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Growth-Inhibiting, Bactericidal, and Urease Inhibitory Effects of *Paeonia lactiflora* Root Constituents and Related Compounds on Antibiotic-Susceptible and -Resistant Strains of *Helicobacter pylori*

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ABSTRACT: An assessment was made of the growth-inhibiting, bactericidal, and urease inhibitory activities of paeonol (PA), benzoic acid (BA), methyl gallate (MG), and 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranose (PGG) identified in *Paeonia lactiflora* root, structurally related compounds, and four antibiotics toward three reference strains and four clinical isolates of *Helicobacter pylori* using broth dilution bioassay and Western blot. BA and PA showed strong bactericidal effect at pH 4, while MG and PGG were effective at pH 7. These constituents exhibited strong growth-inhibiting and bactericidal activity toward the five strains resistant to amoxicillin (minimal inhibitory concentration (MIC) 12.5 mg/L), clarithromycin (64 mg/L), metronidazole (64 mg/L), or tetracycline (15 mg/L), indicating that these constituents and the antibiotics do not share a common mode of action. Structural characteristics, such as types of functional groups and carbon skeleton, and hydrophobicity appear to play a role in determining the anti-*H. pylori* activity. *H. pylori* urease inhibitory activity of PGG was comparable to that of acetohydroxamic acid, while MG was less potent at inhibiting urease than thiourea. The UreB band disappeared at 250 mg/L PGG on Western blot, while the UreA bands were faintly visible at 1000 mg/L PGG. These constituents showed no significant cytotoxicity. Global efforts to reduce the level of antibiotics justify further studies on *P. lactiflora* root-derived materials containing MG, PA, and PGG as potential antibacterial products or lead molecules for the prevention or eradication from humans from diseases caused by *H. pylori*.

KEYWORDS: *Helicobacter pylori, Paeonia lactiflora, natural bactericide, structure−activity relationship, antibiotic resistance, urease*

INTRODUCTION

In humans, *Helicobacter pylori* is highly associated with a number of the most important diseases of the upper gastrointestinal tract, including chronic superficial gastritis, duodenal and gastric ulcers, gastric adenocarcinoma, and non-Hodgkin's lymphomas of the stomach.[1,2] Infections are prevalent worldwide and common in both developed and developing countries. In developing countries, 70−90% of the population carries *H. pylori*, while the prevalence of infection in developed countries is lower, ranging from 25% to 50%.[1,3] Most infections by *H. pylori* are acquired in childhood and persist lifelong if not eradicated effectively. *H. pylori* eradication has been achieved principally by the use of conventional antibacterial drugs, including potent triple therapies consisting of a mixture of two antibiotics such as amoxicillin, clarithromycin, and/or metronidazole with bismuth or a proton pump inhibitor. Triple therapy has a success rate of 80−90%,[4] but sometimes serious side effects occur, such as taste disturbances, nausea, diarrhea, dyspepsia, headache, and angioedema,[2] as well as disturbance of human gastrointestinal microflora.[5] The cost of combination therapy is also significant. In addition, widespread use of antimicrobial agents has often resulted in the development of resistance.[2,3,6] There is therefore a critical need for the development of new improved antibacterial agents with novel target sites to establish a rational resistance management strategy based on all available information on the extent and nature of resistance in *H. pylori* because a commercial vaccine is still not available.

Natural compounds extracted from plants, particularly higher plants, have been suggested as alternative sources for anti-*H. pylori* products. This approach is appealing, in part because they constitute a potential source of bioactive chemicals that have been perceived by the general public as relatively safe and often act at multiple and novel target sites, thereby reducing the potential for resistance.[7] Much effort has been focused on plant preparations and their constituents as potential sources of commercial antibacterial products for prevention or eradication of *H. pylori*. In particular, it was reported that *Paeonia lactiflora* Pallas (Paeoniaceae) root had growth inhibitory activity toward human intestinal bacteria[8] and antitumor activity.[9] *P. lactiflora* root is composed of the monoterpene glycosides (albiflorin, benzoylpaeoniflorin, oxypaeoniflorin, and paeoniflorin), the monoterpenes (lactoflorin, paeoniflorigenone, and paeonilactones), benzoic acid and its esters, and gallotannins.[9] No information has been obtained concerning the potential of *P. lactiflora* root-derived materials to control antibiotic-resistant *H. pylori*, although pharmacological actions of the genus *Paeonia* have been well described by He et al.[9]

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In the present study, an assessment is made of the growth inhibitory and bactericidal activities of the constituents that comprise *P. lactiflora* root toward three reference strains and four clinical isolates of *H. pylori* using a broth dilution bioassay. The activities of the constituents were compared to those of epigallocatechin gallate and four currently available antibiotics, amoxicillin, clarithromycin, metronidazole, and tetracycline. *H. pylori* urease inhibitory activities of the constituents were compared to those of two potent *H. pylori* urease inhibitors acetohydroxamic acid[10] and thiourea.[11] The effect of the constituents on morphological transformation of the bacterial strain was also examined because conversion of the spiral to the coccoid form in *H. pylori* is caused by environmental factors or antibiotic treatment.[12] The quantitative structure−activity relationship (QSAR) of paeonol and 10 structurally related compounds is discussed.

MATERIALS AND METHODS

Materials. Paeonol and 10 structurally related compounds examined in this study are as follows: acetophenone (AP) (>99.5% purity) and 4′-hydroxyacetophenone (4′-HAP) (>98%) purchased from Fluka (Buchs, Switzerland); 2′,4′-dihydroxyacetophenone (2′,4′-DHAP) (99.0%), 2,4-dihydroxycinnamic acid (2,4-DHCA) (97.0%), 2-ethylphenol (2-EP) (99.0%), 2-hydroxybenzyl alcohol (2-HBA) (99.0%), *p*-hydroxypropiophenone (*p*-HPP) (98.0%), paeonol (PA) (99.0%), and salicylaldehyde (SA) (98.0%) purchased from Sigma-Aldrich (St. Louis, MO); 2′,6′-dihydroxy-4′-methoxyacetophenone (2′,6′-DHMAP) (98.0%) purchased from Indofine (Philadelphia, PA); and xanthoxylin (XT) (>98%) purchased from Tokyo Chemical Industry (Tokyo, Japan). For the QSAR analysis, values of molecular weight (MW) and octanol/water partition coefficient (log *P*) for the test compounds were obtained from ACD/log *P* DB v.12.01 [ACD/Laboratories Online (ACD/I-Lab), Advanced Chemistry Development, Inc., Montreal, Canada]. (−)-Epigallocatechin gallate (EGCG), thiourea (TU), and four antibiotics (amoxicillin, clarithromycin, metronidazole, and tetracycline) were purchased from Sigma-Aldrich (St. Louis, MO). Acetohydroxamic acid (AHA) was supplied by Tokyo Chemical Industry (Tokyo). (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) was purchased from Sigma-Aldrich. Brucella broth and newborn bovine serum (NBS) were purchased from Becton, Dickinson and Co. (Sparks, MD) and Hyclone (Longan, UT), respectively. A Bradford protein assay kit was supplied by Sigma-Aldrich. The protein molecular weight standards (Precision Plus Protein all blue standards) were provided by Bio-Rad Life Sciences (Hercules, CA). All of the other chemicals and reagents used in this study were of analytical grade quality and available commercially.

Bacterial Strains and Culture Conditions. The three reference strains (ATCC 700392, ATCC 700824, and ATCC 43504) and four clinical isolates (PED 503G, PED 3582GA, 221, and B) of *H. pylori* were used in this study. The clinical isolates were obtained in 2011 from the Culture Collection of Antimicrobial Resistant Microbes (Seoul, Republic of Korea) from individual patients with gastric or duodenal ulcers. All strains were stored at −80 °C in Brucella broth supplemented with 5% NBS, which contained vancomycin (10 mg/L), polymyxin B (5 mg/L), trimethoprim (5 mg/L), and amphotericin B (2 mg/L) and 20% glycerol[13] until use. The bacterial strains were grown on Brucella agar supplemented with 10% NBS at 37 °C for 3 days in an atmosphere of 5% O_2, 15% CO_2, and 80% N_2 in a Hirayama anaerobic chamber (Tokyo).

Extraction and Isolation. Air-dried root (6 kg) of *P. lactiflora* was pulverized and extracted with methanol (2 × 12 L) at room temperature for 2 days and filtered. The combined filtrate was concentrated to dryness by rotary evaporation at 40 °C to yield 978 g. The extract was sequentially partitioned into hexane- (7.0 g), chloroform- (22.0 g), ethyl acetate- (45.3 g), butanol- (313.8 g), and water-soluble (590.0 g) portions for subsequent bioassay. For isolation of active principles, 5 and 10 mg/paper disk (1 mm thickness, 8 mm diameter) of each *P. lactiflora* root-derived material was tested in a paper-disk diffusion bioassay as described previously.[14]

The hexane-soluble fraction (7 g) was most biologically active and was chromatographed on a 55 × 5 cm silica gel (0.063−0.2 mm) column (550 g) and eluted with a gradient of hexane and ethyl acetate [100:0 (1 L), 99:1 (1 L), 90:10 (2 L), 80:20 (2 L), 70:30 (1 L), and 0:100 (1 L) by volume] and finally with methanol (1 L) to provide 29 fractions (each about 250 mL). Column fractions were monitored by thin-layer chromatography (TLC) on Merck precoated silica gel plates (Kieselgel 60 F_{254}) with hexane and ethyl acetate (85:15 by volume). Fractions with similar R_f values on the TLC plates were pooled. Fractions 6−7 (1.72 g) were rechromatographed on a silica gel column using hexane and ethyl acetate (99.5:0.5 by volume) to give nine fractions and were bioassayed. For further separation of constituents from the active fractions 4−5 (156 mg), an Agilent 1200 series high-performance liquid chromatograph (Santa Clara, CA) was used. The column was a 4.6 mm i.d. × 150 mm Agilent Eclipse XDB-C_{18} (Santa Clara, CA) using a mobile phase of methanol and water (8:2 by volume) at a flow rate of 0.5 mL/min. Chromatographic separations were monitored using a UV detector at 274 nm. Finally, an active principle **1** (94 mg) was isolated at a retention time of 10.9 min.

The active chloroform-soluble fraction (11 g) was chromatographed on a silica gel column and eluted with a gradient of chloroform and methanol [100:0 (1 L), 99:1 (1 L), 95:5 (1 L), 90:10 (2 L), 80:20 (1 L), 70:30 (1 L), and 0:100 (1 L) by volume] to provide eight fractions (each about 1 L). Fractions 3−4 (5.63 g) were rechromatographed on a silica gel column eluted with a gradient of hexane and acetone [100:0 (1 L), 90:10 (1 L), 80:20 (1 L), 70:30 (1 L), and 0:100 (1 L) by volume] to give 11 fractions (each about 450 mL). A preparative HPLC was used for separation of the constituents from the active fractions 2−5 (1.76 g). The column and elution conditions were the same as described above. Chromatographic separations were monitored using a UV detector at 216 nm. Finally, an active principle **2** (109 mg) was isolated at a retention time of 4.03 min.

The ethylacetate-soluble fraction (8 g) was also chromatographed on a silica gel column and eluted with a gradient of chloroform and methanol as stated above to provide eight fractions. Active fractions 1−2 (E1, 550 mg) and 6−7 (E2, 569 mg) were obtained. The E1 fraction was purified by preparative TLC with chloroform and methanol (7:3 by volume) to afford an active principle **3** (245 mg). The active fraction E2 was purified by Merck TLC silica gel 60 RP-18 with chloroform:methanol:water (70:25:5 by volume) to afford an active principle **4** (45 mg).

Microbiological Assay. A broth dilution assay in sterile 96-well plates or 15 mL tubes was used to evaluate the minimal inhibitory concentrations (MICs)[15] and minimal bactericidal concentrations (MBCs)[16] of the test materials toward all *H. pylori* strains, respectively. For MICs, initial test materials were prepared in dimethylsulfoxide (DMSO), and 2-fold serial dilutions were then performed in 75 μL of 10% NBS-supplemented Brucella broth. The final concentration of DMSO in all assays was 2.5% or less. Subsequently, 75 μL of bacterial suspension (5 × 10^6 CFU/mL) of each strain from cultures on Brucella agar was added. The plates were incubated at 37 °C under microaerophilic conditions and shaken at 50 rpm for 48 h. MICs were defined as the lowest concentrations that visibly inhibited bacterial growth using resazurin as an indicator. For MBCs and time killing assay, the bacterial suspensions (200 μL, 1 × 10^7 CFU/mL) were inoculated onto 1.8 mL of 10% NBS-supplemented Brucella broth alone or containing each compound concentration (0, 1, 2, 4, or 8 times the MIC) and incubated with shaking (150 rpm) at 37 °C for 48 h. Samples for viability measurement were taken after 0, 6, 12, 18, 24, 36, and 48 h, and 0.1 mL of 10-fold serial dilutions was plated onto Brucella agar without the test samples. Colonies were counted after 3 days of incubation by a plate colony count technique. Amoxcillin, clarithromycin, metronidazole, and tetracycline served as positive controls and were similarly prepared. Negative controls consisted of the DMSO solution. All bioassays were repeated three times in triplicate.

Measurement of Bactericidal Activity at Various pH Values. The method of Ohno et al.[17] was used with a slight modification to

Table 1. In Vitro Minimal Inhibitory Concentrations (MICs) and Bactericidal Concentrations (MBCs) of Four Commercial Antibiotics toward Three Reference Strains and Four Clinical Isolates of *H. pylori* Using Broth Dilution Bioassay

	MIC[a] (MBC[a])						
antibiotics	ATCC 700392	ATCC 700824	ATCC 43504	PED 503G	PED 3582GA	221	B
amoxicillin	0.03 (0.07)	0.01 (0.03)	0.03 (0.07)	12.5 (15)	0.5 (1)	0.06 (0.12)	2 (4)
clarithromycin	0.07 (0.1)	0.03 (0.07)	0.07 (0.3)	0.6 (1)	64 (128)	0.5 (1)	0.06 (0.06)
tetracycline	0.3 (0.7)	0.1 (0.1)	0.3 (1)	0.06 (0.12)	4 (8)	2 (4)	15 (20)
metronidazole	1 (2)	1 (6)	24 (180)	2 (4)	1 (2)	64 (128)	1 (2)

[a]Unit, mg/L.

assess the effects of pH on the bactericidal activity of the test compounds. The buffer solutions used were 100 mM citrate buffer (pH's 4.0 and 5.0) and 10 mM sodium phosphate buffer (pH's 6.0 and 7.0). The bacterial suspension of *H. pylori* ATCC 43504 (200 μL, 1×10^7 CFU/mL) was added to 1.8 mL of each buffer containing each compound concentration (0, 1, 2, 4, or 8 times the MIC). The cultures were incubated with shaking (150 rpm) at 37 °C. Samples (0.1 mL) were taken at 0, 15, 30, and 60 min and plated onto Brucella agar without the test samples. Colonies were counted after 3 days of incubation as stated above. All bioassays were repeated twice in triplicates.

Cytotoxicity Assay. The cytotoxicity of the test materials to three human cell lines HeLa cells (ATCC CCL2, cervix carcinoma cell line), A549 cells (ATCC CCL-185, lung carcinoma cell line), and MRC5 cells (ATCC CCL-171, lung fibroblast cell line) was evaluated using a MTT assay described previously.[18]

Microscopic Observation. *H. pylori* ATCC43504 was cultured in Brucella broth without or with the test materials (150 and 300 mg/L) in microaerophilic conditions for 24 and 48 h. Bacterial suspension (15 μL) was evenly spreaded and fixed on slides, and then stained with 0.3% methylene blue. The proportion of coccoid versus spiral bacteria was determined using a Carl Zeiss microscope equipped with Zeiss AxioCam HRC camera (Jena, Germany). Counts of 200 bacteria from each slide were done as reported previously.[19]

Inhibition of Urease in Vitro. Urease crude of *H. pylori* ATCC 43504 was prepared according to the method of Icatlo et al.[20] The assay mixtures, containing 0.25 μg of urease crude (0.04 urease units) in 100 μL of the EDTA-sodium phosphate buffer (pH 7.3) and the constituent (10−2000 μg/mL), were preincubated at 37 °C for 2 h with rotation at 50 rpm. Inhibition of urease activity was determined using the indophenol method.[21] The ammonia released by the urease was quantified by measuring absorbance on a Molecular Devices Versa Max microplate reader (Sunnyvale, CA) at 625 nm with ammonium chloride as a standard. AHA and TU served as standard references and were similarly prepared. The protein content was determined using a Bradford protein assay kit. BSA was used as a protein standard. All bioassays were repeated three times in triplicates.

SDS-PAGE and Western Blot Analysis. The mixtures of 50 μg of urease crude (0.08 urease units) in 100 μL of EDTA-sodium phosphate buffer of *H. pylori* ATCC 43504 and the test compound (100−1500 μg/mL) were preincubated for 1 h at 37 °C. The mixtures were loaded onto wells of SDS-PAGE 12% (w/v) polyacrylamide gels.[22] After electrophoresis at 120 V in 2 h, the gels were stained with 0.1% Coomassie brilliant blue R-250 for quantification. For Western blotting, proteins from unstained gels were transferred onto a Pall Corp. polyvinyl difluoride membrane (Pensacola, FL).[23] The membrane was then incubated overnight at 4 °C with 1:2000 dilution of Abcam duck polyclonal to *H. pylori* urease (Cambridge, UK). After being washed with phosphate-buffered saline (PBS) containing 0.5% Tween-20 (v/v) three times, the membrane was further incubated for 2 h with Abcam rabbit polyclonal secondary antibody to Abcam chicken IgY-H&L diluted 1:4000. Finally, the blots were developed using an Amersham Bioscience ECL chemiluminescence reagent (Buckinghamshire, UK) and exposed to an AGFA CP-PU X-ray film (Mortsel, Belgium). Western blot results were analyzed using a Bio-Rad Molecular Imager Gel Doc XR system (Hercules, CA).

Native PAGE and Zymogram Analysis. For native PAGE analysis, samples were mixed with 5× sample buffer without SDS and loaded onto 6% SDS-free PAGE gels.[22] Urease activity in gel was detected by nitroprusside-thiol reaction as described previously.[24]

Instrumental Analyses. ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD on a Bruker AVANCE 600 spectrometer (Karlsruhe, Germany) using tetramethylsilane as an internal standard, and chemical shifts are given in δ (ppm). UV spectra were obtained in methanol with a Kontron UVICON 933/934 spectrophotometer (Milan, Italy), mass spectra on Jeol GMS-600 W or Jeol JMS-700 spectrometer (Tokyo, Japan), and FT-IR spectra on a Midac Nicolet Magna 550 series II spectrometer (Irvine, CA). Optical rotation was measured with a Rudolph Research Analytical Autopol III polarimeter (Flanders, NJ).

Data Analysis. Percent urease inhibition was determined as reported previously.[25] Concentrations of the test compounds causing 50% loss of the urease activity (IC_{50}) were calculated using GraphPad Prism 5 software program. The IC_{50} values of their treatments were considered to be significantly different from one another when 95% confidence limits failed to overlap. Percentages of conversion to the coccoid form were transformed to arcsine square root values for analysis of variance. The Bonferroni multiple-comparison method was used to test for significant differences among the treatments (SAS OnlineDoc, Version 8.01). Resistance to amoxicillin, clarithromycin, metronidazole, and tetracycline was defined by MIC ≥ 1 mg/L, MIC ≥ 1 mg/L, MIC ≥ 8 mg/L, and MIC ≥ 4 mg/L, respectively, as described previously.[26] Cytotoxicity was exposed as 50% cytotoxicity concentration (CC_{50}) of the compound that reduced the viability of cells to 50% of the control, and was calculated using GraphPad Prism 5 software program. The CC_{50} values of their treatments were considered to be significantly different from one another when 95% confidence limits failed to overlap. Correlation coefficient analysis of the growth inhibitory and bactericidal activities of compounds toward *H. pylori* ATCC 43504 was determined using their MIC and MBC values and physical parameter (MW and partition coefficient) values for the test compounds.

RESULTS

Antibiotic Resistance. The antibacterial activity of four commercial antibiotics toward all strains was evaluated by a broth dilution assay (Table 1). On the basis of MIC values, strain PED 503G exhibited a high level of resistance to amoxicillin (12.5 mg/L) as compared to ATCC 700392 and ATCC 700824 susceptible to all test antibiotics. Strain PED 3582GA was resistant to clarithromycin (MIC 64 mg/L) and tetracycline (MIC 4 mg/L). Strains 221 and ATCC 43504 exhibited a high level of resistance to metronidazole (MIC 64 and 24 mg/L). Strain B was resistant to tetracycline (MIC 15 mg/L) and amoxicillin (MIC 2 mg/L). Similar results were likewise observed in the bactericidal activity.

Bioassay-Guided Fractionation and Identification. Fractions obtained from the solvent hydrolyzable of the methanol extract of *P. lactiflora* root were bioassayed toward two *H. pylori* strains by paper-disk diffusion. The hexane-soluble fraction showed the most growth inhibitory activity,

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followed by chloroform- and ethyl actate-soluble fractions. At concentrations of 10, 5, and 1 mg/disk, the inhibitory zone diameters were 29.9, 23.9, and 19.9 mm toward *H. pylori* ATCC 700392 and 31.3, 26.7, and 14.1 mm toward *H. pylori* ATCC 700824, respectively. These fractions were used to identify peak activity fractions for the next step in the purification. The butanol- and water-soluble fractions were ineffective.

Paper-disk diffusion assay-guided fractionation of *P. lactiflora* root extract afforded four antibacterial principles identified by spectroscopic analyses, including MS and NMR. The four antibacterial principles were paeonol (PA) (**1**), benzoic acid (BA) (**2**), methyl gallate (MG) (**3**), and 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranose (PGG) (**4**) (Figure 1). PA (**1**) was

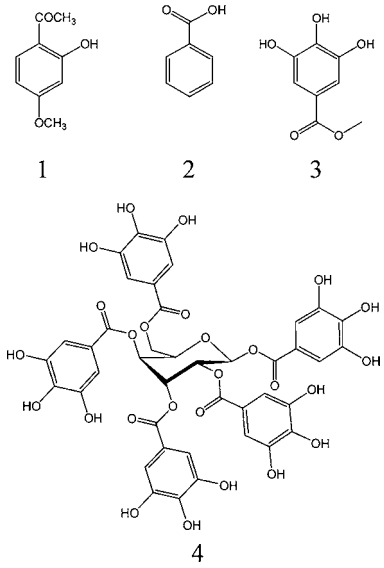

Figure 1. Structures of paeonol (PA) (**1**), benzoic acid (BA) (**2**), methyl gallate (MG) (**3**), and 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranose (PGG) (**4**).

identified on the basis of the following evidence: pale yellow needles. UV (MeOH): λ_{max} nm 274. FT-IR: ν_{max} 3450 cm^{-1} (OH), 1620 cm^{-1} (conjugated C=O), 1576 cm^{-1} (aromatic C=C). EI-MS (70 eV), *m/z* (% rel. int.): 166 $[M]^+$ (48.7), 151 (100), 108 (6.6), 95 (7.5), 80 (1.9), 69 (2.4). ^{1}H NMR (CD_3OD, 600 MHz): δ 2.55 (3H, s), 3.84 (3H, s), 6.41 (1H, d, *J* = 2.4 Hz), 6.50 (1H, dd, *J* = 8.9, 2.4 Hz), 7.77 (1H, d, *J* = 8.9 Hz). ^{13}C NMR (CD_3OD, 150 MHz): δ 26.5, 56.3, 101.9, 108.5, 115.2, 134.2, 166.4, 167.9, 204.7. MG (**3**): white crystals. UV (MeOH): λ_{max} nm 260. FT-IR: ν_{max} 3404−3054 cm^{-1} (OH stretch), 1609 cm^{-1} (aromatic C=C), 1301−1000 cm^{-1} (C−O). EI-MS (70 eV), *m/z* (% rel. int.): 184.1 $[M]^+$ (65.8), 153 (100), 125 (16.7), 124 (2.8), 107 (3.8), 79 (6.1), 78 (2.5). ^{1}H NMR (CD_3OD, 600 MHz): δ 3.80 (3H, s), 4.84 (1H, br), 7.03 (2H, s). ^{13}C NMR (CD_3OD, 150 MHz): δ 52.4, 110.2, 121.6, 139.9, 146.7, 169.2. PGG (**4**): gray powder; $[\alpha]_D^{22}$: +25.8 (*c* 0.004, ethanol). UV (MeOH): λ_{max} nm 280. FT-IR: ν_{max} 3332 cm^{-1} (OH), 1694 cm^{-1} (conjugated C=O), 1610, 1535 cm^{-1} (aromatic C=C). FAB^+-MS, *m/z*: 986 $[M + Na]^+$. FAB-MS: 939 $[M - H]^-$ (8.0), 787 $[M - (HO)_3C_6CO]^-$ (3.4), 769 (M − gallic acid − H)$^-$ (2), 617 (M − digallic acid − H)$^-$ (1.9), 601 $[M - \text{gallic acid} - (HO)_3C_6H_2COO]^-$ (2.9), 447 (M − tetragallic acid − H)$^-$ (1.7). ^{1}H NMR (CD_3OD, 600 MHz): δ 4.36 (2H, m), 4.51 (1H, d, *J* = 10.6 Hz), 5.54 (1H, t, *J* = 9.7 Hz), 5.61 (1H, t, *J* = 9.3 Hz), 5.90 (1H, t, *J* = 9.7 Hz), 6.20 (1H, d, *J* = 8.28 Hz), 6.89 (2H, s), 6.95 (2H, s), 6.97 (2H, s), 7.04 (2H, s), 7.10 (2H, s). ^{13}C NMR (CD_3OD, 150 MHz): δ 63.6, 70.4, 72.6, 74.5, 74.8, 94.2, 110.8−111.1 (each C2′-C6′), 120.6−121.5 (each C1′), 140.4−140.7 (each C4′), 146.5−146.8 (each C3′−C5′), 166.5−168.2 (each C=O). Structure of BA (**2**) was similarly determined. The interpretations of proton and carbon signals of compounds **1**, **3**, and **4** were largely consistent with those of Kim et al.,[27] Ahn et al.,[28] and Zhao et al.,[29] respectively.

Growth Inhibitory Activity of Test Compounds. The growth inhibitory activities of the four constituents and EGCG toward two antibiotic-susceptible strains (ATCC 700392 and ATCC 700824) and five antibiotic-resistant strains (ATCC 43504, PED 503G, PED 3582GA, 221, and B) of *H. pylori* were likewise compared (Table 2). On the basis of MIC values, PGG, MG, PA, and EGCG did not differ in growth inhibitory activity from each other toward *H. pylori* ATCC 700392 and ATCC 700824. The inhibitory activity of BA was the least of any of the constituents. Overall, all of the constituents were less potent at inhibiting microbial growth than either amoxicillin, clarithromycin, metronidazole, or tetracycline (Table 1). Interestingly, all of the compounds were of nearly similar growth-inhibiting activity toward both antibiotic-susceptible and -resistant strains, indicating a lack of resistance in the resistant strains.

Bactericidal Activity of Test Compounds. The MBCs of the five compounds toward all strains examined are recorded in Table 2. MG was the most toxic compound toward *H. pylori* ATCC 700392 and ATCC 700824 but was less toxic than either amoxicillin, clarithromycin, metronidazole, or tetracycline (Table 1). PGG, PA, and EGCG were of equal toxicity. BA was the least of any of the constituents. All of the compounds were of nearly similar toxicity to both antibiotic-

Table 2. In Vitro Minimal Inhibitory Concentrations (MICs) and Bactericidal Concentrations (MBCs) of *P. lactiflora* Root Constituents and Epigallocatechin Gallate toward Three Reference Strains and Four Clinical Isolates of *H. pylori* Using Broth Dilution Bioassay

	MIC[b] (MBC[b])						
compound[a]	ATCC 700392	ATCC 700824	ATCC 43504	PED 503G	PED 3582GA	221	B
PGG	160 (640)	160 (640)	80 (640)	160 (640)	160 (640)	160 (640)	160 (640)
MG	160 (320)	160 (320)	160 (320)	320 (640)	160 (640)	160 (640)	160 (640)
PA	160 (640)	160 (640)	160 (640)	320 (640)	160 (640)	160 (640)	160 (320)
BA	320 (1280)	320 (1280)	320 (1280)	320 (1280)	320 (620)	320 (1280)	320 (1280)
EGCG	160 (640)	160 (640)	80 (640)	160 (640)	160 (640)	160 (640)	160 (640)

[a]Abbreviations are the same as in Figure 1 and in the text. [b]Unit, mg/L.

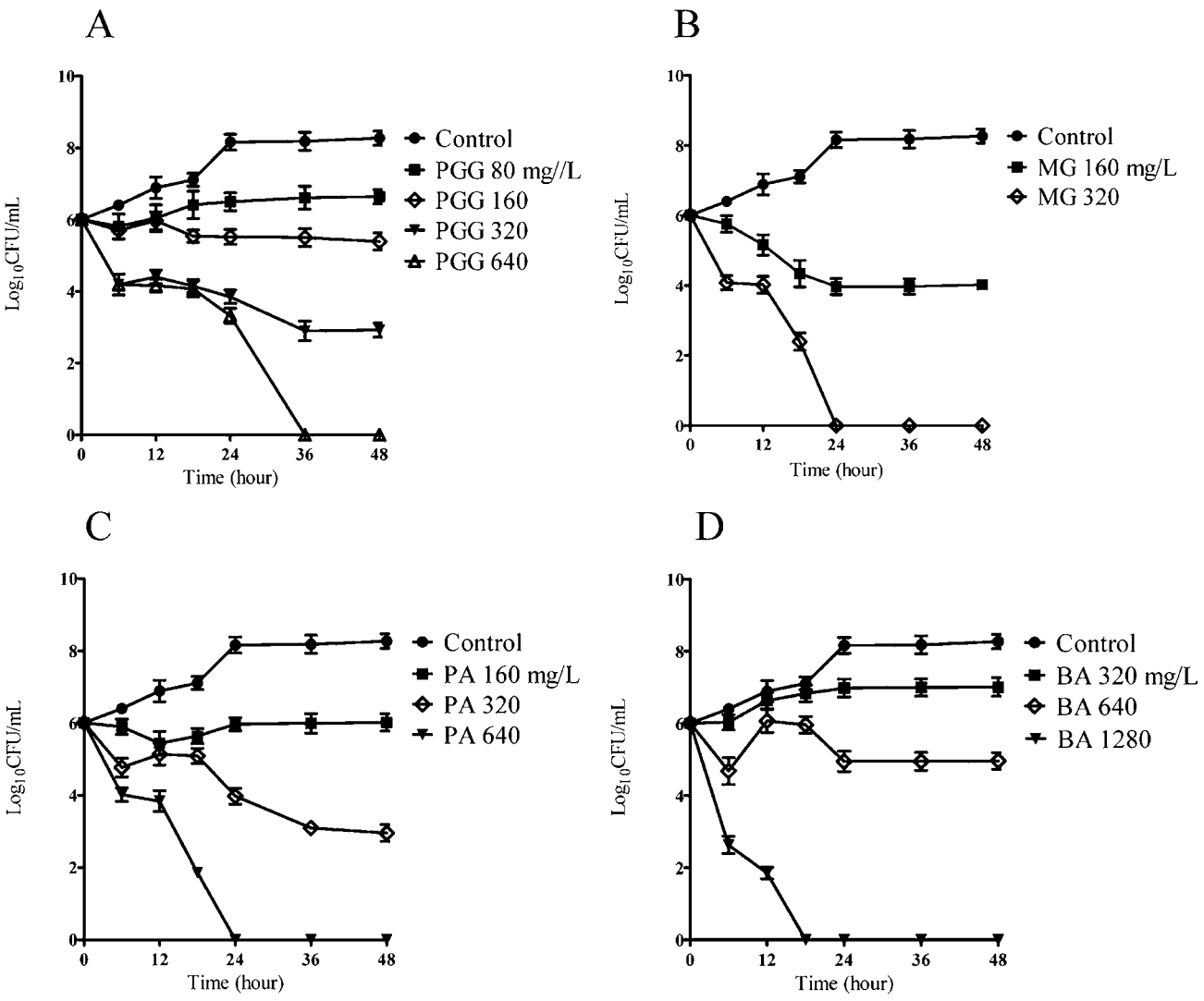

Figure 2. Time-course bactericidal activity of *P. lactiflora* root constituents at different concentrations (0, 1, 2, 4, or 8 times the MIC) toward *H. pylori* ATCC43504. (A) PGG, MIC 80 mg/L; (B) MG, MIC 160 mg/L; (C) PA, MIC 160 mg/L; (D) BA, MIC 320 mg/L. Abbreviations are the same as in Figure 1 and in the text. The mean values (±SD) for the log number of CFU/mL were plotted.

susceptible and -resistant strains, indicating a lack of resistance in the resistant strains.

Time course of bactericidal activity of PGG, MG, PA, and BA at different concentrations toward *H. pylori* ATCC43504 was likewise investigated (Figure 2). The results revealed that viable count of the organism was reduced in a dose-dependent manner. Treatment of *H. pylori* with PGG at 4 times the MIC reduced the viability of *H. pylori* by ~5 $\log_{10}$ over 48 h, while treatment with PGG at the MIC resulted in ~1.5 $\log_{10}$ reduction (Figure 2A). Treatment with MG at the MIC or 2 times the MIC resulted in ~4 and ~3 $\log_{10}$ reductions in 48 and 12 h, respectively (Figure 2B). Treatment with PA at the MIC or 4 times the MIC effected reductions of ~2 and ~3 $\log_{10}$ in 48 and 12 h, respectively (Figure 2C). Treatment with BA at the MIC or 4 times the MIC effected reductions of ~1 and ~5 $\log_{10}$ in 48 and 12 h, respectively (Figure 2D).

Effect of Test Compounds on the Viability of *H. pylori* at Varying pH Values. The bactericidal activity of PGG, MG, PA, and BA toward *H. pylori* ATCC43504 at various pH values was investigated (Figure 3). The activity of the constituents varied according to concentration and pH examined. PA was more effective at pH's 4 and 5 than at pH's 6 and 7, while BA exhibited the strongest activity at pH 4. PGG (640 mg/L) and MG (320 mg/L) were most effective at pH 7.

Structure−Activity Relationship. Comparisons were made to determine growth inhibitory and bactericidal activity differences involving the skeletal structure and functional groups of PA and 10 structurally related compounds (Figure 4) using the MIC and MBC data obtained (Table 3). On the basis of MIC values, 2′,6′-DHMAP and SA showed the most growth-inhibiting activity. The inhibitory activities of PA, 2′,4′-DHAP, XT, *p*-HPP, and 2-EP were the same, and these compounds were more potent at inhibiting microbial growth than either 4′-HAP or AP. The inhibitory activities of 2,4-DHCA and 2-HBA were the least of any of the compounds. Similar results were likewise observed in the bactericidal activity.

Correlation coefficient analysis showed that octanol/water partition coefficient (log *P*) is correlated negatively with MIC ($r = -0.627$) and MBC ($r = -0.685$). MW has no correlation with MIC ($r = -0.096$) and MBC ($r = -0.283$).

Cytotoxicity. To determine the selectivity of the anti-*H. pylori*, the cytotoxic effects of the test materials were examined

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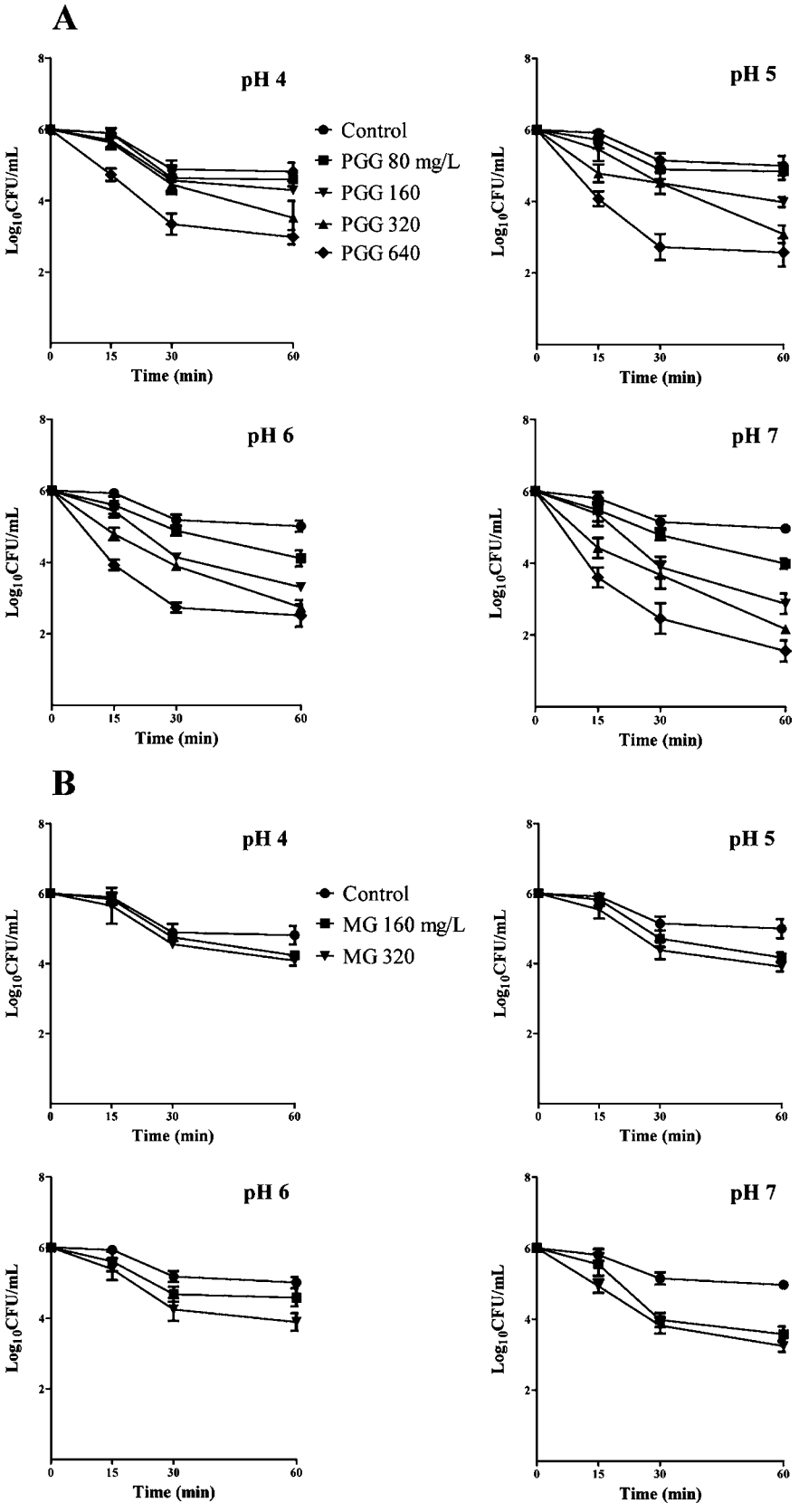

Figure 3. continued

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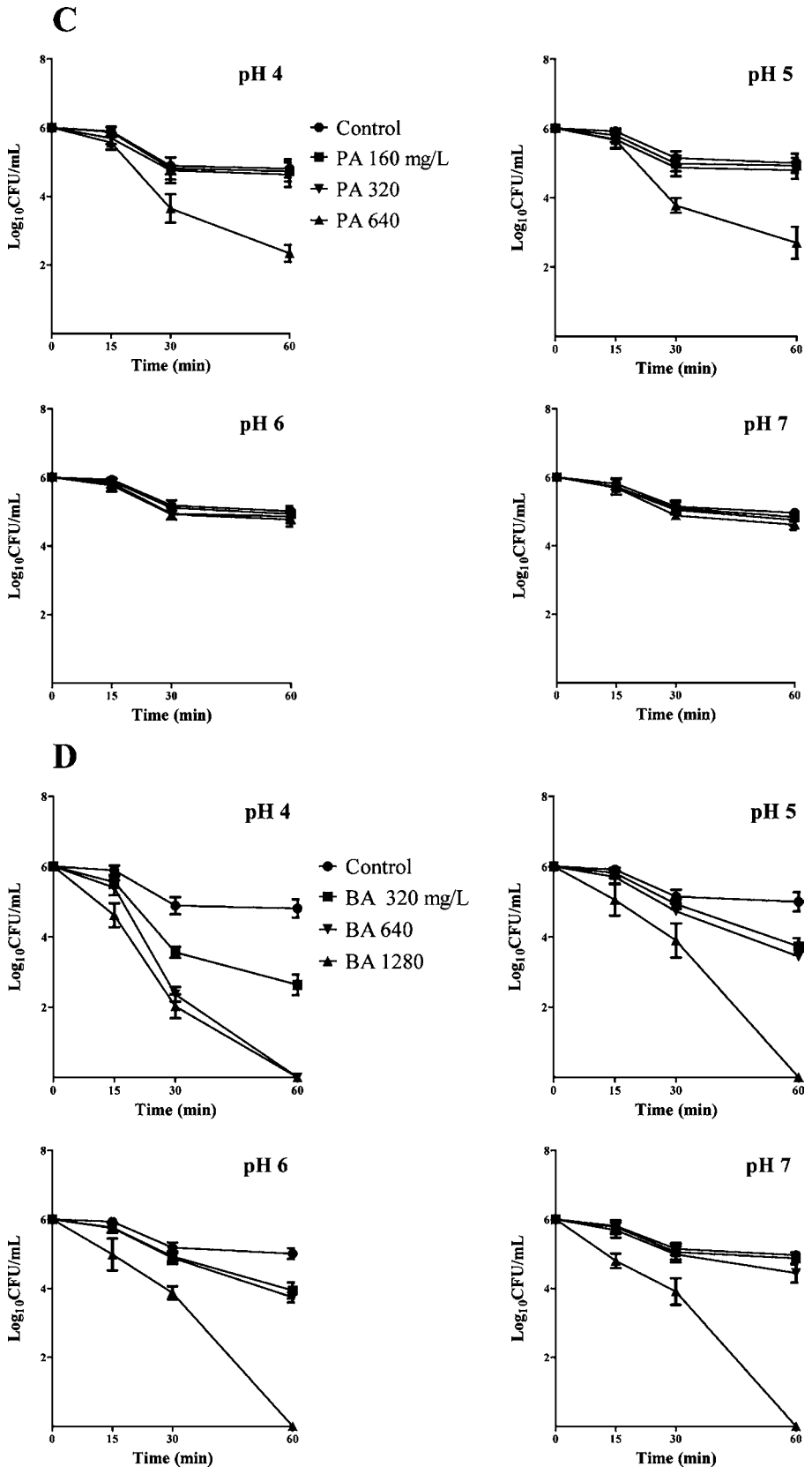

Figure 3. The bactericidal activity of *P. lactiflora* root constituents at concentrations (0, 1, 2, 4, or 8 times the MIC) toward *H. pylori* ATCC43504 at various pH values. (A) PGG, MIC 80 mg/L; (B) MG, MIC 160 mg/L; (C) PA, MIC 160 mg/L; (D) BA, MIC 320 mg/L. Abbreviations are the same as in Figure 1 and in the text. The mean values (±SD) for the log number of CFU/mL were plotted.

(Table 4). PGG, PA, MG, and BA showed no significant cytotoxicity toward HeLa, MRC5, and A549 cell lines.

Effect on Morphology of *H. pylori*. The proportion of coccoid versus spiral form of *H. pylori* ATCC 43504 was

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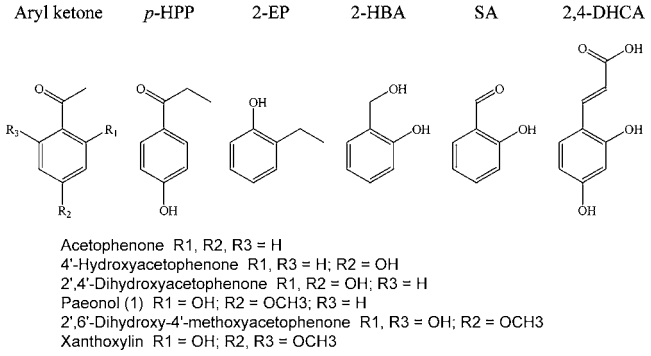

Figure 4. Structures of paeonol and 10 structurally related compounds examined: *p*-HPP, *p*-hydroxypropiophenone; 2-EP, 2-ethylphenol; 2-HBA, 2-hydroxybenzyl alcohol; SA, salicylaldehyde; 2,4-DHCA, 2,4-dihydroxycinnamic acid.

Table 3. Quantitative Structure–Activity Relationship of Paeonol and 10 Structurally Related Compounds in *H. pylori* ATCC 43504 Using Minimal Inhibitory Concentrations (MICs) and Bactericidal Concentrations (MBCs)

compound[a]	MW[b]	log P[c]	MIC (mg/L)	MBC (mg/L)
2′,6′-DHMAP	182.17	2.59 ± 0.38	9	20
SA	122.12	1.61 ± 0.26	9	20
PA	166.17	2.16 ± 0.27	160	640
2′,4′-DHAP	152.15	1.74 ± 0.27	160	640
XT	196.20	2.37 ± 0.37	160	640
p-HPP	150.17	1.96 ± 0.24	160	640
2-EP	122.16	2.47 ± 0.19	160	640
4′-HAP	136.15	1.42 ± 0.24	320	750
AP	120.15	1.66 ± 0.22	640	1500
2,4-DHCA	180.16	1.91 ± 0.51	1280	1280
2-HBA	124.14	0.30 ± 0.22	1280	2000

[a]Abbreviations are the same as in the text. [b]Molecular weight. [c]Octanol/water partition coefficient.

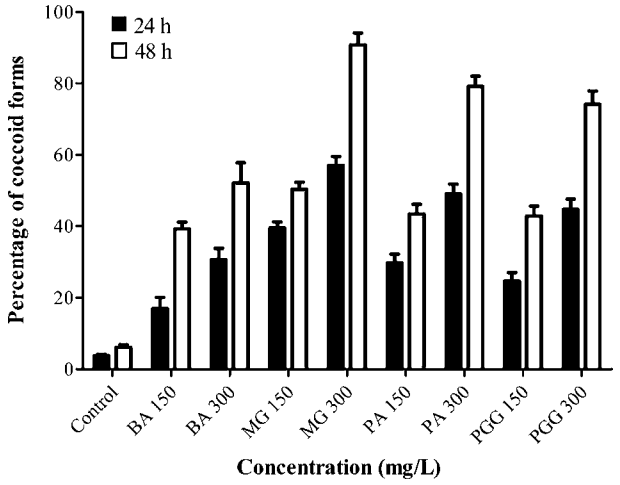

Figure 5. Effect of test compounds at concentrations of 150 and 300 mg/L on *H. pylori* ATCC 43504 morphology 24 (■) and 48 h (□) post-treatment. Abbreviations are the same as in Figure 1 and in the text.

Table 5. Effect of Test Compounds and Two Commercially Available Urease Inhibitors on *H. pylori* ATCC 43504 Urease Inhibition

compound[a]	slope ± SE	IC_{50}, mM (95% CL[b])
PGG	1.6 ± 0.08	0.072 (0.067–0.076)
MG	1.3 ± 0.07	1.330 (1.236–1.430)
BA	1.3 ± 0.06	17.95 (16.93–19.04)
PA		NI[c]
AHA	1.5 ± 0.06	0.049 (0.047–0.052)
TU	1.9 ± 0.10	0.568 (0.540–0.597)

[a]Abbreviations are the same as in Figure 1 and in the text. [b]CL denotes confidence limit. [c]No inhibition.

determined at 150 and 300 mg/L of the constituents for 24 and 48 h (Figure 5). Effect of concentration (F = 104.80; df = 8, 45; P < 0.0001) and exposure time (F = 233.59; df = 1, 45; P < 0.0001) on conversion of *H. pylori* to the coccoid form was significant. The concentration by exposure interaction was also significant (F = 6.59; df = 8, 45; P < 0.0001). MG caused considerable conversion to the coccoid form (91% versus 50% at 300 and 150 mg/L), while BA caused the lowest conversion (52% versus 39% at 300 and 150 mg/L). PA (79% versus 43% at 300 and 150 mg/L) caused as much conversion to the coccoid form as PGG (74% versus 43% at 300 and 150 mg/L).

Urease Inhibitory Activity. The in vitro urease inhibitory activities of the four constituents were compared to those of AHA and TU toward *H. pylori* ATCC 43504 (Table 5). On the basis of IC_{50} values, PGG was slightly less potent at inhibiting urease than AHA but was significantly higher than TU. MG was significantly less potent at inhibiting urease than TU, while BA and PA showed poor and no inhibitory activity, respectively.

Effect on Urease Production. SDS-PAGE analysis revealed reduction of multiple protein bands of *H. pylori* ATCC 43504 in the presence of PGG and MG, although responses varied according to treatment concentration. In this experiment, the UreA and UreB bands, as shown in Figure 6A (lane 1), were confirmed by Western blot with antibodies toward *H. pylori* urease (Figure 6B) as reported previously.[23] The UreB band completely disappeared at 250 mg/L PGG, while the UreA dimer and monomer bands were faintly visible at 1000 mg/L PGG (Figure 6B). The UreB and UreA (dimer and monomer) bands were faintly visible at 1000 mg/L MG (Figure 6B).

Table 4. Cytotoxic Effect of Test Compounds on Three Cell Lines

	CC_{50}, μM (95% CL[b])		
compound[a]	HeLa	MRC5	A549
PGG	108.8 (98.1–120.8)	108.5 (101.8–115.7)	167.7 (166.7–168.7)
PA	>3000	>3000	>3000
MG	357.6 (338.8–69.4)	244.8 (218.0–274.9)	658.2 (645.1–671.6)
BA	>5000	>5000	>5000

[a]Abbreviations are the same as in Figure 1 and in the text. [b]CL denotes confidence limit.

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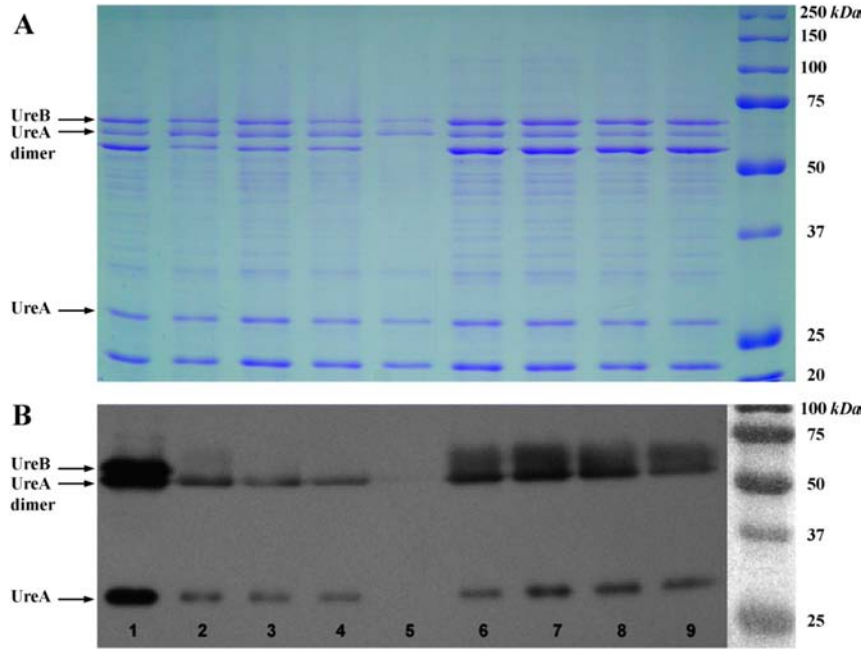

Figure 6. SDS-PAGE (A) and Western blot profiles (B) of *H. pylori* ATCC 43504 urease treated with PGG and MG. Abbreviations are the same as in Figure 1 and in the text. Lanes: (1) ATCC 43504 urease; (2) 250 mg/L PGG; (3) 500 mg/L PGG; (4) 1000 mg/L PGG; (5) 1500 mg/L PGG; (6) 250 mg/L MG; (7) 500 mg/L MG; (8) 1000 mg/L MG; (9) 1500 mg/L MG.

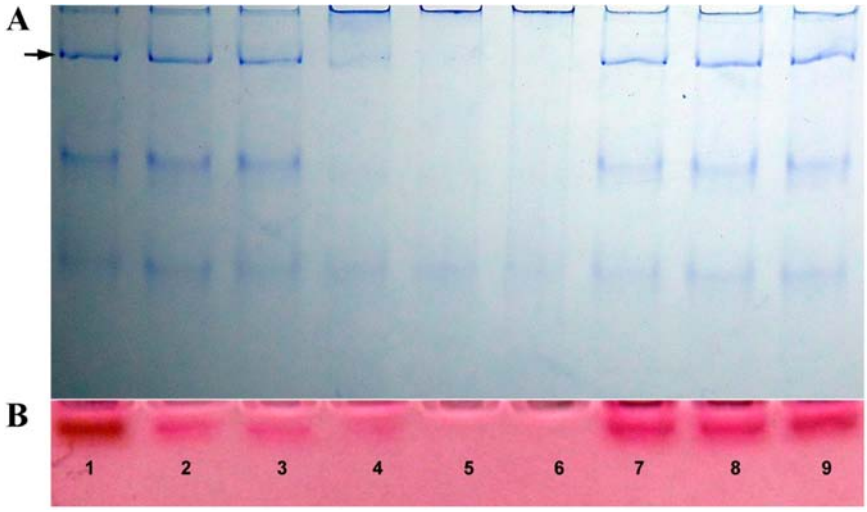

Figure 7. Native PAGE profile and zymogram of *H. pylori* ATCC 43504 urease treated with test compounds. Abbreviations are the same as in Figure 1 and in the text. Lanes: (1) ATCC 43504 protein; (2) 1000 mg/L AHA; (3) 1000 mg/L TU; (4) 250 mg/L PGG; (5) 500 mg/L PGG; (6) 1000 mg/L PGG; (7) 250 mg/L MG; (8) 500 mg/L MG; (9) 1000 mg/L MG. Arrow indicates urease band position.

Native PAGE profile and zymogram of *H. pylori* ATCC 43504 urease treated with the test compounds are given in Figure 7. The urease band disappeared at 250 mg/L PGG but did not disappear even at 1000 mg/L of AHA, TU, or MG on the native gel (Figure 7A). Moreover, the intensity of the urease band at 1000 mg/L PGG obviously decreased on the activity-stained gel as compared to that of AHA and TU (Figure 7B). Treatment with MG did not cause a decrease in the intensity of the urease band at all concentrations tested (Figure 7B).

■ DISCUSSION

Plants and their constituents are a potential for *H. pylori* therapy because some are selective and biodegrade to nontoxic products.[30] Certain plant preparations and their constituents are highly effective toward antibiotic-resistant *H. pylori* strains,[31,32] and are likely to be useful in resistance management strategies. The mode of action and the resistance mechanisms of antimicrobial agents have been well reviewed by Gerrits et al.[33] and Francesco et al.[34] In the present study, the antibacterial principles of *P. lactiflora* root were identified as the aryl ketone PA (**1**), the simple benzoic acids BA (**2**) and MG (**3**), and the *gluco*-hexose PGG (**4**). PGG, MG, and PA exhibited the pronounced growth-inhibiting and bactericidal activity, although the concentration of these constituents was lower than that of the test antibiotics. These four constituents exhibited growth-inhibiting and bactericidal activity toward five strains resistant to amoxicillin (strains PED 503G and B),

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clarithromycin (strain PED 3582GA), metronidazole (starins 221 and ATCC 43504), or tetracycline (strains PED 3582GA and B). These present findings that BA, MG, PA, and PGG are virtually equal in antibacterial activity to both antibiotic-susceptible and -resistant strains of *H. pylori* suggest that these constituents and the penicillin amoxicillin, the macrolide clarithromycin, the nitroimidazole metronidazole, or tetracycline do not share a common mode of action. In addition, these constituents showed no significant cytotoxicity toward HeLa, MRC5, and A549 cell lines, suggesting their anti-*H. pylori* activity might not be due to general toxicity. This original finding indicates that materials derived from *P. lactiflora* root may hold promise for the development of novel and effective antibacterial products even toward currently antibiotic-resistant *H. pylori*. PGG possesses potent antibacterial activity on various strains of *H. pylori*.[35]

Investigations on the modes of action and the antibacterial characteristics of naturally occurring antibacterial agents may contribute to the development of selective *H. pylori* control alternatives with novel target sites and low toxicity. Although the modes of action of phytochmeicals such as alkaloids, phenolics, and terpenoids toward *H. pylori* have not been clearly defined or understood, major mechanisms of phenolics proposed include membrane permeability changes, disruption of proton motive force and membrane-associated functions, and/or intracellular acidification, resulting in disruption of H^+,K^+-ATPase required for ATP synthesis of microbes.[36] In addition, conversion of the spiral to the coccoid form in *H. pylori* is caused by antibiotic treatment.[12] The coccoid form has a lower level of metabolism and of protein and DNA synthesis than the spiral form.[37] The former is less efficient than the latter in adhering to gastric epithelial cells and is considered nonmotile, which may be a disadvantage for colonizing the gastric mucosa.[38,39] It has been suggested that coccoid forms consist of the living bacteria and the dying ones.[40,41] Unlike the former forms, the dying forms are not capable of recovering their virulence and causing the occurrence of diseases.[40,41] In the present study, a proportional relationship between the coccoid number induced by the *P. lactiflora* root constituents and their bactericidal property was observed. This proved that most of the coccoid forms induced by the constituents were the morphologic manifestation of bacterial cell death. In addition, BA and PA showed strong bactericidal effect at pH 4, while MG and PGG were effective at pH 7. Interestingly, at 640 mg/L, PA and BA exhibited more pronounced bactericidal activity than PGG at low pH (4 and 5). This finding suggests that PA and BA may be more effective antibacterial agents than MG and PGG in stomach. The exact anti-*H. pyoli* mechanism of the *P. lactiflora* root constituents remains to be proven, although the partially hydrophobic nature of phenolic phytochemicals allows for accumulation and attachment in the bacterial cytoplasmic membrane, where inhibitory effects may eventually lead to cell death.[25]

It is suggested that *H. pylori* urease functions as both a colonization factor and a virulence factor because of the production of ammonia, which may contribute to the development of gastritis and peptic ulceration.[2,42,43] The native urease is an extracellular cell bound enzyme constituting 5–10% of the total protein with a molecular mass of ∼580 kDa consisting of six 66 kDa (UreB) and six 32 kDa (UreA) subunits.[44] The present finding clearly indicates that PGG and MG inhibited *H. pylori* urease activity. The urease inhibitory activity of PGG was comparable to that of AHA, while MG was less potent at inhibiting urea than TU. Goldie[10] reported that urease inhibitory activity of AHA was only temporary: it showed strong inhibition activity after 4 h, but no activity after 24 h. It has been suggested that the weak urease inhibitory activity of AHA might be explained by prolonged exposure of gels to substrate solution and a consequent degradation of AHA.[45] Moroever, PGG and MG exhibited antibacterial activity toward *H. pylori*, although AHA does not affect the survival of the organism.[10] In addition, urease bands and urease production were faintly visible and disappeared at 250 mg/L PGG on Western blot and native PAGE. The urease inhibitory activity of PGG was higher than that of MG, and the band intensity of PGG was lower than that of MG. The mechanisms by which PG and MG inhibit urease remain to be proven. BA and PA exhibited weak and no urease inhibitory activity, respectively, despite their growth inhibitory and bactericidal activity.

QSAR of phytochemicals in many microorganisms has been well studied. It has been reported that chemical structure, hydrolyzable group, hydrophobicity, hydrogen-bonding, and molecular size parameters of terpenoids are associated with antimicrobial activity.[46] In the present study, introduction of functional groups, such as hydroxyl or methoxy, in AP as basic structure significantly increased the growth inhibitory and bactericidal activities. The existence of hydroxyl group (2′,4′-DHAP) or methyl group (*p*-HPP) in *p*-HAP significantly increased the antibacterial activity. Hydroxyl group (2′,6′-DHMAP) in PA significantly increased the antibacterial activity, whereas methoxy group did not affect the antibacterial activity. Substitution of hydroxyl group in *p*-HPP to methoxy group (PA) significantly increased the antibacterial activity. The aldehyde SA exhibited significantly more antibacterial activity than either the ketones (except for 2′,6′-DHMAP), the phenol 2-EP, the acid 2,4-DHCA, or the alcohol 2-HBA. In addition, log *P* was correlated negatively with MIC and MBC. These present findings indicate that structural characteristics, such as types of functional groups and carbon skeleton, and hydrophobicity rather than MW appear to play a role in determining the growth inhibitory and bactericidal activity toward *H. pylori*.

In conclusion, *P. lactiflora* root-derived preparations containing MG, PA, and PGG could be useful as sources of antibacterial products for prevention or eradication from humans from diseases caused by *H. pylori* in the light of their activity toward antibiotic-resistant *H. pylori* strains. The anti-*H. pylori* action of the constituents may be an indication of at least one of the pharmacological actions of *P. lactiflora*. For practical use of *P. lactiflora* root-derived materials as novel anti-*H. pylori* products to proceed, further studies are needed to establish their human safety and whether this activity is exerted in vivo after consumption of *P. lactiflora* root-derived products by humans. Historically, *P. lactiflora* root (2–4 g of dried root/3 times/day) is used as analgesic, hemostyptic, and bacteriostatic agents.[47] In addition, formulations for improving antibacterial potency (e.g., in combination with other antimicrobials[33]) and stability need to be developed.

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