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***Chapter 10***

# ANTIMICROBIAL AND ANTIVIRAL ACTIVITIES OF GRAPE SEED EXTRACTS

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## ABSTRACT

Grape seed extract (GSE) is a rich source of polyphenols. The polyphenols are important secondary metabolites which play multiple essential roles in plant physiology and which show a broad range of bioactive properties in human organism, mainly as antioxidant, anti-inflammatory, anticancer, cardioprotection, and antiaging. GSE is recognized as a complex mixture of monomeric, oligomeric, and polymeric flavan-3-ols. The principal monomers identified are (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, and (−)-epigallocatechin gallate. The content of flavan-3-ols in seed grapes is influenced by several factors mainly cultivar, irrigation, nitrogen fertilization, delayed harvest, and storage conditions. Antimicrobial and antiviral activities of GSE have been described. Moreover, some researchers showed that seed extracts were more effectively antimicrobial than other parts of grapes. The decrease order of the antimicrobial activity is seed, skin and flesh grape extracts.

We demonstrated, for the first time, a significant correlation between the content of the flavan-3-ols in GSEs, with a polymerization degree ≥4, and antifungal activity. Recently, we also demonstrated a significant inhibition of *Candida albicans,* in an experimental murine model of vaginal candidiasis, using GSE with high content of polymeric flavan-3-ols. Technologies to deliver GSEs for an effective inhibition of pathogens have been reported. GSE is Generally Recognized as Safe (GRAS), approved by Food and Drug Administration (FDA) and also sold as dietary supplement. Antimicrobial activity together with lack of toxicity suggests that GSE could be used for

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the prevention and control of infection diseases without side effects, making greater potential for grape in the field of food and pharmaceutical application.

**Keywords:** *Vitis vinifera*, grape seed extract, proanthocyanidins, antifungal activity, antibacterial activity, antiviral activity

## INTRODUCTION

Phytomedicine, which has historically been an important aspect of traditional medicine in non-industrialized countries, is now becoming an integral part of healthcare in industrialized countries. Plants are the source of thousands of new phytochemicals, and different strategies can be applied to improve the yields of bioactive metabolites in the plant and to obtain chemically standardized extracts [1, 2]. Along with conventional methods, numerous new methods have been established but till now no single method is regarded as standard for extracting bioactive compounds from plants. The efficiencies of conventional and non-conventional extraction methods mostly depend on the critical input parameters; understanding the nature of plant matrix and chemistry of bioactive compounds [3].

Grapes is the second largest fruit crop after orange in the world, cultivated especially in Mediterranean area [4]. As reported by the wide literature, grapes is a rich source of polyphenols, which are important secondary metabolites produced by the higher plants and which play multiple essential roles in plant physiology. Polyphenols from grapes show healthy properties in human organism, mainly as antioxidant, antiallergic, anti-inflammatory, anticancer, antihypertensive, renoprotective, and antimicrobial agents [5-7]. *Vitis vinifera* seeds contain lipids, proteins, carbohydrates, and 5–8% polyphenols, depending on the cultivar of *Vitis vinifera* [8]. Standardized grape seed extracts contain from 74 to 78% oligomeric proanthocyanidins and less than approximately 6% of free flavanol monomers on a dry weight. The content of flavan-3-ols is influenced by several factors mainly cultivar, irrigation, nitrogen fertilization, delayed harvest, and storage conditions [9, 10]. Moreover, the application of an extraction process suitable to efficiently recover the target metabolites and an appropriate analytical method for an accurate qualitative and quantitative determination of extract components are required [11, 12].

Grape seed extract (GSE), rich in proanthocyanidins, shows potential antimicrobial activities in preventing pathogen contamination of food [6, 13, 14].

The phenolic compounds from different parts of grapes displayed different antimicrobial effects [6]. The decrease order of the antimicrobial activity is seed, skin and flesh grape extracts. Anastasiadi et al. [15] suggested that high concentrations of flavonoids and their derivatives by grape seeds, and flavonoids, stilbenes, and phenolic acids by grape stems, were responsible for the antimicrobial activity. Ferrazzano et al. [16] reported that red grape seeds, with high polyphenol content, had anti-cariogenic properties against *Streptococcus mutans.* Moreover, GSEs inhibit the growth of anaerobic bacteria, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, associated with periodontal diseases.

Rhodes et al. [17] demonstrated that polymeric phenolic fractions produced the highest inhibition activity for all *Listeria* species, but not for other bacteria, such as *Bacillus cereus, Salmonella menston, Escherichia coli, Staphylococcus aureus* or *Yersinia enterocolitica.*

Recently, high antifungal activity of GSE, rich in polymeric flavan-3-ols, against broad panel of human fungal pathogens has been demonstrated against *Candida* spp., *Cryptococcus neoformans*, and dermatophytes [10, 18]. The increase and spread of antimicrobial resistance and the infections caused by opportunistic pathogens are the most serious public health problems. Antimicrobial agents for the treatment of infections have become limited, leading frequently to recurrent infections, treatment failure and increase of morbidity and mortality. The decreased effectiveness and toxicity of current antibiotics led to search of alternative antimicrobial substances. Plants are recognized as a source of unexplored chemical structures with high therapeutic potential, including antimicrobial activity against clinically important microorganisms. Main classes of phytochemicals with antimicrobial properties and their mode of action have been studied [6, 19].

In this review, an overview of the activity of GSE against human microbial pathogens is reported. Moreover, novel techniques of GSE are described.

## GSE ACTIVE CONSTITUENTS

Grape is a phenol-rich plant and these molecules are mainly distributed in the skin, stem, leaf and seed. In the grape berry, anthocyanins, flavonols and simple flavan-3-ols are localized in the pericarp (skin), while flavan-3-ols are the most abundant class in the seed. GSEs contain from 74 to 78% oligomeric proanthocyanidins and less than approximately 6% of free flavanol monomers with respect to dry weight [20].

Catechins and proanthocyanidins are located essentially in the seeds, then in the skins and in traces in the pulp [21]. Proanthocyanidins vary in size, ranging from dimers to polymers with more than 40 units. GSE is recognized as a complex mixture of monomeric, oligomeric, and polymeric flavan-3-ols. The principal monomers identified are (+)-catechin, (−)-epicatechin, (−) epicatechin gallate (ECg), (−)-epigallocatechin (EGC), and (−)-epigallocatechingallate (EGCg). Prieur et al. [22] found that 55% of the procyanidins extracted from grape seeds consisted of more than five monomer units and determined that their mean degree of polymerization ranged from 2 to 16. Mattivi et al. [23] demonstrated that upper and extension units of polymeric proanthocyanidins are constituted mainly of epicatechin units, with the co-presence of catechin and epicatechin gallate. Recently we published GSEs HPLC profiles in terms of monomers, oligomers, and polymers from different *V. vinifera* cultivars. Optimizing the chromatographic method, also with the help of an RP 18 Poroshell column, several monomer and oligomer compounds have been separated and quantified in all the fifteen extracts. Moreover, two groups of polymeric procyanidins (Pol 1 and Pol 2) with a polymerization degree ≥4 have been separated and determined by their mass spectra in negative ionization mode [10]. GSE extract can be obtained from viticultural and winemaking supply chain's waste matter, such as seeds and/or pomace and or green seeds. Storage, delayed harvesting and the different kinds of water supply are the variables mostly affecting grape polyphenol content. Polyphenol content diminished by more than 50% after 6 weeks if the grapes was stored in a refrigerator [9]. It is known that to obtain reproducible plant extracts during the years it is of fundamental importance to have a raw material obtained in agronomic controlled conditions [24]. In GSE extracts, a high amount of total phenols and, above all, their optimal distribution between oligomers, polymers and

gallate forms within the extract is obtained by growing the grapevines under moderate hydric and nitrogen stress [10, 18].

Wine grape pomace (WGP), containing both seeds and skins, is a rich source of polyphenols. Dehydration of wet pomace is a first step before developing further applications. However, polyphenolics are sensitive to heat and oxygen. Several studies have evaluated the effects of different drying methods on the biochemical changes of fruit pomace [25, 26].

The minimum loss of bioactive compounds was found at drying temperature not higher than 50°C [27]. Physiochemical properties and chemical composition of dried pomace of Pinot Noir and Merlot, subjected to different drying methods followed by storage at 15 ± 2ºC, were evaluated by Tseng and Zhao [26]. Overall, 40ºC oven and ambient air dry are highly acceptable by considering the amount of retention of most measured bioactive compounds and their much less cost compared with freeze dry, thus may be employed in commercial application of drying large quantity of wine processing by products.

## ANTIBACTERIAL ACTIVITY

### Gram-Positive Bacteria

Some authors reported that GSE was more effective in inhibiting Gram-positive than Gram-negative bacteria.

GSE of *V. vinifera* var. Ribier black table grapes was found to be highly inhibitory towards *Listeria monocytogenes*. *L. monocytogenes* is ubiquitous in the environment and causes the disease listeriosis, which, although rare, has a 20–30% mortality rate. Fractionation of the extracts showed that the antilisterial activity was strongest using the polymeric phenolic fractions [17].

GSE from New Zealand subjected to extraction process using three different solvents (50% aqueous acetone, 50% aqueous ethanol or 50% aqueous methanol) were tested against *Staphylococcus aureus* NCTC 6571. Methanol/water seed extracts exhibited higher antimicrobial activity against *S. aureus* than the other extracts. New Zealand Pinot noir grapes was more effective against *S. aureus* bacteria than extracts from Pinot Meunier grapes, suggesting that extraction solvent, fraction, and grape variety significantly influence antimicrobial properties. The relationship between total phenolic content and antimicrobial activities against *S. aureus* was found to be positively significant [28]. Baydar [29] reported that seed extracts at 2.5% concentration had a bactericidal effect on *S. aureus* during the 24 h.

Both growth and biofilm formation of *S. mutans* UA159 were inhibited by GSE at 4 mg/mL [30]. Cueva et al. [31] reported that extracts from grape seeds were active against *Enterococcus faecalis* V583, *S. aureus* ATCC 25923 and *Streptococcus pneumonia*. Furiga [32] investigated the preventive effects of GSE on dental plaque formation. CLSM observations of biofilms incubated with 2000 $\mu$g/ml of GSE revealed a decrease in the number of microcolonies and thickness of biofilms. These observations suggest the need to evaluate the germ-killing efficacy of grape compounds in oral rinses and chewing gums.

### Gram-Negative Bacteria

GSE extracts prepared from *Vitis rotundifolia*, (cultivar Ison and from cultivar Carlos) exhibited strong antimicrobial activity against *Escherichia coli* O157:H7. *E. coli* O157:H7 is an enteropathogen responsible for hemorrhagic colitis, bloody diarrhea, and hemolytic uremic syndrome. Kim et al. [33] reported that heat treatment of both extracts increased antibacterial activity and total phenolic content.

Quiñones et al. [34] reported that commercial GSEs inhibited Shiga toxin1 and Shiga toxin 2 produced by *E. coli* O157:H7. Chardonnay seed flour extracts at 165 μg seed flour equivalents/mL exhibited bactericidal activity against *E. coli* [35].

Ethanol extracts of grape seeds were found to be effective in inhibiting *Klebsiella pneumoniae* with MIC value of 40 μg/mL [36]. Two grape extracts currently sold as nutritional supplements inhibited cholera toxin and *E. coli* heat-labile toxin activity against cultured cells and intestinal loops by blocking toxin binding to the cell surface [37].

Cholera toxin (CT), produced by *Vibrio cholera*, is an AB5 toxin responsible for the profuse, life-threatening diarrhea of cholera. The MIC of GSE, against *Vibrio vulnificus* was 10 mg/mL. Treatment with 500 mg/mL GSE reduced the initial inherent microbiota in fresh shucked to below the detection level [38].

Anti-*Helicobacter pylori* activity of muscadine (*Vitis rotundifolia*) seed extract was determined. *H. pylori* is considered the etiological agent of peptic ulcer and gastritis. The MIC results indicated that the GSE had significant effects against growth of *H. pylori* strains (MIC range from 256 to 1,024 μg/mL) [39].

Silván et al. [40] examined GSE activity against different *Campylobacter* strains demonstrating the capacity of the GSE to inhibit *Campylobacter* growth in the range from 5.08 to 6.97 log CFU/mL. The analysis of the antibacterial activity against *C. jejuni* of the collected fractions showed that phenolic acids, catechins and proanthocyanidins were the main responsible of the behavior observed.

## ANTIFUNGAL ACTIVITY

The highly fatal fungal systemic infections, associated with immunosuppression, are supported mainly by *Candida* species, *Cryptococcus neoformans* and *Aspergillus* spp. Superficial mucosal and cutaneous infections are mainly caused by *Candida* spp., dermatophytes and *Malassezia* spp. The increase in fungal infections caused an increase in the prescription of antifungal drugs, with a resulting increase of costs. In addition, for some fungal infections, mainly the superficial ones (for example, onychomycosis caused by dermatophytes), the timing of the therapeutical treatment is long and often is not resolving and subsequently they become chronic and recurrent and the remarkable side effects make even more dramatic the already poor health conditions of the individuals who are being treated. Many antifungal agents currently in use bring about undesired effects, they are often ineffective toward new or recurrent agents of opportunistic infections and resistance to antifungal therapy continue to increase [41].

The number of therapeutic options for the treatment of fungal infections is quite limited when compared with those available to treat bacterial infections. Indeed, only three classes of

molecules are currently used in clinical practice and only one new class of antifungal drugs has been developed in the last 30 years [42].

We reported, for the first time, anti-*fungal* activity and chemical analysis of GSEs obtained from different wine and table cultivars of *V. vinifera* L., grown in different agronomic conditions against a broad panel of human fungal pathogen [18].

Cheng et al. [28] have demonstred that extracts obtained from Pinot noir and Pinot meunier seeds showed anti-*Candida* activity with MIC values of 0.39 and 50 mg/mL for Pinot noir and Pinot meunier, respectively.

Recently, we compared the anti-*Candida* activity with respect of phenolic content in GSE obtained by several *V. vinifera* cultivars. We demonstrated a significant negative correlation coefficient of total flavan-3-ols contained in the different extracts and MIC values ($r = -0.648$, $P = 0.00896$). Moreover, we demonstrated, for the first time, that the antifungal activity (MIC) of GSEs is attributable mostly to the polymeric flavan-3-ols (with a polymerization degree ≥4) (Figure 1), with a significative negative correlation coefficient ($r = -0.6974$, $P = 0.0038$). Differently, the content of gallate monomers and oligomers did not seem to be correlated to antifungal activity ($r = -0.4334$, $P = 0.1065$) [10].

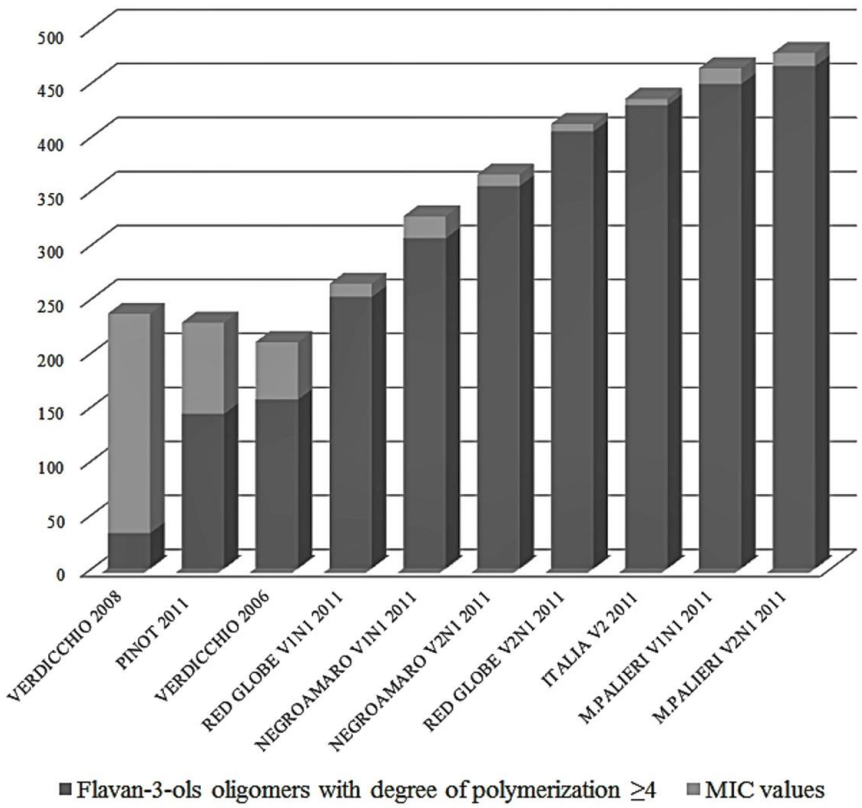


Figure 1. Flavan-3-ols with degree of polymerization ≥4 in grape seed extracts obtained by different cultivars of *Vitis vinifera* L. and geometric mean MIC against *Candida albicans* reference strains (ATCC90028, ATCC3153, ATCC10261, ATCC10231 and ATCC24433).

It is important to emphasize that the typical catechin of green tea, EGCg, known to be responsible of growth-inhibitory effect on clinical isolates of *Candida* spp. [43], was absent in our samples [10].

## ANTIVIRAL ACTIVITY

Several authors reported antiviral activities of resveratrol, as active component in skin grape [44-46]. The effect of a polyphenol-based grape extract (NE) obtained from Portuguese white-winemaking by-products, and resveratrol in pure form, on adenovirus type 5 infection has been evaluated. The NE and resveratrol reduced 4.5 and 6.5 log (TCID50/mL) on total infectious Ad-5 production, respectively [47].

Few papers report antiviral activity of GSE. Hala [48] found that GSE was active upon inhibiting the hepatitis C virus (HCV) replication into HepG2 cells. Feline calicivirus, FCV-F9; murine norovirus, MNV-1; and bacteriophage MS2) and hepatitis A virus (HAV; strain HM175) were treated with commercial GSE (Gravinol-S). At high titers (~7 $\log_{10}$ PFU/ml), FCV-F9 was significantly reduced by 3.64, 4.10, and 4.61 $\log_{10}$ PFU/ml; MNV-1 by 0.82, 1.35, and 1.73 $\log_{10}$ PFU/ml; MS2 by 1.13, 1.43, and 1.60 $\log_{10}$ PFU/ml; and HAV by 1.81, 2.66, and 3.20 $\log_{10}$ PFU/ml after treatment at 37°C with 0.25, 0.50, and 1 mg/ml GSE, respectively in a dose-dependent manner [49]. GSE at 1 mg/ml in apple juice reduced MNV-1 to undetectable levels after 1 h and by 1 log in milk after 24 h. GSE at 1 and 2 mg/ml in AJ reduced HAV to undetectable levels after 1 h, while 2 and 4 mg/ml GSE in milk caused ~1 log reduction after 24 h. GSE at 2 mg/mL in intestinal fluid reduced FCV-F9, MNV-1 and HAV to undetectable levels after 6 h [50].

Nair et al. [51] found that GSE seemed to exert antiviral effects by inducing Th1-derived cytokine γ interferon (IFN-γ) by peripheral mononuclear cells, suggesting that the beneficial immunostimulatory effect of GSE may be mediated through induction of IFN-γ. The enhancing effect of GSE on IFN-γ expression was further supported by a concomitant increase in the number of cells with intracytoplasmic IFN-γ as well as the synthesis and secretion of IFN-γ.

## ANTIMICROBIAL ACTIVITY OF GSE IN DIFFERENT DELIVERY SYSTEMS

Over the past several years, great advances have been made on development of novel drug delivery systems for plant extracts. The variety of novel herbal formulations like polymeric nanoparticles, nanocapsules and liposomes, has been reported using bioactive plant extracts. The novel formulations are reported to have remarkable advantages over conventional formulations of plant extracts which include enhancement of solubility, bioavailability, protection from toxicity, enhancement of pharmacological activity, enhancement of stability, sustained delivery, and protection from physical and chemical degradation [52].

There has been an increasing trend in the application of electrostatic spray in various agricultural and biological systems as it has great potential in distributing the antimicrobial

compounds when applied [53]. Electrostatic spraying is a novel technology that can be used for fine coating of antimicrobials on a biotic surface so that it can provide greater retention and efficient distribution required to interact with pathogens [54]. Ganesh et al. [55] reported that electrostatic spraying (in comparison to conventional treatments) of spinach with GSE and malic acid showed 2.6 and 3.3 log reductions of *L. monocytogenes* and *Salmonella* Typhimurium on days 7 and 14 respectively.

A novel technique of delivering employs nanoparticles that show high interactions in biological systems [56]. Application of nanotechnology has been extensively explored in several bio-medical areas, especially drug delivery and also has demonstrated huge potential in nutraceuticals and functional foods for delivering bioactive compounds [57, 58]. Recently, we demonstrated the ability of polylactic-co-glycolic acid nanoparticles (PLGA NPs) to cross the plant cell wall and membrane of *V. vinifera* cell cultures and grapevine-pathogenic fungi [59]. In grapevine plants PLGA NPs can be absorbed by the roots and transported to the leaves through the vascular tissues. Moreover, PLGA NPs can enter in leaf tissues through stomata openings [59].

Ravichandran et al. [60, 61] have investigated the inhibitory effects of polylactic-co-glycolic acid nanoparticle-encapsulated GSE and MA (1% GSE + 1% MA) against *L. monocytogenes*, *S.* Typhimurium, *E. coli* O157:H7 and *C. jejuni*. GSE-MA nanoparticles showed effective inhibitory action against *L. monocytogenes* with a reduction of 5.5 log CFU/mL and against *E. coli* O157:H7 and *S.* Typhimurium with a reduction of 5.2 log CFU/mL and 4.9 log CFU/mL, respectively.

Edible films containing natural antimicrobials can minimize post packaging pathogen contamination. GSE incorporated in pea starch film inhibited the growth of *S. aureus, E. faecalis, E. faecium*, and *L. monocytogenes* [62].

Soy protein isolates (SPI) are widely used to prepare edible films [63, 64]. Antimicrobial and physical properties of soy protein films with various natural antimicrobials have been demonstrated [65, 66]. SPI films containing grape seed extract (GSE 1% w/w), nisin (10,000 IU/g), ethylenediaminetetraacetic acid (EDTA 0.16% w/w), inhibited the growth of *L. monocytogenes* (reduction of 2.9 log CFU/mL), *E. coli* (1.8 log reduction) and *S.* Typhimurium (0.6 log reduction) [64].

## CONCLUSION

GSE has the potential to provide inexpensive antimicrobial agent for use against a broad range infectious diseases and to protect food contamination. In phytotherapy it is crucial to obtain extracts with reproducible chemical composition, as often the pharmacological activity shows itself thanks to the concurrent presence of molecules, often belonging to different chemical classes that in this way concur to define the phytocomplex. The activity of phytoterapic products is generally performed by the synergic action of different components, comprising the less concentrated ones, which contribute to the modulation of the product's activity. The main industrial interest is to obtain a phytocomplex with a standardized and reproducible content in characterized active principles, starting from low cost and safe plant matrices and by using simple and reproducible extraction procedures. In spite of interesting antimicrobial activity, up to date GSE hasn't been developed as antimicrobial product for

human and animal infections. Besides pharmaceutical application, the recent literature shows positive results on the possibility to use GSE as antimicrobial agent in food conservation systems.

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