In Vitro Anti-Helicobacter pylori Activity of Chinese Chive (Allium tuberosum)

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The anti-Helicobacter pylori (H. pylori) activity of a water extract of Chinese chive (Allium tuberosum) (WCC) was investigated. On disk diffusion assay, WCC effectively inhibited the growth of all 21 strains tested, including isogenic mutants, showing inhibition diameters of 12 to 29 mm, irrespective of drug susceptibility and clinical manifestation. The minimum inhibitory concentration (MIC) of WCC was 2.45 mg dry weight/mL. Killing assay with multiples of MIC confirmed that WCC had bactericidal activity and that the inhibitory effects were dose dependent. In addition, to determine whether the inhibitory activity of WCC under severe stress conditions, such as heat and acidity, is altered, the stability of WCC was evaluated. The inhibitory activity of WCC exposed to acidic conditions (pH 1.0 to 6.4) was stable, while heat-treated WCC (100°C, 10 min) showed slightly decreased inhibition activity. On combination assay with antibiotics frequently used in clinical practice, WCC was found to be an innocuous agent for antibiotic activity. These results suggest that daily intake of WCC is able to prevent H. pylori colonization in the stomach, and that it could be applied as adjuvant therapy in H. pylori eradication.

Keywords: Helicobacter pylori, Chinese chive, Allium tuberosum, antibiotic resistance, growth inhibition effect, bactericidal activity

Introduction

Helicobacter pylori (H. pylori) was recognized as a human carcinogen by IARC in 1994 (Sugiyama et al., 2002), and is a pathogenic gram-negative spiral microaerophilic bacterium that colonizes the human stomach in up to half the world’s population (Rothenbacher and Brenner, 2003). H. pylori plays a major role in the development of gastro-duodenal diseases, including gastritis, peptic ulcer and gastric cancer, and is associated with a wide range of non-gastrointestinal tract conditions such as idiopathic thrombocytopenic purpura. As gastric cancer is one of the most frequent causes of cancer-related death, eradication of H. pylori can, therefore, contribute to the treatment and prevention of these diseases. Generally, H. pylori infection in clinical practice is treated with an effective regimen, a triple therapy consisting of a proton pump inhibitor and combinations of two antibiotics; amoxicillin (AMPC) and clarithromycin (CAM) or AMPC and metronidazole (MNZ) in Japan (Nishimori, et al., 2006). However, unsuccessful eradication therapy is increasing due to the increased occurrence of drug-resistant H. pylori (Nariman, et al., 2004). The appearance rates of drug-resistant H. pylori against AMPC, CAM and MNZ from 2007 to 2009 were 0.4%, 19.4% and 25.7%, respectively (Lyudmila et al., 2010), and these rates are continuing to escalate.

Instead of antibiotics, non-antibiotic substances derived from foods with anti-H. pylori activity are now a focus in Gastroenterology. There have been several reports on natural compounds derived from foods, including sulforaphane (broccoli sprout), allicin (garlic), catechins (green tea) and 6-gingerol (ginger), that inhibit the growth of H. pylori in vitro (Fahey et al., 2002; Jonkers et al., 1999; Canizares et al., 2004; Mabe et al., 1999; Mahady et al., 2003). Furthermore, the actual application of these compounds to humans infected with H. pylori is of interest in the translational research fields of foods technology. However, the stability of these components against exposure to stress conditions, including cooking and the stomach environment, is rarely investigated. The bystander effects between these compounds and anti-
biotics also remain unclear. In particular, the relationship between Chinese chive (Allium tuberosum) and its functional effects on the growth of H. pylori (anti-H. pylori activity) are unknown to date.

In this study, we focused on the antimicrobial activity of Chinese chive against H. pylori. The Chinese chive is a perennial herbaceous plant native to China. In Japan, the Chinese chive is widely consumed as a vegetable. Garlic (Allium sativum), which is widely-recognized as a functional food, and Chinese chive both belong to the genus Allium. Among members of this genus, extracts of garlic showed anti-H. pylori activity (Jonkers et al., 1999; Canizares et al., 2004) and Chinese leek (Allium odorum L), which is closely related to Chinese chive, showed inhibitory activity against the spiral gram-negative Campylobacter species (Lee et al., 2004). To our knowledge, however, there have been no reports on whether water extracts of Chinese chive (WCC) possess anti-H. pylori activity, and whether such effects are stable under severe stress conditions, such as heat and acidity.

Materials and Methods

Reagents  Brucella broth and horse serum were purchased from Becton Dickinson Co. (Cockeysville, MD) and Gibco (Auckland, New Zealand), respectively. Agar was obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of the highest grade commercially available. Milli-Q water or sterilized water was used in all procedures.

Preparation of WCC  Chinese chive was purchased from a local supermarket. A 150-g sample of the edible portion of Chinese chive was minced and added to 100 mL of water, and was then ground with a blender. After water-insoluble substances were removed by centrifugation (36,000 × g, 10 min, 4°C), the obtained extract was passed through a 0.45-μm filter (Sartorius, Goettingen, Germany). The filtrate was used as WCC. All procedures were carried out at room temperature and WCC was stored at −20°C until use. In addition, the dry weight (DW) of Chinese chive was measured after lyophilization.

Bacterial strains and culture conditions  Fourteen H. pylori isolates randomly selected from patients suffering from gastro-duodenal diseases and seven isogenic mutants derived from wild-type strain HPK5 were used in this study (Table 1). In treatment regimens for H. pylori eradication, combinations of antibiotics such as CAM, MNZ and AMPC are frequently used (Cavallaro et al., 2006; Touati et al., 2000). The detailed properties of the strains used were as follows: 5 strains were MNZ resistant; 2 strains were CAM resistant; and 14 strains were antibiotic susceptible. The seven isogenic mutants of H. pylori HPK5 obtained from a Japanese patient with gastric ulcer (Takeuchi et al., 1998) were as follows: cdrA-disrupted (HPKT510) (Takeuchi et al., 1998; Takeuchi et al., 2006),

Table 1. Measurements of inhibition zones (mm) formed with WCC for each H. pylori strain used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Drug-resistance</th>
<th>Inhibition Zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NY13</td>
<td>Japan</td>
<td>CAM</td>
<td>19</td>
</tr>
<tr>
<td>NY31</td>
<td>Japan</td>
<td>CAM</td>
<td>29</td>
</tr>
<tr>
<td>TK1402</td>
<td>Japan</td>
<td>×</td>
<td>17</td>
</tr>
<tr>
<td>KMT52</td>
<td>Japan</td>
<td>×</td>
<td>17</td>
</tr>
<tr>
<td>KMT114</td>
<td>Japan</td>
<td>×</td>
<td>20</td>
</tr>
<tr>
<td>KMT127</td>
<td>Japan</td>
<td>MNZ</td>
<td>20</td>
</tr>
<tr>
<td>KMT130</td>
<td>Japan</td>
<td>×</td>
<td>21</td>
</tr>
<tr>
<td>CPY3401</td>
<td>Japan</td>
<td>×</td>
<td>22</td>
</tr>
<tr>
<td>KYU1</td>
<td>United States</td>
<td>MNZ</td>
<td>21</td>
</tr>
<tr>
<td>NCTC11637</td>
<td>United States</td>
<td>×</td>
<td>23</td>
</tr>
<tr>
<td>J99</td>
<td>United States</td>
<td>×</td>
<td>21</td>
</tr>
<tr>
<td>26695</td>
<td>United Kingdom</td>
<td>×</td>
<td>16</td>
</tr>
<tr>
<td>SS1</td>
<td>Australia</td>
<td>×</td>
<td>19</td>
</tr>
<tr>
<td>HPK5</td>
<td>Japan</td>
<td>×</td>
<td>12</td>
</tr>
<tr>
<td>HPKT510</td>
<td>Japan</td>
<td>MNZ</td>
<td>12</td>
</tr>
<tr>
<td>HPK5BA</td>
<td>Japan</td>
<td>×</td>
<td>13</td>
</tr>
<tr>
<td>HPK5CA</td>
<td>Japan</td>
<td>×</td>
<td>17</td>
</tr>
<tr>
<td>HPK5SA</td>
<td>Japan</td>
<td>MNZ</td>
<td>15</td>
</tr>
<tr>
<td>HPT208</td>
<td>Japan</td>
<td>MNZ</td>
<td>13</td>
</tr>
<tr>
<td>HPT209</td>
<td>Japan</td>
<td>×</td>
<td>13</td>
</tr>
<tr>
<td>HPureI</td>
<td>Japan</td>
<td>×</td>
<td>17</td>
</tr>
</tbody>
</table>

a) ×, drug-susceptibility; MNZ, Metronidazole resistant; CAM, Clarithromycin resistant. Concentration of WCC was 61.3 mg DW/mL.
babA-disrupted (HPK5BA), cagA-disrupted (HPK5CA), sabA-disrupted (HPK5SA), ureA-disrupted (HPT208), ureB-disrupted (HPT209) and ureI-disrupted (HPTureI) (Trang et al., 2009). In addition, 4 strains frequently seen worldwide, NCTC11637 (derived from USA), SS1 (derived from Australia) (Hiratsuka et al., 2005), J99 (derived from USA) (Hiratsuka et al., 2005) and 26695 (derived from United Kingdom) (Kidd et al., 2001; Akopyants et al., 1998), were employed in this study.

All H. pylori strains were grown on Brucella agar plates supplemented with 10% horse serum (HS) and 1.4% agar at 37°C under microaerobic conditions (10% CO₂) (Sanyo CO₂ incubator, Osaka, Japan) for 48 h, as described previously (Trang et al., 2009). Brucella medium supplemented with 10% HS (Brucella-serum medium) was used, unless otherwise stated.

**Growth inhibition assay** The effects of WCC on the growth of H. pylori were examined using the disk diffusion method reported by Canizares et al. (2004) with slight modification. Briefly, bacteria grown on Brucella agar plates for 48 h were harvested and suspended in Brucella medium (OD₆₀₀ = 0.6), and were then spread on the surface of Brucella agar plates for 48 h, corresponding to the late stationary phase, were incubated at 37°C for 72 h under microaerobic conditions. Sterilized water was used as a control for all experiments. Colony forming units (CFU) were determined to assess bacterial viability. MIC was defined as the lowest WCC concentration to give 1 × 10⁴ CFU inhibition, as compared with controls. All tests were performed at least in duplicate.

**Killing assay** In order to determine the bactericidal activity of WCC against H. pylori, killing assay was performed in the presence of 1 or 5 × MIC of WCC, in accordance with previously reported methods (Takeuchi et al., 2006; Morishita et al., 2008). Briefly, bacteria grown on Brucella agar plates for 48 h, corresponding to the late stationary phase, were harvested and suspended in Brucella-serum medium (OD₆₀₀ = 0.6). In 1.5-mL microtubes, 0.4 mL of bacterial suspension was added and centrifuged at 4,100 × g for 1 min (KUBOTA 1120; Kubota Corp., Tokyo, Japan) to remove supernatants. Pellets were re-suspended in Brucella-serum medium with or without WCC, and were then incubated at 37°C under microaerobic conditions with shaking (Bio shaker BR-40LF; Taitec Co., Ltd., Kanagawa, Japan) for 5 h. At 1, 3 and 5 h after incubation, each suspension collected was serially diluted 10-fold, inoculated onto the surface of the WCC-agar plates, and were then kept at 37°C for 72 h under microaerobic conditions. Sterilized water was used as a control for all experiments. Colony forming units (CFU) were determined to assess bacterial viability. MIC was defined as the lowest WCC concentration to give 1 × 10⁴ CFU inhibition, as compared with controls. All tests were performed at least in duplicate.

**Microscopic observations of bacterial morphology and DNA distribution** In order to determine the effects of WCC on bacterial morphology and DNA distribution, bacteria at the edge of the inhibition zone on disk diffusion assay were subjected to morphological examination. Aliquots of the bacteria suspended in phosphate buffered saline (PBS) were dropped onto a slide glass, dried and stained with gram staining (neo-B&M Wako; Wako Co., Osaka, Japan) or 1 μg/mL 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI; Dojindo, Kumamoto, Japan) solution at room temperature for observation of morphology or DNA distribution, respectively.

In addition, for comparison, disk diffusion assay was repeated with seven antibiotics, as follows: AMPC, 0.01 μg; CAM, 0.01 μg; MNZ, 5 μg; levofloxacin (LVFX), 5 μg; ciprofloxacin (CPFX), 5 μg; imipenem (IMP), 10 μg; and amikacin (AMK) 30 μg (Becton Dickinson Co., Cockeysville, MD). Disk diffusion assay with all seven antibiotic disks was performed as described above.

**Determination of minimum inhibitory concentration (MIC)** The minimum inhibitory concentrations (MIC) of WCC were determined using the agar dilution method reported by Voravuthikunchai and Mitchell (2008) and Dore et al. (1999), with slight modification. Briefly, 0.75 mL of WCC was added to dishes containing 14.25 mL of not-yet-solidified Brucella agar (WCC-agar plate) in order to give WCC concentrations ranging from 0 to 3.0 mg DW/mL. Subsequently, H. pylori suspensions (OD₆₀₀ = 0.5-0.6) were serially diluted 10-fold and inoculated onto the surface of the WCC-agar plates, and were then kept at 37°C for 72 h under microaerobic conditions. Sterilized water was used as a control for all experiments. Colony forming units (CFU) were determined to assess bacterial viability. MIC was defined as the lowest WCC concentration to give 1 × 10⁴ CFU inhibition, as compared with controls. All tests were performed at least in duplicate.

**Stability of WCC under acidic conditions** The stability of the anti-H. pylori activity of WCC after exposure to acidic conditions was assessed using a series of WCC solutions (pH 6.4) adjusted to pH 1.0-6.0 with 10 N HCl in disk diffusion assay, as described above. The acidified-WCC was centrifuged at 4,100 × g for 1 min in order to remove the insoluble substances formed by acidification, and was then kept at 4°C.
until use. Sterilized water without WCC was used as a control. All tests were performed at least in duplicate.

Stability of WCC treated by heat  In order to evaluate the stability of anti-\textit{H. pylori} activity of WCC after heat treatment, preparation of heat-treated WCC was performed as follows: in a 1.5-mL microtube, 0.4 mL of WCC was added, followed by heating with a dry heater (Dry Thermo Unit DTU-1C; Taitec Co.) for 3, 5 or 10 min at 100°C. The solution was cooled in an ice bath, followed by centrifugation at 4,100 \( \times g \) for 1 min in order to remove the insoluble substances formed by heating. The anti-\textit{H. pylori} activity of heat-treated WCC was evaluated using the disk diffusion method, as described above. Sterilized water without WCC was used as a control. All tests were performed at least in duplicate.

Stability of WCC exposed to acidity and heat  The stability of anti-\textit{H. pylori} activity of WCC after exposure to both acidity and heat was assessed by adjusting heat-treated WCC solutions (pH 6.4) to pH 1.0-6.0 with 10 N HCl, followed by centrifugation at 4,100 \( \times g \) for 1 min to remove insoluble substances formed by acidification and heating. Anti-\textit{H. pylori} activity of dual-treated WCC was evaluated by disk diffusion assay as described previously. Sterilized water without WCC was used as a control. All tests were performed at least in duplicate.

Results and Discussion  
Preparation of WCC  From 150.0 g of Chinese chive, 178.6 mL of WCC was obtained. The DW of WCC determined after lyophilization was 7.3%. Thus, the concentration of WCC obtained in this study was 61.3 mg DW/mL.

Anti-\textit{H. pylori} activity of WCC  The anti-\textit{H. pylori} activity of WCC was investigated against 21 strains using the disk diffusion method. The WCC prepared showed the anti-\textit{H. pylori} activity against all 21 strains, as the growth inhibitive zone was in the range of 12-29 mm (Table 1). Next, to determine the MIC of WCC against the growth of \textit{H. pylori}, five isolates with different drug susceptibilities, inhibition zones and geographical districts, (NY31, TK1402, KMT127, KMT130 and SS1) were subjected to agar dilution assay. The results showed that MIC for all strains was the same (2.45 mg DW/mL), indicating that WCC possesses inhibitive activity against \textit{H. pylori} and that disk diffusion assay with WCC is not strict assay to determine the MIC value but is sufficient for evaluating the effects of WCC on the growth inhibition of microorganisms.

The MIC values for \textit{H. pylori} strains obtained in the present study were similar to those of a closely related species (Chinese leek) against \textit{Campylobacter} spp (2.0 mg DW/mL) (Lee et al., 2004). Furthermore, the MICs of a water extract of garlic against \textit{H. pylori} and \textit{Campylobacter} spp. were 5.0 mg DW/mL (Cellini et al., 2006) and 4.0 or 5.0 mg DW/mL (Lee et al., 2004), respectively. Thus, the anti-\textit{H. pylori} activity of WCC is more potent than that of garlic, which is widely used as a functional food (Ankri and Mirelman 1999). The compound allicin, which is present in garlic, is known to exhibit a wide spectrum of antimicrobial activity due to its chemical reaction with the thiol groups of various enzymes, e.g., alcohol dehydrogenase and thioredoxin reductase (Yabuki et al., 2010). Allicin is enzymatically generated from S-alk(ен)yl cysteine sulfoxide (alliin), which is a precursor of the sulfur-containing compounds found in members of the genus \textit{Allium}. In Chinese chive, however, the amount of alliiin is lower than in garlic, but the amount of other sulfide compounds derived from S-methyl cysteine sulfoxide (me-thiin), the precursor of the sulphur-containing compounds, is much greater than in garlic (Ankri et al., 1999). Thus, the anti-\textit{H. pylori} properties of WCC reported here may be attributed to sulfide compounds such as S-methyl methan-thiosulfinate, which are distinct from allicin and its synthetic pathway in garlic. Further investigation is necessary in order to identify the components and mechanisms involved in anti-\textit{H. pylori} activity.

Seven isogenic mutants were used in this study. HPK5BA lacking BabA protein and HPK5SA lacking SabA protein strains cannot bind to the sialic acid and Leb expressed on epithelial cells in the human stomach, respectively (Doig and Trust, 1994; Sabarth et al., 2005). These bacterial adhesion proteins localized on the outer membrane are involved in persistent infection. HPT208, HPT209 and HPTureI mutants do not possess UreA, UreB and UreI proteins, respectively (Nam-Chul et al., 2001). Urease proteins consist of dimerized UreA and UreB subunits as apoprotein, and the regulation of urease activity via Urel is essential for adapting \textit{H. pylori} to acidic conditions. These proteins localized on the surface of bacteria are also involved in direct interaction with materials in the micro-environment of \textit{H. pylori}. In this study, the susceptibility of these isogenic mutants to WCC was not different, indicating that these bacterial proteins were not involved in the function of growth inhibition of WCC. HPK5CA lacking CagA protein, one of the most closely related to major pathogens of \textit{H. pylori}, showed the same susceptibility to WCC, indicating that CagA is not associated with WCC function. In addition, HPKT510 lacks the cdrA protein, which suppresses cell division, and is able to tolerate \( \beta \)-lactam antibiotics and more severe microenvironments than HPK5 (Takeuchi et al., 2006). The loss of the cdrA gene in \textit{H. pylori} was observed during persistent infection due to adaptation to the harsh microenvironment of the stomach (Nakazawa and Takeuchi, 2008). Based on the results with...
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HPKT510, WCC may even inhibit such mutant strains possessing high survivability under severe conditions. Taken together, our results suggest that the anti-*H. pylori* activity of WCC exerted against all *H. pylori* strains examined operates via a pathway unrelated to these bacterial proteins and more investigations are thus necessary to clarify the function of WCC within *H. pylori*.

Bystander effect with combinations of WCC and antibiotics  In order to confirm the interference between WCC and antibiotics with regard to the inhibitory effects on growth of *H. pylori*, disk diffusion assay was performed using various combinations of WCC and antibiotics. The combinations tested were as follows: WCC & AMPC, WCC & CAM and WCC & MNZ. With TK1402, a drug-susceptible strain, doses of AMPC, CAM and MNZ were set at 0.008 μg, 0.01 μg and 3 μg, respectively, as these concentrations provided an inhibition zone of equal diameter (17 mm) as when using WCC. Subsequently, solutions of 20 μL of WCC with 1 μL of 0.008 μg/μL (AMPC), 0.01 μg/μL (CAM) or 3 μg/μL (MNZ) were used in disk diffusion assay to determine the cooperative effects of WCC and antibiotics. Similarly, two drug-resistant strains, NY31 (CAM resistant) and KYU1 (MNZ resistant), were examined. With TK1402, as shown in Fig. 1, the size of the inhibition zone for WCC (14 mm) and CAM (23 mm) was larger with the combination (37 mm). All other combinations showed similar results (data not shown). Thus, WCC has an additive or synergistic effect with the tested antibiotics. There have been no other reports on interference between vegetables and antibiotics with regard to anti-*H. pylori* activity. The positive effects observed in this study will therefore provide insight in possible combinations of WCC and antibiotics that could result in lower doses of antibiotics required for *H. pylori* eradication. Furthermore, the present observations may also help reduce the side effects of antibiotics and increase treatment efficacy.

Microscopic observations  The anti-*H. pylori* activity of WCC was examined by analyzing bacterial morphology and DNA synthesis and distribution after disk diffusion assay with WCC and antibiotics. NY31 (CAM-resistant) at the edge of the inhibition zone was studied after gram and DAPI staining (Fig. 2). Cells treated with WCC showed filamentous morphology (approximately 4 μm) (Fig. 2b), as compared with untreated cells (Fig. 2a). The filamentous cells were induced by fluoroquinolones, CPFX (Fig. 2c) and

![Fig. 1. Bystander effect of combinations of WCC (A) and antibiotics (B). Mixed solutions of WCC and antibiotic (C) showed more effective inhibition than either WCC or antibiotic alone.](image)

![Fig. 2. Morphological observation of *H. pylori* strain NY31 after gram staining (a-f) and DAPI (g-j). *H. pylori* cells were exposed to no stimulation (control; a and g), WCC (b and h), ciprofloxacin (c and i), levofloxacin (d and j), amoxicillin (e) or imipenem (f). Cells converted to filamentous shape when exposed to WCC and fluoroquinolones. Nucleoid features were seen in control cells (g) and filamentous cells (h) induced by WCC, but not fluoroquinolones (i and j). Scale bars are 10 μm.](image)
LVFX (Fig. 2d). However, AMPC (Fig. 2e) and IMP (Fig. 2f) caused cell lysis, and cells treated with the other compounds (AMK, CAM and MNZ) were indistinguishable from untreated cells. Fluoroquinolones are well known inhibitors for DNA gyrase and topoisomerase II (Shah, 1991), which are essential enzymes in DNA synthesis and replication in microorganisms. The inhibition of DNA replication triggers a bacterial reaction (SOS response), eventually leading to cell death. The SOS response is a particular feature of the action of 4-quinolone and consists of the induction of non-replicating DNA synthesis and inhibition of cell division leading to filamentation (Mouton and Leroy, 1991; Crosby et al., 1994). Based on our results, the anti-\textit{H. pylori} activity of WCC is similar to that of fluoroquinolones and is distinct from other antibiotics. Next, DNA synthesis, replication and partition were analyzed by DAPI staining, and were compared between cells treated with WCC and fluoroquinolones. Fluorescence microscopy confirmed that fluorescent DNA was uniformly distributed within untreated cells (Fig. 2g) and filamentous cells induced by WCC (Fig. 2h). However, the filamentous cells induced by fluoroquinolones showed significant decreases in fluorescence intensity (Figs. 2i, j) when compared to untreated (Fig. 2g) and WCC-treated (Fig. 2h) cells. DAPI binds to double-stranded DNA fragments and the staining intensity is dependent on DNA synthesis (Steffens et al., 2010), suggesting that fluoroquinolones inhibit DNA synthesis and replication, but that WCC scarcely influenced these processes. The anti-\textit{H. pylori} activity of WCC different from that of fluoroquinolones despite the appearance of filamentous cells induced by both compounds. Therefore, the anti-\textit{H. pylori} activity of WCC seen in this study is distinct from those of the antibiotics examined, suggesting that WCC may target new molecules, as well as the composition of \textit{H. pylori}, to inhibit growth.

In order to investigate the function of WCC against \textit{H. pylori}, Western blotting and microscopic observation by immune-fluorescence staining with \textit{H. pylori}-specific FtsZ antibody prepared in-house were performed. Interestingly, levels of FtsZ expression in filamentous cells were higher than in control cells (data not shown). However, no different distributions of FtsZ were seen between control and filamentous cells (data not shown). This indicates that WCC did not inhibit expression of FtsZ, which is a critical protein in Z-ring formation in cell division. Taken together, the results suggest that WCC inhibits the process of cell division following the Z-ring formation. Increased FtsZ expression in filamentous cell is probably a compensation mechanism against the inhibition of cell division. Further research is needed to identify the novel compounds that substantially act as growth inhibitors or antimetabolites, and to elucidate their action mechanisms within microorganisms.

Bactericidal activity of WCC Growth inhibition of WCC against all 21 \textit{H. pylori} strains was observed by the disk diffusion method. To determine whether the inhibitory effects of WCC were due to bactericidal and/or bacteriostatic activities, killing assay with four isolates (NY31, KMT127, KMT130 and SS1) was performed in the presence of 1 or 5 × MIC of WCC. Killing assay is the most reliable method for determining the susceptibility of microorganisms to active compounds and antibiotics. Figure 3 shows the viability of NY31 cultured with multiples of MIC (1 and 5 × MIC of WCC) and without WCC (control). At 1 × MIC of WCC, NY31 cell counts decreased 10 and 10^2 CFUs within 1 h (p < 0.05) and 3 h (p < 0.01), as compared with control groups, respectively. In the presence of 5 × MIC, WCC killed 10^2 CFU NY31 cells at within 1 h (p < 0.01) and no living cells were seen at 3 h (p < 0.01), indicating that the inhibitory effect is dose and time dependent, and that WCC possessed bactericidal activity. With regard to the other 3 strains (KMT127, KMT130 and SS1), the killing assay results were consistent with the data for NY31 (data not shown), thus suggesting that the activity of WCC is exerted on \textit{H. pylori} irrespective of drug susceptibility (CAM and MNZ). CAM and MNZ are valuable agents in the treatment of several protozoal and bacterial infections, including \textit{H. pylori} (Asaka et al., 2001; Fujioka et al., 2007; Jorgensen et al., 1998). However, the incidence of antibiotic-resistant \textit{H. pylori} is increasing, and this can lead to the failure of eradication therapy with antibiotics (Fujioka et al., 2007; Jorgensen et al., 1998). Our findings indicated that WCC provides an alternative to

![Fig. 3. Bactericidal activity of WCC against \textit{H. pylori} NY31.](image)

- **: p < 0.05 **: p < 0.01

At 1 × MIC, WCC reduced counts of NY31 cells by 10, 10^2 and 10^3 CFUs within 1, 3 and 5 h, as compared with control groups, respectively. In the presence of 5 × MIC, NY31 cells showed a 10^2 CFU reduction within 1 h, and no living cells were seen after 3 h.
conventional therapy.

Stability of WCC activity after exposure to acidity and heat Four isolates (NY31, KMT127, KMT130 and SS1) were used in this study. As H. pylori is able to colonize under the acidic conditions of the human stomach as a result of urease activity (Nam-Chul et al., 2001), the anti-H. pylori activity of acidified-WCC was investigated. WCC (pH 6.4) was exposed to serial acidic conditions (pH 1.0-6.0) and the activity of acidified-WCC is shown in Table 2. For four individual isolates, the size of the growth inhibition zone for WCC at the lowest pH (pH 1.0) was almost that same as at pH 6.4. Furthermore, the inhibition zones were nearly equal under all acidic conditions (pH 1.0 to 6.4), indicating that the anti-H. pylori activity of WCC is acid stable. Next, the effects of heat treatment on the anti-H. pylori activity of WCC were examined, and the results are shown in Table 3. The inhibition zones using heat-treated WCC decreased with the duration of heat treatment up to 10 min, as compared with untreated WCC. However, the heat-treated WCC maintained sufficient anti-H. pylori activity.

The effects of WCC under both conditions (acidic and heat treatment) were then investigated, and the results are shown in Table 4. WCC was boiled for 10 min and was exposed to acidic conditions before use in this assay. Overall, the values of growth inhibition with dual-treated WCC were slightly lower than single-treated WCC. However, the effective inhibition activity of heat-treated WCC was largely conserved under acidic conditions (pH 1.0-6.4), indicating that heated WCC remains active against anti-H. pylori, even under acidic conditions. Stable anti-bacterial effects of WCC against H. pylori, irrespective of drug susceptibility were observed, thus suggesting that the anti-H. pylori activity of WCC would be maintained after cooking and ingestion, thereby providing a potential alternative means of preventing H. pylori colonization and eradication of H. pylori infection.

To our knowledge, antimicrobial activities of Chinese chive extracts against pathogenic bacteria, such as Escherichia coli O157:H7 (Seo et al., 2001), food-borne bacteria (Mau et al., 2001) and Aspergillus spp (Yin and Tsao, 1999), have been reported, but there no such reports for carcinogenic bacteria, such as H. pylori. Furthermore, most anti-H. pylori activity in vegetables has been confirmed by screen-

### Table 2. Measurement of inhibitory zones (mm) formed with WCC under acidic conditions.

<table>
<thead>
<tr>
<th>Strain</th>
<th>pH 6.4</th>
<th>6.0</th>
<th>5.0</th>
<th>4.0</th>
<th>3.0</th>
<th>2.0</th>
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</tr>
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</tr>
<tr>
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<td>25</td>
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<td>24</td>
</tr>
<tr>
<td>KMT130</td>
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<td>21</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>SS-1</td>
<td>27</td>
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<td>27</td>
<td>27</td>
<td>26</td>
<td>27</td>
<td>28</td>
</tr>
</tbody>
</table>

Concentration of WCC was 61.3 mg DW/mL.

### Table 3. Measurement of inhibition zones (mm) formed with heat-treated WCC.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Heating time</th>
<th>Control</th>
<th>3 min</th>
<th>5 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
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<td>25</td>
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<td>16</td>
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</tr>
<tr>
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<td>19</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>SS-1</td>
<td></td>
<td>18</td>
<td>19</td>
<td>19</td>
<td>16</td>
</tr>
</tbody>
</table>

Concentration of WCC was 61.3 mg DW/mL.

### Table 4. Measurement of inhibition zones (mm) formed with heat-treated WCC under acidic conditions.

<table>
<thead>
<tr>
<th>Strain</th>
<th>pH 6.4</th>
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<td>14</td>
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<td>16</td>
</tr>
<tr>
<td>SS-1</td>
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<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

Concentration of WCC was 61.3 mg DW/mL.

All samples were heated at 100°C for 10 min before acidification.
ing tests, such as the disk diffusion method or agar dilution method (Stamatis et al., 2003; Li et al., 2005; Ndip et al., 2007). With regard to the anti-

*Helicobacter pylori* properties of WCC, there have been few investigations regarding the stability of functional materials exposed under severe stress conditions and interference with antibiotics. The failure of eradication therapy with antibiotics is becoming more common (Fujioka et al., 2007; Jorgensen et al., 1998). Therefore, to resolve these issues, we focused on researching alternative compounds that show antibacterial activity. WCC possesses high potential activity to inhibit the growth and viability of all *H. pylori* strains tested, irrespective of susceptibility to antibiotics (CAM and MNZ), clinical outcome and geographical district, indicating that the WCC is a useful alternative adjuvant therapy for eradication of *H. pylori* infection. In addition, our results regarding the stability of functional materials and interference with antibiotics based on translational application between foodstuffs and medicine showed that Chinese chives are a good candidate for preventing *H. pylori* infection. Now, we are focusing on identifying the constituents of WCC involved in this antibacterial activity.

This study is the first to report that water extracts of Chinese chive exhibit growth inhibition of *H. pylori*, and that the compounds responsible for this anti-*H. pylori* activity are stable under severe conditions such as heating and acidity. Moreover, the anti-*H. pylori* activity shows a synergistic effect with antibiotics and acts via unique pathways from these antibiotics.

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References


In Vitro Anti-Helicobacter pylori Activity of Chinese Chive (Allium tuberosum)


