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Comparative antibacterial effect of gallic acid and catechin against *Helicobacter pylori*

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ABSTRACT

The antimicrobial effect of gallic acid and catechin on *Helicobacter pylori* cultures was investigated. The antimicrobial effect was evaluated by measuring the absorbance of liquid culture media at 600 nm, by an agar-well diffusion method and by scoring colony forming units. Both polyphenols displayed strong growth inhibitory effects on two strains of *H. pylori* (26695 and ATCC 43504). The antibacterial effects were dependent on dose, contact time and type of polyphenol. Gallic acid showed a higher inhibitory effect on both *H. pylori* strains compared to catechin. Partially additive growth inhibitory effect between catechin and gallic acid was observed.

1. Introduction

Helicobacter pylori is a neutralophilic Gram-negative spiral-shaped bacterium that colonizes the human stomach (Dunn, Cohen, & Blaser, 1997). Gastric *H. pylori* infection impacts a high percentage of the world population (Dunn et al., 1997; Yamaoka, 2008). Although most of the infected population is asymptomatic and only a fraction presents serious pathologies, it has been estimated that *H. pylori* increases by 10 times the probability to develop gastric cancer (Yamaoka, 2008). Gastric cancer is the second leading cause of cancer-related deaths worldwide, accounting for 10% of cancer deaths during year 2000 (Parkin, 2000). On the other hand, *H. pylori* infection causes a serious transmissible infectious disease that damages gastric structure and function, and is recognized as the causative agent in gastric atrophy, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (Dunn et al., 1997; Tombola et al., 2003). The eradication therapies of *H. pylori* involve the use of tetracycline, amoxicillin, clarithromycin and metronidazole (Bonacorsi, Raddi, Carlos, Sannomiya, & Vilegas, 2009; Toledo & López-Solís, 2009). However, the eradication of *H. pylori* is not always successful due to the increased prevalence of antibiotic resistance, which in the case of metronidazole can reach 100% (Aboderin et al., 2007). Recent epidemiological evidence has shown that tetracycline resistance among clinical isolates of *H. pylori* is almost 30% (Vallejos, Cerda, Valenzuela, & Toledo, 2003). Considering that eradication therapies can be ineffective, costly, based on the use of drugs that may be unavailable in some geographic areas or may produce undesirable secondary effects, the search for new drugs and the development of alternative therapies has become critical (Bonacorsi et al., 2009). Plants are attractive sources of polyphenols, a family of compounds showing promising results in the treatment of a variety of diseases. Several studies have shown that phenolic compounds found in cranberries, green tea, apple and wine, affect *H. pylori* (Mabe, Yamada, Oguni, & Takahashi, 1999; Mahady, Pendland, & Chadwick, 2003; Pastene et al., 2010; Vattem, Lin, Ghaedian, & Shetty, 2005; Yahiro et al., 2005). Almost without exception, these studies were based on the analysis of the effect of complex polyphenol plant extracts. In that regard, limited information exists about the effect of pure polyphenols on *H. pylori*. Gallic acid and catechin are two abundant phenolic compounds that are widely distributed among plants (Obreque-Slier et al., 2010). Not much is known about the effect of pure gallic acid and pure catechin, or about any eventual synergy or interference between them, on *H. pylori* growth. The aim of this study was to compare the antimicrobial effect of gallic acid and catechin, alone or in combination, against *H. pylori* cultures.

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2. Materials and methods

2.1. Materials

Gallic acid and catechin standards (Sigma–Aldrich, Santiago, Chile), cellulose acetate filters (Asahi Glass, Tokyo, Japan), sodium chloride and bacto agar (JT Baker, Mexico), dimethyl sulfoxide (DMSO) (Winkler, Santiago, Chile), horse serum (Thermo Scientific HyClone, Utah, USA), antibiotic supplements Dent and Vitox (Oxoid, Hampshire, England) and pro-analysis grade chemicals (Merck Ltd., Santiago, Chile) were purchased.

2.2. Polyphenol solutions

Stock solutions of both gallic acid and catechin were prepared by dissolving 50 mg of the polyphenol in 1 mL of 10% DMSO in phosphate-buffered saline (PBS). Both solutions were sterilized by filtration through cellulose acetate filters (0.2 μm pore size; 25 mm diameter).

2.3. Bacterial strains and growth conditions

Two *H. pylori* strains were used: *H. pylori* ATCC 43504, isolated from Australian gastric antrum patient, and *H. pylori* 26695 (ATCC 700392), isolated from United Kingdom patient with gastritis, whose genome has been sequenced.

Frozen stocks of *H. pylori* were recovered and routinely grown for 48 h at 37 °C, 5.5% CO_2, 70–80% humidity on Trypticase soy agar (TSA) (Becton–Dickinson, Sparks, MD 21152, USA) with 0.4% *H. pylori* selective supplement Dent (Oxoid Basingstoke, Hampshire, England), 0.3% Isovitalex (Oxoid) and 5% horse serum (Cerda, Rivas, & Toledo, 2003; Toledo, Valenzuela, Rivas, & Jerez, 2002). For liquid growth experiments, cells were grown in Trypticase soy broth (TSB) (Becton–Dickinson) with 5% horse serum, supplemented with Isovitalex and Dent (Oxoid). Bacteria were first grown to an optical density of 0.6–1.0 at 600 nm (OD_{600}) at pH 7.0 and subsequently diluted to a starting OD_{600} of 0.05. To measure the growth of *H. pylori* in liquid medium, a serial dilution was prepared, aliquots of the various dilutions were plated on Trypticase soy agar plates and the number of colonies (CFU) was determined (Toledo & López-Solís, 2009).

2.4. H. pylori growth assay in liquid medium

H. pylori (3×10^7 cells/mL) were inoculated in 5 mL of TSB and supplemented with a range of concentrations (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) of gallic acid or catechin. After incubation at 37° C for 48 h with constant shaking at 250 rpm in a controlled atmosphere (5.5% CO_2 and 70% relative humidity), bacterial growth was determined by turbidimetry at 600 nm or by counting colony forming units on trypticase soy agar plates (Stevens, Sheldon, Klapes, & Klaenhammer, 1991).

2.5. H. pylori viability assay

From each of the experimental culture tubes described in the previous section, 100 μL-aliquots were taken at the end of the incubation period to prepare serial dilutions in PBS. Aliquots of 10 μL from each of these dilutions were plated on TSA and incubated for 48 h at 37 °C (Eydelnant & Tufenkji, 2008). The number of colony forming units per mL (CFU/mL) corresponding to each experimental condition was determined.

2.6. Inhibition kinetics of bacterial growth

The assay was conducted basically as described by Romero, Medina, Vargas, Brenes, and De Castro (2007) with minor modifications. Briefly, 3×10^7 CFU/mL were inoculated into Eppendorf tubes containing 1 mL of TSB medium supplemented with 1 mg/mL of gallic acid or catechin and incubated at 37 °C. From this mixture, 100 μL-aliquots were removed at 0, 15, 30, 45, 60 and 90 min. The numbers of viable cells at various time points were determined as described above.

2.7. Inhibition halo test on agar plates

The procedure was performed as described by Rodríguez, Alberto, and Manca de Nadra (2007). Three-millimeter diameter wells were made in TSA plates and 30 μL of a series of gallic acid or catechin solutions were deposited in the wells (corresponding to 0–1 mg/well). Then, 100 μL of a *H. pylori* suspension containing 3×10^7 cells/mL were evenly spread over the plates with a metal handle loop. After 48 h of incubation at 37 °C the diameter of the growth inhibition halos were determined.

2.8. Studies on possible synergistic action between gallic acid and catechin

H. pylori strains were grown on TSA plates with a series of wells containing a fixed concentration of catechin (0.4 mg/well) plus increasing doses of gallic acid (0.0, 0.05, 0.1, 0.2, 0.25 and 0.4 mg/well). After 48 h of incubation at 37 °C the diameter of the growth inhibition halos were measured.

2.9. Statistical analysis

Data were analyzed using the analysis of variance (ANOVA) and the Tukey test to evaluate differences.

3. Results

3.1. Antibacterial activity of gallic acid against H. pylori

3.1.1. Growth assay in liquid medium

As shown in Fig. 1 the OD_{600} values ranged between 0.2 for control and 1.8 for the medium supplemented with 1 mg/mL of gallic acid. However, because such increase in the absorbance was

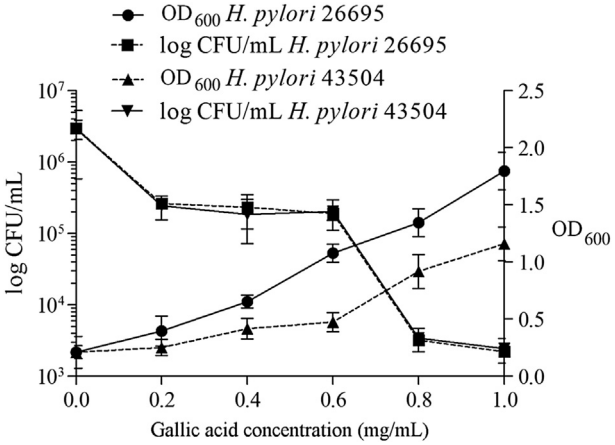

Fig. 1. OD_{600} and CFU/mL values of *Helicobacter pylori* cultures in presence of gallic acid.

also observed in the absence of microorganisms we finally concluded that phenomenon might derive from the product of a chemical reaction between gallic acid and some of the components of the culture medium.

On the other hand, the values of CFU/mL ranged from 5×10^6 to 3×10^3 for the same extreme gallic acid concentrations. Thus, the OD_{600} values showed a tendency to increase with the content of gallic acid, while the CFU/mL values showed a significant decrease with increasing contents of the polyphenol in the culture medium.

3.1.2. Effect of gallic acid on the inhibition halo of bacterial growth

As shown in Fig. 2, the diameters of the inhibition zones ranged from 0 for the control condition to 14 mm for the presence of 1 mg of gallic acid. In this assay, the diameters of the inhibition halos increased gradually with the content of gallic acid. Also, the diameters of the inhibition halos observed in the presence of doses of gallic acid equal to or greater than 0.2 mg were significantly higher ($p < 0.05$) than those produced in the absence of gallic acid. Both *H. pylori* strains used in this study showed no differences in this assay.

3.1.3. Kinetics of bacterial growth inhibition by gallic acid

As shown in Fig. 3, during the 90 min of exposure to gallic acid the CFU/mL values decreased continuously from 2×10^6 to 8×10^1 (*H. pylori* strain 26695) and from 2×10^7 to 10^0 (*H. pylori* strain 43504). According to this study, decrease in *H. pylori* viability became statistically significant ($p < 0.05$) after 30 min of incubation in the presence of 1 mg/mL gallic acid.

3.2. Antibacterial activity of catechin against H. pylori

3.2.1. Growth assay in liquid medium

As shown in Fig. 4, the OD_{600} values ranged from 0.25 for control to 0 for the medium supplemented with 1 mg/mL of catechin. This observation was fully consistent with CFU/mL values, which ranged from 5×10^6 (control condition) to 0 (at 0.8 mg/mL of catechin). Both the OD_{600} values and the CFU/mL values first became significantly lower ($p < 0.05$) compared to the control condition at 0.4 mg/mL of catechin.

3.2.2. Effect of catechin on the inhibition halo of bacterial growth

As shown in Fig. 5, the diameters of the inhibition halos ranged from 0 for the control condition to 16 mm for the presence of 1 mg of catechin. As it was expected, a gradual increase in the diameters

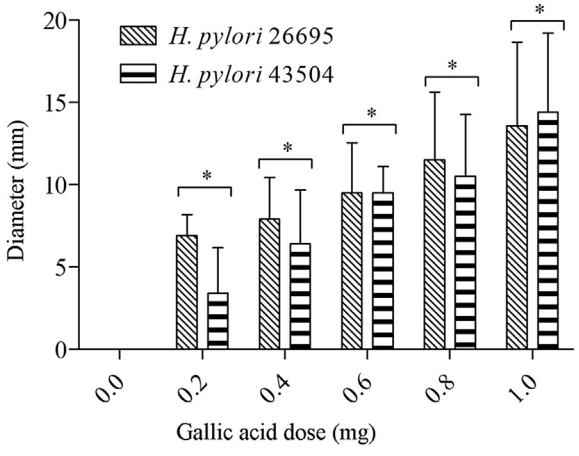

Fig. 2. Inhibition halos on *H. pylori* growth in presence of gallic acid. * Statistical significance with respect to control (no gallic acid) ($p < 0.05$).

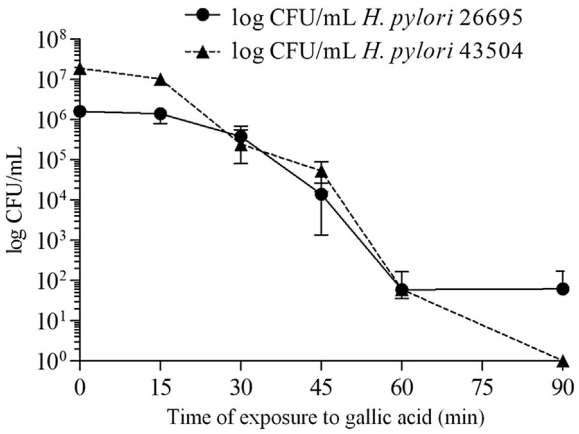

Fig. 3. Kinetics of growth inhibition of *Helicobacter pylori* in presence of gallic acid.

of the inhibition halos was observed when the *H. pylori* strains were incubated in the presence of increasing doses of catechin. Doses equal to or greater than 0.4 mg of catechin produced inhibition halos significantly higher ($p < 0.05$) than those observed in the absence of polyphenols.

3.2.3. Kinetics of bacterial growth inhibition by catechin

Fig. 6 shows that the CFU/mL values decreased from 3.5×10^7, at the start of the incubation, to 5×10^2, at the end of the incubation. Decrease in the CFU/mL values became statistically significant ($p < 0.05$) after 15 min of incubation in the presence of 1 mg/mL catechin.

3.3. Growth inhibitory effect of combinations of gallic acid and catechin in H. pylori strains

H. pylori strains were grown on TSA plates with a series of wells containing either a fixed concentration of catechin (0.4 mg/well) plus increasing doses of gallic acid (from 0 to 0.4 mg/well) or the same doses of gallic acid alone. After 48 h of incubation at 37 °C the diameter of the growth inhibition halos were measured. A dose of 0.4 mg of only catechin produced inhibition halos of 6.16 ± 0.28 mm and 8.33 ± 1.66 mm in *H. pylori* strains 26695 and 43504, respectively (Fig. 7). When inhibition halos were examined in relation to the presence of a range of doses of only gallic acid, no inhibition was observed with doses of gallic acid less than 0.20 mg

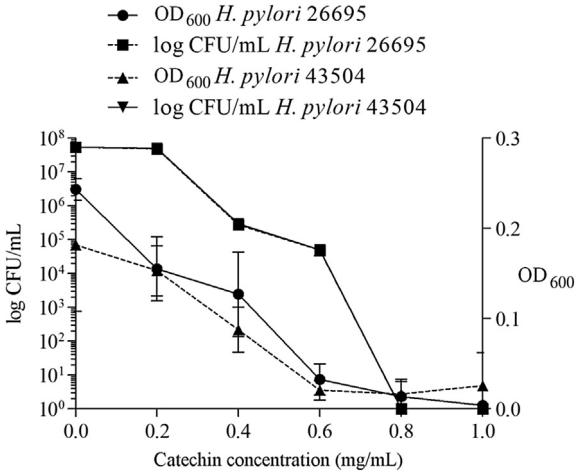

Fig. 4. OD_{600} and CFU/mL values of *Helicobacter pylori* cultures in presence of catechin.

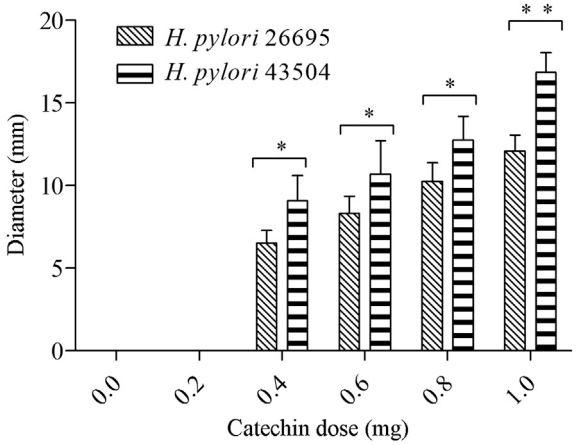

Fig. 5. Inhibition halos on *H. pylori* growth in presence of catechin. * Statistical significances with respect to control (no gallic acid) ($p < 0.05$). ** Statistical significance with respect to control and between *H. pylori* strains.

in either *H. pylori* strain. When both polyphenols (fixed catechin and varying gallic acid) were placed together in single wells, inhibition halos were equal to or larger than those produced in presence of the corresponding doses of only gallic acid, and equal to or smaller than the ones observed in presence of only catechin (except with 0.4 mg of gallic acid, a condition resulting in a significantly larger inhibition halo) (Fig. 7A and B).

4. Discussion

Phenolic compounds are one of the most abundant groups of substances in the plant kingdom. These compounds have been associated with important sensory, taxonomic, nutritional and pharmacological properties of foods (Monagas, Bartolomé, & Gómez-Cordovés, 2005). Several authors have demonstrated the bactericidal effect of those compounds against *H. pylori*, a bacterium associated with a large number of gastrointestinal diseases (Mabe et al., 1999; Mahady et al., 2003; Pastene et al., 2010; Vattem et al., 2005; Yahiro et al., 2005). Most of those studies were based on the use of complex mixtures of polyphenols that were extracted with organic solvents from olive oil, cranberries, blackberry leaves, grapes and wines. Thus, limited information is available about the individual effects of these compounds (Brown, Huang, Haley-Zitlin, & Jiang, 2009; Mahady et al., 2003; Martinia et al., 2009; Romero et al., 2007; Tombola et al., 2003; Vattem et al., 2005; Yahiro et al., 2005).

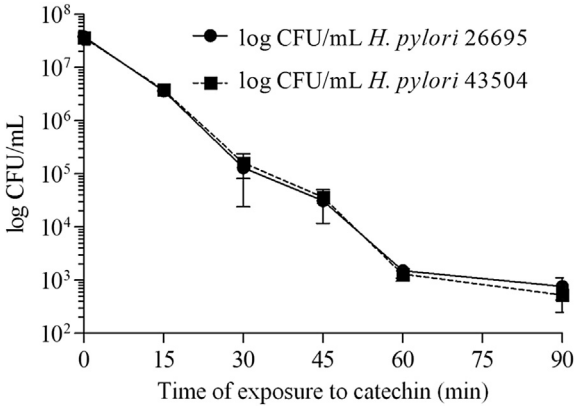

Fig. 6. Kinetics of growth inhibition of *Helicobacter pylori* in presence of catechin.

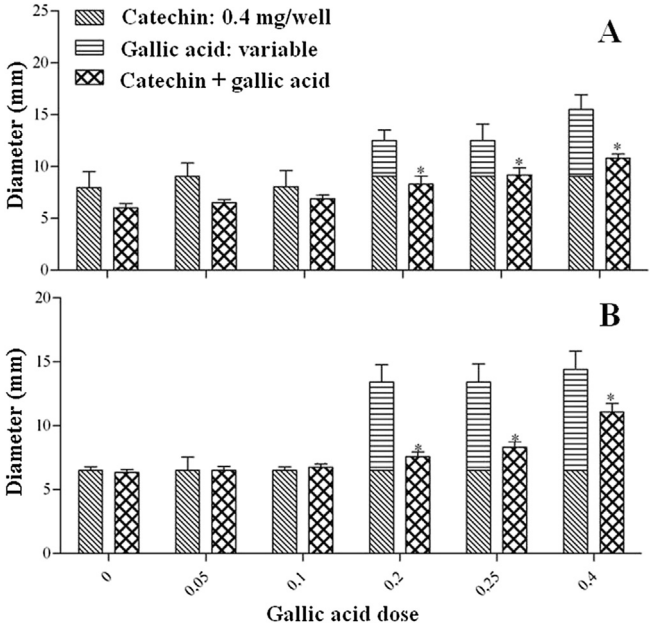

Fig. 7. Inhibition halos on *H. pylori* growth (A, strain 43504; B, strain 26695) in presence of either constant catechin, varying gallic acid or combinations of both. * Statistical significance between individual and corresponding combined application ($p < 0.05$).

Among the most studied phenolic compounds, resveratrol, ellagic acid, myricetin, epicatechin-3-*O*-gallate and epicatechin have been found to display antibacterial effects against *H. pylori* (Brown et al., 2009; Mahady et al., 2003; Tombola et al., 2003; Yahiro et al., 2005). Gallic acid and catechin, two polyphenols occurring in high concentration in some foods, such as grapes and wine, are much less studied in this respect (Monagas et al., 2005; Ribéreau-Gayon et al., 1959). Despite their quantitative relevance, there is limited information about their individual or combined antibacterial effects against *H. pylori*. In this study, we evaluated the effect of both gallic acid and catechin on two *H. pylori* strains (26695 and ATCC 43504).

Gallic acid is a non-flavonoid polyphenol whose concentrations may reach 220 mg/kg in some foods (Obreque-Slier et al., 2010). In this study, we observed that gallic acid provokes a dose-dependent decrease in the CFU/mL value and also a dose-dependent increase in the diameters of the inhibition halos in agar plate cultures of both bacterial strains. Both observations highly suggest that gallic acid has a significant inhibitory effect on the growth of *H. pylori*. Such conclusion is also consistent with the observation that the CFU/mL value decreased with increasing times of exposure of the *H. pylori* strains to gallic acid, thus confirming that time of exposure to the polyphenol has a major influence on the viability of these microorganisms.

Likewise, catechin is a flavonoid phenolic compound occurring in a large number of foods, whose concentrations may reach levels of 3000 mg/kg (Obreque-Slier et al., 2010). In this study we observed that the inhibitory effect of catechin on *H. pylori* growth had similar behaviour to that observed for gallic acid. However, this time we found that increasing amounts of catechin caused a significant decrease in the absorbance at 600 nm. Accordingly, with no exception, all our observations indicated that catechin inhibits the growth of both *H. pylori* strains, which fully coincides with reports by other authors (Tombola et al., 2003). It is important to note that, except for the maximum concentration of catechin in the measurement of the inhibition halo diameter, there were no statistically significant differences in the sensitivities of the two *H. pylori* strains

to catechin and gallic acid. This observation suggests that the antibacterial effects of catechin and gallic acid are not dependent on the bacterial strain.

Considering that both gallic acid and catechin showed inhibitory actions against the growth of *H. pylori* and that both polyphenols are usually part of the complex composition of natural foods, beverages and juices, we also evaluated the effect of combining gallic acid and catechin on the growth of the bacterium. To this end, we performed the inhibition halo test by combining increasing amounts of gallic acid with a constant amount of catechin in both *H. pylori* strains. In this experiment, the inhibition halos were similar or bigger than the inhibition halos produced after the addition of equivalent amounts of gallic acid alone. In addition, the highest dose of gallic acid resulted in an inhibition halo that was significantly higher than the inhibition halo produced by catechine alone. This partially additive effect between catechin and gallic acid is in agreement with observations by Yanagawa, Yamamoto, Hara, and Shimamura (2003), that polyphenols in plant extracts are less effective than the corresponding pure compounds. Anyhow, such partially additive effect of gallic acid and catechin on growth inhibition of *H. pylori* needs to be further studied because different pharmacological properties of both polyphenols may account for it.

In conclusion, both gallic acid and catechin display growth inhibitory effects in *H. pylori*, those effects are partially additive in a dose-dependent manner over the range of doses in the study and growth inhibition is dependent on contact time and type of polyphenol.

Acknowledgments

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