Anti-\textit{Helicobacter pylori} Activity of the Metabolites of Poncirus from \textit{Poncirus trifoliata} by Human Intestinal Bacteria

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Poncacin was isolated from water extract of the fruits of \textit{Poncirus trifoliata} and metabolized by human intestinal bacteria. The inhibitory effect of poncacin and its metabolites by these bacteria on the growth of \textit{Helicobacter pylori} (HP) was investigated. Among them, poncacin (5,7-dihydroxy-4'-methoxyflavanone), the main metabolite most potently inhibited the growth of HP, with a minimum inhibitory concentration (MIC) of 10–20 μg/ml. However, poncacin and its metabolites except poncacin did not inhibit the growth of HP, nor did they inhibit HP urease.

Key words \textit{Helicobacter pylori}; \textit{Poncirus trifoliata}; 5,7-dihydroxy-4'-methoxyflavanone

\textit{Helicobacter pylori} (HP) was isolated from the gastric antrum of chronic gastritis patients by Warren and Marshall in 1983 (Lancet, 1, 1273–1275, ref. 1). HP stimulates gastritis to a condition of gastric cancer. Pathogenic HP strongly produces urease, which hydrolyzes urea to CO\textsubscript{2} and ammonia. HP urease is believed to play a critical role in the pathogenesis of gastritis and peptic ulcer. Therefore, eradication of HP and inhibition of its urease are important for the treatment of patients with gastroduodenal diseases.\textsuperscript{2} Here, we attempted to isolate poncacin from the fruits of \textit{Poncirus trifoliata} (family Rutaceae) which has been used to treat gastric and duodenal ulcers in folk cures of our country. Poncacin was incubated with human intestinal bacteria and its metabolites were isolated. The inhibitory effects of poncacin and its metabolites by human intestinal bacteria on the growth and urease activity of HP in vitro were investigated.

MATERIALS AND METHODS

Materials Brucella agar and brucella broth were purchased from Difco, Co. (U.S.A.). Horse serum was from Sigma Chem. Co. (U.S.A.). AnaeroPak Campylo was from Mitsubishi Gas Chemical Co., Inc. (Japan). The other chemicals were of analytical grade.

Bacterial Strain HP ATCC43504, NCTC11637 and NCTC11638 were purchased from ATCC and NCTC, respectively. The other HPs (HP82516, 82548, 4) were clinical isolates selected from Korean gastrointestinal samples. They were inoculated on brucella agar plates supplemented with 7% horse serum and cultured for 3 d at 37°C in an anaerobic jar with AnaeroPak Campylo.

Isolation of Poncacin from Ponciri Fructus The fruits of \textit{Poncirus trifoliata} (\textit{Ponciri Fructus}) was purchased from Heungin Yakuco, Seoul, Korea and identified. It was extracted twice with distilled water at 80°C for 6 h. The water extract was fractionated stepwise with ether, ethylacetate and butanol. To isolate the main component, poncacin, from the ethylacetate fraction of \textit{Ponciri Fructus}, the extract (15 g) was subjected to silica-gel column chromatography (5×80 cm) and was eluted with chloroform–methanol (10:1 to 2:1). The main compound was isolated, crystallized with MeOH, and identified as poncacin (1.2 g) by instrumental analysis.

Poncacin Colorless needles from MeOH. m.p. 211–213°C. [α\textsubscript{D}]+75.6 (c=1.2 in DMSO). FAB-MS \textit{m/z} 595 (M+1). 1\textsuperscript{H}-NMR (500 MHz, CD\textsubscript{3}OD) δ: 2.50 (1H, d, J=2.2 Hz, cis) 2.80 (1H, d, J=9.1Hz, trans), 3.77 (3H, s), 4.55 (1H, s), 5.10 (1H, d, J=9.0Hz), 5.57 (1H, dd, J=2.2, 9.1 Hz), 6.59 (1H, d, J=2.1Hz), 6.3 (1H, d, J=2.2Hz), 6.97 (2H, d, J=8.7Hz), 7.45 (2H, d, J=8.7Hz), 12.7 (1H, s, OH).

\textsuperscript{13}C-NMR (125 MHz, CD\textsubscript{3}OD) δ: 78.31 (C2), 40.25 (C3), 197.12 (C4), 162.88 (C5), 96.33 (C6), 164.85 (C7), 95.15 (C8), 162.65 (C9), 103.29 (C10), 130.35 (C1'), 128.42 (C2'), 113.90 (C3'), 159.50 (C4'), 113.90 (C5'), 128.42 (C6'), 100.35 (C1''), 77.10 (C2''), 76.08 (C3''), 69.58 (C4''), 76.87 (C5''), 60.41 (C6''), 97.43 (C1''), 70.44 (C2''), 73.04 (C3''), 71.79 (C4''), 68.23 (C5''), 17.97 (C6''), 56.44 (−OCH\textsubscript{3}).

Metabolism of Poncacin by Human Intestinal Bacteria

This was performed according to our previous method.\textsuperscript{3} Poncacin was transformed to poncacin and some phenolic compounds by human intestinal microflora.

Ponciretin (5,7-Dihydroxy-4'-methoxyflavanone) Colorless needles from MeOH. m.p. 193–194. [α\textsubscript{D}]-3 (c=0.7 in DMSO). FAB-MS \textit{m/z}: 287 (M+1), \textsuperscript{1}H-NMR (500 MHz, CD\textsubscript{3}OD) δ: 2.69 (1H, d, J=2.2Hz, cis) 2.80 (1H, d, J=9.1 Hz, trans), 3.60 (3H, s), 5.34 (1H, dd, J=2.2, 9.1 Hz), 5.89 (1H, d, J=2.1Hz), 5.90 (1H, d, J=2.2Hz), 6.94 (2H, d, J=8.7Hz), 7.39 (2H, d, J=8.7Hz), 12.10 (1H, s, OH).

\textsuperscript{13}C-NMR (125 MHz, CD\textsubscript{3}OD) δ: 80.24 (C2), 44.00 (C3), 197.59 (C4), 165.46 (C5), 97.12 (C6), 168.36 (C7), 96.21 (C8), 164.78 (C9), 103.38 (C10) 57.50 (−OCH\textsubscript{3}), 132.32 (C1'), 128.91 (C2'), 115.02 (C3'), 161.43 (C4'), 115.02 (C5'), 128.91 (C6').

Growth Inhibition Assay of HP One ml of each extract or isolated compound was added to petri dish containing un-
solidified 7 ml brucella agar supplemented with 7% horse serum. Final concentration of each extract was 1 mg/ml.
Final concentrations of each isolated compound were 100, 80, 40, 20, 10, 5, 2.5, 1, 0.5, 0.25 and 0.1 µg/ml. And then approximately 5×10⁶ CFU of the HP was then inoculated onto the agar plates, cultured microaerobically for 3 d at 37°C in an anaerobic jar (85% N₂, 10% CO₂, 5% O₂) and then the minimum inhibitory concentration (MIC) was determined. Ampicillin was used as a positive control. All experiments were conducted in triplicate.

Preparation of HP Urease: HP was inoculated from an agar plate into 30 ml of brucella broth supplemented with 10% fetal bovine serum in 100 ml flask, which was placed in an anaerobic jar. The harvested cells were washed with 10 ml of 20 mM phosphate buffer, pH 7.0, sonicated and centrifuged at 5000 g for 30 min. The resulting supernatant was used as the crude enzyme.

Assay of Urease Activity: Urease activity was determined according to the method of Gutmann and Bergmeyer.¹³

RESULTS

Effects of Ponciri and Its Metabolites on Human Intestinal Microflora on HP Growth: Ponciri Fructus was extracted with distilled water, and from this water extract, ponciri was isolated as the main component. This ponciri was anaerobically incubated with human intestinal microflora and six metabolites were observed by TLC. Two of these were isolated by silica-gel column chromatography. FAB-MS of the isolated main metabolite showed that a quasi-molecular ion peak at m/z (M⁻) was 287. TLC chromatogram and ¹H- and ¹³C-NMR spectra of the metabolite showed that the flavanone skeleton of ponciri was intact but a rhamnoglycosyl moiety was missing. Comparison with authentic ponciretin¹³ identified it as ponciretin. FAB-MS of the other isolated metabolite showed that a molecular ion peak at m/z (M⁻) was 449. TLC chromatogram and ¹H- and ¹³C-NMR spectra of the metabolite showed that the flavanone skeleton of ponciri was intact, and only a rhamnose moiety was missing. Comparison with authentic poncirexin identified this as poncirexin (5,7-dihydroxy-4'-methoxy flavanone-7-glucopyranoside).¹³ The other metabolites were identified using TLC (physico-chemical properties) and were 2,4-dihydroxyacetophenone, 4-hydroxybenzoic acid, phloroglucinol and pyrogallol. The inhibitory effect of ponciri and its metabolites on the growth of HP was measured (Table 1). Among these metabolites, ponciretin had the most potent inhibitory effect on HP growth. Ponciri and the other metabolites, poncirexin and phenolic compounds, did not inhibit the HP growth, however. MICs of ponciretin transformed from ponciri by human intestinal microflora on the growth of HP were 10–20 µg/ml.

Effects of Ponciri and Its Metabolites on HP Urease: The water extract of Ponciri Fructus was fractionated stepwise with ether, ethylacetate and butanol, and the inhibitory effects of these fractions on HP urease were tested (Table 2). Most fractions weakly inhibited HP urease at 0.3 mg/ml. Polar fractions, butanol fraction and residual fraction, of the extract have the weak inhibitory activity of HP urease. Therefore, the active components were not isolated from these fractions. The inhibitory effect of ponciri and its metabolites on the activity of HP urease was weak. Most of the tested compounds weakly inhibited HP urease. Among them, phloroglucinol most potently inhibited the urease activity. The other metabolites did not inhibit it.

DISCUSSION

Ponciri Fructus has been used as a folk cure to treat gastritis in Korea. However, we reported earlier that the water extract of Ponciri Fructus did not inhibit the growth of HP.¹³ Therefore, we isolated the main component, ponciri, from Ponciri Fructus. When it was anaerobically incubated with human intestinal microflora, its metabolites were ponciretin,

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**Table 1. MICs of Compounds Separated from Ponciri and Its Metabolites on the Growth of Helicobacter pylori**

<table>
<thead>
<tr>
<th>Compound</th>
<th>HP ATCC43504</th>
<th>HP NCTC11637</th>
<th>HP NCTC11638</th>
<th>HP 82516</th>
<th>HP 82548</th>
<th>HP 4</th>
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<tbody>
<tr>
<td>Ponciri Fructus water extract</td>
<td>&gt;1000</td>
<td>-</td>
<td>-</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Ponciri</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Poncirexin</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Ponciretin</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2,4-Dihydroxyacetophenone</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Phloroglucinol</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*a* Not detected.

**Table 2. Inhibitory Effects of Ponciri and Its Metabolites on the Urease of Helicobacter pylori**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition (%)</th>
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<tr>
<td>Ponciri Fructus</td>
<td>8</td>
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<tr>
<td>Ethylacetate fraction</td>
<td>10</td>
</tr>
<tr>
<td>Butanol fraction</td>
<td>33</td>
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<tr>
<td>Residual fraction</td>
<td>33</td>
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<tr>
<td>Ponciretin</td>
<td>24</td>
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<tr>
<td>Poncirexin</td>
<td>18</td>
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<tr>
<td>Ponciretin</td>
<td>21</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>0</td>
</tr>
<tr>
<td>2,4-Dihydroxyacetophenone</td>
<td>15</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>76</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>23</td>
</tr>
</tbody>
</table>

*a* Final concentration of each herbal extract was 0.3 mg/ml.
poncirenin and phenolic acids. Measurement of their inhibitory effect showed that ponciretin potently inhibited the growth of HP, and the other metabolites hardly inhibited it at all. Ponciretin inhibited HP growth more strongly than the previously isolated components, thiosulfinate, (+)-protolichiesterinic acid, decursin and decrucinol angelate.5—9) However, ponciretin lacked inhibition of the urease of HP. These results suggest that the mechanism to inhibit HP growth does not extend to inhibition of urease. The eradication of HP has been known to cure gastritis and to prevent relapse of duodenal ulcer. Therefore, antibiotics, such as amoxicillin, whose spectrum is in vitro same to that of ampicillin, and tetracyclins, are used in clinics.10) Amoxicillin, which is used for eradication of HP, was tested under the same procedure, its MIC was 0.5—2 μg/ml medium. Compared with these antibiotics, the isolated compounds showed inhibitory effects on growth of HP at one-order higher concentration. Pathogens resistant to these antibiotics and their side effects have appeared, however. Therefore, ponciretin and its derivatives are believed to contribute to the prevention of gastritis in some degree, even if they are not potent growth inhibitors of HP.

REFERENCES