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Original Article

Inhibitory effect of plumbagin on *Helicobacter pylori* growth and urease activity

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Abstract: *Helicobacter pylori* primarily colonizes in the human stomach and induces various gastric diseases, including gastric cancer. *H. pylori* utilizes various bacterial proteins, such as toxins, ureases, and adhesion and flagella proteins, for successful colonization and pathogenesis. In particular, urease is an indispensable virulence factor for the pathogenesis and survival of *H. pylori* in the acidic conditions of the stomach. In this study, therefore, we investigated the anti-urease activity of a natural compound, plumbagin, and found that it inhibited urease activity of various urease-producing bacteria. We determined the minimal inhibitory concentration of plumbagin against *H. pylori*, and demonstrated the inhibitory effect of plumbagin on *H. pylori* urease by using a urease activity assay. The inhibitory effect of plumbagin on urease was confirmed by using purified jack bean urease as the reference molecule, with acetohydroxamic acid as the reference inhibitor. Furthermore, we also confirmed the binding affinity of plumbagin to urease proteins. Collectively, our results suggest that plumbagin is a potent inhibitor of urease, which may possess potential therapeutic efficacy as a supportive agent against *H. pylori* infection.

Keywords: *Helicobacter pylori*, plumbagin, urease, anti-microbial effect, natural compound

Introduction

Helicobacter pylori is a gram-negative curved bacterium that primarily colonizes on the human stomach; approximately half of the world's population is reported to be infected with *H. pylori* [1]. Infection with *H. pylori* is associated with various gastric diseases, including inflammation of the gastric mucosa, peptic ulcers, and gastric adenocarcinoma [2]. In particular, the WHO has classified *H. pylori* as a group I carcinogen based on the epidemiological evidence in gastric cancer development [3].

Urease is a major virulence factor of *H. pylori*, because it is indispensable for colonization on the gastric epithelium. Typically, *H. pylori* colonizes at the acidic mucosa of the stomach, and urease produces NH_3 to increase the pH where it is colonized to survive in the hostile environment [4]. The structure of *H. pylori* urease is known to be a dodecameric complex of the form $((\alpha\beta)_3)_4$, which is composed of two subunits, α (61-66 kDa) and β (26-31 kDa) [4].

Plumbagin is a natural compound found in *Plumbago indica*. Many reports have suggested that plumbagin inhibits the growth of various tumor cell lines through the induction of apoptosis or cell cycle arrest [5, 6]. Moreover, plumbagin exhibits an anti-bacterial effect [7]. In particular, some studies have reported that plumbagin has an inhibitory effect on *H. pylori*, although the mechanism was not clarified [8, 9].

In this study, we determined the minimal inhibitory concentration (MIC) of plumbagin against *H. pylori* (ATCC 49503) and investigated the inhibitory effect of plumbagin on the urease expression and activity, which is indispensable for the colonization of the bacteria on the gastric mucosa.

Materials and methods

Materials

H. pylori reference strain was purchased from ATCC (ATCC49503, Manassas, VA, USA). Mue-

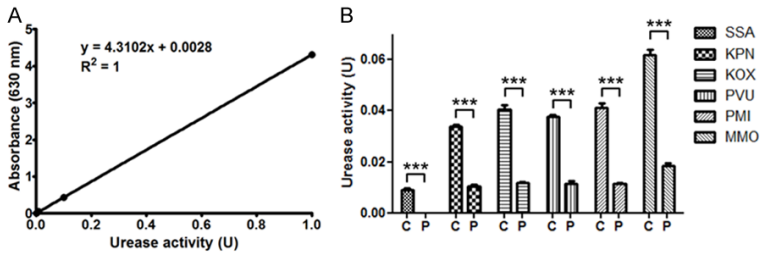

Figure 1. Effect of plumbagin on bacterial urease activity. A. Standard curve of the absorbance of the standard jack bean ureases. A solution of standard jack bean urease was prepared at 1 U and subjected to 10-fold serial dilutions to 0.001 U. Five microliters of 20% urea was added to the standard ureases and incubated at 25°C for 1 min. After incubation, the ammonia production was measured by colorimetric assay. The data were obtained from triplicate experiments. B. The effect of plumbagin on various bacterial urease activity. The bacteria were incubated in BHI broth for 24 h and the supernatant was collected. The collected supernatants were incubated with 10 μM plumbagin for 1 h and urease activity was assayed. The urease activity was calculated in comparison with the activity of the reference compound (jack bean urease) and presented in terms of unit activity. (C: control group, P: plumbagin treated group, SSA: *Staphylococcus saprophyticus*, KPN: *Klebsiella pneumoniae*, KOX: *Klebsiella oxytoca*, PVU: *Proteus vulgaris*, PMI: *Proteus mirabilis*, MMO: *Morganella morganii*).

Table 1. Absorbance of standard urease activity measured by colorimetric assay

Urease (U)	Mean Abs. ± SD	% CV
0.001	0.0043 ± 0.0002	4.7
0.01	0.0489 ± 0.0015	3.1
0.1	0.4335 ± 0.0072	1.7
1	4.313 ± 0.0322	0.7

ller-Hinton broth, Brucella agar and brain heart infusion (BHI) broth were purchased from Becton-Dickinson (Braintree, MA, USA). Bovine serum was purchased from Gibco (Long Island, NY, USA). Plumbagin, jack bean urease, acetohydroxamic acid (AHA) and protease inhibitor cocktail were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Antibodies to detect urease A and urease B were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and polyclonal antibody against whole *H. pylori* was produced as previously described [10].

Bacterial culture

H. pylori were grown on the Brucella agar plate supplemented with 10% bovine serum at 37°C for 72 h in a humidified atmosphere with 5% CO_2. Then the bacterial colonies were collected and suspended in BHI broth supplemented with 10% bovine serum. The number of bacterial particles in the *H. pylori* suspension was adjusted to MacFarland 0.33 and incubated at 37°C for 72 h in a humidified atmosphere with 5% CO_2. Other bacteria were inoculated and grown in BHI broth at 37°C for 24 h.

Urease activity assay

Supernatant of the bacterial culture was collected. The supernatant was incubated with indicated concentrations of plumbagin at 37°C for 1 h. Five microliters of 20% urea (Duksan Pure Chemical, Seoul, South Korea) was added and further incubated at 25°C for 1 min. After incubation, the amount of ammonia produced was measured by ammonia assay kit (Asan Pharmaceutical, Seoul, South Korea). Briefly, 400 μL of deproteinization solution was added, vortexed and centrifuged at 2500 RPM for 5 min. One hundred microliters of supernatant was mixed with 100 μL of phenol (40 g/L), 50 μL of NaOH (35.6 g/L) and 100 μL of sodium hypochlorite (10%) were incubated at 37°C for 10 min. The absorbance was measured at 630 nm wavelength. Jack bean urease was used as a reference compound to calculate the urease activity. Jack bean urease was dissolved in 0.2 M sodium phosphate buffer (pH 7.0) and standard curve was drawn by measuring the activity of 10-fold serially diluted (0.001, 0.01, 0.1 and 1 U) jack bean urease (**Figure 1A** and **Table 1**). The unit activity of ureases was calculated by using equation derived from the standard curve.

Broth dilution method to determine MIC

H. pylori grown on the Brucella agar plate were collected and suspended in Mueller-Hinton broth supplemented with 10% bovine serum. The number of bacterial particles in the *H. pylori* suspension was adjusted to MacFarland 0.33. The bacteria were treated with indicated concentrations of plumbagin and incubated for 72 h. The bacterial growth was examined by absorbance at 600 nm wavelength.

Western blotting

Cultured *H. pylori* were washed twice with PBS and then lysed with RIPA buffer containing pro-

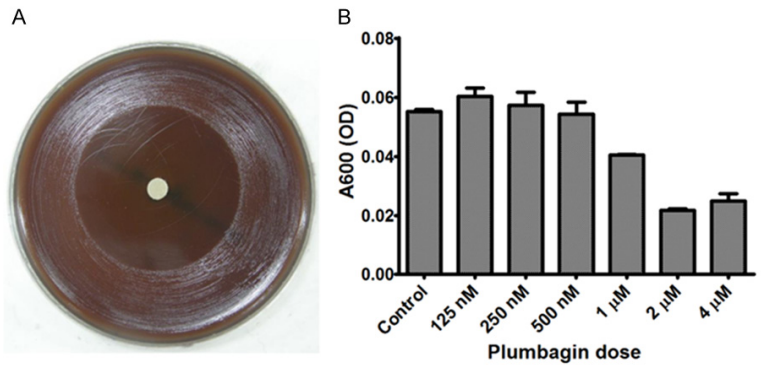

Figure 2. Inhibitory effect of plumbagin on *H. pylori* growth. A. The disk diffusion test of plumbagin on *H. pylori*. One hundred microliters of bacterial suspension was adjusted to McFarland scale 5.0 (15×10^8/mL) and spread on Mueller-Hinton blood agar. A 10 μM plumbagin disc was attached to the middle of the plate and incubated for 72 h. B. The broth dilution test was conducted to determine the inhibitory concentration of plumbagin on *H. pylori*. The bacterial suspension of *H. pylori* was prepared in Mueller-Hinton broth supplemented with 10% bovine serum. The indicated concentration of plumbagin was treated with 2 mL of bacterial suspension previously adjusted to McFarland scale 0.33 (1×10^8/mL). The absorbance was measured after incubation for 72 h. The data were obtained from three independent tests.

tease inhibitor cocktail. The cell lysates were sonicated for 1 min and incubated on ice for 10 min. The lysates were then centrifuged and the supernatants were subjected to westernblot as described previously [11]. In brief, protein samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane and the membrane incubated with primary antibodies overnight and then with appropriate secondary antibodies for 1 h. Bands were visualized using ECL (Thermo, Waltham, MA, USA).

Statistical analysis

Data in the graphs are presented as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism 5.02 software (GraphPad Software, San Diego, CA, USA). The data were analyzed by unpaired Student's t-test and $P < 0.05$ was considered to be statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

Results

Inhibitory effect of plumbagin on urease activity of various bacteria

Ureases from various bacteria (*Staphylococcus. saprophyticus, Klebsiella pneumoniae, K. oxytoca, Proteus vulgaris, P. mirabilis,* and *Morganella morganii*) were isolated, incubated with plumbagin *in vitro*, and assayed for their activity. The results showed that plumbagin inhibited urease activity from various bacteria, although the inhibitory effects were dependent on the bacteria (**Figure 1B**).

Inhibitory effect of plumbagin on H. pylori growth and urease activity

As urease activity is particularly important for *H. pylori* colonization and pathogenesis, we investigated the effect of plumbagin on *H. pylori*. We performed the disc diffusion test to examine the inhibition of *H. pylori* by plumbagin. The *H. pylori* reference strain ATCC 49503 was used in this study, and the bacteria were grown on Mueller-Hinton agar plate supplemented with 5% sheep blood. A clear inhibition zone, with a diameter of 43 mm, was observed around the 10 mm disc (**Figure 2A**). We then investigated the MIC by using the broth dilution test, and found that the MIC of plumbagin against *H. pylori* was 1 μM (**Figure 2B**). Our data confirmed the anti-microbial effect of plumbagin on *H. pylori*, and we also determined the MIC by using the broth dilution test. Moreover, these results were concordant with previous reports that suggested an inhibitory effect on several Western-type *H. pylori* strains [12].

The function of urease is particularly important for the pathogenesis of *H. pylori*, because the bacteria require urease to colonize the gastric mucosa. To investigate whether plumbagin influenced the synthesis of ureases, the protein levels of the urease subunits (urease α and urease β) were measured by using western blotting. We observed that the protein levels of the urease subunits were unaffected by plumbagin treatment (**Figure 3A**). However, although plumbagin did not affect the protein level of the ureases, it did result in the inhibition of urease function. In an *in vitro* assay of urease activity, 10 μM plumbagin reduced the activity of urease isolated from *H. pylori* from 0.026 U to 0.006 U (75.4% inhibition) (**Figure 3B**). To con-

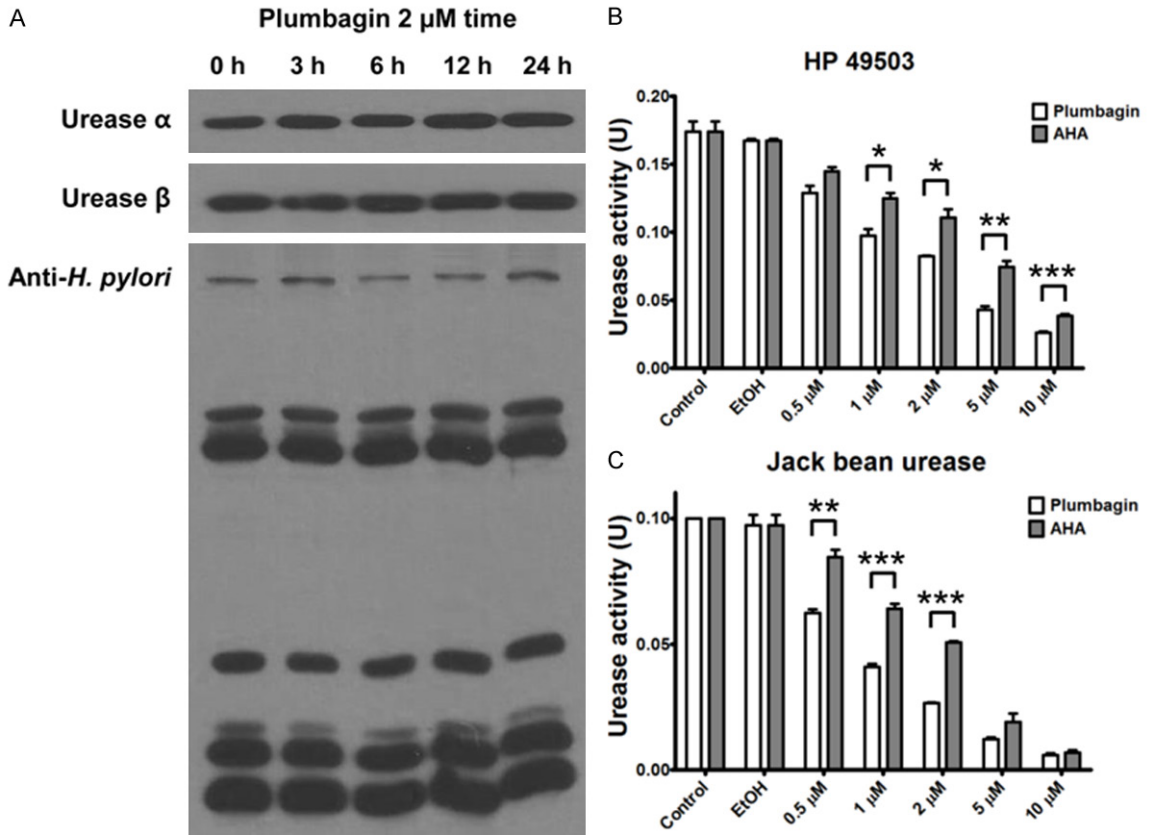

Figure 3. Effect of plumbagin on urease activity and expression of subunits of *H. pylori* urease. A. The protein levels of urease subunits after plumbagin treatment. *H. pylori* suspension was adjusted to McFarland scale 0.33 (1×10^8/mL) and treated with plumbagin (2 μM) for the indicated time periods. The cell lysates were collected and subjected to western blot. Non-specific bacterial proteins detected by anti-*H. pylori* antibody were used as an internal control. B. The inhibition of *H. pylori* urease activity by plumbagin or AHA. *H. pylori* was incubated in BHI broth supplemented with 10% bovine serum for 72 h and the supernatant, which included the urease, was collected. The supernatant was incubated with indicated the concentration of plumbagin or AHA for 1 h and the urease activity was measured. The urease activity was calculated through comparison with the activity of a reference compound (jack bean urease) and presented in terms of unit activity. C. The inhibition of purified jack bean urease activity by plumbagin or AHA. The indicated concentrations of plumbagin or AHA were incubated with 0.1 U of jack bean urease for 1 h and the urease activity was measured. The data were obtained from three independent experiments and analyzed by unpaired Student's *t*-test ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

Table 2. The percentage inhibition of urease activity by plumbagin and AHA

Concentration (μM)	% Inhibition (Mean ± SD)					
	HP49503			Jack bean urease		
	Plumbagin	AHA	*p* value	Plumbagin	AHA	*p* value
0.5	25.84 ± 1.25	16.22 ± 8.04	0.0549	37.67 ± 2.52	15.33 ± 4.73	0.0019**
1	43.47 ± 8.38	27.70 ± 8.02	0.0108*	59.00 ± 2.00	35.83 ± 3.33	0.0005***
2	52.32 ± 3.34	35.87 ± 10.01	0.0102*	73.33 ± 0.58	49.23 ± 0.67	< 0.0001***
5	74.96 ± 3.6	56.97 ± 6.50	0.0042**	87.67 ± 1.15	80.92 ± 5.83	0.1207
10	84.78 ± 1.73	77.59 ± 1.43	0.0008***	94.00 ± 1.00	92.97 ± 1.46	0.371

$^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

firm the inhibitory function of plumbagin on urease activity, purified jack bean urease was used as a reference molecule. Plumbagin markedly reduced the activity of jack bean urease from 0.1 U to 0.006 U (93.9% inhibition) (**Figure 3C**). The *in vitro* urease inhibitory activity of

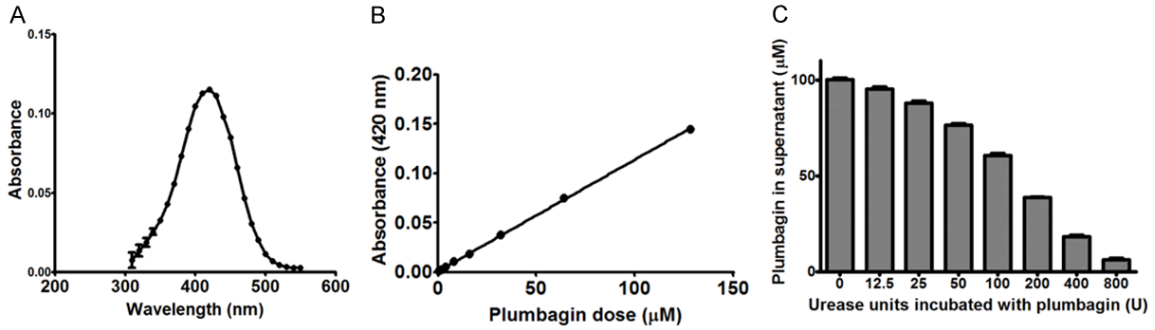

Figure 4. Binding affinity of plumbagin to jack bean urease protein. A. The optimal wavelength for the measurement of plumbagin concentration. The absorbance of 100 μM plumbagin was measured between 310 and 550 nm at 10 nm intervals. The graph was constructed from data collected from triplicate experiments. B. The standard curve illustrating the absorbance of the plumbagin standard. Plumbagin solution was prepared at 128 μM and subjected to 2-fold serial dilutions to 2 μM. The absorbance of the standard plumbagin solutions was measured at 420 nm. The graph was constructed by using data collected from triplicate experiments. C. The binding affinity of plumbagin to jack bean urease protein. Various doses of jack bean urease (12.5-800 U) were incubated with 500 μM plumbagin for 1 h. After incubation, the urease proteins were precipitated and the plumbagin concentration remaining in the supernatant was measured.

Table 3. Absorbance of 100 μM plumbagin at various wavelength

Wavelength (nm)	Mean Abs. ± SD	% CV
310	0.0076 ± 0.0067	87.5
320	0.0136 ± 0.0051	37.2
330	0.0188 ± 0.0038	20.3
340	0.0256 ± 0.0026	10.3
350	0.0326 ± 0.0022	6.7
360	0.0429 ± 0.0017	4.0
370	0.0557 ± 0.0010	1.8
380	0.0731 ± 0.0004	0.5
390	0.0905 ± 0.0004	0.4
400	0.1048 ± 0.0003	0.3
410	0.1129 ± 0.0002	0.2
420	0.1152 ± 0.0002	0.2
430	0.1113 ± 0.0003	0.3
440	0.0979 ± 0.0003	0.3
450	0.0850 ± 0.0004	0.4
460	0.0658 ± 0.0004	0.6
470	0.0466 ± 0.0004	0.9
480	0.0305 ± 0.0005	1.7
490	0.0203 ± 0.0005	2.6
500	0.0116 ± 0.0005	4.3
510	0.0069 ± 0.0004	5.2
520	0.0047 ± 0.0001	2.7
530	0.0034 ± 0.0000	1.4
540	0.0029 ± 0.0002	8.4
550	0.0028 ± 0.0003	11.7

plumbagin was also compared with that of acetohydroxamic acid (AHA), a well-known urease inhibitor. The percentage inhibition of urease activity by plumbagin and AHA is listed in **Table 2**. For both ureases, the inhibition of urease activity of plumbagin was significantly higher than that of AHA (**Figure 3B**, **3C** and **Table 2**), and the inhibitory effect of plumbagin was stronger against purified jack bean urease than *H. pylori* urease (**Figure 3B** and **3C**). This presumably occurred because various substances present in the bacteria-cultured media interfered with the interaction between urease and plumbagin, whereas the purified urease could interact directly with plumbagin without any interference. Thus, our results indicate that plumbagin inhibited the function of ureases.

Binding affinity of plumbagin to urease

To demonstrate the optimal wavelength at which the concentration of plumbagin should be measured, the absorbance of 100 μM plumbagin was measured between 310 and 550 nm at 10 nm intervals. The peak absorbance was observed at 420 nm (**Figure 4A** and **Table 3**). We then measured the absorbance of the various doses of plumbagin at the optimal wavelength to construct a standard curve. Standard plumbagin was subjected to 2-fold serial dilutions from 128 μM to 2 μM. The absorbance of the plumbagin standards was measured at 420 nm and a standard curve was constructed (**Figure 4B** and **Table 4**). Various doses of jack bean urease (12.5-800 U) were incubated with 500 μM of plumbagin at 37°C for 1 h. After incubation, the urease proteins were precipi-

Table 4. Absorbance of standard plumbagin measured at 420 nm wavelength

Plumbagin (μM)	Mean Abs. at 420 nm ± SD	% CV
2	0.0025 ± 0.0001	5.7
4	0.0052 ± 0.0001	2.4
8	0.0107 ± 0.0005	4.3
16	0.0187 ± 0.0002	1.3
32	0.0376 ± 0.0006	1.6
64	0.0748 ± 0.0002	0.3
128	0.1446 ± 0.0015	1.1

Table 5. Concentration of plumbagin remaining in the supernatant after precipitation with urease

Urease (U)	Mean concentration of plumbagin (μM) ± SD	% CV
0	100.1 ± 1.51	1.5
12.5	95.4 ± 1.55	1.6
25	87.9 ± 1.64	1.9
50	76.6 ± 1.04	1.4
100	60.6 ± 1.35	2.2
200	38.6 ± 0.51	1.3
400	18.2 ± 1.32	7.3
800	6.2 ± 1.1	17.7

tated with 400 μL of 50 mg/mL sodium tungstate (Na_2Wo_4) and centrifuged at 2500 rpm for 5 min. The remaining plumbagin concentration in the supernatant was measured at 420 nm. We found that the plumbagin concentration remaining in the supernatant decreased in a urease dose-dependent manner, which indicated that plumbagin bound to and precipitated with the jack bean urease proteins (**Figure 4C** and **Table 5**). These results demonstrate the binding affinity of plumbagin to urease protein.

Discussion

Bacterial ureases are associated with the pathogenesis of several diseases. For example, *P. mirabilis* often induces urinary stones during its infection of the urinary tract, because the ammonia produced from the hydrolysis of urea causes polyvalent ions to become insoluble, thereby resulting in stone crystallization [13]. In particular, urease is a major virulence factor of *H. pylori* and considered indispensable for the colonization of the gastric mucosa. The decreased synthesis or abnormal function of urease is closely associated with survival of *H. pylori*, because urease activity is necessary to overcome the hostile environment of the gastric mucosa. Furthermore, *H. pylori* infection in patients with liver cirrhosis can cause hepatic coma and hepatic encephalopathy, because the bacteria convert urea to ammonia, which is then readily absorbed from the gastric lumen into circulation, thereby elevating ammonia levels in the blood [14]. Thus, urease inhibitors have the potential to be used as a supportive agent for the prevention of *H. pylori* infection.

Previously, synthetic compounds such as bismuth complexes, boric and boronic acids, hydroxamic acid derivatives, imidazole derivatives and phosphoramidites have been widely studied as potential urease inhibitors [15, 16]. Some studies have highlighted the possibility of AHA for clinical use [17-20]. However, AHA had severe side effects, such as teratogenicity and, psychoneurological and musculo-integumentary symptoms [18, 19, 21]. Therefore, the search for novel urease inhibitors with promising levels of activity is ongoing.

In the present study, we confirmed that plumbagin exerted an anti-microbial effect on *H. pylori* and determined that the MIC of plumbagin against *H. pylori* was 1 μM. Furthermore, we found that plumbagin inhibited urease activity of various bacteria, including *H. pylori*. The urease inhibitory function of plumbagin was confirmed through the inhibition of purified jack bean urease and the binding affinity of plumbagin to urease was established. The urease activity of *H. pylori* is crucial for colonization of the bacteria on gastric mucosa, which subsequently leads to various gastric diseases. Therefore, this study suggested that plumbagin inhibited the colonization of *H. pylori* through both its anti-urease and anti-microbial activity. We expect that our results will help understand the inhibitory function of plumbagin on *H. pylori*. However, further studies appear to be necessary for other various virulence factors of *H. pylori*, to determen whether they are regulated by plumbagin. Moreover, the use of a *H. pylori*-infected cell line or animal model will further enhance the understanding of plumbagin for use as a treatment for *H. pylori*.

Currently, triple therapy using clarithromycin, amoxicillin, and a proton pump inhibitor has been proposed as the first-line treatment for

eradication of *H. pylori* [22]. However, the resistance of *H. pylori* to clarithromycin and amoxicillin has increased owing to the increased use of these antibiotics, and the continued increase in the anti-microbial resistance of *H. pylori* will significantly limit the effective eradication of *H. pylori* in the future [23]. Therefore, it is necessary to use natural compounds, such as plumbagin, as a supportive agent for the prevention and eradication of *H. pylori*.

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Disclosure of conflict of interest

None.

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