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RESEARCH NOTE

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Inhibitory Effect of 7-O-butyl Naringenin on Growth of *Helicobacter pylori* ATCC 26695

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Abstract The antimicrobial effect of a novel flavonoid (7-O-butyl naringenin) on *Helicobacter pylori* ATCC 26695 and its inhibitory effects on the urease activity of the strain were evaluated by comparing with quercetin and naringenin. *H. pylori* was cultured with brain heart infusion supplemented with 5% horse serum at 37°C under 10% CO_2 atmosphere and the inhibitory effects of flavonoids against the strain were detected using micro-plate methods. During 12 hr of incubation time, the optical densities of phenol red reduced (pink color) in the urea broth by producing ammonia were detected at 560 nm with a spectrophotometer. The results indicated that both quercetin and 7-O-butyl naringenin were effective against the growth of *H. pylori*. Moreover, inhibitory effect of 7-O-butyl naringenin on the growth of *H. pylori* was about two-fold higher than quercetin at the same concentration. With regard to *H. pylori* urease activity, 7-O-butyl naringenin had a greater inhibitory effect than did naringenin or quercetin at the same concentration.

Keywords: flavonoid, *Helicobacter pylori*, 7-O-butyl naringenin, antimicrobial effect, urease

Introduction

Helicobacter pylori is a motile, micro-aerophilic gram negative bacterium that is a pathogenic agent which produces large amounts of urease and vacuolating toxin (VacA toxin) in the human stomach (1, 2). Previous studies have shown that ammonia generated from urea by *H. pylori* urease from protects the bacterium from gastric acid in the stomach, and that the apoptosis is induced by the VacA toxin (1, 2). Therefore the inhibition of *H. pylori* infection, growth, urease, and VacA toxin activity are important for the treatment of patients with gastro duodenal diseases such as gastric adenocarcinoma, chronic gastritis, and peptic ulcer.

For many years, inhibitory effects on the growth of *H. pylori* have been tested by using natural compounds such as propolis, herbs, and other plants (3-9). Flavonoids are natural compounds ubiquitous to green plant cells. Flavonoids appear to have antimicrobial, anti-oxidative, anti-inflammatory, and anti-carcinogenic effects and played major roles in successful medical treatments in ancient times, and their use has continued to this day (10). There have been various studies on the functional effects of flavonoids with regard to the health food and pharmaceutical industries (11, 12). In particular, it has been shown that some flavonoids have antimicrobial effects against *H. pylori*. Although the minimum inhibitory concentration (MIC) of some flavonoids against the growth of *H. pylori* has been determined, the nature of the inhibitory effects has not been sufficiently studied (8). In addition, a new flavonoid chemically derived from its natural flavonoid has been studied to determine its functional activities as a medicinal compound (11).

Therefore, the objective of this research is to examine the *in vitro* inhibitory effects of some flavonoids that are mostly popular in nature (quercetin and naringenin), and 7-O-butyl naringenin, a new derived flavonoid, on the growth as well as the urease activity of *H. pylori*.

Materials and Methods

Bacterial strains *H. pylori* ATCC 26695 was purchased from American Type Culture Collection. *H. pylori* was inoculated into brain heart infusion (Difco Laboratories, Detroit, MI, USA) agar plates supplemented with 5% horse serum and was cultured under micro-aerophilic conditions (10% CO_2 atmosphere) for 3 days. For these studies, the strains were then inoculated in brain heart infusion broth (Difco) supplemented with 5% horse serum and were cultured at 37°C for 3 days before use.

Materials Three kinds of flavonoids were used for these studies; naringenin, quercetin, and 7-O-butyl naringenin. Naringenin and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of 7-*O*-butyl naringenin A solution of naringenin (500 mg, 1.84 mmol), butyl bromide (1.5 equiv), and K_2CO_3 (1.0 equiv) in dimethyl formamide (DMF) (10 mL) was stirred for 12 hr at room temperature.

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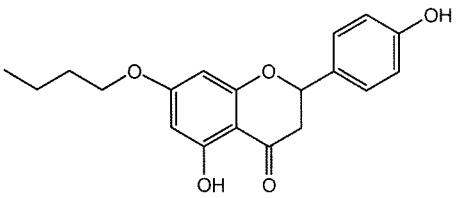

Fig. 1. Chemical structure of 7-*O*-butyl naringenin.

The mixture was quenched by addition of saturated aqueous NH_4Cl and extracted with ethyl acetate, and the extracts were dried over anhydrous $MgSO_4$. After evaporation, the resulting crude product was purified by flash column chromatography to afford the product as colorless oil in 35% yield. ^{1}H-NMR ($CDCl_3$, 400 MHz) 7.27 (d, J = 8.4 Hz, 2H), 6.87 (d, J = 8.4 Hz, 2 H), 6.02 (d, 2H), 5.31 (dd, J = 2.7, 13.0 Hz, 1H), 3.93 (t, J = 6.5 Hz, 2H), 3.11-2.73 (m, 2H), 1.73 (m, 2H), 1.44 (m, 2H), 0.95 (t, J = 7.3 Hz, 3H) (Fig. 1)

Assay of antimicrobial effects on *H. pylori* The microplate culture method was used to test the effect of flavonoids on the growth of *H. pylori*. The initial cell number of *H. pylori* was adjusted to 2.6×10^6 colony forming units (CFU)/mL of broth in the microplate well. One hundred ninety microliters of brain heart infusion supplemented with 5% horse serum, 50 μL of cultured broth, and 10 μL of flavonoid solution was added per well and cultured at 37°C under 10% CO_2 atmosphere with the CO_2 incubator (MCO-18AIC; Sanyo, Japan). The flavonoid concentration of was adjusted to 0.4, 4, and 40 ppm (7-O-butyl naringenin), or 4, 40, 80 ppm (naringenin and quercetin) in total broth per well. For the blank and control wells, 10 μL of distilled water and methanol were added instead of a flavonoid solution, respectively. During the 72 hr incubation, the turbidities of each sample were measured at 620 nm with a microplate reader (EL311; Bio-Tek Instruments Inc., Seoul, Korea).

Inhibition effects of flavonoids on urease of *H. pylori* *H. pylori* were cultured with brain heart infusion broth supplemented with 5% horse serum for 3 days and used for these studies. Seven milliliters of urea broth (urea 20 g, NaCl 5 g, KH_2PO_4 2 g, peptone 1 g, glucose 1 g, phenol red 0.012 g, in distilled water 1 L), 700 μL of the cultured broth and 280 μL of the flavonoid solutions were mixed. The initial cell number of *H. pylori* was 2.0×10^7 CFU/mL of total reaction mixture and the concentration of flavonoids was adjusted to be 80 ppm in each reaction mixture. As a control, 10 μL of methanol was added instead of a flavonoid solution. During 12 hr of incubation at 37°C, the optical densities of phenol red reduced (pink color) in urea broth by producing ammonia were measured at 560 nm with a spectrophotometer.

Satistical analysis Analysis of variance was performed for each group of three samples using the SAS program. Duncan's test also was used to verify the significance of the difference for each treatment.

Results and Discussion

Inhibitory effects on the growth of *H. pylori* As shown in Fig. 2, a 4% of methanol solution had no effect on the growth of *H. pylori* as compared with a blank and a control. In addition, naringenin up to 80 ppm also did not affect cell growth (Fig. 2). However, it appeared that quercetin and 7-O-butyl naringenin had inhibitory effects on cell growth at 80 and 40 ppm (Fig. 3 and 4), respectively. From these results, it appears that 7-O-butyl naringenin has a two-fold higher inhibitory effect on the growth of *H. pylori* than quercetin at the same concentration. In addition, it appears that the antimicrobial effect of 7-O-butyl naringenin against *H. pylori* is much greater than that of the natural naringenin.

Inhibitory effects on urease activity of *H. pylori* Urease, a nikel metalloenzyme is an essential virulent factor in *H. pylori* and major actor in the resistance to gastric acidity (1). Additionally, it has been known that urease also provides ammonium for nitrogen assimilation mediated by glutamine synthase, an essential protein in *H. pylori* (13).

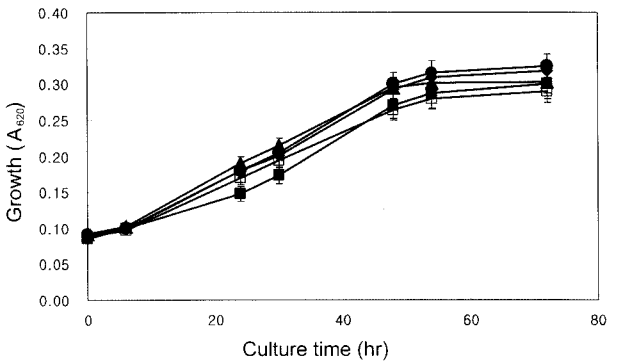

Fig. 2. The antimicrobial effects of naringenin on the growth of *H. pylori*. Samples were adjusted to 4.0 ppm (●), 40 ppm (▲), and 80 ppm (□) of flavonoids in broth and, control (◆) and blank (■) were used same volumes of methanol and d-water instead of flavonoid solution, respectively.

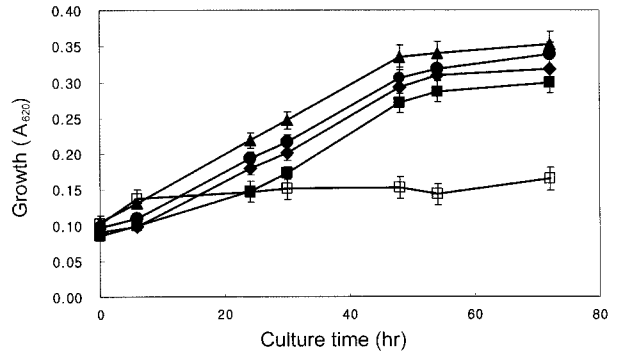

Fig. 3. The antimicrobial effects of quercetin on the growth of *H. pylori*. Samples were adjusted to 4.0 ppm (●), 40 ppm (▲), and 80 ppm (□) of flavonoids in broth and, control (◆) and blank (■) were used same volumes of methanol and d-water instead of flavonoid solution, respectively.

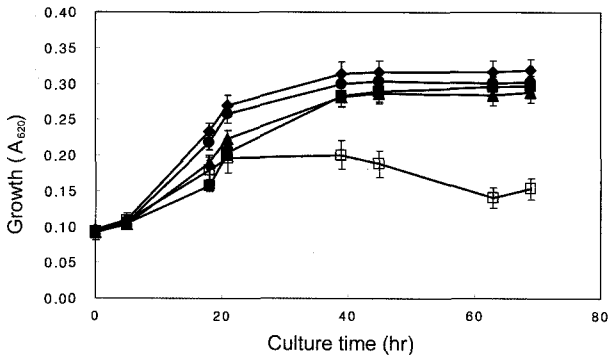

Fig. 4. The antimicrobial effects of 7-O-naringenin on the growth of *H. pylori*. Samples were adjusted to 4.0 ppm (●), 40 ppm (▲), and 80 ppm (□) of flavonoids in broth and, control (◆) and blank (■) were used same volumes of methanol and d-water instead of flavonoid solution, respectively.

Table 1. The inhibitory effect of various flavonoids at 80 ppm on urease activity of *H. pylori* cells after incubation at 37°C for 12 hr

Flavonoids	Growth (A_{560})
Control	0.726±0.051[1)]
Naringenin	0.631±0.021
Quercetin	0.457±0.032
7-O-butyl Naringenin	0.402±0.023

[1)]The values are mean±SD.

At pH 6.8-7.0, the color of phenol red changes from brown to pink and the pink-color intensity can be detected at 560 nm as an assay method for urease activity in the cultured broth. In these studies, during the incubation of *H. pylori*, ammonia is produced from urea by urease in cells and efflux into the broth raises its pH. As shown in Table 1, naringenin had little affect on urease activity at a concentration of 80 ppm, while both quercetin and 7-O-butyl naringenin had significant inhibitory effects ($p<0.05$) on *H. pylori* urease activity. Furthermore, this shows that 7-O-butyl naringenin has a stronger inhibitory effect on urease than the other two flavonoids ($p<0.05$). Since urea is a small uncharged molecule, it has long been considered to passively diffuse through the membrane of ureolytic bacteria (1). Therefore, it presumed that a hydrophobic interaction of 7-O-naringenin with urease might be related with inhibitory effect, although the mechanism has not clearly known yet.

Based on these studies, the inhibitory effects of flavonoids on the growth of *H. pylori* differ according to the kinds of molecular groups and the positions of their side chains on the flavonoid back-bone structure. Although the mechanism of their inhibition of urease activity has still been studied, the greater inhibitory effects of 7-O-butyl naringenin compared with natural naringenin may be helpful for examining the anti-microbial effects of other flavonoids against *H. pylori* and be useful in the development of the drugs for patients with gastritis and peptic ulcer.

Acknowledgments

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