gallic acid or quercetin equivalent, respectively, are reported in Figure 2. Total phenolic content varied widely among analyzed parts of the plant and the highest value was found in leaves in both aqueous and ethanolic extracts. In fact, the amount of phenolic compounds in leaves aqueous extract (680 mg GAE/g dw) was about 6- and 2.3-fold higher than the same extract from stems (102.1 mg GAE/g dw) and flowers (289.2 mg GAE/g dw), respectively (Figure 2(a)). Moreover, these results showed that ethanolic extract of leaves is rich in polyphenols content (815.7 mg GAE/g dw) with a value about 1.5-fold

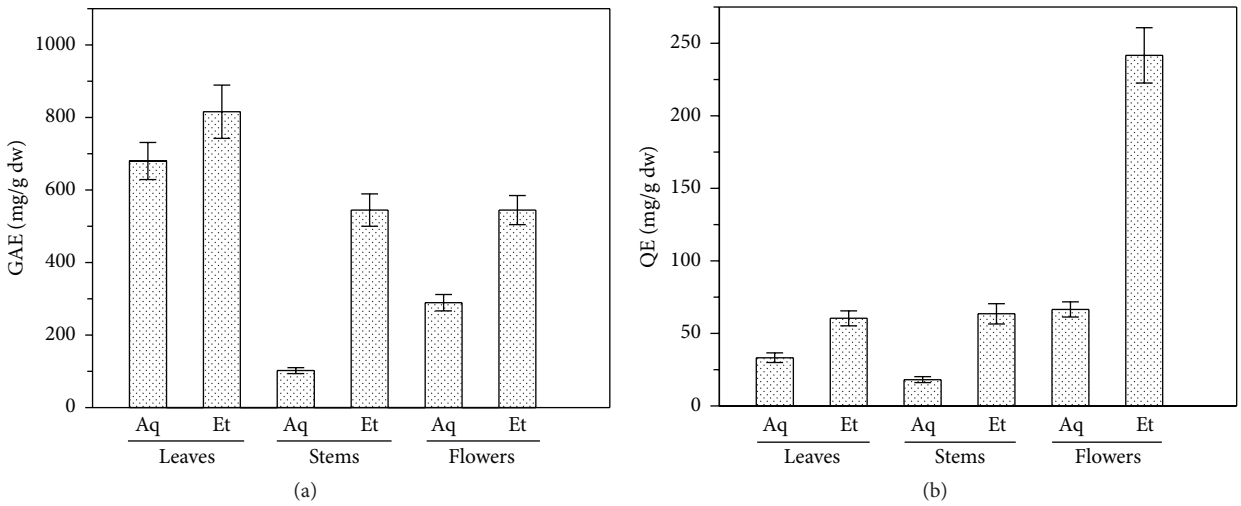


Figure 2: Polyphenol and flavonoid content in aqueous (Aq) and ethanolic (Et) leaves, stems, and flowers extracts from *E. characias*. (a) Polyphenol amount is expressed as mg of gallic acid equivalent (GAE) per g of dry weight (dw); (b) the amount of flavonoids is expressed as mg of quercetin equivalent (QE) per g of dry weight (dw). All data are expressed as mean of three measurements ± standard deviation.

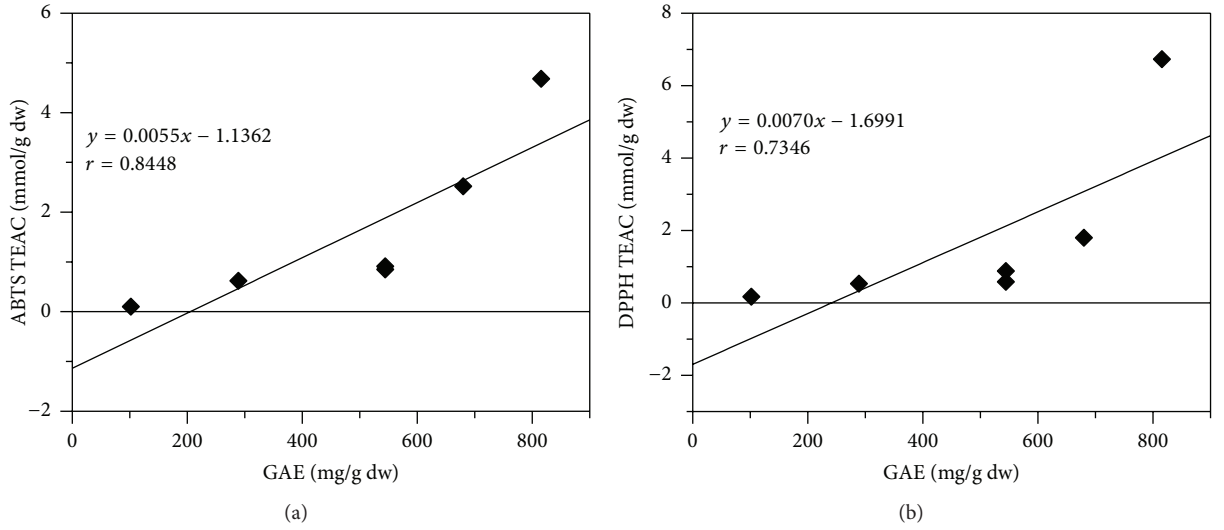


Figure 3: Correlation between phenolic content and antioxidant capacity of *E. characias* extracts. (a) ABTS assay; (b) DPPH assay. TEAC: Trolox equivalent antioxidant capacity; GAE: gallic acid equivalents.

higher than the same extract from stems and flowers (about 544.6 mg GAE/g dw for each extract). Polyphenols have been reported to have antioxidant activity mainly based on their redox properties which have a key role in scavenging free radicals and chelating oxidant metal ions. High phenolic content in all *E. characias* extracts resulted in high TEAC values determined by ABTS or DPPH methods, which could indicate that phenolic compounds were capable of functioning as free radical scavengers. Good correlation was found between TEAC values for both ABTS and DPPH ($r = 0.8448$ and $r = 0.7346$, resp.) of the different plant extracts and their phenolic contents (Figure 3), confirming a strong relationship between antioxidant capacity and phenolic content.

Among phenolic compounds, flavonoids are ubiquitous plant compounds with an important role as attractants to pollinators, as sunscreens to protect against UV irradiation, and as antimicrobial and antiherbivory factors [37]. Content of flavonoids is shown in Figure 2(b). Flavonoid content of flowers ethanolic extract was about 2-fold higher (241.7 mg QE/g dw) than the correspondent extract from the other part of the plant (~62 mg QE/g dw). This extract also showed the highest ratio of total flavonoids (TFC) and phenolic content (TPC) being 0.44, indicating that flavonoids were almost 40% of the total phenolic content. However, aqueous extracts of both leaves and stems exhibited the lowest amounts of total flavonoids (33.2 mg and 18.1 mg QE/g dw, resp.), with aqueous extract from leaves showing the lowest TFC/TPC ratio of 0.05.

Compounds like flavonoids, alkaloids, terpenoids, and coumarins are known to have the capacity to inhibit

Table 2: Inhibition of AChE and BChE by *E. characias* extracts.

Extracts		IC_{50} values (mg/mL)	
		AChE	BChE
Leaves	Aqueous	4.2 ± 0.25^{c}	—
	Ethanolic	0.6 ± 0.056^{d}	0.39 ± 0.04^{c}
Stems	Aqueous	6.9 ± 0.71^{a}	—
	Ethanolic	5.8 ± 0.43^{b}	—
Flowers	Aqueous	5.25 ± 0.35^{b}	4.2 ± 0.39^{a}
	Ethanolic	0.6 ± 0.045^{d}	1.22 ± 0.08^{b}
Galantamine		$0.27 \pm 0.07\ \mu$g/mL	$8.12 \pm 0.61\ \mu$g/mL

Results are expressed as mean ± standard deviation of three independent experiments. Means followed by distinct letters in the same column are significantly different ($p < 0.05$).

cholinesterase types, key enzymes in the cholinergic nervous system [38]. This inhibitory activity is usually the first of a number of requirements for the development of medicines for treating some neurological disorders such as Alzheimer's disease.

Table 2 shows the acetylcholinesterase and butyrylcholinesterase inhibitory activities of the *E. characias* extracts, compared with those of standard inhibitor galantamine. A survey on IC_{50} values revealed that all the extracts exhibited acetylcholinesterase inhibitory activity while only few of them inhibited butyrylcholinesterase. This is not surprising because AChE and BChE enzymes display distinct substrate and inhibitor specificities. Most of the other IC_{50} values were about three orders of magnitude higher compared to the results obtained with galantamine. However, ethanolic leaves extract showed the significantly highest ($p < 0.05$) butyrylcholinesterase inhibition that is about 50 times less than the effect of galantamine. This is a good result since the standard inhibitor is a single molecule, whereas plant extracts are a mixture of numerous compounds and might contain only few active components.

3.2. Antimicrobial and Anti-HIV Activities. Tables 3(a) and 3(b) report the antagonistic activity of *E. characias* extracts against a panel of microorganisms including Gram-negative, Gram-positive, and spore-forming bacteria, yeast, and mold species. As can be observed, the leaves extracts appeared to have the strongest antibacterial activity followed by flowers and stems extracts. Gram-negative bacteria, yeasts, and molds were the least sensitive, being resistant to all extracts at the maximum concentration tested (5000 μg/mL). Leaves extracts exhibited antibacterial activity towards all Gram-positive bacteria tested. *B. cereus* ATCC 11778 was the most susceptible strain being totally inhibited by the ethanolic leaves extract at the concentration of 312.5 μg/mL (MIC equivalent to MBC). *L. monocytogenes* ATCC 19115 and *S. aureus* ATCC 6538 also exhibited a good sensitivity to the ethanolic leaves extract as they both showed MIC values of 1250 μg/mL. Higher MIC values were observed for the aqueous leaves extracts towards these bacterial strains. As regards the flowers extracts, all Gram-positive strains tested were moderately inhibited by both aqueous and ethanolic extracts with MIC values equal to or greater than 2500 μg/mL with the exception of *B. cereus* strain which was more sensitive to the ethanolic extract (MIC 1250 μg/mL). Stems extracts showed the lowest activity, with ethanolic extract slightly inhibiting the abovementioned bacterial strains (MIC 5000 μg/mL). Our findings are in agreement with those reported by Lin et al. for *Euphorbia macrorrhiza* species [39], who observed inhibitory activity against *S. aureus* but no effect towards *E. coli* and *C. albicans* strains tested. In contrast, Perumal et al. [40] observed antimicrobial effect for *Euphorbia hirta* ethanolic extracts towards both Gram-positive and Gram-negative bacteria with MIC values lower than those observed in this work. On the other hand, the antagonistic effect of plants extracts even within the same species is variable and depends on several factors such as the concentration of active components due to different tissue composition, variation in the extraction protocol and technique used to detect antimicrobial activity, and resistance of the test microorganisms [41–43].

Finally, *E. characias* extracts were also tested for their ability to inhibit the HIV-1 RT-associated RDDP and RNase H functions using the known nonnucleoside RT inhibitor efavirenz [27] and the diketo acid derivative RDS1759 [33] as control for RDDP and RNase H inhibition, respectively. Interestingly, all extracts were able to inhibit both RT functions (Table 4). In all cases, ethanolic extracts were more active than aqueous extracts and the flower extracts were the most potent on both enzyme activities.

3.3. LC-ESI-Orbitrap-MS, LC-ESI-Orbitrap-MS/MS, and LC-DAD Analysis of E. characias Ethanolic Leaves Extract. Considering all the antioxidants and biological activities of the extracts, ethanolic extract from leaves appeared to be the most active extract. Thus, this extract was selected for further study by liquid chromatography (LC). It was analyzed by an analytical method developed in LC-ESI-Orbitrap-MS and LC-ESI-Orbitrap-MS/MS, in negative ion mode. The negative LC-MS profile highlighted the presence of a large group of compounds corresponding to the deprotonated molecular ions of different flavonoids and ellagitannin derivatives (Figure 4). Individual components were identified by comparison of their m/z values in the Total Ion Current (TIC) profile with those of the selected compounds described in literature (Table 5). Additional LC-ESI-Orbitrap-MS/MS experiments were carried out in order to select and submit these ions to fragmentation experiments using the parameters previously chosen by ESI-MS and ESI-MS/MS direct infusion experiments. By matching experimental MS/MS spectra with those reported in literature and/or with those reported in a public repository of mass spectral data called MassBank [35], compounds **1**–**16** were identified, with the exception of compounds **3**, **5**, and **8** (unknown compounds).

Table 5 reports identification of compounds, based on high resolution mass spectrometric data, chemical formula derived by accurate mass maturation, retention times, MS/MS results, and references used for identification. Compounds **1** and **2** were identified by the diagnostic $[M - H]^{-}$ ion shown in HR ESI-MS analysis, compared with

Table 3: Antimicrobial activity of *E. characias* aqueous (a) and ethanol (b) extracts. MIC and MBC/MFC values are expressed in μg/mL.

(a)

Target microorganisms	Leaves		Stems		Flowers	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
E. coli	>5000	—	>5000	—	>5000	—
S. typhimurium	>5000	—	>5000	—	>5000	—
S. aureus	5000	>5000	>5000	—	5000	>5000
B. cereus	1250	1250	5000	>5000	2500	5000
L. monocytogenes	2500	2500	>5000	—	2500	>5000
C. albicans	>5000	—	>5000	—	>5000	—
S. cerevisiae	>5000	—	>5000	—	>5000	—
A. flavus	>5000	>5000	>5000	—	>5000	>5000
P. chrysogenum	>5000	>5000	>5000	—	>5000	>5000

(b)

Target microorganisms	Leaves		Stems		Flowers	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
E. coli	>5000	—	>5000	—	>5000	—
S. typhimurium	>5000	—	>5000	—	>5000	—
S. aureus	1250	2500	5000	>5000	5000	>5000
B. cereus	312.5	312.5	5000	>5000	1250	2500
L. monocytogenes	1250	1250	5000	>5000	5000	>5000
C. albicans	>5000	—	>5000	—	>5000	—
S. cerevisiae	>5000	—	>5000	—	>5000	—
A. flavus	>5000	—	>5000	—	>5000	>5000
P. chrysogenum	>5000	—	>5000	—	>5000	>5000

Positive controls: ampicillin MICs (*S. aureus* ATCC 6538: 2.5 μg/mL, *B. cereus* ATCC 11778: 10 μg/mL, and *L. monocytogenes* ATCC 19115: 2.5 μg/mL); gentamicin MICs (*E. coli* ATCC 35150: 10 μg/mL, *S. typhimurium* ATCC 14028: 10 μg/mL); ketoconazole MICs (*C. albicans* ATCC 10231: 2.5 μg/mL, *S. cerevisiae* ATCC 2601: 2.5 μg/mL); amphotericin B MICs (*A. flavus* ATCC 46283 and *P. chrysogenum* ATCC 10135: 5 μg/mL).

Table 4: Effects of *E. characias* extracts on HIV-1 RT-associated functions.

Extracts		IC_{50} (μg/mL)*	
		HIV-1 RDDP	HIV-1 RNase H
Leaves	Aqueous	0.785 ± 0.003^{c}	$1.95 \pm 1.03^{a,b}$
	Ethanolic	0.75 ± 0.028^{c}	$0.685 \pm 0.155^{b,c}$
Stems	Aqueous	6.87 ± 0.81^{a}	2.235 ± 0.245^{a}
	Ethanolic	3.05 ± 0.2^{b}	$1.615 \pm 0.035^{a,b,c}$
Flowers	Aqueous	1.03 ± 0.0^{c}	$1.51 \pm 0.53^{a,b,c}$
	Ethanolic	0.26 ± 0.08^{c}	0.33 ± 0.1^{c}
Efavirenz		0.0016 ± 0.0003**	ND***
RDS1759		ND	7.1 ± 0.5**

*Extracts concentration required to inhibit HIV-1 RT-associated functions by 50%.
**Values expressed in μM concentration.
***Not done.
Means followed by distinct letters in the same column are significantly different ($p < 0.05$).

standards and literature, and from the MS/MS data obtained working in LC-ESI-Orbitrap-MS/MS in Product Ion Scan in negative ion mode, and the compounds were identified by MassBank as gallic acid and catechin and confirmed by standard analysis. Compounds **7**, **9**, **10**, **11**, and **15** were identified by the diagnostic $[M - H]^-$ ions shown in HR ESI-MS analysis, their fragmentation profiles obtained in LC-ESI-(Orbitrap)-MS/MS in Product Ion Scan in negative ion mode, compared with literature data, and resulting compounds previously reported in *E. characias* leaves [25]. They are derivatives of quercetin. Compounds **4** and **6** were tentatively identified by the diagnostic $[M - H]^-$ ions shown in HR ESI-MS analysis, their fragmentation profiles obtained in LC-ESI-Orbitrap-MS/MS in Product Ion Scan in negative ion mode, compared with MassBank data. They were proposed as myricetin derivatives. The identity of compound **12** was hypothesized from the MSMS data obtained working in LC-ESI-Orbitrap-MS/MS in Product Ion Scan in negative ion mode, and the compound was tentatively identified by MassBank, as dicaffeoylquinic acid. Compounds **13** and **14** were tentatively identified by the diagnostic $[M - H]^-$ ion shown in HR ESI-MS analysis and from the MS/MS data obtained working in LC-ESI-Orbitrap-MS/MS in Product Ion Scan in negative ion mode, compared with literature. These compounds were not described in *E. characias*, but in another plant of the same genus, *Euphorbia pekinensis* [36].

From a quantitative point of view, the most significant polyphenolic compounds were flavonoids, mainly quercetin

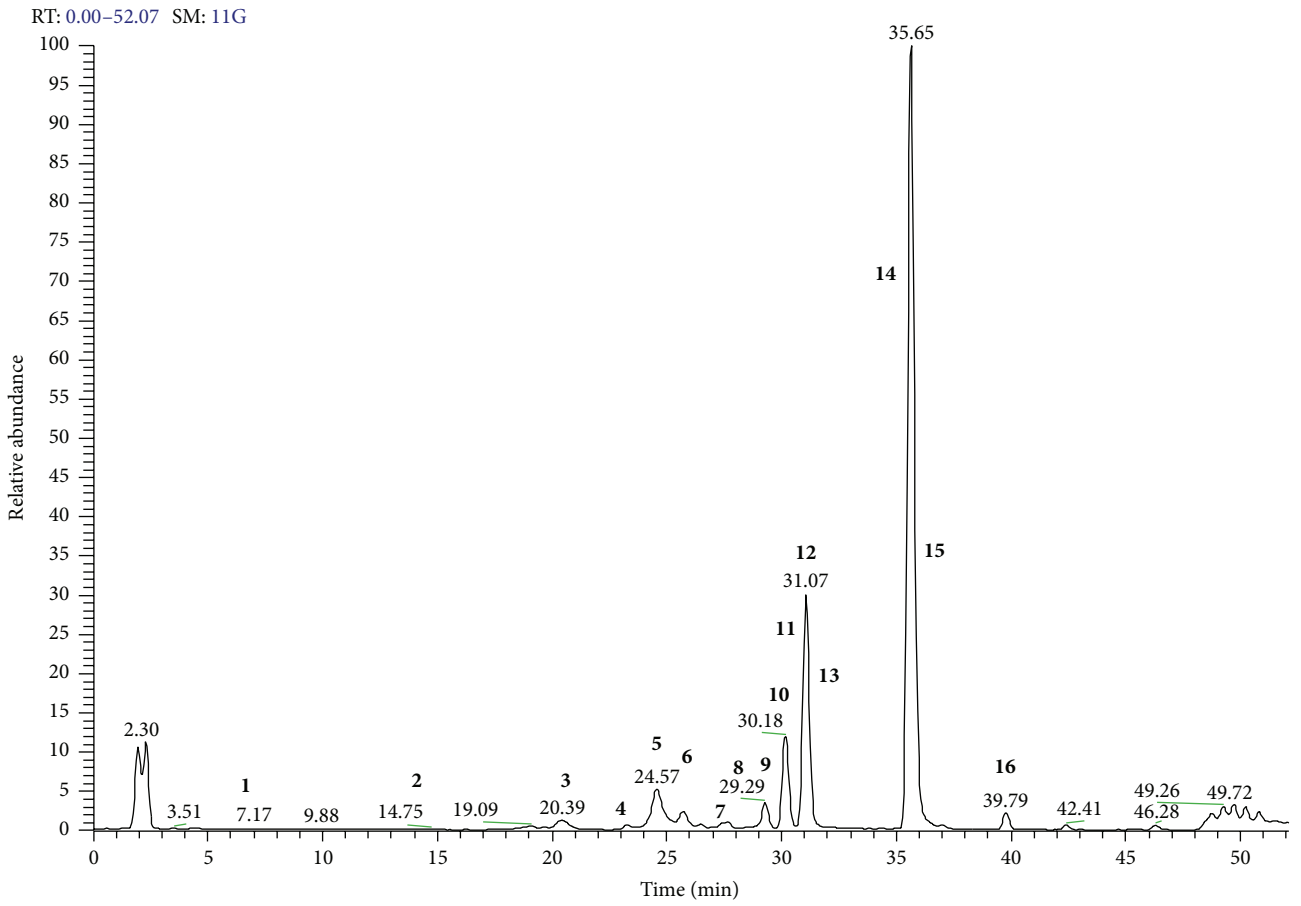


Figure 4: Identification of polyphenolic compounds in *E. characias* leaves using LC-ESI-Orbitrap-MS/MS in negative ion mode. Chromatographic conditions are described in the text. A list of compounds is reported in Table 5.

derivatives (Table 5). Quercetin-3-(2-*O*-acetyl)-arabinoside (**15**) and quercetin-3-*O*-rhamnoside (**11**) were the most abundant compounds (39.99 ± 2.52 and 31.88 ± 2.75 g/L, resp.), followed by quercetin-3-*O*-arabinoside (**10**) and quercetin-3-*O*-xyloside (**9**). These findings are in agreement with the results of previous investigations on the aerial part of *E. characias* [25]. For the first time other compounds such as gallic acid (**1**), catechin (**2**), myricetin derivatives, and ellagic acid derivatives were quantified in *E. characias* leaves extracts. Some of these compounds such as gallic acid, catechin, and quercetin-3-*O*-rhamnoside were isolated from other plant sources and displayed cholinesterase inhibitory activity [44–46]. Several compounds are derivatives of myricetin (**4** and **6**) and quercetin (**7**, **9**–**11,** and **15**), two flavonoids which showed anti-HIV-1 activity [8, 47]. Finally, previous studies indicated that quercetin derivatives possess antimicrobial properties and the mechanisms of these compounds were attributed to their ability to form complexes with extracellular and soluble proteins and bacterial cell walls [48]. This might explain the lack of activity or the minor susceptibility of the Gram-negative bacteria and the greater inhibition of *E. characias* extract against Gram-positive bacteria. In fact, Gram-negative bacteria possess an effective permeability barrier represented by the outer lipidic membrane which could restrict the penetration of plant extracts. The next step will be the isolation of pure compounds of the extract and the evaluation and characterization of their biological activities in order to find new bioactive compounds from a natural source.

4. Conclusions

The results of this study indicate that *E. characias* aerial parts are a good source of antioxidant, antimicrobial, antiviral, and anti-ChE compounds. Leaves extracts exhibited the strongest antioxidant activities and the highest amount of polyphenols and cholinesterase inhibitors molecules, while flowers extracts seemed to be the best sources of flavonoids and exerted the best antiviral activity. Extracts also exhibited acetyl- and butyrylcholinesterase inhibition and these are promising results since ChE inhibitors represent the standard therapeutic approach to the treatment of Alzheimer's disease.

Analyzing all results, extraction with ethanol was more efficient than use of plain water. In fact ethanol extracts were found to provide the highest antioxidant and anti-ChE activity and phenolic and flavonoids contents compared with the correspondent aqueous extract. The same result was found for antimicrobial assays since ethanol extracts were found to be more effective towards the food-borne pathogens

Table 5: Identification of polyphenolic compounds in *E. characias* leaves ethanolic extract using LC-ESI-Orbitrap-MS/MS in negative ion mode and quantification by LC-DAD.

	Putative identification	RT (min)	g/L (mean ± SD)	MW	$[M-H]^-$	Molecular formula	MS/MS	References
1	Gallic acid[a]	7.17	0.94 ± 0.23	170.0215	169.1195	$C_7H_6O_5$	125.02	[35]
2	Catechin[a]	14.75	0.65 ± 0.04	290.2680	289.0715	$C_{15}H_{13}O_6$	245.05 205.04 125.02	[35]
3	Unknown	20.39	NQ		951.0734		932.70	—
4	Myricetin-hexose[b]	23.16	0.02 ± 0.00	480.0904	479.0824	$C_{21}H_{19}O_{13}$	317.06	[35]
5	Unknown	24.56	NQ		960.789		913.01	—
6	Myricetin-deoxyhexose[b]	26.49	0.01 ± 0.00	464.0954	463.0873	$C_{21}H_{19}O_{12}$	317.06	[35]
7	Quercetin-3-*O*-glucoside[a]	27.52	1.94 ± 0.09	464.0954	463.0873	$C_{21}H_{19}O_{12}$	301.07	[25]
8	Unknown	28.97	NQ		469.0516	$C_{22}H_{13}O_{12}$	393.07	—
9	Quercetin-3-*O*-xyloside[c]	29.20	2.25 ± 0.16	434.0849	433.0771	$C_{20}H_{17}O_{11}$	301.07	[25]
10	Quercetin-3-*O*-arabinoside[c]	30.18	9.54 ± 0.36	434.0849	433.0771	$C_{20}H_{17}O_{11}$	301.07	[25]
11	Quercetin-3-*O*-rhamnoside[a]	31.02	31.88 ± 2.75	448.1005	447.0924	$C_{21}H_{19}O_{11}$	301.25	[25]
12	Di-*O*-caffeoylquinic acid[d]	31.07	0.02 ± 0.00	516.0962	515.0800	$C_{17}H_{23}O_{18}$	353.10 191.02	[35]
13	3,3′-Dimethyl ellagic acid pentose[e]	31.53	0.01 ± 0.00	462.0798	461.0877	$C_{22}H_{19}O_{12}$	329.02	[36]
14	3,3′-Dimethyl ellagic acid deoxyhexose[e]	35.70	0.01 ± 0.00	476.0954	475.0877	$C_{22}H_{19}O_{12}$	329.02	[36]
15	Quercetin-3-(2-*O*-acetyl)-arabinoside[a]	35.74	39.99 ± 2.52	476.0954	475.0877	$C_{22}H_{19}O_{12}$	300.08	[25]
16	Acacetin glucuronide[f]	39.84	0.50 ± 0.02	460.1005	459.0923	$C_{22}H_{19}O_{11}$	283.27	[35]

[a]Quantified using corresponding authentic standard; [b]quantified as equivalent of myricetin-3-*O*-glucoside; [c]quantified as equivalent of quercetin-3-*O*-glucoside; [d]quantified as equivalent of chlorogenic acid; [e]quantified as equivalent of ellagic acid; [f]quantified as equivalent of acacetin; NQ: not quantified.

L. monocytogenes, *S. aureus*, and *B. cereus*. HPLC-DAD and LC-ESI-MS chromatogram revealed that leaves ethanolic extract showed the presence of several phenolic and flavonoid compounds known for their antioxidant activity and that could be responsible for the biological activity of the extract. Further studies will be performed for the isolation and characterization of single active compounds, which could be used for pharmaceutical application as well as in food system for the prevention of the growth of food-borne bacteria.

Abbreviations

AChE: Acetylcholinesterase
AD: Alzheimer's disease
ABTS: 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BChE: Butyrylcholinesterase
DPPH•: 2,2-Diphenyl-1-picrylhydrazyl radical
FRAP: Ferric reducing antioxidant power
GAE: Gallic acid equivalent
MBCs/MFCs: Minimum bactericidal/fungicidal concentrations
MICs: Minimum inhibitory concentrations
QE: Quercetin equivalent
RDDP: RNA-dependent DNA polymerase.

Competing Interests

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

Acknowledgments

The authors thank Professor Annalena Cogoni (Department of Sciences of Life and Environment, Section of Botany and Botanical Garden, University of Cagliari, Italy) for plant species identification. This work was partially supported by grants from Università di Cagliari (Contributo di Ateneo per la Ricerca, CAR, 2013-14).

References

[1] M. García-Alonso, S. De Pascual-Teresa, C. Santos-Buelga, and J. C. Rivas-Gonzalo, "Evaluation of the antioxidant properties of fruits," *Food Chemistry*, vol. 84, no. 1, pp. 13–18, 2004.

[2] S. Kumar and A. K. Pandey, "Chemistry and biological activities of flavonoids: an overview," *The Scientific World Journal*, vol. 2013, Article ID 162750, 16 pages, 2013.

[3] Y. Xie, W. Yang, X. Chen, and J. Xiao, "Inhibition of flavonoids on acetylcholine esterase: binding and structure–activity relationship," *Food & Function*, vol. 5, no. 10, pp. 2582–2589, 2014.

[4] S. Cosentino, C. I. G. Tuberoso, B. Pisano et al., "*In-vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils," *Letters in Applied Microbiology*, vol. 29, no. 2, pp. 130–135, 1999.

[5] S. Cosentino, A. Barra, B. Pisano, M. Cabizza, F. M. Pirisi, and F. Palmas, "Composition and antimicrobial properties of Sardinian *Juniperus* essential oils against foodborne pathogens and spoilage microorganisms," *Journal of Food Protection*, vol. 66, no. 7, pp. 1288–1291, 2003.

[6] A. Upadhyay, I. Upadhyaya, A. Kollanoor-Johny, and K. Venkitanarayanan, "Combating pathogenic microorganisms using

plant-derived antimicrobials: a minireview of the mechanistic basis," *BioMed Research International*, vol. 2014, Article ID 761741, 18 pages, 2014.

[7] F. Esposito, L. Zinzula, A. Maxia, E. Tramontano, and C. Sanna, "Inhibition of HIV-1 reverse transcriptase associated activities by the hydroalcoholic extract of *Casimiroa edulis* seeds," *Natural Product Research*, vol. 25, no. 11, pp. 1067–1073, 2011.

[8] F. Esposito, C. Sanna, C. Del Vecchio et al., "*Hypericum hircinum* L. components as new single-molecule inhibitors of both HIV-1 reverse transcriptase-associated DNA polymerase and ribonuclease H activities," *Pathogens and Disease*, vol. 68, no. 3, pp. 116–124, 2013.

[9] B.-S. Min, N. Nakamura, H. Miyashiro, Y.-H. Kim, and M. Hattori, "Inhibition of human immunodeficiency virus type 1 reverse transcriptase and ribonuclease H activities by constituents of *Juglans mandshurica*," *Chemical & Pharmaceutical Bulletin*, vol. 48, no. 2, pp. 194–200, 2000.

[10] L. Xu, N. Grandi, C. Del Vecchio et al., "From the traditional Chinese medicine plant *Schisandra chinensis* new scaffolds effective on HIV-1 reverse transcriptase resistant to non-nucleoside inhibitors," *Journal of Microbiology*, vol. 53, no. 4, pp. 288–293, 2015.

[11] J. T. Mwine and P. Van Damme, "Why do Euphorbiaceae tick as medicinal plants? A review of Euphorbiaceae family and its medicinal features," *Journal of Medicinal Plants Research*, vol. 5, no. 5, pp. 652–662, 2011.

[12] Q.-W. Shi, X.-H. Su, and H. Kiyota, "Chemical and pharmacological research of the plants in genus *Euphorbia*," *Chemical Reviews*, vol. 108, no. 10, pp. 4295–4327, 2008.

[13] D. Spanò, F. Pintus, C. Mascia et al., "Extraction and characterization of a natural rubber from *Euphorbia characias* latex," *Biopolymers*, vol. 97, no. 8, pp. 589–594, 2012.

[14] D. Spanò, F. Pintus, F. Esposito, D. Loche, G. Floris, and R. Medda, "Euphorbia characias latex: micromorphology of rubber particles and rubber transferase activity," *Plant Physiology and Biochemistry*, vol. 87, pp. 26–34, 2015.

[15] E. Dainese, A. Sabatucci, F. Pintus et al., "Domain mobility as probed by small-angle X-ray scattering may account for substrate access to the active site of two copper-dependent amine oxidases," *Acta Crystallographica Section D: Biological Crystallography*, vol. 70, no. 8, pp. 2101–2110, 2014.

[16] F. Pintus, D. Spanò, A. Bellelli, F. Angelucci, G. Floris, and R. Medda, "Nucleotide pyrophosphatase/phosphodiesterase from *Euphorbia characias* latex: purification and characterization," *Plant Science*, vol. 177, no. 6, pp. 636–642, 2009.

[17] F. Pintus, D. Spanò, A. Bellelli et al., "*Euphorbia* peroxidase catalyzes thiocyanate oxidation in two different ways, the distal calcium ion playing an essential role," *Biochemistry*, vol. 49, no. 40, pp. 8739–8747, 2010.

[18] F. Pintus, R. Medda, A. C. Rinaldi, D. Spanò, and G. Floris, "*Euphorbia* latex biochemistry: complex interactions in a complex environment," *Plant Biosystems*, vol. 144, no. 2, pp. 381–391, 2010.

[19] F. Pintus, D. Spano, S. Corongiu, G. Floris, and R. Medda, "Purification, primary structure, and properties of *Euphorbia characias* latex purple acid phosphatase," *Biochemistry*, vol. 76, no. 6, pp. 694–701, 2011.

[20] D. Spanò, K. Pospiskova, I. Safarik et al., "Chitinase III in *Euphorbia characias* latex: purification and characterization," *Protein Expression and Purification*, vol. 116, pp. 152–158, 2015.

[21] A. Mura, F. Pintus, A. Fais et al., "Tyramine oxidation by copper/TPQ amine oxidase and peroxidase from *Euphorbia characias* latex," *Archives of Biochemistry and Biophysics*, vol. 475, no. 1, pp. 18–24, 2008.

[22] F. Pintus, D. Spanò, G. Floris, and R. Medda, "*Euphorbia characias* latex amine oxidase and peroxidase: interacting enzymes?" *Protein Journal*, vol. 32, no. 6, pp. 435–441, 2013.

[23] M. Palomino-Schätzlein, P. V. Escrig, H. Boira, J. Primo, A. Pineda-Lucena, and N. Cabedo, "Evaluation of nonpolar metabolites in plant extracts by 13C NMR spectroscopy," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 21, pp. 11407–11416, 2011.

[24] F. Pintus, D. Spanò, A. Corona, and R. Medda, "Antityrosinase activity of *Euphorbia characias* extracts," *PeerJ*, vol. 3, article e1305, 2015.

[25] P. V. Escrig, D. J. Iglesias, A. Corma, J. Primo, E. Primo-Millo, and N. Cabedo, "*Euphorbia characias* as bioenergy crop: a study of variations in energy value components according to phenology and water status," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 42, pp. 10096–10109, 2013.

[26] A. Corona, T. Masaoka, G. Tocco, E. Tramontano, and S. F. J. Le Grice, "Active site and allosteric inhibitors of the ribonuclease H activity of HIV reverse transcriptase," *Future Medicinal Chemistry*, vol. 5, no. 18, pp. 2127–2139, 2013.

[27] F. Esposito, A. Corona, and E. Tramontano, "HIV-1 reverse transcriptase still remains a new drug target: structure, function, classical inhibitors, and new inhibitors with innovative mechanisms of actions," *Molecular Biology International*, vol. 2012, Article ID 586401, 23 pages, 2012.

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

[29] F. Pintus, D. Spanò, C. Mascia, A. Macone, G. Floris, and R. Medda, "Acetylcholinesterase inhibitory and antioxidant properties of *Euphorbia characias* latex," *Records of Natural Products*, vol. 7, no. 2, pp. 147–151, 2013.

[30] C. I. G. Tuberoso, M. Boban, E. Bifulco, D. Budimir, and F. M. Pirisi, "Antioxidant capacity and vasodilatory properties of Mediterranean food: the case of *Cannonau* wine, myrtle berries liqueur and strawberry-tree honey," *Food Chemistry*, vol. 140, no. 4, pp. 686–691, 2013.

[31] G. L. Delogu, M. J. Matos, M. Fanti et al., "2-Phenylbenzofuran derivatives as butyrylcholinesterase inhibitors: synthesis, biological activity and molecular modeling," *Bioorganic & Medicinal Chemistry Letters*, vol. 26, no. 9, pp. 2308–2313, 2016.

[32] F. Esposito, A. Corona, L. Zinzula, T. Kharlamova, and E. Tramontano, "New anthraquinone derivatives as inhibitors of the HIV-1 reverse transcriptase-associated ribonuclease H function," *Chemotherapy*, vol. 58, no. 4, pp. 299–307, 2012.

[33] A. Corona, F. S. Di Leva, S. Thierry et al., "Identification of highly conserved residues involved in inhibition of HIV-1 RNase H function by diketo acid derivatives," *Antimicrobial Agents and Chemotherapy*, vol. 58, no. 10, pp. 6101–6110, 2014.

[34] A. Corona, A. Schneider, K. Schweimer, P. Rösch, B. M. Wöhrl, and E. Tramontano, "Inhibition of foamy virus reverse transcriptase by human immunodeficiency virus type 1 RNase H inhibitors," *Antimicrobial Agents and Chemotherapy*, vol. 58, no. 7, pp. 4086–4093, 2014.

[35] H. Horai, M. Arita, S. Kanaya et al., "MassBank: a public repository for sharing mass spectral data for life sciences," *Journal of Mass Spectrometry*, vol. 45, no. 7, pp. 703–714, 2010.

[36] P. Hou, Y. Zeng, B. Ma et al., "A fast, sensitive, and high-throughput method for the simultaneous quantitation of three

ellagitannins from Euphorbiae pekinensis Radix in rat plasma by ultra-HPLC-MS/MS," *Journal of Separation Science*, vol. 36, no. 15, pp. 2544–2551, 2013.

[37] R. A. Dixon and G. M. Pasinetti, "Flavonoids and isoflavonoids: from plant biology to agriculture and neuroscience," *Plant Physiology*, vol. 154, no. 2, pp. 453–457, 2010.

[38] P. J. Houghton, Y. Ren, and M.-J. Howes, "Acetylcholinesterase inhibitors from plants and fungi," *Natural Product Reports*, vol. 23, no. 2, pp. 181–199, 2006.

[39] J. Lin, J. Dou, J. Xu, and H. A. Aisa, "Chemical composition, antimicrobial and antitumor activities of the essential oils and crude extracts of *Euphorbia macrorrhiza*," *Molecules*, vol. 17, no. 5, pp. 5030–5039, 2012.

[40] S. Perumal, R. Mahmud, S. Pillai, W. C. Lee, and S. Ramanathan, "Antimicrobial activity and cytotoxicity evaluation of *Euphorbia hirta* (L.) extracts from Malaysia," *APCBEE Procedia*, vol. 2, pp. 80–85, 2012.

[41] Y. Deng, G. Yang, J. Yue et al., "Influences of ripening stages and extracting solvents on the polyphenolic compounds, antimicrobial and antioxidant activities of blueberry leaf extracts," *Food Control*, vol. 38, no. 1, pp. 184–191, 2014.

[42] H. Ikigai, T. Nakae, Y. Hara, and T. Shimamura, "Bactericidal catechins damage the lipid bilayer," *Biochimica et Biophysica Acta (BBA)—Biomembranes*, vol. 1147, no. 1, pp. 132–136, 1993.

[43] A. M. Janssen, J. J. C. Scheffer, and A. Baerheim Svendsen, "Antimicrobial activity of essential oils: a 1976–1986 literature review. Aspects of the test methods," *Planta Medica*, vol. 53, no. 5, pp. 395–398, 1987.

[44] T. Kulisic-Bilusic, V. Katalinic, V. Dragovic-Uzelac et al., "Antioxidant and AChE inhibiting activity of tea," *Food Technology and Biotechnology*, vol. 46, pp. 368–375, 2008.

[45] N. Suganthy and K. Pandima Devi, "*In vitro* antioxidant and anti-cholinesterase activities of *Rhizophora mucronata*," *Pharmaceutical Biology*, vol. 54, no. 1, pp. 118–129, 2016.

[46] M. A. Aderogba, A. R. Ndhlala, K. R. R. Rengasamy, and J. Van Staden, "Antimicrobial and selected *in vitro* enzyme inhibitory effects of leaf extracts, flavonols and indole alkaloids isolated from *Croton menyharthii*," *Molecules*, vol. 18, no. 10, pp. 12633–12644, 2013.

[47] S. Pasetto, V. Pardi, and R. M. Murata, "Anti-HIV-1 activity of flavonoid myricetin on HIV-1 infection in a dual-chamber in vitro model," *PLoS ONE*, vol. 9, no. 12, Article ID e115323, 18 pages, 2014.

[48] M. M. Senthamilselvi, D. Kesavan, and N. Sulochana, "An anti-inflammatory and anti-microbial flavone glycoside from flowers of *Cleome viscosa*," *Organic and Medicinal Chemistry Letters*, vol. 2, article 19, pp. 1–5, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 7945675, 6 pages
http://dx.doi.org/10.1155/2016/7945675

Research Article

Improvement of the Texture of Yogurt by Use of Exopolysaccharide Producing Lactic Acid Bacteria

Xue Han,[1] Zhe Yang,[1] Xueping Jing,[1] Peng Yu,[2] Yingchun Zhang,[1] Huaxi Yi,[1] and Lanwei Zhang[1]

[1] *School of Chemical Engineering & Technology, Harbin Institute of Technology, Harbin 150090, China*
[2] *Dairy Research Institute, Bright Dairy & Food Co., Ltd., Building 2, No. 1518, West Jiangchang Road, Shanghai 200436, China*

Correspondence should be addressed to Huaxi Yi; yihuaxi@hit.edu.cn and Lanwei Zhang; zhanglw@hit.edu.cn

Received 14 March 2016; Accepted 13 April 2016

Academic Editor: Yiannis Kourkoutas

19 *Streptococcus thermophilus* with high exopolysaccharide production were isolated from traditional Chinese fermented dairy products. The exopolysaccharide and viscosity of milk fermented by these 19 isolates were assayed. The strains of *Streptococcus thermophilus* zlw TM11 were selected because its fermented milk had the highest exopolysaccharide content (380 mg/L) and viscosity (7716 mpa/s). Then *Streptococcus thermophilus* zlw TM11 was combined with *Lactobacillus delbrueckii* subsp. *bulgaricus* 3 4.5 and the combination was named SH-1. The quality of the yogurt fermented by SH-1 and two commercial starter cultures (YO-MIX 465, YF-L711) were compared. It was shown that the exopolysaccharide content of yogurt fermented by SH-1 was similar to that of yogurt fermented by YF-L711 and significantly higher than YO-MIX 465 ($p < 0.05$). In addition, the yogurt fermented by SH-1 had the lowest syneresis (8.5%) and better texture and sensory than the samples fermented by YO-MIX 465 and YF-L711. It manifested that the selected higher exopolysaccharide production starter SH-1 could be used as yogurt starter and reduce the amount of adding stabilizer, which can compare with the imported commercial starter culture.

1. Introduction

Yogurt has been an integral part of everyday diet for centuries, rising as the second most popular snack among children in the world [1]. Textural properties of yogurt, such as viscosity [2], smoothness and thickness [3], and structural resistance to stress [4], are important attributes to determine its consumer acceptance, and these attributes nowadays are accompanied with certain health benefits [5]. Many methods have been used to improve the quality of the yogurt, such as increasing the solids in milk (adding fat, proteins, or sugars such as sucrose and fructose), addition of stabilizers (pectin, starch, alginate, and gelatin) [6, 7]. However, these approaches did not satisfy the consumers demand for products with as few food additives as possible. Exopolysaccharide (EPS) produced by LAB with GRAS (generally recognized as safe) status is an important source of natural alternatives. Recently, EPS produced by LAB have gained considerable attention in the fermented dairy industry because of their potential application as viscosifiers, texturizers, and emulsifying agents [8]. It had been reported that EPS produced by yogurt starter cultures could affect the texture of yogurt and improve sensory characteristics such as mouthful, shininess, clean cut, ropiness, and creaminess [5]. Furthermore, the yogurt cultures producing EPS may decrease the extent of syneresis (the lower whey separation) [9]. Syneresis is considered as a major defect in yogurt and connected with extensive rearrangements of the gel network [10]. Presently, EPS plays a major role in the production of fermented dairy products in Northern Europe, Eastern Europe, and Asia [6]. However, the EPS of LAB are in a great variety, which depends on the type of LAB strains, culture conditions, and medium composition [9, 11]. It is significant to select the strains with higher EPS production.

Streptococcus thermophilus and *Lactobacillus delbrueckii* subsp. *bulgaricus* are the mainly strains used in the yogurt starter culture and used for yogurt production. Unfortunately, some of strains of *Lactobacillus delbrueckii* subsp.

bulgaricus and *Streptococcus thermophilus* did not produce EPS or produce only low yields of EPS, which may affect the end products quality [12–14]. Presently, most of the yogurt starter cultures were imported from Denmark Danisco or Chr Hansen in China. And Chinese own authority starter culture was scarcity.

In China, there are many traditional fermented dairy products. Therefore, screening LAB from natural sources has been one of the powerful means to obtain strains for the food industry. We have already screened LAB from Chinese traditional fermented dairy products and some properties have been characterized in our previous work [15, 16]. In this study, it was mainly focused on the exopolysaccharide production strains by *Streptococcus thermophilus*. The aim of this work was to select the starter culture with higher exopolysaccharide production to improve the yogurt quality and develop our own authority yogurt starter culture. The selected higher exopolysaccharide production starter will reduce the amount of additives addition and can be comparable with the imported starter culture.

2. Materials and Methods

2.1. Materials. The commercial yogurt starters, YO-MIX 465 and YF-L711, were obtained from Danisco and Chr. Hansen (Denmark). The whole cow raw milk was purchased from supermarket (Harbin, China). Skim milk was purchased from Nestle Company, Heilongjiang, China. All the chemicals used in this paper were of analytical grade.

2.2. Exopolysaccharide Producing Strains Selection. 19 *Streptococcus thermophilus* were screened from traditional Chinese fermented dairy products. The above 19 isolates were inoculated into the sterilized (121°C, 15 min) reconstituted skim milk (12%), respectively. The inoculate rate was 2% and incubated at 42°C until pH attained to 4.7. The fermented samples were cooled at 4°C for 12 h. Then the content of exopolysaccharide and viscosity in fermented milks were detected. The *Streptococcus thermophilus*, which produced higher exopolysaccharide and had higher viscosity, was selected. The pH of each sample was determined directly using a digital pH meter (PB-10, Sartorius, Germany) calibrated with standard buffer solutions. Three replicates were performed.

2.3. Yogurt Starter Culture. The selected *Streptococcus thermophilus* with higher exopolysaccharide production was combined with *Lactobacillus delbrueckii* subsp. *bulgaricus* 3 4.5, which was screened from Chinese traditional fermented dairy products and identified by Biolog identification system and analysis of 16S rDNA gene sequence. The direct vat starter (DVS) SH-1 which was composed by these two stains (the fermented characteristic about the combined starter culture was reported in our previous work [16]) was used to ferment the cow milk. The fermented cow milk produced by the commercial yogurt starter, YO-MIX 465 and YF-L711, was used as contrasts. Then the exopolysaccharide content, texture, syneresis, and sensory of fermented cow milk were evaluated.

2.4. Yogurts Preparation. Raw fresh cow milk consisted of 3.14% protein, 3% fat, lactose 4.92%, and 11.81% total solids, was analyzed by MilkoScan FT1 (Denmark), and then preheated to 65°C and homogenized. The homogenized milk was pasteurized at 65°C for 30 minutes and cooled to 42°C and portioned to three equal batches. The batches were inoculated with about 3 mg/L starter cultures (YO-MIX 465, YF-L711, and SH-1) that were determined by the initial viable counts of yogurt. The initial viable counts of each batches were about 10^7 CFU/mL measured by spread plate count method and incubated at 42°C until the pH reached 4.6 (about 4 to 4.5 h). Then yogurt samples were put into the refrigerating chamber (4°C) for 12 h to detect the EPS production, appearance viscosity, texture, syneresis, and sensory of yogurts. Each test has three replications.

2.5. Exopolysaccharides Detection. EPS was isolated from the fermented sample, according to a modified method of Lin and Chien [8]. The fermented sample was added with an equal volume of trichloroacetic acid (40%); the precipitated protein and bacteria were removed by centrifugation (2200 g for 35 min at 4°C). The supernatant was then mixed with an equal volume of ethanol, stored at 4°C for 24 h, and centrifuged as described above to collect the precipitated EPS. The EPS was then dissolved in water. The total neutral glycoside content was determined by the phenol-sulphuric acid method [17].

2.6. Viscosity Measurement. Viscosity measurement was carried out at 4 ± 1°C by Brookfield Programmable DV-E Viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) equipped with S63 spindle at 20 rpm.

2.7. Texture Analysis. The texture of yogurt was determined by penetration measurements (TA.XT plus, Texture Analyzer, Stable Micro Systems Ltd., England). The instrument was adjusted to the following conditions: cylindrical probe, probe diameter: 35 mm; penetration speed: 1.0 mm/s; penetration distance, 20 mm into surface. The software used was Exponent (Stable Micro Systems, 2006, version 5.0). 100 mL of yogurt sample was analyzed in each cup. Four parameters were evaluated, the firmness (g) (maximum force, i.e., exerted on the sample), defined as the force necessary to attain a given deformation; the cohesiveness (g/s) (adhesive force), defined as forces of internal bonds, which keep the product complete; the viscosity (g); and the adhesiveness (g/s) (total negative area); the work is necessary for overcoming the force of attraction between the area of foodstuff and other solids coming to contact with them.

2.8. Syneresis Measurement. Syneresis index of different yogurt samples was determined according to the methodology proposed by Farnsworth et al. [18] with modifications. Yogurt (20 g) was prepared in centrifuge cups and centrifuged at 350 ×g (model K-24; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) for 10 min at 4°C. The clear

supernatant was collected and weighed and syneresis was calculated according to the following equation [18, 19]:

$$\text{Syneresis}\,(\%) = \frac{\text{weight of supernatant}\ (\text{g})}{\text{weight of yogurt sample}\ (\text{g})} \times 100\%. \quad (1)$$

2.9. Sensory Evaluation. Twenty trained panelists (fourteen women and six men, aged 22–45) were asked to evaluate the sensory attributes of yogurt. The ratings were presented on a 9-point hedonic scale ranging from 9 ("like extremely") to 1 ("dislike extremely"). Yogurt sensory parameters were evaluated by thickness, smoothness, fermented odor, finished flavor, and taste quality. To minimize bias, all groups were three digits coded. The yogurts were served to panelists after the cooling process. Result was given on averages of the three trials for each type of yogurt [20].

2.10. Statistical Analysis. Statistical analyses were performed using SPSS 14.0 software (SPSS Inc.; Chicago, IL, USA). Significant differences among treatments were tested by ANOVA followed by Tukey's test with a level of significance at $\alpha = 0.05$. Data were expressed as mean values ± standard deviation (S.D.). For analyzing the relationship of exopolysaccharide production and viscosity of fermented milk, the correlate bivariate analysis was used to determine the correlation coefficient. All experiments were performed in duplicate and repeated three times.

3. Results and Discussion

3.1. Exopolysaccharide Production Strains Selection. The exopolysaccharide production and the viscosity of fermented cow milk produced by the 19 *Streptococcus thermophilus* isolated from traditional Chinese fermented dairy products were assayed. In Table 1, it showed that different strains had different exopolysaccharide production and viscosity. For the fermented milk produced by *Streptococcus thermophilus* zlw TM11, the exopolysaccharide and viscosity were significantly higher than that of the other strains ($p < 0.05$). The relationship of exopolysaccharide production and viscosity of fermented milk was analyzed by SPSS software correlate bivariate analysis. The results showed that the correlation coefficient between exopolysaccharide production and viscosity was 0.841 ($p < 0.01$). It suggested the strains producing higher exopolysaccharide might contribute to the higher viscosity of fermented milk. Guzel-Seydim et al. [9] reported that exopolysaccharide filaments attached mucous bacteria to the protein matrix and thus caused more viscous-like behavior. Many researchers also reported that yogurts made with polysaccharide producing cultures had higher viscosity values than the yogurts made with none of the polysaccharide production cultures [21, 22]. Therefore, the higher exopolysaccharide producing strains of *Streptococcus thermophilus* zlw TM11 were selected to combine with *Lactobacillus delbrueckii* subsp. *bulgaricus* 3 4.5. The combination was named SH-1 and was used as starter to make yogurt.

Table 1: The content of exopolysaccharide and viscosity for different *Streptococcus thermophilus* fermented milk (values are means ± SD for $n = 3$).

Strains	EPS (mg/L)	Viscosity (mPa/s)
zlwTM11	384 ± 22^{a}	7716 ± 608.80^{A}
zlwB9-3	255 ± 43^{b}	5960 ± 1716.77^{B}
zlwQ	175 ± 54^{c}	4360 ± 685.85^{C}
zlwA1	155 ± 2^{cd}	4240 ± 58.89^{C}
zlwA2M17	141 ± 29^{cde}	4198 ± 340.33^{C}
zlw17YA	128 ± 5^{de}	3450 ± 42^{CDE}
zlwCH9.9 4.0	127 ± 7^{de}	3804 ± 27.50^{CD}
zlw3	121 ± 5^{de}	3562 ± 101.17^{CD}
zlwLBH	120 ± 5^{de}	2920 ± 203.15^{DEF}
zlw1703Ca	115 ± 3^{de}	2870 ± 84.92^{DEF}
zlwCH9-94.5	101 ± 57^{dfg}	2210 ± 206.14^{F}
zlw94.3	99 ± 2^{efg}	2726 ± 97.73^{EF}
zlwSP1.1	93 ± 10^{fgh}	2726 ± 383.26^{EF}
zlw1703F	85 ± 34^{fgh}	2722 ± 1084.76^{EF}
zlw1	73 ± 6^{ghi}	1206 ± 64.90^{G}
zlwDV	56 ± 12^{ghi}	2194 ± 272.35^{F}
zlw1703D	54 ± 16^{hi}	2556 ± 33.41^{EF}
zlwSH94.3	47 ± 13^{i}	2303 ± 121.39^{F}
zlwSH94.5	45 ± 7^{i}	1092 ± 43.27^{G}

a, b, c, d, e, f, g, h, and i: values with different letters within the same column differ significantly ($p < 0.05$) for exopolysaccharide. A, B, C, D, E, F, and G: values with different letters within the same column differ significantly ($p < 0.05$) for viscosity.

3.2. Content of Exopolysaccharides in Yogurt. The exopolysaccharide content in each yogurt samples was showed in Figure 1. For yogurt sample produced by SH-1, the contents of exopolysaccharide and viscosity were significantly higher than that of sample produced by YO-MIX 465 ($p < 0.05$) and had no significant difference with YO-MIX 711 ($p > 0.05$). Starter YO-MIX 711 had the higher exopolysaccharide production and also had higher viscosity in fermented milk compared with YO-MIX 465, although there was no significant difference for the fermented milk viscosity ($p > 0.05$). It was verified in the result of Table 1 that the strains with higher exopolysaccharide production had the higher viscosity. And it also indicated that the exopolysaccharide production was different from the types of starter culture [11].

3.3. Texture Properties. The textures properties of the yogurts fermented by different starter cultures were showed in Figure 2. The concentration of exopolysaccharide of SH-1 was the highest (423.05 mg/L), corresponding to the highest firmness (55.77 g), highest cohesiveness (715.31 g·s), highest viscosity (27.83 g), and highest adhesiveness (364.30 g·s), respectively. The YF-L711 has the lowest firmness (36.70 g), cohesiveness (457.86 g·s), viscosity (18.63 g), and adhesiveness (212.10 g·s). These factors are significantly lower than starter SH-1 ($p < 0.05$). For YF-L711, the firmness (36.70 g) and cohesiveness had no significantly different with YO-MIX 465 ($p > 0.05$). While viscosity (18.63 g) and adhesiveness were significantly lower than YO-MIX 465 fermented yogurt

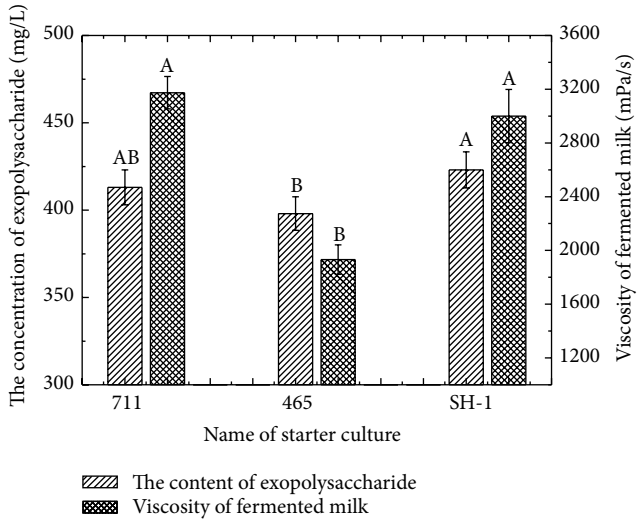


Figure 1: The concentration of exopolysaccharide in yogurt fermented by different starter culture. Mean values ($n = 3$), 465 was the starter of YO-MIX 465, 711 was the starter of YF-L711, and SH-1 was the screened starter. A, B, and treatments with different letters are different at $p < 0.05$. Error bars indicate standard deviation.

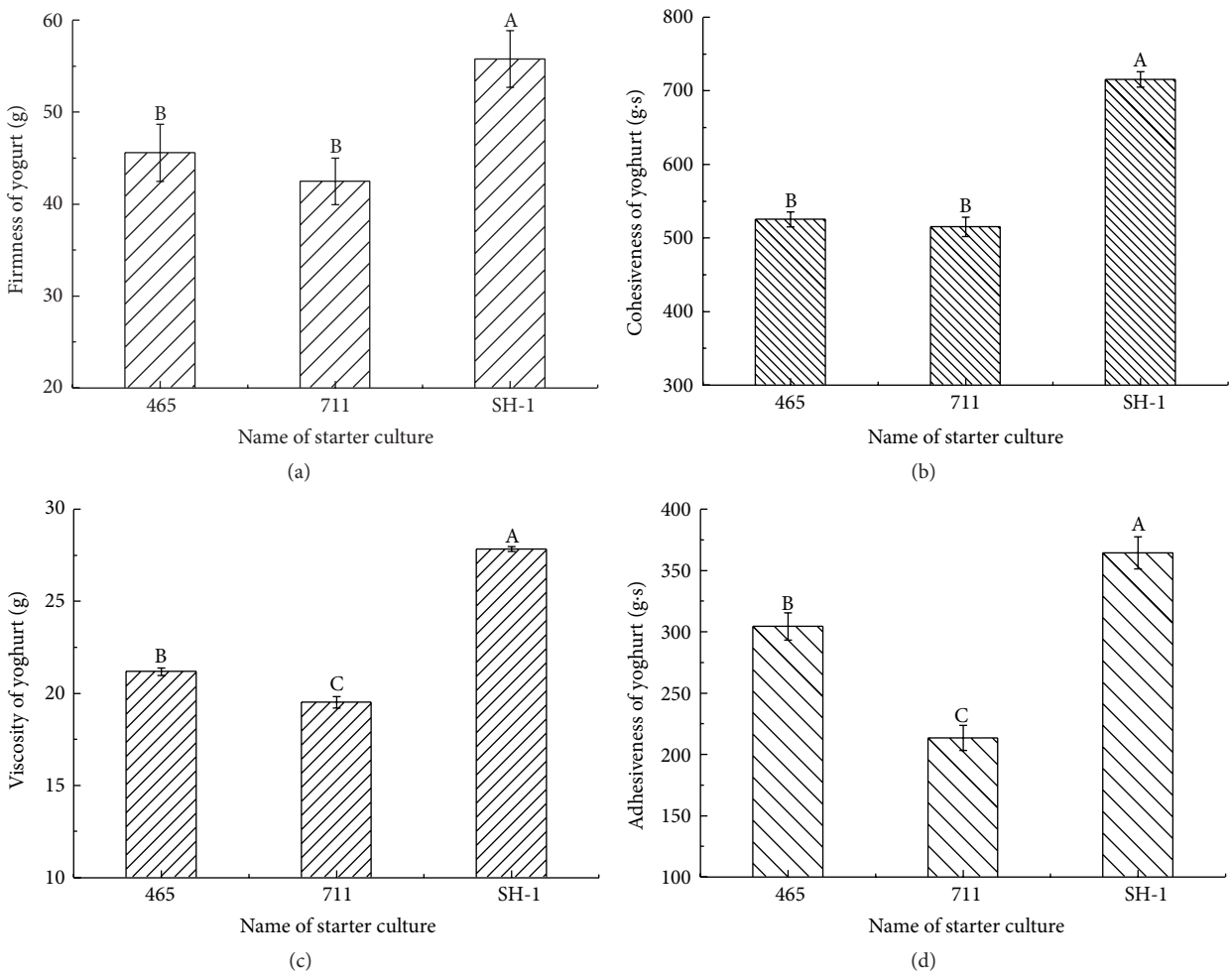


Figure 2: The texture of different starter cultures fermented yogurt. (a) The firmness of yogurt; (b) the cohesiveness of yogurt; (c) the viscosity of yogurt; (d) the adhesiveness of yogurt. 465 was the starter of YO-MIX 465, 711 was the starter of YF-L711, and SH-1 was the screened starter. A, B, and C: treatments with different letters are different at $p < 0.05$. Error bars indicate standard deviation.

($p < 0.05$). The content of exopolysaccharide in YF-L711 fermented yogurt was a little higher than that of YO-MIX 465 fermented yogurt, though no significant difference ($p > 0.05$) was observed. It was inferred that a little increase of exopolysaccharide might have great effect on viscosity and adhesiveness.

Many researchers reported that the exopolysaccharide could improve the texture of yogurt, because exopolysaccharide produced by LAB interacts with the free water in the gel-like structure [9, 23, 24]. In this study, the yogurt fermented by SH-1 had the highest content of exopolysaccharide and the best texture than the yogurt fermented by the other starter cultures. It suggested that the selected starter SH-1 could reduce some stabilizer addition and replace the imported commercial starters. Similar results were found by Patel et al. [22]. They reported that exopolysaccharide could improve the quality of yogurt, while having no effect on flavor of yogurt. Therefore, it could replace some stabilizer. However, the concentration of polysaccharide and the texture of yogurt had no linear correlation. It was assumed that the texture of yogurt could be affected by starter types and the structure of exopolysaccharide [25].

3.4. Syneresis of Yogurt. The syneresis of samples fermented by different starters was showed in Figure 3. There were no statistical differences for these three samples ($p > 0.05$). Among them, starter SH-1 had the highest exopolysaccharide content (423 mg/L), corresponding to the lowest whey separation (8.51%). For commercial starters YO-MIX 465 and YF-L711, the syneresis was 10.70% and 12.12%, respectively. It seemed that the higher content of exopolysaccharide would contribute to the lower whey separation. Similar results had also been reported by other studies [2]. This might be due to the high water-binding capacity of EPS as well as modifications of yogurt microstructure by EPS cultures [24, 26]. Thus, the yogurts made from EPS producing starters showed better textural characteristics.

3.5. Sensory Evaluation. The scores for sensory characteristics of the yogurt samples were presented in Figure 4. For thickness, the selected starter culture SH-1 was better than YO-MIX 465 and similar to YF-L 711. For smoothness, the starter SH-1 was similar to YO-MIX 465 and YF-L 711. For taste quality, the scores of selected starter SH-1 were higher than YF-L 711 but lower than YO-MIX 465. For the finished flavor, selected starter SH-1 has no significant difference from YF-L 711 and YO-MIX 465 ($p > 0.05$). As regards to the fermented odor, the starter SH-1 was no better than YO-MIX 465 and YF-L 711. The results of sensory evaluation indicated that the selected starter SH-1 had the potential to replace the imported commercial starter.

4. Conclusion

The starter culture type affected the qualities of yogurt samples. The selected starter SH-1 had the similar content of exopolysaccharides to the commercial starter YF-L 711 and significantly higher than YO-MIX 465. The higher exopolysaccharides could provide better texture of yogurt and lower whey separation. On the other hand, the sensory evaluation of yogurt using selected culture SH-1 was better than or similar to the other commercial starter cultures, except for the fermented odor which was a little lower than the other two commercial starters. These implied that our own authority starter SH-1 could produce the yogurt with similar or better quality compared with the commercial starters.

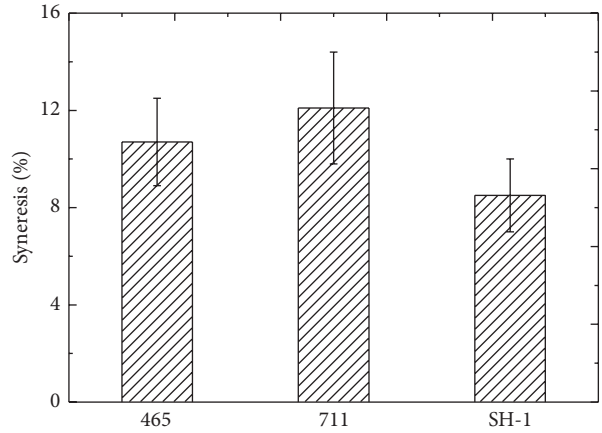


Figure 3: The syneresis of yogurt fermented by different starter culture. 465 was the starter of YO-MIX 465, 711 was the starter of YF-L711, and SH-1 was the screened starter. There are no significant differences at $p > 0.05$. Error bars indicate standard deviation.

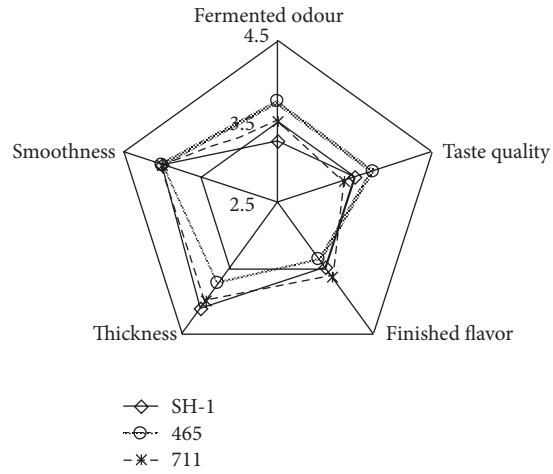


Figure 4: Sensory value for different starter fermented milk. 465 was the starter of YO-MIX 465, 711 was the starter of YF-L711, and SH-1 was the screened starter.

Competing Interests

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

Acknowledgments

This work was financially supported by the Youth Innovation Foundation of Harbin, Heilongjiang Province (2013RFQXJ147) and National Natural Science Foundation of China (31101317). It was also supported by Postdoctoral Science-Research Developmental Foundation of Heilongjiang Province (LBH-Q12108).

References

[1] U. Purwandari, N. P. Shah, and T. Vasiljevic, "Effects of exopolysaccharide-producing strains of *Streptococcus thermophilus* on technological and rheological properties of set-type yoghurt," *International Dairy Journal*, vol. 17, no. 11, pp. 1344–1352, 2007.

[2] V. M. Marshall and H. L. Rawson, "Effects of exopolysaccharide-producing strains of thermophilic lactic acid bacteria on the texture of stirred yoghurt," *International Journal of Food Science and Technology*, vol. 34, no. 2, pp. 137–143, 1999.

[3] D. Jaworska, B. Waszkiewicz-Robak, W. Kolanowski, and F. Swiderski, "Relative importance of texture properties in the sensory quality and acceptance of natural yoghurts," *International Journal of Dairy Technology*, vol. 58, no. 1, pp. 39–46, 2005.

[4] A. Skriver, J. Holstborg, and K. B. Qvist, "Relation between sensory texture analysis and rheological properties of stirred yogurt," *Journal of Dairy Research*, vol. 66, no. 4, pp. 609–618, 1999.

[5] P. V. Behare, R. Singh, S. K. Tomar, R. Nagpal, M. Kumar, and D. Mohania, "Effect of exopolysaccharide-producing strains of *Streptococcus thermophilus* on technological attributes of fat-free lassi," *Journal of Dairy Science*, vol. 93, no. 7, pp. 2874–2879, 2010.

[6] P. Duboc and B. Mollet, "Applications of exopolysaccharides in the dairy industry," *International Dairy Journal*, vol. 11, no. 9, pp. 759–768, 2001.

[7] W. J. Lee and J. A. Lucey, "Formation and physical properties of yogurt," *Asian-Australasian Journal of Animal Sciences*, vol. 23, no. 9, pp. 1127–1136, 2010.

[8] T. Y. Lin and M.-F. C. Chien, "Exopolysaccharides production as affected by lactic acid bacteria and fermentation time," *Food Chemistry*, vol. 100, no. 4, pp. 1419–1423, 2007.

[9] Z. B. Guzel-Seydim, E. Sezgin, and A. C. Seydin, "Influences of exopolysaccharide producing cultures on the quality of plain set type yogurt," *Food Control*, vol. 16, no. 3, pp. 205–209, 2005.

[10] W.-J. Lee and J. A. Lucey, "Rheological properties, whey separation, and microstructure in set-style yogurt: effects of heating temperature and incubation temperature," *Journal of Texture Studies*, vol. 34, no. 5-6, pp. 515–536, 2003.

[11] B. Ismail and K. M. Nampoothiri, "Production, purification and structural characterization of an exopolysaccharide produced by a probiotic *Lactobacillus plantarum* MTCC 9510," *Archives of Microbiology*, vol. 192, no. 12, pp. 1049–1057, 2010.

[12] F. Vaningelgem, M. Zamfir, F. Mozzi et al., "Biodiversity of exopolysaccharides produced by *Streptococcus thermophilus* strains is reflected in their production and their molecular and functional characteristics," *Applied and Environmental Microbiology*, vol. 70, no. 2, pp. 900–912, 2004.

[13] A. Gürsoy, F. Durlu-Özkaya, F. Yildiz, and B. Aslim, "Set type yoghurt production by exopolysaccharide producing Turkish origin domestic strains of *Streptococcus thermophilus* (W22) and *Lactobacillus delbrueckii ssp. Bulgaricus* (B3)," *Kafkas Universitesi Veteriner Fakultesi Dergisi*, vol. 16, pp. S81–S86, 2010.

[14] G. J. Grobben, M. R. Smith, J. Sikkema, and J. A. M. De Bont, "Influence of fructose and glucose on the production of exopolysaccharides and the activities of enzymes involved in the sugar metabolism and the synthesis of sugar nucleotides in *Lactobacillus delbrueckii* subsp. bulgaricus NCFB 2772," *Applied Microbiology and Biotechnology*, vol. 46, no. 3, pp. 279–284, 1996.

[15] X. Han, L. W. Zhang, M. Du, H. Yi, J. Li, and L. Zhang, "Effects of copper on the post acidification of fermented milk by *St. thermophilus*," *Journal of Food Science*, vol. 77, no. 1, pp. M25–M28, 2012.

[16] X. Han, L. W. Zhang, P. Yu, H. Yi, and Y. Zhang, "Potential of LAB starter culture isolated from Chinese traditional fermented foods for yoghurt production," *International Dairy Journal*, vol. 34, no. 2, pp. 247–251, 2014.

[17] Z. L. Urshev, Z. P. Dimitrov, N. S. Fatchikova, I. G. Petrova, and D. I. Ishlimova, "Partial characterization and dynamics of synthesis of high molecular mass exopolysaccharides from *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*," *World Journal of Microbiology and Biotechnology*, vol. 24, no. 2, pp. 171–179, 2008.

[18] J. P. Farnsworth, J. Li, G. M. Hendricks, and M. R. Guo, "Effects of transglutaminase treatment on functional properties and probiotic culture survivability of goat milk yogurt," *Small Ruminant Research*, vol. 65, no. 1-2, pp. 113–121, 2006.

[19] C. Gauche, T. Tomazi, P. L. M. Barreto, P. J. Ogliari, and M. T. Bordignon-Luiz, "Physical properties of yoghurt manufactured with milk whey and transglutaminase," *LWT—Food Science and Technology*, vol. 42, no. 1, pp. 239–243, 2009.

[20] N. Sahan, K. Yasar, and A. A. Hayaloglu, "Physical, chemical and flavour quality of non-fat yogurt as affected by a β-glucan hydrocolloidal composite during storage," *Food Hydrocolloids*, vol. 22, no. 7, pp. 1291–1297, 2008.

[21] T. Amatayakul, A. L. Halmos, F. Sherkat, and N. P. Shah, "Physical characteristics of yoghurts made using exopolysaccharide-producing starter cultures and varying casein to whey protein ratios," *International Dairy Journal*, vol. 16, no. 1, pp. 40–51, 2006.

[22] S. Patel, A. Majumder, and A. Goyal, "Potentials of exopolysaccharides from lactic acid bacteria," *Indian Journal of Microbiology*, vol. 52, no. 1, pp. 3–12, 2012.

[23] L. De Vuyst and B. Degeest, "Heteropolysaccharides from lactic acid bacteria," *FEMS Microbiology Reviews*, vol. 23, no. 2, pp. 153–177, 1999.

[24] A. N. Hassan, M. Corredig, and J. F. Frank, "Capsule formation by nonropy starter cultures affects the viscoelastic properties of yogurt during structure formation," *Journal of Dairy Science*, vol. 85, no. 4, pp. 716–720, 2002.

[25] Z. Ahmed, Y. Wang, N. Anjum, A. Ahmad, and S. T. Khan, "Characterization of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir—part II," *Food Hydrocolloids*, vol. 30, no. 1, pp. 343–350, 2013.

[26] L. De Vuyst and B. Degeest, "Heteropolysaccharides from lactic acid bacteria," *FEMS Microbiology Reviews*, vol. 23, no. 2, pp. 153–177, 1999.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 5201879, 11 pages
http://dx.doi.org/10.1155/2016/5201879

Research Article

Phytochemical Composition and Antibacterial Activity of Hydroalcoholic Extracts of *Pterospartum tridentatum* and *Mentha pulegium* against *Staphylococcus aureus* Isolates

Alfredo Aires,[1] Eduardo Marrinhas,[2] Rosa Carvalho,[2] Carla Dias,[3] and Maria José Saavedra[3]

[1]*Centre for the Research and Technology for Agro-Environment and Biological Sciences, CITAB, University of Trás-os-Montes e Alto Douro, Quinta de Prados, 5001-801 Vila Real, Portugal*
[2]*University of Trás-os-Montes e Alto Douro, UTAD, Quinta de Prados, 5001-801 Vila Real, Portugal*
[3]*Animal and Veterinary Research Centre, CECAV, University of Trás-os-Montes e Alto Douro, Quinta de Prados, 5001-801 Vila Real, Portugal*

Correspondence should be addressed to Alfredo Aires; alfredoa@utad.pt

Received 8 February 2016; Revised 8 March 2016; Accepted 16 March 2016

Academic Editor: Nikos Chorianopoulos

Pterospartum tridentatum and *Mentha pulegium* are largely used in Portuguese folk medicine to treat several human disorders and inflammatory processes but without any consistent evidence for those beneficial pointed properties. Thus, the aim of the current work is to evaluate its benefits and phytochemicals related to those beneficial properties. A distinct polyphenol profile between *P. tridentatum* and *M. pulegium* was found. Taxifolin, myricetin, ginestin, ginestein, and ginestein derivatives, biochanin A-glucoside, and biochanin A were identified in *P. tridentatum*, whilst in *M. pulegium* the luteolin-7-rutinoside, diosmin, and apigenin and respective derivatives were most representative polyphenols. These variations had implications in the antiradical and antibacterial activity and the *P. tridentatum* exhibited the highest antibacterial activity against methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* MSSA, which was mainly dose-dependent. This antibacterial activity seems to be related to high content of flavonols, flavones, and isoflavones, which can act synergistically with each other against this type of bacteria. Our results showed consistent evidence that *Pterospartum tridentatum* and *Mentha pulegium* are an important reservoir of phytochemicals with antiradical activity and antibacterial capacity and thus they might be used in a preventive way or in a combined pharmaceutical and antibiotic therapy against pathogenic bacteria.

1. Introduction

Nowadays, one key problem in human health is the less effectiveness of commercial antibiotics against several pathogenic bacterial isolates. One of them is the *Staphylococcus aureus*, a gram-positive bacterium from Staphylococcaceae family, and considered one of the world's most important infectious agents causing disease outbreaks related to food consumption, badly treated wounds, and hospital-associated infections [1, 2]. *S. aureus* is often reported as being for a variety of human and animal diseases and its epidemiological relevance is mainly due to their ability of becoming highly resistant to common antimicrobials such as tetracycline, vancomycin, penicillin G, and methicillin [3, 4] and to a less degree to oxacillin, lincomycin, clindamycin, erythromycin, streptomycin, cefoxitin, kanamycin, chloramphenicol, and gentamicin [5, 6].

In the last decades, evolution of resistance, for example, to methicillin, has become an enormous problem for treatment of *S. aureus* infections. Thus, the health authorities have increased the research programs to develop new and more effective antimicrobial molecules and several plants have been used in different ways to extract potential antimicrobial compounds. Different authors have shown that plants have naturally bioactive compounds that could act alone or in synergy with antibiotics against bacterial isolates [7, 8] and

the aromatic and medicinal plants have been one of the most studied plants and found useful as antibacterial, antifungal, and antihelminthic [9–11] among other beneficial properties. However there is still a lack of information about either their phytochemical composition, their antimicrobial activity, or even how the phytochemicals act against microorganisms. Two common plants highly present in native flora of Mediterranean areas particularly in the Iberian Peninsula are the *Mentha pulegium*, normally called European pennyroyal, and *Pterospartum tridentatum* (L.) W. K. & Lge. (Syn.: *Genista tridentata* L. subsp. *cantabrica* (Spach) Nyman), frequently named as "*Carqueja*." *M. pulegium* is an aromatic herb that belongs to the family Lamiaceae, naturalized in America, and thrives in western, southern, and central Europe, Asia, Iran, Arab countries, and Ethiopia [12]. Its essential oil and dry parts have been traditionally used in medicine (digestive, liver, and gallbladder disorders, amenorrhea, gout, colds, increased micturition, skin diseases, and abortifacient), gastronomy (culinary herb), aromatherapy, and cosmetics [13]. *P. tridentatum* is a small shrub belonging to the Leguminosae family and Papilionoideae subfamily [14], and its flowers are traditionally used in folk medicine as depurative and hypoglycaemic and for throat irritation conditions [15]. Most studies performed so far on *M. pulegium* and related *Mentha* species were carried out with their essential oil in different regions of the world, including Tunisia [16], Greece [17], Turkey [18], and Portugal [19], and focused mainly on their chemical composition. There is still a lack of information about their phytochemical composition related to functional capacity and antimicrobial activity. Thus, we set this study in which we evaluate the phytochemical composition of *P. tridentatum* and *M. pulegium* and its effect on the antioxidant activity and antimicrobial potential against different isolates of *Staphylococcus aureus*, an important pathogen highly associated with outbreak diseases and antibiotic resistance phenomena.

2. Material and Methods

2.1. Sampling. The sampling process was done according to previous works conducted in our lab and already published [20], but with the same modifications. One kilogram of fresh *Pterospartum tridentatum* and *Mentha pulegium* was collected in natural open fields in Portugal. The *P. tridentatum* (*Carqueja*) samples were collected in Vila Real Region (altitude of 400 meters) near the Natural Park of Alvão (Northern Portugal) (N 41°17′35.538″, W 7°44′29.6268″), whilst *M. pulegium* (pennyroyal) samples were collected in Santarem Region (Central Portugal). These species are largely present in open fields of Portugal as native flora, but in the northern region *Carqueja* is more common whilst in the south the predominance goes to *Pennyroyal*. After harvest, the samples were botanically identified by the Botany Services of University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal. After this identification, fresh samples were dried in a freeze-dryer system (UltraDry Systems™, USA), milled and reduced to a fine powder, and stored in dark flasks at 4°C in a dark environment until extraction. Fresh and dry weights were registered and the level of dry matter was determined.

2.2. Extraction. Dried powder of each sample (200 g) was extracted in triplicate with methanol 70% (methanol : water, v/v) in a warm bath at 70°C in 30 minutes with intermittent agitation. After that, methanolic extracts were filtered (Whatman No. 1), centrifuged at 4000 rpm during 15 min 4°C (Kubota, 2000). Hydroalcoholic extracts were then evaporated until complete dryness in a rotary evaporator under vacuum (40°C, 178 mbar). Yields of extraction were calculated. The final concentration achieved was 5 mg·mL^{-1} dry weight. The concentration was prepared diluting the solid residue with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Taufkirchen, Germany). These extracts were used for the phytochemical analysis and in vitro bioassays of antioxidant and antimicrobial activity.

2.3. Phytochemical Analysis HPLC-DAD-UV/VIS. Polyphenols generally named phenolic compounds are the group of secondary metabolites frequently found in extracts of aromatic and medicinal plants and they are often reported as having antimicrobial and antioxidant activity. The identification and quantification of phenolics in *P. tridentatum* and *M. pulegium* samples were performed using HPLC-DAD-UV/VIS. From the previous extracts, an aliquot of 1 mL was overnight evaporated until complete dryness under continuous nitrogen flux. The dried extracts were then resuspended with 1 mL of 70% methanol (methanol : water, v/v) and filtered (Spartan Ø 0.13) to HPLC amber vials (to avoid degradation by light) and stored at −20°C until injection in HPLC. The eluent was composed of water with 1% of trifluoroacetic acid (TFA) (solvent A) and acetonitrile with 1% TFA (solvent B). Elution was performed at a flow rate of solvent of 1 mL·min^{-1}, with gradient starting with 100% of water, and the injection volume of 20 μL. Chromatograms were recorded at 280, 320, 370, and 520 nm with a C18 column (250 × 46 mm, 5 μm). Phytochemicals were identified using peak retention time, UV spectra, and UV max absorbance bands and through comparison with external commercial standards (Extrasynthese, France). The external standards were freshly prepared in 70% methanol (methanol : water) in a concentration of 1.0 mg·mL^{-1} and analysed by HPLC-DAD-UV immediately before the samples.. Methanol and acetonitrile were purchased from Panreac Chemistry (Lisbon, Portugal) and Sigma-Aldrich (Taufkirchen, Germany), respectively. The aqueous solutions were prepared using ultrapure water (Milli-Q, Millipore).

2.4. Evaluation of Functional Properties

2.4.1. Free Radical Scavenging of 2,1-Diphenyl-2-picrylhydrazyl Free Radical (DPPH$^{\bullet}$). The scavenging capacity of DPPH of methanolic *P. tridentatum* and *M. pulegium* extracts was determined using a spectrophotometric 96-well microplate assay [21] with several modifications as follows: 20 μL of sample extract and 280 μL of 60 μM methanolic radical DPPH solution freshly prepared added to each well. Then the plate was left to stand at room temperature in 30 min. After that, the reduction in absorbance was measured at 517 nm with a spectrophotometer (Multiskan™ FC Microplate Photometer, USA). The free radical scavenging activity as

Table 1: Isolates of *Staphylococcus aureus* used in the in vitro antibacterial assay.

Reference	Type	Source
MJMC021	Methicillin-resistant strains	Clinical
MJMC024	Methicillin-resistant strains	Clinical
MJMC026	Methicillin-resistant strains	Clinical
MJMC025	Methicillin-sensitive strains	Clinical
MJMC027	Methicillin-sensitive strains	Clinical
MJMC029	Methicillin-sensitive strains	Clinical

expression of antioxidant activity (AA) was calculated as percentage inhibition of the DPPH radical, according to the following equation: I (%) = [((solvent absorbance − sample absorbance)/solvent absorbance) × 100]. The compound butylated hydroxytoluene (BHT) (Sigma-Aldrich, Taufkirchen, Germany), a synthetic analog of vitamin E, was used as positive control of AA in order to compare the results for the samples. Also the inhibition concentration at 50% inhibition (IC_{50}) was determined in order to compare the AA between the extracts themselves and extracts and positive control, and lower IC_{50} means better free radical scavenging activity, thus higher AA. All determinations were performed in triplicate.

2.4.2. In Vitro Antibacterial Activity

(1) Bacterial Isolates. Seven gram-positive isolates of *Staphylococcus aureus* (3 methicillin-resistant *Staphylococcus aureus* and 3 methicillin-sensitive *Staphylococcus aureus* MSSA, plus one standard control strain from the American type culture collection (ATTC)) were obtained from Maria José Saavedra (Ph.D.) core collection located in Microbiology Laboratory of Veterinarian Science Department of UTAD (Table 1). The isolates were previously and properly identified by standard biochemical classification techniques [22] using API 20E, API 20NE, API Staph-Ident, and API Step (BioMerieux), according to the procedure previously described [23], followed by genetic identification through 16S rRNA sequencing. When tested, the isolates were prepared freshly, sowed in Petri plates (92 × 16 mm, Sarstedt, Germany) with BHI (Brain Heart Infusion, Oxoid, England) media, and incubated at 37°C overnight in order to obtain fresh and pure bacteria cultures. Then, bacterial suspension (5×10^6 cfu·mL^{-1}) of each isolate was prepared adjusting to an optical density range of 0.5–1.0 measured at $OD_{620\,nm}$ and used in the bioassays in order to obtain in each well 5×10^5 cfu·mL^{-1} inoculum final concentration.

(2) Determination of a Minimum Inhibitory Concentration (MIC). Phytochemicals are routinely classified as antimicrobials on the basis of susceptibility assays that produce a MIC within the range of 100–1000 μg·mL^{-1} [24] and the MIC was defined as the lowest concentration of an antimicrobial compound which can maintain or reduce the growth of a microorganism after 24 hours of incubation [25]. In the current study, the antibacterial activity was assessed by MIC and the *P. tridentatum* and *M. pulegium* methanolic extracts were prepared with a maximum concentration of 5000 μg·mL^{-1} dry weight in 10% DMSO in a microplate bioassay [26]. After that, 100 μL of each extract and 1000 μg of standard antibiotic were added into the first row of 96-well microplates, followed by serial dilutions on the additional wells containing 100 μL of nutrient broth. Positive and negative controls were included: a column with gentamicin (Oxoid, England) as positive control and three negative controls (a column without bioactive compounds, a column without the bacterial solution (20 μL of nutrient broth instead), and a column with DMSO solution). After this mixture the optical densities (OD) were measured at 620 nm (Multiskan FC Microplate Photometer, USA) that were automatically recorded. Then, the microplates were placed at 37°C during 24 hours and then the OD were measured again at 620 nm. These absorbance values were subtracted from those obtained before incubation to deplete the effect of color interference. The MIC was considered the lowest concentration in which the final OD was inferior to the initial OD. To classify the antibacterial effect we adopted the following scale: strong (+++), if MIC values ≤100 μg·mL^{-1}, moderate (++) when 100 < MIC ≤ 500 μg·mL^{-1}, weak (+) when 500 < MIC ≤ 1000 μg·mL^{-1}, and null (−) (without effect) when MIC > 1000 μg·mL^{-1}.

2.5. Statistical Analysis. The results were expressed as mean values and standard deviation (SD) of three replicates. The results were analyzed using one-way ANOVA followed by Duncan multiple range test, based on confidence level equal to or higher than 95% ($p < 0.05$). Software SPSS V.17 (SPSS-IBM, Orchard Road, Armonk, New York, NY, USA) was used to carry out this analysis.

3. Results and Discussion

The polyphenol profile and respective chemical structures of phenolics identified in the current study of hydroalcoholic extracts from *P. tridentatum* and *M. pulegium* are presented in Figures 1 and 2. The respective data (retention time, λ_{max} in the visible region) and average content of each polyphenol, expressed as mg·g^{-1} dry weight (dw), are presented in Tables 2 and 3. It was possible to assess that *P. tridentatum* had high content taxifolin, ginestin, ginestein, and ginestein derivatives, biochanin A-glucoside, and biochanin A, whilst *M. pulegium* exhibited higher diversity with luteolin-7-rutinoside, diosmin, and apigenin and respective derivatives have the most representative phenolics identified. *P. tridentatum* was richer in isoflavones, whilst *M. pulegium* present high but similar content in flavones and hydroxycinnamic acids. These results have shown an important class of phytochemicals in both plant species. The presence of high levels of taxifolin, ginestin, and ginestein, all frequently associated with antioxidant, antimicrobial, and anthelmintic activity [27–31], made this plant extract very interesting from bioactive point of view. Taxifolin, a flavanol subclass of flavonoids [28], is abundant in several types of plants and is an interesting potential component of dietary supplements or antioxidant-rich functional food [29]. Genistein is an isoflavone [28] known by its anti-inflammatory and antioxidant properties [30] and has been shown to

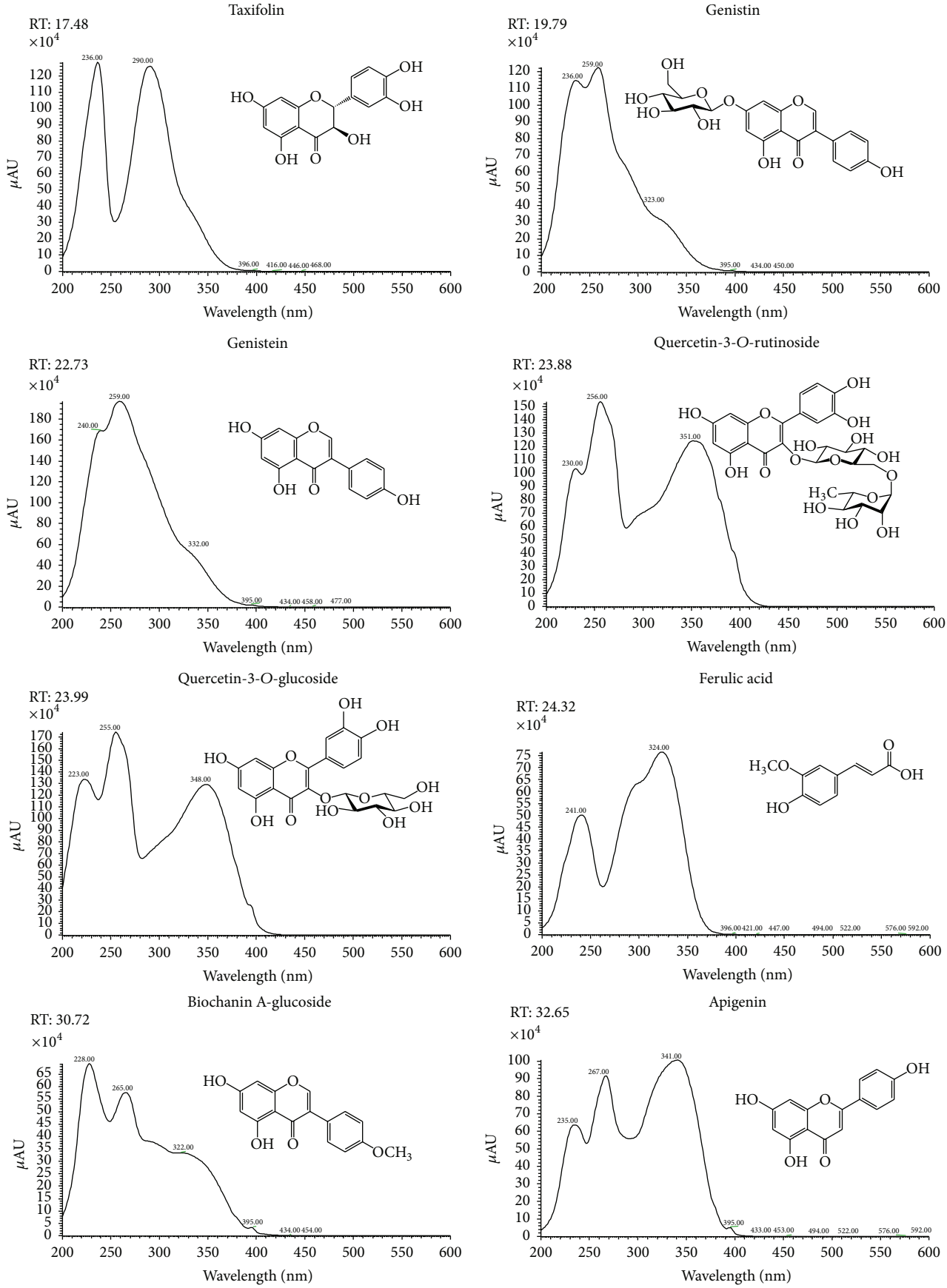


Figure 1: Continued.

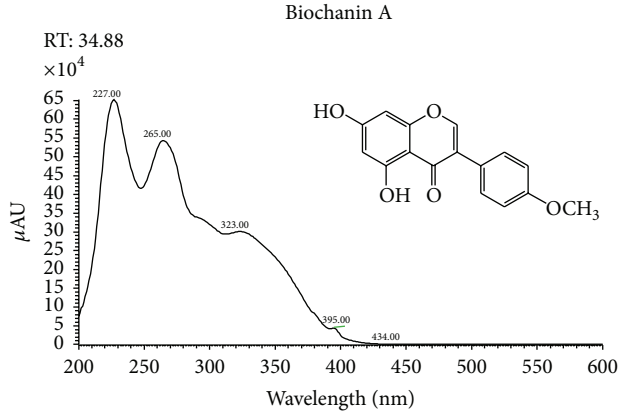


Figure 1: UV spectra of polyphenols detected in *Pterospartum tridentatum*.

interact with animal and human estrogenic receptors. This compound is often mentioned as responsible for wound healing properties [31], which is one of the main reasons because plants with high content of such compounds are largely used in traditional medicine, even if in the majority of the cases there is no scientific evidence for that. In fact, native floras have been used in folk medicine for thousands years, even if their biological effects and chemicals responsible for those properties were poorly understood. Only in the recent years, the therapeutic value of several herbs and the direct relation between their bioactive potential and their content in some specific essential oils (EOs), alkaloids, terpenoids, glucosinolates, and phenolics, among other compounds, were properly established. Our results showed that *P. tridentatum* and *M. pulegium* have important phenolics often associated with anti-inflammatory, antioxidant, and antimicrobial properties [32], reinforcing the scarce information available until now about these two herbs. Works about the phytochemical composition of these two native plants are very scarce but some of them [32, 33] seem to be in agreement with our results. Quercetin, genistein, and bioachanin-A and related isomers have been found by other authors in *P. tridentatum* [32], whilst in different types of *Mentha* [33] the presence of catechin and catechins derivatives, rosmarinic acid, quercetin, luteolin, and apigenin was detected as we found in this study. This difference could be related not only to genetic factors but also to agroclimatic conditions, since these herbs were collected in two different Portuguese regions: the *P. tridentatum* was collected in Northern Portugal (more temperate and wet) whilst *M. pulegium* was collected in Centre-South of Portugal (more dry and hot). As consequence, both profile and average content of phenolics of these two herbs were different. This difference may explain the difference noted in their bioactivity.

The results for the evaluation of functional properties expressed as free radical (2,1-diphenyl-2-picrylhydrazyl free radical (DPPH$^{\bullet}$)) activity and in vitro antibacterial activity are presented in Figure 3 and in Table 4, respectively. We observed that biological *P. tridentatum* and *M. pulegium* have high levels of AA, even higher than the positive control (BHT) used in this assay (Figure 3). Despite this similarity, a different trend in the phenolic influence on AA was observed. In the *P. tridentatum* the high AA seems to be explained by the high content of isoflavones and flavanols (49 and 42% of total phenolics identified, resp.), whilst in *M. pulegium* the AA seems to be explained by the synergism between phenolics acids (32% of total phenolics), flavones, and flavanones (31 and 17% of total phenolics, resp.). This difference is understandable since all of these compounds are often reported [27, 29, 30, 32, 33] as having important antioxidant activities. The presence of several phenolic acids, flavones, isoflavones, and flavanols in both extracts, with potential to scavenge free radicals such as superoxide and nitric oxide [29], can thus explain the high antioxidant potential exhibited by these two herbs. However, based on the average content of phenolics (Tables 2 and 3) the expectation was to have higher AA in *P. tridentatum* extracts but was not the case. In fact, based on the values of IC_{50} (Figure 3) the AA in *M. pulegium* was higher compared to *P. tridentatum*, which seems to contradict the previous finding, but we must be aware that other compounds, besides the phenolics such as pigments, alkaloids, and carotenoids (not determined in this work), often reported as being present in high concentrations in these two herbs [34], might have contributed to high levels of AA observed in this type of extract. In addition, the higher presence of phenolic acids such as hydroxybenzoic acid, chlorogenic acid, caffeic acid, ferulic acid, and rosmarinic acid, all based on –CH=CH–COOH groups, widely recognizable for forming easily complexes with DPPH [35] seems to be one of the main reasons for *M. pulegium* extracts having the lowest IC_{50} and thus the highest AA. Therefore, based on the current work it seems that the higher proportion of phenolic compounds with hydroxyl groups on the aromatic ring is responsible for the higher AA exhibited by *M. pulegium* extracts, which is in agreement with the previous findings [35] in which the positions of hydroxyl groups were found extremely important for the bioactivity of polyphenols, including their antioxidant capacity. Despite these differences, the high AA found for both extracts are critical and determinant for their therapeutic value and this may be in part responsible for their reputation as anti-inflammatory, hypotensive, hypoglycemic, and depurative agent. It seems that they can reduce

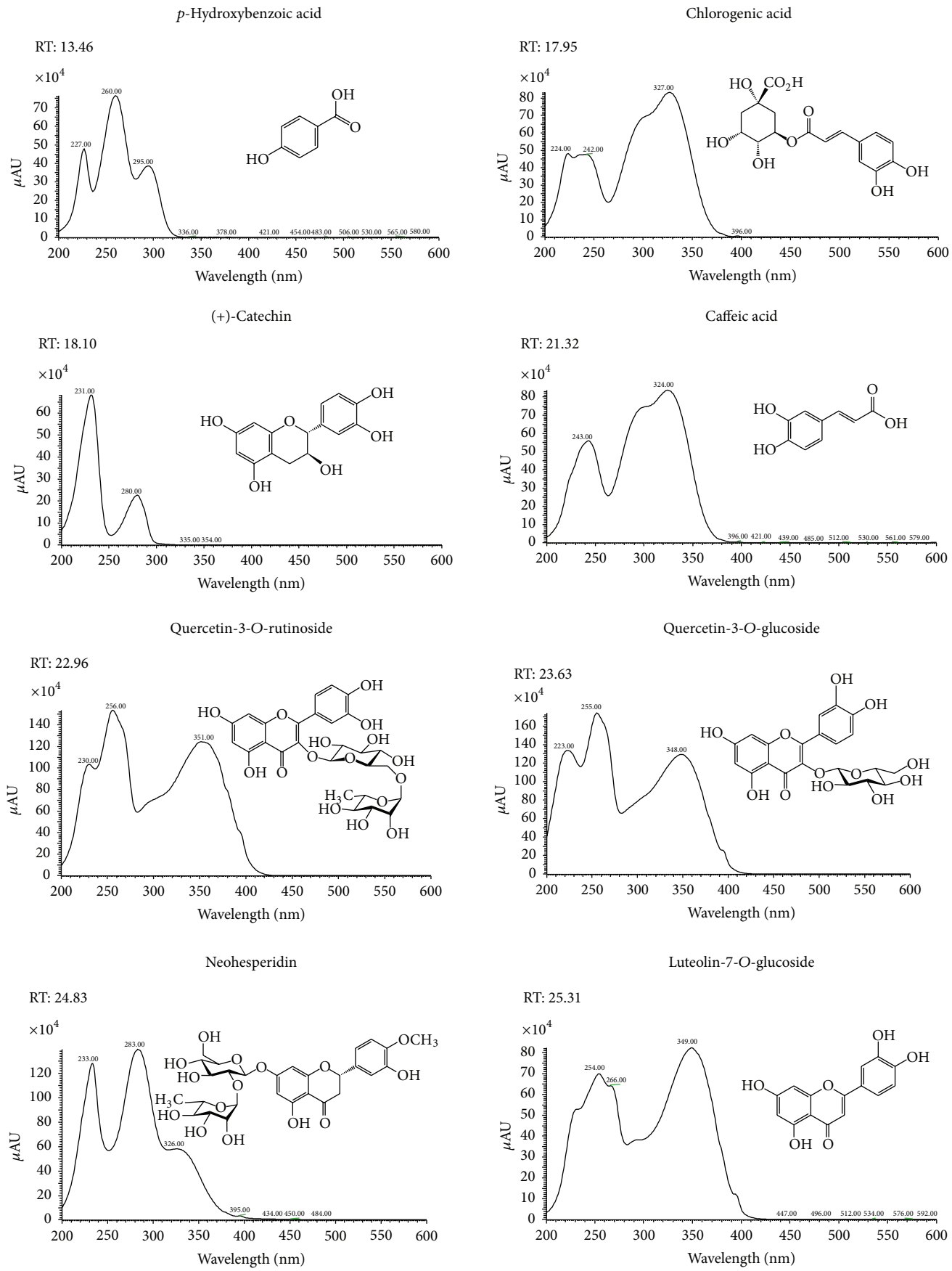


Figure 2: Continued.

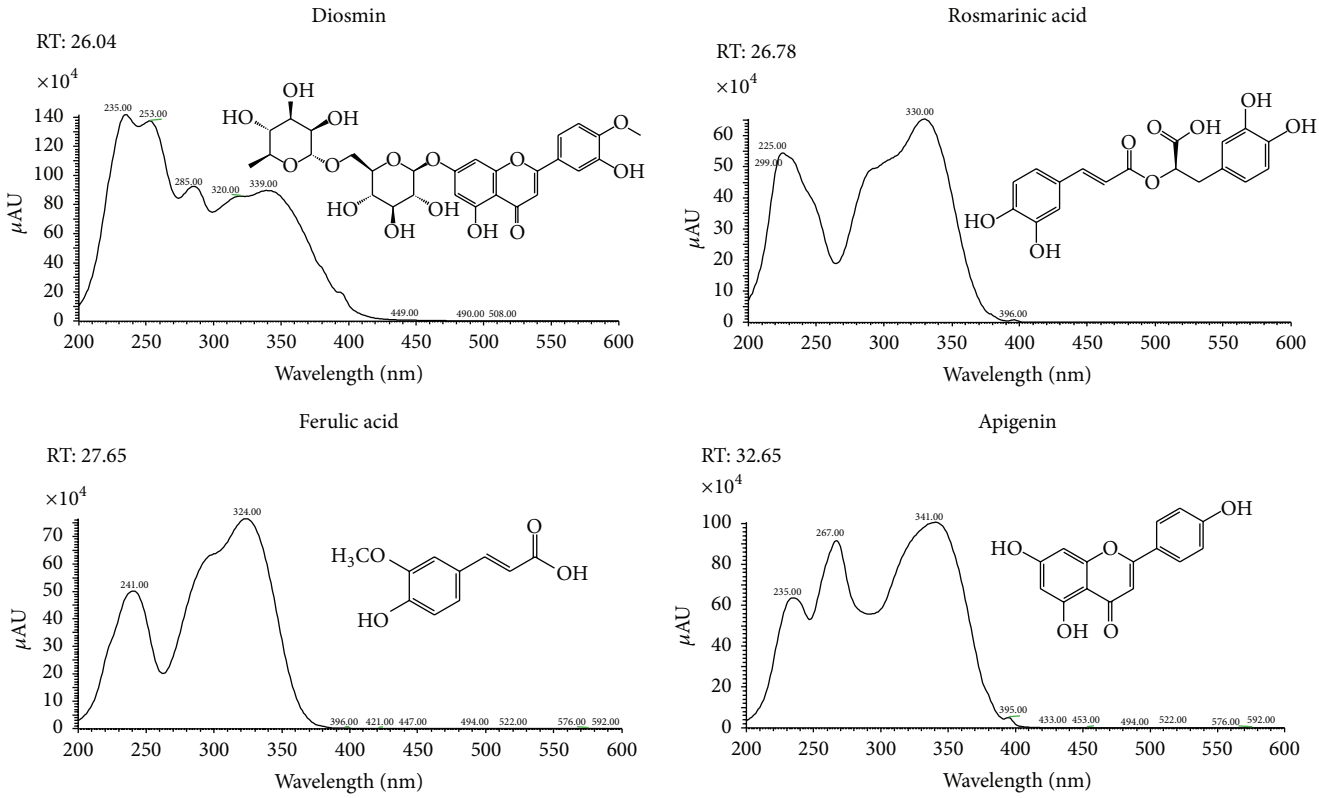


Figure 2: UV spectra of polyphenols detected in *Mentha pulegium*.

Table 2: Average content of polyphenols and respective retention time (Rt) and wavelengths of maximum absorption in the visible region (λ_{max}) in *P. tridentatum* extracts (by elution order)[†].

Polyphenols	Rt (min)	UV (nm)	UV-DAD/VIS bands (nm) in 70% methanol	Class	Mg·g^{-1} dry weight
Taxifolin	17.48	280	290, 327sh	Flavanol	21.76 ± 0.030
Genistin	19.79	320	259, 323sh	Isoflavone	16.75 ± 0.040
Genistein	22.73	320	259, 332sh	Isoflavone	12.01 ± 0.030
Quercetin-3-O-rutinoside	23.88	370	256, 266sh, 351	Flavonol	1.58 ± 0.030
Quercetin-3-O-glucoside	23.99	370	255, 266sh, 348	Flavonol	1.23 ± 0.010
Ferulic acid	24.32	320	244, 296sh, 324	Hydroxycinnamic acid	0.27 ± 0.002
Biochanin A-glucoside	30.66	320	265, 322sh	Flavone	1.37 ± 0.002
Apigenin	32.65	370	229, 267sh, 341	Flavone	0.44 ± 0.002
Biochanin A	34.88	320	265, 323sh	Flavone	2.89 ± 0.004
Total of hydroxycinnamic acids					0.27 ± 0.002
Total of flavonols					24.57 ± 0.100
Total of flavone					4.70 ± 0.008
Total of isoflavone					28.76 ± 0.070
Total of polyphenols identified					58.30 ± 0.180

[†]Values expressed as mean ± standard deviation of three replicates.

Table 3: Average content of polyphenols and respective retention time (Rt) and wavelengths of maximum absorption in the visible region (λ_{max}) in *M. pulegium* extracts (by elution order)[†].

Polyphenols	Rt (min)	UV (nm)	UV-DAD/VIS bands (nm) in 70% methanol	Class	Mg·g^{-1} dry weight
p-Hydroxybenzoic acid	13.46	280	227, 260sh, 295	Hydroxybenzoic acid	0.212 ± 0.004
Chlorogenic acid	17.95	320	242, 300sh, 327	Hydroxycinnamic acid	0.387 ± 0.004
(+)-Catechin	18.10	280	231, 280	Flavan-3-ols	0.212 ± 0.004
Caffeic acid	21.32	320	243, 296sh, 324	Hydroxycinnamic acid	0.230 ± 0.001
Quercetin-3-*O*-rutinoside	22.96	370	256, 266sh, 351	Flavonol	0.144 ± 0.003
Quercetin-3-*O*-glucoside	23.63	370	255, 266sh, 348	Flavonol	0.140 ± 0.002
Neohesperidin	24.84	280	283, 326	Flavanone	0.628 ± 0.001
Luteolin-7-*O*-glucoside	25.31	370	254, 266, 349	Flavone	0.201 ± 0.004
Diosmin	26.04	370	253, 268, 339	Flavone	0.623 ± 0.006
Rosmarinic acid	26.78	320	249, 299sh, 330	Hydroxycinnamic acid	0.287 ± 0.006
Ferulic acid	27.55	320	241, 296sh, 324	Hydroxycinnamic acid	0.277 ± 0.003
Apigenin	32.65	370	235, 267sh, 341	Flavone	0.323 ± 0.002
Total of hydroxybenzoic acids					0.212 ± 0.004
Total of hydroxycinnamic acids					1.181 ± 0.014
Total of flavan-3-ols					0.212 ± 0.004
Total of flavonols					0.284 ± 0.005
Total of flavones					1.147 ± 0.012
Total of flavanones					0.628 ± 0.001
Total polyphenols identified					3.664 ± 0.040

[†]Values expressed as mean ± standard deviation of three replicates.

Table 4: Minimum inhibitory concentration (MIC) of *P. tridentatum* and *M. pulegium* aqueous and methanolic extracts expressed as μg·mL^{-1}[†].

Isolate	Reference	Type	Gentamicin (commercial antibiotic)	*P. tridentatum*	*M. pulegium*
S. aureus	ATCC 13565	Standard	<39 (+++)	312.5 (++)	2500 (−)
S. aureus	MJMC021	MRSA	<39 (+++)	78.1 (+++)	2500 (−)
S. aureus	MJMC024	MRSA	<39 (+++)	78.1 (+++)	2500 (−)
S. aureus	MJMC026	MRSA	<39 (+++)	78.1 (+++)	2500 (−)
S. aureus	MJMC025	MSSA	<39 (+++)	39.1 (+++)	39.1 (+++)
S. aureus	MJMC027	MSSA	<39 (+++)	39.1 (+++)	78.1 (+++)
S. aureus	MJMC029	MSSA	<39 (+++)	39.1 (+++)	39.1 (+++)

[†]Inside brackets there is the classification of the antibacterial activity effect: strong (+++), if MIC values ≤ 100 μg·mL^{-1}, moderate (++) when 100 < MIC ≤ 500 μg·mL^{-1}, weak (+) when 500 < MIC ≤ 1000 μg·mL^{-1}, and null (−) (without effect) when MIC > 1000 μg·mL^{-1}.

oxidative stress, a key factor in the progression of chronical inflammatory diseases. Further investigations should be done in order to determine the action keys on the pathways of the inflammatory mechanisms. Based on these results it seems very clear that the traditional usage of *P. tridentatum* and *M. pulegium* as medicinal plants associated with anti-inflammatory and depurative processes is correct and thereby these plants serve as natural sources of antioxidants for food and medicinal purposes.

Although the results with DPPH$^{\bullet}$ free radical scavenging activity have shown that *P. tridentatum* and *M. pulegium* have similar values of AA, the antibacterial activity was very different. *P. tridentatum* exhibited the highest antibacterial activity due to lower minimum inhibitory concentration levels found. The MSSA isolates were more affected than MRSA isolates, as we expected. This activity was mainly dose-dependent and in general according to the classification criteria adopted (Table 4) the antibacterial activity was strong and in some isolates similar to the antibacterial activity observed for the antibiotic used as positive control. As similar to AA, the higher antimicrobial activity for *P. tridentatum* can be the consequence of two effects: (i) the higher content of flavonols and isoflavones and (ii) the additive effect of different types of phenolics, which seems to boost the antimicrobial efficacy of such extracts. Isoflavones such as genistin and genistein (and respective isomers) have been reported as having anti-inflammatory, antiproliferative, and antibacterial effects [36]. Also, phenolics like rutin, isoquercetin, and quercetin can

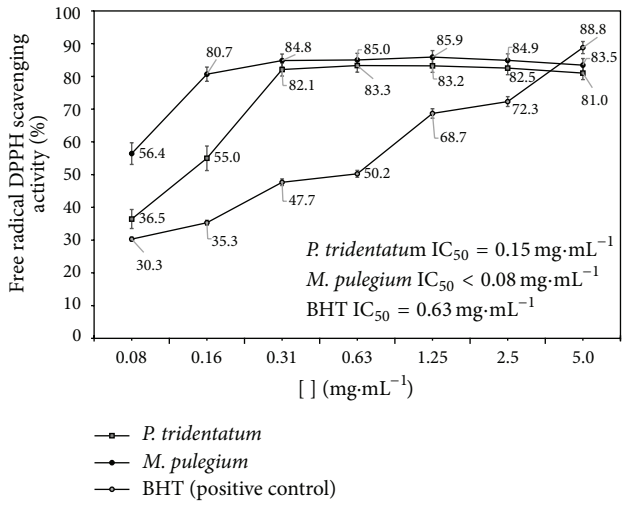


Figure 3: % free radical DPPH scavenging activity of *Pterospartum tridentatum* and *Mentha pulegium* methanolic extracts.

play an important role as antimicrobial agents due to their capacity of interference on bacterial mechanisms of nucleic acid synthesis, cytoplasmic membrane, energy metabolisms, and being particularly effective against gram-positive bacteria [37] such as *S. aureus* studied in the current work. The antimicrobial activity of *P. tridentatum* extracts and in a less extension *M. pulegium* might be due to one of the mechanisms of action mentioned above. The richness of flavonols and isoflavones on *P. tridentatum* can be responsible for the increment in antibacterial activity exhibited for this extract compared to the *M. pulegium*. In fact, the high presence of compounds such as taxifolin, genistin, and biochanin often reported as having antibacterial activity [38] can be responsible for depletion of bacteria resistance mechanisms leading to increment in their susceptibility to these compounds. Also, it was observed that taxifolin and respective isomers extracted from *Hypericum japonicum* Thunb. ex. Murray (Guttiferae) delayed the protein synthesis of *S. aureus* (including the MRSA strains), affecting the synthesis of nucleic acids and enzymatic systems needed for bacteria growth [39]. This action is responsible for increasing the membranes permeability to drugs, leading to a decrease in bacteria survival, suggesting that these compounds might have a bacteriostatic effect rather than a bactericidal activity. Moreover it was noted that, in general, flavonoids and oligomers of flavonoids, particularly those with high grade of hydroxylation (such as flavonols, flavones, and flavanones), have a strong ability to link with bacteria cell walls from complexes [40, 41], affecting the bacteria growth and survival. Thus, plant extracts with high content of such compounds, like *P. tridentatum*, can be very useful when used in a complementary therapy with commercial drugs due to their bacteriostatic effect.

The majority of the studies available in literature about the antimicrobial activity of *P. tridentatum* and *M. pulegium* report mainly the effects of their essential oils (EOs) [19, 39–41] and very few about the effect of their hydroalcoholic extracts [41–43], and thus they attribute their antibacterial efficacy essentially to the EOs, and fewer conclusions are made about the importance of other bioactive compounds such as polyphenols. Moreover, their effects against *S. aureus* MSSA and MRSA have been scarcely explored. Thus, our results seem to be important because they not only reinforce the idea that richness of polyphenols is also critical for the antimicrobial capacity of any plant, but also show the bioactivities of these two herbs, proving that they can be used to extract bioactive compounds with antimicrobial activity against MSSA and MRSA.

4. Conclusion

P. tridentatum and *M. pulegium* are two important herbs from Mediterranean native flora and might be used to extract important and effective bioactive compounds against epidemiological important pathogenic bacteria, particularly against *S. aureus*, one of the most important pathogenic bacteria, often associated with foodborne outbreak diseases and hospital/clinical environment infections. Our results have shown that both extracts can be effective against MRSA and MSSA due to high content of different class of flavonoids, particularly flavonols, flavones, and isoflavones compounds, which can act synergistically with each other against those bacteria. Further research is needed to elucidate accurately the pathways and mechanisms used by these compounds against bacteria.

Competing Interests

The authors declare no conflict of interests.

Acknowledgments

The authors acknowledge the financial support provided by the Portuguese Foundation for Science and Technology (FCT) (Alfredo Aires-SFRH/BPD/65029/2009) under Project UID/AGR/04033/2013.

References

[1] S. Y. C. Tong, L. F. Chen, and V. G. Fowler Jr., "Colonization, pathogenicity, host susceptibility, and therapeutics for *Staphylococcus aureus*: what is the clinical relevance?" *Seminars in Immunopathology*, vol. 34, no. 2, pp. 185–200, 2012.

[2] A. Fetsch, M. Contzen, K. Hartelt et al., "*Staphylococcus aureus* food-poisoning outbreak associated with the consumption of ice-cream," *International Journal of Food Microbiology*, vol. 187, pp. 1–6, 2014.

[3] J. Gao, M. Ferreri, F. Yu et al., "Molecular types and antibiotic resistance of *Staphylococcus aureus* isolates from bovine mastitis in a single herd in China," *Veterinary Journal*, vol. 192, no. 3, pp. 550–552, 2012.

[4] H. Jamali, M. Paydar, B. Radmehr, S. Ismail, and A. Dadrasnia, "Prevalence and antimicrobial resistance of *Staphylococcus aureus* isolated from raw milk and dairy products," *Food Control*, vol. 54, pp. 383–388, 2015.

[5] A. Dhand and G. Sakoulas, "Daptomycin in combination with other antibiotics for the treatment of complicated Methicillin-resistant *Staphylococcus aureus* bacteremia," *Clinical Therapeutics*, vol. 36, no. 10, pp. 1303–1316, 2014.

[6] S. M. Mandal, A. K. Ghosh, and B. R. Pati, "Dissemination of antibiotic resistance in methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *S aureus* strains isolated from hospital effluents," *American Journal of Infection Control*, vol. 43, no. 12, pp. e87–e88, 2015.

[7] S. Chusri, T. Siriyong, P. Na-Phatthalung, and S. P. Voravuthikunchai, "Synergistic effects of ethnomedicinal plants of Apocynaceae family and antibiotics against clinical isolates of *Acinetobacter baumannii*," *Asian Pacific Journal of Tropical Medicine*, vol. 7, no. 6, pp. 456–461, 2014.

[8] P. Bellio, B. Segatore, A. Mancini et al., "Interaction between lichen secondary metabolites and antibiotics against clinical isolates methicillin-resistant *Staphylococcus aureus* strains," *Phytomedicine*, vol. 22, no. 2, pp. 223–230, 2015.

[9] M. E. I. Badawy and S. A. M. Abdelgaleil, "Composition and antimicrobial activity of essential oils isolated from Egyptian plants against plant pathogenic bacteria and fungi," *Industrial Crops and Products*, vol. 52, pp. 776–782, 2014.

[10] X. P. Zhang, W. X. Li, T. S. Ai, H. Zou, S. G. Wu, and G. T. Wang, "The efficacy of four common anthelmintic drugs and traditional Chinese medicinal plant extracts to control *Dactylogyrus vastator* (Monogenea)," *Aquaculture*, vol. 420-421, pp. 302–307, 2014.

[11] Z. Chen, B. He, J. Zhou, D. He, J. Deng, and R. Zeng, "Chemical compositions and antibacterial activities of essential oils extracted from *Alpinia guilinensis* against selected foodborne pathogens," *Journal of Ethnopharmacology*, vol. 178, pp. 125–136, 2016.

[12] J. Gruenwald, T. Brendler, and C. Jaenicke, *PDR for Herbal Medicines*, Medical Economic Company, Montvale, NJ, USA, 2000.

[13] V. K. Agnihotri, S. G. Agarwal, P. L. Dhar et al., "Essential oil composition of *Mentha pulegium* L. growing wild in the northwestern Himalayas India," *Flavour and Fragrance Journal*, vol. 20, no. 6, pp. 607–610, 2005.

[14] S. Talavera, *Flora Iberica*, vol. 7, CSIC, Madrid, Spain, 1999.

[15] M. Pardo-de-Santayana, J. Tardío, E. Blanco et al., "Traditional knowledge of wild edible plants used in the northwest of the Iberian Peninsula (Spain and Portugal): a comparative study," *Journal of Ethnobiology and Ethnomedicine*, vol. 3, article 27, 2007.

[16] L. Riahi, M. Elferchichi, H. Ghazghazi et al., "Phytochemistry, antioxidant and antimicrobial activities of the essential oils of *Mentha rotundifolia* L. in Tunisia," *Industrial Crops and Products*, vol. 49, pp. 883–889, 2013.

[17] A. Alexopoulos, A. C. Kimbaris, S. Plessas et al., "Antibacterial activities of essential oils from eight Greek aromatic plants against clinical isolates of *Staphylococcus aureus*," *Anaerobe*, vol. 17, no. 6, pp. 399–402, 2011.

[18] M. K. Erhan, Ş. C. Bölükbaşi, and H. Ürüşan, "Biological activities of pennyroyal (*Mentha pulegium* L.) in broilers," *Livestock Science*, vol. 146, no. 2-3, pp. 189–192, 2012.

[19] B. Teixeira, A. Marques, C. Ramos et al., "European pennyroyal (*Mentha pulegium*) from Portugal: chemical composition of essential oil and antioxidant and antimicrobial properties of extracts and essential oil," *Industrial Crops and Products*, vol. 36, no. 1, pp. 81–87, 2012.

[20] E. Freitas, A. Aires, E. A. D. S. Rosa, and M. J. Saavedra, "Antibacterial activity and synergistic effect between watercress extracts, 2-phenylethyl isothiocyanate and antibiotics against 11 isolates of *Escherichia coli* from clinical and animal source," *Letters in Applied Microbiology*, vol. 57, no. 4, pp. 266–273, 2013.

[21] S. Dudonné, X. Vitrac, P. Coutiére, M. Woillez, and J.-M. Mérillon, "Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 5, pp. 1768–1774, 2009.

[22] P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken, Eds., *Manual of Clinical Microbiology*, American Society for Microbiology, Washington, DC, USA, 6th edition, 1995.

[23] L. B. Reller, M. Weinstein, J. H. Jorgensen, and M. J. Ferraro, "Antimicrobial susceptibility testing: a review of general principles and contemporary practices," *Clinical Infectious Diseases*, vol. 49, no. 11, pp. 1749–1755, 2009.

[24] M. Simões, R. N. Bennett, and E. A. S. Rosa, "Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms," *Natural Product Reports*, vol. 26, no. 6, pp. 746–757, 2009.

[25] J. M. Andrews, "Determination of minimum inhibitory concentrations," *Journal of Antimicrobial Chemotherapy*, vol. 48, supplement 1, pp. 5–16, 2001.

[26] S. D. Sarker, L. Nahar, and Y. Kumarasamy, "Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals," *Methods*, vol. 42, no. 4, pp. 321–324, 2007.

[27] J. Vrba, V. Kren, J. Vacek, B. Papouskova, and J. Ulrichova, "Quercetin, quercetin glycosides and taxifolin differ in their ability to induce AhR activation and cyp1a1 expression in HepG2 cells," *Phytotherapy Research*, vol. 26, no. 11, pp. 1746–1752, 2012.

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

[29] F. Topal, M. Nar, H. Gocer et al., "Antioxidant activity of taxifolin: an activity–structure relationship," *Journal of Enzyme Inhibition and Medicinal Chemistry*, pp. 1–10, 2015.

[30] N.-Y. Sung, E.-B. Byun, D.-S. Song et al., "Anti-inflammatory action of γ-irradiated genistein in murine peritoneal macrophage," *Radiation Physics and Chemistry*, vol. 105, pp. 17–21, 2014.

[31] A. R. Ndhlala, H. M. Ghebrehiwot, B. Ncube et al., "Antimicrobial, anthelmintic activities and characterisation of functional phenolic acids of achyranthes aspera linn.: a medicinal plant used for the treatment of wounds and Ringworm in East Africa," *Frontiers in Pharmacology*, vol. 6, article 274, 2015.

[32] C. L. Roriz, L. Barros, A. M. Carvalho, C. Santos-Buelga, and I. C. F. R. Ferreira, "Scientific validation of synergistic antioxidant effects in commercialised mixtures of *Cymbopogon citratus* and *Pterospartum tridentatum* or *Gomphrena globosa* for infusions preparation," *Food Chemistry*, vol. 185, pp. 16–24, 2015.

[33] R. I. Moldovan, R. Oprean, D. Benedec et al., "LC-MS analysis, antioxidant and antimicrobial activities for five species of Mentha cultivated in Romania," *Digest Journal of Nanomaterials and Biostructures*, vol. 9, no. 2, pp. 559–566, 2014.

[34] N. Candan and L. Tarhan, "Relationship among chlorophyll-carotenoid content, antioxidant enzyme activities and lipid peroxidation levels by Mg^{2+} deficiency in the *Mentha pulegium* leaves," *Plant Physiology and Biochemistry*, vol. 41, no. 1, pp. 35–40, 2003.

[35] D. K. Maurya and T. P. A. Devasagayam, "Antioxidant and prooxidant nature of hydroxycinnamic acid derivatives ferulic and

caffeic acids," *Food and Chemical Toxicology*, vol. 48, no. 12, pp. 3369–3373, 2010.

[36] H. Hong, M. R. Landauer, M. A. Foriska, and G. D. Ledney, "Antibacterial activity of the soy isoflavone genistein," *Journal of Basic Microbiology*, vol. 46, no. 4, pp. 329–335, 2006.

[37] H. Kuspradini, T. Mitsunaga, and H. Ohashi, "Antimicrobial activity against *Streptococcus sobrinus* and glucosyltransferase inhibitory activity of taxifolin and some flavanonol rhamnosides from kempas (*Koompassia malaccensis*) extracts," *Journal of Wood Science*, vol. 55, no. 4, pp. 308–313, 2009.

[38] J. An, G. Y. Zuo, X. Y. Hao, G. C. Wang, and Z. S. Li, "Antibacterial and synergy of a flavanonol rhamnoside with antibiotics against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA)," *Phytomedicine*, vol. 18, no. 11, pp. 990–993, 2011.

[39] A. C. Grosso, M. M. Costa, L. Ganço et al., "Essential oil composition of *Pterospartum tridentatum* grown in Portugal," *Food Chemistry*, vol. 102, no. 4, pp. 1083–1088, 2007.

[40] M. Mahboubi and G. Haghi, "Antimicrobial activity and chemical composition of *Mentha pulegium* L. essential oil," *Journal of Ethnopharmacology*, vol. 119, no. 2, pp. 325–327, 2008.

[41] K. Morteza-Semnani, M. Saeedi, and M. Akbarzadeh, "Chemical composition and antimicrobial activity of the essential oil of mentha pulegium L," *Journal of Essential Oil-Bearing Plants*, vol. 14, no. 2, pp. 208–213, 2011.

[42] M. T. Coelho, J. C. Gonçalves, V. Alves, and M. M. Martins, "Antioxidant activity and phenolic content of extracts from different *Pterospartum tridentatum* populations growing in Portugal," *Procedia Food Science*, vol. 1, pp. 1454–1458, 2011.

[43] H. Ghazghazi, A. Chedia, M. Weslati et al., "Chemical composition and in vitro antimicrobial activities of mentha pulegium leaves extracts against foodborne pathogens," *Journal of Food Safety*, vol. 33, no. 3, pp. 239–246, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 6505383, 8 pages
http://dx.doi.org/10.1155/2016/6505383

Research Article

Polyphenols from Chilean Propolis and Pinocembrin Reduce MMP-9 Gene Expression and Activity in Activated Macrophages

Nicolás Saavedra,[1] Alejandro Cuevas,[1] Marcela F. Cavalcante,[2] Felipe A. Dörr,[2] Kathleen Saavedra,[1] Tomás Zambrano,[1] Dulcineia S. P. Abdalla,[2] and Luis A. Salazar[1]

[1]*Center of Molecular Biology and Pharmacogenetics, Department of Basic Sciences, Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Avenida Francisco Salazar 01145, 4811230 Temuco, Chile*
[2]*Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, Universidade de São Paulo, Avenida Professor Lineu Prestes 580, 05508-000 São Paulo, SP, Brazil*

Correspondence should be addressed to Luis A. Salazar; luis.salazar@ufrontera.cl

Received 16 December 2015; Accepted 7 March 2016

Academic Editor: Nikos Chorianopoulos

Polyphenols from diverse sources have shown anti-inflammatory activity. In the context of atherosclerosis, macrophages play important roles including matrix metalloproteinases synthesis involved in degradation of matrix extracellular components affecting the atherosclerotic plaque stability. We prepared a propolis extract and pinocembrin in ethanol solution. Propolis extract was chemically characterized using LC-MS. The effect of treatments on gene expression and proteolytic activity was measured *in vitro* using murine macrophages activated with LPS. Cellular toxicity associated with both treatments and the vehicle was determined using MTT and apoptosis/necrosis detection assays. MMP-9 gene expression and proteolytic activity were measured using qPCR and zymography, respectively. Thirty-two compounds were identified in the propolis extract, including pinocembrin among its major components. Treatment with either ethanolic extract of propolis or pinocembrin inhibits MMP-9 gene expression in a dose-dependent manner. Similarly, an inhibitory effect was observed in proteolytic activity. However, the effect showed by ethanolic extract of propolis was higher than the effect of pinocembrin, suggesting that MMP-9 inhibition results from a joint contribution between the components of the extract. These data suggest a potential role of polyphenols from Chilean propolis in the control of extracellular matrix degradation in atherosclerotic plaques.

1. Introduction

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes involved in physiological processes associated with homeostasis regulation, host defense, and tissue repair. These proteins belong to a family of calcium-dependent, zinc-containing endopeptidases that degrade proteins and proteoglycan components of extracellular matrix (ECM) [1]. Diverse cellular types, including connective tissue cells, proinflammatory cells, osteoblasts, endothelial cells, neutrophils, lymphocytes, and macrophages, express MMPs. Regularly, the expression of these enzymes in physiological mechanisms is under strict control, playing an important role in ECM remodeling under normal conditions such as fetal tissue development and postnatal tissue repair [2]. In pathological events, deregulation of MMPs is frequent [3], and excessive breakdown of ECM is observed in connective tissue destruction and remodeling associated with cancer invasion and metastasis [4], cartilage destruction in arthritis [5], and atherosclerotic plaque rupture [6]. More specifically, the deregulation of MMP-9 expression has been associated with tumor invasiveness [4, 7, 8], atherosclerotic plaque rupture in animals with advanced lesions [9], and acute coronary syndrome in humans [10]. MMP-9 or 92-kDa gelatinase is expressed by activated macrophages and foam cells in atheroma plaque [11] and is specialized in the digestion of basement membrane collagens and elastin, facilitating macrophage extravasation [12, 13]. MMP-9 expression is increased in inflammatory, malignant, and degenerative diseases, particularly in acute coronary syndrome in humans,

where circulating MMP-9 levels are increased [10], suggesting that inhibition of MMP-9 activity might have a therapeutic potential.

Propolis is a polyphenol-rich resinous substance collected by honeybees from a variety of plant sources as trees and shrubs. Its colour is variable depending on the plant from which is collected, and its smell is intense and aromatic [14]. It is generally composed by fats, aliphatic and aromatic hydrocarbons, flavonoids, alcohols, terpenes, sugars, and esters. Its chemical composition is very complex and varies according to geographic origin depending on the local flora from which it was produced [15, 16], as well as bee species that performed the collection [17]. This variability results in differences between the biological properties showed by different extracts [18]. Propolis has been used as a complementary medicine since ancient times [19], demonstrating biological activity such as lipid lowering effects and antibacterial, antitumor, and anti-inflammatory effects [20–24]. In our country, there are reports of antifungal activity against *Candida* spp. [25] and hepatoprotective [26] and antioxidant activities [27]. Chemical characterization of Chilean propolis has identified pinocembrin among its main components, which also showed biological activity as an isolated compound [28–30]. Pinocembrin (5,7-dihydroxyflavanone) is one of the primary flavonoids in propolis, can be extracted as a pure compound, and has been incorporated in pharmaceutical industry for its wide range of pharmacological effects [30], including antimicrobial, anti-inflammatory, antioxidant, and anticancer activities [31–33].

Considering these antecedents, the aim of the present study was to evaluate the effect of polyphenols from Chilean propolis and pinocembrin on MMP-9 gene expression and gelatinolytic activity in activated macrophages.

2. Materials and Methods

2.1. Ethanolic Extract of Propolis (EEP) and Pinocembrin. An ethanolic extract was prepared from a propolis sample obtained from southern Chile (Cunco, La Araucanía). The sample was desiccated and frozen at −20°C. Then, the propolis (150 g) was pounded and macerated in 80% ethanol (v/v) protected from light for 30 minutes at 60°C under constant shaking. The resulting solution (EEP) was centrifuged (5000 ×g for 5 minutes) and filtered using first an AP20 prefilter (Millipore, USA) and then a 0.2 μm hydrophobic filter (Millipore, USA), both in a vacuum filtration system. Later, the solvent was removed using a rotary evaporator. Finally, the resulting material was dissolved in a reduced volume of 80% ethanol (v/v) in order to obtain an EEP in which the solvent does not exceed 0.02% v/v of final concentration in the culture medium. Pinocembrin (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) was dissolved using the same solvent as EEP.

2.2. EEP Chemical Characterization. The EEP was characterized by liquid chromatography coupled to diode array detection and mass spectrometry (LC-DAD-MS). Chromatographic separation was achieved in a Shimadzu Prominence (Kyoto, Japan) using a C-18 column (250 × 3.0 mm, 5 μm; Luna C-18(2), Phenomenex, CA, USA) at 40°C. Gradient elution employed (A) water and (B) methanol both with 0.1% formic acid (30 to 60% B in 80 min, 0.5 mL/min). Mass spectrometry data was acquired with an Esquire HCT (Bruker Daltonics, MA, USA) after electrospray ionization in positive and negative modes. UV absorption profiles and fragmentation results (MS/MS) were compared to literature for compound assignments.

2.3. Cell Culture. RAW 264.7 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2.0 g/L sodium bicarbonate, 16.5 mmol/L HEPES, and 1x antibiotic/antimicotic mixture at 37°C in a 5% CO_2 humidified atmosphere.

2.4. Cell Viability. To evaluate the effect of treatments on cell viability, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. RAW 264.5 cells (5.0×10^3/well) were seeded in a 96-well plate in the conditions above described, 12 hours before the experiment. The cells were treated with EEP or pinocembrin at concentrations within 1–10 μg/mL in presence or absence of LPS stimuli (100 ng/mL) during 12 hours. The effect of vehicle was also tested using it like treatments. Then, the medium was replaced by RPMI-without phenol red containing MTT (1 mg/mL, Sigma Chemical Company, St. Louis, MO, USA) and the cells were incubated for 3 hours. After supernatant discarding, the precipitate was dissolved with dimethyl sulfoxide and the optical density was measured at 580 nm using a spectrophotometric microplate reader. Relative viability percentage with respect to the control cells was calculated.

2.5. Necrosis/Apoptosis Detection. As a complementary assay to evaluate cell viability, we performed necrosis/apoptosis detection in cells exposed to EEP, pinocembrin, or vehicle. RAW 264.5 cells (3.0×10^5/well) were seeded in a 24-well plate in the conditions described above, 12 hours before the experiment. The cells were treated with EEP or pinocembrin at concentrations between 1 and 10 μg/mL under LPS stimuli (100 ng/mL) during 12 hours. The effect of the vehicle was also tested. As a positive control of cell death, we used cells exposed to 5% dimethyl sulfoxide. Treated cells were washed and then resuspended in reaction buffer. Annexin V-FITC and propidium iodide were added following the manufacturer's instructions (Sigma Chemical Company, St. Louis, MO, USA). The cells were incubated for 15 minutes at room temperature and were protected from light. Finally, cells were analyzed using a FACS Canto flow cytometer (BD Biosciences, San José, CA, USA). Data analysis was performed using FlowJo version 9.5.1 software (TreeStar). Cells without fluorescence emission were considered to calculate the relative viability percentage with respect to the control.

2.6. Gene Expression. RAW 264.7 cells (4.0×10^5 cells/well) were plated and incubated in the conditions above described during 12 hours to allow cells adherence. Then, the fetal bovine serum content on culture media was reduced to 1%

and the cells were stimulated with LPS 100 ng/mL (Sigma, USA) during 12 hours and were coincubated with noncytotoxic concentrations of EEP, pinocembrin, and the corresponding concentration of vehicle. At the end of treatment period, total RNA was obtained using Trizol® reagent following the manufacturer's indications (Invitrogen, Life Technologies, USA) and then quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, USA). 1 μg of total RNA was reverse-transcribed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems. Inc., USA). PCR assays were performed using a 7300 Real-Time PCR System (Applied Biosystems. Inc., USA) in a reaction containing 50 ng of reverse-transcribed RNA, 200 nM concentration of each primer, and 10 μL of 2x Fast SYBR® Green Master Mix (Applied Biosystems. Inc., USA) on a final volume of 20 μL under cycling conditions recommended by the manufacturer. Primers used were MMP-9F 5′-TG CCC ACC GTC CTT TCT TGT T-3′, MMP-9R 5′-TGC TCG GAT GCA TCT GCA ACT-3′, Rpl13aF 5′-TCC TCA AGA CCA ACG GAC TCC T-3′, and Rpl13aR 5′-AAC CTT TGG TCC CCA CTT CCC T-3′ for MMP-9 and Rpl13, respectively. The relative gene expression was analyzed using the qPCR database [28] in which the corresponding Cq was obtained using the Miner algorithm [29]. Rpl13a was used as reference gene.

2.7. Gelatinolytic Activity. RAW 264.7 cells (4.0×10^5 cells/well) were plated and incubated in standard conditions during 12 hours. Then, the cells were washed and culture medium was replaced by serum-free media. The cells were activated with LPS (100 ng/mL) and coincubated with EEP, pinocembrin, or vehicle during 24 hours. The supernatant medium was collected and the protein concentration was measured using Modified Lowry Protein Assay Kit (Pierce Biotechnology Inc.). Then, 50 μg of proteins was electrophoresed in a 10% acrylamide gel containing 1 mg/mL gelatin. After separation, gels were washed with 2.5% Triton X-100 and incubated for 18 hours in a reaction buffer (0.05 M Tris–HCl (pH 8), 5 mmol/L $CaCl_2$, and 5 mmol/L $ZnCl_2$). Finally, gels were stained with Coomassie Brilliant Blue R250. Clear areas indicating gelatin lysis were quantified using the imageJ 1.46r software (National Institute of Health, USA).

2.8. Statistical Analysis. Results were analyzed using GraphPad Prism version 5.0a (GraphPad Software, San Diego CA, USA). Data are presented as mean ± SD. Differences between groups involving continuous variables were evaluated by one-way ANOVA with Dunnett's posttest in those comparisons when significant differences were detected. Statistical significance was set at $\alpha = 0.05$.

3. Results

3.1. Ethanolic Extract of Propolis Content and LC-DAD-MS Analysis. An ethanolic extract of propolis (EEP) was prepared from a propolis sample obtained from southern Chile (Cunco, La Araucanía). The chemical characterization of EEP by liquid chromatography coupled to diode array detection and mass spectrometry (LC-DAD-MS) detected the presence of 36 compounds, successfully identifying 32 of them. The major components found in the extract were pinocembrin and derivatives of caffeic acid and pinobanksin (Figure 1).

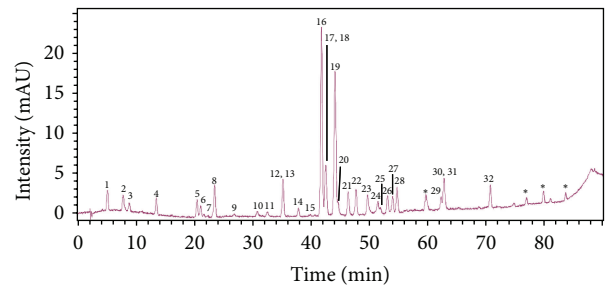


Figure 1: Chromatogram at 290 nm showing the main components found in the ethanolic extract of Chilean propolis. 1: caffeic acid; 2: p-coumaric acid; 3: ferulic/isoferulic acid; 4: 3,4-dimethylcaffeic acid; 5: pinobanksin-5-methyl ether; 6: p-coumaric methyl ester; 7: quercetin; 8: pinobanksin; 9: quercetin-3-methyl ether; 10: pinocembrin-5-methyl ether; 11: apigenin; 12: luteolin-5-methyl ether; 13: cinnamyliden acetic acid; 14: pinobanksin derivative; 15: isorhamnetin; 16: pinocembrin; 17: caffeic acid benzyl ester; 18: caffeic acid isoprenyl ester; 19: pinobanksin-3-*O*-acetate; 20: caffeic acid isoprenyl ester; 21: chrysin; 22: caffeic acid phenethyl ester (CAPE); 23: galangin; 24: chrysin methyl ether; 25: p-coumaric benzyl ester; 26: caffeic acid derivative; 27: pinobanksin-3-O-propionate; 28: caffeic acid cinnamyl ester; 29: pinobanksin-3-O-pentenoate; 30: p-coumaric cinnamyl ester; 31: pinobanksin-3-O-butyrate; 32: pinobanksin-3-O-pentanoate/2-methylbutyrate; ∗: unknown.

3.2. EEP Treatment Does Not Affect Cell Viability in RAW 264.7 Cells. RAW 264.5 cells were treated with EEP or pinocembrin at concentrations within 1–10 μg/mL in presence or absence of lipopolysaccharide (LPS, 100 ng/mL) stimuli. The effect of treatment on cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. Figure 2 shows the relative viability of RAW 264.7 cells under experimental conditions described above. The activation of macrophages using LPS did not show variations with respect to control cells. Similarly, vehicle exposition did not alter the cell viability in either presence or absence of LPS stimuli. Regarding treatment exposition of activated RAW 264.7 cells, EEP and pinocembrin did not show significant variations at explored concentrations. Additionally, we assessed cell viability relative to control using a necrosis/apoptosis detection assay, in which no differences were observed up to 7.5 μg/mL of EEP. In pinocembrin and vehicle treated cells, no significant changes were observed (Figure 3).

3.3. Inhibition of MMP-9 Expression by EEP Treatment in RAW 264.7 Cells. The mRNA expression of MMP-9 in RAW 264.7 cells was evaluated by quantitative real-time PCR (qRT-PCR). The vehicle did not affect MMP-9 gene expression in conditions of LPS-stimulation and unstimulated cells. Moreover, treatment with EEP showed a significant reduction in a dose dependent manner with higher inhibition at the highest

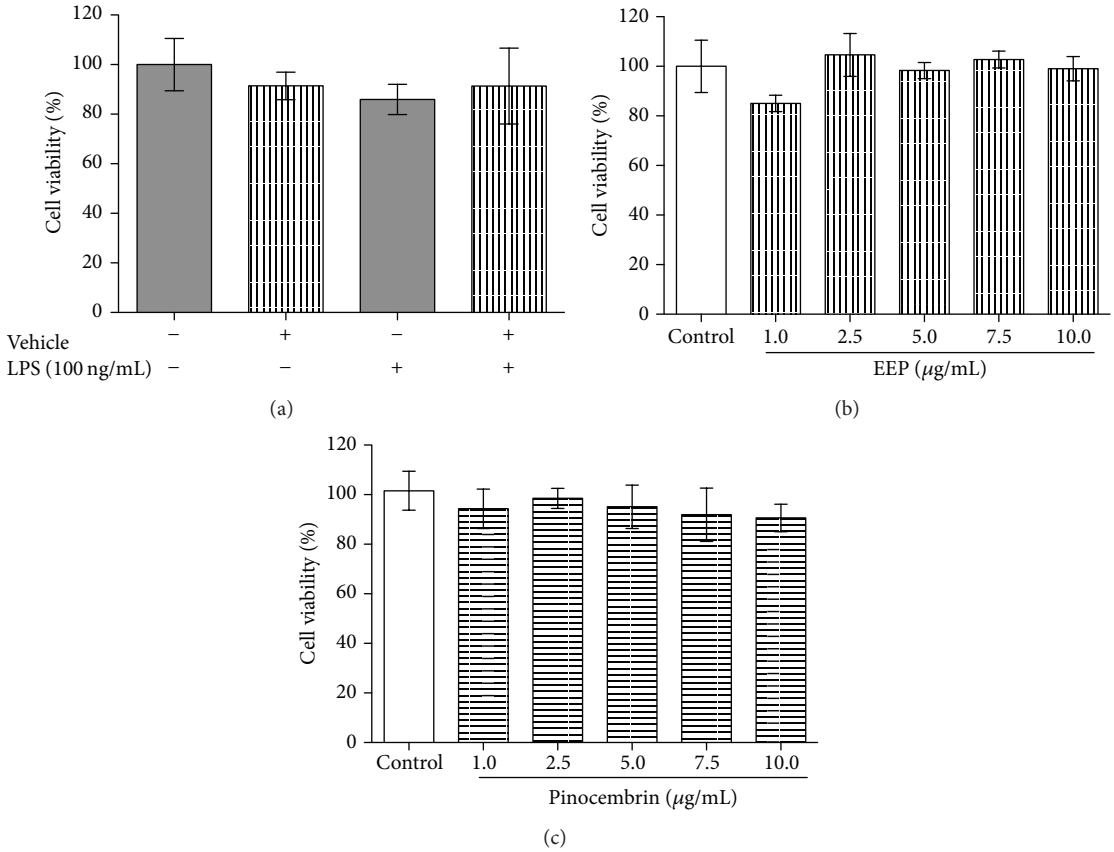


Figure 2: MTT cell viability assay for ethanolic extract of polyphenols, pinocembrin, or vehicle exposed cells. (a) Cell viability of vehicle exposed RAW 264.7 cells in presence or absence of LPS stimuli (100 ng/mL). (b) and (c) Effect of EEP and pinocembrin on cell viability of RAW 264.7 cells under LPS stimulus.

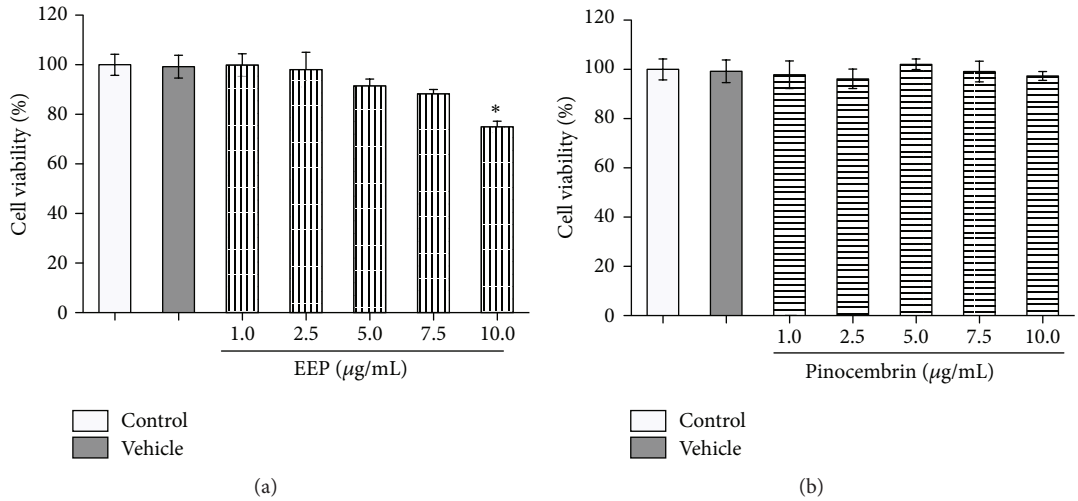


Figure 3: Necrosis/apoptosis detection in activated RAW 264.7 cells exposed to ethanolic extract of propolis, pinocembrin, and vehicle. (a) Effect of EEP (1–10 μg/mL) on cell viability by necrosis/apoptosis detection. (b) Effect of pinocembrin treatment (1–10 μg/mL) on cell viability by necrosis/apoptosis detection in RAW 264.7 cells under LPS stimulus. *ANOVA: $p = 0.004$; Dunnett's multiple comparison test: $p < 0.05$.

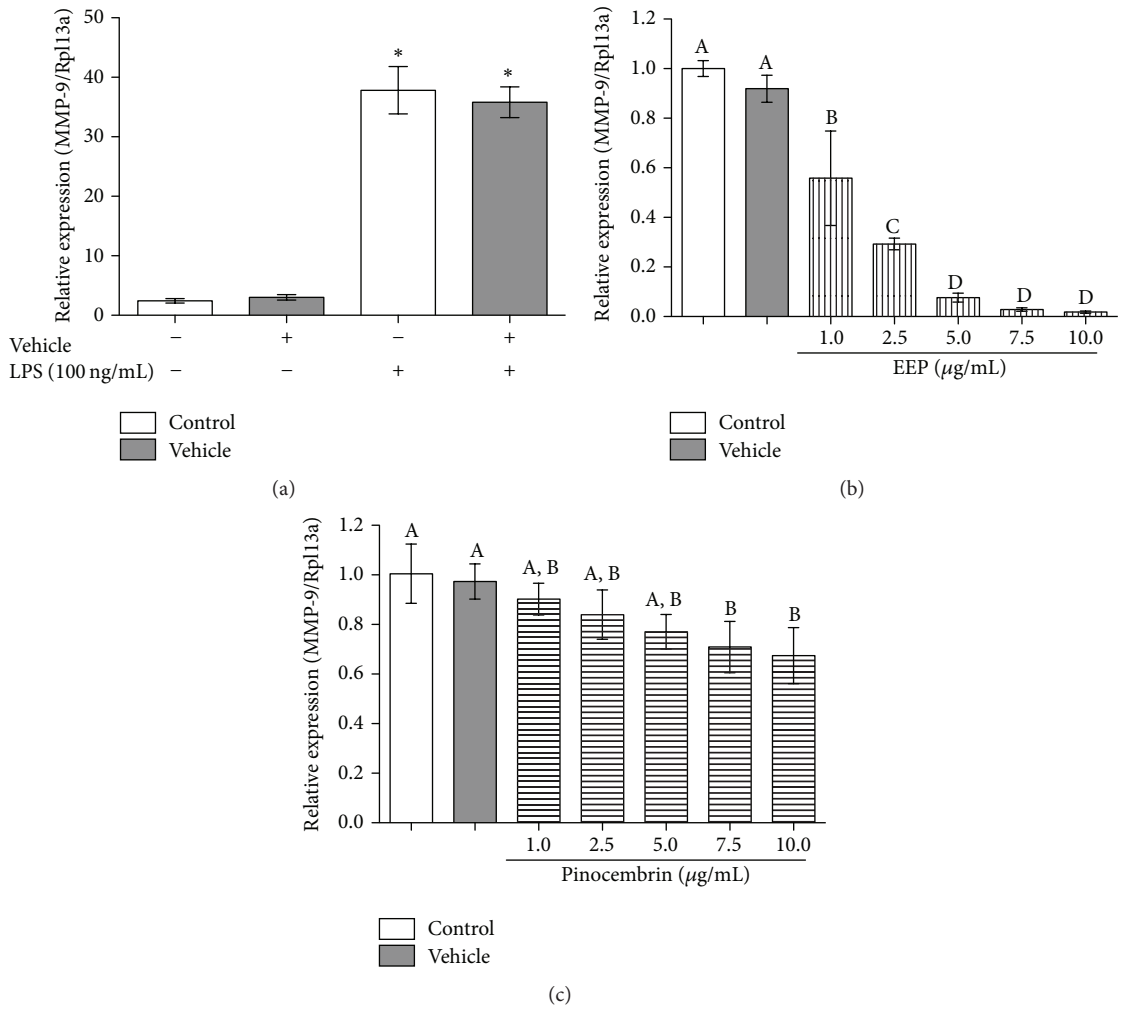


Figure 4: Effect of ethanolic extract of propolis, pinocembrin, and vehicle on relative gene expression of MMP-9. (a) Vehicle induced effect on MMP-9 gene expression in presence or absence of LPS stimulus. (b) Effect of EEP on MMP-9 relative gene expression. (c) Effect of pinocembrin on MMP-9 relative gene expression. Different letters indicate significant differences. *ANOVA: $p < 0.0001$; Tukey's Multiple Comparison Test: $p < 0.05$.

concentrations of treatment. Similarly, pinocembrin treated cells showed an inhibitory effect on MMP-9 mRNA expression. However, the effect exerted by pinocembrin treatment was lesser than the effect showed by the EEP treatment (Figure 4).

3.4. Inhibition of MMP-9 Activity by EEP Treatment. Finally, we evaluated the effect of EEP and pinocembrin on the gelatinolytic activity of MMP-9 secreted by activated macrophages. In cells treated with EEP, we observed a significant reduction of collagen degradation starting at 2.5 μg/mL, with an increasing effect at higher EEP concentrations. Pinocembrin treated cells also showed a significant reduction of collagen degradation, but to a lesser extent than EEP, affecting the gelatinolytic activity from 7.5 μg/mL of treatment (Figure 5).

4. Discussion

Biologic activity of polyphenols from several sources has been widely studied. Among the common sources, propolis offers complex mixtures to evaluate the joint effect of its constituents. The chemical composition of propolis samples is determined by factors as botanical and geographical origin [34]. These factors define a characteristic pattern of compounds, referred to as propolis fingerprinting [35]. South American propolis contains certain predominant compounds as Artepillin C and p-Coumaric acid found in Brazilian propolis [36, 37] and pinocembrin in Chilean propolis [28, 29]. In the present study, we used a propolis sample collected from southern Chile. As aforementioned, pinocembrin was one of the predominant components. However, considering the large amount of caffeic acid and pinobanksin derivatives, probably these two compounds could also influence its biological activities and should be studied separately.

Evidence of biological activity of Chilean propolis has been shown in previous works by our group [38–41]. The present study demonstrates an inhibitory effect of EEP and pinocembrin on both gene expression and gelatinolytic

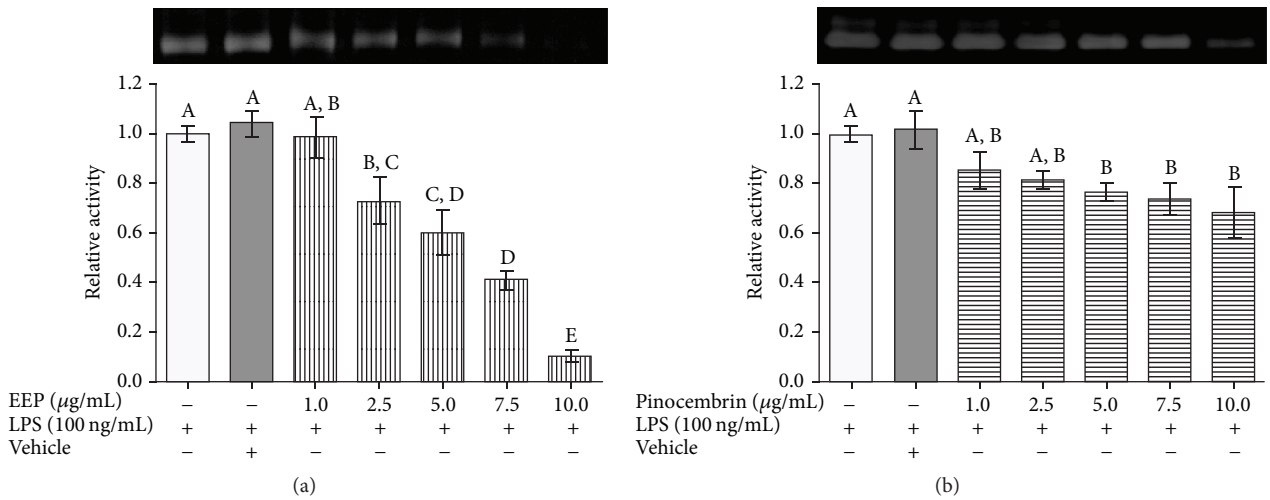


Figure 5: Effect of ethanolic extract of propolis, pinocembrin, and vehicle on gelatinolytic activity of MMP-9. (a) Effect of EEP on MMP-9 relative proteolytic activity. (b) MMP-9 relative proteolytic activity of pinocembrin treated cells. Different letters indicate significant differences.

activity of MMP-9 using a cellular model of activated macrophages (LPS 100 ng/mL). LPS stimuli result in the induction of numerous inflammatory mediators as cytokines and chemokines including TNF-α, IL-1, IL-6, and MCP-1. This effect is associated with the activity of inflammation-related transcription factors as NF-kappa B [42], also involved in MMP-9 expression [43]. In atherosclerosis development, matrix metalloproteinases are mainly secreted by macrophages. These enzymes are involved in vascular remodeling allowing the adaptation of affected vessel to the vascular injury in order to maintain the lumen diameter, mechanism modulated by wall components and extracellular matrix, especially by its degradation. However, MMP-9 proteolytic activity has been associated with the progression of atherosclerotic plaques to a vulnerable state and consequently to the development of ischemic events [10, 44–47]. Matrix metalloproteinases are also involved in other disease-associated processes such as cell invasion and metastasis in cancer [48]. In this context, using an *in vitro* model of hepatocellular carcinoma, treatment with polyphenols from propolis in concentrations similar to those used in the present work did inhibit the activity of MMP-9, similar to the effect associated with caffeic acid phenethyl ester treatment obtained from the propolis sample [49]. This effect on MMP-9 has also been demonstrated by other isolated compounds as kaempferol, apigenin, resveratrol, and quercetin [50, 51]. Pinocembrin exhibits antibacterial, anti-inflammatory, anticancer, and neuroprotective activities [30], and the anti-inflammatory effect exerted by pinocembrin has been associated with suppression of IκBα, JNK, and p38MAPK activation [30, 52], signaling pathways involved in MMP-9 induction in LPS-stimulated macrophages [53, 54]. Our study compared the effect of pinocembrin and EEP, obtaining an inhibitory effect on both gene expression and proteolytic activity. However, the modulation demonstrated using this particular flavonoid as treatment was lesser than that exhibited by EEP, which has, among its components, detectable amounts of all compounds listed above except for resveratrol. So, the observed effect can be a product of the joint activity of identified compounds. In conclusion, polyphenolic components of Chilean propolis show a significant inhibition of MMP-9 gene expression and activity, suggesting a potential role in the control of extracellular matrix degradation in atherosclerotic plaques and subsequently on plaque stability.

5. Conclusion

In summary, our results indicate that components of Chilean propolis showed a significant inhibition of MMP-9 gene expression and activity, suggesting a potential role in the control of extracellular matrix degradation in atherosclerotic plaques and subsequently on plaque stability.

Competing Interests

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

Acknowledgments

Nicolás Saavedra, Alejandro Cuevas, Kathleen Saavedra, and Tomás Zambrano were the recipients of fellowships from CONICYT, Chile. This study was supported by grants from CONICYT (Chile), FAPESP-Brazil, UFRO-FAPESP 2014/50238-6, and CAPES-Brazil. Nicolás Saavedra is the recipient of Postdoctoral Fellowship from Convenio de Desempeño, Universidad de La Frontera.

References

[1] I. Massova, L. P. Kotra, R. Fridman, and S. Mobashery, "Matrix metalloproteinases: structures, evolution, and diversification," *The FASEB Journal*, vol. 12, no. 12, pp. 1075–1095, 1998.

[2] J. F. Strauss III, "Extracellular matrix dynamics and fetal membrane rupture," *Reproductive Sciences*, vol. 20, no. 2, pp. 140–153, 2013.

[3] L. Nissinen and V.-M. Kähäri, "Matrix metalloproteinases in inflammation," *Biochimica et Biophysica Acta*, vol. 1840, no. 8, pp. 2571–2580, 2014.

[4] A. Merdad, S. Karim, H.-J. Schulten et al., "Expression of matrix metalloproteinases (MMPs) in primary human breast cancer: MMP-9 as a potential biomarker for cancer invasion and metastasis," *Anticancer Research*, vol. 34, no. 3, pp. 1355–1366, 2014.

[5] L. Lipari and A. Gerbino, "Expression of gelatinases (MMP-2, MMP-9) in human articular cartilage," *International Journal of Immunopathology and Pharmacology*, vol. 26, no. 3, pp. 817–823, 2013.

[6] A. C. Newby, "Metalloproteinases promote plaque rupture and myocardial infarction: a persuasive concept waiting for clinical translation," *Matrix Biology*, vol. 44–46, pp. 157–166, 2015.

[7] J. Chen, X. Liu, H. Jiao et al., "Prognostic and clinical significance of STAT3 and MMP9 in patients with gastric cancer: a meta-analysis of a Chinese cohort," *International Journal of Clinical and Experimental Medicine*, vol. 8, no. 1, pp. 546–557, 2015.

[8] G. Shay, C. C. Lynch, and B. Fingleton, "Moving targets: emerging roles for MMPs in cancer progression and metastasis," *Matrix Biology*, vol. 44–46, pp. 200–206, 2015.

[9] P. J. Gough, I. G. Gomez, P. T. Wille, and E. W. Raines, "Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice," *The Journal of Clinical Investigation*, vol. 116, no. 1, pp. 59–69, 2006.

[10] I. M. Loftus, A. R. Naylor, S. Goodall et al., "Increased matrix metalloproteinase-9 activity in unstable carotid plaques. A potential role in acute plaque disruption," *Stroke*, vol. 31, no. 1, pp. 40–47, 2000.

[11] Z. S. Galis, G. K. Sukhova, R. Kranzhöfer, S. Clark, and P. Libby, "Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 2, pp. 402–406, 1995.

[12] G. Cimmino, M. Ragni, P. Cirillo et al., "C-reactive protein induces expression of matrix metalloproteinase-9: a possible link between inflammation and plaque rupture," *International Journal of Cardiology*, vol. 168, no. 2, pp. 981–986, 2013.

[13] F. Chen, P. Eriksson, G. K. Hansson et al., "Expression of matrix metalloproteinase 9 and its regulators in the unstable coronary atherosclerotic plaque," *International Journal of Molecular Medicine*, vol. 15, no. 1, pp. 57–65, 2005.

[14] P. G. Pietta, C. Gardana, and A. M. Pietta, "Analytical methods for quality control of propolis," *Fitoterapia*, vol. 73, supplement 1, pp. S7–S20, 2002.

[15] A. Daugsch, C. S. Moraes, P. Fort, and Y. K. Park, "Brazilian red propolis—chemical composition and botanical origin," *Evidence-Based Complementary and Alternative Medicine*, vol. 5, no. 4, pp. 435–441, 2008.

[16] M. I. Nieva Moreno, M. I. Isla, N. G. Cudmani, M. A. Vattuone, and A. R. Sampietro, "Screening of antibacterial activity of Amaicha del Valle (Tucuman, Argentina) propolis," *Journal of Ethnopharmacology*, vol. 68, no. 1–3, pp. 97–102, 1999.

[17] S. Silici and S. Kutluca, "Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region," *Journal of Ethnopharmacology*, vol. 99, no. 1, pp. 69–73, 2005.

[18] V. S. Bankova, S. L. de Castro, and M. C. Marcucci, "Propolis: recent advances in chemistry and plant origin," *Apidologie*, vol. 31, no. 1, pp. 3–15, 2000.

[19] S. Castaldo and F. Capasso, "Propolis, an old remedy used in modern medicine," *Fitoterapia*, vol. 73, supplement 1, pp. S1–S6, 2002.

[20] Y.-B. Ghasem, A. Ownagh, and M. Hasanloei, "Antibacterial and antifungal activity of Iranian propolis against *Staphylococcus aureus* and *Candida albicans*," *Pakistan Journal of Biological Sciences*, vol. 10, no. 8, pp. 1343–1345, 2007.

[21] A. Uzel, K. Sorkun, Ö. Önçağ, D. Çoğulu, Ö. Gençay, and B. Salih, "Chemical compositions and antimicrobial activities of four different Anatolian propolis samples," *Microbiological Research*, vol. 160, no. 2, pp. 189–195, 2005.

[22] I. Ichi, H. Hori, Y. Takashima et al., "The beneficial effect of propolis on fat accumulation and lipid metabolism in rats fed a high-fat diet," *Journal of Food Science*, vol. 74, no. 5, pp. H127–H131, 2009.

[23] H. Pratsinis, D. Kletsas, E. Melliou, and I. Chinou, "Antiproliferative activity of Greek propolis," *Journal of Medicinal Food*, vol. 13, no. 2, pp. 286–290, 2010.

[24] M. Viuda-Martos, Y. Ruiz-Navajas, J. Fernández-López, and J. A. Pérez-Álvarez, "Functional properties of honey, propolis, and royal jelly," *Journal of Food Science*, vol. 73, no. 9, pp. R117–R124, 2008.

[25] C. L. Herrera, M. Alvear, L. Barrientos, G. Montenegro, and L. A. Salazar, "The antifungal effect of six commercial extracts of Chilean propolis on *Candida* spp," *Ciencia e Investigación Agraria*, vol. 37, no. 1, pp. 75–84, 2010.

[26] C. L. Herrera, O. Fritz, G. Montenegro, M. Alvear, M. del Sol, and L. A. Salazar, "El Propóleos Reduce la Esteatosis Hepática Inducida por Dieta en Ratones," *International Journal of Morphology*, vol. 28, no. 1, pp. 75–84, 2010.

[27] A. Russo, V. Cardile, F. Sanchez, N. Troncoso, A. Vanella, and J. A. Garbarino, "Chilean propolis: antioxidant activity and antiproliferative action in human tumor cell lines," *Life Sciences*, vol. 76, no. 5, pp. 545–558, 2004.

[28] S. Kumazawa, T. Hamasaka, and T. Nakayama, "Antioxidant activity of propolis of various geographic origins," *Food Chemistry*, vol. 84, no. 3, pp. 329–339, 2004.

[29] C. L. Herrera, M. Alvear, L. Barrientos, G. Montenegro, and L. A. Salazar, "The antifungal effect of six commercial extracts of *Chilean propolis* on *Candida* spp.," *Ciencia e Investigacion Agraria*, vol. 37, no. 1, pp. 75–84, 2010.

[30] A. Rasul, F. M. Millimouno, W. Ali Eltayb, M. Ali, J. Li, and X. Li, "Pinocembrin: a novel natural compound with versatile pharmacological and biological activities," *BioMed Research International*, vol. 2013, Article ID 379850, 9 pages, 2013.

[31] M. A. Saad, R. M. Abdel Salam, S. A. Kenawy, and A. S. Attia, "Pinocembrin attenuates hippocampal inflammation, oxidative perturbations and apoptosis in a rat model of global cerebral ischemia reperfusion," *Pharmacological Reports*, vol. 67, no. 1, pp. 115–122, 2015.

[32] C.-L. Hsu, Y.-S. Yu, and G.-C. Yen, "Anticancer effects of Alpinia pricei Hayata roots," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 4, pp. 2201–2208, 2010.

[33] X. Lan, W. Wang, Q. Li, and J. Wang, "The natural flavonoid pinocembrin: molecular targets and potential therapeutic applications," *Molecular Neurobiology*, vol. 53, no. 3, pp. 1794–1801, 2016.

[34] V. Bankova, "Recent trends and important developments in propolis research," *Evidence-Based Complementary and Alternative Medicine*, vol. 2, no. 1, pp. 29–32, 2005.

[35] J. Zhou, Y. Li, J. Zhao, X. Xue, L. Wu, and F. Chen, "Geographical traceability of propolis by high-performance liquid-chromatography fingerprints," *Food Chemistry*, vol. 108, no. 2, pp. 749–759, 2008.

[36] Y. K. Park, J. F. Paredes-Guzman, C. L. Aguiar, S. M. Alencar, and F. Y. Fujiwara, "Chemical constituents in *Baccharis dracunculifolia* as the main botanical origin of southeastern brazilian propolis," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 5, pp. 1100–1103, 2004.

[37] M. C. Marcucci, F. Ferreres, C. García-Viguera et al., "Phenolic compounds from Brazilian propolis with pharmacological activities," *Journal of Ethnopharmacology*, vol. 74, no. 2, pp. 105–112, 2001.

[38] L. Barrientos, C. L. Herrera, G. Montenegro et al., "Chemical and botanical characterization of chilean propolis and biological activity on cariogenic bacteria *Streptococcus mutans* and *Streptococcus sobrinus*," *Brazilian Journal of Microbiology*, vol. 44, no. 2, pp. 577–585, 2013.

[39] N. Saavedra, L. Barrientos, C. L. Herrera, M. Alvear, G. Montenegro, and L. A. Salazar, "Effect of Chilean propolis on cariogenic bacteria *Lactobacillus fermentum*," *Ciencia e Investigación Agraria*, vol. 38, no. 1, pp. 117–125, 2011.

[40] A. Pacheco, J. B. Daleprane, V. S. Freitas et al., "Effect of chilean propolis on glucose metabolism in diabetic mice," *International Journal of Morphology*, vol. 29, no. 3, pp. 754–761, 2011.

[41] J. B. Daleprane, V. da Silva Freitas, A. Pacheco et al., "Anti-atherogenic and anti-angiogenic activities of polyphenols from propolis," *The Journal of Nutritional Biochemistry*, vol. 23, no. 6, pp. 557–566, 2012.

[42] M. Guha and N. Mackman, "LPS induction of gene expression in human monocytes," *Cellular Signalling*, vol. 13, no. 2, pp. 85–94, 2001.

[43] W. R. Jae, K.-W. Lee, D. Kim et al., "NF-κB-dependent regulation of matrix metalloproteinase-9 gene expression by lipopolysaccharide in a macrophage cell line RAW 264.7," *Journal of Biochemistry and Molecular Biology*, vol. 40, no. 1, pp. 88–94, 2007.

[44] I. M. Loftus, A. R. Naylor, P. R. F. Bell, and M. M. Thompson, "Matrix metalloproteinases and atherosclerotic plaque instability," *British Journal of Surgery*, vol. 89, no. 6, pp. 680–694, 2002.

[45] J. D. Raffetto and R. A. Khalil, "Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease," *Biochemical Pharmacology*, vol. 75, no. 2, pp. 346–359, 2008.

[46] G. Pasterkamp, A. H. Schoneveld, D. J. Hijnen et al., "Atherosclerotic arterial remodeling and the localization of macrophages and matrix metalloproteases 1, 2 and 9 in the human coronary artery," *Atherosclerosis*, vol. 150, no. 2, pp. 245–253, 2000.

[47] S. Blankenberg, H. J. Rupprecht, O. Poirier et al., "Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease," *Circulation*, vol. 107, no. 12, pp. 1579–1585, 2003.

[48] E. I. Deryugina and J. P. Quigley, "Matrix metalloproteinases and tumor metastasis," *Cancer and Metastasis Reviews*, vol. 25, no. 1, pp. 9–34, 2006.

[49] U.-H. Jin, T.-W. Chung, S.-K. Kang et al., "Caffeic acid phenyl ester in propolis is a strong inhibitor of matrix metalloproteinase-9 and invasion inhibitor: isolation and identification," *Clinica Chimica Acta*, vol. 362, no. 1-2, pp. 57–64, 2005.

[50] J. Majtan, J. Bohova, R. Garcia-Villalba et al., "Fir honeydew honey flavonoids inhibit TNF-α-induced MMP-9 expression in human keratinocytes: a new action of honey in wound healing," *Archives of Dermatological Research*, vol. 305, no. 7, pp. 619–627, 2013.

[51] E. Scoditti, N. Calabriso, M. Massaro et al., "Mediterranean diet polyphenols reduce inflammatory angiogenesis through MMP-9 and COX-2 inhibition in human vascular endothelial cells: a potentially protective mechanism in atherosclerotic vascular disease and cancer," *Archives of Biochemistry and Biophysics*, vol. 527, no. 2, pp. 81–89, 2012.

[52] L. W. Soromou, X. Chu, L. Jiang et al., "In vitro and in vivo protection provided by pinocembrin against lipopolysaccharide-induced inflammatory responses," *International Immunopharmacology*, vol. 14, no. 1, pp. 66–74, 2012.

[53] C.-H. Woo, J.-H. Lim, and J.-H. Kim, "Lipopolysaccharide induces matrix metalloproteinase-9 expression via a mitochondrial reactive oxygen species-p38 kinase-activator protein-1 pathway in raw 264.7 cells," *Journal of Immunology*, vol. 173, no. 11, pp. 6973–6980, 2004.

[54] Y.-S. Lee, H. T. L. Tran, and V. T. Quang, "Regulation of expression of matrix metalloproteinase-9 by JNK in Raw 264.7 cells: presence of inhibitory factor(s) suppressing MMP-9 induction in serum and conditioned media," *Experimental and Molecular Medicine*, vol. 41, no. 4, pp. 259–268, 2009.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 1068528, 10 pages
http://dx.doi.org/10.1155/2016/1068528

Research Article

Dietary Flavonoid Hyperoside Induces Apoptosis of Activated Human LX-2 Hepatic Stellate Cell by Suppressing Canonical NF-κB Signaling

Liwen Wang,[1,2] Zhiwei Yue,[3] Mengzheng Guo,[2] Lianying Fang,[2] Liang Bai,[1] Xinyu Li,[1] Yongqing Tao,[1] Suying Wang,[1] Qiang Liu,[2] Dexian Zhi,[1] and Hui Zhao[1]

[1]*Tianjin Key Laboratory of Food and Biotechnology, School of Biotechnology and Food Science, Tianjin University of Commerce, Tianjin 300134, China*
[2]*Tianjin Key Laboratory of Radiation Medicine and Molecular Nuclear Medicine, Institute of Radiation Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300192, China*
[3]*Department of Hematology and Tangshan Key Laboratory, Translational Medical Center, North China University of Science and Technology, Tangshan, Hebei 063000, China*

Correspondence should be addressed to Qiang Liu; liuqiang@irm-cams.ac.cn, Dexian Zhi; zdx@tjcu.edu.cn, and Hui Zhao; zhaohui@tjcu.edu.cn

Received 4 January 2016; Accepted 1 March 2016

Academic Editor: Yiannis Kourkoutas

Hyperoside, an active compound found in plants of the genera *Hypericum* and *Crataegus*, is reported to exhibit antioxidant, anticancer, and anti-inflammatory activities. Induction of hepatic stellate cell (HSC) apoptosis is recognized as a promising strategy for attenuation of hepatic fibrosis. In this study, we investigated whether hyperoside treatment can exert antifibrotic effects in human LX-2 hepatic stellate cells. We found that hyperoside induced apoptosis in LX-2 cells and decreased levels of α-smooth muscle actin (α-SMA), type I collagen, and intracellular reactive oxygen species (ROS). Remarkably, hyperoside also inhibited the DNA-binding activity of the transcription factor NF-κB and altered expression levels of NF-κB-regulated genes related to apoptosis, including proapoptotic genes *Bcl-Xs*, *DR4*, *Fas*, and *FasL* and anti-apoptotic genes *A20*, *c-IAP1*, *Bcl-*X_L, and *RIP1*. Our results suggest that hyperoside may have potential as a therapeutic agent for the treatment of liver fibrosis.

1. Introduction

Liver fibrosis is a major cause of morbidity and mortality worldwide due to chronic viral hepatitis and, more recently, from fatty liver disease associated with obesity [1]. Hepatic fibrosis is largely asymptomatic. However, progressive fibrosis resulting in cirrhosis can cause distortion of the liver parenchyma and vascular architecture [2], of which major clinical consequences are impaired liver function, an increased portal hypertension, and the development of hepatocellular carcinoma [3]. An epidemiological analysis has identified liver cirrhosis as a leading cause of disease-related death worldwide [4].

Liver fibrosis is a reversible, progressive pathological process characterized by excess accumulation of extracellular matrix (ECM) proteins. Hepatic stellate cells (HSCs) are quiescent, nonproliferative, vitamin-A storing cells; they are localized to the space of Disse and function as the principal storage sites of retinoids in normal liver. HSCs are the principal cell type involved in liver fibrogenesis, and the survival of activated HSCs is the hallmark feature of liver fibrosis. Upon activation, HSCs proliferate and undergo transdifferentiation from quiescent cells to activated myofibroblast-like cells secreting excess ECM proteins [5]. Antifibrotic drug research focuses on the inhibition of HSC activation or proliferation and the promotion of apoptosis in activated HSCs [6, 7]. Given the current lack of successful treatment options for liver fibrosis, new strategies for slowing this process are urgently needed.

In addition to the activation of fibroblasts and immune cells, fibrosis-inducing events also cause release of profibrotic metabolites such as reactive oxygen species (ROS). ROS are critical intermediates in both liver physiology and pathology. Recent research indicates that oxidative stress and the antioxidant system may also be critical for the development and persistence of fibrosis [8]. In addition to ROS, the transcription factor nuclear factor-kappa B (NF-κB) is also essential for liver cell survival and liver homeostasis [9]. The regulation of cell death, inflammation, and wound healing by NF-κB not only emphasizes the role of this transcription factor in the progression of liver diseases, but also highlights the mechanistic links between liver injury, inflammation, fibrosis, and hepatocellular carcinoma [10]. Several studies have indicated that NF-κB inhibition is a potent mechanism for the induction of HSC apoptosis [11, 12]. Hence, when NF-κB activation is prevented or inhibited, apoptosis of activated HSCs is enhanced.

Natural products have recently attracted much attention in drug research because they have frequently served as major sources of chemical diversity for novel biomedical agents and pharmaceutical discovery. Flavonoids are plant polyphenols found in vegetables, fruits, and plant-based beverages and are well known for their physiological antipyretic, analgesic, and anti-inflammatory activities [13]. Hyperoside (also called quercetin 3-O-b-d-galactoside; Figure 1(a)), a major pharmacologically active component from the genera *Hypericum* and *Crataegus* [14], has been demonstrated to possess numerous biological functions, including cardioprotective [15], antiredox [16], and anti-inflammatory activities [17]. Hyperoside also displays antiviral activity against hepatitis B in HepG2 cells transfected with hepatitis B viral genome, via the suppression of hepatitis B antigen secretion [18]. However, little research has been conducted on the potential roles and mechanisms of hyperoside in the treatment of liver fibrosis. In the present study, we utilized the human HSC line LX-2, which preserves key features of primary HSCs critical for liver fibrosis research [19], to investigate the molecular mechanisms of the proapoptotic effects of hyperoside.

2. Materials and Methods

2.1. Cell Culture. LX-2 cells were gifted by Dr. Zhigang Bai, Liver Research Center, Beijing Friendship Hospital, Beijing, China. Cells were cultured in RPMI-1640 medium (Thermo Fisher, Beijing, China) containing 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. Hyperoside (Biopurify, Chengdu, China) was dissolved in dimethyl sulfoxide (DMSO, Solarbio, Beijing, China) and added at the concentrations indicated.

2.2. Cell Viability Assay. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, M5655, USA) was used as an indicator of cell viability. Cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were washed with fresh medium, followed by treatment with hyperoside. After a 24 h or 48 h incubation period, the cells were washed, and 100 μL of MTT (1 mg/mL) was added, followed by incubation for 4 h. Finally, DMSO (150 μL) was added in order to solubilize the formazan salt formed, and the amount of formazan salt was determined by measuring the OD at 540 nm using a microplate reader (Synergy HT; BioTek Instruments).

2.3. Apoptosis Quantification. Detection of apoptosis was performed with an Annexin V-FITC/PI kit (BD Biosciences, New Jersey, USA) according to the manufacturer's instructions. Cells were seeded in 6-well plate (1×10^6 cells/well) and treated with different concentrations (0.5 μM, 1.0 μM, or 2.0 μM) of hyperoside at 37°C for 24 h or 48 h. Cells were harvested, washed twice with ice-cold PBS, and resuspended in 1 mL binding buffer. Resuspended cells (100 μL) were transferred to a 1.5 mL EP tube and 5 μL Annexin V-FITC plus 5 μL PI was added. The tube was gently vortexed and incubated for 15 min at room temperature in the dark. Binding buffer (400 μL) was then added and the cells were analyzed immediately by flow cytometry. Percentage of Annexin V-FITC$^+$ cells was represented as apoptosis rate [20].

2.4. Western Blotting Analysis. Protein was extracted from LX-2 cells prepared with ice-cold lysis buffer (P1016, Solarbio Science and Technology, Beijing, China). Protein concentration in cell lysates was determined with a BCA protein kit (Beyotime, Shanghai, China). Samples containing equal amounts of protein (20 g) were mixed with loading buffer containing 5% 2-mercaptoethanol, heated for 5 min at 95°C, loaded onto a 10% SDS-PAGE gel, and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% milk and 0.1% Tween 20 in Tris-buffered saline (TBS), membranes were incubated overnight at 4°C with primary antibody. Antibodies against α-SMA (BM0002) and collagen I (BA0325) were purchased from Boster (Wuhan, China); β-tubulin (CW0098) was purchased from CWBIO (Beijing, China). Membranes were then incubated with the appropriate horseradish peroxide conjugated secondary antibody at room temperature. Protein bands were detected using ECL (WBKLS0100, Millipore, Billerica, MA, USA), according to the manufacturer's protocols. Experiments were repeated three times.

2.5. Intracellular Reactive Oxygen Species Assay. The production of intracellular reactive oxygen species was measured by DCFH oxidation. The DCF-DA reagent passively enters cells, where it is deacetylated by esterases into nonfluorescent DCFH. Inside the cell, DCFH reacts with ROS to form DCF, a fluorescent product. For this assay, 10 mM DCF-DA (S0033, Beyotime, Jiangsu, China) was dissolved in methanol and diluted 1000-fold in RPMI-1640 medium to give a final concentration of 10 μM DCF-DA. Hyperoside treated LX-2-enriched cultures seeded (5×10^4/well) in 96-well plates were then incubated with DCFH-DA for 20 minutes at 37°C. Immediately after incubation, DCF fluorescence was read at 485 nm excitation and 530 nm emission by flow cytometry.

2.6. NF-κB DNA-Binding Activity. The Trans-AM NF-κB p65 Transcription Factor Assay kit (Active Motif North America, Carlsbad, CA) was used to quantify the DNA-binding activity

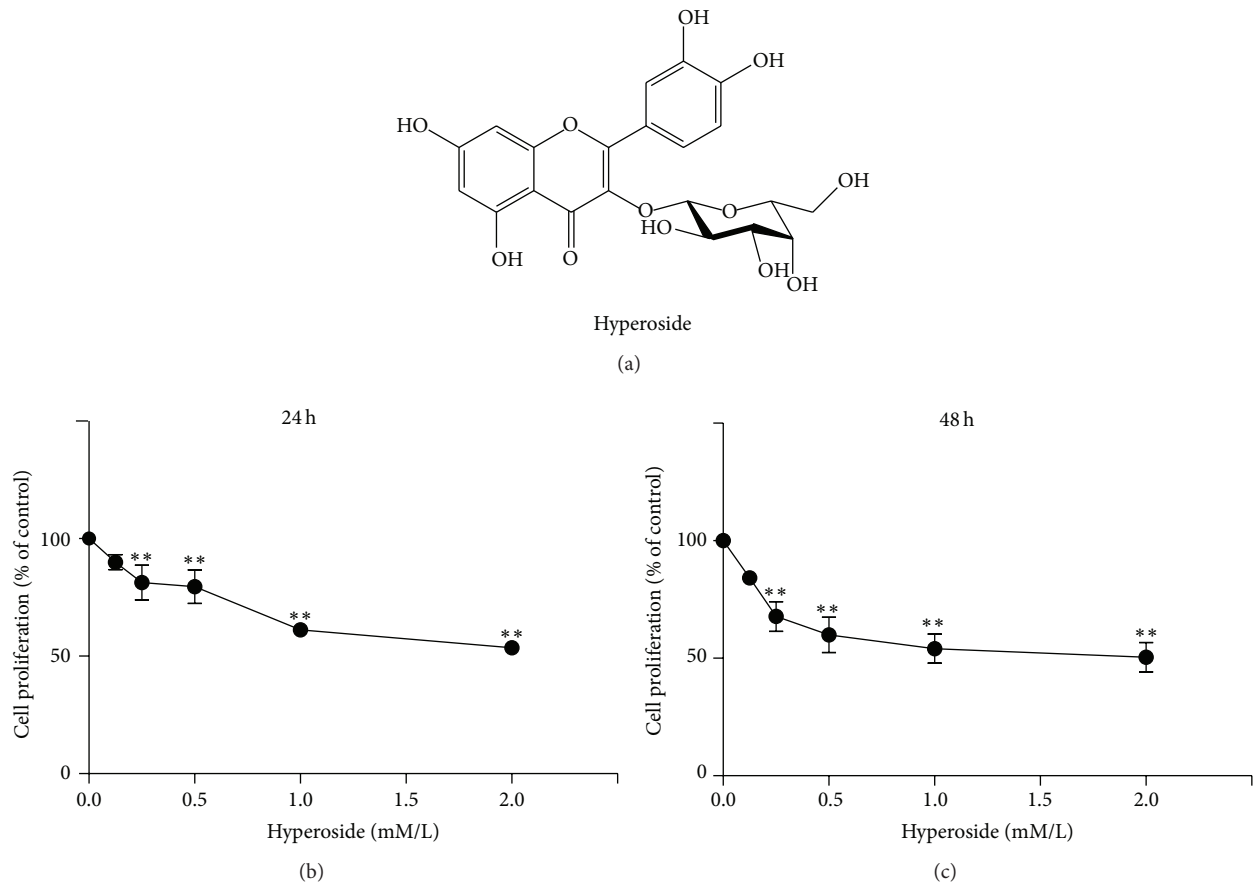


Figure 1: Structure of hyperoside and its inhibitory proliferation effect on LX-2 cells. (a) Chemical structure of hyperoside. (b) Effect of hyperoside on the growth of LX-2 cell lines for 24 h. (c) Effect of hyperoside on the growth of LX-2 cell lines for 48 h. Cell proliferation was analyzed using MTT assay. Cells were treated with different concentration of hyperoside (0, 0.125, 0.25, 0.5, 1.0, and 2.0 mM/L). Results represent the mean ± SEM from three independent experiments (** means compared with the control group, $P < 0.001$).

of NF-κB in LX-2 cells, according to the instructions of the manufacturer. Briefly, nuclear extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology Rockford, IL). Protein content in the two fractions was quantitated using a Bradford assay. Nuclear extracts were incubated in 96-well plates coated with immobilized oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) containing a consensus (5′-GGGACTTTCC-3′) binding site for the p65 subunit of NF-κB. NF-κB binding to the target oligonucleotide was detected by incubation with primary antisera specific for the activated form of p65. The ELISA assay was developed with anti-IgG horseradish peroxidase conjugate and developing solution provided with the kit. Optical density (OD) was determined at 450 nm with a reference wavelength of 655 nm. Background binding was subtracted from the value obtained for binding to the consensus DNA sequence.

2.7. Quantitative RT-PCR. Total RNA was extracted from LX-2 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA). A 10 μg aliquot of each RNA sample was reverse transcribed into cDNA using oligo-dT random primers and reverse transcriptase. Quantitative real-time PCR was performed using SYBR Premix Ex Taq™ II (Takara, Japan). The primers used are shown in Table 1. 18s was used as a housekeeping gene.

2.8. Statistical Analysis. Data are expressed as mean ± SEM, representing at least three independent experiments. Statistical analysis was conducted using SPSS software (SPSS version 14.0, SPSS Science, Chicago, IL, USA). One-way analysis of variance (ANOVA) was followed by least significant difference (LSD) test. A P value of less than 0.05 ($P < 0.05$) was considered to indicate statistical significance.

3. Results

3.1. Hyperoside Inhibits LX-2 Proliferation. The chemical structure of hyperoside is shown in Figure 1(a). The fibrogenic activities of HSCs are based on their activation and

Table 1: Primers of quantitative real-time PCR.

Primers	Sequence		Annealing temperature (°C)
	Forward (from 5′ to 3′)	Reverse (from 5′ to 3′)	
18s	CAGCCACCCGAGATTGAGCA	TAGTAGCGACGGGCGGTGTG	60
α-SMA	AGGTAACGAGTCAGAGCTTTGGC	CTCTCTGTCCACCTTCCAGCAG	60
Collagen I	GAGCGGAGAGTACTGGATCG	GTTCGGGCTGATGTACCAGT	60
A20	GTCCGGAAGCTTGTGGCGCT	CCAAGTCTGTGTCCTGAACGCCC	55
Bcl-Xs	GCAGTAAAGCAAGCGCTGAG	GTTCCACAAAAGTATCCTGTTCAAAG	60
DR4	CTGAGCAACGCAGACGCGCTGTCCAC	ACAGCATCAGAGTCTCAGTGGGGTCAGC	60
Fas	ATTCTGCCATAAGCCCTGTC	TTGGTGTTGCTGGTGAGTGT	55
FasL	GTTCTGGTTGCCTTGGTAGG	TGTGCATCTGGCTGGTAGAC	55
c-IAP1	TTGTCAACTTCAGATACCACTGGAG	CAAGGCAGATTTAACCACAGGTG	60
Bcl-X_L	AGTTCCCTTGGCCTCAGAAT	AGGGTTGCACCAATCAGGTA	55
Rip1	GTCAAATTCAGCCACAGAACAGCC	CCCTTTAGCCTTCCCTCATCACC	55

proliferation. To explore the potential antifibrotic effects of hyperoside, the viability of LX-2 cells following hyperoside treatment was determined by MTT assay. As shown in Figures 1(b) and 1(c), hyperoside treatment for both 24 h and 48 h significantly reduced LX-2 cell viability in a dose-dependent manner. The 50% inhibitory concentration (IC_{50}) values for hyperoside were determined as 1.16 mM for 24 h treatment and 0.78 mM for 48 h treatment.

3.2. Hyperoside Promotes Apoptosis in LX-2 Cells. To determine whether the decrease in cell viability we observed in hyperoside treated LX-2 cells was attributable to the induction of apoptosis, we examined the rate of LX-2 apoptosis using Annexin V-FITC/PI labeling. Figure 2 showed that hyperoside treatment induced apoptosis in a concentration-dependent manner in LX-2 cells. The rate of apoptosis did not increase significantly in cells treated for only 24 h, while 48 h of hyperoside treatment significantly increased the rate of apoptosis in LX-2 cells. After 48 h of hyperoside treatment, the curvilinear response had an inflection point at the 0.5 mM dose, at which apoptosis rate was significantly increased. Because of these findings, we used hyperoside at a concentration of 1 mM in subsequent experiments involving the LX-2 cell line. These results indicate that growth inhibition of LX-2 cells by hyperoside is associated with apoptosis.

3.3. Hyperoside Downregulates Endogenous α-SMA and ECM Protein Levels in LX-2 Cells. The major phenotypical transformation that follows HSC activation is the transdifferentiation into α-smooth muscle actin- (α-SMA-) positive myofibroblasts that have increased cell proliferation and produce large amounts of ECM proteins such as collagen I [5]. The effect of hyperoside on expression of the HSC activation markers α-SMA and type I collagen was evaluated by western blotting. LX-2 cells were exposed to hyperoside for 48 h, and total protein was isolated for subsequent analysis. As depicted in Figure 3(a), secretion of α-SMA was inhibited by hyperoside, but the effect was not statistically significant until treated with 2.0 μM hyperoside. Treatment with 0.5 μM hyperoside slightly suppressed the expression of collagen I levels, and the protein levels markedly decreased when the hyperoside is 1.0 μM. As depicted in Figure 3(a), treatment with 0.5 μM or 1.0 μM hyperoside slightly decreased α-SMA and collagen I levels. Treatment with 2.0 μM hyperoside markedly decreased α-SMA and collagen I protein levels.

Next, we quantified mRNA levels of α-SMA and collagen I using quantitative RT-PCR. As illustrated in Figure 3(b), α-SMA mRNA levels were significantly decreased in LX-2 cells treated with 2.0 μM hyperoside. A significant decrease in collagen I gene expression was also observed. These results are consistent with the decreased protein levels shown in Figure 3(a).

3.4. Hyperoside Attenuates Intracellular ROS Production in LX-2 Cells. Flavonoids are broadly recognized for their natural antioxidant properties [21], while ROS have been associated with fibrosis in several organs, especially in the liver [22, 23]. When we treated LX-2 cells with hyperoside at multiple concentrations and measured intracellular ROS levels by DCF-DA fluorescence, we found that hyperoside drastically reduced intracellular ROS production in activated liver fibrosis cells (Figure 4(a)). Quantitative evaluation of DCF fluorescence (Figure 4(b)) found that this inhibitory effect on intracellular ROS production was reduced by approximately 60% compared to control levels. We are the first to report that hyperoside attenuates intracellular ROS production in LX-2 cells.

3.5. Hyperoside Blocks NF-κB Activation in LX-2 Cells. To evaluate the NF-κB-inhibiting effects of hyperoside, nuclear extracts were prepared from pretreated LX-2 cells and the subcellular localization of the NF-κB RelA/p65 DNA-binding complex was examined. As shown in Figure 5(a), TNF-α was effective in inducing NF-κB DNA-binding activity. Nuclear extracts from control LX-2 cells demonstrated high levels of NF-κB DNA-binding activity, which was reduced by nearly 50% after hyperoside treatment when compared to the TNF-α induced group. This finding demonstrates that hyperoside can markedly attenuate TNF-α induced NF-κB activation in LX-2 cells.

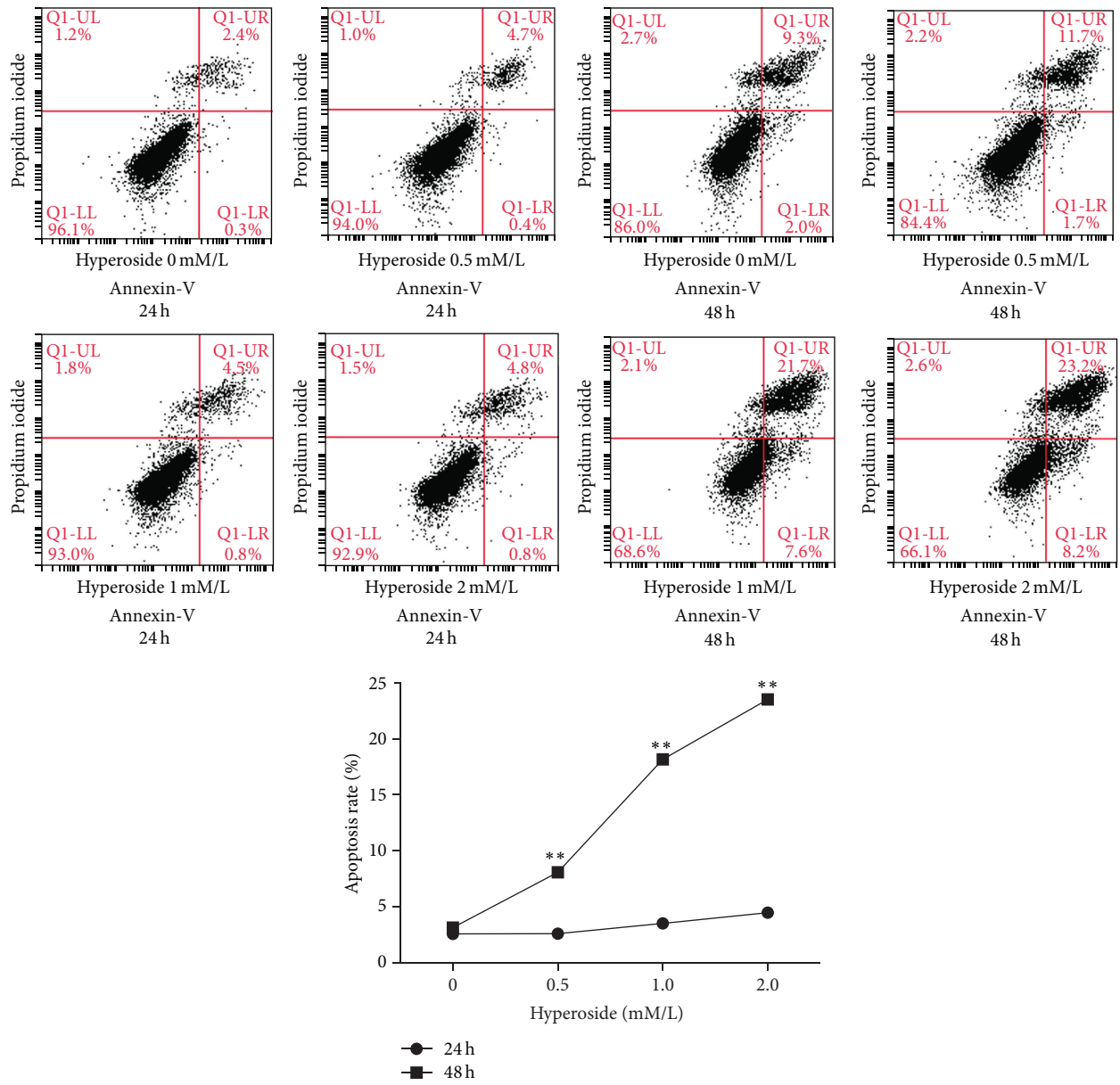


Figure 2: Hyperoside induced proapoptosis effect and its statistical representation of data. The apoptosis rate of Lx-2 cells was analyzed by flow cytometry (Annexin V-FITC/PI). Cells were treated with different dose of hyperoside (0, 0.5, 1.0, and 2.0 mM/L) for 24 h and 48 h, respectively. Results represent the mean ± SEM from three independent experiments (**means compared with the control group, $P < 0.001$, $n = 3$).

3.6. Hyperoside Induces HSC Apoptosis by Mediating NF-κB-Dependent Genes. The NF-κB transcription factor complex regulates genes governing a wide range of biological functions; these genes include regulators of apoptosis and cell proliferation such as *Fas*, *FasL*, *DR4*, and *Bcl-X_L* [24, 25], and NF-κB mediates cell death by the upregulation or downregulation of these target genes. To gain further insight into the effects of hyperoside on the regulation of NF-κB target genes in LX-2 cells, we assayed the expression of several proapoptotic genes (*Bcl-Xs*, *DR4*, *Fas*, *FasL*) and antiapoptotic genes (*A20*, *c-IAP1*, *Bcl-X_L*, *RIP1*) by real-time PCR following hyperoside treatment. As shown in Figure 5(b), we detected a dose-dependent upregulation of proapoptotic genes after 48 h of hyperoside treatment, with the largest increases in mRNA levels observed in *DR4* and *FasL*. Conversely, the expression of antiapoptotic genes *Bcl-X_L*, *c-IAP1*, and *Rip1* decreased with increasing doses of hyperoside.

4. Discussion

Recently, naturally occurring plant compounds have become attractive subjects of research into novel therapeutic strategies for liver fibrosis. In particular, dietary flavonoids such as morin [26], hesperidin [27], and silymarin [28] have

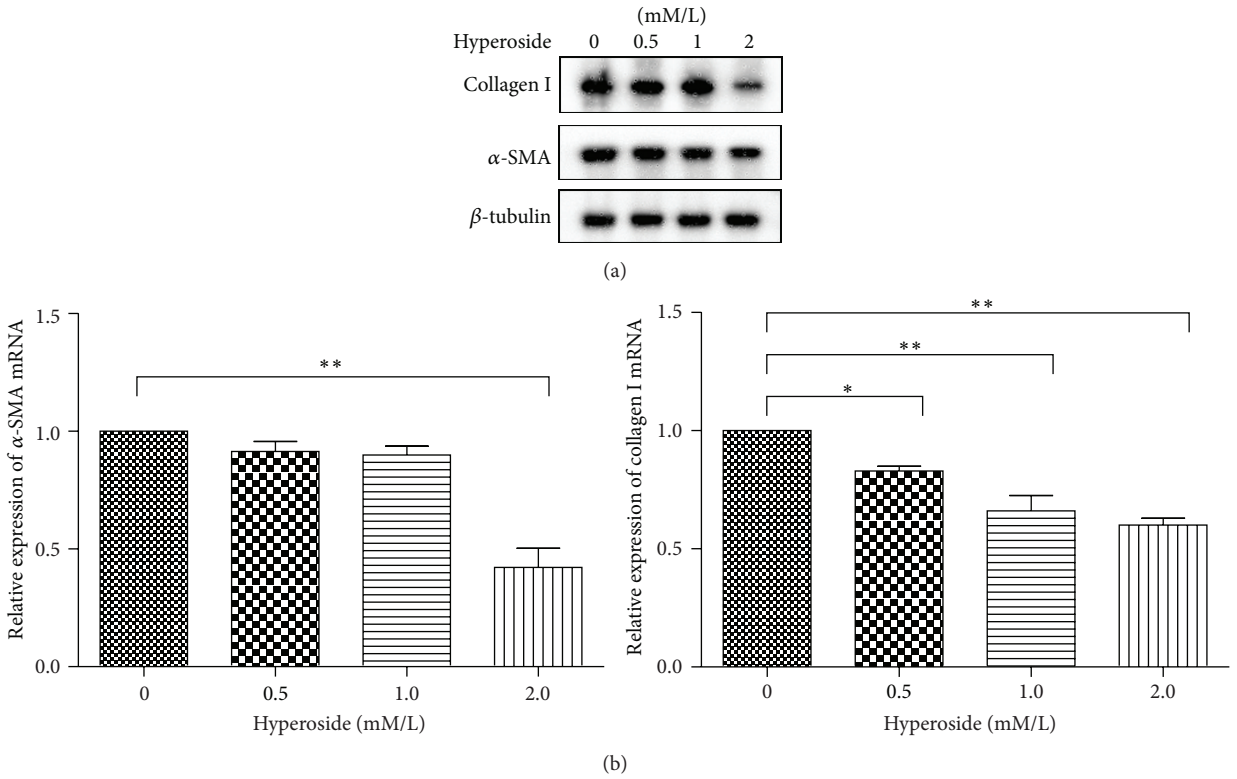


FIGURE 3: Hyperoside attenuated LX-2 activation. (a) Western blotting analysis of α-SMA and collagen I. The relative expression of proteins was calculated according to the reference band of β-tubulin. (b) mRNA levels were quantitated by real-time PCR. The expression was analyzed by $2^{-\Delta\Delta CT}$ method. 18s mRNA was used as a housekeeping gene (*means compared with the control group, $P < 0.05$; **means compared with the control group, $P < 0.001$, $n = 3$).

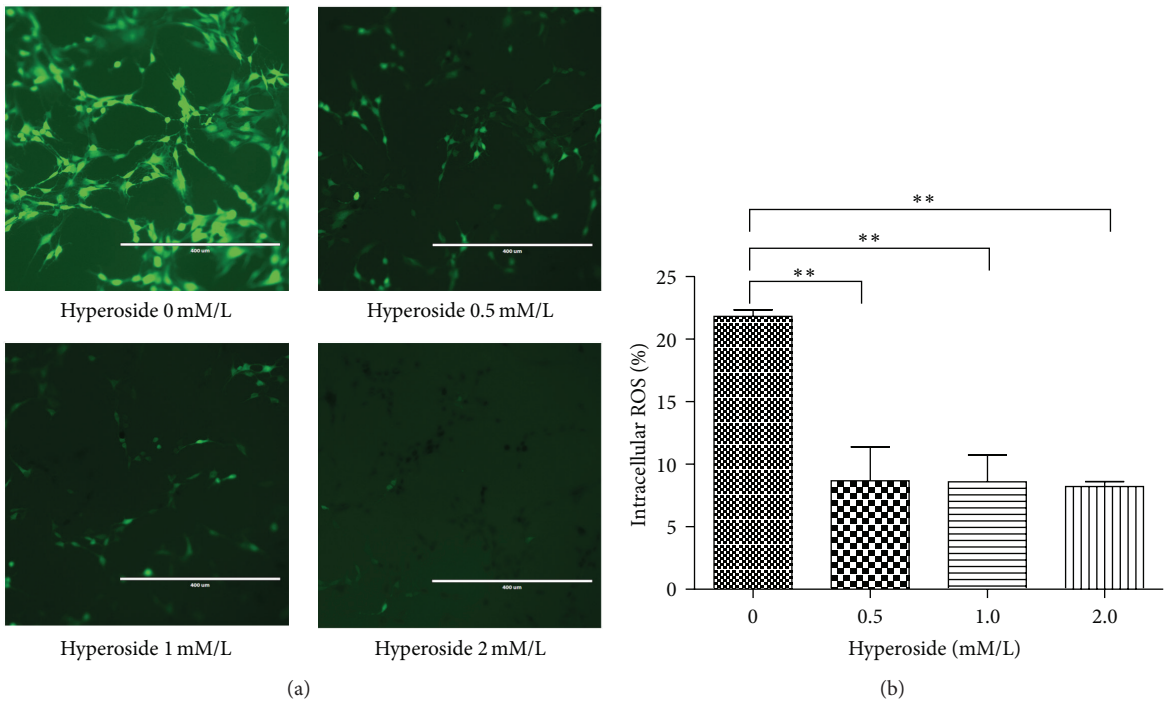


FIGURE 4: (a) LX-2 cells were treated in the absence or presence of hyperoside with different concentration for 24 h. The cells were then stained with DCF-DA and subjected to fluorescence microscopy. (b) Intracellular ROS was detected with flow cytometry.

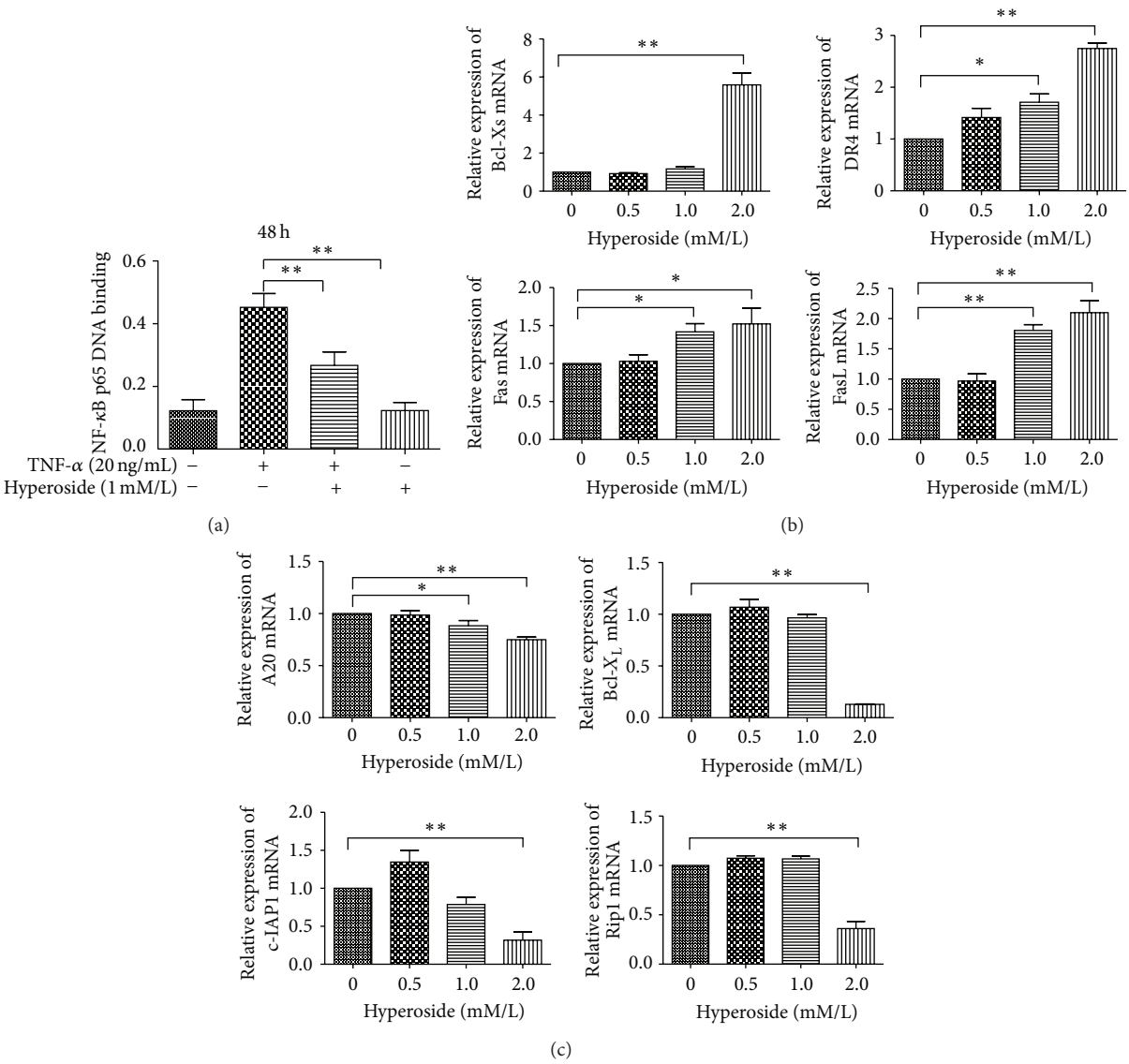


Figure 5: Hyperoside induces HSC apoptosis by blocking NF-κB activation and mediating NF-κB-dependent genes. (a) Hyperoside blocks NF-κB activation in Lx-2 cells. The effect of hyperoside on NF-κB DNA-binding activity in LX-2 cells was evaluated by ELISA. LX-2 cells were incubated for 48 hours in the absence or presence of TNF-α (20 ng/mL) and hyperoside (1 mM/L). Nuclear protein was extracted and subjected to ELISA for measurement of NF-κB DNA-binding activity. (b) Relative expression of NF-κB mediating proapoptotic target genes. (c) Relative expression of NF-κB mediating antiapoptotic target genes (*means compared with the control group, $P < 0.05$; **means compared with the control group, $P < 0.001$, $n = 3$).

been reported to attenuate the process of fibrosis. Hyperoside has been shown to have pleiotropic pharmacological effects, which include antithrombotic activities [29], protective effects on cardiomyocytes [30], and anti-inflammatory activities [31]. A recent report suggested that hyperoside exhibits hepatoprotective effects against CCl_4-induced liver injury and suppresses inflammation, but the mechanisms by which it protects against liver cirrhosis still need to be elucidated [32]. In the present study, we demonstrated that hyperoside induced culture-activated HSCs to undergo apoptosis due to downregulation of intercellular ROS and thereby inhibition of NF-κB.

NF-κB signaling plays an active role in a number of chronic liver diseases [10, 33]. There is evidence of NF-κB activity in activated HSCs [6, 34] and the inhibition of HSC apoptosis promotes liver fibrosis [7, 11, 35]. Inhibition of NF-κB signaling is usually connected with the induction of apoptosis in activated HSCs and the resolution of experimentally induced liver fibrosis [36]. For example, gliotoxin [7] and resveratrol [37] can induce HSC apoptosis both *in vitro* and *in vivo* through the inhibition of NF-κB signaling and altered regulation of NF-κB-dependent gene transcription [38]. Consistent with these reports, we observed that when hyperoside was administered to LX-2 cells for 48 h, late

apoptotic and necrotic cells (Figure 2) were increased significantly. In the early apoptosis stage, cells trigger ultimate loss of the mitochondrial membrane potential and translocation of phosphatidylserine. Early apoptotic cells can be rescued from the apoptotic program if the apoptotic stimulus is removed [39]. In our study, for early and median apoptotic cells, there is slighter change in 24 h than in 48 h perhaps for LX-2 cells 24 h hyperosdie treatment is not as effective as 48 h, which is consistent with the result of cell viability assay that for 48 h IC_{50} is lower than that in 24 h. As a consequence of NF-κB signaling inhibition, active markers for HSCs α-SMA and type I collagen were reduced by hyperoside in LX-2 cells.

Theoretically, ROS represent a serious hazard for the cell, as not only can they oxidize macromolecules—thus damaging proteins, lipids, and DNA [40]—but they are also key secondary messengers in numerous signaling pathways including proliferation, metabolism, and apoptosis [41]. In the liver, ROS contribute to hepatic fibrosis triggered by numerous liver injuries, including alcohol abuse, HCV infection, iron overload, and chronic cholestasis [42, 43]. Importantly, several studies have found that ROS influence intracellular NF-κB signaling [44] and stimulate collagen gene induction in HSCs, contributing to the pathogenesis of liver fibrosis [45]. Interestingly, studies have revealed the existence of a reciprocal, negative feedback loop between NF-κB and ROS [41]. Hyperoside is a quercetin-3-O-galactoside compound with a catechol group in the B-ring. Based on established structure-antioxidant activity relationships [46], we hypothesized that hyperoside would exert the suppressive capacity of intercellular ROS in HSCs. As expected, our experimental results indicate that hyperoside attenuates the generation of intracellular ROS and decreases the activation of NF-κB.

Collectively, our results suggest that the antifibrotic effects of hyperoside on cultured LX-2 cells are mediated by the inhibition of canonical NF-κB signaling and the induction of apoptosis in activated HSCs. Hyperoside can be regarded as a potential candidate in the search for pharmacological agents to combat liver fibrosis, although the precise mechanisms involved remain to be discovered, and extensive preclinical experiments are still required. Plants possessing the hyperoside compound are abundant in China [47], and many of these are considered to be atoxic and edible according to traditional Chinese medicine. This study adds support to the notion that the development of novel medicines and health-promoting foods based on hyperoside is a viable strategy, especially in China, and represents a valuable research goal because liver cirrhosis arising from viral hepatitis remains a serious global health issue.

Competing Interests

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

Authors' Contributions

Liwen Wang and Zhiwei Yue have equal contribution.

Acknowledgments

This study was supported by National Natural Science Foundation of China (Grant nos. 81172837 and 31571832), Tianjin Innovative Research Team Grant (TD-12-5049), Tianjin Municipal Science and Technology Commission (Grant no. 13JCQNJC12200), and National Student Innovation Programme (201510069043).

References

[1] P. Angulo, D. E. Kleiner, S. Dam-Larsen et al., "Liver fibrosis, but no other histologic features, is associated with long-term outcomes of patients with nonalcoholic fatty liver disease," *Gastroenterology*, vol. 149, no. 2, pp. 389.e10–397.e10, 2015.

[2] V. Hernandez-Gea and S. L. Friedman, "Pathogenesis of liver fibrosis," *Annual Review of Pathology: Mechanisms of Disease*, vol. 6, pp. 425–456, 2011.

[3] D. Schuppan and N. H. Afdhal, "Liver cirrhosis," *The Lancet*, vol. 371, no. 9615, pp. 838–851, 2008.

[4] H. B. El-Serag and K. L. Rudolph, "Hepatocellular carcinoma: epidemiology and molecular carcinogenesis," *Gastroenterology*, vol. 132, no. 7, pp. 2557–2576, 2007.

[5] R. I. A. Rippe and D. A. A. Brenner, "From quiescence to activation: gene regulation in hepatic stellate cells," *Gastroenterology*, vol. 127, no. 4, pp. 1260–1262, 2004.

[6] A. Anan, E. S. Baskin-Bey, S. F. Bronk, N. W. Werneburg, V. H. Shah, and G. J. Gores, "Proteasome inhibition induces hepatic stellate cell apoptosis," *Hepatology*, vol. 43, no. 2, pp. 335–344, 2006.

[7] M. C. Wright, R. Issa, D. E. Smart et al., "Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats," *Gastroenterology*, vol. 121, no. 3, pp. 685–698, 2001.

[8] E. Crosas-Molist, E. Bertran, and I. Fabregat, "Cross-talk between TGF-β and NADPH oxidases during liver fibrosis and hepatocarcinogenesis," *Current Pharmaceutical Design*, vol. 21, no. 41, pp. 5964–5976, 2015.

[9] T. D. Gilmore, "Introduction to NF-κB: players, pathways, perspectives," *Oncogene*, vol. 25, no. 51, pp. 6680–6684, 2006.

[10] T. Luedde and R. F. Schwabe, "NF-κB in the liver—linking injury, fibrosis and hepatocellular carcinoma," *Nature Reviews Gastroenterology and Hepatology*, vol. 8, no. 2, pp. 108–118, 2011.

[11] F. Oakley, M. Meso, J. P. Iredale et al., "Inhibition of inhibitor of κb kinases stimulates hepatic stellate cell apoptosis and accelerated recovery from rat liver fibrosis," *Gastroenterology*, vol. 128, no. 1, pp. 108–120, 2005.

[12] A. M. Elsharkawy, F. Oakley, and D. A. Mann, "The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis," *Apoptosis*, vol. 10, no. 5, pp. 927–939, 2005.

[13] D. F. Romagnolo and O. I. Selmin, "Flavonoids and cancer prevention: a review of the evidence," *Journal of Nutrition in Gerontology and Geriatrics*, vol. 31, no. 3, pp. 206–238, 2012.

[14] D. Sampietro, E. Quiroga, M. Sgariglia, J. Soberón, and M. A. Vattuone, "A thermostable α-galactosidase from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947: purification and properties," *Antonie van Leeuwenhoek*, vol. 102, no. 2, pp. 257–267, 2012.

[15] S. Trumbeckaite, J. Bernatoniene, D. Majiene, V. Jakštas, A. Savickas, and A. Toleikis, "The effect of flavonoids on rat heart mitochondrial function," *Biomedicine and Pharmacotherapy*, vol. 60, no. 5, pp. 245–248, 2006.

[16] W. Zhou, J. Oh, W. Li, D. W. Kim, S. H. Lee, and M. K. Na, "Phytochemical studies of Korean endangered plants: a new flavone from *Rhododendron brachycarpum* G. Don," *Bulletin of the Korean Chemical Society*, vol. 34, no. 8, pp. 2535–2538, 2013.

[17] S.-K. Ku, S. Kwak, O.-J. Kwon, and J.-S. Bae, "Hyperoside inhibits high-glucose-induced vascular inflammation in vitro and in vivo," *Inflammation*, vol. 37, no. 5, pp. 1389–1400, 2014.

[18] L.-L. Wu, X.-B. Yang, Z.-M. Huang, H.-Z. Liu, and G.-X. Wu, "In vivo and in vitro antiviral activity of hyperoside extracted from *Abelmoschus manihot* (L) medik," *Acta Pharmacologica Sinica*, vol. 28, no. 3, pp. 404–409, 2007.

[19] L. Xu, A. Y. Hui, E. Albanis et al., "Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis," *Gut*, vol. 54, no. 1, pp. 142–151, 2005.

[20] D. Diaz, A. Prieto, E. Reyes, H. Barcenilla, J. Monserrat, and M. Alvarez-Mon, "Flow cytometry enumeration of apoptotic cancer cells by apoptotic rate," *Methods in Molecular Biology*, vol. 1219, pp. 11–20, 2015.

[21] C. Zhu, Z. Lei, and Y. Luo, "Studies on antioxidative activities of methanol extract from *Murraya paniculata*," *Food Science and Human Wellness*, vol. 4, no. 3, pp. 108–114, 2015.

[22] S. De Minicis and D. A. Brenner, "NOX in liver fibrosis," *Archives of Biochemistry and Biophysics*, vol. 462, no. 2, pp. 266–272, 2007.

[23] Y.-H. Paik, K. Iwaisako, E. Seki et al., "The nicotinamide adenine dinucleotide phosphate oxidase (NOX) homologues NOX1 and NOX2/gp91phox mediate hepatic fibrosis in mice," *Hepatology*, vol. 53, no. 5, pp. 1730–1741, 2011.

[24] J. Kucharczak, M. J. Simmons, Y. Fan, and C. Gélinas, "To be, or not to be: NF-κB is the answer—role of Rel/NF-κB in the regulation of apoptosis," *Oncogene*, vol. 22, no. 56, pp. 8961–8982, 2003.

[25] S. K. Radhakrishnan and S. Kamalakaran, "Pro-apoptotic role of NF-κB: implications for cancer therapy," *Biochimica et Biophysica Acta—Reviews on Cancer*, vol. 1766, no. 1, pp. 53–62, 2006.

[26] P. MadanKumar, P. NaveenKumar, S. Manikandan, H. Devaraj, and S. NiranjaliDevaraj, "Morin ameliorates chemically induced liver fibrosis in vivo and inhibits stellate cell proliferation in vitro by suppressing Wnt/β-catenin signaling," *Toxicology and Applied Pharmacology*, vol. 277, no. 2, pp. 210–220, 2014.

[27] S. M. Elshazly and A. A. A. Mahmoud, "Antifibrotic activity of hesperidin against dimethylnitrosamine-induced liver fibrosis in rats," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 387, no. 6, pp. 559–567, 2014.

[28] S. Clichici, D. Olteanu, A.-L. Nagy, A. Oros, A. Filip, and P. A. Mircea, "Silymarin inhibits the progression of fibrosis in the early stages of liver injury in CCl4-treated rats," *Journal of Medicinal Food*, vol. 18, no. 3, pp. 290–298, 2015.

[29] S.-K. Ku, T. H. Kim, S. Lee, S. M. Kim, and J.-S. Bae, "Antithrombotic and profibrinolytic activities of isorhamnetin-3-O-galactoside and hyperoside," *Food and Chemical Toxicology*, vol. 53, pp. 197–204, 2013.

[30] Z.-L. Li, J. Hu, Y.-L. Li et al., "The effect of hyperoside on the functional recovery of the ischemic/reperfused isolated rat heart: potential involvement of the extracellular signal-regulated kinase 1/2 signaling pathway," *Free Radical Biology and Medicine*, vol. 57, pp. 132–140, 2013.

[31] S. Lee, H. J. Sang, S. L. Yeon et al., "Antiinflammatory activity of hyperin from *Acanthopanax chiisanensis* roots," *Archives of Pharmacal Research*, vol. 27, no. 6, pp. 628–632, 2004.

[32] J.-H. Choi, D.-W. Kim, N. Yun et al., "Protective effects of hyperoside against carbon tetrachloride-induced liver damage in mice," *Journal of Natural Products*, vol. 74, no. 5, pp. 1055–1060, 2011.

[33] V. Sivaramakrishnan and S. Niranjali Devaraj, "Morin regulates the expression of NF-κB-p65, COX-2 and matrix metalloproteinases in diethylnitrosamine induced rat hepatocellular carcinoma," *Chemico-Biological Interactions*, vol. 180, no. 3, pp. 353–359, 2009.

[34] A. Lang, R. Schoonhoven, S. Tuvia, D. A. Brenner, and R. A. Rippe, "Nuclear factor κB in proliferation, activation, and apoptosis in rat hepatic stellate cells," *Journal of Hepatology*, vol. 33, no. 1, pp. 49–58, 2000.

[35] A. M. Elsharkawy, M. C. Wright, R. T. Hay et al., "Persistent activation of nuclear factor-κB in cultured rat hepatic stellate cells involves the induction of potentially novel rel-like factors and prolonged changes in the expression of IκB family proteins," *Hepatology*, vol. 30, no. 3, pp. 761–769, 1999.

[36] S. L. Friedman, "Evolving challenges in hepatic fibrosis," *Nature Reviews Gastroenterology and Hepatology*, vol. 7, no. 8, pp. 425–436, 2010.

[37] E. Chávez, K. Reyes-Gordillo, J. Segovia et al., "Resveratrol prevents fibrosis, NF-κB activation and TGF-β increases induced by chronic CCl_4 treatment in rats," *Journal of Applied Toxicology*, vol. 28, no. 1, pp. 35–43, 2008.

[38] F. Habens, N. Srinivasan, F. Oakley, D. A. Mann, A. Ganesan, and G. Packham, "Novel sulfasalazine analogues with enhanced NF-kB inhibitory and apoptosis promoting activity," *Apoptosis*, vol. 10, no. 3, pp. 481–491, 2005.

[39] S. Elmore, "Apoptosis: a review of programmed cell death," *Toxicologic Pathology*, vol. 35, no. 4, pp. 495–516, 2007.

[40] J. F. Curtin, M. Donovan, and T. G. Cotter, "Regulation and measurement of oxidative stress in apoptosis," *Journal of Immunological Methods*, vol. 265, no. 1-2, pp. 49–72, 2002.

[41] C. Bubici, S. Papa, K. Dean, and G. Franzoso, "Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance," *Oncogene*, vol. 25, no. 51, pp. 6731–6748, 2006.

[42] M. Parola and G. Robino, "Oxidative stress-related molecules and liver fibrosis," *Journal of Hepatology*, vol. 35, no. 2, pp. 297–306, 2001.

[43] G. Poli and M. Parola, "Oxidative damage and fibrogenesis," *Free Radical Biology and Medicine*, vol. 22, no. 1-2, pp. 287–305, 1997.

[44] A. Szuster-Ciesielska, K. Plewka, J. Daniluk, and M. Kandefer-Szerszeń, "Zinc supplementation attenuates ethanol- and acetaldehyde-induced liver stellate cell activation by inhibiting reactive oxygen species (ROS) production and by influencing intracellular signaling," *Biochemical Pharmacology*, vol. 78, no. 3, pp. 301–314, 2009.

[45] N. Nieto, P. Greenwel, S. L. Friedman, F. Zhang, A. J. Dannenberg, and A. I. Cederbaum, "Ethanol and arachidonic acid increase α2(I) collagen expression in rat hepatic stellate

cells overexpressing cytochrome P450 2E1. Role of H_2O_2 and cyclooxygenase-2," *The Journal of Biological Chemistry*, vol. 275, no. 26, pp. 20136–20145, 2000.

[46] K. E. Heim, A. R. Tagliaferro, and D. J. Bobilya, "Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships," *Journal of Nutritional Biochemistry*, vol. 13, no. 10, pp. 572–584, 2002.

[47] S. Donnapee, J. Li, X. Yang et al., "*Cuscuta chinensis* Lam.: a systematic review on ethnopharmacology, phytochemistry and pharmacology of an important traditional herbal medicine," *Journal of Ethnopharmacology*, vol. 157, pp. 292–308, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 4302706, 7 pages
http://dx.doi.org/10.1155/2016/4302706

Research Article

Polyphenol-Rich Extract from Propolis Reduces the Expression and Activity of *Streptococcus mutans* Glucosyltransferases at Subinhibitory Concentrations

Jorge Jesús Veloz,[1,2] Nicolás Saavedra,[1] Marysol Alvear,[3] Tomás Zambrano,[1] Leticia Barrientos,[1] and Luis A. Salazar[1]

[1]*Center of Molecular Biology and Pharmacogenetics, Scientific and Technological Bioresource Nucleus (BIOREN), Universidad de La Frontera, Avenida Francisco Salazar 01145, 4811230 Temuco, Chile*
[2]*Departamento de Ciencias Biológicas y Químicas, Facultad de Ciencias, Universidad San Sebastián, Campus Los Leones, Lota 2465, 7510157 Providencia, Santiago, Chile*
[3]*Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Avenida Francisco Salazar, 01145 Temuco, Chile*

Correspondence should be addressed to Luis A. Salazar; luis.salazar@ufrontera.cl

Received 10 December 2015; Accepted 8 March 2016

Academic Editor: Kimon A. Karatzas

Tooth decay is an infectious disease, whose main causative agent identified is *Streptococcus mutans* (*S. mutans*). Diverse treatments have been used to eradicate this microorganism, including propolis. To date, it has been shown that polyphenols from Chilean propolis inhibit *S. mutans* growth and biofilm formation. However, the molecular mechanisms underlying this process are unclear. In the present study, we assessed the effect of Chilean propolis on the expression and activity of the glycosyltransferases enzymes and their related genes. Polyphenol-rich extract from propolis inhibited gene expression of glycosyltransferases (GtfB, GtfC, and GtfD) and their related regulatory genes, for example, *VicK*, *VicR*, and *CcpA*. Moreover, the treatment inhibited glucosyltransferases activity measured by the formation of sucrose-derived glucans. Additionally, an inhibitory effect was observed in the expression of SpaP involved in sucrose-independent virulence of *S. mutans*. In summary, our results suggest that Chilean propolis has a dose-dependent effect on the inhibition of genes involved in *S. mutans* virulence and adherence through the inhibition of glucosyltransferases, showing an anticariogenic potential of polyphenols from propolis beyond *S. mutans* growth inhibition.

1. Introduction

Streptococcus mutans (*S. mutans*) is considered to be the principal causative agent of human dental caries among a wide variety of microorganisms detected in the oral cavity [1, 2]. Although the etiology and factors related to dental caries development are well known, this pathology remains a public health issue due to its increased prevalence in recent reports [3]. Therefore, the search for new therapeutic strategies holds great interest. Polyphenols from different botanical sources have emerged as promissory therapeutic agents due to their wide range of biological activities [4]. Propolis is a resinous substance collected by bees (*Apis mellifera*) from different vegetable species [5]. Several pharmacological properties have been described for the extracts of propolis, such as antidiabetogenic, antiatherogenic, antimicrobial, and antifungal properties, which are related mainly to the polyphenols content of propolis samples [6–9]. Since chemical studies have determined a relationship between propolis composition and the region from where propolis is collected together with the plant source of each area [10, 11], the biological activities of propolis can vary depending on the same conditions aforementioned. We have previously reported antimicrobial activity of Chilean propolis for *S. mutans* [7]

and the inhibition of biofilm formation by these microorganisms [12]. However, the underlying molecular mechanism involved in this inhibitory effect by Chilean propolis remains unclear.

The capacity of *S. mutans* to form a biofilm on the teeth surface is considered to be the most important virulence mechanism related to its cariogenicity, which involves sucrose-dependent and sucrose-independent processes. Glucosyltransferases (Gtfs) are involved in the sucrose-dependent mechanism, allowing *S. mutans* the synthesis of both water-soluble and water-insoluble glucans from sucrose [13, 14]. These sucrose-derived glucans favor bacterial adhesion to the tooth enamel by binding to hydroxyapatite minerals (HA) and enabling the interaction between microorganisms. Gtfs from *S. mutans* differ on the types of glucans they can synthetize and the roles performed in biofilm formation. GtfB synthetizes insoluble glucans with α-1,3-linkages that facilitates cell aggregation in stable biofilms by mediating the interaction between *S. mutans* bacteria. In contrast, GtfD forms mainly soluble glucans with α-1,6-linkages that contain an hydrophobic domain, allowing the interaction with salivary proteins in the pellicle. GtfC produces both soluble and insoluble glucans showing the highest affinity for HA among other Gtfs [15]. The expression of Gtfs is influenced by environmental factors of the oral cavity, for example, pH and carbohydrate availability, but it is also affected by the expression of other regulatory genes such as *vicR*/*vicK*, which belongs to the *vicRKX* operon in the *S. mutans* chromosome [16], and the catabolite control protein A (CcpA) [17]. Regarding the sucrose-independent mechanism, it is less important than sucrose-dependent mechanisms, but it also participates in the primary adherence process in biofilm development, involving the interaction between salivary agglutinins and the surface protein adhesin SpaP (I/II antigen) on the *S. mutans* bacterial wall, which is encoded by the *SpaP* gene [18, 19].

The present study aimed to evaluate the effect of subbactericide concentrations of polyphenol-rich extract from Chilean propolis on the expression of glucosyltransferases and regulatory genes as a possible mechanism underlying the propolis inhibitory effect on *S. mutans*.

2. Materials and Methods

2.1. Polyphenol-Rich Extract from Propolis. Propolis was collected in spring of 2008 from La Araucanía, Chile. The sample was crushed in cold, 30 grams was dissolved in 100 mL of ethanol (70%) and mixed in constant agitation for 7 days at room temperature. Subsequently, polyphenol-rich extract of propolis (PEP) was filtered with Whatman 2.0 and centrifuged at 327 g. for 20 minutes at 4°C. Later the solvent evaporated in 60°C for 2 hours in a Rotavapor evaporator (Büchi, R-210, Germany) and dissolved for 24 h with sterile DMSO to obtain 50% w/w extract concentrate propolis extract (EP).

2.2. Determination of Total Phenolic Content. To quantify the total phenolic content of polyphenol-rich extract from propolis, we used the colorimetric Folin-Ciocalteu assay. Briefly, 100 μL of sample (PEP or calibrator solution) was mixed with 100 μL of distilled water and 2 mL of Folin-Ciocalteu reagent (Merck, Germany). The resultant solution was gently mixed and incubated during 8 minutes. Finally, 3 mL of sodium carbonate 20% (w/v) was added in a 25 mL flask and incubated for 2 hours. The absorbance of the solution was at measured 760 nm in a microplate reader. For the calibration curve, we used standard solutions of pinocembrin-galangin in a proportion of 2 : 1 [11]. Thus, the results of quantification are expressed in equivalents of pinocembrin-galangin mixture (μg mL^{-1}).

2.3. Streptococcus mutans Isolation and Culture Conditions. *Streptococcus mutans* strains were obtained from salivary samples of children with active tooth decay. The samples were cultured in petri plates with Columbia agar enriched with sucrose (1%) in an anaerobic container (Becton, Dickinson and Company, NY, USA). Then, culture plates were incubated at 37°C and 5% of CO_2 for 24 hours. Finally, *S. mutans* was identified using polymerase chain reaction (PCR) as previously described [20].

2.4. Quantification of Relative Gene Expression in S. mutans Cultures under Polyphenol-Rich Extract Treatment. To evaluate the activity of the PEP on glucosyltransferases and other virulence-related genes, *S. mutans* was cultured in trypticase soy broth and treatments were applied using a macrodilution sensitivity test scheme with PEP concentrations ranging between 0.1 and 1.6 μg mL^{-1}. Moreover, cultures exposed to DMSO and chlorhexidine were used as a vehicle control and positive control, respectively. *S. mutans* cultures were incubated during 24 hours in the conditions above described [21]. Total RNA was isolated from cultures using TRIzol reagent (Ambion, USA) according to manufacturer's instructions. Then, cDNA was synthesized using a reverse transcriptase reaction starting from 1 μg of total RNA and using 200 ng of random primers and 200 U of RevertAid M-MuLV Reverse Transcriptase (Promega, Madison, WI, USA) in a final volume of 20 μL. Glucosyltransferases B, C, and D and *VicK*, *VicR*, *SpaP*, and *CcpA* genes were amplified using real time PCR in a StepOne Real Time PCR System (Life Technologies, USA).

For real time PCR amplification, we used 0.5 μL of each forward and reverse primers (200 nM); 12.5 μL of Fast SYBR® Green Master Mix (Life Technologies, USA); 1 μL of cDNA sample and RNAse-free water up to complete 25 μL of final volume. The amplification program was performed in the following conditions: initial denaturation at 95°C for 10 minutes and 40 cycles of 15 seconds at 95°C, with an extension stage at 60°C for 1 minute. Results were analyzed by the comparative cq method ($2^{-\Delta\Delta Cq}$) [22] using the 16S rRNA of *S. mutans* as reference gene. All these experiments were completed by triplicate and results were expressed as relative expression in arbitrary units. The sequence of primers used for this analysis was: GtfB: 5′-AGC AAT GCA GCC AAT CTA CAA AT-3′ and 5′-ACG AAC TTT GCC GTT ATT GTC A-3′; GtfC: 5′-CTC AAC CAA CCG CCA CTG TT-3′ and 5′-GGT TTA ACG TCA AAA TTA GCT GTA TTA-3′;

GtfD: 5′-CTT TGG TTC AGA CGG TGT TG-3′ and 5′-CTG CTT TTG ACT TGT TTT CCG-3′; SpaP: 5′-CAG TAC CTG ACT TGA TAA TAA CAC C-3′ and 5′-TCC CTG CAA GAA TCA CTC AGA A-3′; VicR: 5′-CGC AGT GGC TGA GGA AAA TG-3′ and 5′-ACC TGT GTG TGT CGC TAA GTG ATG-3′; VicK: 5′-CAC TTT ACG CAT TCG TTT TGC C-3′ and 5′-CGT TCT TCT TTT TCC TGT TCG GTC-3′; CcpA: 5′-CCG TGA AGC GGG AGT GTC CA-3′ and 5′-TGC CAA ACC ACG CGC CAC AG-3′; 16S rRNA: 5′-TGG AAA CGA TAG CTA ATA CCG CAT A-3′ and 5′-TAA TAC AAC GCA GGT CCA TCT ACT A-3′.

2.5. Estimation of Glucosyltransferases Enzymatic Activity. To obtain a crude extract of glucosyltransferases from cultures, the cells were suspended in a potassium phosphate buffer (20 mM) at pH 6.8 and then centrifuged at 10 000 g at 4°C. The enzymes were precipitated with 80% sulfate of ammonium solution. The pellet was resuspended in 500 μL of buffer potassium phosphate for later ultrafiltration in a 10 KDa and 3000 MWCO Vivaspin500 (GE Healthcare Life Science, USA). Finally, a phenylmethanesulfonyl fluoride solution PMSF (1 mM) and sodium azide at concentration of 0.02% were added [23]. For the enzymatic reaction, 50 μL of crude glucosyltransferases extract and 50 μL of sterile sucrose (0.1 M) in potassium phosphate buffer containing PEP in concentrations ranging from 0.1 to 1.6 μg mL^{-1} were added. These solutions were incubated at 37°C for 6 hours in 96-well microplates. After incubation, the content of each well was transferred to a microtube and centrifuged at 10 000 g during 10 minutes. For estimation of water-soluble glucans, the centrifugated liquid was transferred into a microplate and mixed with 10 μL of concentrate sulfuric acid and 10 μL of phenol. Finally, the microplates were incubated at 95°C and the absorbance was quantified at 490 nm in a microplate reader. For water-insoluble glucans assessment, the pellet obtained after centrifugation was dissolved in 300 μL of sodium hydroxide (1 M) and the absorbance was quantified in the same conditions [24]. These assays were performed by triplicate and results were expressed as means ± standard deviation.

2.6. Statistical Analysis. Statistical analyses were performed using computational software package Prism 5 (Graph Pad Software Inc., San Diego, USA). The values for comparison of multiple means of individual experiments were estimated by statistical analysis of variance (ANOVA). Significant differences were considered at $p < 0.05$.

3. Results

3.1. Total Polyphenols Content of Polyphenol-Rich Extract from Propolis. The total phenolics content in PEP was quantified in equivalence of pinocembrin-galangin mixture by the Folin-Ciocalteu reaction to obtain the starting concentration and perform appropriate treatment dilutions for gene expression and glucosyltransferases assays. Thus, PEP solution was determined to contain a concentration of 137753 μg mL^{-1} of total polyphenols.

3.2. Effect of Polyphenol-Rich Extract from Propolis on Relative Gene Expression in S. mutans Cultures. To evaluate the effect of treatment with polyphenols on gene expression of glucosyltransferases and regulatory genes, cultured *S. mutans* was treated using four noninhibitory concentrations of PEP (0.1, 0.2, 0.4, and 0.8 μg mL^{-1}) and one bactericide concentration (1.6 μg mL^{-1}) as death control. Values were selected considering the minimum inhibitory and bactericide concentrations obtained for the same extract [12]. Moreover, chlorhexidine was used as positive control due to its well-known bactericide effect on *S. mutans* strains. As expected, chlorhexidine and bactericide concentration of PEP showed a significant reduction on glucosyltransferases or regulatory genes expression, and no effect of vehicle was observed on the expression of all evaluated genes. Moreover, all remaining concentrations of PEP used as treatments inhibited the expression of GtfB, GtfC, GtfD, and SpaP (Figure 1). Similarly, the expression of regulatory genes *VicK* and *CcpA* was reduced in *S. mutans* under PEP treatment at all tested concentrations. However, that inhibitory effect was significant only from 0.2 μg mL^{-1} for VicR gene (Figure 2).

3.3. Effect of Polyphenol-Rich Extract from Propolis on Formation of Water-Soluble and Water-Insoluble Glucans by S. mutans Cultures. The formation of water-soluble and water-insoluble glucans by *S. mutans* under PEP treatment was assessed in supernatant and pellet of treated cultures. In both cases, glucans formation was significantly reduced at all tested concentrations compared with vehicle-treated cells used as control (Figure 3).

4. Discussion

Different studies have been conducted evaluating the effect of polyphenolic compounds with the ability to inhibit *Streptococcus mutans* growth to describe novel therapeutic alternatives for dental caries treatment. These polyphenols have been obtained from several botanical resources including plants [25], fruits [26], green tea [27], and propolis [28, 29]. The antibacterial effect of Chilean propolis has been previously determined, showing minimal inhibitory concentrations (MIC) and minimal bactericide concentrations (MBC) of about 0.9 and 1.3 μg mL^{-1}, respectively. Moreover, it was reported that polyphenols from propolis inhibited biofilm formation by *S. mutans* when using subinhibitory concentrations, which probably involves the regulation of physiological mechanisms related to *S. mutans* virulence [12]. In the present study, we evaluated the effect of subinhibitory concentrations of a polyphenol-rich extract from propolis, previously used to inhibit biofilm growth and formation by *S. mutans*, on the expression of virulence genes and glucan formation by Gtfs enzymes. The most important virulence factor of *S. mutans* is its ability to accumulate on dental plaque by formation of biofilm, which is mediated by three distinct Gtfs (GtfB, GtfC, and GtfD) acting by extracellular water-soluble and water-insoluble glucans synthesis from diet sucrose. Teeth colonization occurs by using different pathways, in which the bacteria can generate an attachment on the tooth surface by

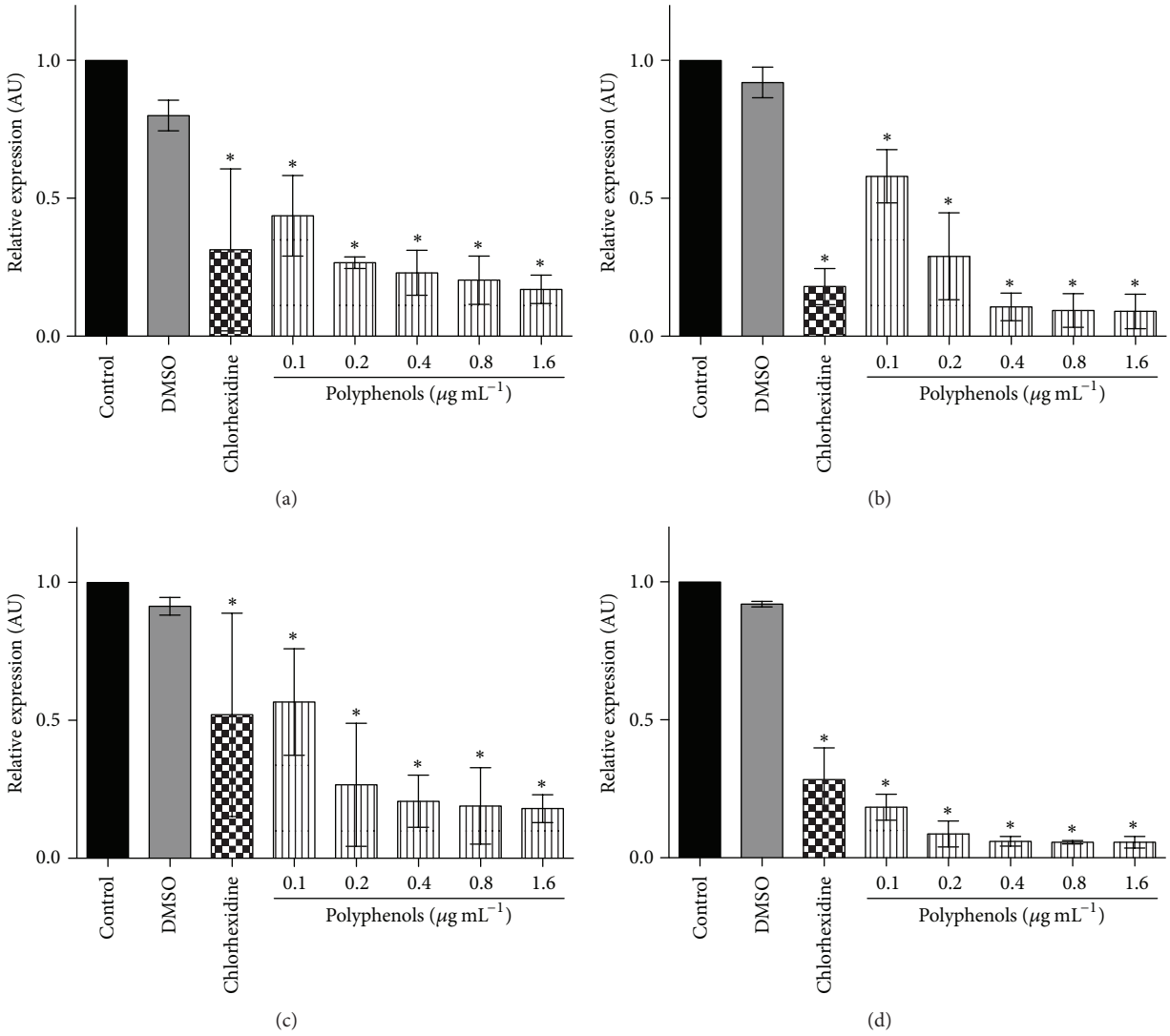


Figure 1: Effect of polyphenol-rich extract from propolis on the expression of genes related with *S. mutans* virulence. (a) GtfB; (b) GtfC; (c) GtfD; (d) SpaP. Statistical significance was determined by ANOVA and Dunnett's multiple comparison test using nontreated cells as control. $^{*}p < 0.05$.

the antigenic protein SpaP (cPAc or antigen I/II) together with the development of acidic conditions in the oral cavity through Gtfs activities by glucan synthesis, allowing mineral tooth dissolution and cellular aggregation in biofilm structure [30, 31]. After exposition to polyphenols, gene expression of glucosyltransferases showed different levels of repression depending on the concentration of polyphenols tested. The expression of genes encoding GtfB and GtfC showed a stronger inhibition in our model, being repressed more than 40% and 60%, respectively, at concentrations between 0.1 and up to 0.8 μg mL^{-1}, with a dose-dependent effect. Similarly, *S. mutans* cultures exposed to polyphenols showed that GtfD was inhibited in approximately 40% or more at subinhibitory concentrations starting from 0.2 μg mL^{-1}. Since these Gtfs are decisive in biofilm formation through the synthesis of insoluble glucans in virulent phenotypes, its inhibition can result in reduced biofilm formation, which is consistent with what is previously published for Chilean propolis treatment [12, 32, 33]. Gtfs are regulated at the transcriptional level in response to environmental conditions such as pH, carbohydrate availability, and cell density. Moreover, *S. mutans* displays open reading frames (Orf) for Gtfs regulation; these regulatory factors are associated with two-component regulatory systems (TCSs) as VicK/VicR [34, 35]. Concordantly, PEP treatment results in the inhibition of *VicK* and *VicR* expression; however, the effect was not dose-dependent, suggesting that Gtfs inhibition might involve other regulatory mechanisms. VicK, that corresponds to a membrane-bound sensor histidine kinase (HK), showed a highly decreased expression in approximately more than 50% compared to control in gene expression quantification assays. HKs components are bifunctional proteins having both kinase and phosphatase activities. VicR belongs to the OmpR family of RRs; it is conformed by a helix-turn-helix DNA binding motif

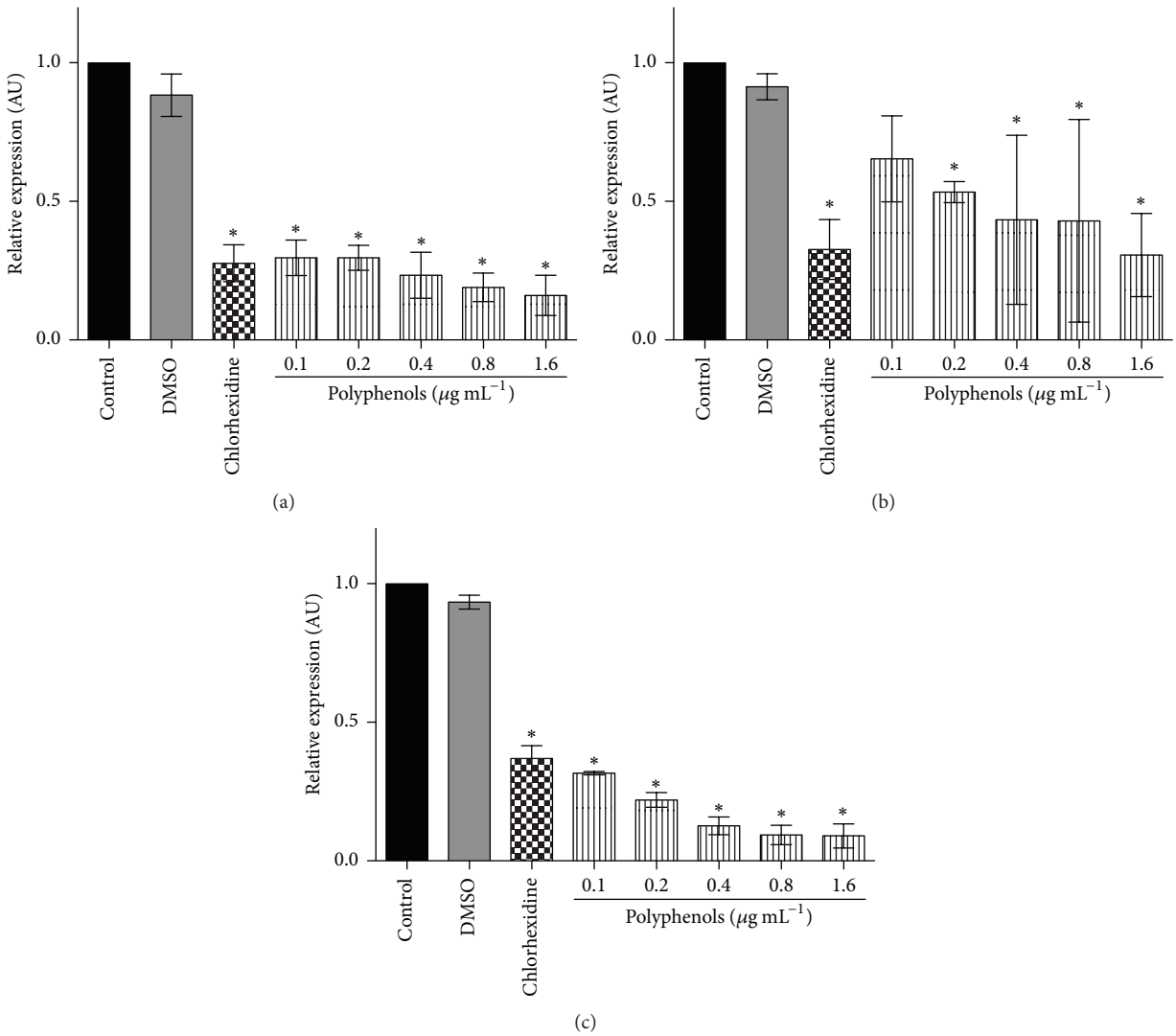


Figure 2: Effect of polyphenol-rich extract from propolis on the expression of regulatory genes involved in *S. mutans* virulence mechanisms. (a) *VicK*; (b) *VicR*; (c) *CcpA*. Statistical significance was determined by ANOVA and Dunnett's multiple comparison test using nontreated cells as control. $^{*}p < 0.05$.

and it is involved upstream in virulence genes, including *GtfB*, *GtfC*, and *GtfD*. The biochemistry of the VicR/VicK TCS is important to understand antimicrobial resistance mechanisms and for the development of new potential therapies [36]. Carbon Catabolite Repression (CCR) genes such as CcpA can also regulate *S. mutans* metabolism and adherence on tooth colonization and biofilm formation, integrating complex regulatory networks that activate or silence some genes in response to carbon source and availability [37]. Gene expression of *S. mutans* showed important *CcpA* inhibition following PEP treatment, reaching under 30% of expression in relation to control cells, at low concentrations of PEP in a dose-dependent manner. CcpA is critical for *gtfB* and *gtfC* expression, because CcpA inactivation results in a marked decrease in the levels of Gtfs promoter activity [38], suggesting that inhibitory dose-dependent effect of PEP on Gtfs expression might be triggered by its repression. Moreover, enzymatic activity of Gtfs estimated by insoluble and soluble glucans formation was also affected by PEP at subinhibitory concentrations, confirming a functional effect of Gtfs inhibition. SpaP expression was also reduced by PEP treatment, contributing to biofilm inhibition by a sucrose-independent mechanism, and may lead to the anticariogenic action of polyphenols [18, 19].

The polyphenolic compounds contained in Chilean propolis are diverse and include a variety of flavonoids and phenolic acids [12]. Among the principal components found within Chilean propolis, pinocembrin stands out for its high content [7]. This compound has been identified as a promising pharmacological candidate, mainly for being described with numerous biological activities including antimicrobial, anti-inflammatory, antioxidant, and anticancer effects [39]. Since the present study did not include the evaluation of individual polyphenols, additional studies are required to

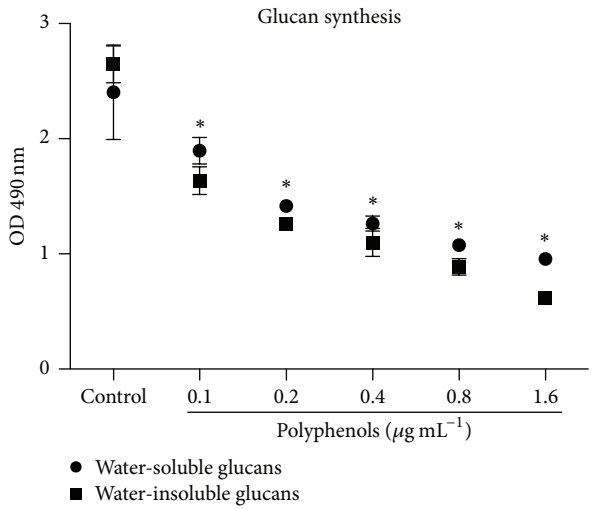


Figure 3: Effect of polyphenol-rich extract from propolis on the metabolic activity of glucosyltransferases. Statistical significance was determined by ANOVA and Dunnett's multiple comparison test using nontreated cells as control. $^{*}p < 0.05$.

identify the compound responsible of the modulatory effects shown by PEP treatment.

5. Conclusion

In summary, our results suggest that Chilean propolis has a dose-dependent effect on the inhibition of genes involved in *S. mutans* virulence and adherence through the inhibition of glucosyltransferases, showing an anticariogenic potential of polyphenols from propolis beyond *S. mutans* growth inhibition.

Competing Interests

The authors declare that they have no competing interests

Acknowledgments

The authors would like to express their gratitude to CONICYT and the financial support of DIUFRO Project DI10-0031.

References

[1] M. Matsumoto-Nakano, K. Fujita, and T. Ooshima, "Comparison of glucan-binding proteins in cariogenicity of *Streptococcus mutans*," *Oral Microbiology and Immunology*, vol. 22, no. 1, pp. 30–35, 2007.

[2] J. A. Aas, B. J. Paster, L. N. Stokes, I. Olsen, and F. E. Dewhirst, "Defining the normal bacterial flora of the oral cavity," *Journal of Clinical Microbiology*, vol. 43, no. 11, pp. 5721–5732, 2005.

[3] R. A. Bagramian, F. Garcia-Godoy, and A. R. Volpe, "The global increase in dental caries. A pending public health crisis," *American Journal of Dentistry*, vol. 22, no. 1, pp. 3–8, 2009.

[4] D. Vauzour, A. Rodriguez-Mateos, G. Corona, M. J. Oruna-Concha, and J. P. E. Spencer, "Polyphenols and human health: prevention of disease and mechanisms of action," *Nutrients*, vol. 2, no. 11, pp. 1106–1131, 2010.

[5] S. Castaldo and F. Capasso, "Propolis, an old remedy used in modern medicine," *Fitoterapia*, vol. 73, supplement 1, pp. S1–S6, 2002.

[6] J. B. Daleprane, V. da Silva Freitas, A. Pacheco et al., "Anti-atherogenic and anti-angiogenic activities of polyphenols from propolis," *Journal of Nutritional Biochemistry*, vol. 23, no. 6, pp. 557–566, 2012.

[7] L. Barrientos, C. L. Herrera, G. Montenegro et al., "Chemical and botanical characterization of chilean propolis and biological activity on cariogenic bacteria *Streptococcus mutans* and *Streptococcus sobrinus*," *Brazilian Journal of Microbiology*, vol. 44, no. 2, pp. 577–585, 2013.

[8] N. Saavedra, L. Barrientos, C. L. Herrera, M. Alvear, G. Montenegro, and L. A. Salazar, "Effect of Chilean propolis on cariogenic bacteria Lactobacillus fermentum," *Ciencia e Investigacion Agraria*, vol. 38, no. 1, pp. 117–125, 2011.

[9] H. Koo, P. L. Rosalen, J. A. Cury, Y. K. Park, M. Ikegaki, and A. Sattler, "Effect of *Apis mellifera* propolis from two Brazilian regions on caries development in desalivated rats," *Caries Research*, vol. 33, no. 5, pp. 393–400, 1999.

[10] V. Bankova, "Chemical diversity of propolis and the problem of standardization," *Journal of Ethnopharmacology*, vol. 100, no. 1-2, pp. 114–117, 2005.

[11] M. Popova, V. Bankova, D. Butovska et al., "Validated methods for the quantification of biologically active constituents of poplar-type propolis," *Phytochemical Analysis*, vol. 15, no. 4, pp. 235–340, 2004.

[12] J. J. Veloz, N. Saavedra, A. Lillo, M. Alvear, L. Barrientos, and L. A. Salazar, "Antibiofilm activity of chilean propolis on *Streptococcus mutans* is influenced by the year of collection," *BioMed Research International*, vol. 2015, Article ID 291351, 6 pages, 2015.

[13] T. Ooshima, M. Matsumura, T. Hoshino, S. Kawabata, S. Sobue, and T. Fujiwara, "Contributions of three glucosyltransferases to sucrose-dependent adherence of Streptococcus mutans," *Journal of Dental Research*, vol. 80, no. 7, pp. 1672–1677, 2001.

[14] Z. T. Wen and R. A. Burne, "Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*," *Applied and Environmental Microbiology*, vol. 68, no. 3, pp. 1196–1203, 2002.

[15] W. Krzyściak, A. Jurczak, D. Kościelniak, B. Bystrowska, and A. Skalniak, "The virulence of Streptococcus mutans and the ability to form biofilms," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 33, no. 4, pp. 499–515, 2014.

[16] M. D. Senadheera, B. Guggenheim, G. A. Spatafora et al., "A VicRK signal transduction system in Streptococcus mutans affects gtfBCD, gbpB, and ftf expression, biofilm formation, and genetic competence development," *Journal of Bacteriology*, vol. 187, no. 12, pp. 4064–4076, 2005.

[17] L. Zeng, S. C. Choi, C. G. Danko, A. Siepel, M. J. Stanhope, and R. A. Burne, "Gene regulation by CcpA and catabolite repression explored by RNA-Seq in *Streptococcus mutans*," *PLoS ONE*, vol. 8, no. 3, Article ID e60465, 2013.

[18] S.-J. Ahn, S.-J. Ahn, Z. T. Wen, L. J. Brady, and R. A. Burne, "Characteristics of biofilm formation by *Streptococcus mutans* in the presence of saliva," *Infection and Immunity*, vol. 76, no. 9, pp. 4259–4268, 2008.

[19] L. J. Brady, S. E. Maddocks, M. R. Larson et al., "The changing faces of *Streptococcus* antigen I/II polypeptide family adhesins," *Molecular Microbiology*, vol. 77, no. 2, pp. 276–286, 2010.

[20] L. A. Salazar, C. Vásquez, A. Almuna et al., "Molecular detection of cariogenic streptococci in saliva," *International Journal of Morphology*, vol. 26, no. 4, pp. 951–958, 2008.

[21] CLSI, "Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically; approved standard—eight edition," CLSI Document M07-A8, Clinical and Laboratory Standards Institute (CLSI), Wayne, Pa, USA, 2009.

[22] T. D. Schmittgen and K. J. Livak, "Analyzing real-time PCR data by the comparative CT method," *Nature Protocols*, vol. 3, no. 6, pp. 1101–1108, 2008.

[23] H. Koo, P. L. Rosalen, J. A. Cury, Y. K. Park, and W. H. Bowen, "Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity," *Antimicrobial Agents and Chemotherapy*, vol. 46, no. 5, pp. 1302–1309, 2002.

[24] B. Islam, S. N. Khan, I. Haque, M. Alam, M. Mushfiq, and A. U. Khan, "Novel anti-adherence activity of mulberry leaves: inhibition of *Streptococcus mutans* biofilm by 1-deoxynojirimycin isolated from *Morus alba*," *Journal of Antimicrobial Chemotherapy*, vol. 62, no. 4, pp. 751–757, 2008.

[25] J. Smullen, G. A. Koutsou, H. A. Foster, A. Zumbé, and D. M. Storey, "The antibacterial activity of plant extracts containing polyphenols against *Streptococcus mutans*," *Caries Research*, vol. 41, no. 5, pp. 342–349, 2007.

[26] S. Duarte, S. Gregoire, A. P. Singh et al., "Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms," *FEMS Microbiology Letters*, vol. 257, no. 1, pp. 50–56, 2006.

[27] W. Shumi, M. A. Hossain, D.-J. Park, and S. Park, "Inhibitory effects of green tea polyphenol epigallocatechin gallate (EGCG) on exopolysaccharide production by *Streptococcus mutans* under microfluidic conditions," *Biochip Journal*, vol. 8, no. 3, pp. 179–186, 2014.

[28] S. A. Libério, A. L. A. Pereira, M. J. A. M. Araújo et al., "The potential use of propolis as a cariostatic agent and its actions on mutans group streptococci," *Journal of Ethnopharmacology*, vol. 125, no. 1, pp. 1–9, 2009.

[29] S. Duarte, P. L. Rosalen, M. F. Hayacibara et al., "The influence of a novel propolis on mutans streptococci biofilms and caries development in rats," *Archives of Oral Biology*, vol. 51, no. 1, pp. 15–22, 2006.

[30] R. O. Mattos-Graner, M. H. Napimoga, K. Fukushima, M. J. Duncan, and D. J. Smith, "Comparative analysis of Gtf isozyme production and diversity in isolates of *Streptococcus mutans* with different biofilm growth phenotypes," *Journal of Clinical Microbiology*, vol. 42, no. 10, pp. 4586–4592, 2004.

[31] Y. Terao, R. Isoda, J. Murakami, S. Hamada, and S. Kawabata, "Molecular and biological characterization of gtf regulation-associated genes in *Streptococcus mutans*," *Oral Microbiology and Immunology*, vol. 24, no. 3, pp. 211–217, 2009.

[32] S. Biswas and I. Biswas, "Regulation of the glucosyltransferase (*gtfBC*) operon by CovR in *Streptococcus mutans*," *Journal of Bacteriology*, vol. 188, no. 3, pp. 988–998, 2006.

[33] P.-M. Chen, J.-Y. Chen, and J.-S. Chia, "Differential regulation of *Streptococcus mutans gtfBCD* genes in response to copper ions," *Archives of Microbiology*, vol. 185, no. 2, pp. 127–135, 2006.

[34] A. Yoshida and H. K. Kuramitsu, "Multiple *Streptococcus mutans* genes are involved in biofilm formation," *Applied and Environmental Microbiology*, vol. 68, no. 12, pp. 6283–6291, 2002.

[35] R. N. Stipp, R. B. Gonçalves, J. F. Höfling, D. J. Smith, and R. O. Mattos-Graner, "Transcriptional analysis of gtfB, gtfC, and gbpB and their putative response regulators in several isolates of *Streptococcus mutans*," *Oral Microbiology and Immunology*, vol. 23, no. 6, pp. 466–473, 2008.

[36] E. Ayala, J. S. Downey, L. Mashburn-Warren, D. B. Senadheera, D. G. Cvitkovitch, and S. D. Goodman, "A biochemical characterization of the DNA binding activity of the response regulator VicR from streptococcus mutans," *PLoS ONE*, vol. 9, no. 9, Article ID e108027, 2014.

[37] J. Abranches, M. M. Nascimento, L. Zeng et al., "CcpA regulates central metabolism and virulence gene expression in *Streptococcus mutans*," *Journal of Bacteriology*, vol. 190, no. 7, pp. 2340–2349, 2008.

[38] C. M. Browngardt, Z. T. Wen, and R. A. Burne, "RegM is required for optimal fructosyltransferase and glucosyltransferase gene expression in *Streptococcus mutans*," *FEMS Microbiology Letters*, vol. 240, no. 1, pp. 75–79, 2004.

[39] A. Rasul, F. M. Millimouno, W. Ali Eltayb, M. Ali, J. Li, and X. Li, "Pinocembrin: a novel natural compound with versatile pharmacological and biological activities," *BioMed Research International*, vol. 2013, Article ID 379850, 9 pages, 2013.