

Relation between Antibacterial Activity against Food Transmitted Pathogens and Total Phenolic Compounds in Grape Pomace Extracts from Cabernet Sauvignon and Syrah Varieties

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Abstract

Grape pomace is the main by-product of wine production that concentrates bioactive metabolites of polyphenolic nature with antibacterial activity. Since grape pomace composition varied depending on grape variety, climate, vineyard location, and winemaking technology, it is important to study the composition and antibacterial activity of each variety separately. In this study, antibacterial activity against different food pathogens was evaluated and its relation with polyphenols content was determined. Grape pomace from Cabernet Sauvignon and Syrah varieties was extracted with methanol/HCl 1% (v/v), followed by sequential extractions with hexane, chloroform and ethyl acetate. Ethyl acetate fraction had the highest antibacterial activity determined through the microdilution method, reaching over 90% of inhibition at 500 $\mu\text{g}\cdot\text{ml}^{-1}$ with the exception of *Salmonella* Typhi (70% of inhibition). *Staphylococcus aureus* and *Escherichia coli* were the most susceptible strains, exceeding 50% of inhibition at 62.5 $\mu\text{g}\cdot\text{ml}^{-1}$. Ethyl acetate fraction contains the highest phenolic concentration in both Cabernet Sauvignon (132.2 mg of GAE g^{-1}) and Syrah (102.6 mg of GAE g^{-1}) pomace, as determined by the Folin-Ciocalteu method. Antibacterial activity present in grape pomace extracts is in direct relation to the polar phenolic content, in particular

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that from Cabernet Sauvignon.

Keywords

Antibacterial Activity, Grape Pomace Extract, Ethyl Acetate Fraction, Total Phenolic Content

1. Introduction

Contamination of food by multiresistant infectious bacteria has become a serious problem, because they limit the treatment and effectiveness of the antibiotics currently in clinical use. This has stimulated the search for compounds with antibacterial activity from natural sources. Fruit peel and seeds are rich in bioactive substances such as phenolic acids and flavonoids [1]-[3] which are known to possess antibacterial activity [4]-[6]. Grapes (*Vitis vinifera*) are one of the world's largest fruit crops, with an annual world production of 70 million tons in 2010 (<http://faostat.fao.org>). It is estimated that more than 80% of total grape production is used in winemaking [7]. The main organic waste produced in wine industries is grape pomace [3], representing 13% to 25% of the total weight of processed fruit [1]-[8]. This waste is produced after pressing the crushed grapes in white wine processing or after fermentation in red wine production. Its main components are fragmented skin, broken cells, pulp remains, stalks, and seeds [9]-[11] and it contains high amount of phenolic compounds due to their poor extraction during the winemaking process [12]. Anthocyanins, catechins, flavonol glycosides, phenolic acids, alcohols and stilbenes have been identified among the compounds present in grape pomace [8] [13]-[15].

Antibacterial activity has also been determined in extracts obtained from whole fruits [16], seeds [17]-[20], pomaces [17]-[21] and skins [18]. Organic extracts from seeds showed bacteriostatic activity against anaerobic bacteria responsible for periodontal diseases [19], and they were also able to inhibit hydrofolate reductase activity in *Staphylococcus aureus* [20]. Thimothe *et al.* (2007) showed inhibition of glucosyltransferases B and C in *Streptococcus mutans* by all the grape extracts studied. Furthermore, it has been determined that seeds and pomace extracts obtained with different solvents have distinct polyphenolic content [21] [22]. As grape pomace is a food waste containing antibacterial activity, it represents a huge potential for controlling pathogens transmitted through food, however their ability to control these pathogens has not been explored previously. Since grape pomace composition varies depending on grape variety, climate, vineyard location, and winemaking technology [8]-[10], it is important to investigate the composition and antibacterial activity of each variety separately and thus explore more deeply the biotechnological applications of this residue of the wine industry. In this study, the presence of antibacterial activity against food pathogens was evaluated (*Escherichia coli*, *Salmonella* Typhi, *S. aureus*, and *Listeria monocytogenes*) in organic extracts obtained from two varieties of grape pomace. We also evaluated the relationship between this activity and the total content of phenolic compounds.

2. Experimental

2.1. Grape Pomace Extracts

The grape pomace samples were obtained after the fermentation process from the 2009 harvest season of the Cabernet Sauvignon and Syrah varieties of the Miguel Torres vineyard (Curicó, Chile). The pomace was kept at -20°C before use. Each pomace sample (450 g) was ground and extracted with 1 L of methanol/HCl 1% (v/v) for 4 h at 4°C with constant agitation (100 rpm), concentrated in a rotary evaporator at 50°C , and subjected to sequential liquid-liquid extraction with hexane, chloroform and ethyl acetate [15]. Finally, the fractions were concentrated to dryness and kept at -20°C . For the experiments described below, each extract was dissolved in methanol.

2.2. Determination of Total Phenolic Content

The total phenolic content (TPC) determination in each pomace extract was performed using the Folin-Ciocalteu method [23]. The Folin-Ciocalteu reagent used in these experiments was Sigma-Aldrich and the absorbance was measured at 765 nm on a Shimadzu model UV-VIS-1240 spectrometer. The estimation of phenolic compounds in the extract was calculated from a calibration curve obtained with gallic acid equivalents. TPC was

expressed in milligrams of gallic acid per gram of extract (mg GAE g⁻¹ extract). All samples were analyzed in triplicate.

2.3. Bacterial Strains and Culture Media

Two Gram negative bacteria, *E. coli* ATCC 25922 and *S. Typhi* STH-2370, and the Gram positive bacteria *S. aureus* ATCC 6538, were grown in Mueller-Hinton media (MH-B, Merck). Gram positive *L. monocytogenes* ATCC 15313 was grown in a 1:1 mixture of Brain Heart Infusion (BHI, Merck) and Tryptic Soy Broth (TSB, Becton Dickinson). All bacterial cultures were maintained in solid agar media at 4°C, and for each experiment a single colony was inoculated in the corresponding liquid medium and incubated at 37°C for 18 h.

2.4. Antibacterial Activity Determination

The antibacterial activity was determined in 96-well microdilution plates following the Clinical Laboratory Standards Institute (CLSI, www.clsi.org) recommendations. Grape pomace extracts dissolved in methanol at different final concentrations were assayed (62.5, 125, 250 and 500 µg·ml⁻¹). The plates were incubated at 37°C for 18 h and the absorbance at 600 nm was determined in an Elisa reader (Thermos Labsystems Multiskan FC Model). The inhibition equation was used to calculate the inhibition percentage of each extract, where, TA₆₀₀ is the absorbance of the sample (microdilution assays with different fractions of grape pomace extract), EA₆₀₀ is absorbance control and SA₆₀₀ is growth control.

$$(\%) = \left(100 - \left(\left(100 \times (TA_{600} - EA_{600}) \right) (SA_{600})^{-1} \right) \right)$$

3. Results

3.1. Total Phenolic Content

Table 1 summarizes the TPC of the different fractions of grape pomace extracts obtained from the Cabernet Sauvignon and Syrah grape varieties. The TPC varied depending on the polarity of solvents used for the fractionation (hexane, chloroform and ethyl acetate), showing with the most non polar solvent (hexane) the lowest value among the fractions of both grape varieties. By other hand, highest TPCs were obtained with the more polar solvent, reaching the ethyl acetate fraction 132.229 and 102.592 mg GAE g⁻¹ extract for Cabernet Sauvignon and Syrah, respectively. These results show that TPC concentration in the different fractions depends directly on the solvents polarity.

Moreover, the total phenolic content in grape pomace extract is also dependent on the grape variety, as shown in **Table 1**. The fractions obtained with hexane, chloroform and ethyl acetate from the Cabernet Sauvignon variety showed significant higher ($P < 0.001$) TPC concentrations compared to the Syrah variety.

3.2. Antibacterial Activity of Grape Pomace Extracts

All fractions of grape pomace extracts showed antibacterial activity against all the tested strains of Gram positive and Gram negative bacteria, indicating that they have a broad spectrum action.

In general, *S. aureus* and *E. coli* were the most sensitive strains to the antibacterial activity of the compounds present in grape pomace extract, as shown in **Figure 1** and **Figure 2**. For all the bacteria tested, hexane extracts were less active, with 80% maximum inhibition against *S. aureus* and *L. monocytogenes* at 500 µg·ml⁻¹, while

Table 1. Total phenolics in grape pomace fractions.

Fraction	Total phenolic content (mg GAE g ⁻¹ extract)	
	Cabernet Sauvignon	Syrah
Hexane	12.577 ± 1.66	6.989 ± 0.4*
Chloroform	68.592 ± 4.4	42.032 ± 3.3*
Ethyl acetate	132.229 ± 4.8	102.592 ± 3.7*

*t-test $P < 0.001$.

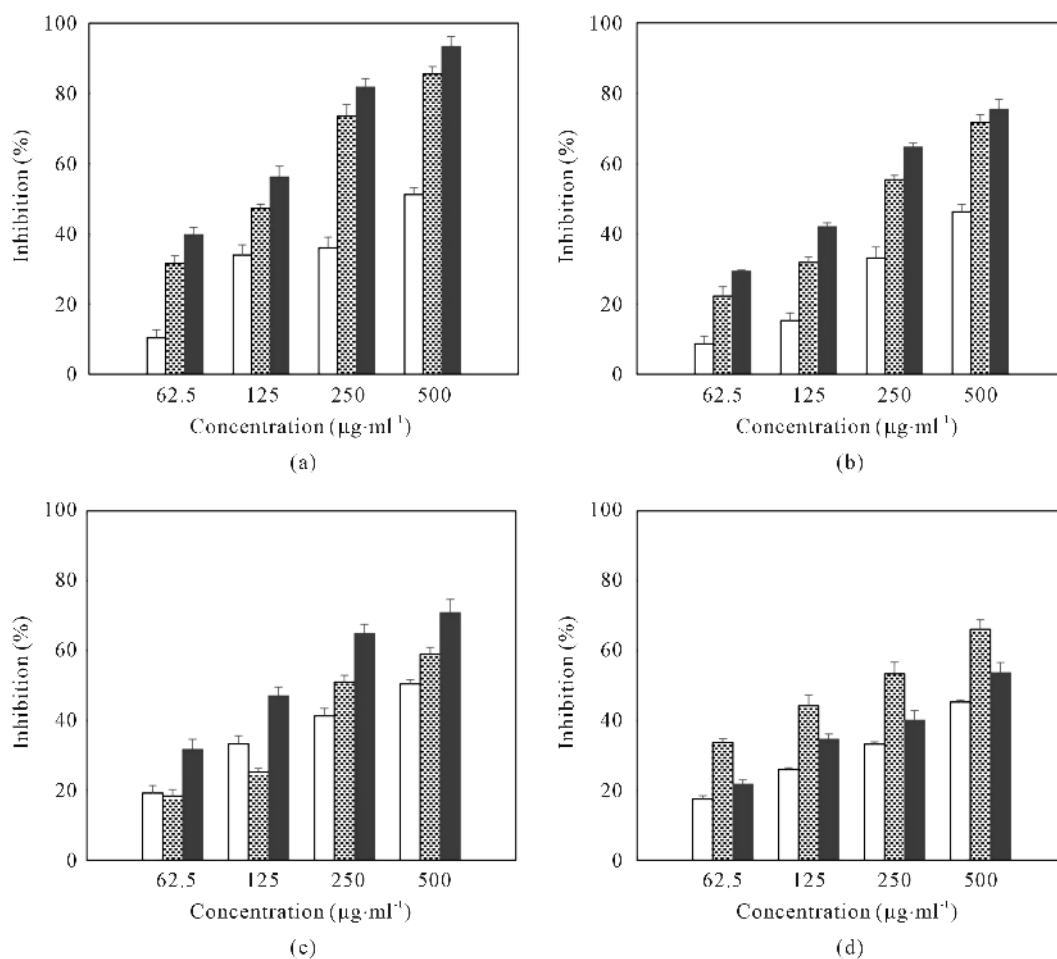


Figure 1. Antibacterial activity of grape pomace extracts against Gram positive bacteria. The figure shows the effect of grape pomace extracts obtained using hexane (white bars), chloroform (hatched bars) and ethyl acetate (gray bars) on bacterial growth of *S. aureus* (a) and (b) and *L. monocytogenes* (c) and (d). Extracts from Cabernet Sauvignon (a) and (c) and Syrah (b) and (d) varieties were analyzed.

extracts from the Cabernet Sauvignon variety fractionated with chloroform and ethyl acetate showed greater antibacterial activity on all tested bacteria. The growth inhibition of *S. aureus* reached 90% and 98% for the chloroform and ethyl acetate fractions, respectively, at the highest concentration used. Moreover, at the lowest concentration, 62.5 µg·ml⁻¹, inhibition was 50% and 60%, respectively. Although *L. monocytogenes* was less susceptible than *S. aureus* to the extracts fractionated with chloroform and ethyl acetate from both grape varieties, inhibition values were close to 90%. Furthermore, extracts from the Syrah variety showed less activity than Cabernet Sauvignon extracts, with inhibition against *S. aureus* and *L. monocytogenes* not exceeding 90% and 45% at concentrations of 500 and 62.5 µg·ml⁻¹, respectively.

On the other hand, the Gram negative bacteria *E. coli* and *S. Typhi* showed higher growth inhibition with Cabernet Sauvignon extracts (Figure 2). Again, all fractions of the Syrah variety extracts showed lower antibacterial activity compared to Cabernet Sauvignon. Of the two Gram negative bacteria tested, both *E. coli* and *S. Typhi* showed the same trend at all the concentrations used, but *S. Typhi* was less susceptible, reaching 70% maximum inhibition at a concentration of 500 µg·ml⁻¹.

4. Discussion

4.1. Total Phenolic Content

In this work, the concentration of polyphenolic compounds found in the different fractions are in agreement with

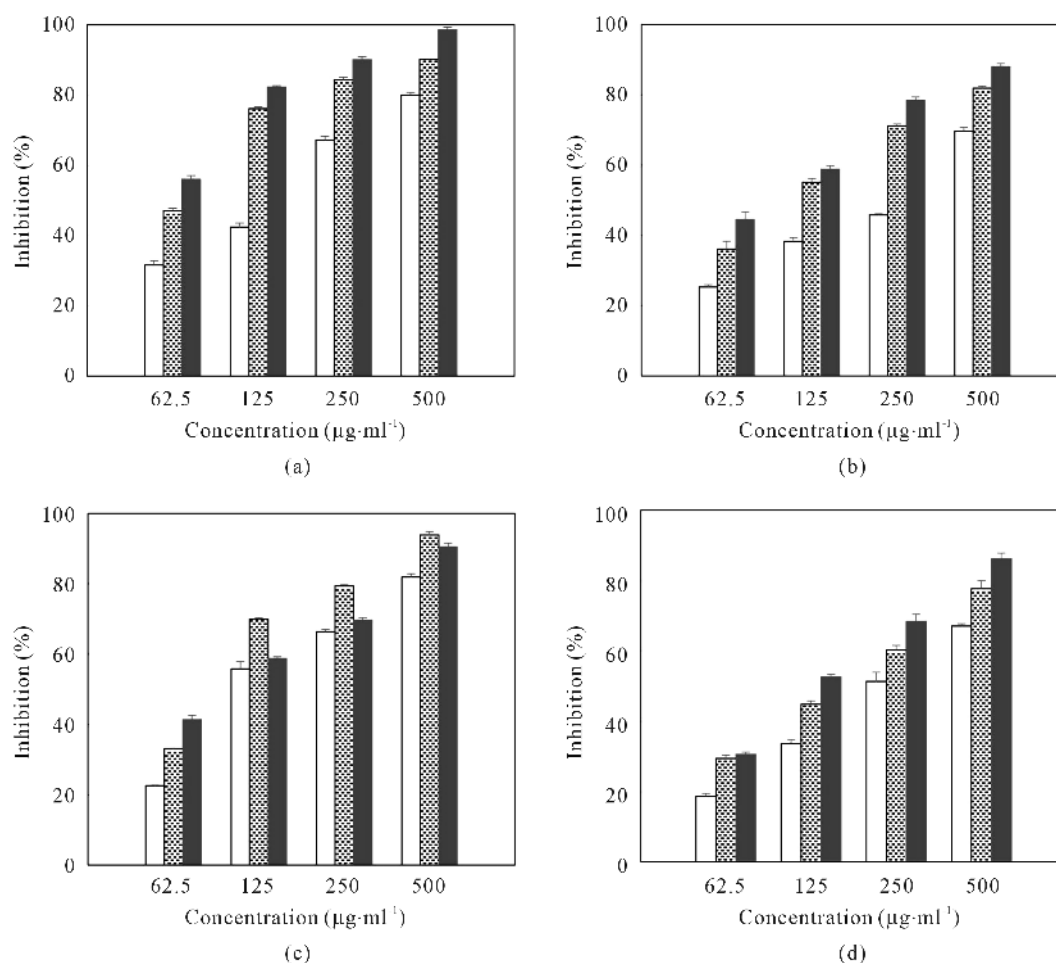


Figure 2. Antibacterial activity of grape pomace extracts against Gram negative bacteria. The figure shows the effect of grape pomace extracts obtained using hexane (white bars), chloroform (hatched bars) and ethyl acetate (gray bars) on bacterial growth of *E. coli* (a) and (b) and *S. Typhi* (c) and (d). Extracts from Cabernet Sauvignon (a) and (c) and Syrah (b) and (d) varieties were analyzed.

results of Jayaprakasha *et al.* (2003), who reported that an acetone:water:acetic acid (90:9.5:0.5) solvent mixture is more efficient than one of methanol:water:acetic acid (90:9.5:0.5) in the extraction of phenolic compounds from the grape seeds. On the other hand, Baydar *et al.* (2004) analyzed TPC in grape pomace extracts of the Narince variety (white grape), and found that an ethyl acetate:methanol:water (60:30:10) mixture was more efficient than ethanol:water (95:5) in the extraction of phenolic compounds, with quantities reaching 45.44 and 29.55 mg GAE g⁻¹ extract, respectively. Özcan *et al.* (2004) determined the TPC in pomace extracts from the Emir (white grape) and Kalecik karasi (red grape) varieties, and got values that varied between 68.77 and 96.25 mg GAE g⁻¹ extract, respectively. The authors used the same extraction method described by Jayaprakasha *et al.* (2003) for grape seeds, acetone:water:acetic acid (90:9.5:0.5). On the other hand, Anastasiadi *et al.* (2009) determined the TPC in grape pomace extracts from Voidomato and Mandilaria (red grape), and Asyrtiko and Aidani (white grape) varieties, all cultivated on the Greek islands, getting concentrations of 376.71, 207.79, 465.3, 107.12 mg GAE g⁻¹ extract, respectively. Sagdic *et al.* (2011) determined TPC from grape pomace extracts of two white grape varieties, Emir and Narince, and three red grape varieties, Gamay, Kalecik karasi, and Okuzgozu, all from Turkey, and got 75.5 - 281.4 mg GAE g⁻¹ extract. These results agree with our results in terms of the variables involved in the TPC yield, which are the kind and polarity of the solvents used in the extraction process, the extraction process itself, and the grape variety. Furthermore, our results of the TPC analysis of each of the fractions agreed with the identification of the compounds present in the grape pomace extracts obtained by the same methodology. According to HPLC studies, fractions obtained with hexane showed a low concentra-

tion and diversity of the identified compounds, while the fraction extracted with ethyl acetate exhibited a greater concentration and diversity of phenolic compounds [15].

4.2. Antibacterial Activity of Grape Pomace Extracts

Respect to the antibacterial activity, our results indicate the existence of a broad spectrum activity present in the grape pomace of both varieties tested, in agreement with the results obtained by other authors [10] [21] [22] [24] [25]. Our results also indicate that grape pomace from Cabernet Sauvignon exhibits greater antibacterial activity than that from Syrah grapes. Oliveira *et al.* (2013) studied the antibacterial activity of grape pomace extracts of Syrah and Merlot. They determined that the extracts obtained from Syrah have lower antibacterial activity against all the bacteria tested, which is consistent with our findings.

Although the antibacterial activity of grape pomace extracts has been reported previously, this study shows the direct relation between the total content of phenolic compounds and antibacterial activity, concluding that the higher the amount of TPCs in the fractions, the greater the antibacterial activity [16] [22]. Oliveira *et al.* (2013) classified antibacterial plant extracts as “strong” if their MIC values are $\leq 500 \mu\text{g}\cdot\text{ml}^{-1}$.

The ethyl acetate fraction possesses a wide variety of phenolic compounds, identifying the following: gallic acid, vanillin, syringic acid, ellagic acid, myricetin, and quercetin, among others [15]. In the literature it has been reported that some of these phenolic acids and flavonoids possess antibacterial activity [14] [26] [27], which would explain the increased activity of the ethyl acetate obtained from the grape pomace varieties obtained in this work. Our results indicate that grape pomace is a rich source of antibacterial compounds that could be potentially added to animal food as bacterial growth control.

5. Conclusion

In this study it was determined that the grape pomace extracts obtained with chloroform and ethyl acetate from the Cabernet Sauvignon variety have a broad spectrum antibacterial activity against food transmitted pathogens. Our results suggest that the residue from Cabernet variety offers greater potential as a source of these compounds. In spite of the promising activity and the high amount of TPCs, studies on the identification of bioactive compounds and on the assessment of their mechanism of action are scarce. Furthermore, studies need to be directed toward the enhancement of antibacterial activity of grape pomace extracts and its possible synergistic effect with antibiotics used for the treatment of multiresistant bacterial strains.

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