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**Research Article**

# Effect of Phenolic Compounds on Viability of Wine Spoilage Lactic Acid Bacteria. A Structure-Activity Relationship Study

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**Abstract:** The effect of wine phenolic compounds (PCs) such as hydroxybenzoic acids, hydroxycinnamic acids and a flavanol on growth of *Lactobacillus hilgardii and Pediococcus pentosaceus*, both isolated from argentine red wine, was for the first time assayed in synthetic wine-like medium. At a concentration of 400 mg/L and after 96 h of incubation, highest inhibition of *L. hilgardii* 6F, *L. hilgardii* $X_1$B and *P. pentosaceus* 12p occurred with *trans*-caffeic and *trans*-*p*-coumaric acid, producing damage of cell integrity. *P. pentosaceus* was most sensitive to PCs. A structure-antibacterial activity relationship (SAR) study of the PC revealed that *trans*-caffeic and *trans*-*p*-coumaric acid have similar electronic distribution with an interatomic distance of 8 Å between the catechol hydroxyls and the carboxylic group. These structural requirements are necessary to determine a pattern of pharmacophores responsible for antibacterial activity. The current study of the molecular and electronic properties of PCs allowed to establish a pharmacophoric pattern responsible for antibacterial activity to control wine spoilage lactic acid bacteria.

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**Key words:** antibacterial activity, hydroxycinnamic acid, lactic acid bacteria, polyphenol, structure-activity relationship, wine

## Introduction

During the winemaking process, the metabolic activity of certain lactic acid bacteria (LAB), mainly belonging to the genera *Lactobacillus*, *Pediococcus* and *Oenococcus*, can have beneficial or detrimental effects on wine quality (Lonvaud-Funel, 1999). Malolactic fermentation (MLF), usually carried out by *Oenococcus oeni*, is a desirable process that generally takes place after alcoholic fermentation producing decarboxylation of *L*-malic acid into *L*-lactic acid; MLF reduces the wine acidity and contributes to microbial stability and organoleptic quality of the final product (Moreno-Arribas and Polo 2005). However, during industrial winemaking, development of undesirable LAB can occur during alcoholic fermentation or after MLF (even during storage or ageing). This bacterial metabolism can produce changes in the wine composition resulting in depreciation of the wine (Ribéreau-Gayón et al. 2006). Several *Lactobacillus hilgardii*, *Pediococcus pentosaceus* and *Pediococcus parvulus* strains have been reported for their potential wine deterioration (Ribéreau-Gayón et al 2006), including exopolysaccharide production (Manca de Nadra and Strasser de Saad 1995, Pastorkova et al. 2013) and biogenic amine formation (Farías et al. 1993, Landete et al. 2007, Aredes Fernández et al. 2010). Therefore, a common practice in wineries to minimize the possibility of alterations associated with microbial spoilage is addition of sulfite to minimize LAB growth and metabolism after complete malic acid degradation. In general, the sulfur concentration required for LAB inhibition is in the order of 30 mg/L of free $SO_2$ for acidic wines (pH between 3.2 and 3.6), but up to 100 mg/L for wines with higher pH. Nevertheless, the sulfiting practice is actually strictly controlled, since high doses of $SO_2$ can cause organoleptic alterations in the final product (Ribéreau-Gayón et al. 2006) and can produce health problems like anaphylaxis, a severe sensitivity or allergic reaction that causes the collapse of the circulatory system and throat swelling

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in humans (Taylor et al. 1986; Romano and Suzzi 1993). Nowadays, there is a growing tendency to reduce the maximum permitted concentration of $SO_2$ in musts and wines. A potential alternative to the use of $SO_2$ are PCs, which are natural constituents of grapes and wines. These compounds have previously been reported to exert a dose-dependent negative effect on LAB development (Campos et al. 2009, Rodriguez et al. 2009, García-Ruíz et al. 2013, Pastorkova et al. 2013, Stivala et al. 2014, 2015). Low molecular weight PCs, phenolic acids, have previously been reported to produce strong inhibition of wine LABs (García-Ruíz et al. 2012, Stivala et al. 2014, 2015). In this way, Sánchez-Maldonado et al. (2011) have studied the structure–function relationships of the antibacterial activity of phenolic acids on LAB, demonstrating that the presence of double bond of hydroxycinnamic acids plays an important role in antibacterial activity of these compounds. In addition, the PC concentration required for an efficient antibacterial effect against LAB would be lower in an adverse medium such as wine (Stead 1993). Thus far, scientific studies have focused on assessment of inhibitory activity of PCs in optimal growth media and at much higher concentrations than in wine (Stead 1993, Campos et al. 2003, García-Ruíz et al. 2011, 2013).

The aim of this study was evaluate the antibacterial activity of wine PCs against two recognized wine spoilage bacteria, *L. hilgardii* 6F and $X_1$B and *P. pentosaceus* 12p in a synthetic wine-like medium (SWM). By first time a structure–activity relationship (SAR) by computational studies was carried out.

## Materials and Methods

**Bacterial strains and growth conditions.** *L. hilgardii* 6F and *L. hilgardii* $X_1$B were selected by their ability to produce biogenic amines (Farías et al. 1993, Aredes Fernández et al. 2010) and *P. pentosaceus* 12p by its ability to produce exopolysaccharide (Manca de Nadra and Strasser de Saad 1995). All these LABs were isolated from Cafayate wines, Salta, Argentina (Strasser de Saad and Manca de Nadra 1987). The strains were kept at -20°C in a sterilized mixture of culture medium and glycerol (50:50, v/v).

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Microorganisms were activated in MRS broth supplemented with tomato juice (15%) at pH 5.0. A synthetic wine-like medium (SWM) was used for growth experiments. SWM contained 1.7% Yeast Nitrogen Base (YNB -Difco™ & BBL™) solution, supplemented with (in g/L): glucose 5.0; fructose 3.0; L-malic acid 3.0; tartaric acid 4.0; $K_2SO_4$ 0.1; $MgSO_4$ 0.025; $MnSO_4$ 1.0; adenine 0.05. The medium was also supplemented with 5% (v/v) ethanol (99.5% v/v) and the corresponding amino acids required for microorganism growth (Aredes Fernandez et al. 2003, Stivala et al. 2014, 2015). The pH was adjusted to 4.5 and media were sterilized by filtration through a 0.22 µm pore size nylon membrane. The composition of SWM aims to simulate the natural conditions of wine (Stivala et al. 2014, 2015).

**Phenolic compounds.** Hydroxybenzoic acids (gallic and protocatechuic acid) and hydroxycinnamic acids (*trans*-*p*-coumaric and *trans*-caffeic acid) and the flavanol (+)-catechin were purchased from Sigma-Aldrich (St. Luis, MO). Individual PC solutions were prepared by dissolving each compound in ethanol (99.5% v/v) at the appropriate concentration (8 g/L). Solutions were prepared immediately prior to use to minimize PC oxidation.

**Effect of phenolic compounds on cell viability.** Previous screenings were performed with each tested PC in order to establish the concentration that produces growth inhibition for the most LABs studied (data not shown), being 400 mg/L the selected concentration for the experimental procedures and SAR analysis. Thus, SWM was individually supplemented with 400 mg/L of gallic acid, protocatechuic acid, *trans*-caffeic acid, *trans*-*p*-coumaric acid and (+)-catechin. Bacteria were inoculated at $10^7$ cfu/mL approximately and incubated for 96 h under the different assay conditions. Immediately after inoculation and after 96 h, bacterial viability was determined by viable cell counts on MRS-agar medium. Plates were incubated at 28°C under microaerophilic conditions during 120 h.

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In order to establish the inhibitory effect of PCs, the change in cell viability vs time (A) (i.e. viable cells after inoculum vs viable cells at the end of the incubation time) of each microorganism was determined in SWM and SWM supplemented with each PC. A was determined as follows:

$$A\ [\text{Log cfu/mL}] = \text{Log}\ (x - x_0) \qquad \text{Eq.1}$$

Where, x is the viable cell concentration at the end of the incubation time and $x_0$ the viable cell concentration at the start of the inoculum.

**Transmission electron microscopy.** In order to detect changes in cell morphology produced by PCs in SWM by transmission electron microscopy (TEM), *L. hilgardii* 6F, *L. hilgardii* $X_1B$ and *P. pentosaceus* 12p cells of each assay condition were collected through centrifugation immediately after inoculation and at the end of the incubation time. The cells were washed with sterile isotonic saline solution and then fixed in Karnovsky solution (Karnovsky 1965) and incubated overnight at 4ºC. The fixed samples were washed three times with sodium phosphate buffer and then fixed with a 1:1 solution of sodium phosphate buffer/2% osmium tetroxide ($OsO_4$). Afterwards, samples were washed three times with distilled water and subsequently stained with a 1:1 solution of phosphate buffer/uranyl acetate. After incubation for 30 min in the dark, the solution was discarded and the samples were progressively washed with 70%, 90% and 100% ethanol and dehydrated with 100% acetone. The bacterium was embedded in Spur resin, and heated at 60ºC for 24 h. Ultrathin sections were obtained with an ultramicrotome, mounted on copper grids and contrasted with uranyl acetate and lead citrate (Venable and Coggeshall 1965). Samples were examined under a Zeiss EM 109 transmission electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany).

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**Chemical structure-antibacterial activity relationship.** To improve the understanding of the experimental results, a conformational and electronic study of the compounds reported here was carried out. All calculations were carried out using the Gaussian 03 program (Frisch et al. 2003). The search for low-energy conformations on the potential energy surface for the compounds reported here was performed by using a combination of *ab initio* (RHF/3-21G) and Density Functional Theory (DFT) (B3LYP/6-31G(d)) calculations. Final DFT geometries were obtained from the geometry optimization calculations. Minima were characterized through harmonic frequency analysis calculated at DFT level. Correlation effects were included using DFT according to the functional by Lee, Yang and Parr (Lee et al. 1988, Miehlich et al. 1989) as proposed and parameterized by Becke (1993) (RB3LYP), and the 6-31G(d) basis set. Molecular electrostatic potentials (MEPs) were calculated using B3LYP/6-311G (d,p) wave functions from the MOLEKEL program (Flükiger et al. 2000). In an attempt to find potential reactive sites the electronic distribution of the lower energy conformations obtained for the different compounds was evaluated. From the calculated electron distribution, properties such as net atomic charge and bond polarity can be predicted, which helps to characterize the nature of the interactions at specific receptor sites. The electron distribution can also be used to quantitatively map the electrostatic potential generated by a molecule in all regions surrounding it (Srebenik et al. 1973). Thus, the electronic study of these compounds was carried out using MEPs. Considering that the electrostatic potential has primarily been used for predicting sites, relative reactivity towards electrophilic attack in biological recognition and hydrogen bonding interactions (Greeling et al. 1996, Polilzer and Truhlar 1981), the emphasis of these studies has been put on negative V(r) regions.

In the great majority of MEPs, the regions of negative values for the local minima energy are site candidates for electrophilic attacks in the molecule. The positive regions only possess energy maxima at the nuclear positions (Yang et al. 2005) indicating that there is no affinity for nucleophilic reagents.

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**Statistical analysis.** The means and reproducibility of data were calculated based on three independent experiments performed in duplicate. The experimental data of bacterial viability were analyzed by one-way analysis of variance test. Variable means showing statistical significance were compared using Tukey's test (Minitab student R12). All statements of significance are based on the 0.05 level of probability

## Results and Discussion

**Effect of the phenolic compounds on growth of wine lactic acid bacteria.** Table 1 shows the PC effect on viability of the three LAB strains (*L. hilgardii* 6F, *L. hilgardii* $X_1B$ and *P. pentosaceus* 12p) in SWM control medium and in SWM supplemented with 400 mg/L of each of the five PCs individually assayed. PCs used in this work were selected because were previous reported by their antibacterial effect against wine spoilage LAB (Campos et al. 2009 García-Ruíz et al. 2009) and sometimes are naturally present in wines from different countries (García-Ruíz et al. 2009; Stivala et al. 2015). In control medium the viable cell count of *L. hilgardii* 6F and *L. hilgardii* $X_1B$ inocula increased, reaching 9.04 and 8.34 Log cfu/mL after 96 h of incubation, respectively. In SWM, viability of the *P. pentosaceus* 12p inoculum remained stable with a viable cell count of 7.15 Log cfu/mL after 96 h.

The hydroxycinnamic acids (*trans*-caffeic and *trans-p*-coumaric acid) showed an inhibitory effect against both *L. hilgardii* strains assayed (Table 1). Highest inhibition of *L. hilgardii* 6F occurred in the presence of *trans*-caffeic and *trans-p*-coumaric acid, showing a decrease in viable cell count with values below the inoculum (A= -0.12 and 0.14 Log cfu/mL, respectively). With the exception of gallic acid, all PCs assayed showed inhibition of *L. hilgardii* $X_1B$; *p*-coumaric acid demonstrated the highest inhibitory effect (A = -0.43 Log cfu/mL). In general, *P. pentosaceus* 12p was the most sensitive strain to action of the PCs assayed. For this microorganism, *trans*-caffeic and *trans-p*-coumaric acid showed the highest inhibitory effect after 96 h of incubation (A=-2.18 and -1.97Log cfu/mL, respectively).

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Previous results have demonstrated that phenolic acids of low molecular weight have higher antibacterial activity against wine LAB (Stead 1993, García Ruíz et al. 2012). Similarly, other authors have shown that *p*-coumaric, ferulic, caffeic and sinapic acid were also effective antibacterial agents against *L. hilgardii* and *P. pentosaceus* strains (García-Ruíz et al. 2011) and other LAB species (Stead 1993, Landete et al. 2007). In addition, and consistent with our results, Campos et al. (2009), García-Ruíz et al. (2009) and Sánchez-Maldonado et al. (2011) reported that hydroxycinnamic acids generally showed greater effectiveness than hydroxybenzoic acids with regard to inhibition of LABs in MRS medium. However, concentrations of pure PCs in previous studies were higher than 1000 mg/L, which is higher than those found in the natural environment (Rodriguez et al. 2009). Marsilio and Spear (1998) demonstrated that 1000 mg/L of *p*-coumaric acid significantly decreased growth of *L. plantarum*. Campos et al. (2003) found that addition of 500 mg/L of *p*-coumaric, protocatechuic or gallic acid to MRS medium supplemented with tomato juice (complex medium) produced growth inhibition of *L. hilgardii* strains isolated from Porto wine (Portugal). García-Ruíz et al. (2011) showed that (+)-catechin and (-)-epicatechin exhibited a low inhibitory effect on the growth of various *L. hilgardii* and *P. pentosaceus* strains at concentrations higher than those normally found in wine (2000 mg/L). Despite previous reports describing antibacterial PC activity (García-Ruíz et al. 2011; Pastorkova et al. 2013), this is the first study on antibacterial PC activity using synthetic medium to simulate the natural conditions of wine.

**Microscopy study.** TEM was used to evaluate the effect of PCs on the integrity of the microorganisms. In SWM all microorganisms assayed presented a homogeneous cytoplasm and an intact cell wall after 96 h of incubation (Figure 1A, 2A and 3A). In media supplemented with pure PCs the two *L. hilgardii* strains and *P. pentosaceus* 12p showed alterations in the cell wall at the end of the incubation period. Changes in the microorganism cell wall were most evident after incubation of *L. hilgardii* 6F (Figure 1B) and $X_1$B (Figure 2B) with *trans*-caffeic and *trans-p*-coumaric acid, respectively. TEM images of *P.*

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193 *pentosaceus* 12p (Figure 3B) revealed that *trans*-caffeic acid produced the most evident alterations in cell
194 integrity. These results are in agreement with observations reported by Campos et al. (2009), who
195 demonstrated that *p*-coumaric and caffeic acid produced the highest growth inhibitory effect on *L.*
196 *hilgardii* strain 5 through cell damage. Currently, the mechanisms through which polyphenols inhibit
197 growth of LAB from wine are still being studied. Several authors have reported that polyphenols can
198 modify the structure of the cell membrane, producing the drain of intracellular constituents (Johnston et
199 al. 2003, Rodriguez et al. 2009).
200
201 **Structure-activity relationship of phenolic compounds acting as antibacterial agents.** To study the
202 structure-activity relationship of PCs against LAB, different structures and the effects of structural
203 changes in different regions of the PCs were considered. The compounds assayed in this study occur
204 naturally in wine at different concentrations. They were chosen because of their different functional
205 group(s) and/or ring substituents in an attempt to relate the phenolic chemical structure to their inhibitory
206 effects on growth of enological LAB. To enhance the understanding of the above experimental results, a
207 conformational and electronic study of the compounds was carried out. In general, such compounds are
208 conformationally rigid and have restricted molecular flexibility.
209 The results given in Table 1 show that in general, maximum inhibitory of bacterial growth was
210 produced by *trans*-caffeic and *trans*-*p*-coumaric acid.
211 In order to find the potential reactive sites the electronic distribution in the lower-energy
212 conformations of *trans*-caffeic acid, *trans*-*p*-coumaric acid and (+)-catechin was evaluated. The electron
213 distribution can also be used to quantitatively map the electrostatic potential generated by a molecule in
214 all regions surrounding it.
215 Figure 4 gives the MEPs obtained for *trans*-caffeic acid, *trans*-*p*-coumaric acid and (+)-catechin.
216 These compounds were selected because they showed highest antibacterial activity.

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The MEPs obtained for *trans*-caffeic (Figure 4A) and *trans-p*-coumaric acid (Figure 4B) show only one region with a negative potential V(r) (orange zone near the catechol OH group) of -0.14166 and -0.14179 el/au3, respectively. The MEP of (+)-catechin (Figure 4C) displays two regions with negative potential: an orange zone located near the oxygen atom of the pyran ring with V(r) = -0.14179 el/au3 and a second minimum in the third ring of the molecule with V(r) = -0.15875 el/au3.

Furthermore, it is important to mention that although the spatial disposition of (+)-catechin is rather similar to that of *trans*-caffeic and *trans-p*-coumaric acid, certain differences can be observed in the electronic behavior of these compounds, which may explain the lower antibacterial activity of (+)-catechin with respect to *trans*-caffeic and *trans-p*-coumaric acid. The most striking feature of the MEPs is the existence of only one region with negative potentials for the compounds with higher antibacterial activity. It should be noticed that the most active compounds (*trans*-caffeic and *trans-p*-coumaric acid) displayed closely related MEPs.

At this point, SAR analysis requires evaluation of a possible pharmacophoric pattern to produce an antibacterial effect. In the next step of our study we evaluated the interatomic distances between the potential reactive sites. Figure 5 shows the interatomic distances obtained for the potentially reactive sites of the PCs assayed here. An interesting fact is that the distance between the catechol hydroxyls and carboxylate group in protocatechuic acid is significantly shorter than that of the active compounds in their extended forms. Thus, the notably lower activity and in some cases absence of activity observed for gallic and protocatechuic acid could be explained in terms of their smaller molecular size compared to the size of the active compounds (*trans*-caffeic and *trans-p*-coumaric acid). The interatomic distance of the reactive groups of gallic and protocatechuic acid is about 6.2 and 6.4 Å, respectively (Figure 5A), while the distance in *trans*-caffeic and *trans-p*-coumaric acid reaches values of 8.5 and 8.8 Å (Figure 5B). This would indicate that a distance of 8 Å or more between catechol hydroxyls and the carboxylic group might be a structural requirement for compounds to perform higher antibacterial activity. In this way, Sánchez-

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Maldonado et al. (2011) have reported that antibacterial activity of hidroxycinnamic acids is strongly dependent on the double bond of the side chain.

Cueva et al. (2010) reported that antibacterial activity of phenolic acids is determined by their chemical structure, particularly through the number and position of the substituents on the benzene ring and the length of the saturated chain. Other authors have reported that damage to the wine LAB cell membrane could be a result of hydrophobic interactions between PCs and the membrane lipids. This would lead to the disintegration of the bacterial membrane, causing cell death after alterations in processes dependent on energy transport and metabolic pathways that are essential for the viability of the bacteria (Ibrahim et al. 1996, García-Ruíz et al. 2009). The computational studies performed in this paper allow a deduction of the structural requirements necessary for PCs to exhibit significant antibacterial activity. These studies may provide guidance for the design of novel compounds.

## Conclusion

Results reported in the present study contribute to the knowledge of the potential use of PCs as antibacterial agents against wine spoilage LAB. *Trans*-caffeic and *trans-p*-coumaric acid showed the highest growth inhibition of the bacteria assayed. These compounds produced damage to the cellular integrity, evidenced by TEM micrographs. The SAR study provided specific information on the minimal structural requirements necessary to obtain a greater antibacterial activity (pharmacophore pattern). These results represent a promising approach to wine preservation regarding future use of PCs against LAB spoilage and, consequently, elimination or at least a reduction in the use of $SO_2$ in wineries. However, further studies are required in order to assess the impact on wine organoleptic properties.

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**Table 1** Effect of phenolic compounds (at 400 mg/L) on viability of lactic acid bacteria.

| **Phenolic compounds** | **A [Log cfu/mL]** | | |
|---|---|---|---|
| | ***L. hilgardii* 6F** | ***L. hilgardii* $X_1$B** | ***P. pentosaceus* 12p** |
| **Control** | 1.38[a] | 0.84[a] | 0.33[a] |
| **Gallic acid** | 1.64[b] | 1.35[b] | -1.68[b] |
| **Protocatechuic acid** | 1.55[a,b] | 0.39[c] | -1.10[c] |
| ***trans*-caffeic acid** | -0.12[c] | 0.08[d] | -2.18[d] |
| ***trans-p*-coumaric acid** | 0.14[d] | -0.43[e] | -1.97[d] |
| **(+)-catechin** | 1.62[b] | 0.11[d] | -0.63[e] |

Values with the same letter in the same column are not significantly different ($p < 0.05$).

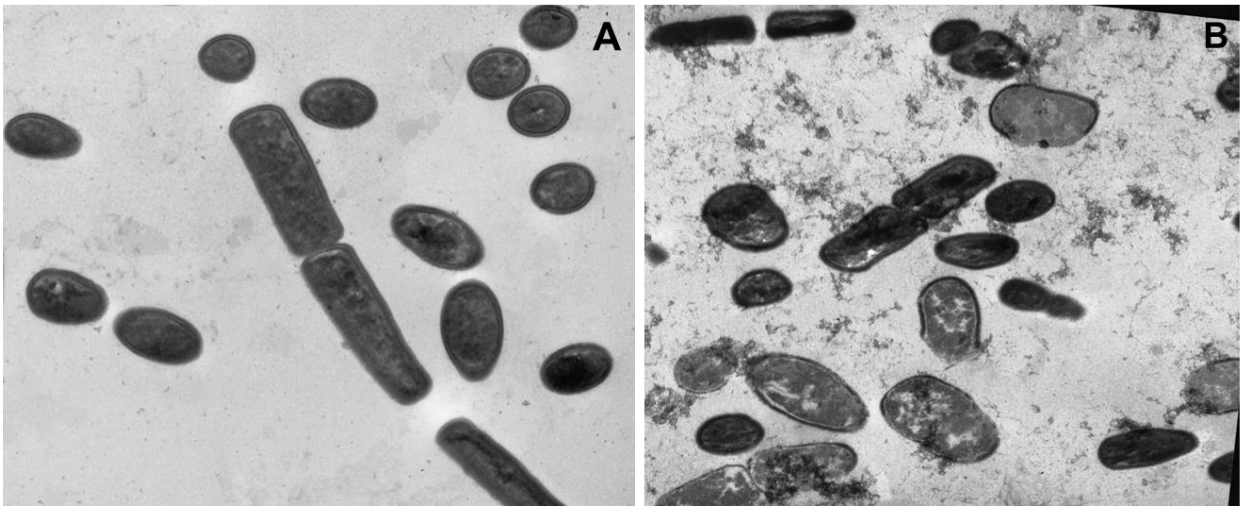


**Figure 1** Electron micrographs of ultrathin sections of *L. hilgardii* 6F obtained after 96 h incubation in SWM (**A**); and SWM supplemented with 400 mg/L of *trans*-caffeic acid (**B**).

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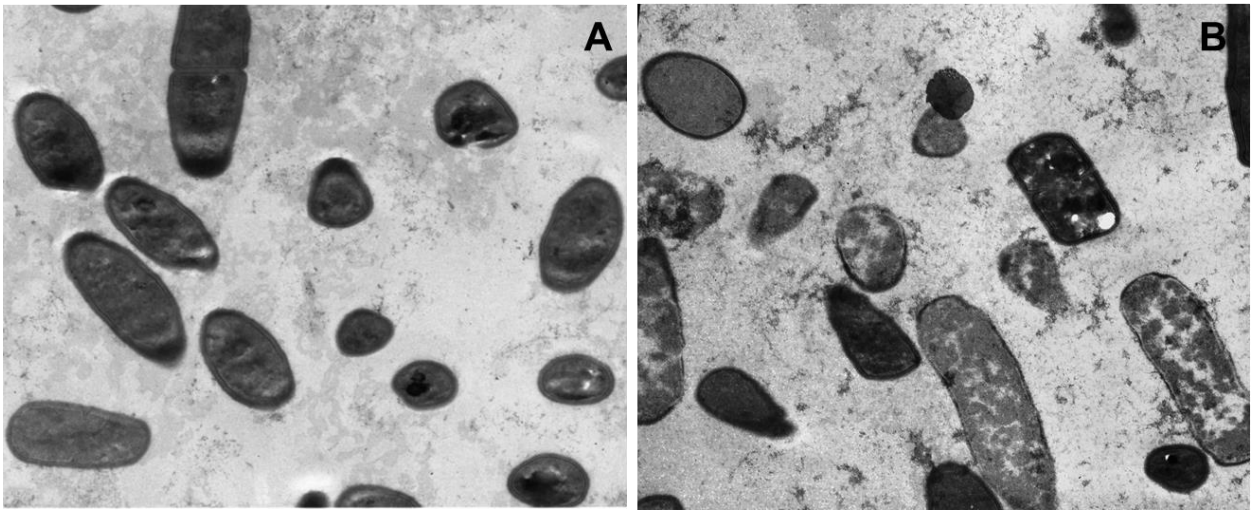


**Figure 2** Electron micrographs of ultrathin sections of *L. hilgardii* $X_1B$ obtained after 96 h incubation in SWM (**A**); and SWM supplemented with 400 mg/L of *trans-p*-coumaric acid (**B**).

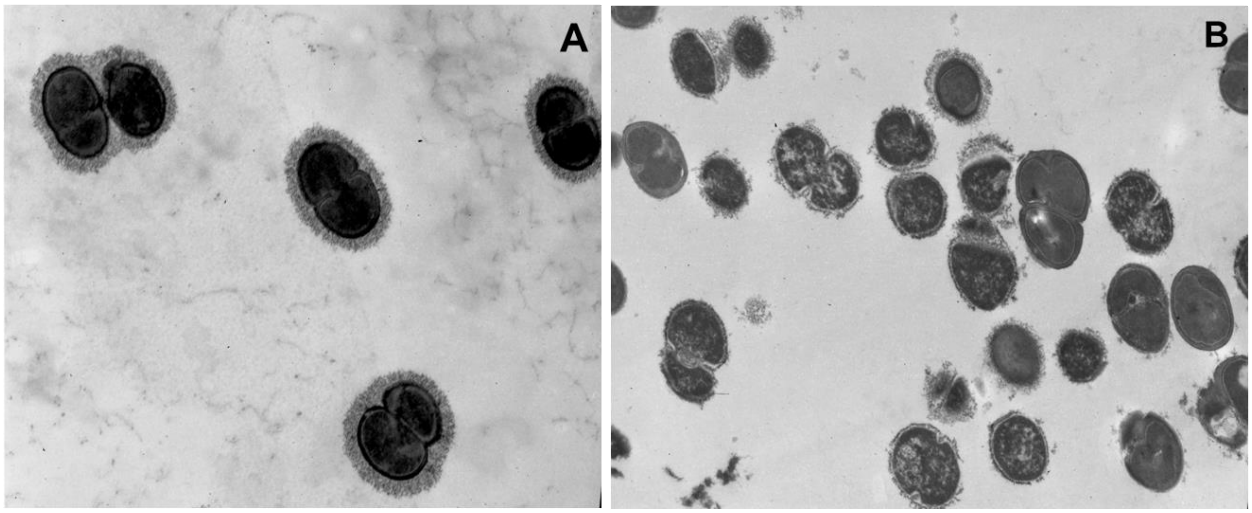


**Figure 3** Electron micrographs of ultrathin sections of *P. pentosaceus* 12p obtained after 96 h incubation in SWM (**A**); and SWM supplemented with 400 mg/L of *trans-p*-coumaric acid (**B**) and after incubation at 30°C for 96 h.

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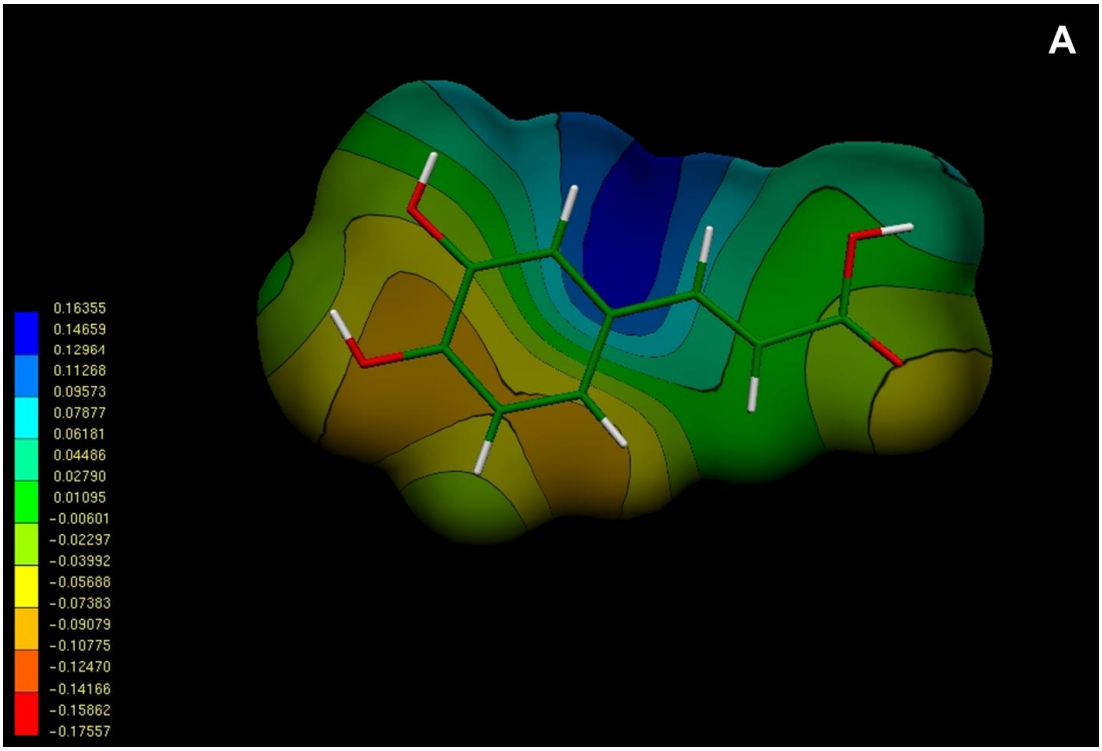


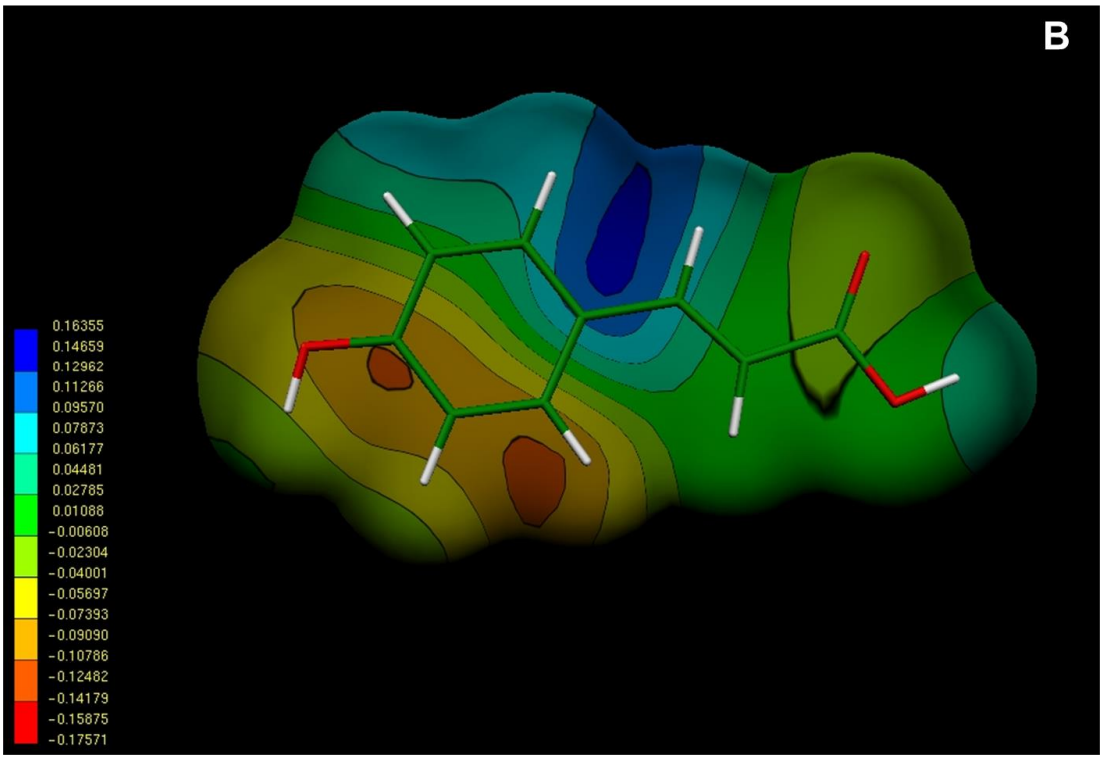

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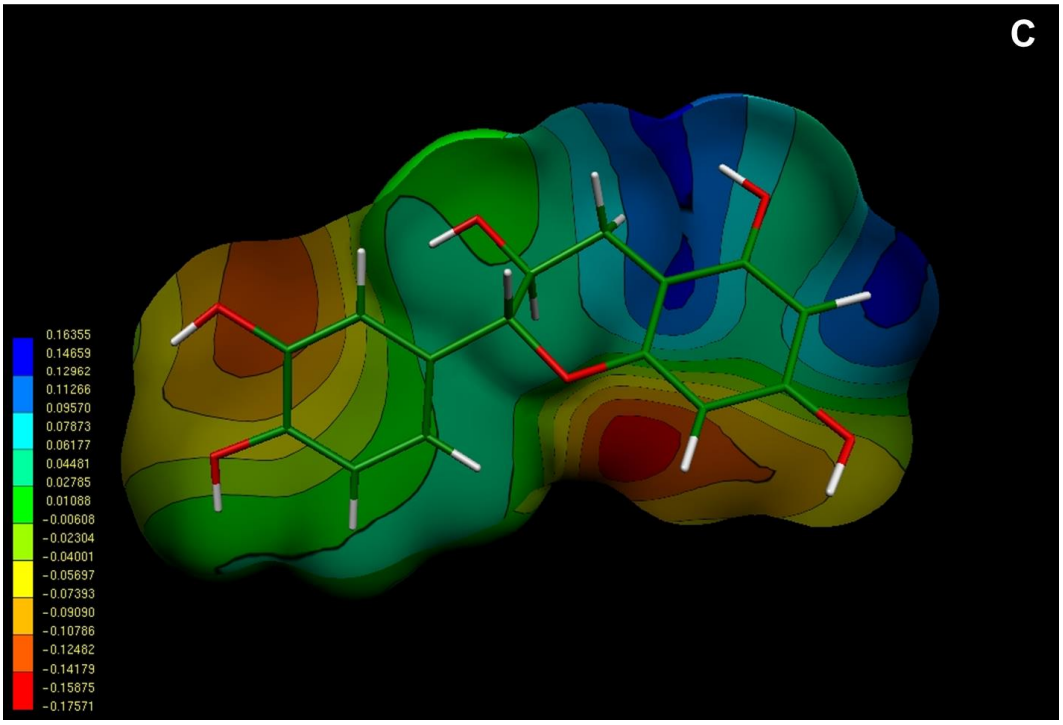


**Figure 4** The molecular electrostatic potential of *trans*-caffeic acid (**A**), *trans-p*-coumaric acid (**B**) and (+)-catechin (**C**). The surfaces were generated with Gaussian 03 after B3LYP minimization using single point B3LYP / 6-311 ++ G (d, p) calculations. The coloring represents the electrostatic potential with red indicating the strongest attraction to a positive point charge and blue indicating the strongest repulsion. The color code is shown at the left of each figure.

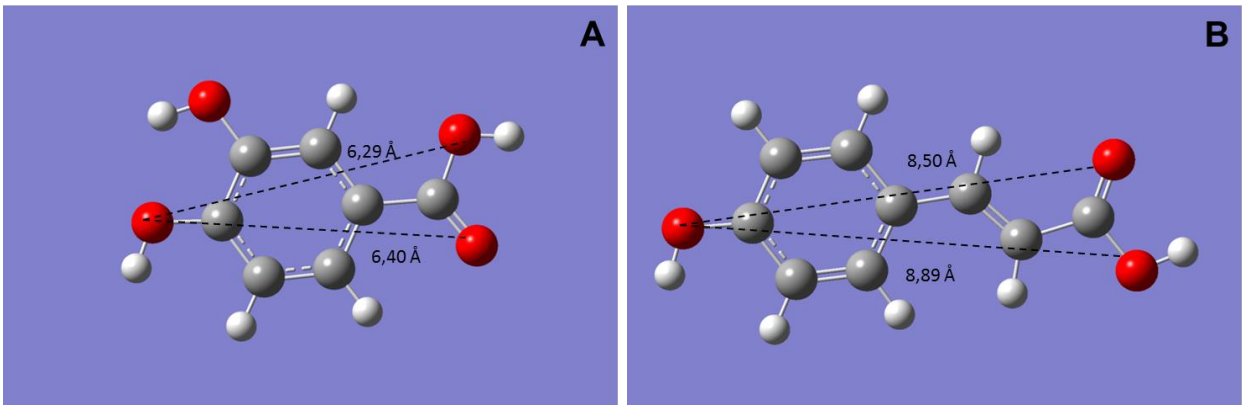


**Figure 5** Interatomic distances between the reactive groups of protocatechuic acid (**A**) (similar to gallic acid) and *trans-p*-coumaric acid (**B**) (similar to *trans*-caffeic acid).