

Inhibitory Effect of Ponciretin on *Helicobacter pylori* VacA Toxin-induced Vacuolation in HeLa Cells

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Abstract The inhibitory effects of flavanone derivatives on *Helicobacter pylori* (HP) growth, infection and VacA toxin-induced vacuolation were investigated. Among flavanones tested, ponciretin potently inhibited the growth of HP with a MIC value of 0.01 mg/ml and VacA toxin-induced vacuolation in HeLa cells with IC₅₀ value of 0.078 mM. However, other flavanones inhibited neither HP growth nor VacA toxin-induced vacuolation. All flavanones tested did not inhibit HP infection to KATO III cells. Ponciretin also inhibited activation of procaspase-3 to caspase-3 in HeLa cell induced by HP VacA toxin, but did not affect Bax and Bcl-2 protein levels. These findings indicate that ponciretin inhibits growth as well as vacuolation by HP VacA toxin, which induces cell death *via* proteolytic activation of a cascade of caspases.

Key words: *Helicobacter pylori*, ponciretin, VacA toxin, vacuolation, caspase-3

Helicobacter pylori (HP) was first isolated from the gastric antrum of chronic gastritis patients by Warren and Marshall in 1983 [28]. Pathogenic HP produces urease and vacuolating toxin, and HP urease hydrolyzes urea to CO₂ and ammonia. This ammonia generated by HP protects itself from the environment of gastric acid in stomach, and directly damages the gastric mucosal cell.

Jones *et al.* [16] demonstrated that HP induced epithelial gastric cell apoptosis, and Boquet *et al.* [4] reported that the apoptosis originated from its VacA toxin and was related to caspase-3 activation. Maeda *et al.* [25] reported that Bcl-2 and Bax, which are associated with mitochondria related apoptosis, were not affected by VacA toxin of HP. Furthermore, evidence suggests that HP vacuolating toxin (VacA toxin) may be potentiated by urease-mediated ammonia

production [7, 10, 27]. Therefore, the inhibitions of HP growth, infection, urease, and VacA toxin vacuolation appear to be important for the treatment of patients with gastritis and peptic ulcer.

To eradicate the HP, antibiotics, ampicillin and tetracyclines, have been used. However, the occurrence of pathogens resistant to these antibiotics and their side-effects, implies a need for other effective agents [13]. Recently, researchers have isolated anti-HP compounds, such as ponciretin, catechins, magnolol, and decursin, from herbal sources [1, 2, 20, 24, 29]. However, the inhibitory effects of these compounds on infection and vacuolation of HP have not thoroughly been studied. Therefore, we examined *in vitro* inhibitory effects of flavanone derivatives, particularly ponciretin, on the growth, infection and VacA toxin-induced vacuolation of HP.

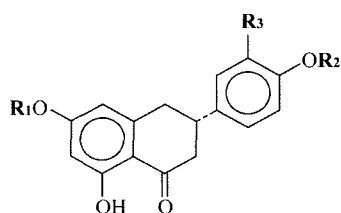
MATERIALS AND METHODS

Materials

Bacto agar and Brucella broth were purchased from Difco laboratories (Detroit, MI, U.S.A.), fetal bovine serum (FBS) and antibiotic-antimycotic solution were from Gibco BRL (Rockville, MD, U.S.A.), naringin, naringenin, hesperidin, hesperetin, cell culture medium, neutral red and horse serum were from Sigma Chemical Co. (St. Louis, U.S.A.), and protease inhibitor cocktail reagent was purchased from Roche Co. (Mannheim, Germany). The anti-mouse Bax, Bcl-2, procaspase-3 and β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Enhanced chemiluminescence (ECL) immunoblot system was from Pierce Co. (Rockford, IL, U.S.A.). AnaeroPack Campylo was from Mitsubishi Gas Chemical Co., Inc. (Tokyo, Japan). Poncirin, poncirenin and ponciretin (Fig. 1) were isolated according to the methods previous reported [2].

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	R1	R2	R3
Hesperidin	Rha (β 1 \rightarrow 6) Glc-	CH ₃	OH
Neohesperidin	Glc-	CH ₃	OH
Hesperetin	H	CH ₃	OH
Naringin	Rha (β 1 \rightarrow 2) Glc-	H	H
Prunin	Glc-	H	H
Naringenin	H	H	H
Poncirin	Rha (β 1 \rightarrow 2) Glc-	CH ₃	H
Poncirenin	Glc-	CH ₃	H
Ponciretin	H	CH ₃	H

Fig. 1. Structure of flavanone derivatives.

Bacterial Strains and Isolation of VacA Toxin from HP

HP strain ATCC 49503 and ATCC 43504 were purchased from American Type Culture Collection (Manassas, VA, U.S.A.). They were inoculated to Brucella agar plates supplemented with 7% horse serum and transferred into Brucella broth containing 10% FBS after 3 days. The bacteria were cultured for a further 3 days at 37°C in a thermostatic rotary shaker under microaerophilic conditions (AnaeroPack Campylo: 85% N₂, 10% CO₂ and 5% O₂).

Vacuolating cytotoxin (VacA) was purified according to the modified method of Cover and Blaser [8]. HP 60190 (ATCC 49503) was used as the source of VacA toxin purification. HP was cultured for 72 h at 37°C in Brucella broth containing 10% FBS in an ambient atmosphere containing 5% oxygen. The culture was centrifuged at 6,000 \times g for 30 min, and proteins present in the supernatant were precipitated with 50% saturated ammonium sulfate solution. After centrifugation at 10,000 \times g for 40 min, the pellet was resuspended in 60 mM Tris-HCl (pH 7.5). Hydrophobic interactive chromatography was performed on Q-Sepharose column (1 \times 5 cm) with the same buffer containing 0.6 M ammonium sulfate and eluted with the same buffer containing 0.4 M ammonium sulfate. The vacuolation-active fractions were dialyzed against the same buffer.

Assay for Cytotoxicity of VacA

HeLa cells were cultured as a monolayer in plastic flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1% antibiotic-antimycologic solution and 3.5 g/l sodium bicarbonate under 5% CO₂ at 37°C. Attached cells

were released with trypsin/EDTA and seeded at a density of 7.0 \times 10³ cells/well (180 μ l volume) in 96-well tissue culture plates 1 day before experiments. Seeded HeLa cells were incubated with partially purified VacA 0.05 mg/ml. Cytotoxicity of VacA in HeLa cells was measured by MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [5]. All assays were performed in triplicate.

Assay for the Inhibitory Activity of HP-induced Vacuolation

HeLa cells were cultured as a monolayer in plastic flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1% antibiotic-antimycotic solution and 3.5 g/l sodium bicarbonate under 5% CO₂ at 37°C. Attached cells were released with trypsin/EDTA and seeded at a density of 7.0 \times 10³ cells/well in 96-well tissue culture plates 1 day before experiments.

Inhibitory effect of test agents on VacA-induced vacuolation in HeLa cells was measured by neutral red uptake assay [9]. Briefly, seeded HeLa cells were incubated for 16 h with partially purified toxin and serial dilutions of samples in a microtiter plate. To detect the vacuoles, cells were incubated for 8 min at room temperature with 100 μ l of 0.05% neutral red in phosphate-buffered saline (PBS) and washed twice with 0.9% NaCl containing 0.1% BSA. After the addition of 100 μ l of acidified ethanol solution (70% ethanol, 0.36% HCl), optical density of extracted neutral red was measured at 540 nm using a microtiter plate reader (Molecular Devices Emax, CA, U.S.A.). All assays were performed in triplicate. Each inhibition rate was obtained at each concentration using the following Equation 1, and the values of IC₅₀ were then calculated from the data using a probit method.

Equation 1:

Inhibition rate (%)

$$= \frac{\text{Absorbance of control group} - \text{Absorbance of sample group}}{\text{Absorbance of control group} - \text{Absorbance of normal group}} \times 100$$

Immunoblot Analysis

Immunoblot assay for procaspase-3, Bcl-2 and Bax protein expression levels was performed according to the method of Maeda *et al.* [25]. The HeLa cells were treated with VacA toxin and/or ponciretin and collected. The collected cells were suspended in lysis buffer (10 mM Tris HCl, pH 8.0, buffer containing 1.5 mM MgCl₂, 1 mM DTT, 0.1% NP40 and protease inhibitor cocktail), lysed and then centrifuged. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the supernatant solution was performed and then transferred electrophoretically to a PVDF membrane. The membrane was probed with the antibodies for procaspase-3, Bcl-2 and Bax proteins, and an ECL detection assay was then performed according to the manufacturer's instructions.

Inhibition Assay of HP Growth and Urease

Activity. Growth and urease inhibition assay of HP ATCC 43504 was performed according to the method previously reported [14, 18].

Assay of HP Infection-Inhibitory Activity

KATO III cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% antibiotic-antimycotic solution and 2.2 g/l sodium bicarbonate under 5% CO₂ at 37°C. The cells were harvested with trypsin/EDTA for bacterial infection experiment [17]. Serial dilutions of samples were incubated with an equal volume of HP suspension in PBS for 30 min in a 37°C water bath and mixed with KATO III cells (5.0×10⁶ cells/ml). After further 1 h of incubation, incubation mixture was loaded onto 15% sucrose and centrifuged. The precipitate cells were washed once with PBS and then the amount of ammonia released from urea was determined by the phenol-hypochlorite urease assay as previously described [14].

Statistical Analysis

All the data were expressed as mean±standard deviation, and the statistical significance was determined using Student's *t*-test.

RESULTS

Inhibitory Activity of Flavanone Derivatives on HP Growth, Infection and VacA-Induced Vacuolation

To evaluate anti-HP activity of flavanone derivatives, their inhibitory activities against HP growth were measured (Table 1). Ponciretin potently inhibited HP growth with a

Table 1. Inhibitory effects of some flavanones on the growth, infection and urease activity of *H. pylori*.

Agent	MIC ^a (mg/ml)	IC ₅₀	
		Infection (mg/ml)	Urease activity
Poncirin	>0.1	>0.1	>0.1
Poncirenin	>0.1	>0.1	>0.1
Ponciretin	0.01	>0.1	>0.1
Naringin	>0.1	>0.1	>0.1
Prunin	>0.1	>0.1	>0.1
Naringenin	0.08	>0.1	>0.1
Hesperidin	>0.1	>0.1	>0.1
Neohesperidin	>0.1	>0.1	>0.1
Hesperetin	0.08	>0.1	>0.1
Ampicillin	0.001	- ^b	>0.1
Acetohydroxamic acid	-	-	0.04

^aHP ATCC 43504 was used for minimum inhibitory concentration (MIC) assay.

^bnot determined.

Table 2. Inhibitory effects of some flavanones on HP VacA-induced vacuolation.

Agent	IC ₅₀ (mM)
Poncirin	>0.4
Poncirenin	>0.4
Ponciretin	0.078
Naringin	>0.4
Prunin	>0.4
Naringenin	0.36
Hesperidin	>0.4
Neohesperidin	>0.4
Hesperetin	>0.4

MIC value of 10 µg/ml. Naringenin and hesperetin weakly inhibited the growth. However, other flavanone derivatives tested did not inhibit HP growth. We also measured the inhibitory effect of these flavanone derivatives on HP infection into KATO cells and urease activity. However, all flavanone derivatives tested did not inhibit HP infection and urease activity.

The inhibitory effect of these flavanone derivatives on VacA toxin-induced vacuolation in HeLa cells was investigated (Table 2). Ponciretin inhibited VacA-induced vacuolation in HeLa cells with IC₅₀ value of 0.078 mM. Naringenin weakly inhibited it, however, other flavanones tested did not inhibit it.

Inhibitory Effect of Ponciretin on Apoptosis of HeLa Cells by VacA-induced Vacuolation

VacA toxin was partially purified from HP ATCC 49503 by ammonium sulfate precipitation and Q-Sepharose column chromatography, and cytotoxicity of VacA toxin against HeLa cells was then measured (Fig. 2). Its cytotoxicity was increased time-dependently. Ponciretin inhibited the VacA

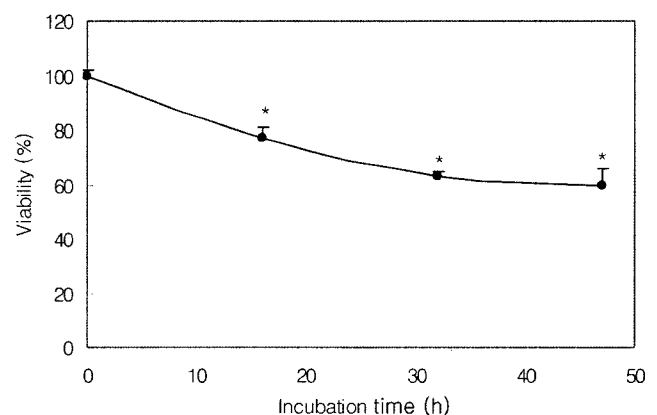


Fig. 2. Cytotoxicity of HP VacA toxin.

Cytotoxicity assayed by MTT assay. Results are expressed as percentages of viable cells. A concentration of VacA toxin treated was 0.05 mg/ml. Values are mean±standard deviation of three independent experiments (**p*<0.05).

toxin-induced vacuolation (Fig. 3), however, its glycoside poncirin did not inhibit the VacA toxin vacuolation (Fig. 4).

To understand the inhibitory mechanism of ponciretin against HP VacA toxin vacuolation, an apoptotic marker, procaspase-3, level in HeLa cells treated with VacA toxin was measured (Fig. 5). VacA toxin significantly decreased procaspase-3 protein level, however, ponciretin significantly inhibited the decrease of procaspase-3 protein level induced by VacA toxin. Therefore, we also measured other apoptotic markers such as Bcl-2 and Bax expression levels in the cytosol of HeLa cells treated with VacA toxin and/or ponciretin, however, VacA toxin did not significantly affect these expression levels of HeLa cells.

DISCUSSION

Flavonoids exhibit antiinflammatory, anticancer, and antioxidant activities [6, 15, 26]. Recently, it has been

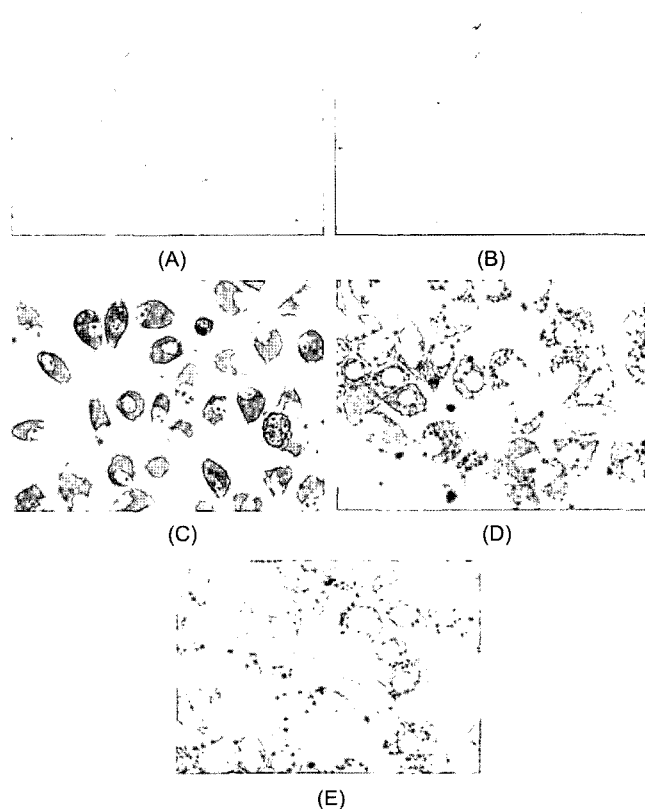


Fig. 3. Inhibitory effect of ponciretin on VacA-induced vacuolation in HeLa cells.

HeLa cells were cultured in DMEM containing 10% FBS. VacA toxin (0.05 mg) and sample (0.075 mM and 0.15 mM) were treated, and then incubated for 16 h. (A) treated with medium alone and stained with neutral red; (B) treated with 0.15 mM ponciretin alone; (C) treated with VacA toxin alone and stained with neutral red; (D) treated with VacA toxin and 0.075 mM ponciretin, and stained with neutral red; (E) treated with VacA toxin and 0.15 mM ponciretin, and stained with neutral red. Arrow indicates vacuolations in HeLa.

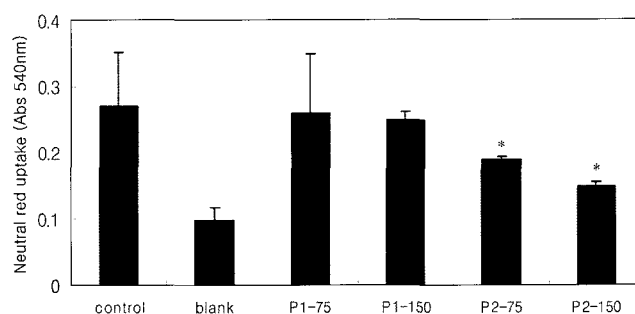


Fig. 4. Inhibitory effects of ponciretin and poncirin on neutral red dye uptake by VacA-treated HeLa cells.

HeLa cells were incubated with VacA toxin and test compounds, and stained with 0.05% neutral red solution. Poncirin and ponciretin were applied at concentrations of 0.075 mM and 0.15 mM: blank, treated with vehicle alone; control, treated with VacA toxin; P1-75, treated with 0.075 mM poncirin and VacA toxin; P1-150, treated with 0.150 mM poncirin with VacA toxin; P2-75, treated with 0.075 mM ponciretin with VacA toxin; P2-150, treated with 0.15 mM ponciretin and VacA toxin. A concentration of VacA toxin treated was 0.05 mg/ml. Values are mean±standard deviation of three independent experiments (n=3). *Significantly different to control ($p < 0.05$).

reported that these compounds show the inhibition against HP growth [2, 3, 11, 21, 24]. However, the effect of the flavonoids on infection and vacuolation of HP have not thoroughly been studied. Therefore, the present study examined whether flavanone derivatives exhibited HP growth-inhibitory activity, and investigated their inhibitory effects *in vitro* on the growth and vacuolation of HP VacA toxin as well as infective properties of HP against HeLa cells. All these compounds did not inhibit the infection of HP into KATO III cells. However, among flavanone derivatives tested, ponciretin potently inhibited HP vacuolation into HeLa cells: ponciretin showed the most potent inhibition against VacA toxin-induced vacuolation with IC_{50} value of 0.078 mM. It has been reported that ponciretin exhibits an antioxidant action. Therefore, we first investigated VacA vacuolation-inhibitory effect of antioxidant agents ascorbic acid and glutathione. However, these antioxidants did not exhibit antivacuolation activity (Data not shown), and did not affect cell viability under the present experimental condition. Second, the effect of VacA toxin on the production of nitric oxide in HeLa cells was examined, and glutathione content assay was then performed by an enzymatic recycling technique. However, no significant change of glutathione content by VacA was observed (Data not shown).

Jones *et al.* [16], Maeda *et al.* [25], and Kim *et al.* [19] demonstrated that HP VacA toxin-induced apoptosis is related to caspase-3 activity. Therefore, to investigate the effect of ponciretin on apoptosis of HeLa cells by VacA toxin, we measured procaspase-3 level in HeLa cells. VacA toxin significantly decreased procaspase-3 protein level, in support by the report of Maeda *et al.* [7] that VacA toxin

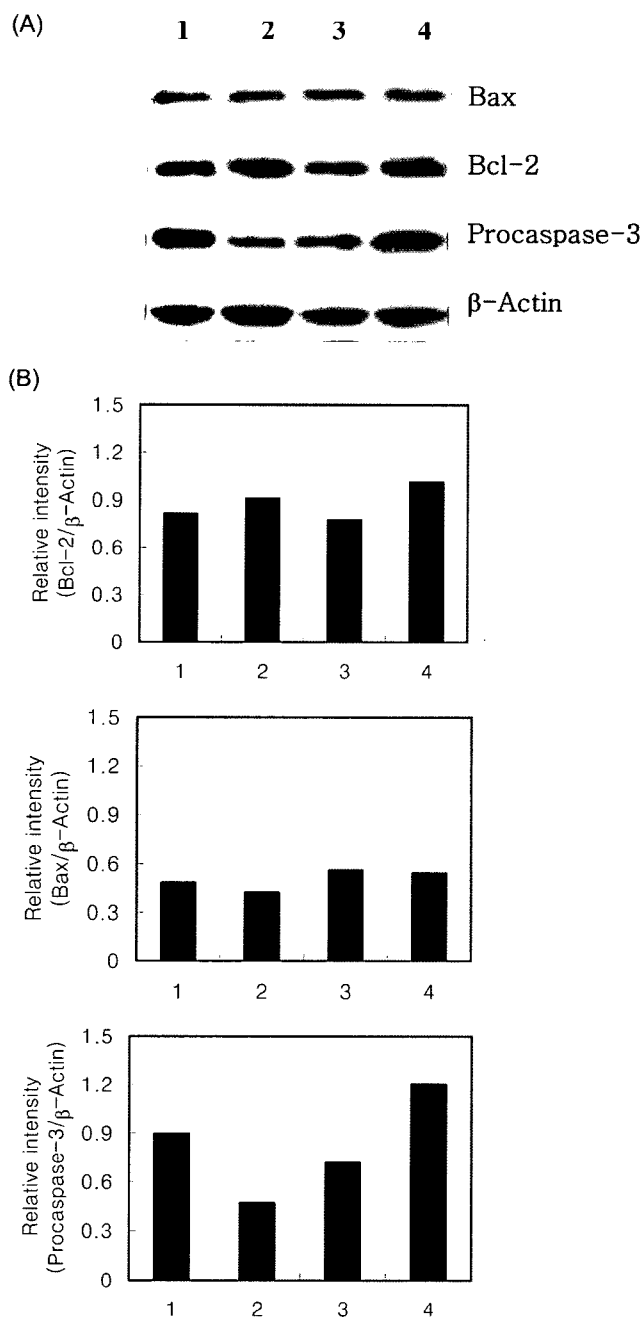


Fig. 5. Inhibitory effect of ponciretin on Bcl-2, Bax and procaspase-3 protein levels of HeLa cells by VacA toxin.

(A) Immunoblot of Bcl-2, Bax and procaspase-3 proteins from protein samples, treated in Materials and Methods. (B) Relative intensity of (A) protein immunoblots (Bcl-2, Bax or procaspase-3 protein/ β -Actin). 1, HeLa cell treated with vehicle alone; 2, HeLa cell treated with VacA toxin (0.05 mg); 3, HeLa cell treated with VacA toxin (0.05 mg) and ponciretin (0.075 mM); 4, HeLa cell treated with VacA toxin (0.05 mg) and ponciretin (0.15 mM).

activates procaspase-3 to caspase-3. The antivacuolating agent, ponciretin, potently inhibited the reduction of procaspase-3 protein level induced by VacA.

VacA toxin, which had mainly been regarded as a cytotoxin of the gastric epithelial cell layer, now turned out to be a potent immunomodulatory toxin, targeting the adapted immune system [9, 23]. VacA toxin is able to induce apoptosis in epithelial cells [12]: Apoptosis is characterized by plasma membrane blebbing, shrinkage, chromatin condensation, chromosomal DNA fragmentation, and formation of membrane-bound apoptotic bodies [4, 22]. Two major pathways leading to apoptosis have been described. One pathway involves apoptosis mediated by death receptors, such as CD95 (Fas) and tumor necrosis factor receptors. It processes effector caspases in the last step. In the other pathway, various proapoptotic signals converge at the mitochondria, provoking translocation of cytochrome c from the mitochondria to the cytoplasm. Bcl-2 family members are associated with mitochondria related apoptosis. While cell survival-promoting molecules such as Bcl-2 and Bcl-X, localized at the outer mitochondrial membrane, prevent translocation of cytochrome c from the mitochondria, induced expression or enforced dimerization of Bax results in mitochondrial dysfunction leading to cytochrome c release. These results support that the decrease of procaspase-3 protein may be due to activation of procaspase-3 to caspase-3. The antivacuolating agent ponciretin potently inhibited the reduction of procaspase-3 protein level induced by VacA. Nevertheless, no significant change was detected in the expression of Bcl-2 and Bax. These results suggest that HP VacA toxin may induce cell death transmitted via the proteolytic activation of a cascade of caspases, and that ponciretin can inhibit the apoptotic signaling. Ponciretin also exhibited the inhibitory effect against HP growth, as previously reported [2]. Based on these findings, we suggest that ponciretin may improve gastric cell death by HP via antivacuolating and growth-inhibitory activities.

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