Solanum lyratum Extract Inhibits *Helicobacter pylori*-mediated Apoptosis in Human Gastric Epithelial Cells

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Abstract. *Helicobacter pylori infection is associated with chronic gastritis, peptic ulcers, and gastric cancer. The effects of Solanum lyratum extract (SLE) on anti-H. pylori activity and H. pylori-induced apoptosis were investigated. SLE showed a moderate ability in inhibiting growth of H. pylori and also in interrupting the association of bacteria with host cells. SLE was also able to suppress H. pylori-induced apoptosis. SLE inhibited caspase-8 activation, thereby preventing the release of cytochrome c from mitochondria and activation of the subsequent downstream apoptotic pathway. Thus, SLE may offer a new approach for the treatment of H. pylori by down-regulation of apoptosis in the H. pylori infected gastric epithelium. As it does not directly target bacteria, SLE treatment might not cause development of resistant strains.*

Helicobacter pylori is a Gram-negative microaerophilic bacterium and is associated with chronic gastritis, peptic ulcers, and gastric cancer (1, 2). There is increasing evidence showing that apoptosis plays an important role in the pathogenesis of infectious diseases, carcinogenesis and autoimmune diseases (3). *H. pylori* infection affects the balance between gastric epithelial proliferation and apoptosis in the stomach (4). Several studies have indicated that the infection can induce apoptosis in gastric epithelial cells and subsequently results in cell proliferation in order to maintain the homeostasis of the gastrointestinal mucosa (4, 5). *H. pylori*-induced apoptosis occurs mainly through the death receptor, leading to the cleavage of pro-caspase-8 and *Bid*, release of cytochrome *c* from mitochondria, and activation of the subsequent downstream apoptotic pathway (6, 7).

Several treatments are used as a means of eradicating *H. pylori* infection, all include at least one antibiotic in combination with a proton pump inhibitor (1, 8). The effectiveness of these treatments has been impaired by increasing drug resistant strains (9, 10). Therefore, finding a safe and efficient treatment to eradicate infection in humans is important.

Many herbal extracts of Solanaceae plants have been shown to be potent in the treatment of various infections (11-15). The methanolic extract of *Solanum torvum* fruit showed antimicrobial activity against *Actinomyces pyogenes*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pyogenes* (15). The aqueous and methanolic extract of the berries of *S. aculeastrum* showed moderate antimicrobial activity against *S. aureus* and *B. subtilis* (12). *S. palinacanthum* prevented the growth of *A. aeruginosa*, *B. subitilis* and *S. aureus* (13).

S. lyratum is one of the most valued Chinese traditional medicines for regulating body immune function (16), and for its antianaphylactic activity (17). Moreover, the antitumor activity of its extracts has been observed both *in vitro* and *in vivo* (18-22). Therefore, *S. lyratum* is usually used as an anticancer drug to treat cancer of the liver, lung and esophagus (23). Yet there has been no report as to whether it can inhibit *H. pylori*-induced gastric caner. This study evaluates the antimicrobial activity of *S. lyratum* extract (SLE) and also the effects of SLE on suppressing *H. pylori*-induced apoptosis of human gastric epithelial AGS cells.

Materials and Methods

Plant material and preparation of the SLE. S. lyratum was collected from Nantou County Sinyi Township Dongpu located in the middle of Taiwan in September 2002. Voucher specimens (CMU SL 0222) were deposited in the School of Chinese Medicine Resources, China

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Key Words: Helicobacter pylori, *Solanum lyratum*, human gastric epithelial cells, apoptosis.

Medical University, Taichung, Taiwan. *S. lyratum* (600 g) was extracted repeatedly with 50% ethanol at room temperature. The combined ethanol extracts were filtered and evaporated under reduced pressure to yield a brownish viscous residue (58.44 g). For the present experiments, the crude extracts were dissolved in phosphate-buffered saline (PBS).

Bacterial and cell culture. H. pylori strain 26695 (ATCC 700392), the reference strain, was obtained from the American Type Culture Collection (ATCC). The antibiotic resistant strains V633, V1254, V1354, and V2356 were clinical isolates at Taichung Veterans General Hospital, Taichung, Taiwan from a previous study (24, 25) and they are all resistant to both metronidazole and clarithromycin, the antibiotics used nowadays to treat *H. pylori* infection. *H. pylori* was grown on blood agars under microaerophilic conditions at 37˚C for 48-72 h. AGS cell line (ATCC CRL 1739; human gastric adenocarcinoma cell line) was purchased from the ATCC and was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin).

Antimicrobial activity. Disc diffusion method was applied to determine the antimicrobial activity of plant extracts. A suspension of the *H. pylori* (1×10^5 cells) was spread on Mueller-Hinton agar plates supplemented with 5% sheep blood. Filter paper discs (6 mm in diameter) were impregnated with 10 μl of SLE (100-200 mg/ml), clarithromycin, or metronidazole and placed on the inoculated plates. These plates were incubated at 37˚C for 48 h. The diameters of the inhibition zones were measured in mm. All of the tests were replicated four times and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced.

Cell viability assay. AGS cells were seeded onto 24-well plates at a density of 5×10^4 cells/well for 24 h. SLE (0-500 μg/ml) was then added to the cells, while only PBS was used in the control group and cells were grown at 37˚C for 24 h. For determination of cell viability, the trypan blue exclusion protocol was used. Briefly, approximately 10 μl of cell suspension in PBS, pH 7.4, were mixed with 40 μl of trypan blue, and the numbers of stained (dead cells) and unstained cells (live cells) were counted using a hemocytometer. The cell viability was calculated as the percentage of cells surviving after the treatment.

Association assay. AGS cells co-cultured with PBS-resuspended *H. pylori* at a multiplicity of infection (MOI) of 100 were treated with SLE (0-50 μg/ml) in antibiotic-free RPMI-1640 supplemented with 10% fetal bovine serum (FBS). Cell-associated bacteria were quantified 6 h after infection by osmotic lysis of host cells. Cell culture supernatants were removed gently, cells were washed with PBS, and osmotic lysis was performed to calculate the total number of bacteria. For this purpose, sterile water was added to infected cells after washing, cell lysates were resuspened with PBS, and bacterial numbers were determined by plating serial dilutions on chocolate agar plates. The association activity was determined as the mean of triplicates. The results are expressed as the percentage of relative association of *H. pylori* as compared with the control (untreated) group.

Preparation of cell extracts and Western blot analysis. AGS cells were seeded onto 6-well plates at a density of 5×10^5 cells/well for 24 h. Infected cells were treated with SLE (0-50 μg/ml) for 3 h and were then lysed with ice-cold lysis buffer (0.5 M Tris-HCl, pH 7.4, 10% sodium dodecyl sulfate, 0.5 M dithiothreitol). Protein concentration was determined by Bradford method (Bio-Rad, Hercules, CA, USA). Protein sample (20 mg) was loaded and separated on SDS-PAGE using a Hoefer mini VE system (Amersham Biosciences, Piscataway, NJ, USA). Proteins were transferred to a polyvinylidine fluoride (PVDF) membrane (Hybond-P; Amersham) according to the manufacturer's instructions. Following the transfer, the membrane was washed with PBS and blocked for 1 h at 37˚C with 5% fat free milk in PBS and 0.1% of Tween 20 (PBST). The primary antibody (β-actin, caspase-8, Bid, Bad, Bax, cytochrome *c*, caspase-9, and caspase-3; Santa Cruz Biotechnology, CA, USA) was added at a dilution of 1/10000. Blots were incubated with peroxidase-conjugated secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG; Santa Cruz Biotechnology) at a dilution of 1/1000. Following removal of the secondary antibody, blots were washed with PBST and developed by ECL-Western blotting system (Pierce, Rockford, IL, USA). Densities of the obtained immunoblots were quantified by Kodak Digital Science 1D (ver. 2.03) (Kodak, Rochester, NY, USA).

Statistical analysis. The differences between mean values for SLE-treated and control groups were evaluated by Student's *t*-test using the SPSS software program (SPSS Inc., Chicago, IL, USA). Differences with a $p<0.05$ were considered significant.

Table I. *Effect of SLE against H. pylori strains.*

Treatment (mg/ml)	Inhibition zone (mm) *H. pylori* strain				
	26695[c]	V633[d]	V1254[d]	V1354[d]	V2356[d]
SLE (200)	11	10	10.5	13	9
SLE (100)	9.5	–	8	8.5	–
CLR[a] (0.05)	44.25	–	–	–	–
MTZ[b] (0.8)	31	–	–	–	–

[a]Clarithromycin; [b]metronidazole; [c]*H. pylori* strain 26695 (ATCC 700392), the reference strain; [d]dantibiotic-resistant strains, clinical isolates; –no inhibition observed.

Results

SLE inhibited H. pylori growth. SLE was tested for its anti-*H. pylori* activity based on the disc diffusion method. PBS was used as negative control and showed no effect. As shown in Table I, SLE was able to inhibit *H. pylori* reference strain at the concentration of 100 mg/ml with 9.5 mm of inhibition. Clinical isolates were also examined in this study: the strains were isolated from *H. pylori*-positive patients who failed in a triple treatment (lansoprazole, clarithromycin, and metronidazole) (24) and these strains had been tested for multidrug resistance as well (25). As we expected, all the clinical isolates were resistant to clarithromycin (CLR) at 0.05 mg/ml and metronidazole (MTZ) at 0.8 mg/ml, while the reference strain was sensitive

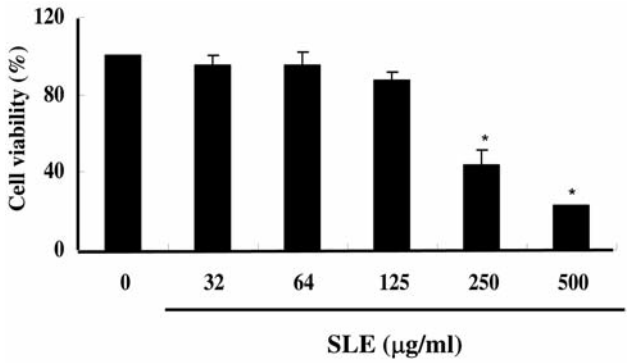

Figure 1. *Effect of SLE on the viability of AGS cells. *The difference was significant at p<0.05.*

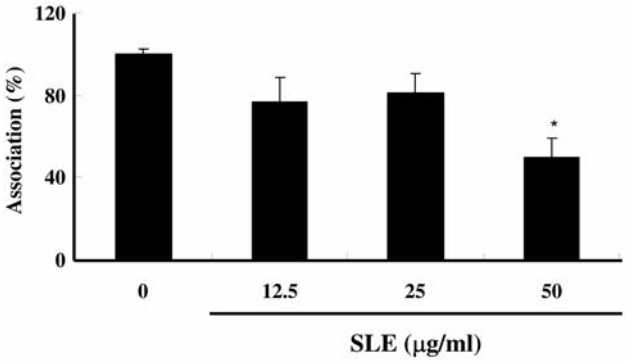

Figure 2. *The effect of SLE on H. pylori association with AGS cells. *The difference was significant at p<0.05.*

to both antibiotics. SLE was able to inhibit the growth of all *H. pylori* strains at 200 mg/ml. V1254, V1354, and the reference strains were still sensitive to SLE at a concentration of 100 mg/ml with inhibition zone of 8-9.5 mm.

Cytotoxicity of SLE on AGS cells. In order to determine the treatment dosage of SLE, the viability of AGS cells on treatment with SLE was assayed. SLE at 250 μg/ml significantly reduced viability by 56% (Figure 1). At a concentration of 64 μg/ml, 95% of AGS cells were not affected after 24 h treatment. For us to investigate the effect of SLE on interaction between AGS cells and *H. pylori*, nontoxic dosages of SLE (<64 μg/ml) were chosen for further assays.

SLE inhibited the association of H. pylori with AGS cells. Because SLE was able to inhibit *H. pylori* growth, we further analyzed the effect of SLE on the association of *H. pylori* with AGS cells (Figure 2). The bacteria associated with host cells included those adhered to and invaded into cells. SLE treatment significantly reduced the association of *H. pylori* with AGS cells. At 50 μg/ml, it caused a 50% decrease in bacterial association as compared with untreated controls. Even at the concentrations of 12.5 and 25 μg/ml, the association activity of *H. pylori* dropped by 20%, although this was not statistically significant. In the range of 12.5-50 μg/ml, SLE did not affect the cell viability.

SLE suppressed the expression of proteins associated with apoptosis. Several studies indicated that the molecular mechanism of *H. pylori*-induced apoptosis in epithelial cells was *via* caspase-8 activation, release of cytochrome *c* from mitochondria, and activation of the subsequent downstream apoptotic pathway (6, 7). Hence, we then further examined the effect of SLE on *H. pylori*-mediated apoptosis in AGS cells. The expressions of caspase-8, Bid, Bad, Bax, cytochrome *c*, caspase-9, and caspase-3 in infected cells were monitored (Figure 3). Compared with untreated cells, SLE significantly suppressed the expression of the above apoptotic proteins in a dose-dependent manner. This indicated that SLE treatment was able to suppress *H. pylori*-mediated apoptosis in AGS cells.

Discussion

S. lyratum is one of the most valued Chinese traditional medicines for regulating body immune function (16), and is also used as an anticancer drug to treat liver, lung, and esophageal cancers (23). This is the first report to show its anti-*H. pylori* activity and anti-apoptotic activity in *H. pylori*-infected cells.

H. pylori is associated with chronic gastritis, peptic ulcers, and gastric cancer. In order to eliminate *H. pylori* infection, several treatments including at least one antibiotic in combination with a proton pump inhibitor have been applied (8). Unfortunately increased antibiotic resistant strains have become more prevalent, increasing the risk of serious health consequences (10). Therefore, finding a safe and efficient treatment to decrease the need for or even replace antibiotics for eradicating *H. pylori* infection is very important.

In this study, SLE showed a moderate ability in inhibiting the growth of *H. pylori*, including multidrug-resistant strains which were isolated from *H. pylori*-positive patients who failed in a triple treatment. The association of *H. pylori* with AGS cells was also disrupted by SLE in a dose-dependent manner. However, the concentrations of SLE used in anti-*H. pylori* activity were much higher than that in association assay. These data suggested that the major action of SLE on treating *H. pylori* infection was to disrupt the interaction between bacteria and cells. Furthermore, studies have showed that apoptosis plays an important role in the pathogenesis of various infectious diseases (3). *H. pylori*

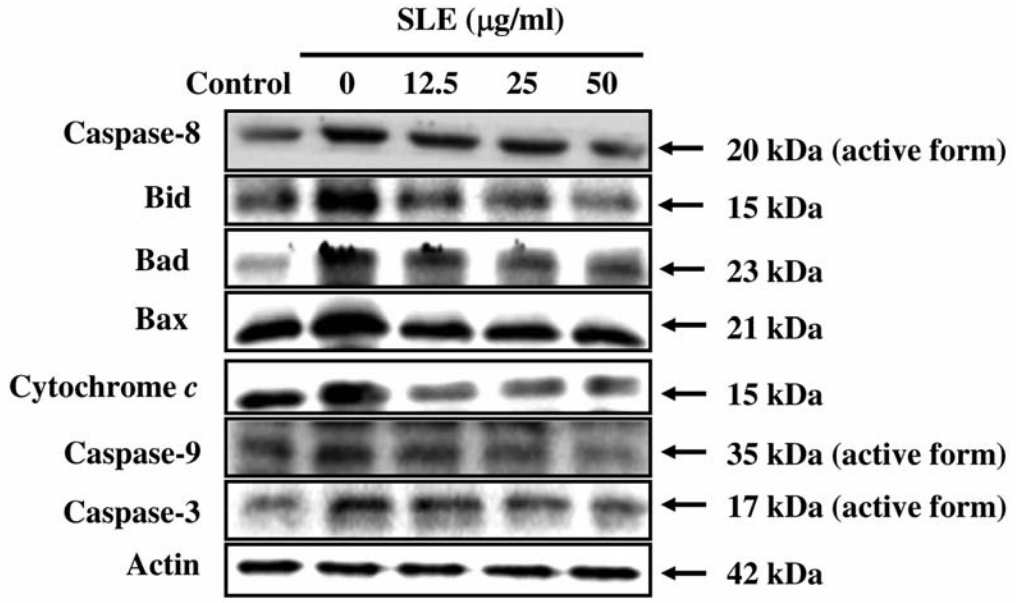

Figure 3. *Effect of SLE on the expression of caspase-8, Bid, Bad, Bax, cytochrome c, caspase-9, and caspase-3 of H. pylori-infected AGS cells. Control group was uninfected and untreated.*

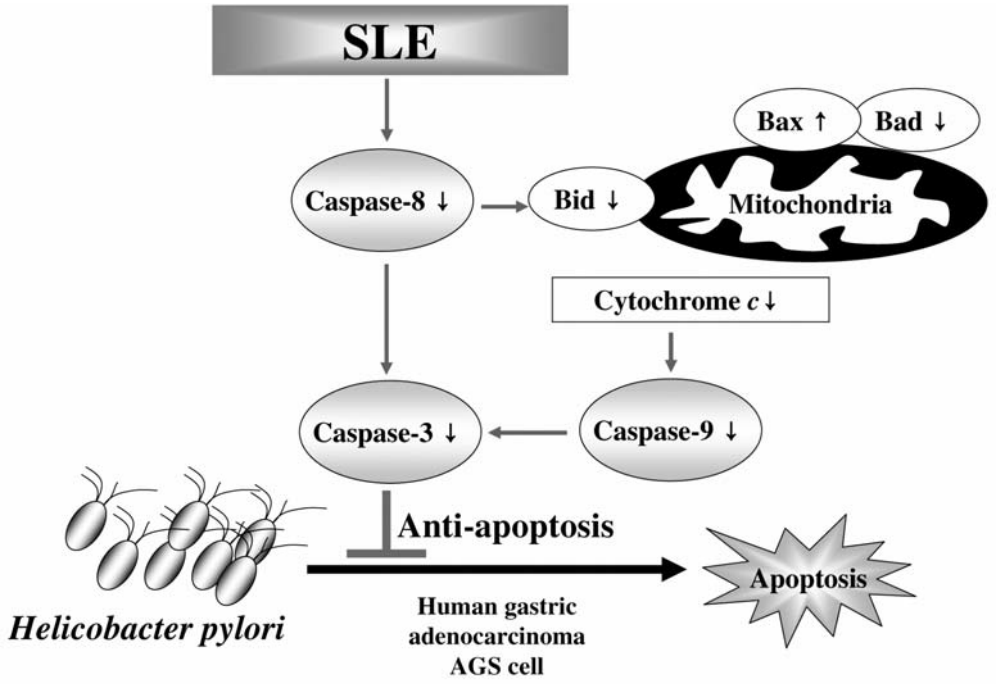

Figure 4. *Hypothetical inhibitory mechanisms of SLE action in H. pylori-mediated apoptosis in AGS cells.*

infection causes apoptosis of gastric epithelial cells (4-7, 26, 27). The apoptotic effect induced by *H. pylori* is an important factor in the pathogenesis of *H. pylori*-induced gastric diseases. *H. pylori* infection might trigger apoptosis in AGS *via* death receptor in the plasma membrane. Activation of death receptors results in the cleavage of pro-caspase-8 to generate caspase-8 which cleaves *Bid*. Increased levels of Bad and Bax facilitate a change in mitochondrial membrane potential, leading to the release of cytochrome *c* and activation of subsequent downstream apoptotic events

(Figure 4). SLE treatment was able to remarkably suppress the apoptotic activity induced by *H. pylori*. Thus, SLE may offer a new approach for the treatment of *H. pylori* by down-regulation of apoptosis in the *H. pylori*-infected gastric epithelium. Since it does not directly targets bacteria, SLE treatment might not cause resistant strains to develop.

Acknowledgements

This investigation was supported by research grants from the Committee on Chinese Medicine and Pharmacy, Department of Health, Executive Yuan, Taiwan (CCMP96-RD-210 and CCMP97-RD-210) and China Medical University (CMU95-339, CMU96-089, CMU96-065, and CMU97-275).

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Received July 2, 2009
Revised March 16, 2010
Accepted March 18, 2010