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Antibacterial effect of phenolic compounds from different wines

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Abstract

The antimicrobial properties of pure phenolic compounds and polyphenols of different wines against pathogens were investigated. It was observed that bacterial species exhibited different sensitivities towards the different concentrations of phenolic compounds. *Escherichia coli* was the most sensitive bacterium and *Flavobacterium* sp. was resistant against all phenolic compounds tested. All wine samples showed antimicrobial properties and the inhibition increased when the polyphenols concentration of wines increased. Clarified wines were inactive against all bacteria, indicating that polyphenolic compounds present in red wines, are responsible for the antimicrobial effects observed. The different concentrations of polyphenols in wines could have an important impact on consumers with the consequent increase in wine commercialization.
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Keywords: Phenolic compounds; Wine; Antimicrobial activity

1. Introduction

In nature there are a large number of different types of antimicrobial compounds. Food contamination and spoilage by microorganisms have attracted increased attention because they are problems that have not yet been brought under adequate control despite the preservation techniques available.

Grapes and wines contain a large array of phenolic compounds. Derived from the basic structure of phenol (hydroxybenzene), the term "phenolic" refers to any compound with a phenol-type structure. Singleton (1980) described three classes of phenolics in terms of chemical structures that range from relatively simple to complex: non-flavonoids, flavonoids and tannins.

The specific amounts and types of phenolics present in grapes and wines depend on a number of factors, including variety of grape and the vinification process: phenol carboxylic acids, 100–200 mg/l, catechin, 10–400 mg/l, quercetin, 5–20 mg/l (Cheyner & Teissedre, 1998) (Fig. 1). The complexity of the phenolic composition and the importance of phenolic constituents to color, flavor and stability characteristics of grape juices and wines are recognized (Singleton & Esau, 1969). The concentration of total phenols varied from 1800 to 4059 mg/l gallic acid equivalents (GAE), averaging 2567 mg/l GAE and from 165 to 331 mg/l, averaging 239 mg/l GAE for red and white wines, respectively (Frankel, Waterhouse, & Teissedre, 1995).

The flavonoids constitute a large group of secondary plant metabolites. Dietary flavonoids have attracted much interest recently because in vitro and in vivo studies suggest that they have a variety of beneficial biological properties, which may play an important role in the maintenance of human health. Flavonoids are potent antioxidants, free radical scavengers and metal chelators; they inhibit lipid peroxidation and exhibit various physiological activities

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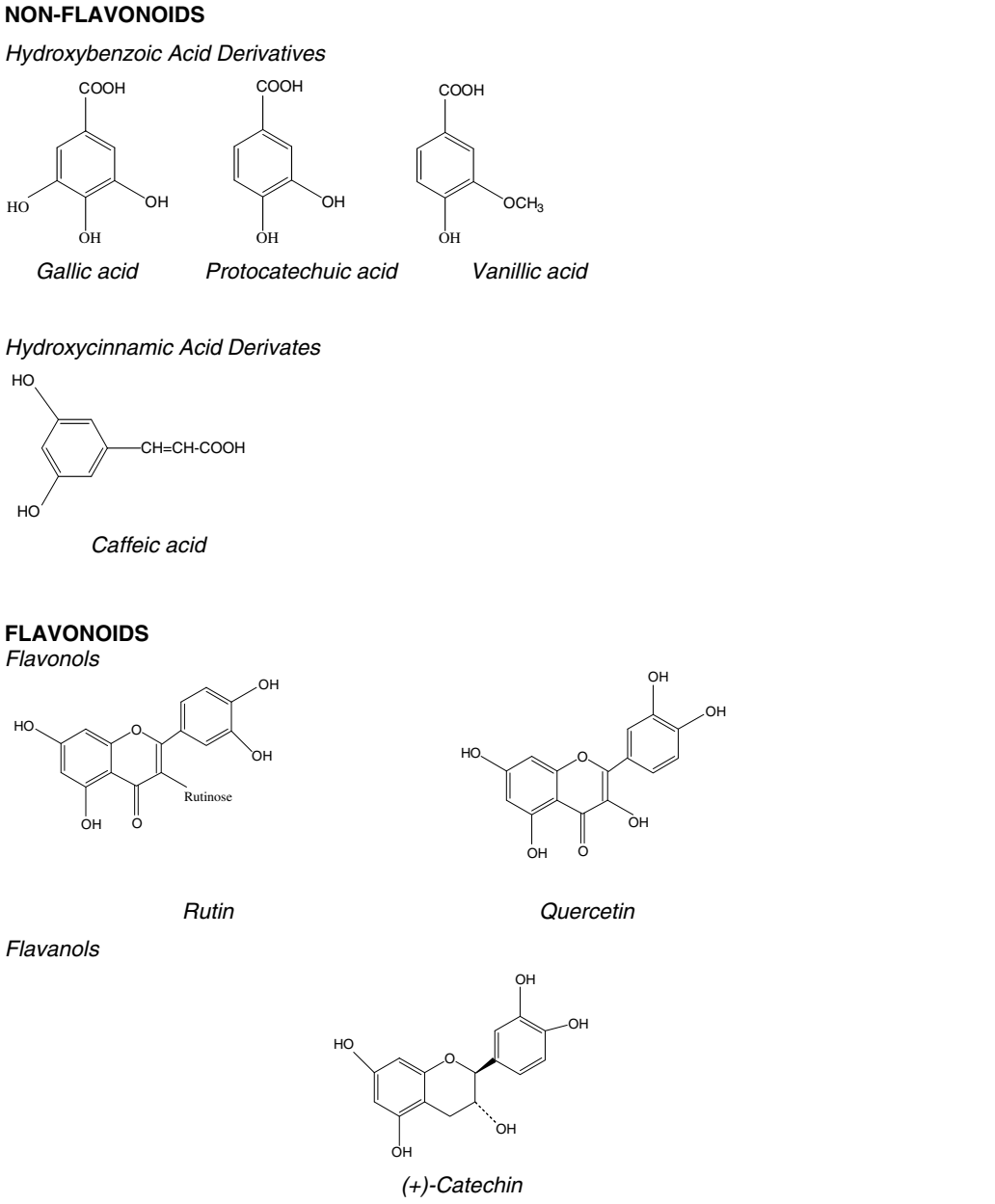

Fig. 1. Chemical structures of phenolic compounds.

including anti-inflammatory, antiallergic, anticarcinogenic, antihypertensive, antiarthritic and antimicrobial activities. Epidemiological studies have indicated that high flavonoid consumption is associated with reduced risk of chronic diseases like cardiovascular diseases (Hertog, Kromhout, & Aravanis, 1995; Middleton & Kandaswami, 1994).

Phenolic compounds may affect growth and metabolism of bacteria. They could have an activating or inhibiting effect on microbial growth according to their constitution and concentration (Alberto, Farías, & Manca de Nadra, 2001, 2002; Reguant, Bordons, Arola, & Rozés, 2000).

The aim of this work was to investigate the antimicrobial properties of pure phenolic compounds (flavonoids and phenolic acids) and total polyphenols of different Argentinean wines, Cabernet Sauvignon, Malbec and Merlot against food borne pathogens that are widely distributed in the environment and frequently detected in fresh and processed foods.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used as test organism were *Escherichia coli*, *Proteus mirabilis*, *Serratia marcescens*, *Flavobacterium* sp. and *Klebsiella pneumoniae*, obtained from human origin and *Escherichia coli* ATCC35218, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC

25923, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. All bacteria were cultured aerobically at 37 °C in nutrient broth and agar medium (contain in g/l: beef extract, 3; peptone, 5; sodium chloride, 8 and for solid medium, agar 15).

Before experimental use, cultures from solid medium were subcultivated in liquid media, incubated for 24 h and used as the source of inoculums for each experiment.

2.2. Chemicals

Gallic acid was obtained from Merck, catechin was obtained from Sigma, vanillic acid, protocatechuic acid, caffeic acid, quercetin and rutin were purchased from ICN. Ciocalteu's phenol reagent and sodium carbonate were from Merck.

2.3. Samples

2.3.1. Pure phenolic compounds

For agar diffusion assays, all phenolic compounds were dissolved in ethanol 99.8% and filter-sterilization through a 0.22 μm membrane filter. The range of phenolic compounds concentrations used includes the concentration normally present in wines.

2.3.2. Wines

Different Argentinean red table wines (Cabernet Sauvignon, Malbec and Merlot) were used. Wine samples were protected against sunlight and stored at 4 °C. Control (without concentration), twofold and fourfold concentrated (rotary evaporator) wines were clarified by the addition of 30, 60 and 120 mg/l of activated charcoal, respectively. All wines samples were filter-sterilized.

Table 1
Antimicrobial activity of non-flavonoids compounds against pathogenic bacteria

Phenolic compounds (mg/l)		*Serratia marcescens*	*Proteus mirabilis*	*Escherichia coli*	*Klebsiella pneumoniae*	*Flavobacterium* sp.	Mean of inhibition zone
Gallic acid	5	–	–	w	w	–	0.4
	10	–	–	w	+	–	0.6
	25	–	–	+	+	–	0.8
	50	–	–	+	+	–	0.8
	100	–	–	+	++	–	1.2
	200	–	–	+	++	–	1.2
	500	–	–	+	+++	–	1.6
	1000	–	–	+	+++	–	2.0
Vanillic acid	5	–	–	w	w	–	0.4
	10	–	–	w	w	–	0.4
	25	–	–	+	+	–	0.8
	50	–	–	+	+	–	0.8
	100	–	–	+	+	–	0.8
	200	–	–	++	++	–	1.6
	500	–	–	++	++	–	1.8
	1000	–	–	++	++	–	1.8
Protocatechuic acid	5	–	–	w	–	–	0.2
	10	–	–	w	–	–	0.2
	25	–	–	w	–	–	0.2
	50	–	–	+	–	–	0.4
	100	–	–	+	–	–	0.4
	200	–	–	+	–	–	0.4
	500	–	–	+	–	–	0.4
	1000	–	–	+	–	–	0.4
Caffeic acid	1	w	–	+	–	–	0.6
	5	+	–	+	–	–	1.0
	20	+	–	+	–	–	1.0
	50	+	–	++	–	–	1.2
	100	+	+	++	–	–	1.6
	500	+	+	++	–	–	1.6
Control (+) chloramphenicol 1000 mg/l		++++	++++	++++	++++	++++	
Mean of inhibition zone		0.4	0.1	2.2	1.7	–	

No antimicrobial activity (−), inhibition zone <1 mm. Weakly antimicrobial activity (w), inhibition zone 1 mm. Slight antimicrobial activity (+), inhibition zone 2–3 mm. Moderate antimicrobial activity (++), inhibition zone 4–5 mm. High antimicrobial activity (+++), inhibition zone 6–9 mm. Strong antimicrobial activity (++++), inhibition zone >9 mm. Standard deviation ± 0.5 mm.

2.4. Colorimetric determination of total phenolic compounds

Colorimetric determination of total phenolics was based on the procedure of Singleton and Rossi (1965). A standard curve of gallic acid was used. Results are expressed as milligram per liter gallic acid equivalents (GAE).

2.5. Antibacterial test

The agar diffusion test was used to investigate antibacterial effects of phenolic compounds.

Soft agar medium was inoculated with liquid overnight culture to a cell density of 2.0×10^9 cfu/ml, and plates containing 10 ml of agar media were overlaid with 10 ml of this inoculated soft agar. Equidistant holes were made in the agar. A 30-μl volume of each sample was pipetted into the agar wells. Chloramphenicol (1000 mg/l) was used as a positive control and the negative control was ethanol. After 24 h incubation the diameter of the inhibition zones, (no growth) around the holes in the bacterial lawn was measured with an accuracy of 0.5 mm using a ruler.

2.6. Statistical analysis

All experiments were carried out at least in triplicate. Statistical analysis was performed using MS-Excel software.

3. Results and discussion

3.1. Antibacterial activity of pure phenolic compounds

Tables 1 and 2 shows the antimicrobial screening of seven pure phenolic compounds: four phenolic acids, gallic, vanillic, protocatechuic and caffeic and three flavonoids, rutin, catechin and quercetin against *Ser. marcescens*, *Pr. mirabilis*, *E. coli*, *Kl. pneumoniae* and *Flavobacterium* sp.

Caffeic acid was the only non-flavonoid compound with an inhibitory effect on the growth of *Ser. marcescens*. It was inhibited by all flavonoids from 1 mg/l rutin, 2 mg/l quercetin and 10 mg/l catechin. The highest antimicrobial activity was observed with 500 mg/l quercetin.

Table 2
Antimicrobial activity of flavonoids compounds against pathogenic bacteria

Phenolic compounds (mg/l)		*Serratia marcescens*	*Proteus mirabilis*	*Escherichia coli*	*Klebsiella pneumoniae*	*Flavobacterium* sp.	Mean of inhibition zone
Rutin	1	w	–	w	–	–	0.4
	10	+	–	w	–	–	0.6
	25	+	–	+	–	–	0.8
	50	+	–	++	–	–	1.2
	100	+	–	++	–	–	1.2
	500	+	–	++	–	–	1.2
	1000	+	–	++	–	–	1.2
Quercetin	2	+	–	–	–	–	0.4
	10	+	+	–	–	–	0.6
	25	++	+	+	+	–	2.0
	50	++	+	++	++	–	2.8
	100	++	++	++	+++	–	3.4
	500	+++	++	++	+++	–	3.8
	1000	+++	++	+++	+++	–	4.2
Catechin	10	w	–	w	–	–	0.4
	50	w	–	+	–	–	0.6
	100	+	–	+	–	–	0.8
	200	+	–	+	–	–	0.8
	500	+	+	+	–	–	1.2
	1000	++	+++	++	–	–	2.8
Control (+) chloramphenicol 1000 mg/l		++++	++++	++++	++++	++++	
Mean of inhibition zone		2.5	1.3	2.6	1.2	–	

No antimicrobial activity (–), inhibition zone <1 mm. Weakly antimicrobial activity (w), inhibition zone 1 mm. Slight antimicrobial activity (+), inhibition zone 2–3 mm. Moderate antimicrobial activity (++), inhibition zone 4–5 mm. High antimicrobial activity (+++), inhibition zone 6–9 mm. Strong antimicrobial activity (++++), inhibition zone >9 mm. Standard deviation ± 0.5 mm.

Table 3
Antimicrobial activity of non-flavonoids compounds against collection bacteria

Phenolic compounds (mg/l)		*Escherichia coli* ATCC 35218	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*Staphylococcus aureus* ATCC 29213	*Staphylococcus aureus* ATCC 25923	Mean of inhibition zone
Gallic acid	5	w	–	–	–	–	0.2
	10	w	–	–	–	–	0.2
	25	w	w	–	–	–	0.4
	50	w	w	w	–	–	0.6
	100	w	w	w	–	–	0.6
	200	+	w	w	w	–	1.0
	500	+	+	+	+	–	1.6
	1000	+	+	+	+	–	2.0
Vanillic acid	5	–	–	–	–	–	–
	10	–	–	–	–	–	–
	25	–	–	–	–	–	–
	50	–	–	–	–	–	–
	100	w	–	–	–	–	0.2
	200	w	w	–	–	–	0.4
	500	+	w	–	–	–	0.6
	1000	+	w	–	–	–	0.6
Protocatechuic acid	5	–	–	–	–	–	–
	10	–	–	–	–	–	–
	25	–	–	–	–	–	–
	50	–	–	–	–	–	–
	100		w	–	–	–	0.2
	200	w	w	–	–	–	0.4
	500	+	w	w	–	–	0.8
	1000	+	+	w	–	–	1.0
Caffeic acid	1	+	–	–	–	–	0.4
	5	+	w	–	–	–	0.6
	20	+	w	–	–	–	0.6
	50	+	w	–	w	–	1.0
	100	+	+	w	+	–	1.6
	500	++	+	+	+	–	2.4
Control (+) chloramphenicol 1000 mg/l		++++	++++	++++	++++	++++	
Mean of inhibition zone		1.3	0.8	0.4	0.4	–	

No antimicrobial activity (−), inhibition zone <1 mm. Weakly antimicrobial activity (w), inhibition zone 1 mm. Slight antimicrobial activity (+), inhibition zone 2–3 mm. Moderate antimicrobial activity (++), inhibition zone 4–5 mm. High antimicrobial activity (+++), inhibition zone 6–9 mm. Strong antimicrobial activity (++++), inhibition zone >9 mm. Standard deviation ± 0.5 mm.

On the growth of *Pr. mirabilis*, caffeic acid was also the only non-flavonoid compound with an inhibitory effect and its sensitivity was observed from 100 mg/l. With respect to flavonoid compounds, 100 and 1000 mg/l of quercetin and catechin, were necessary to produce a moderate and a high inhibition, respectively.

E. coli was the most sensitive bacterium. Its growth was inhibited from 5 to 1000 mg/l of gallic, vanillic and protocatechuic acids, being maximally effective at 200 mg/l vanillic acid. The inhibitory effect of caffeic acid was observed from 1 to 500 mg/l achieving at 50 mg/l its maximal effect. Among flavonoid compounds 50 mg/l rutin or quercetin produced a moderate antimicrobial activity on *E. coli*. The same effect was observed with 1000 mg/l catechin indicating that the microorganism is more tolerant to this flavonoid.

Kl. pneumoniae was inhibited by gallic and vanillic acids as well as quercetin. Gallic acid was more effective than vanillic acid achieving a strong antimicrobial activity from 500 mg/l and the same effect was observed with 100 mg/l quercetin.

Flavobacterium sp. was resistant to all pure phenolic compounds tested.

Of the four non-flavonoid compounds, the hydroxycinnamic acid derivate, caffeic acid, has been shown to possess more effective antibacterial activity against the bacteria investigated than hydroxybenzoic acids derivatives. Quercetin was the best antibacterial flavonoid with a mean inhibition zone of 4.2 mm.

Inhibition against collection strains by the phenolic compounds is presented in Tables 3 and 4. *Staphylococcus aureus* ATCC 25923 was resistant to all pure phenolic acids

Table 4
Antimicrobial activity of flavonoids compounds against collection bacteria

Phenolic compounds (mg/l)		*Escherichia coli* ATCC 35218	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*Staphylococcus aureus* ATCC 29213	*Staphylococcus aureus* ATCC 25923	Mean of inhibition zone
Rutin	1	–	–	–	–	–	–
	10	–	–	–	–	–	–
	25	–	–	–	–	–	–
	50	–	–	–	–	–	–
	100	w	–	–	–	–	0.2
	500	w	w	w	–	–	0.6
	1000	+	w	w	–	–	0.8
Quercetin	2	–	–	–	–	w	0.2
	10	–	–	–	w	+	0.6
	25	–	–	–	+	+	1.0
	50	w	w	–	+	+	1.4
	100	+	w	w	++	++	2.4
	500	+	+	+	++	++	3.4
	1000	+	+	+	++	++	3.8
Catechin	10	–	–	w	–	w	0.4
	50	–	–	+	–	+	0.8
	100	w	–	+	–	+	1.2
	200	+	–	++	–	+	1.8
	500	+	–	+++	–	+	2.6
	1000	++	–	++++	–	+	3.4
Control (+) chloramphenicol 1000 mg/l		++++	++++	++++	++++	++++	
Mean of inhibition zone		1.1	0.3	1.9	1.0	1.8	

No antimicrobial activity (−), inhibition zone <1 mm. Weakly antimicrobial activity (w), inhibition zone 1 mm. Slight antimicrobial activity (+), inhibition zone 2–3 mm. Moderate antimicrobial activity (++), inhibition zone 4–5 mm. High antimicrobial activity (+++), inhibition zone 6–9 mm. Strong antimicrobial activity (++++), inhibition zone >9 mm. Standard deviation ± 0.5 mm.

Table 5
Total phenolic compounds concentration

Wines		Grape variety		
		Cabernet Sauvignon	Malbec	Merlot
Not clarified	Control	2300 ± 90*	2522 ± 80	2704 ± 100
	2×	4494 ± 130	4848 ± 142	5010 ± 150
	4×	8209 ± 410	9393 ± 470	9883 ± 494
Clarified	Control	35.2 ± 1.8	25.1 ± 1.3	50.3 ± 2.9
	2×	40.1 ± 2.0	34.9 ± 1.7	70.4 ± 3.5
	4×	50.0 ± 2.5	48.4 ± 2.4	74.1 ± 3.7

* mg/l GAE. Each value represents the average of three determinations ± SD.

assayed although *E. coli* ATCC 35218 was inhibited by all of them. Caffeic acid was the most effective non-flavonoid compound, achieving the highest antimicrobial activity with 500 mg/l (Table 3).

Among flavonoid compounds all bacteria showed a growth inhibition, at least by one of them (Table 4). *Pseudomonas aeruginosa* ATCC 27853 was the bacterium most sensitive to flavonoid compounds. *E. coli* ATCC 25922 was more resistant than the other bacteria. Quercetin was the only phenolic which exhibited antibacterial effect against all bacteria assayed. The flavonoid glycoside, rutin was the less effective; no inhibitory effect was observed against *Staphylococcus aureus* strains in its presence.

Puupponen-Pimiä et al. (2001), reported that different bacterial species (Gram-positive and Gram-negative) exhibit different sensitivities towards phenolics and that phenolic acids such as cinnamic, coumaric, caffeic and ferulic inhibited *E. coli* and *Salmonella enterica* at high concentrations. In our study low concentrations of caffeic acid (from 1 mg/l) were inhibitory to the growth of an *E. coli* strain isolated from a human.

Hydroxycinnamic acids, due to their propenoic side chain, are much less polar than the corresponding hydroxybenzoic acids. Campos, Couto, and Hogg (2003) reported that in *Oenococcus oeni*, this property might facilitate the transport of these molecules across the cell membrane, which might be related in turn to the stronger inhibitory effect of hydroxycinnamic acids. On the other hand hydroxycinnamic acid derivatives are known to interact with membrane lipids of taste papillae in the tongue by a neutralization of the membrane's electric potential, following penetration of the molecule (Macheix, Fleuriet, & Billot, 1990). A similar effect could occur in the bacterial cell membrane, affecting their energy metabolism.

Whiting and Carr (1959), Whiting (1975) and Stead (1993) reported that there are organisms able to tolerate

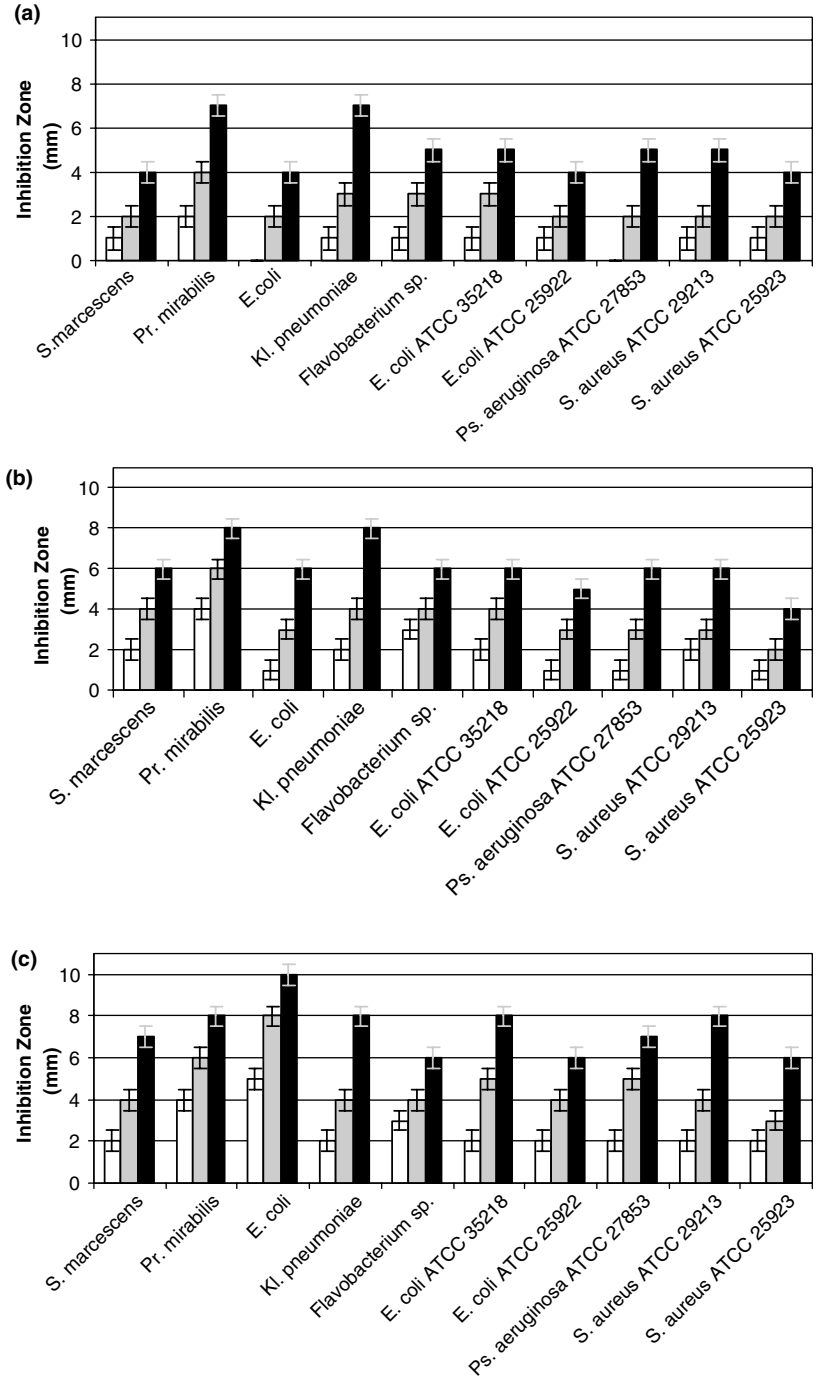

Fig. 2. Antimicrobial activity of total phenolic compounds present in different wines against selected bacteria. (a) Cabernet Sauvignon (b) Malbec and (c) Merlot wine samples. (□) Control wine; (▒) twofold concentrated wine and (■) fourfold concentrated wine. The values are the average of three determinations.

and even metabolize hydroxycinnamic acids by reduction of their side chain to yield the corresponding 2-hydroxyphenylpropionic acids, which can subsequently be decarboxylated to substituted *p*-ethyl phenols. This may be the reason why caffeic acid did not affect the growth of *Kl. pneumoniae* and *Flavobacterium* sp.

Considering the chemical structure of gallic and protocatechuic acids the only difference is one more hydroxyl group in the first. So the higher inhibitory effect of gallic acid than protocatechuic acid could be related with this property.

Among flavonoids tested, the flavonol quercetin, aglycone form, was more effective than the glycosidic form rutin. Rauha et al. (2000) reported that quercetin inhibited the growth of all the prokaryotic species studied (*Staphylococcus aureus*, *Staph. epidermidis*, *Bacillus subtilis*, *Micrococcus luteus*, *E. coli* and *Ps. aeruginosa*) and that rutin was inactive on all of them. Our results show that both

flavonols were inhibitory against determined microorganisms: quercetin against *Ser. marcescens*, *Pr. mirabilis*, *E. coli*, *Kl. pneumoniae*, *Ps. aeruginosa* and *Staph. aureus* and rutin against *Ser. marcescens*, *E. coli* and *Ps. aeruginosa.*

On the other hand, Puupponen-Pimiä et al. (2001) found that the flavonoids catechin, rutin and quercetin did not affect the growth of *E. coli*.

In our study quercetin was the strongest inhibitor which was active against nine of the ten bacteria assayed.

3.2. Phenolic composition of different wines

We determined the total phenolic compounds of the different wines to find out the relationship between antimicrobial activity and phenolic compound content. Table 5 shows the total concentration of phenolic compounds in Cabernet Sauvignon, Malbec and Merlot Argentinean red wines.

The phenolic compounds concentrations in Malbec and Merlot wines were higher than in Cabernet Sauvignon wine. In decolorized wines, used as control, the phenolic concentrations ranged from 25.1 mg/l in the control sample of Malbec to a maximum of 74.1 mg/l in Merlot, fourfold concentrated.

3.3. Antimicrobial activity of wine phenolic compounds

Fig. 2 shows the antimicrobial activities of three Argentinean wines measured by the agar diffusion method against selected bacteria.

The mean inhibition zone for all bacteria for samples of Cabernet Sauvignon wine increased from 0.9 to 5.0 mm when the concentration increased from 2300 to 8209 mg/l, from 1.9 to 6.1 mm when the polyphenolic concentration of Malbec wine increased from 2522 to 9393 mg/l and from 2.6 to 7.4 mm when the polyphenolic concentration of Merlot wine increased from 2704 to 9883 mg/l.

In the three wine varieties evaluated for their effect on bacterial growth, the inhibition zones around the bacteria increased with the concentration of polyphenolic compounds. The controls carried out with the clarified wines samples (without concentration and twofold and fourfold concentrated) were inactive against all bacteria, indicating that polyphenolic compounds were responsible of the antimicrobial effects.

Cabernet Sauvignon without concentration failed to show any activity against *E. coli* and *Ps. aeruginosa* ATCC 27853, although all Malbec and Merlot wine samples were active against the tested bacteria. The lowest antimicrobial activity showed with samples of Cabernet Sauvignon wine could be related with its lower phenolic concentration.

Pr. mirabilis was the bacterium most sensitive to Cabernet Sauvignon and Malbec wine samples, whereas *E. coli* was the bacterium most sensitive to Merlot wine samples followed by *Pr. mirabilis*. The largest inhibition zone diameter was 10.0 mm against *E. coli* for Merlot wine four fold concentrated, no such effect was found with the others wine samples.

Baydar, Özkan, and Sağdiç (2004) reported that acetone: water: acetic acid and methanol: water: acetic acid grape seed extracts inhibited the fifteen bacteria used as test organisms (*Aeromonas hydrophila*, *Bacillus brevis*, *B. cereus*, *B. megaterium*, *B. subtilis*, *Enterococcus faecalis*, *E. coli*, *Kl. pneumoniae*, *Listeria monocytogenes*, *Mycobacterium smegmatis*, *Pr. vulgaris*, *Ps. aeruginosa* and *Staph. aureus*) and they attributed the inhibitory effect to their phenolic composition. The grape seed extracts had high total phenolics compared with those of bagasse (berry without seed and juice), which did not inhibit any of the bacteria tested.

The inhibitory effect of phenolic compounds could be explained by adsorption to cell membranes, interaction with enzymes, substrate and metal ion deprivation (Scalbert, 1991).

4. Conclusion

The present work contributes to the knowledge of the beneficial properties of phenolic compounds present in different wines against bacteria that affect the human health.

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