JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 7743–7749 7743
DOI:10.1021/jf9006592

In Vitro Inhibitory Effect of Apple Peel Extract on the Growth of *Helicobacter pylori* and Respiratory Burst Induced on Human Neutrophils

Edgar Pastene,*,†,# Hernán Speisky,† Miriam Troncoso,‡ Julio Alarcón,§ and Guillermo Figueroa‡

†Laboratory of Antioxidants, Institute of Nutrition and Food Technology, Universidad de Chile, Av. Macul 5540, Santiago, P.O. Box 138-11, Chile, ‡Laboratory of Microbiology, Institute of Nutrition and Food Technology, Universidad de Chile, Av. Macul 5540, Santiago, P.O. Box 138-11, Chile, and §Laboratory of Synthesis and Natural Products, Department of Basic Sciences, Faculty of Sciences, University of Bío-Bío, Av. Andrés Bello s/n P.O. Box 447 Chillán, Chile. # Current address: Faculty of Pharmacy, Universidad de Concepción, Barrio Universitario s/n, P.O. Box 237, Concepción, Chile.

In the present work, the in vitro effect of a standardized extract of apple peel APPE (60% of total polyphenols; 58% of flavonoids; 30% of flavan-3-ols and procyanidins) was evaluated with regard to the viability of *Helicobacter pylori*. The cytotoxic effect of APPE on *H. pylori* was also evaluated through the resazurin assay and ATP level determination. In both assays, APPE showed an early cytotoxic effect, which was both concentration and time-dependent. Additionally, the effect of APPE on the intra and extracellular production of reactive oxygen species (ROS) was evaluated in human neutrophils stimulated by *H. pylori*, phorbol myristate acetate (PMA), and formyl-methionyl-leucyl-phenylalanine (fMLP). The extracellular and intracellular production of ROS was evaluated through chemiluminiscence with the isoluminol−horseradish peroxidase (HRP) and luminol−superoxide dismutase (SOD)−catalase systems, respectively. APPE showed an inhibiting effect on the multiplication of two *H. pylori* strains (ATCC 43504 and TX136) with a miminnum inhibitory concentration (MIC) value of 112.5 μg gallic acid equivalent (GAE)/mL. APPE inhibited the respiratory burst of neutrophils induced by *H. pylori*, PMA, and fMLP in concentration-dependent form. Interestingly, this effect was observed on both the interior and exterior of the neutrophil. This result suggests that apple peel polyphenols have an attenuating effect on the damage to gastric mucosa caused by neutrophil generated ROS and, particularly, when *H. pylori* displays its evasion mechanisms.

KEYWORDS: *Helicobacter pylori*; apple peel; *Malus domestica*; polyphenols; antioxidants; respiratory burst; free radicals; neutrophils

INTRODUCTION

Helicobacter pylori (*H. pylori*) infects the gastric mucosa of half of the world's population, and it is the only microorganism known to successfully inhabit the human stomach (*1*). Many studies have established *H. pylori* as an etiologic agent of gastric cancer, mucosa-associated lymphoid tissue lymphoma (MALT), and peptic ulcers (*2*). In Chile, *H. pylori* infection has a high prevalence (~80%), and the treatment requires the use of an antibiotic combination with a proton pump inhibitor (*3*). The high rates of resistance of *H. pylori* to antibiotics have led to an increased search for new treatment alternatives (*4*). Among these, a number of different products from the vegetable kingdom have been tested, including antimicrobials belonging to different phytochemical groups such as essential oils and polyphenols (*5*, *6*). Polyphenols are ubiquitous constituents of medicinal and nutritional plants. The main advantage of such compounds is their low toxicity with respect to other substances. Polyphenols are natural constituents with recognized antioxidative and antiinflammatory properties. These molecules are abundant in apples and are especially concentrated in the peel. In fact, the concentration of polyphenols in apple peel could be up to three times higher than that found in the pulp. The primary structural classes of whole apple polyphenols include flavonoids, glycosides, phenol carboxylic acid esters, dihydrochalcones, catechins, and procyanidins (*7*−*9*). Growing evidence demonstrates the gastroprotective effects of apple polyphenols on chemically induced injuries (*10*, *11*). Apple peel polyphenols may also contribute to the amelioration of chronic gastro-intestinal infections induced by *H. pylori*. One of the hallmarks of *H. pylori* infection is the high grade of lymphocytic infiltration (*12*). Neutrophils and mononuclear inflammatory cells infiltrate the *H. pylori*-infected stomach mucosa causing inflammation and production of reactive species of oxygen (ROS). However, such respiratory bursts can be evaded because *H. pylori* can turn the production of ROS toward the host, with resulting damage to healthy tissues (*13*). In the present work, a standardized apple peel extract (APPE) is assessed for its anti-*H. pylori* properties. Additionally, the effect of APPE upon the respiratory burst induced by nonopsonized *H. pylori* on human neutrophils is described.

*To whom correspondence should be addressed. Tel: +56-412204523. E-mail: edgar.pastene@gmail.com.

Published on Web 08/19/2009

MATERIALS AND METHODS

Standards, Chemicals, and Solvents. Gallic acid, tannic acid ($C_{76}H_{52}O_{46}$), quercetin 3-*O*-rhamnoglucoside (rutin), and Folin−Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other solvents were of HPLC grade and were purchased from Merck (Darmstadt, Germany).

***Helicobacter pylori* Strains.** *Helicobacter pylori* (ATCC 43504) was kindly provided by Professor Apolinaria García (Universidad de Concepción, Chile). *Helicobacter pylori* (strain TX136) was a clinically isolated culture from a gastric biopsy specimen (Laboratory of Microbiology and Probiotics, INTA, Universidad de Chile).

Preparation of Apple Peel Extract (APPE). Apple peels (82 ± 8% humidity) from ripe fruits of the Granny Smith variety (*Malus domestica* cv. Granny Smith) were kindly provided by SURFRUT Ltd. (Santiago, Chile). Preparation of apple peel extract (APPE) was carried out using a styrene−divinylbenzene copolymerisate adsorption resin according to previously published work (*14*−*17*). Despite the fact that similar results could be achieved with different polymeric resins, in the present work Sepabeads SP-850 (Supelco, Bellefonte, USA) was chosen. Because of its adsorption surface (∼1000 m^2/g), it was possible to achieve better extract yields with this resin. Concentration of polyphenols from apple peels was carried out as described elsewhere (*17*). Dried apple peel polyphenol-rich extract (APPE) was obtained after concentrating through evaporative rotation (< 40 °C) and drying under vacuum in a desiccator (3.50 ± 0.7 g was obtained from 1000 g of fresh apple peel). APPE was stored at − 70 °C until use. Total phenolic content in APPE was carried out using Folin−Ciocalteau reagent and gallic acid as standard for calibration (gallic acid equivalent, GAE). Additionally, low molecular weight (LMW) and high molecular weight (HMW) fractions were prepared from APPE according with the procedure described elsewhere (*17*).

HPLC-DAD/ESI-MS. Quantitative analysis of APPE flavonols was done by high performance liquid chromatography with diode array detection (HPLC-DAD) according to previously published methodology and using rutin as standard for calibration (*17*). For liquid chromatography−mass spectrometry (LC-MS) analysis, an Agilent 1100 (Palo Alto, CA, USA) system equipped with a binary pump, an online degasser, an autosampler, and an UV−vis detector was used. UV detection was recorded at 280 nm. Separation of phenolic compounds was performed on a Supelcosil C18 column (300 × 4.0 mm id.; particle size, 5 μm) equipped with a guard column C18 20 × 4.0 mm. Injection loop was 20 μL, and the flow-rate was 1.0 mL/min. The solvent system was composed of solvent A (double distilled water containing 0.1% formic acid, v/v) and solvent B (acetonitrile containing 0.1% formic acid). The following gradient system was used: 0−25 min, 10−30% B; 25−30 min, 30−75% B; 30−35 min; 75−10%. LC/MS detection was performed directly after UV−vis measurements. Analyses were performed using a Bruker Esquire 4000 (Bruker Daltonics, Bremen, Germany) ion trap mass spectrometer ESI-IT, using the following conditions: nebulizer pressure, 30 psi; nitrogen flow rate, 10 L/min with a temperature of 325 °C; capillary voltage, −3 kV; and cone voltage, −30 V. The ESI was operated in negative mode scanning from *m*/*z* 50 to 1500. Collision-induced dissociation (CID) was performed by applying 30% of energy level. The software package used for the electrospray ionization−mass spectrometry (ESI-MS) control was esquire Control 5.2 (Bruker Daltonics, Breme, Germany). The instrument parameters were optimized using rutin prior to analysis of apple peel extract.

Screening Disk Diffusion Test. The disk diffusion test was used in the primary screening for the susceptibility of *Helicobacter pylori* ATCC 43504 and TX136 strains to APPE. Bacterial suspensions, adjusted to yield approximately 1.0 × 10^9 CFU/mL, were disseminated onto agar plates containing 7% of defibrinated horse blood. Filter paper discs (6 mm diameter) were placed on the inoculated agar surfaces and impregnated with 40 μL of stock solutions of APPE. The plates were incubated during 5−7 days at 37 °C under microaerophilic conditions (15% CO_2, 5% O_2, and 80% N_2). Disks containing amoxicillin (25 μg) and erythromycin (15 μg) (SDA, Santiago, Chile) were used as controls. All tests were performed in triplicate, and the antibacterial activity observed was expressed as the mean of inhibition diameters (mm) produced by APPE.

Quantitative Susceptibility Test: Minimal Inhibitory Concentration. The minimal inhibitory concentration (MIC) of APPE against *H. pylori* ATCC 43504 and TX136 was determined using a broth dilution method. A bacterial inoculum, standardized at ∼10^7 CFU/mL and APPE, prepared as a stock solution in sterile PBS 1× at a concentration of 10 mg GAE/mL and PBS 1×, was added in appropriate concentrations to Brucella broth supplemented with 0.1% cyclodextrin. The cultures were incubated for 72 h before 10 μL aliquots were seeded on blood agar plates. The MIC was defined as the lowest concentration of APPE-inhibiting growth when the broth cultures were read after 72 h of incubation under microaerophilic conditions at 37 °C. The minimal bactericidal concentration (MBC) was defined as the minimal concentration of APPE required to kill 99.9% of the organisms in the medium after 72 h of incubation.

Resazurin-Based Microtiter Assay. Standardized innocula were prepared from early to midlog-phase cultures of 10^8 CFU/mL in the appropriate media. Bacteria were incubated for 3−4 days in microaerophilic conditions (15% CO_2, 5% O_2 and 80% N_2) in agar plates containing 7% horse blood and 0.1% β-cyclodextrin. To evaluate the early effect of APPE upon *H. pylori* viability, the resazurin assay was used. Resazurin is an oxidation−reduction indicator used for the evaluation of cell growth. It is a blue nonfluorescent and nontoxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable bacteria. In the assay, a modification to the recently published protocols was used (*18*). Briefly, in white 96-well sterile plates serial dilutions of APPE extract were undertaken beginning with a 10 mg/mL GAE/mL stock solution. Plates were then dried under vacuum. *H. pylori* inoculum was adjusted with Brucella broth to 0.8 × 10^6 CFU/mL, and 100 μL was added to each well. Plates were incubated with moderate agitation under conditions of microaerophilia at 37 °C. To evaluate the early effect of APPE upon *H. pylori*, plates were removed at 15, 30, and 120 min. Ten microliters of 0.01% resazurin was added to each well, and then the wells were quickly sealed (< 5 min) with 50 μL of heavy mineral oil to minimize the effect of oxygen during the reading time. Plates were incubated at 37 °C inside the HT Synergy apparatus during 120 min, continuously recording the fluorescence emission (530 nm excitation and 590 nm emission). The grade of cytotoxicity of APPE was calculated as a percent of the control, which was prepared without APPE. Autofluorescence of the samples or controls also was subtracted.

Measurement of Intracellular ATP Levels in *H. pylori*. As an equivalent of antibacterial energy loads, intracellular ATP was measured according to Schweinitzer and co-workers (*19*) with minor modifications. Bacterial cells were taken directly from colonies obtained during the exponential growth phase and were gently resuspended in Brucella broth without prior washing. The bacterial cells were then incubated with different concentrations of APPE for 1 h at 37 °C before starting the ATP assays. For determining the ATP content of cells, BacTiter-Glo reagent (an ATP-dependent luciferase−luciferin reagent mixture; Promega Inc., Madison, WI) was added directly to the live bacterial suspensions at a ratio of 1:1 after the desired incubation times. The suspensions were incubated for 5 min at 37 °C to lyse the bacteria and to initiate the enzymatic reaction. The emission of photons was measured with a Synergy HT multilector (BioTek Instruments, Vermont, USA) in luminescence mode. All experiments were performed at least three times on separate days in quadruplicate measurements.

Isolation of Human Peripheral Neutrophils. Neutrophils were isolated from heparinized blood obtained from healthy adult donors. Polymorphonuclear netrophils (PMNs) were isolated using a double gradient of Histopaque-1119 and Histopaque-1077 according to the Sigma procedure (Sigma). The neutrophils were washed twice and resuspended (10^5 cells/mL) in a Hank's Balanced Salt Solution (HBSS) buffer containing 10 mM glucose, 1 mM Ca^{2+}, and 1.5 mM Mg^{2+} (pH 7.3). They were kept on ice until use. The final concentration of purified cells was determined by counting with a hemocytometer. The cell viability was confirmed in every cell preparation by using the trypan blue exclusion assay. Values higher than 99% of purified cells containing > 95% neutrophils were considered acceptable. Samples were drawn and used in the experiments within 5 h of preparation.

APPE Effect on the Neutrophil Respiratory Burst Induced by Nonopsonized-*H. pylori*, PMA, and fMLP. The luminol-enhanced chemiluminiscence of human neutrophils was measured using microtiter plate-reader Synergy HT (Biotek Instruments, Vermont, USA), as described previously (*20*, *21*). Luminol (5-amino-2,3-dihidro-1,4-ftalazinedione) and isoluminol (6-amino-2,3-dihidro-1,4-ftalazinedione)

Table 1. Effect of APPE against Two *H. pylori* Strains

	H. pylori ATCC 43504	*H. pylori* TX136	*H. pylori* ATCC 43504	*H. pylori* TX136
simple	halo[a](mm)	halo[a](mm)	MIC_{90} (μg/mL)	MIC_{90} (μg/mL)
APPE	11	12	112.5[b]	112.5[b]
LMW	15	18	89.5[b]	89.5[b]
HMW	R[c]	R	>1500[b]	>1500[b]
tannic acid	R	R	900.0	900.0
amoxicilin	54	58	0.06	0.02
erythromycin	40	45	0.15	0.20

[a] Halo of inhibition was measured using 6 mm discs impregnated with 600 μg of GAE of APPE, LMW, and HMW; 600 μg of tannic acid; 25 μg of amoxicillin, and 15 μg of erythromycin. [b] μg GAE/mL of APPE. [c] R = resistant.

were used as permeable and impermeable probes, respectively, and were purchased from Sigma-Aldrich (St. Louis, USA). Luminol and isoluminol were dissolved (18 mg) in 100 mL of PBS 1× (pH 7.4). An aliquot of 100 μL of neutrophil suspension (1×10^5 cells per well) was mixed with 10 μL of a stock solution of luminol or isoluminol. To evaluate the intracellular ROS production, a mixture of 50 U/mL of superoxide dismutase (SOD, Sigma) and 2000 U/mL of catalase (CAT, Sigma) was used. To evaluate the extracellular production of ROS, the isoluminol chemiluminiscence was amplified through the addition of 4 U/mL of horseradish peroxidase (HRP, Calbiochem). Chemiluminiscence was recorded during 10 min at 37 °C prior to the addition of activators. Chemiluminiscence was measured during 15 to 60 min according to the stimulus used. To assess the APPE effect, different concentrations were preincubated with neutrophils prior to the activation with PMA (200 nM), fMLP (1 μM), and nonopsonized *H. pylori* (MOI 25:1, or $2{,}5 \times 10^6$ UFC/mL) in accordance with Allen et al. (*12*). The area under the peak signal of chemiluminiscence was used to calculate the ROS inhibition percentage relative to samples without APPE. All of the assays were run in triplicate.

RESULTS AND DISCUSSION

Certain polyphenols exhibit anti-*H. pylori* effects interacting with multiple molecular targets. Thus, polyphenols may act as neutralizing agents for *H. pylori* proteins such as VacA and the enzyme urease (*17*, *22*, *23*). However, the effect of such compounds on the *H. pylori* viability remains unclear. According to Funatogawa and co-workers (*24*), the membrane damage is one of the potential mechanisms by which polyphenols could display a bactericidal effect against *H. pylori*. However, such a mechanism only has been demonstrated for hydrolyzable tannins with ranges of MIC values between 12.5 and 50 μg/mL. In the same study for pure procyanidins and flavonoids, MIC_{90} values were always around 100 μg/mL. In the present work APPE shows a MIC value of 112.5 μg GAE/mL for two *H. pylori* strains (**Table 1**). This value was coincident with the minimal bactericidal concentration (MBC). However, MIC for APPE was lower than those displayed for the antibiotics amoxicillin and erythromycin. Along the same line, previous studies suggest that the anti-*H. pylori* effect of flavan-3-ols such as catechin and epicatechin is modest. For instance, MIC_{90} for epicatechin was around 800−1600 μg/mL, whereas for epigallocatechin gallate (EGCG), this value was 83−167 μg/mL (*25*). Moreover, in our study, discs impregnated with 600 μg of tannic acid did not produce an inhibition halo for both *H. pylori* strains assayed. In a previous study (*17*), we demonstrate that APPE possesses primarily quercetin glycosides (58%) and, to lesser extent, epicatechin and procyanidins (30%). Interestingly, as is shown in **Table 1**, a low molecular weight fraction (LMW; MIC = 89.5 μg/mL) was more effective against *H. pylori* than a polymeric fraction (HMW; MIC > 1500 μg/mL). This finding in conjunction with literature reports suggests that quercetin glycosides, at least in part, could be playing a role against *H. pylori*. Because the anti-*H.pylori* activity of APPE seems to be linked to the presence of flavonol, it was identified by means of HPLC-ESI/MS/MS and comparison with standards (**Figure 1a**,**b**). The latter allow us to confirm the identity of the main quercetin glycosides of APPE as rutin (peak **6**), hyperoside (peak **7**), isoquercitrin (peak **8**), and quercitrin (peak **11**). Two quercetin-*O*-pentosides (peaks **9** and **10**) only could be tentatively assigned according to their elution times previously reported in the literature (*26*, *27*). According to these studies, these compounds could be assigned as quercetin-*O*-xyloside (reynutrin) and quercetin-3-*O*-arabinoside (avicularin). Nonetheless, in our study confirmation with standards was not possible. Along with these compounds, other low molecular weight polyphenols such as epicatechin, phloridzin, and chlorogenic acid (**Figure 1b**; insert) were concentrated in the LMW fraction. In **Table 2** is shown a list of the main phenolic constituents identified in APPE by means of LC-UV-MS/MS (negative mode). All of the identified molecules have been previously reported in apples (*9*, *27*, *28*).

From the results showed in **Table 3**, it can be inferred that an increased period of *H. pylori*-APPE contact produces a higher bacterial toxicity which is reflected by the diminution of the IC_{50} values (63.6 μg GAE/mL at 15 min versus 32.8 μg GAE/mL at 2 h). Such time dependence would be associated with the polarity of APPE constituents, which is determinant of its cell membrane permeability. The majority of the APPE constituents have low solubility in lipids. Therefore, a critical issue is the time of contact among polyphenols and the bacterium; longer times of contact could favor the entrance of certain polyphenols such as chalcones and quercetin glycosides. Indeed, many studies conclude that modifications tending to improve the lipid solubility of flavonoids increase its bactericidal effects (*29*,*30*). In the current study, the early effect exerted by APPE on *H. pylori* viability was also verified by thorough analysis of the ATP levels after 1 h of incubation. As is observed in **Figure 2**, APPE causes a depletion of *H. pylori* ATP levels in a concentration-dependent manner.

Although the anti-*H. pylori* effect of flavonoids is considered moderate, these molecules have recently been used as a template for the development of new, more powerful derivatives. For instance, a resulting synthetic derivative of the antibiotic−antioxidant combination (metronidazol−genistein) showed a very powerful anti-*H. pylori* activity with a MIC_{90} = 0.39 μg/mL (*31*).

Table 4 shows the IC_{50} values of APPE upon the induction of intracellular and extracellular ROS production using three activators (*H. pylori*, fmLP, and PMA). PMA is a direct activator of protein kinases (PKC), which does not require binding to a receptor. PKC activation caused calcium mobilization and reduced nicotinamide adenine dinucleatide phosphate (NADPH) oxidase assemblage. The NADPH oxidase complex first generates ROS within the neutrophil (phagosome) and then a flux to the outside (*32*). Since in our experiment the ROS occurring extracellularly was deliberately suppressed by SOD/CAT addition, the observed diminution of luminescence could be attributed to intracellular changes (e.g., a greater superoxide radical-scavenging capacity) induced by the exposure of neutrophils to APPE. While the concentrations of APPE used in the activated neutrophil experiments did not compromise the viability of such cells, some of the polyphenols present in APPE (*17*) have been previously shown to be effective as scavengers of superoxide (*33*) and peroxyl radicals (*9*). Polyphenols or their metabolites generated during the respiratory burst (oxidation and/or hydrolysis products) not only would act as free radical scavengers but also may inhibit some enzymes such as PKC or MPO. Nevertheless, it is not known whether or not such molecules (metabolites) are capable of entering the neutrophil during the inflammatory process.

When examining the effect of APPE upon extracellular production of ROS with an isoluminol−HRP system, a concentration-dependent effect was observed with an IC_{50} value of 1.1 μg/mL

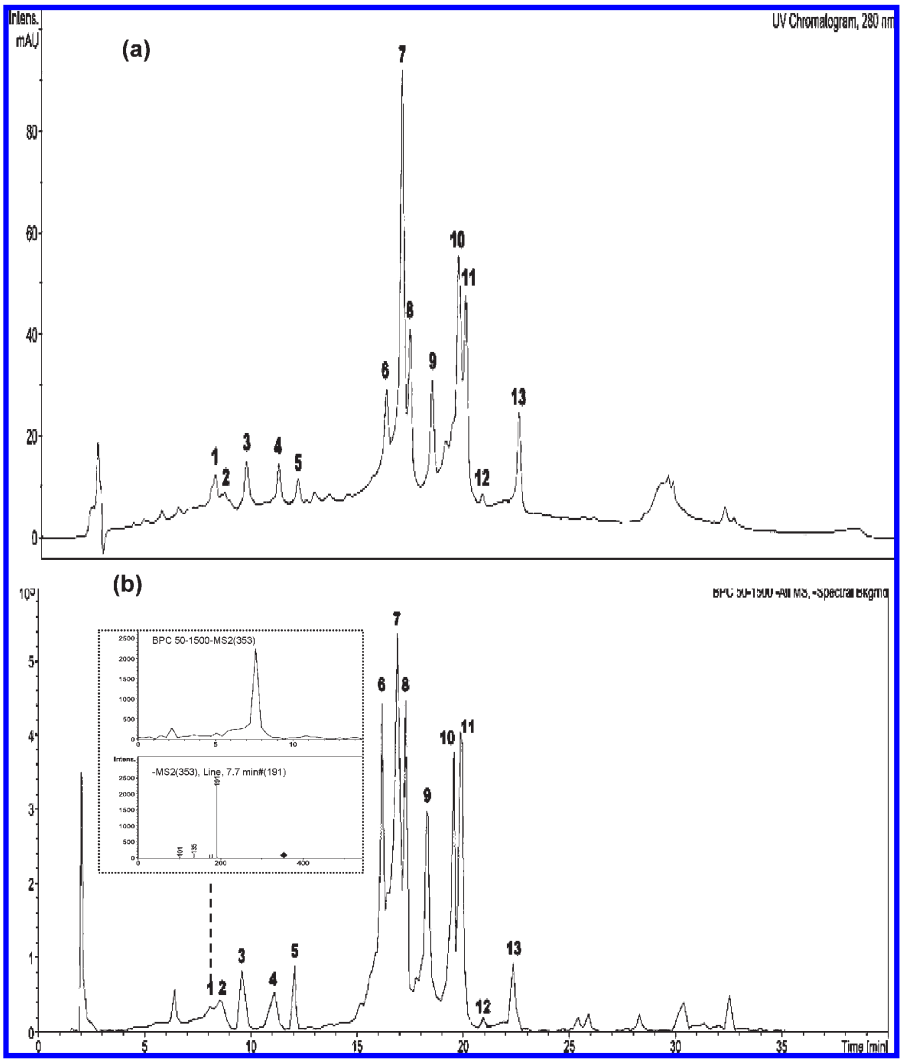

Figure 1. Reverse-phase HPLC-UV and ESI/MS profiles of APPE: (**a**) HPLC profiles of APPE recorded at 280 nm showing the peaks of (**1**) chlorogenic acid (**2**) procyanidin B1, (**3**) procyanidin B2, (**4**) (−)-epicatechin, (**5**) procyanidin C1, (**6**) rutin (quercetin-3-*O*-rutinoside), (**7**) hyperoside (quercetin-3-*O*-galactoside), (**8**) isoquercitrin (quercetin-3-*O*-glucoside), (**9**) quercetin-3-*O*-pentoside, (**10**) quercetin-3-*O*-pentoside, (**11**) quercitrin (quercetin-3-*O*-rhamnoside), (**12**) phloretin-2-*O*-xyloglucoside, and (**13**) phloridzin (phloretin-2-*O*-glucoside). (**b**) Total ion chromatogram (TIC) in full scale mode (in the negative ion mode). Insert: multiple reaction monitoring (MRM) of peak (**1**) (precursor ion, *m*/*z* 353).

Table 2. Data Obtained from the Analysis of APPE by HPLC-ESI/MS (Negative Mode)

compound	peak (n°)	t_R (min)	HPLC-ESIMS (*m*/*z*)
chlorogenic acid	1	7.7	353, 191, 135, 101
procyanidin B1	2	8.5	577, 425, 289
procyanidin B2	3	9.4	577, 425, 407, 451, 289
(−)-epicatechin	4	10.9	289, 245, 205, 137, 161
procyanidin C1	5	11.8	865, 695, 577, 713, 287
quercetin-3-*O*-rutinoside	6	15.9	609, 301, 343, 255, 179
quercetin-3-*O*-galactoside	7	16.7	463, 301, 179, 151, 343
quercetin-3-*O*-glucoside	8	17.1	463, 301, 179, 151
quercetin-3-*O*-pentoside	9	18.1	433, 301, 151, 179
quercetin-3-*O*-pentoside	10	19.3	433, 301, 151, 179
quercetin-3-*O*-rhamnoside	11	19.7	447, 301, 179, 151, 271
phloretin-*O*-xyloglucoside	12	20.5	567, 435, 273
phloretin-2-*O*-glucoside	13	22.1	435, 273, 167, 301

(**Table 4**). This value for APPE is approximately 30 times lower than that found inside the neutrophil. In human neutrophils, PMA mainly stimulates the intracellular production of ROS; therefore, oxidative challenge for polyphenols is lower on the outside of the neutrophil than inside it (*34*).

Table 3. APPE Effect upon *H. pylori* Viability Determined by Resazurin-Based Microtiter Assay

time (min)	IC_{50} (μg GAE/mL[a] (μg APPE/mL)[b]
15	63.6 (106.0)
30	50.8 (84.7)
120	32.8 (54.6)

[a] Total polyphenolic contents were determined by the Folin−Ciocalteau assay. [b] Dry weight APPE extract.

Since fMLP is an analogue of bacterial peptides, in neutrophils it could act as a chemoattractant and inducer of the respiratory burst. Therefore, chemiluminiscence generation mediated by fMLP could be a suitable model for the study of ROS generation during an infectious process. In human neutrophils, fMLP mainly stimulates the production of ROS at the intracellular level by means of its interaction with a G-protein-coupled receptor (FPRs) (*35*). In fact, the maximum luminescence signal was less than a half of that observed at the intracellular level (1200 RFU versus 3000 RFU). However, although marginal, the extracellular production of ROS stimulated by fMLP could be amplified by

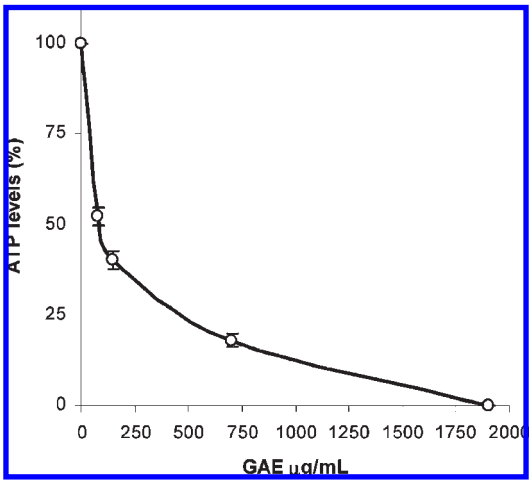

Figure 2. APPE effect upon the *H. pylori* ATP levels. CFU/mL was incubated in the presence of APPE (0−1800 μg GAE/mL), under microaerophilic conditions during 1 h. ATP levels were analyzed by chemiluminiscence as described in Materials and Methods section. Data are the average ± SD of four experiments performed in triplicate.

Table 4. Antioxidant Effect of APPE on Neutrophils Activated by *H. Pylori* (ATCC 43504), fMLP, and PMA

stimulus	luminol-SOD/CAT IC_{50} (μg/mL)[a] (μmol/L)[b]	isoluminol-HRP IC_{50} (μg/mL)[a] (μmol/L)[b]
H. pylori	31.4 (29.5)	7.1 (6.7)
fmLP	Nd[c]	0.064 (0.061)
PMA	30.0 (26.9)	1.1 (1.0)

[a] Total polyphenolic content calculate as gallic acid equivalents (μg GAE/mL), determined by Folin−Ciocalteau methodology. [b] Total flavonoids expressed as rutin equivalents (μmol RutE/L), analyzed by HPLC-DAD. [c] Nd, not detected.

HRP. **Table 4** shows that APPE is able to inhibit the extracellular production of ROS with an IC_{50} of 0.064 μg/mL. This low IC_{50} value can imply a direct influence on fMLP binding to its receptor, altering some of the signaling events that promote NADPH oxidase assemblage (e.g., activation of PKC). The cellular model used for assessing the inhibitory effect of 34 polyphenols on the ROS production was reported earlier by Limasset and co-workers (*36*). The authors concluded that, depending on the type of stimulus for the production of ROS, the inhibition shown by certain polyphenols could be opposed, which is indicative of different action mechanisms for these substances. In another work, Krol and co-workers (*37*) studied 14 flavonoids, confirming that the hydroxyl group on C-3, two hydroxyls in the B ring, and the presence of a double bound between C-2 and C-3 are of vital importance for the inhibition of ROS production in neutrophils. The authors also suggested that liposolubility would be one of the preponderant properties for the antioxidant activity of flavonoids. Later, Limasset and collaborators (*38*) evaluated a group of 18 polyphenols constituted by flavonoids and some of their putative metabolites such as some phenolic acids. These results showed an inhibiting activity on ROS production (induced by PMA and fMLP) less powerful and specific than the parental flavonoids. Recently, Moreira and collaborators (*39*) studied the effect of four flavonoids (myricetin, quercetin, kaempferol, and galangin) on ROS production by rabbit neutrophils, stimulated by two complement receptors (FcγR and CR). Interestingly, the inhibitory activity of flavonoids on ROS generation by neutrophils is directly linked to the flavonoid liposolubility. Moreover, in several of the mentioned studies, similar results were also found for some flavonoids identified by us in APPE (*40*).

Certain *H. pylori* water-soluble proteins are able to prime neutrophils for enhanced production of ROS and chemokines when PMA and fMLP are used (*20*,*41*). Satin and colleagues (*42*) reported that protein HP-NAP is one of the factors that stimulate NADPH oxidase in neutrophils, although the response pattern of luminol-dependent chemiluminescence is delayed. Specifically, the authors observed that the ROS production reached a maximum between 40 and 60 min after incubation. Allen et al. (*13*) have reported that *H. pylori* can evade the bactericidal effect of the ROS when neutrophils are continuously stimulated, causing damage to the host gastric mucosa. The latter would be explained by the fact that *H. pylori* cause an anomalous assembly of the functional NADPH oxidase complex in the cellular membrane and not in the phagosome. Specifically, while flavocytochrome *b*558 can be acquired by phagosomes, the $p47^{phox}$ subunits or $p67^{phox}$ cannot be recruited efficiently. The factors responsible of this type of evasion have not been identified. The evidence suggests that these molecules are sensible to the heat, resistant to the treatment with formalin, and located in the surface of *H. pylori.*

In our study, the ATCC 43504 strain, which has some of the classical neutrophil activating factors such as LPS, urease, HP-NAP, and HP(2−20), was employed. Nevertheless, it was established that these latter factors are not required to stimulate the neutrophil respiratory burst. Other authors suggest that the *H. pylori* adhesin SabA could play a preponderant role as a neutrophil activating factor (*43*). **Figure 3a** (left),**b** shows the concentration-dependent inhibiting effect of APPE upon intracellular production of ROS in neutrophils stimulated with nonopsonized *H. pylori.* The IC_{50} value of 31.4 of μg/mL for APPE was similar to that found when PMA was used as the inductor. In the isoluminol−HRP system, the ROS production is directly related to the NADPH oxidase activity and therefore to the amount of superoxide generated. As previously indicated, because of the disruption of NADPH oxidase targeting, extracellular ROS production induced by *H. pylori* would be responsible for the gastric mucosa damage. In the present work, APPE concentration-dependently inhibited the extracellular production of ROS (**Figure 3a** and **c**) in *H. pylori*-stimulated neutrophils, with an IC_{50} value of 7.1 μg/mL. In absence of stimulus, APPE did not induce production of ROS in neutrophils (data not presented). APPE has an important content of quercetin glycosides (58% of the total polyphenolic content). Rutin, hyperosid, isoquercitrin, and quercitrin are the main flavonoids in APPE (**Table 1**). As indicated above, the presence of phenolic groups in C-5, C-7, C-3′, and C-4′ in quercetin glycosides favors antioxidant activity. However, in spite of the existing evidence it has not been possible to date to establish how different sugars attached to C-3 determine the degree of inhibition of ROS production by neutrophils. As shown in **Table 4**, IC_{50} values obtained with the three activators are similar to those published previously in terms of rutin equivalent (RutE μmol/L), where the pure polyphenols were assayed (*44*, *45*). Parallel to its superoxide scavenging capacity, some flavonoids such as quercetin and luteolin inhibit, in concentration-dependent form, the protein phosphorylation that follows the stimulation of neutrophils with fMLP (*46*). Nonetheless, fMLP is habitually used to activate the neutrophil response in the same manner as bacteria. It must be noted that in *H. pylori*−neutrophil interaction, fMLP type 1 receptors (FPLR1) do not act alone (*34*).

In conclusion, in the present work we demonstrate that flavonoids present in APPE possess an anti-*H. pylori* activity. Additionally, this effect is linked to an inhibition of extracellular ROS production induced by nonopsonized *H. pylori.* The results suggest that APPE exerts a protective effect, inhibiting the

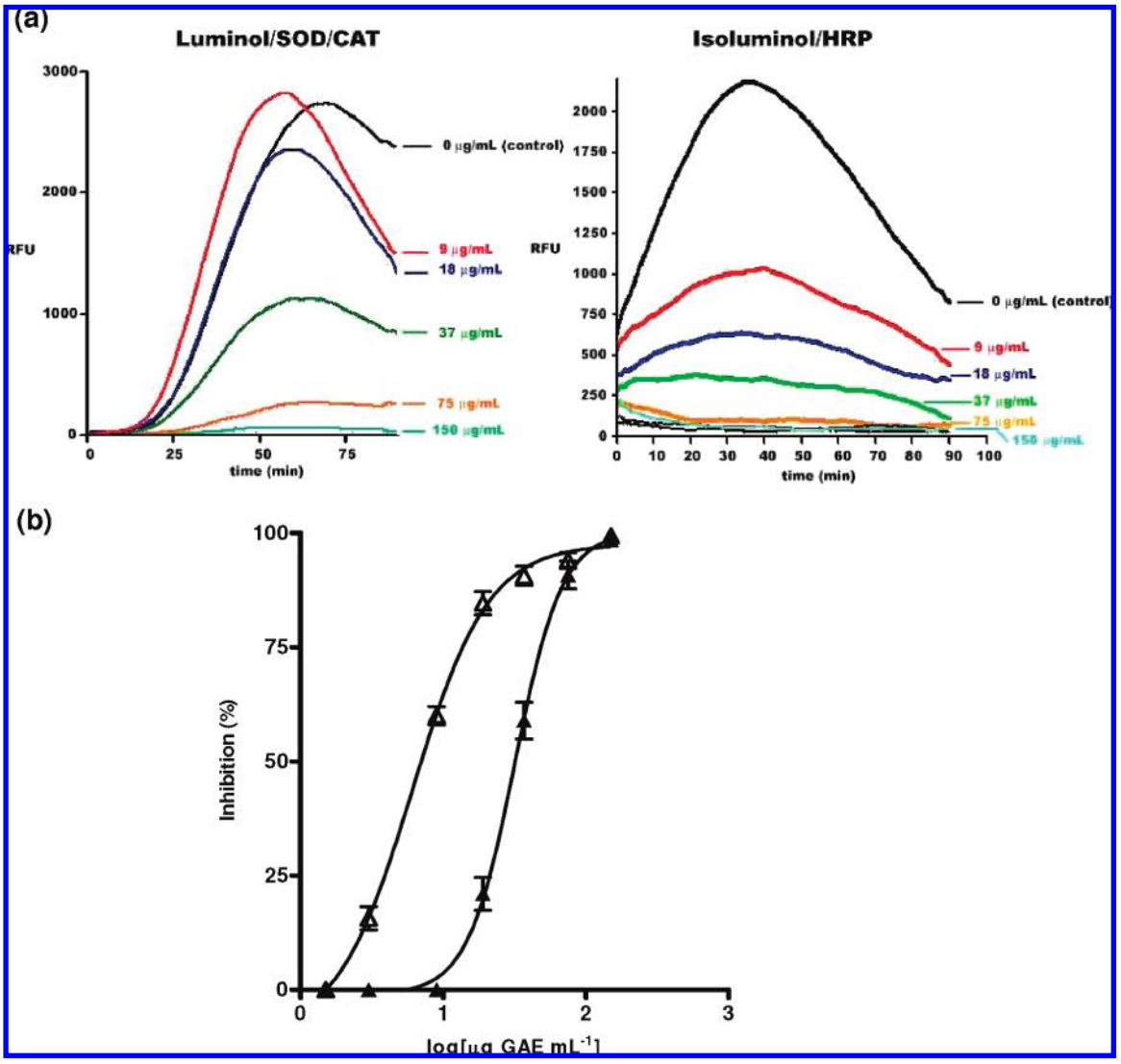

Figure 3. Influence of APPE on nonopsonized *Helicobacter pylori*-induced chemiluminescence of human neutrophils. (**a**) Chemiluminiscence of nonopsonized *H. pylori* stimulated neutrophils evaluated by means of luminol/SOD/CAT and isoluminol/HRP systems. Graphics showed the representative kinetics of three independent experiments. (b) Semilog plots for luminol/SOD/CAT (▲) and isoluminol/HRP (△) systems. Data are the average ± SD of three experiments performed in triplicate.

mechanism by which *H. pylori* and neutrophils collaborate to cause gastric mucosa damage.

ACKNOWLEDGMENT

E.P. thanks Isabel Mella (Laboratory of Microbiology, Institute of Nutrition and Food Technology, Universidad de Chile) and Andrés Barriga (Mass Spectrometry Laboratory, CEPEDEQ, Universidad de Chile) for technical assistance.

LITERATURE CITED

(1) Dunn, B. E.; Cohen, H.; Blaser, M. J. *Helicobacter pylori*. *Clin. Microbiol. Rev*. **1997**, *10*, 720–741.
(2) Atherton, J. C. The pathogenesis of *Helicobacter pylori*-induced gastro-duodenal diseases. *Annu. Rev. Pathol. Mech. Dis*. **2006**, *1*, 63–96.
(3) Figueroa, G.; Troncoso, M.; Portell, D. P.; Toledo, M. S.; Acuña, R.; Arellano, L. Prevalence of IgG antibodies to *Helicobacter pylori* in Chilean individuals. *Eur. J. Clin. Microbiol Infect. Dis*. **1993**, *12*, 795–797.
(4) Megraud, F. *Helicobacter pylori* and antibiotic resistance. *Gut* **2007**, *56*, 1502.
(5) Graca, M.; Faleiro, L.; Cavaleiro, C.; Salgueiro, L.; Casanova, J. Suceptibility of *Helicobacter pylori* to Essential oil of *Dittrichia viscosa* subsp. revolute. *Phytother. Res*. **2008**, *22* (2), 259–263.
(6) Romero, C.; Medina, E.; Vargas, J.; Brenes, M.; De Castro, A. In vitro activity of Olive Oil polyphenols against *Helicobacter pylori*. *J. Agric. Food Chem*. **2007**, *55* (3), 680–686.
(7) Tsao, R.; Yang, R.; Young, J. C.; Zhu, H. Polyphenolics profiles in eight apple cultivars using high-performance liquid liquid (HPLC). *J. Agric. Food Chem*. **2003**, *51*, 6347–6353.
(8) Lee, K. W.; Kim, Y. J.; Kim, D. O.; Lee, H. J.; Lee, C. Y. Major phenolics in apple and their contribution to the total antioxidant capacity. *J. Agric. Food Chem*. **2003**, *51*, 6516–6520.
(9) Vanzani, P.; Rossetto, M.; Rigo, A.; Vrhovsek, U.; Mattivi, F.; ĎAmato, E.; Scarpa, M. Major phytochemicals in apple cultivars: Contribution to peroxyl radical trapping efficienty. *J. Agric. Food. Chem*. **2005**, *53*, 3377–3382.
(10) D'Argenio, G.; Mazzone, G.; Tuccillo, C.; Grandote, I.; Gravina, A. G.; Graziani, G.; Fogliano, V.; Romano, M. Apple polyphenol extracts prevent aspirin-induced damage to the rat gastric mucosa. *Br. J. Nutr*. **2008**, *16*, 1–9.
(11) Graziani, G.; ĎArgento, G. D.; Tuccillo, C.; Loguercio, C.; Ritieni, A.; Morisco, F.; Del Vecchio, C.; Fogliano, V.; Romano, M. Apple polyphenols extracts prevent damage to human gastric epithelial cells in Vitro and to rat gastric mucosa in vivo. *Gut* **2005**, *2*, 193–200.
(12) Allen, L. A. The role of neutrophil and phagocytosis in infection caused by *Helicobacter pylori*. *Curr. Opin. Infect. Dis*. **2001**, *14* (3), 273–277.

(13) Allen, L. A.; Beecher, B. R.; Lynch, J. T.; Rohner, O. V.; Wittine, L. M. *Helicobacter pylori* disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide relase. *J. Immunol.* **2005**, *174*, 3658–3667.

(14) Kameyama, M. O.; Yanagida, A.; Kanda, T.; Nagata, T. Identification of catechin oligomers from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. *Rapid Commun. Mass. Spectrom.* **1997**, *11*, 31–36.

(15) Yanagida, A.; Kanda, T.; Shoji, T.; Kameyama, O.; Nagata, T. Fractionation of apple procyanidins by size-exclusion chromatography. *J. Chromatogr., A* **1999**, *855*, 181–190.

(16) Schieber, A.; Hilt, P.; Streker, P.; EndreB, H.-U.; Rentschler, C.; Carle, R. A new process for the combined recovery of pectin and phenolic compounds from apple pomace. *Innov. Food. Sci. Emerg. Technol.* **2003**, *4*, 99–107.

(17) Pastene, E.; Troncoso, M.; Figueroa, G.; Alarcón, J.; Speisky, H. Association between polymerization degree of apple peel polyphenols and inhibition of *Helicobacter pylori* urease. *J. Agric. Food Chem.* **2009**, *57* (2), 416–424.

(18) Matsumoto, T.; Takahashi, T.; Haruki, Y. A novel approach for screening of new anti-*Helicobacter pylori* substances. *Biol. Pharm. Bull.* **2008**, *31* (1), 143–145.

(19) Scheweinitzer, T.; Mizote, T.; Ishikawa, N.; Dudnik, A.; Inatsu, S.; Schreiber, S.; Suerbaum, S.; Aizawa, S.-I.; Josenhans, C. Functional characterization and mutagenesis of the proposed behavioral sensor TlpD of *Helicobacter pylori*. *J. Bacteriol.* **2008**, *190* (9), 3244–3255.

(20) Shimoyama, T.; Fukuda, S.; Liu, Q.; Nakaji, S.; Fukuda, Y.; Sugawara, K. *Helicobacter pylori* water soluble surface proteins prime human neutrophils for enhanced production of reactive oxygen species and stimulate chemokine production. *J. Clin. Pathol.* **2003**, *56* (5), 348–351.

(21) Viera, J.; Katarina, D.; Radomir, N.; Racková, L.; Májeková, M. Dagmar, H. The combined luminol/isoluminol chemiluminescence method for differentiating between extracellular and intracellular oxidant production by neutrophils. *Redox Rep.* **2006**, *11* (3), 110–116.

(22) Tombola, F.; Campello, S.; De Luca, L.; Ruggiero, P.; Del Giudice, G.; Papini, E.; Zoratti, M. Plant polyphenols inhibit VacA, a toxin secreted by gastric pathogen *Helicobacter pylori*. *FEBS Lett.* **2003**, *543*, 184–189.

(23) Xiao, Z.-P.; Shi, D.-H.; Li, H.-Q.; Zhang, L.-N.; Chen Xu, Ch.; Zhu, H.-L. Polyphenols based on isoflavones as inhibitors of *Helicobacter pylori* urease. *Bioorg. Med. Chem.* **2007**, *15* (11), 3703–3710.

(24) Funatogawa, K.; Hayashi, Sh.; Shimomura, H.; Yoshida, T.; Hatano, T.; Ito, H.; Hirai, Y. Antibacterial activity of hydrolizable tannins derived from medicinal plants against *Helicobacter pylori*. *Microbiol. Immunol.* **2004**, *48* (4), 251–261.

(25) Yee, Y.-K.; Koo, M. W-L. Anti-*Helicobacter pylori* activity of Chinese Tea: in vitro study. *Aliment. Pharmacol. Ther.* **2000**, *14* (5), 635–638.

(26) Schieber, A.; Keller, P.; Carle, R. Determination of phenolics acids and flavonoids of apple and pear by high-performance liquid chromatography. *J. Chromatogr., A* **2001**, *910*, 265–273.

(27) Schieber, A.; Hilt, P.; Conrad, J.; Beifuss, U.; Carle, R. Elution order of quercetin glycosides from apple pomace extracts on a new HPLC stationary phase with hydrophilic endcapping. *J. Sep. Sci.* **2002**, *25*, 361–364.

(28) Sánches-Rabaneda, F.; Jáuregui, O.; Lamuela-Raventós, R. M.; Viladomat, F.; Bastida, J.; Codina, C. Qualitative analysis of phenolic compounds in apple pomace using liquid chromatography coupled to mass spectrometry in tandem mode. *Rapid Commun. Mass. Spectrom.* **2004**, *18*, 553–563.

(29) Shin, J. E.; Kim, J. M.; Bae, E. A.; Hyun, Y. J.; Kim, D. H. In vitro inhibitory effect of flavonoids on growth, infection and vacuolation of *Helicobacter pylori*. *Planta Med.* **2005**, *71* (3), 197–201.

(30) Zhu, Y.; Zhang, P.; Yu, H.; Li, J.; Wang, M.-W.; Zhao, W. Anti-*Helicobacter pylori* and thrombin inhibitory components from Chinese Dragon's blood, *Dracaena cochinchinensis*. *J. Nat. Prod.* **2007**, *70* (10), 1570–1577.

(31) Li, H.-Q.; Xu, Ch.; Li, H.-S.; Xiao, Z.-P.; Shi, L. S.; Zhu, H.-L. Metronidazol-flavonoid derivatives as anti-*Helicobacter pylori* agents with potent inhibitory activity against HPE-induced interleukin-8 production by AGS cells. *ChemMedChem.* **2007**, *2*, 1361–1369.

(32) Dahlgren, C.; Karlsson, A. Respiratory Burst in human neutrophils. *J. Immunol. Methods* **1999**, *232*, 3–14.

(33) Ding, M.; Lu, Y.; Bowman, L.; Huang, C.; Leonard, S.; Wang, L.; Vallyathan, V.; Castranova, V.; Shi, X. Inhibition of AP-1 and neoplastic transformation by fresh apple peel extract. *J. Biol. Chem.* **2004**, *279*, 10670–10676.

(34) Bylund, J.; Karlsson, A.; Boulay, F.; Dahlgren, C. Lipopolysaccharide-induced granule mobilization and priming of the neutrophil response to *Helicobacter pylori* peptide Hp(2−20), which activates formyl peptide receptor-like 1. *Infect. Immun.* **2002**, *70* (6), 2908–2914.

(35) Bylund, J.; Samuelsson, M.; Collins, L. V.; Karlsson, A. NADPH-oxidase activation in murine neutrophils via formyl peptide receptors. *Exp. Cell Res.* **2003**, *282* (2), 70–77.

(36) Limasset, B.; Le Doucen, C.; Dore, J. C.; Ojasoo, T.; Damon, M.; Crastes, D. P. Effects of flavonoids on the release of reactive oxygen species by stimulated human neutrophils: Multivariate analysis of structure-activity relationships (SAR). *Biochem. Pharmacol.* **1993**, *46*, 1257–1271.

(37) Krol, W.; Shani, J.; Czuba, Z.; Scheller, S. Modulating luminol-dependent chemiluminescence of neutrophil by flavones. *Z. Naturforsch., C: Biosci.* **1992**, *47* (11−12), 889–892.

(38) Limasset, B.; Ojasoo, T:; le Doucen, C.; Doré, J. C. Inhibition of chemiluminiscence in human PMNs by monocyclic phenolic acids and flavonoids. *Planta Med.* **1999**, *65* (1), 23–29.

(39) Moreira, M. R.; Kanashiro, A.; Kabeya, L. M.; Polizello, A. C.; Azzolini, A. E.; Curti, C.; Oliveira, C. A.; T-do Amaral, A.; Lucisano-Valim, Y. M. Neutrophil effector functions triggered by Fc-gamma and/or complement receptors are dependent on B-ring hydroxylation pattern and physicochemical properties of flavonols. *Life Sci.* **2007**, *81* (4), 317–326.

(40) Chen, G.; Lu, H.; Wang, C.; Yamashita, K.; Manabe, M.; Meng, Z.; Xu, S.; Kodama, H. Effect of five flavonoid compounds isolated from leaves of *Diospyros kaki* on stimulus-induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophils. *Clin. Chim. Acta* **2002**, *326*, 169–175.

(41) Nørgaard, A.; Andersen, L. P.; Nielsen, H. Neutrophil degranulation by *Helicobacter pylori* proteins. *Gut* **1995**, *36*, 354–357.

(42) Satin, B.; Del Guidice, G.; Della Bianca, V.; Dusi, S.; Laudanna, C.; Tonello, F.; Kelleher, D.; Rappuoli, R.; Montecucco, C.; Rossi, F. The neutrophil-activating protein (HP-NAP) of *Helicobacter pylori* is a protective antigen and major virulence factor. *J. Exp. Med.* **2000**, *191* (9), 1467–1476.

(43) Unemo, M.; Aspholm-Hurting, M.; Ilver, D.; Bergström, J.; Borén, T.; Danielsson, D.; Teneberg, S. The sialic acid binding SabA adhesin of *Helicobacter pylori* is essential for Nonopsonic activation of human neutrophils. *J. Biol. Chem.* **2005**, *280* (15), 15390–15397.

(44) Kanashiro, A.; Kabeya, L. M.; Polizello, C. M.; Lopes, N. P.; Lopes, J. L.C.; Lucisano-Valim, Y. M. Inhibitory activity of flavonoids from *Lychnophora sp*. On generation of reactive oxygen species by neutrophils upon stimulation by immune complexes. *Phytother. Res.* **2004**, *18*, 61–65.

(45) Souza, J. G.; Tomei, R. R.; Kanashiro, A.; Kabeya, L. M.; Azzolini, A. E. C. S.; Dias, D. A.; Salvador, M. J.; Lucisano-Valim, Y. M. Ethanolic crude extract and isolated *Alternanthera maritime*: Neutrophil chemiluminiscence inhibition and free radical scavenging activity. *Z. Naturforsch.* **2007**, *62c*, 339–347.

(46) Lu, H. W.; Sugahara, K.; Sagara, Y.; Masuoka, N.; Asaka, Y. Manabe, M.; Kodama, H. Effect of three flavonoids, 5,7,3́,4́-tetrahydroxy-3-methoxy flavone, luteolin, and quercetin, on the stimulus-induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophil. *Arch. Biochem. Biophys.* **2001**, *393*, 73–77.

Received February 26, 2009. Revised manuscript received July 4, 2009. Accepted July 15, 2009. This study was supported by grant no 24080071 from CONICYT.