In vitro Antibacterial and Morphological Effects of the Urushiol Component of the Sap of the Korean lacquer tree (*Rhus vernicifera* Stokes) on *Helicobacter pylori*

Eradication regimens for *Helicobacter pylori* infection have some side effects, compliance problems, relapses, and antibiotic resistance. Therefore, alternative anti-*H. pylori* or supportive antimicrobial agents with fewer disadvantages are necessary for the treatment of *H. pylori*. We investigated the pH-(5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) and concentration (0.032, 0.064, 0.128, 0.256, 0.514, and 1.024 mg/mL)-dependent antibacterial activity of crude urushiol extract from the sap of the Korean lacquer tree (*Rhus vernicifera* Stokes) against 3 strains (NCTC11637, 69, and 219) of *H. pylori* by the agar dilution method. In addition, the serial (before incubation, 3, 6, and 10 min after incubation) morphological effects of urushiol on *H. pylori* were examined by electron microscopy. All strains survived only within pH 6.0-9.0. The minimal inhibitory concentrations of the extract against strains ranged from 0.064 mg/mL to 0.256 mg/mL. Urushiol caused mainly separation of the membrane, vacuolization, and lysis of *H. pylori*. Interestingly, these changes were observed within 10 min following incubation with the 1 × minimal inhibitory concentrations of urushiol. The results of this work suggest that urushiol has potential as a rapid therapeutic against *H. pylori* infection by disrupting the bacterial cell membrane.

Key Words : *Urushiol;* Helicobacter pylori*; Anti-Infective Agents*

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INTRODUCTION

Helicobacter pylori infection is associated with chronic gastritis, gastric and duodenal ulcers, and gastric cancer in humans (1, 2). Eradication of *H. pylori* results in ulcer healing, reduction of recurrence, and complete cure of lymphomas. Several drug treatment regimens have been developed to eradicate *H. pylori* with a cure rate of up to 90% (3). Antibiotics, proton pump inhibitors, H₂ blockers, and bismuth salts are the typical modalities of treatment (4). However, these regimens have some side effects, compliance problems, relapses, and antibiotic resistance. Therefore, alternative anti-*H. pylori* or supportive antimicrobial agents with fewer disadvantages are necessary for the treatment of *H. pylori*.

Urushiol is an oil found in plants of the Family Anacardiaceae and a major organic component of the sap of the lacquer tree (*Rhus vernicifera* Stokes). It causes an allergic skin rash, inflammation, and irritation on contact, known as urushiol-induced contact dermatitis. Urushiol is a yellow liquid with a boiling point of 200-210℃. It is miscible partially in alcohol and ether, but nearly immiscible in water. Chemically, urushiol is a mixture of several closely related organic compounds. Each consists of a catechol substituted with an alkyl chain that has 15 or 17 carbon atoms (Fig. 1). The alkyl group may be saturated or unsaturated; urushiol oil is a mixture of saturated and unsaturated molecules (5).

Traditionally, despite an allergic reaction to urushiol and lack of rigorous documentation, the lacquer tree was used as a folk
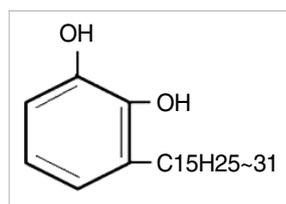
OH

OH

C15H25~31

Fig. 1. Chemical structure of uru-shiol.

remedy for the relief of abdominal discomfort or dyspepsia when administered mixed with boiled chicken in Korea. More-over, some reports have demonstrated that urushiol has both anti-microbial and anti-oxidative activities (6, 7). In this study, we evaluated the pH- and concentration-dependent antibac-terial activity of urushiol from the sap of the lacquer tree against 3 strains of *H. pylori*.

MATERIALS AND METHODS

Isolation of urushiol

The sap (40 mL) of lacquer tree was diluted to a volume of 1 L by the addition of distilled water and extracted with 1 L of n-hexane twice. The hexane extract was concentrated under reduced pressure to yield a brownish oil (26.9 g). The oil was then purified by silica gel column chromatography (Merck 7734) and eluted with 20% acetone/hexane. It was further purified by the same method (Merck 9385), followed by octadecyl silica gel column chromatography (YMC GEL ODS-A) using a gradient of methanol in water to give urushi-ols. The final concentration of extracted urushiol was 10 mg/mL.

Bacterial isolates

Three strains of *H. pylori* (*H. pylori* NCTC11637, *H. pylori* 69 [containing plasmid pBHP69KH: kanamycin resistant strain] and *H. pylori* 219 [ggt isogenic mutant: chlorampheni-col resistant and urease-negative strain]) were obtained from the *H. pylori* Korean Type Culture Collection (HpKTCC, Department of Microbiology, Gyeongsang University Col-lege of Medicine, Jinju, Korea).

H. pylori was isolated from antral mucosal biopsies of pati-ents with chronic gastritis and peptic ulcers. The *H. pylori* strain was identified by colony morphology, Gram staining, motility tests, and biochemical assays such as the urease test, the catalase test, or the oxidase test. *H. pylor* was cultured in an atmosphere 10% CO_2, 5% O_2, and 100% humidity at 37°C. For transport, stab cultures were grown in Wang's media (7.0 g brucella broth, 1.25 g agar [Difco] and 250 mL distilled water) and then frozen in cryomedia in the deep freezer (-70°C). For our study, the frozen strains were processed by thawing, revi-talizing, and lyophilizing. Every 72 hr, we subcultured *H. pylori* strains on Muller-Hinton agar with aged (>2 weeks

old) 5% sheep blood and incubated in a CAMP pouch (7% CO_2) in a 37°C/5% CO_2 incubator. After subculturing twice, we checked the minimal inhibitory concentration of urushi-ol at different pH conditions and evaluated the morphologi-cal effects of urushiol on *H. pylori*.

Minimal inhibitory concentration of urushiol

The agar dilution method was used. Various concentrations of urushiol liquid (0.032, 0.064, 0.128, 0.256, 0.514, and 1.024 mg/mL) were mixed with Mueller-Hinton agar with aged (>2 weeks old) 5% sheep blood. A saline suspension equivalent to a 2.0 McFarland standard (containing 1×10^7 to a 1×10^8 CFU/mL) was prepared from a 72-hr-old subcul-ture from a blood agar plate. The inoculum (1 to 3 L per spot) was replicated directly onto the urushiol-containing agar dilu-tion plates. The plates were kept in a micro-aerobic atmo-sphere produced by the CAMP pouch (7% CO_2) in a 37/5% CO_2 incubator for 3 days. After 3 days incubation, we checked the minimal inhibitory concentration. We then eval-uated the minimal inhibitory concentration values of urush-iol following this same procedure under different pH (5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) conditions.

Ultrastructural analysis of *H. pylori* with electron microscopy

For the ultra-structural analysis, *H. pylori* was incubated in urushiol (0.256 mg/mL) mixed agar. Initial (before incuba-tion) and serial fixations (3, 6, and 10 min after incubation) were done in phosphate-buffered saline containing 2.5% glu-taraldehyde.

For scanning electron microscopy observations, the skin spec-imens were fixed in 2.5% glutaraldehyde for 2 hr at 4°C, wash-ed in 0.1 M phosphate-buffered saline (pH 7.4), postfixed in 1% osmium tetroxide in the same buffer for 90 min at 4°C, dehydrated in a series of ethyl alcohol, exchanged through isoamylacetate, and critical-point dried. The specimens were coated with gold-palladium and then examined under a scan-ning electron microscope (Tabletop microscope, TM-1000, Hitachi high technologies, Tokyo, Japan) at 30 kV.

For transmission electron microscopy observations, the spe-cimens were fixed in 2.5% glutaraldehyde for 2 hr at 4°C, washed in 0.1 M phosphate-buffered saline (pH 7.4), and then postfixed in 1% osmium tetroxide in the same buffer for 90 min. The specimens were then dehydrated through a grad-ed series of ethanol, exchanged through propylene oxide, and embedded in a mixture of Epon. Subsequently, ultrathin sec-tions were obtained by an ultramicrotome (Richert-Jung, RMC Inc., Fresno, CA, USA) with a diamond knife. Ultra-thin sections were double stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (JEOL-1200EX II, JEOL LTD., Tokyo, Japan) at 80 kV.

Table 1. Antimicrobial activity and MIC of urushiol against 3 strains of *H. pylori* according to pH

	Strains		
	HP NCTC11637	HP 69	HP 219
pH 5			
Survival	No	No	No
pH 6			
Survival	Yes	Yes	Yes
MIC (mg/mL)	0.256	0.256	0.256
pH 7			
Survival	Yes	Yes	Yes
MIC (mg/mL)	0.256	0.256	0.256
pH 8			
Survival	Yes	Yes	Yes
MIC (mg/mL)	0.256	0.256	0.256
pH 9			
Survival	Yes/No	Yes	Yes
MIC (mg/mL)	0.064	0.128	0.256
pH 10			
Survival	No	No	No

HP, *H. pylori*; MIC, Minimal inhibitory concentration.

RESULTS

Antibacterial activity of urushiol on *H. pylori*

The minimal inhibitory concentration (mg/mL) of the urushiol against 3 *H. pylori* strains ranged from 0.064 to 0.256. The minimal inhibitory concentration values of kanamycin-resistant *H. pylori* (*H. pylori* 69) and other strains (*H. pylori* NCTC11637 and *H. pylori* 219) were nearly equal. All strains survived only within pH 6.0-9.0. In media of pH 5 or 10, *H. pylori* did not survive after 3 days incubation, irrespective of the concentration of urushiol (Table 1).

Morphological changes of *H. pylori* by urushiol

In the results of initial (before incubation) and serial fixations (3, 6, and 10 min after incubation), separation of the membrane, vacuolization, and lysis of *H. pylori* were the serial morphological changes of *H. pylori* under 1×minimal inhibitory concentration of urushiol. After 3 min exposure, separation of the cell wall and vacuole and bleb formation were observ-
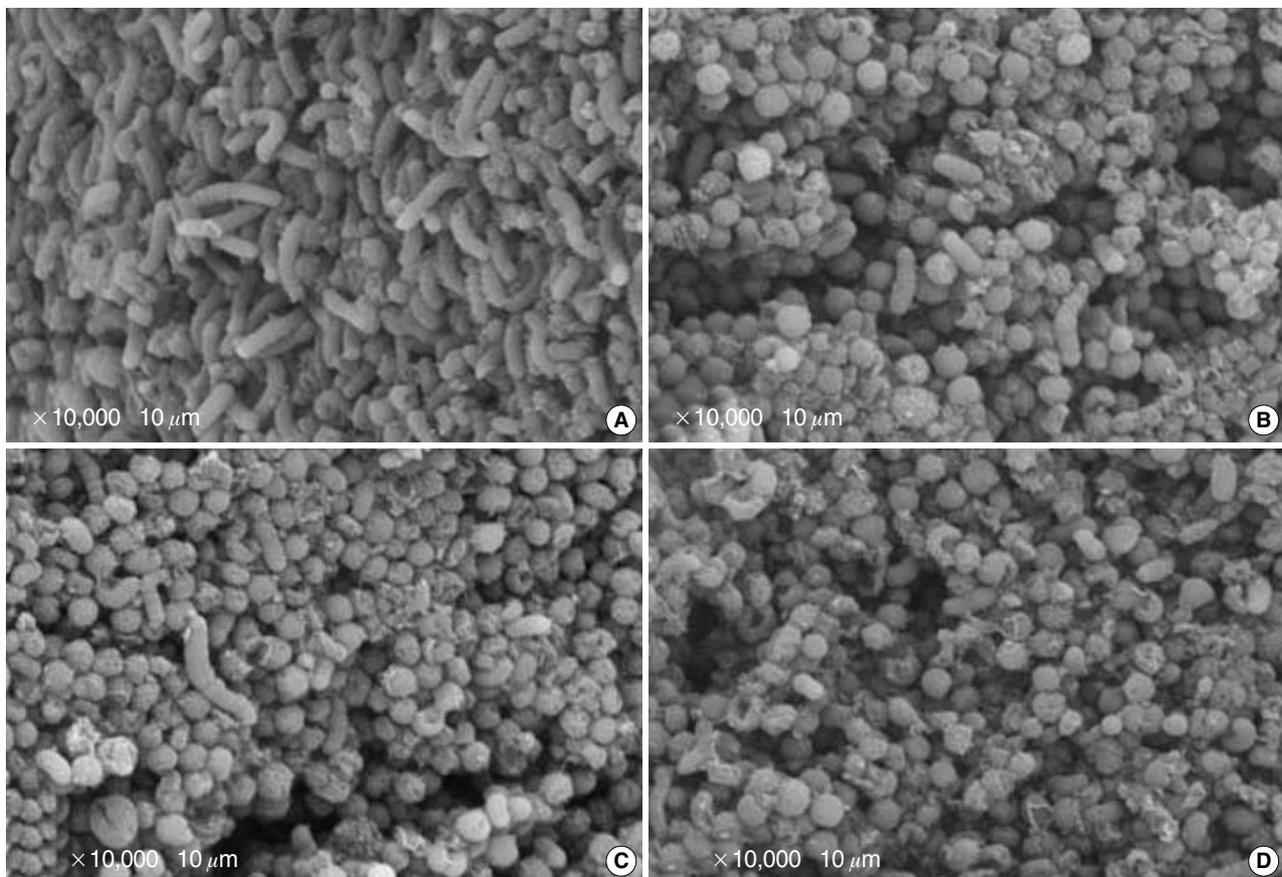

Fig. 2. Serial scanning electron micrographs of *H. pylori* exposed to 1×minimal inhibitory concentration of urushiol. (A) Without urushiol, most of the *H. pylori* bacteria are in spiral forms. (B) After 3 min exposure to urushiol, some of the *H. pylori* bacteria are still in the spiral form. (C) At 6 min after exposure, most of the *H. pylori* bacteria have changed to a round shape and are lysed. (D) After 10 min exposure, almost complete lysis of *H. pylori* bacteria is observed.
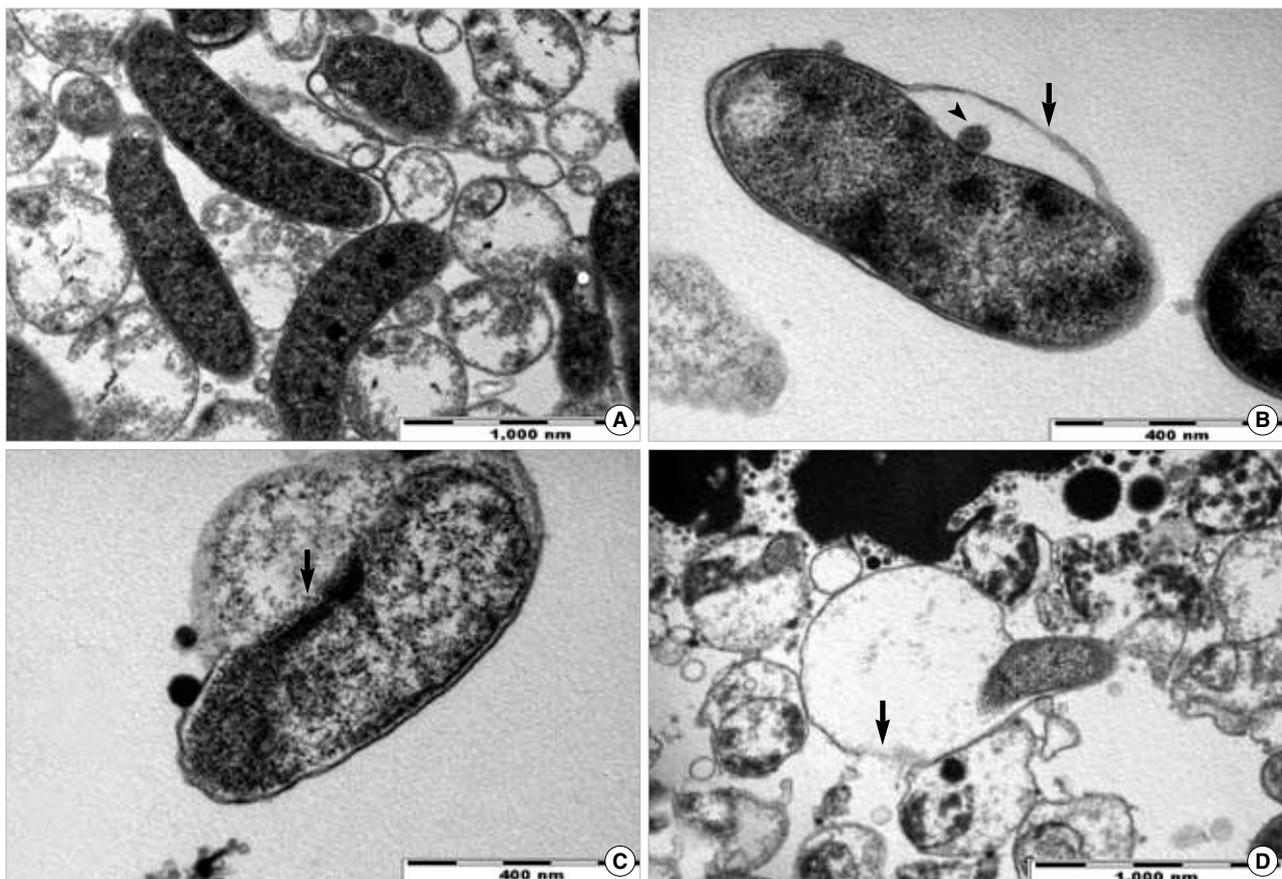
Fig. 3. Serial transmission electron micrographs of *H. pylori* exposed to 1×minimal inhibitory concentration of urushiol. (**A**) In control without urushiol exposure, *H. pylori* bacteria are in normal bacillary form. (**B**) After 3 min exposure, separation of the cell wall and vacuole and bleb formation are observed (arrow). Leakage of some cellular material from the cytoplasmic membrane is observed (arrow head). (**C**) At 6 min after exposure, significant separation with secretory granule loss and lysis of the cytoplasmic membrane are observed (arrow). (**D**) After 10 min exposure, the bacteria are almost complete lysed (arrow).

ed. Leakage of some cellular material from the cytoplasmic membrane was observed. At 6 min after exposure, significant separation with secretory granule loss and lysis of the cytoplasmic membrane were observed. After 10 min exposure, the bacteria are almost complete lysed (Figs. 2, 3).

DISCUSSION

Because antibiotic resistance is a major factor affecting the outcome of treatment, many non-antibiotic therapies including phytomedicines, probiotics, and antioxidants have been investigated as potential alternatives for the treatment of *H. pylori* infections (8-12). The minimal inhibitory concentrations of these alternatives against *H. pylori* have been reported as ranged from 0.075 to 12.5 mg/mL. In our study, the minimal inhibitory concentration of the urushiol against 3 *H. pylori* strains ranged from 0.064 to 0.256 mg/mL. When compared with previous reports of minimal inhibitory concentration of methanol, myrobalan, and plants extracts (9,

13, 14), The result of the present study suggest that urushiol could be a new candidate in the treatment of *H. pylori* infections.

When survival of *H. pylori* was examined under different pH conditions, all strains survived in pH range 6.0-9.0. Because antibiotics have different minimal inhibitory concentrations depending upon pH (15, 16), *H. pylori* eradication regimens using antibiotics need to include proton pump inhibitors. However, the minimal inhibitory concentration values of urushiol revealed minimal differences under different pH conditions. As a result, urushiol may not be influenced by pH and could be a supplementary agent for an anti-*H. pylori* regimen without proton pump inhibitor.

We evaluated the effect of urushiol on *H. pylori* with kanamycin resistance. The minimal inhibitory concentration of urushiol in kanamycin-resistant *H. pylori* and other two strains were nearly equal. In addition, the minimal inhibitory concentration of urushiol in *H. pylori* 219 (urease-negative strain) was not different from that of urease positive *H. pylori*. Though *H. pylori* survive in the stomach due to its urease activity (17),

the presence of urease activity is not related in antibacterial effect of urushiol on *H. pylori* in this study. These findings confirm that urushiol has a different mechanism on *H. pylori* eradication.

At 6 min following incubation with $1 \times$ minimal inhibitory concentration of urushiol, most of the *H. pylori* bacteria have changed to a round shape and are lysed. Within 10 min after exposure, separation of the membrane, vacuolization, and lysis of *H. pylori* were sequentially observed. Peroxynitrite was reported to cause morphological changes of *H. pylori* within 15 min (10), while some antibiotics were reported to act on the *H. pylori* within 10 hr (8). Based on these reports and the present study results, relative activity of urushiol against *H. pylori* seems to be quick. The results of our work suggest that urushiol has the potential to be used as a rapid acting therapeutic against *H. pylori* infection. In addition, we could expect that urushiol acts by disrupting the *H. pylori* cell membrane. For the further evaluation of action mechanism, studies using gold tagging urushiol or fluorescent microscope are warranted.

Based on results from the present study, we would suspect that urushiol is effective in *H. pylori* eradication and reduction of *H. pylori* gastritis or ulcer. However, a key problem of urushiol compared with other new treatment modalities (18-21) is a hypersensitive or allergic reaction. Some studies reported that allergic adverse effect of urushiol could be prevented by modification or polymerization of chemical structure (22, 23). Therefore, further preclinical or clinical studies using chemically modified non-allergic urushiol are needed. In this point, this in-vitro study is a basic proposal that urushiol maybe an alternative therapeutic agent for the *H. pylori* eradication in the future.

In conclusion, urushiol has the potential to act rapidly against *H. pylori* infection by disrupting the *H. pylori* cell membrane.

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