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Molecular Docking, Kinetics Study, and Structure−Activity Relationship Analysis of Quercetin and Its Analogous as *Helicobacter pylori* Urease Inhibitors

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ABSTRACT: It was disclosed in our group for the first time that the flavonoids in *Lonicera japonica* Thunb. are related to its therapy for gastric ulcer. Based on this finding, 20 flavonoids were selected for *Helicobacter pylori* urease inhibitory activity evaluation, and quercetin showed excellent potency with IC_{50} of 11.2 ± 0.9 μM. Structure−activity relationship analysis revealed that removal of the 5-, 3-, or 3′-OH in quercetin led to a sharp decrease in activity. Thus, 3- and 5-OH as well as 3′,4′-dihydroxyl groups are believed to be the key structural characteristics for active compounds, which was supported by the molecular docking study. Meanwhile, the results obtained from molecular docking and enzymatic kinetics research strongly suggested that quercetin is a noncompetitive urease inhibitor, indicating that quercetin may be able to tolerate extensive structural modification irrespective of the shape of the active site cavity and could be used as a lead candidate for the development of novel urease inhibitors.

KEYWORDS: *Lonicera japonica Thunb., quercetin, urease inhibitor, noncompetitive inhibition*

INTRODUCTION

Lonicera japonica Thunb. (family: Caprifoliaceae), also known as *Japanese honeysuckle*, *Jin Yin Hua*, or *Ren Dong*, is native to the East Asian countries. Dry buds or flowers of *L. japonica* are commonly used as a traditional Chinese medicine (TCM) herb, and more than 500 prescriptions containing *L. japonica* have been used to treat various diseases including arthritis, diabetes mellitus, allergy, infections, sores, and swelling.[1,2] *L. japonica* has been therefore listed in the Pharmacopoeia of the People's Republic of China since 1995. In China, *L. japonica* is extensively planted for this end and also extensively used as a health tea. In addition, modern pharmacological studies further disclosed that the extract of *L. japonica* exhibited a broad spectrum of biological activities such as hepatoprotective, neuroprotective, cytoprotective, antimicrobial, antiviral, and antioxidative effects.[3,4]

Recently, some research suggested that *L. japonica* shows good therapeutic potential for gastric ulcer and inhibitory activity against *Helicobacter pylori*. Zhang et al. used a powder of *L. japonica* flowers to treat gastric ulcer caused by *H. pylori*, and a *H. pylori* eradication rate of 72.9% was found after 2 weeks of treatment.[5] Wang et al. reported an *H. pylori* eradication rate of 95.9% with a low recurrence rate (1.6%) by oral administration of a decoction of *WuWeiXiaoDuYin*, a prescription drug containing *L. japonica*, combined with Omeprazole.[6] In 2008, Li et al. demonstrated that an extracting solution (1g/mL of crude drug) of Bee Propolispro displayed inhibitory activity against *H. pylori* in vitro with a MIC of 12.5 mg/mL, while the MIC (0.78 mg/mL) significantly decreased when it was combined with that of *L. japonica* flowers.[7] Extensive studies on the chemical compounds in *L. japonica* demonstrated that chlorogenic acid and luteolin glycoside showed good pharmacological effects, and they are believed to be the main active compounds.[1] Therefore, chlorogenic acid and luteolin glycoside have been officially used as the indicator compounds to characterize the quality of this herb in the current Chinese Pharmacopoeia (Committee for the Pharmacopoeia of PR China, 2010).[8]

In *L. japonica* flower, luteolin mainly occurs in glycosylated forms with the content being 0.1−0.9%, while that of luteolin aglycone is much lower (<0.01%).[9] Nevertheless, like other flavonoid glycosides, luteolin glycoside is easy hydrolyzed to luteolin by enterobacterial enzymes after oral administration.[10] It is well known that flavonoids have attracted much attention because of their various beneficial biological activities like preventing cancer, suppressing inflammation, antioxidation, and decreasing fat absorption.[11] From the flowers and rhizomes of *Pueraria thunbergiana*, a folk medicine for the treatment of gastroduodenal diseases in Korea, Bae et al. found the first flavonoid urease inhibitor, daidzein, showing weak activity against *H. pylori* urease with an IC_{50} of 0.43 mg/mL.[12] Subsequently, Matsubara's[13] and our research[14,15] has proved that tea polyphenols ((+)-gallocatechin with an IC_{50} of 8.7 μM against *H. pylori* urease) and some open-ring derivatives of isoflavones (the most active compound with an IC_{50} of 1.5 μM) have moderate to good inhibitory activity. On the basis of these interesting findings and the effectiveness of *L. japonica* for

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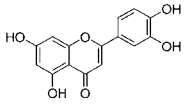
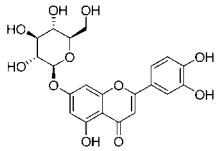
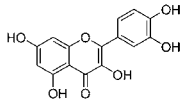
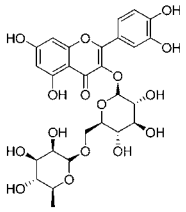
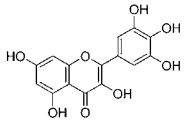
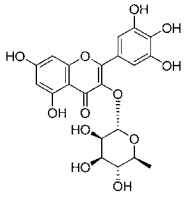
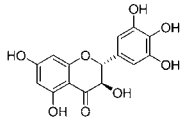
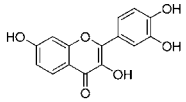
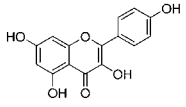
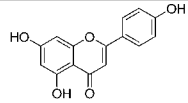
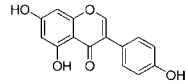
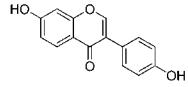
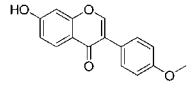
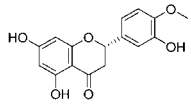
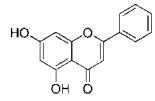
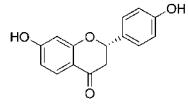
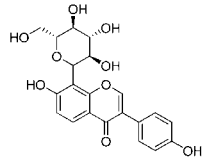
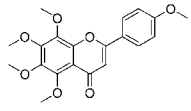
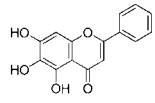
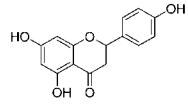
Table 1. In Vitro Inhibitory Activity Data of *L. japonica* Flower Extract, Crude Flavonoid from *L. japonica*, and the Selected Flavonoids against *H. pylori* Urease

entry	compound		IC_{50} (μM)
	name	structure	
1	*L. japonica* flower extract	-----	2195±128
2	crude flavonoid from *L. japonica*	-----	946±54
3	luteolin		35.4±2.0
4	Luteolin glycoside		55.8±4.4
5	quercetin		11.2±0.9
6	rutin		67.6±6.5
7	myricetin		77.2±4.8
8	myricitrin		98.7±9.3
9	dihydromyricetin		156±16
10	fisetin		118±11
11	kaempferol		105±9.7
12	apigenin		138±12
13	genistein		151±18
14	daidzein		556±31
15	formononetin		2003±115
16	hesperetin		324±27
17	chrysin		302±37
18	liquiritigenin		862±74
19	puerarin		922±82
20	tangeretin		4628±215
21	baicalein		291±29
22	naringenin		347±41
23	acetohydroxamic acid		19.4±2.0

treating gastric ulcers caused by *H. pylori*, we therefore examined the inhibitory activities of *L. japonica* extract, crude flavonoids, luteolin, and its analogues against *H. pylori* urease in vitro.

MATERIALS AND METHODS

Chemical and Reagents. Flavonoids were acquired from Shanxi Huike Botanical Development Co., Ltd. (Shanxi, China). Protease inhibitors (Complete mini EDTA-free) were purchased from Roche Diagnostics GmbH (Mannheim, Germany), and Brucella broth was from Becton-Dickinson (Cockeysville, MD). Horse serum was from Hyclone (Utah, American). Dry flowers of *L. japonica* were supplied by Longhui Jinchun *L. japonica* Planting Professional Cooperative (Hunan, China). All other chemicals (reagent grade) used were purchased from Sinopharm Chemical Reagent Co., Ltd. (China).

Preparation of *L. japonica* Flower Extract. The *L. japonica* flowers were dried under vacuum at 30 °C for 20 h and then ground into a fine and uniform powder. To the *L. japonica* powder was added 10 times the weight of water. The resulted mixture was soaked at room temperature for 60 min and then refluxed for 30 min. After filtration, the residue was decocted once more with water and filtrated. The combined filtrates were concentrated to give a *L. japonica* extract under reduced pressure.

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Isolation of Crude Flavonoids from *L. japonica*. The crude flavonoids from *L. japonica* were obtained according to the reference methods with some modifications.[16,17] To be specific, the *L. japonica* powder was extracted with 10 times the weight of ethanol ($V_{ethanol}/V_{water}$ = 70%) under reflux for 2 h. After repeating three times, the combined filtrates were concentrated under reduced pressure to give a viscous residue, which was partitioned between petroleum ether (bp 60−90 °C) and water. The water layer was extracted three times with ethyl acetate. The combined organic layers were washed with saturated brine and condensed under reduced pressure to yield a brownish yellow crude flavonoid powder with the content of total flavonoids being 57%.

Determination of Total Flavonoid Content. The total flavonoid content in samples was determined according to the protocol of Loizzo and his co-workers.[18]

Bacteria. *H. pylori* (ATCC 43504; American Type Culture Collection, Manassas, VA) was grown in Brucella broth supplemented with 10% heat-inactivated horse serum for 24 h at 37 °C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2), as previously described.[14,15]

Preparation of *H. pylori* Urease. For urease inhibition assays, 50 mL broth cultures (2.0×10^8 CFU/mL) were centrifuged (5000*g*, 4 °C) to collect the bacteria, and after washing twice with phosphate-buffered saline (pH 7.4), the *H. pylori* precipitation was stored at −80 °C. *H. pylori* was returned to room temperature, and after the addition of 3 mL of distilled water and protease inhibitors, sonication was performed for 60 s. Following centrifugation (15,000*g*, 4 °C), the supernatant was desalted through a Sephadex G-25 column (PD-10 columns, Amersham Pharmacia Biotech, Uppsala, Sweden). The resultant crude urease solution was added to an equal volume of glycerol and stored at 4 °C until use in the experiment.

Urease Activity Determination. One unit of urease activity is defined as the amount of enzyme needed to liberate 1.0 μmol of NH_3 from urea per min at pH 7.0 at 25 °C. According to the enzymatic assays of Sigma-Aldrich,[19] we determined the activity of the obtained *H. pylori* urease. Briefly, 1 mL of 0.5 M urea with 0.05% (w/v) bovine serum albumin solution was pipetted into a test tube and warmed to 25 °C. Then, 0.20 mL of urease solution containing 0.02 M sodium phosphate buffer (pH 7.0 at 25 °C) was added. The obtained mixture was incubated at 25 °C for exactly 5 min with magnetic stirring and followed by the addition of 0.20 mL of indicator (4.0 mg/mL of *p*-nitrophenol solution), which was titrated immediately with 0.1 M standardized HCl until the color turned from yellow to colorless. As for the blank, the only difference was that the urease solution was added after incubation. The urease activity was calculated based on the following formula:

$$\text{U/mL urease} = \frac{0.1\ \text{mmol/mL} \times \Delta V_{HCL}\ \text{mL} \times 1000\ \mu\text{mol/mmol}}{5\ \text{min} \times 0.2\ \text{mL}}$$

Measurement of Urease Inhibitory Activity. The assay mixture, containing 25 μL (10 U) of *H. pylori* urease and 25 μL of the test compound, was preincubated for 1.5 h at room temperature in a 96-well assay plate. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn.[20]

Kinetics Study. Lineweaver−Burk plots of 1/absorbance versus 1/urea were used to determine the type of enzyme inhibition. Urease inhibition was measured by varying the concentration of urea in the presence of different concentrations of quercetin (**5**). Inhibitory constants (K_i) were determined as the intersection on the *x*-axis of the plots of $1/V_{max\ app}$ vs different concentrations of inhibitor, in which $V_{max\ app}$ was obtained from the Lineweaver−Burk lines or was directly measured from Dixon plots as an intercept on the *x*-axis. All experiments were conducted in triplicate.

Protocol of the Docking Study. The automated docking studies were carried out using AutoDock version 4.2. First, AutoGrid component of the program precalculates a three-dimensional grid of interaction energies based on the macromolecular target using the AMBER force field. The cubic grid box of 110 Å size (x, y, z) with a spacing of 0.375 Å and grid maps were created representing the catalytic active target site region where the native ligand was embedded. Then automated docking studies were carried out to evaluate the binding free energy of the inhibitor within the macromolecules. The GALS search algorithm (genetic algorithm with local search) was chosen to search for the best conformers. The parameters were set using the software ADT (AutoDockTools package, version 1.5.4) for PCs, which is associated with AutoDock 4.2. Default settings were used with an initial population of 50 randomly placed individuals, a maximum number of 2.5×10^6 energy evaluations, and a maximum number of 2.7×10^4 generations. A mutation rate of 0.02 and a crossover rate of 0.8 were chosen. Results differing by less than 0.5 Å in positional root-mean-square deviation (RMSD) were clustered together, and the results of the most favorable free energy of binding were selected as the resultant complex structures.

RESULTS AND DISCUSSION

Urease Inhibitory Activity. It is well known that *H. pylori* is an etiological factor of gastritis and peptic ulcers. Urease of the pathogen enables it to survive in the acidic pH of gastric juice and to spread infection to the inner layers of gastroduodenal mucosa.[21] Effectiveness of *L. japonica* in treating gastric ulcers is sure enough,[5−7,22] and we putatively attributed it to the inhibition of *L. japonica* against *H. pylori* urease. On the basis of this consideration, the inhibitory activity of the *L. japonica* flower extract against *H. pylori* urease was therefore determined (Table 1). Just as we expected, a positive result was observed. As mentioned above, it was proved that several flavonoids showed moderate to good urease inhibitory activity, and luteolin is believed to be one of the main active compounds in *L. japonica* flower. Therefore, we further evaluated the urease inhibitory activity of the crude flavonoid extracted from *L. japonica* flowers (Table 1). In comparison with the *L. japonica* flower extract, the increased inhibitory activity of the crude flavonoid provides a strong support for our hypothesis.

It was recently reported that the flower buds of Chinese *L. japonica* contain 15 flavonoids with quercetin (**5**), rutin (**6**), luteolin (**3**), luteolin glycoside (**4**), and kaempferol (**11**) being the most common.[9] These flavonoids together with some of their analogues were selected to assay urease inhibitory activity for structure−activity relationship analysis, and their chemical structures (the atom numbers are shown in Figure 1) and assay

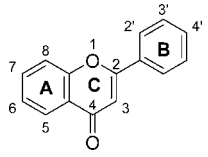

Figure 1. Atom numbers of the flavonoid skeleton.

results are listed in Table 1. Out of these flavonoids, quercetin (**5**) is the most active with an IC_{50} of 11.2 ± 0.9 μM against *H. pylori* urease, which is slightly more potent than the positive control, acetohydroxamic acid (IC_{50} = 19.4 ± 2.0 μM). Replacement of the 3-hydroxyl group in quercetin (**5**) with rutinoside resulted in loss of activity over 4-fold, which indicated that too high hydrophilicity may weaken the binding energy between flavone and the enzyme. This is confirmed by the comparison of luteolin glycoside (**4**), myricitrin (**8**), and puerarin (**19**) with luteolin (**3**), myricetin (**7**), and daidzein (**14**), respectively. Removal of the 3-hydroxyl group in quercetin (**5**) also resulted in a mild decrease in potency

(luteolin (**3**), 35.4 ± 2.0 μM). In comparison with quercetin (**5**), the addition of a hydroxyl group in the 5′ position caused a significant loss in potency (myricetin (**7**), 77.2 ± 4.8 μM). However, removal of the 5- or 3′-hydroxyl group in quercetin (**5**) gives fisetin (**10**) or kaempferol (**11**), also showing a sharp decrease in activity and importantly to an even less efficient inhibitor (genistein (**13**) vs daidzein (**14**); liquiritigenin (**18**) vs Naringenin (**22**)). This clearly suggested that the 3- and 5-hydroxyl groups as well as the 3′,4′-dihydroxyl groups are key structural characteristics for active compounds. In the comparison of myricetin (**7**) or apigenin (**12**) with dihydromyricetin (**9**) or naringenin (**22**), an approximately 2-fold decrease in activity was observed when the double bond between C3 and C4 changed into a single bond. Converting a hydroxyl group into a methoxy group (formononetin (**15**)) led to a pronounced loss of activity relative to daidzein (**14**). As expected, methylation of all hydroxyl groups in a flavonoid resulted in a complete loss (tangeretin (**20**)) of urease inhibitory activity likely due to the significant increased hydrophobicity. Unexpectedly, introduction of a hydroxyl group between 5- and 7-hydroxyl groups caused no change in potency. Finally, apigenin (**12**), a flavone, with an IC_{50} of 138 ± 12 μM was slightly more potent than the corresponding isoflavone analogue (genistein (**13**), IC_{50} = 151 ± 18 μM).

Kinetics of Urease Inhibition by Quercetin. Since the first isoflavone (daidzein (**14**)) was reported as a *H. pylori* urease inhibitor by Bae and his co-workers,[12] several polyphenols such as Schiff bases derived from polyphenol,[23] *C*-glycosylflavonoids,[24] resveratrol,[25] catechol derivatives,[15] and oximes derived from deoxybenzoins[26] were documented. Among these reported polyphenols, resveratrol has been determined as a reversible inhibitor of *H. pylori* urease with a noncompetitive inhibition mechanism,[25] while no flavonoids have been done so far. Therefore, the most active compound, quercetin (**5**), was selected to determine the inhibition mechanism of this kind of inhibitor, and the data were plotted in a Lineweaver−Burk fashion (Figure 2A). Obviously, these plots tend to a common intercept on the negative *x*-axis (1/S axis), indicating a pure noncompetitive inhibition toward *H. pylori* urease (K_m = 0.258 mM), which means that quercetin (**5**) binds to the enzyme at a site other than its catalytic site to produce an inactive complex, irrespective of substrate binding.[27] The K_i value was calculated directly from Dixon plots (Figure 2B) and was confirmed by plotting the $1/V_{max\ app}$ against different concentrations of quercetin (**5**),[28] where $1/V_{max\ app}$ values were calculated at each intersection point of lines on the *y*-axis of the Lineweaver−Burk plots (Figure 2C). The obtained K_i value was 9.34 μM, demonstrating the affinity of quercetin (**5**) toward *H. pylori* urease.

Computational Analysis of the Binding of Quercetin to Urease. In order to rationalize the noncompetitive inhibition mechanism revealed by the kinetics study, molecular docking of quercetin (**5**) on the crystal structure of *H. pylori* urease (entry 1E9Y in the Protein Data Bank) was performed to give the binding mode, and the results are shown in Figure 3. In order to validate the docking reliability, the ligand acetohydroxamic acid was removed from the active site, and the Grid Box was set large enough to do blind docking. As expected by the Lineweaver−Burk plots, quercetin (**5**) binds at a region outside the urea binding pocket (Figure 3A). Obviously, in this binding mode, quercetin (**5**) tightly anchors the flap, a helix-turn-helix motif composed of residues α313−α346,[29] over the active site cavity through N−H···O,

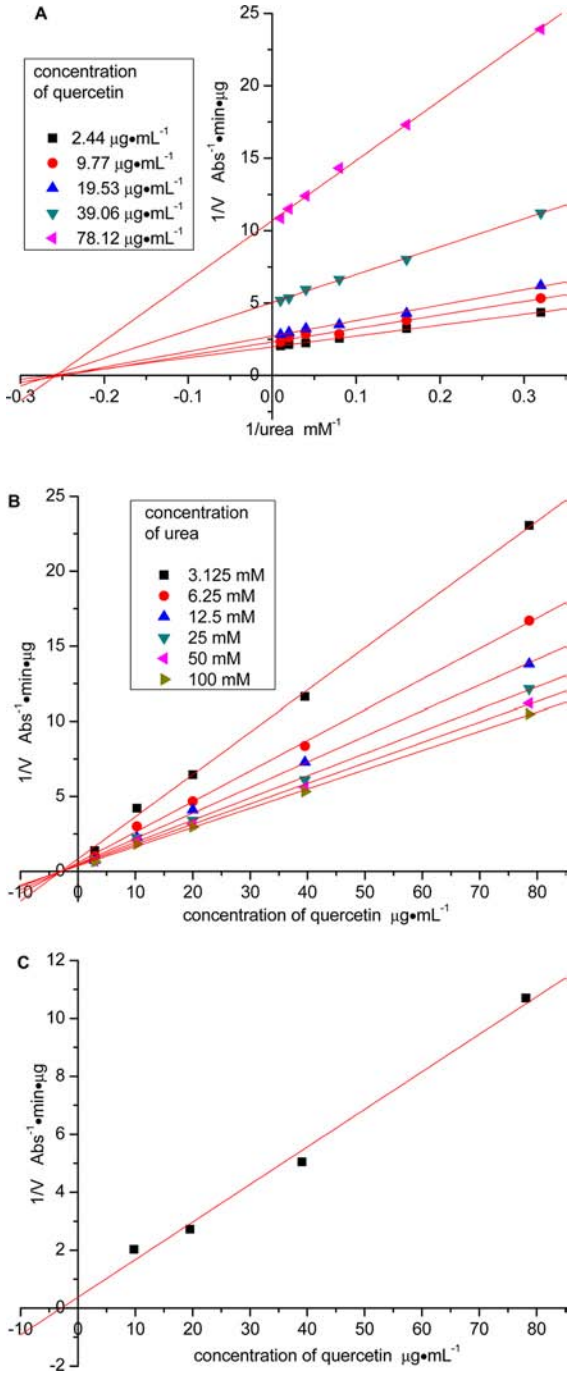

Figure 2. (A) Double-reciprocal Lineweaver−Burk plot of the inhibition of the *H. pylori* urease activity by quercetin (**5**). (B) Dixon plot of the reciprocal of the initial velocities vs various concentrations of quercetin (**5**) at fixed substrate concentrations. (C) Secondary replot of the Lineweaver−Burk plot, $1/V_{max\ app}$ vs various concentrations of quercetin (**5**).

O−H···O, and O−H···S hydrogen bonding interactions, which prevent the flap from backing to the close position. According to Benini et al.,[31] the highly mobile flap is in the open conformation and can allow extensive access of the substrate to the active site having the best fit. Then, the urea molecule within the active site induces a change in the conformation of the flexible flap back to a closed position by rearranging the Alaα365. In the closed conformation, the C−N bond of the urea molecule is broken, and urea collapses into ammonia and a

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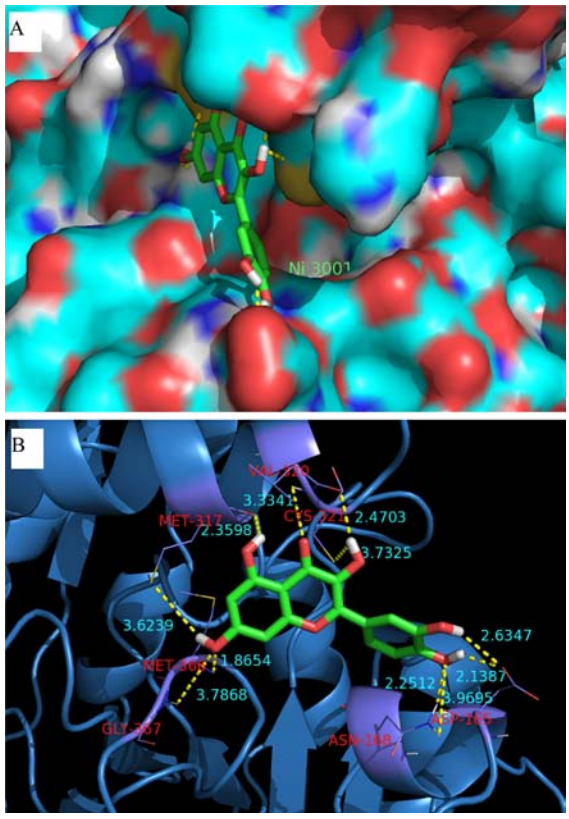

Figure 3. (A) Binding mode of quercetin (**5**) with *H. pylori* urease. The enzyme is shown in surface view, while **5** docked structures are shown as sticks. (B) Binding mode of quercetin (**5**) with *H. pylori* urease. For clarity, only interacting residues are labeled. Hydrogen bonding interactions are shown by dashes. These figures were created using PyMol.

Ni-bound carbamate. Finally, ammonia is released from the active site, assisted by the movement of Hisα322, and the mobile flexible flap then opens.[30,31] From this catalytic mechanism, the binding complex of urease and quercetin (**5**) with the flap in an open conformation will not effectively activate the inert urea without the coordinations of Alaα365 and Hisα322, which leads to an inactive enzyme. This docking mode with the inhibitor binding at a site other than the active site provides a rational explanation for the noncompetitive inhibition mechanism from kinetic studies.

In the best possible binding mode (Figure 3B), the quercetin (**5**) molecule engaged a cleft beside the active site cavity, using 11 typical hydrogen bonds to anchor the flap tightly with the backbone of the enzyme. 3-OH of quercetin (**5**) forms a strong O−H···O hydrogen bond (H···O distance = 2.4703 Å) to the backbone CO of Val320 and forms a relatively weak O−H···S hydrogen bond (H···S distance = 3.7325 Å) to the backbone SH of Cys321. Kühler et al. reported that covalent modification of Cys321 will lead to an inactive enzyme,[32] which indicated that Cys321 may be one of the key residues for the catalytic activity of *H. pylori* urease. Thus, these interactions provide a support for the significant decrease in urease inhibitory activity by the removal of 3-OH of a flavonoid (**5** vs **3**). Similarly, the absence of 5-OH causing a sharp decrease in activity may be related to the strong hydrogen bond (H···N distance = 2.3598 Å) between 5-OH of quercetin (**5**) and CO of Met317. In addition, the 5-CO of quercetin (**5**) binds via a weak hydrogen bond to NH of Val320 with a H···N distance of 3.3341 Å. The 7-OH of quercetin (**5**) is involved in three important interactions considered as hydrogen bonds: one is in between the O atom and the backbone amino group of Met366 with a H···O bond length of 1.8654 Å, and the second is relatively weak and observed between the O atom and the Gly367 backbone NH group with a H···O bond length of 3.7868 Å. On the contrary, the third using 7-OH as the hydrogen bond donor is found between the OH and the backbone S atom of Met317. The above-mentioned seven hydrogen bonding events mediated by A- and C-rings of quercetin (**5**) have solidified the flap in the open conformation, which is further strengthened by the hydrogen bonding mediated by the B-ring. To be specific, 3′-OH as a donor generates a hydrogen bond (H···O distance = 2.6347 Å) with the carbonyl moiety of Asp165, while 4′-OH as a donor also forms a hydrogen bond with the carbonyl moiety of Asp165 but is relatively strong on the one hand (H···O distance = 2.1387 Å). On the other hand, 4′-OH as an acceptor accepts two hydrogen bonds from the amino group of Asn168 with a H···O distance of 2.2512 and 3.9695 Å, respectively. Therefore, the B-ring with 3′,4′-dihydroxyl groups is important to the binding affinity and is a typical character for active compounds (**3**, **4**, **5**, and **6**). This binding mode also gives a rational explanation for the findings that removal of 3′-OH or methylation of 4′-OH led to a significant decrease in urease inhibitory activity (**3** vs. **12**, **5** vs. **11**, and **14** vs. **15**).

In conclusion, 20 flavonoids were selected based on the active component in *L. japonica*, luteolin glycoside, for evaluation of *H. pylori* urease. Out of them, quercetin is the most active with an IC_{50} of 11.2 ± 0.9 μM, showing slightly higher potency than the positive control, acetohydroxamic acid (IC_{50} = 19.4 ± 2.0 μM), and being comparable to that of (+)-gallocatechin reported by Matsubara.[13] Both the molecular docking study and structure−activity relationship analysis proved that 3-OH, 5-OH, and 3′,4′-dihydroxyl groups are very important for urease inhibitory activity and are believed to be the typical structural character of the active flavonoid. The kinetics study revealed that quercetin, the most active flavonoid assayed, is a reversible inhibitor of *H. pylori* urease with a noncompetitive inhibition mechanism. This indicates that quercetin may be able to tolerate an extensive structural modification irrespective of the shape of the active site cavity. Therefore, it may be a good lead for the development of urease inhibitors.

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Notes

The authors declare no competing financial interest.

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