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In Vitro and in Vivo Effects of Apple Peel Polyphenols against *Helicobacter pylori*

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The inhibitory effects of a standarized apple peel polyphenol-rich extract (APPE) against *Helicobacter pylori* infection and vacuolating bacterial toxin (VacA) induced vacuolation were investigated. Apple peel polyphenols significantly prevented vacuolation in HeLa cells with an IC_{50} value of 390 μg of gallic acid equivalents (GAE)/mL. APPE also displayed an in vitro antiadhesive effect against *H. pylori*. A significant inhibition was observed with a 20−60% reduction of *H. pylori* attachment at concentrations between 0.250 and 5 mg of GAE/mL. In a short-term infection model (C57BL6/J mice), two levels of APPE doses (150 and 300 mg/kg/day) showed an inhibitory effect on *H. pylori* attachment. Orally administered apple peel polyphenols also showed an anti-inflammatory effect on *H. pylori*-associated gastritis, lowering malondialdehyde levels and gastritis scores.

KEYWORDS: *Malus domestica*; apple peel; polyphenols; *Helicobacter pylori*; VacA; C57BL6/J

INTRODUCTION

Gastric *Helicobacter pylori* infection affects almost half of the world's population (*1*). This pathogen is responsible of several gastrointestinal clinical disorders including duodenal ulcer, mucosa-associated lymphoid tissue lymphoma (MALT), and gastric carcinoma (*2*, *3*). The initial steps of *H. pylori* infection imply the penetration of the bacteria into the mucin layer and its adherence to epithelial gastric cells by means of an array of different adhesins (*4*). As a result, adhered *H. pylori* can damage the gastric mucosa, producing oxidative stress, inflammation, and host cell death via apoptosis and/or autophagy (*1*). The severity of the clinical outcomes depends on factors linked to the host gastric environment, the *H. pylori* strain, and bacteria−host interplay (*5*). Among the main bacterial pathogen virulence determinants are urease, VacA, and CagA proteins (*6*).

Although the use of a combination of multiple antibiotics and a proton pump inhibitor has proven effective to eradicate *H. pylori* infections (*7*), the development of resistance to antibiotics has led to the search for new prophylactic and/or therapeutic alternatives. In this regard, plant-derived extracts that display inhibitory activity in vitro against *H. pylori* (*8*−*11*) have been proposed to be potentially useful in protecting the host against *H. pylori* colonization. In addition to their bactericidal effects, some such extracts, like those rich in procyanidins, appear to inhibit the adherence of *H. pylori* to the gastric mucosa, the urease activity, and/or the inflammatory and cytotoxic effects of VacA (*12*−*14*). Furthermore, it has been proposed that procyanidins bind to VacA, neutralizing its cytotoxic effects on cultured cells (*15*). A similar effect has been observed for catechins and procyanidins extracted from green tea, red wine, and hop bracts (*15*, *16*). Recently, the urease inhibitory capacity of an apple peel polyphenol-rich extract (APPE), the composition and polyphenolic profile of which match those of fresh apple peel (*17*), was reported by our group. In such work, urease inhibition was greater for procyanidins with higher molecular weight compared to monomeric polyphenols. Additionally, APPE inhibited the in vitro proliferation of *H. pylori* and also the oxidative burst induced by exposure of *H. pylori* in neutrophils (*18*). The antiadherence properties of procyanidins have been investigated by Burger et al. (*19*), who reported that high molecular weight procyanidins of cranberry juice inhibited the adhesion of *H. pylori* to immobilized human mucus and to cultured gastric epithelial cells.

Prompted by our previous results, in the present study we examined in vivo the potential of APPE to exert cytoprotective, antiattachment, and anti-inflammatory effects in a C57BL6/J mice model of short-term *H. pylori* infection.

In addition, the antiadherence and efficacy of APPE to inhibit the vacuolation of HeLa cells induced by VacA cytotoxin was evaluated.

MATERIAL AND METHODS

Standards, Chemicals, and Solvents. Gallic acid, chlorogenic acid, caffeic acid, (+)-catechin, (−)-epicatechin, phloridzin, quercetin, quercetin 3-*O*-rutinoside, procyanidins B1 and B2, sodium carbonate, and the Folin−Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO).

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Flavonol glycosides (quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, and quercetin 3-*O*-rhamnoside) were from Roth (Karlsruhe, Germany). All other solvents were of HPLC grade and purchased from Merck (Darmstadt, Germany).

Apple Peel Extract. Apple peels from ripe fruits (*Malus domestica* cv. Granny Smith) were kindly provided by SURFRUT Ltd. (Santiago, Chile). According to previous work, standardized APPE was prepared by retention on absorber resin Sepabeads SP-850 (Supelco, Bellefonte, PA) that was packed in a glass column (50 mm i.d. × 300 mm) (*17*). This extract was characterized by HPLC according to previously published methodology (*17*, *18*). Total polyphenolic contents (TPC) were determined with Folin−Ciocalteau reagent and expressed as milligrams of gallic acid equivalents (GAE) per gram of dried extract.

Stability of APPE Polyphenols in Acidic Medium. The stability of APPE flavonol glycosides in simulated gastric juice was assessed using procedures previously published with minor modifications (*20*, *21*). APPE (250 mg) was dissolved in 25 mL of simulated gastric juice without pepsin (2.0 g of NaCl and 3 mL of concentrated HCl diluted to 1 L, pH ∼2) and incubated by 4 h at 37 °C with stirring (100 rpm). Aliquots of 200 μL were periodically taken at 15, 30, 60, 120, 180, and 240 min and filtered through a 0.45 μm membrane. Reverse-phase high-performance liquid chromatography (HPLC) was used to evaluate flavonol glycosides, whereas normal phase HPLC was used for procyanidins analysis.

Analysis of Flavonol Glycosides of APPE in Gastric Fluid by Reverse-Phase High-Performance Chromatography (HPLC-DAD-ESI/MS). After incubation of APPE in gastric simulated fluid, hydrolysis products were analyzed by HPLC-DAD according to previously published methodology and using rutin and quercetin as standards for calibration (*21*). Flavonoids were quantified using their absorbances at 355 nm. In these conditions, all flavonoid glycosides eluted between 12 and 20 min. Therefore, any effect on their stability could be evidenced as a decrease in the sum of peak areas in this zone. Thus, an increasing area of the quercetin peak (t_R = 24 min) could be interpreted as a marker of hydrolysis. The presence of quercetin was confirmed by comparison with standard and by HPLC-ESI-MS using a previously published methodology (*18*).

Analysis of Procyanidins of APPE in Gastric Fluid by Normal-Phase HPLC. To assess the effect of the gastric juice on APPE procyanidin stability, we used the same aliquots sampled in the above-mentioned experiments. Procyanidins were separated in a HPLC Waters system 600 (Waters, Milford, MA) by normal-phase HPLC (NP-HPLC) according to the method of Gu and co-workers (*22*) with a 250 × 4.6 mm (5 μm) Lichrospher 100 Diol column (Merck). The solvent system was composed of solvent A (acetonitrile/acetic acid, 98:2 v/v) and solvent B (methanol/water/acetic acid, 95:3:2 v/v). Procyanidins were eluted with the following gradient system: 0−35 min, 0−40% B; 35−55 min, isocratic 40% B; 55−60 min, 40−0% B with 5 min of column reconditioning at a flow rate of 0.6 mL/min. Elution was monitored by fluorescence detection (Waters 2475) with excitation at 230 nm and emission detection at 321 nm. The sum of peak area between 16 and 50 min was compared with the increase of monomers peak area (epicatechin, t_R = 9.5 min), which is considered a marker of hydrolysis.

Cell Cultures. Human cervical epithelial cells (HeLa) were obtained from American Type Culture Collection (Manasas, VA). Cells were cultured as monolayers (ATCC) in plates of 55 cm^2 [(1−2) × 10^5 cells/cm^2], until 80% confluence was reached. Cultured medium was 90% Earle-modified minimal essential medium (MEM) containing 10% fetal bovine serum, 2 mM GlutaMAX, 100 U/mL penicillin, and 100 μg/mL streptomycin (humidified atmosphere, 5% CO_2, 37 °C). Medium was changed twice a week. Cells were detached with 0.5 mg/mL trypsin containing 0.2 mg/mL EDTA in PBS. For the experiments, cells (15×10^3) were seeded in 6-, 12-, or 24-well titration plates, 24−48 h before the experiments. The viability of HeLa cells was determined by trypan blue dye exclusion method (*23*).

VacA in *H. pylori* Supernatants. *H. pylori* strain TX 146, which is a positive form vacuolating cytotoxin, was used to obtain VacA-enriched supernatants. *H. pylori* TX 136 was cultured in liquid medium during 48 h (37 °C, 10% O_2, 6% CO_2). Afterward, biomass was removed by centrifugation (Hettlich, Tuttlingen, Germany) at 14000*g*, and the supernatants were sterilized by membrane filtration (cellulose acetate, 0.22 μm) (*24*). Supernatant was brought to 50% saturation by the addition of solid ammonium sulfate. The suspension was left to rest for 12 h at 4 °C and further centrifuged at 11000*g* for 20 min, and the precipitated proteins were resuspended in 60 mM Tris, pH 7.5, with 100 mM NaCl and dialyzed extensively against the same buffer. Finally, the proteins were concentrated 40 times using an ultrafiltration in a Centricon tube (cutoff M_r 10 kDa; Millipore, Eschborn, Germany). Protein content was determined by means of the Bradford assay using BSA as standard (*25*). The presence of VacA was confirmed by Western blot according to previous work (*24*).

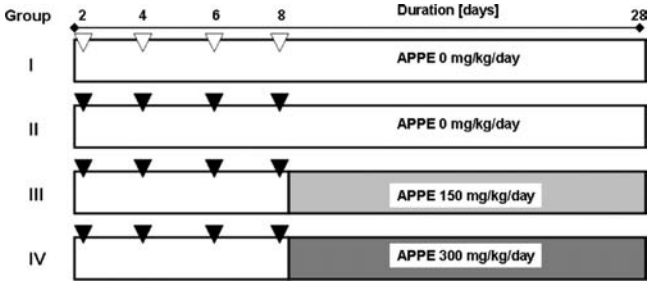

Figure 1. Experimental design. Five-week-old male C57BL/6 mice received intragastric inoculations of *H. pylori* (▼) or vehicle (▽). APPE at 150 mg/kg/day (light gray bar) or 300 mg/kg/day (dark gray bar) was administered through an oral catheter (n = 7).

Vacuolation Assay. HeLa cells were incubated for 16 h at 37 °C in 5% CO_2 with either (i) *H. pylori* supernatants containing VacA (500 μg/mL), (ii) uninoculated Brucella broth, (iii) APPE, or (iv) *H. pylori* supernatants containing VacA plus APPE. Vacuolation in HeLa cells was quantitated spectrophotometrically with a Sunrise microplate reader (Tecan, Männedorf, Switzerland) by neutral red assay as previously reported (*26*) and by using a phase constrast microscope (Carl Zeiss, Oberkochen, Germany) according to the method of Harris and colleagues (*27*). Viable cells were defined as cells having well-defined cytoplasmic and nuclear outlines, and vacuolation was defined as the presence of one or more intracytoplasmic vacuoles in viable cells. For each determination, 100 HeLa cells were counted, and the percentage of vacuolated cells was expressed as the mean ± the standard error of the means and compared by ANOVA test.

Antiadhesion Assay by Urea Phenol Red Method. For assaying the antiadhesion properties of APPE the Lee and co-workers protocol was used with minor modifications (*28*). In brief, HeLa cells (100 μL) were seeded in 6-well plates (Corning, Cambridge, MA) at a density of 5 × 10^4 cells/mL. After achieving confluence in 24 h, the confluent monolayers on the plates were washed three times with PBS buffer. Nonspecific binding was blocked by incubation with 0.5% BSA, prior to two rinses with PBS. Fifty microliters of *H. pylori* suspensions ($OD_{600\ nm}$ = 1.0; 1 × 10^8 UFC/mL) was incubated with APPE at various concentrations for 30 min. Subsequently, 100 μL of the mixture (bacteria plus APPE) was added to the HeLa cells and incubated for 1 h. A solution of 0.03% phenol red in 2% urea (pH 5.2) was added, and absorbance was measured at 560 nm with a Sunrise microplate reader (Tecan). The percentage of attached *H. pylori* was calculated as follows: attached % = 100 − [($OD_{experimental}$ − $OD_{negative}$)/($OD_{positive}$ − $OD_{negative}$) × 100]. The negative control contained only HeLa cells, and the positive control contained the epithelial cells and bacteria, which were used to establish 100% attachment.

Animals. Five-week-old C57BL6/J mice were used. Mice were purchased from ISP (Instituto de Salud Pública, Santiago, Chile). The animals were housed with a 12-h light−dark schedule, fed with standard rodent chow, and given water ad libitum. The rodent diets were kept in a special ventilated room for food storage in the animal house. Approval for this study was obtained prior to experimentation from the animal ethics committee, and all procedures were performed in compliance with Guidelines for the Care and Use of Laboratory Animals at the University of Concepción.

Experimental Protocol. *H. pylori* (ATCC 43504, CagA+, VacA+, American Type Culture Collection, Rockville, MD) was grown in Brucella broth (Becton Dickinson, Cockeysville, MD) containing 10% v/v horse serum at 37 °C under microaerophilic conditions (15% CO_2) at high humidity for 40 h with gentle shaking (150 rpm). Twenty-eight C57BL/6 mice (20−25 g) were divided in four groups according with the scheme displayed in **Figure 1**. After each mouse had fasted for 24 h, samples containing 1 × 10^9 colony-forming units (CFU)/mL were used as the

Table 1. PCR Primers

primer	target gene	sequence	product length	accession no.
Hp	16S rRNA	TGCGAAGTGGAGCCAATCTT (forward) GGAACGTATTCACCGCAACA (reverse)	119 pb	U00679

inoculums, which were orally gavaged using a feeding tube (outer diameter = 0.1 cm) four times at 2-day intervals with 0.3 mL of either bacterial suspension or PBS. The gastric *H. pylori* colonization was followed at different time points by sacrificing the animals. The stomachs were collected for culture, real-time PCR, and histopathology. On day 8 after oral inoculation animals were fed 200 μL of APPE (150 or 300 mg/kg/day for 20 days). Four weeks after *H. pylori* inoculation, the mice were euthanized, and their stomachs were excised and dissected along the lesser curvature, obtaining two equivalent parts (pyloric antrum and corpus). Samples were frozen at −20 °C until their use in the real-time PCR and malondialdehyde (MDA) level analyses.

Histological Examination. A portion of the gastric tissue was fixed in 10% neutral buffered formalin solution and embedded in paraffin, and various sections were stained with hematoxylin-eosin (H&E). All sections were examined by an experienced pathologist who was blind to the treatment groups and to the other results of this study. Gastric mucosal lesions were evaluated according to the histological scoring based on the Sydney system (*29*, *30*). *H. pylori* density was evaluated with the Warthin−Starry stain as modified by Kerr (*31*).

Gastric Tissue MDA Levels. Determination of gastric tissue MDA level was performed by HPLC according to previously published methods (*32*, *33*). Tissue samples were extracted by sonication with 10 volumes of 50 mM phosphate buffer during 30 min after the addition of ice-cold 10% trichloroacetic acid (TCA). Samples were mixed during 3 min and centrifuged at 10000*g* for 5 min. The supernatant was added to 700 μL of thiobarbituric acid (TBA) prepared using TBA in 2 M acetate buffer, pH 3, degassing by vacuum pump (5 min), and flushing the final solution with nitrogen for 10 min. The mixtures were degassed and then incubated for 30 min at 90 °C. At the end of the incubation period, samples were cooled, centrifuged (5 min, 10000*g*) to remove particulate material and, finally, sample aliquots (50 μL) were analyzed by HPLC (Waters) equipped with a 250 × 4.6 mm, 5 μm, Kromasil KR100-5C18 column (Eka Chemicals AB, Bohus, Sweden). Quantitation of the MDA−TBA adduct was carried out using an isocratic system with the solvent system methanol/50 mM phosphate (60 + 40 v/v, pH 6.8). The fluorescence detector was set at λ_{EX} = 515 and λ_{EM} = 545 nm. The MDA standard was prepared from tetramethoxypropane (Sigma), and the results were expressed as nanomoles per milligram of tissue. Analyses were performed in triplicate and presented as the mean ± SD.

Detection of *H. pylori* with Quantitative Real-Time PCR. *H. pylori* genomic DNA was extracted from mice stomach samples according to the method of Downset et al. (*34*), using a commercial kit (Wizard SV Genomic DNA Purification System Promega). As negative amplification controls, RNase/DNase free water and *Lactobacillus* spp. were used. As positive control, genomic DNA from *H. pylori* ATCC 43504 was used. TaqMan Master Mix (Roche) preparation was 4 μL of TaqMan Universal master mix, 9.1 μL of PCR grade H_2O, 0.5 μL of 10 μM TaqMan probe, 0.7 μL of 10 μM 16S forward primer, 0.7 μL of 10 μM 16S reverse primer, and 5 μL of sample DNA template. Amplification and detection were performed in a LightCycler 2.0 system (Roche Diagnostics, Mannheim, Germany). DNA was amplified using the following cycling parameters: heating at 95 °C for 10 min followed by 50 cycles of denaturation (95 °C, 10 s), annealing (55 °C, 10 s), and extension (72 °C, 8 s). The primer sequences for 16S rRNA are given in **Table 1**.

Statistical mMethods. A GraphPad Prism 4 software package (GraphPad Software, Inc., La Jolla, CA) was utilized for statistical analysis. Results of this study were compared by ANOVA followed by the Dunnett's post hoc test. Significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Inhibitory Effect of APPE on the Vacuolating Activity of *H. pylori* VacA in HeLa Cells. VacA cytotoxin induces the formation of specific anion channels in the plasma membrane that are associated with the release of bicarbonate and organic ions, thus permitting adequate bacterial growth (*35*). In the current study, APPE inhibited the *H. pylori*-induced vacuolation of HeLa cells.

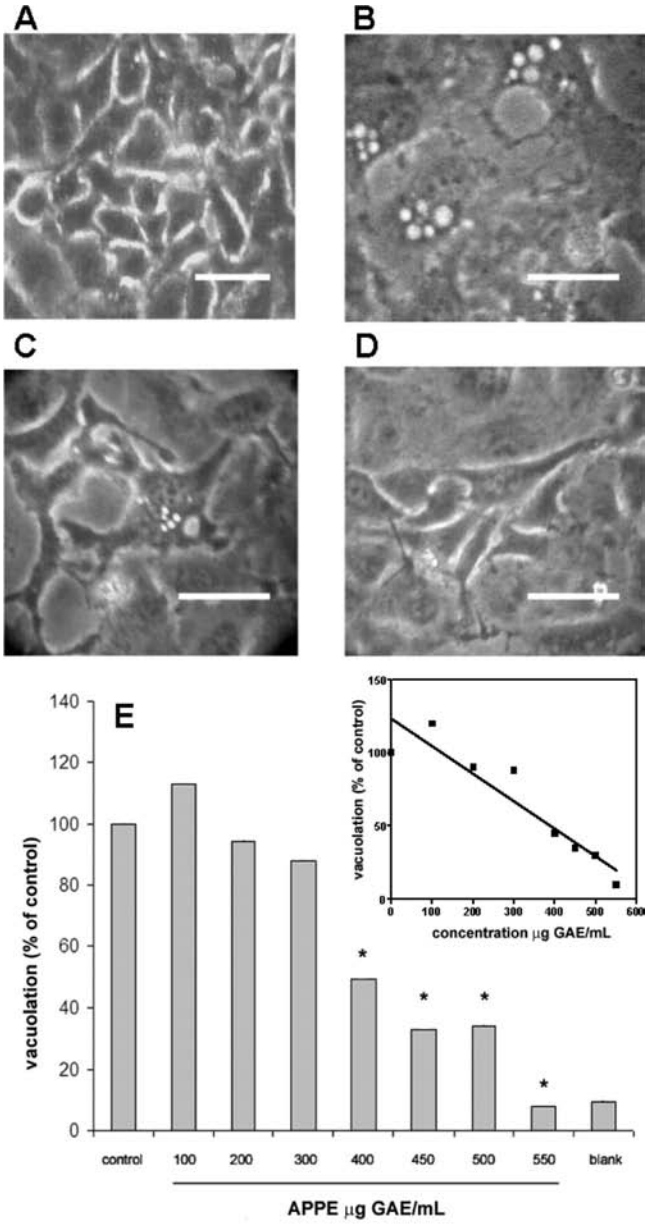

Figure 2. Inhibition of APPE peel extract on VacA toxin-induced vacuolation in HeLa cells. HeLa cells were cultured in MEM containing 5% FBS. *H. pylori* supernatants containing VacA toxin (500 μg/mL) were obtained as described under Material and Methods. Samples were incubated for 16 h at 37 °C, and vacuole formation was evaluated by means of light microscopy: HeLa cells treated (**A**) without *H. pylori* supernatants, (**B**) with *H. pylori* supernatants alone, (**C**) with *H. pylori* supernatants and 100 μg of GAE/mL of APPE, or (**D**) with *H. pylori* supernatants and 500 μg of GAE/mL of APPE (bar = 10 μm); (**E**) vacuolation index in HeLa cells treated with *H. pylori* supernatants and 100−550 μg of GAE/mL of APPE; (inset) linear graphic of vacuolation percentage versus APPE concentration. Data are representative of three different experiments performed in triplicate; ∗, $P < 0.05$ (versus control).

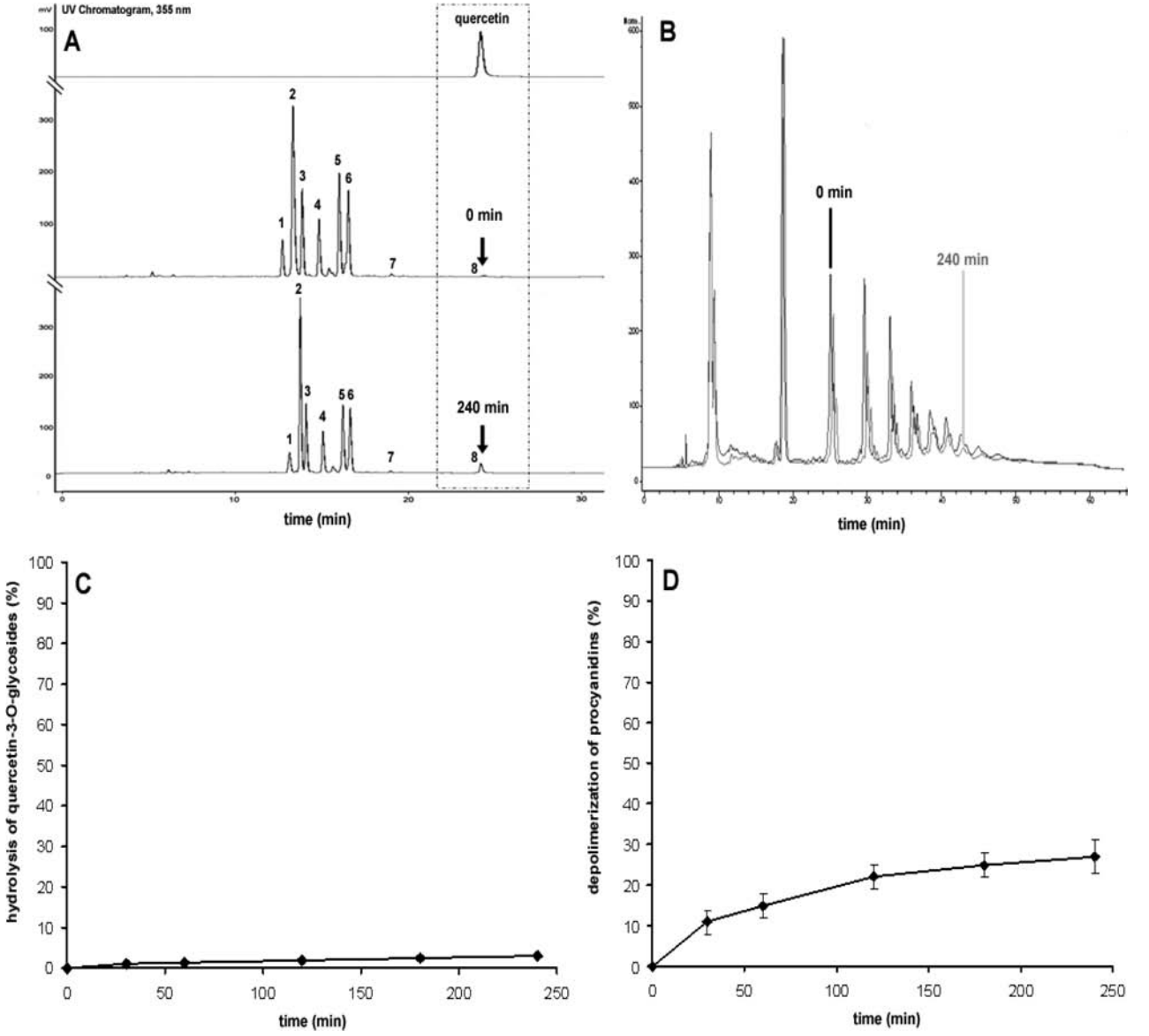

Figure 3. Stability of APPE polyphenols in acidic conditions: (**A**) Flavonoid glycosides analysis by reversed-phase HPLC of APPE before (middle) and after (bottom) 4 h of incubation in artificial gastric juice at 37 °C. The top trace corresponds to a standard of quercetin. The HPLC-DAD chromatograms were recorded at 355 nm showing the peaks of (1) rutin (quercetin-3-*O*-rutinoside), (2) hyperoside (quercetin-3-*O*-galactoside), (3) isoquercitrin (quercetin-3-*O*-glucoside), (4) quercetin-3-*O*-pentoside, (5), quercetin-3-*O*-pentoside, (6) quercitrin (quercetin-3-*O*-rhamnoside), (7) phloridzin (phloretin-2-*O*-glucoside, and (8) quercetin. (**B**) Flavan-3-ols and procyanidins by normal-phase HPLC of APPE before (black trace) and after (gray trace) 4 h of incubation in artificial gastric juice at 37 °C. The HPLC-FLD chromatograms were recorded with excitation at 230 nm and emission detection at 321 nm. (**C**, **D**) Degradation of quercetin glycosides and procyanidins from APPE in simulated acidic gastric environment.

As shown in **Figure 2A**−**D**, HeLa cells challenged with *H. pylori* supernatants (500 μg/mL) during 16 h in the presence of APPE presented a significantly lower vacuolation grade and showed no differences when compared with uninoculated Brucella broth treated controls. The vacuolating-inhibiting effect of APPE was concentration-dependent, with an IC_{50} = 390 μg of GAE/mL (**Figure 2E**). According to previous studies which showed that certain polyphenols can neutralize VacA (*36*), the vacuolating-inhibiting effect of APPE could involve the action of some of its polyphenols. Two potential mechanisms may be involved: The first one is due to a direct VacA−polyphenol interaction, which precludes the insertion of VacA into the eukaryotic membrane. This mechanism has been demonstrated for hop bracts and wine procyanidins, which bind to VacA, inhibiting its in vitro and in vivo effects (*15*, *16*). A second mechanism to be considered may involve the previously established ability of such compounds (and possibly other polyphenols) to interfere with the vacuolization caused by VacA toxin (*24*). APPE is particularly rich in quercetin glycosides (*17*). However, among all polyphenols, flavonoid glycosides seem to be weaker VacA inhibitors than procyanidins and hydrolyzable tannins. For instance, the IC_{50} needed to inhibit vacuolation in HeLa cells by rutin (3-*O*-rhamnoglucosyl quercetin) was > 400 μM (equivalent to 266 μg/mL), whereas for quercetin this value was 46 μM (16 μg/mL) (*24*).

On the other hand, an inhibitory effect of polyphenols against VacA-induced channel formation and urea flux in HeLa cells cultures has also been described (*33*). For instance, a concentration of 200 μM rutin (equivalent to 133 μg/mL) produced only a 15% urea flux inhibition, whereas for the channel formation an IC_{50} = 106 μM (70 μg/mL) was required. According to Shin et al. (*24*), the IC_{50} for quercetin is 19 μM, suggesting that glycosylation would have a negative

effect on the inhibitory activity of flavonoids against the functional effects of VacA.

In APPE, quercetin is almost undetectable by HPLC (**Figure 3A**). Therefore, in the present work we calculated an equivalent value of $IC_{50} = 28\ \mu M$ on a rutin basis. According with the above-mentioned, it is unlikely that this concentration could account for the observed inhibition of VacA effects. To predict the potential gastric hydrolysis of quercetin glycosides, we incubated APPE in a simulated gastric juice for 4 h. As shown in **Figure 3A**,**C**, after 4 h of incubation, stability monitoring of quercetin glycosides from APPE gave 3% of hydrolysis, rendering 0.20 μM free quercetin. In agreement with our results, Goh et al. (*21*) also reported low levels of hydrolysis in simulated gastric fluid for flavonol glycosides of *Gingko biloba*. Hence, one could hypothesize that the amount of quercetin released from APPE during such time could partially play an anti-VacA effect role in vivo. Therefore, compounds other than quercetin, such as procyanidins and chalcones, could be better candidates to explain both the in vitro and in vivo effects of APPE. With regard to the latter, the main chalcone identified in APPE was phloridzin (a phloretin glycoside), which at 390 μg of GAE/mL (IC_{50} of APPE) represents a concentration of 117 μM, considering that this compound corresponds to 11.5% of total polyphenols (*17*). Although this value represents only half of the IC_{50} reported as effective to inhibit vacuolation in HeLa cells (*36*), this compound could partially contribute to the APPE effect.

In contrast, procyanidins represents around 24% of the total polyphenol content in APPE (*17*); thereby, we presume that these compounds would play a role in the antivacuolating activity. Previously, Yahiro et al. (*15*) reported that these compounds bind efficiently to VacA, forming a complex that could not interact with both RPTPα and RPTPβ membrane receptors. In this study, several polyphenolic extracts were assayed. Among them, hop bract extract (HBE) was the most active. Indeed, 10 μg/well of HBE (final volume of 100 μL) was able to inhibit 90% of the vacuolation of HeLa cells. In the same study, 10 μg/well of an apple extract (final volume of 100 μL) produced only a 50% inhibition. As shown in **Figure 2C**, this same concentration of polyphenols (0.1 μg/μL) was reached in our experiments when we used 100 μg of GAE/mL of APPE. Conversely, in our experimental conditions 0.1 μg/μL of polyphenols failed to inhibit HeLa cell vacuolation. The reason for such discrepancy would originate in different experimental conditions and some aspects associated with the cellular model. Nevertheless, in a previous work we reported that the APPE used in the present study has a greater concentration of flavonol glycosides (58%) than B-type procyanidins (24%), which besides possess a low mean degree of polymerization (DPm = 3) (*17*). Such a structural aspect seems to be pivotal to explain the capability of procyanidins to neutralize VacA. Therefore, it seems that the natural origin of these substances deserves attention. In fact, in the Yahiro work, B-type procyanidins with higher molecular size were better VacA inhibitors (*15*). Thus, HBE high molecular weight procyanidins (DPm = 10−30) were 5-fold more effective than those present in the low molecular weight fractions. Besides, we found that after 4 h of incubation in simulated gastric juice, APPE procyanidins undergo a 27% depolymerization (**Figure 3B**,**D**). Thereby, the effect of pH upon APPE seems to be quite different for flavonol glycosides and procyanidins and might affect the in vivo APPE properties.

Effect of APPE on the Adhesion of *H. pylori* to HeLa Cells. Adhesion of *H. pylori* to its host epithelial cells, in addition to being a requisite first step in the colonization process, can also serve to induce the expression of other bacterial virulence factors. As shown in **Figure 4**, APPE was able to diminish adherent bacteria with an IC_{50} = 5.3 mg of GAE/mL. It is unlikely that

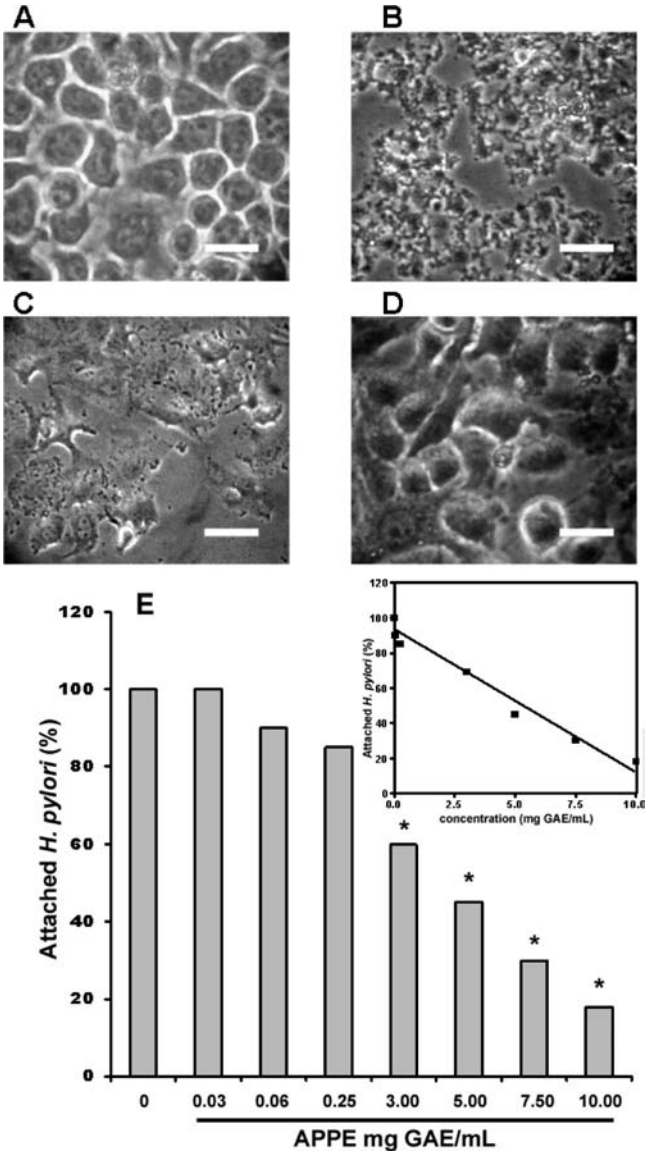

Figure 4. Apple peel extract inhibits *H. pylori* adherence to HeLa cells. The activity was determined by urea phenol red method: HeLa cells (**A**) without *H. pylori*, (**B**) with *H. pylori* alone, (**C**) with *H. pylori* and 5 mg of GAE/mL of APPE, or (**D**) with *H. pylori* and 10 mg of GAE/mL of APPE (bar = 10 μm). (**E**) Antiadhesive activities, after preincubation of APPE (0.03−10 mg of GAE/mL) and *H. pylori* followed by addition to HeLa cells. The positive control without inhibitor was used to establish 100% attachment. (Inset) Linear graphic of vacuolation percentage versus APPE concentration. Data are representative of three different experiments performed in triplicate; ∗, $P < 0.05$ (versus control).

such an effect would be linked to the anti-urease properties of APPE described previously by our group (*17*). As the urease inhibition by APPE is reversible and competitive, exhaustive washing of the cells should be enough to remove any trace of APPE. To confirm this latter hypothesis, we evaluated the unspecific adsorption of APPE to HeLa cell membranes and the penetration of some polyphenols into HeLa cells. After analysis of both pellets and lysates from HeLa cells, no traces of APPE constituents were detected by HPLC (data not shown). These results suggested that in the cell cultures, the remaining urease activity reflected only adhered bacteria. It has been reported that almost 50% of the VacA produced by *H. pylori* remains integrated to the plasmatic membrane of the bacterium; thereby, this VacA fraction could induce the formation of channels and vacuoles more efficiently

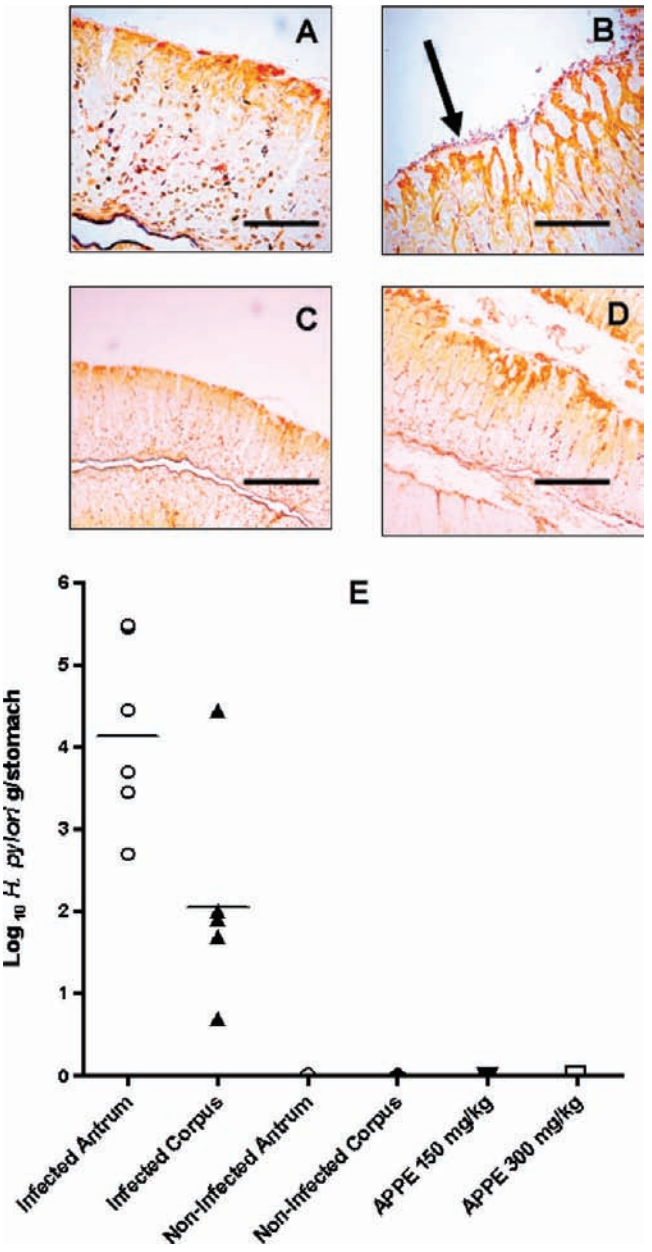

Figure 5. APPE precludes the *H. pylori* colonization of the C57BL6/J mice gastric mucosa: (**A**) Warthin−Starry staining of antral portion of an uninfected mouse; (**B**) antrum of *H. pylori* infected mice, 28 days postinoculation, showing bacteria near the epithelial surface (arrow); (**C**−**D**) antral portion of mice treated with 150 and 300 mg/kg/day of APPE, respectively (bar = 100 μm). (**E**) Quantitative real-time PCR detection of *H. pylori* in the antrum of mice treated with 150 and 300 mg/kg/day of APPE during 20 days.

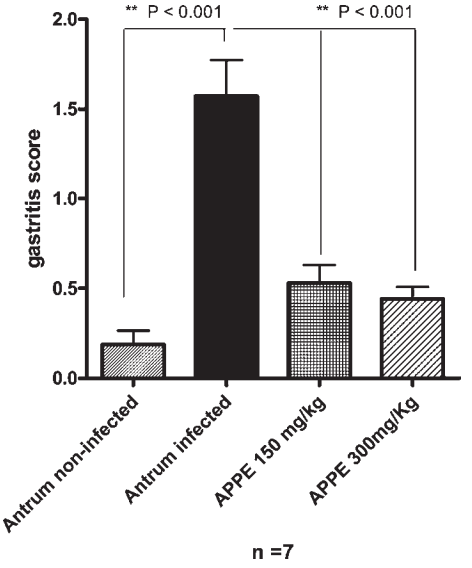

Figure 6. Gastritis score of *H. pylori* infected mice treated with APPE at 150 and 300 mg/kg/day.

than the fraction secreted to the extracellular medium (*37*). Importantly, the observed antiadherence property of APPE polyphenols precludes the direct interaction between *H. pylori* and the host mucosa, which also contributes to avoid the deleterious activity of VacA and CagA proteins.

Effect of APPE against *H. pylori* Attachment to Mice Gastric Mucosa. In agreement with previous studies (*38*, *39*) using rodent models of *H. pylori* infection our experimental short-term protocol also showed, by means of real-time PCR, that most bacteria were selectively located in the pyloric antrum. Additionally, the presence of *H. pylori* in both corpus and antrum was confirmed by culture (during 7 days). No signs of acute toxicity and body loss weight were observed in mice during the experimental period (28 days, data not shown). In **Figure 5A**−**D** is shown through Warthin−Starry (W−S) some *H. pylori* bacteria located in the pyloric antrum of noninfected (**A**), infected (**B**), and APPE treated mice (**C**, **D**). With this stain, *H. pylori* was observed as a brown curved bacillus, located near the surface of epithelial mucosa often associated with mucus and detritus (**B**). These results suggested that with the short-term APPE administration schedule most bacterial adherence in the surface of antral mucosa was inhibited. Real-time PCR analysis of the stomachs from animals treated with APPE (150 and 300 mg/kg/day) did not show significant differences with noninfected control mice (**E**). Additionally, after 7 days of culture it was not possible to recover bacteria with the typical *H. pylori* morphology either in the groups treated with APPE or in the controls. As is shown in **Figure 6**, gastritis scores suggest that APPE significantly protected the mucosa of *H. pylori* infected mice. However, the gross appearance of the stomachs did not reveal significant detrimental injury in both groups (data not shown). Also, histological examination of antral portions of the two APPE-treated groups did not show significant differences in infected animals (H&E stain, data not presented). These results led us to presume that the proposed short-term infection protocol could effectively reproduce the first inflammatory stages of the *H. pylori* colonization process. Also, in all groups of mice, neither leukocyte infiltration nor bacteria attached to the epithelial surface were observed in the corpus. To achieve high colonization levels and visible signs of gastric injury, probably long-term infection protocols would be necessary (*40*). These models involve the use of mouse-adapted *H. pylori* Sydney 1 strain (SS1), which colonizes the C57BL/6 mouse heavily and leads to the development of clinically evident levels of gastritis, closely mimicking human infection. However, strain SS1 is a mutant of *H. pylori*, and its virulence is considerably lower compared with ATCC 43504 strain (*41*).

We found that APPE was able to diminish the *H. pylori* viability and its ability to colonize the mice stomach. However, it is not possible to conclude that the expression of some *H. pylori* virulence genes in the samples from APPE-treated mice was solely due to APPE bactericidal activity. Such a result could be linked to the synergistic activity of various compounds present in APPE.

Despite the short-term host−pathogen interaction lapse and the low gastritis scores observed in the infected animals, MDA

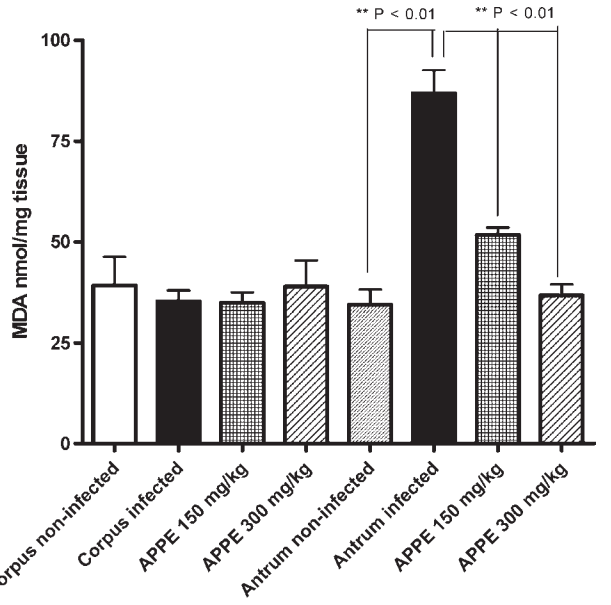

Figure 7. APPE inhibit MDA formation and inflammation of mouse stomach infected with *H. pylori*. MDA levels in antrum samples are from infected and noninfected mice.

levels (**Figure 7**) evaluated in the antrum samples of *H. pylori* inoculated animals were significantly higher than those found in noninfected mice (ANOVA, $p < 0.001$). In the early steps of *H. pylori* infection, increased MDA level is the result of oxidative changes occurring as a primary event, which contributes significantly to mucosal damage (*42*). Interestingly, in this phase APPE displayed a protective and statistically significant (**Figure 7**; $p = 0.001$) effect. As expected, no significant differences were observed in the MDA levels found in the corpus of both infected and noninfected mice. Previously, Graziani and co-workers (*32*) demonstrated the gastroprotective activity of an apple extract against the indirect damage induced by indomethacin in rats. Although active substances were not further characterized, the authors concluded that the observed effect might be associated with the antioxidant capacity of the polyphenols contained in the apple extract. Using Mongolian gerbils as the infection model, Takabayashi et al. demonstrated that a nondialyzable fraction of black tea was capable of diminishing the *H. pylori* colonization density and gastric inflammation (*43*). More recently, Brown et al. (*44*) demonstrated that some structural features of the polyphenols obtained from Muscadine grape skin and seeds were more relevant than their total content in these extracts. Akai et al. (*45*) found that green tea polyphenols inhibited the expression of the pro-inflammatory cytokine KC, which was coincident with a 50% lowering of *H. pylori* colonization ability. According to a recent study, using a combination of green tea catechins with sialic acid (a recognized antiadherent agent), the *H. pylori* colonization capability in BALB/c mice was significantly reduced (up to 60%), regardless of the antibiotic resistance of these isolates (*46*). In line with these studies, the *H. pylori* density was significantly decreased (89% in antrum) when pure quercetin was orally administered to guinea pigs (200 mg/kg/day, during 15 days) (*47*).

In the present work it was demonstrated that APPE could exert dual anti-*H. pylori* effects, inhibiting the process of adherence of the bacteria to the gastric mucosa and also the activity of VacA toxin. The anti-VacA effect was observed with APPE concentrations (IC_{50} = 390 μg of GAE/mL) much lower than those required for adhesion inhibition (IC_{50} = 5.3 mg of GAE/mL). Accordingly, the oral ingestion of two doses of APPE (150 and 300 mg/kg/day for 20 days) would be sufficient to avoid the initial attachment of *H. pylori* to the antral mucosa of C57BL6/J mice. As expected, the antioxidant and anti-VacA properties of APPE probably played a role in diminishing the in vivo inflammatory damage caused by *H. pylori* to the gastric mucosa of mice. As the isolated constituents of APPE were not evaluated in the current study, it is difficult to associate the in vitro and in vivo effects of APPE with a particular compound. In the same line, as nonphenolic constituents of APPE reach up to 40%, more investigation is needed to elucidate if some of these molecules could also interfere with the initial phase of *H. pylori* colonization.

In summary, from our study we observed that APPE can effectively prevent the initial steps in the *H. pylori* colonization process and suppress inflammation, two major outcomes of their pathological consequences. Further studies are needed to confirm if such a preventive role could be observed in humans.

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