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Genistein Attenuates *Helicobacter pylori*-associated Gastritis and Reduces Levels of Inflammatory Mediators in A Rat Model

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ABSTRACT

Background: Gastritis is an invariable finding in patients infected with *Helicobacter pylori* (*H. pylori*). Genistein is the predominant isoflavone in soybean, a specific inhibitor of tyrosine specific-protein kinase that has been demonstrated an anti-inflammatory property. The aim of this study was to determine the effects of genistein on *H. pylori*-associated gastritis in a rat model.

Methods: Male Sprague-Dawley rats were randomLy divided into three groups: group 1 (Control group), group 2 (*H. pylori* infection group), and group 3 (Genistein treatment group). The control group was treated with 0.1% DMSO (1 mL/rat, b.i.d.) for 17 day. In the *H. pylori* infection group and the genistein treatment group, pre-treatment with streptomycin suspended in drinking water (at dose 5 mg/mL) for 3 days, then the rats were inoculated with *H. pylori* suspension (10^{8-10} CFU/mL ; 1 mL/rat, b.i.d.) for 3 consecutive days. Thereafter, rats in the genistein treatment group were treated with genistein (16 mg/kg BW b.i.d.) for 14 days. The rats were then sacrificed at the end of the experimental protocol. Serum samples were collected for measurement of TNF-α level and CINC-1 level. The stomach was removed for detection *H. pylori* infection by urease test and for pathological examination by a pathologist.

Results: The levels of serum TNF-α and CINC-1 were significantly higher in the *H. pylori* infection group than in the control group (43.50 ± 16.51 vs. 20.89 ± 8.90 pg/mL, 138.10 ± 43.56 vs. 81.27 ± 19.89 pg/mL, $p<0.05$, respectively). As expected, the levels of serum TNF-α and CINC-1 were significantly lower in the genistein group than in the *H. pylori* infection group (29.33 ± 10.77 vs. 43.50 ± 16.51 pg/mL, 103.26 ± 23.76 vs. 138.10 ± 43.56 pg./mL, $p<0.05$, respectively). In *H. pylori* infection group, almost all of the stomach tissues showed moderate gastric inflammation and moderate to mark *H. pylori* colonization score. In genistein treatment group, stomach histopathology was improved when compared to *H. pylori* infection group, especially in the reduction of inflammatory cells infiltration.

Conclusion: Administration of genistein can attenuate *H. pylori*-induced gastritis in rats, possibly by reducing the inflammatory mediators and thereby improving gastric pathology.

Key words: *Helicobacter pylori*, Genistein, Gastritis associated *H. pylori* infection

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INTRODUCTION

H. pylori is a spiral gram-negative microaerophilic bacterium that colonizes approximately half of the world's population. Poor hygiene is a predisposing factor to infection, presumably via an oral - oral and a fecal-oral transmission[1-3]. Infection with *H. pylori* causes chronic active gastritis and significantly increases the risk for development of duodenal and gastric ulceration, gastric cancer and mucosal-associated lymphoid tissue lymphoma. In most persons, *H. pylori* colonization does not cause any symptoms. However, long-term carriage of *H. pylori* significantly increases the risk of developing site-specific diseases[4]. In a nation-wide study of gastric biopsy from 3,776 dyspeptic Thai patients from six different geographic regions, the overall incidence of gastritis 48.2%, in 98.2% of those infected *H. pylori*, there was an associated gastritis[5]. Gastric immune and inflammatory responses have emerged as key elements in the pathogenesis of gastritis and epithelial cell damage. Tumor necrosis factor alpha (TNF-α) is a major proinflammatory cytokine and plays an important role in the development of acute inflammation, including neutrophil infiltration of the gastric mucosa in response to *H. pylori*. TNF-α also stimulates transcription factors, induces the synthesis of various inflammatory cytokines including interleukin-1 (IL-1), IL-6 and IL-8[6-9,11,12]. Interleukin 8 is a prototypic human chemokine pertaining to the CXC family, which exerts chemotactic effects on polymorphonuclear leukocytes to the site of inflammation. It also has effects on cell proliferation, cell migration and tumor angiogenesis[8,12]. *H. pylori* infection rapidly up-regulates the expression of IL-8 in human gastric epithelial cell. Therefore, IL-8 mRNA expression was up-regulated within 1 hour after H. pylori infection, reaching a maximal increase of ~120-fold at 8 hours post-infection, and then decreasing[9]. Cytokine-induced neutrophil chemoattractant 1 (CINC-1), a counterpart of the human growth-regulated gene product (GRO) of the interleukin-8 family, has a potent neutrophil chemotactic activity in rats, similar to the effect of IL-8 in human[10,13,14]. Various cells have been reported to produce CINC-1 in response to inflammatory mediators, such as TNF-α, Interleukin 1 beta (IL1β), and lipopolysaccharide (LPSs)[15-17]. Thus, reduction of these various inflammatory cytokines could lower the degree of gastritis induced by their expression, and could be a promising target for prevention and for adjuvants of allopathic anti-*H. pylori* eradication therapy. Genistein (4',5,7-trihydroxyisoflavone) is one of the naturally occurring isoflavones with three phenol hydroxyl residues pertaining to the flavonoid family. Soybeans and most soy products are the major foods containing nutritionally relevant amounts of one hundred grams of soybean supplies 5.72 mg of genistein[18-20]. In particular, Genistein exerts various effects, including estrogen-like[21,22], anti-inflammatory anti-oxidant[23-29] and anti-cancer effect[30,31]. Genistein as a key tyrosine-specific-protein kinase inhibitor has been demonstrated to be anti-inflammatory[29,32,33]. Several studies have proposed that genistein acts as a tyrosine kinase inhibitor to reduce inflammatory cytokines and may be useful in the prevention and eradication of *H. pylori*-associated gastric diseases[9,6,15,34,60]. Genistein has been widely used as a protein tyrosine kinase inhibitor to block LPS-induced release of TNF in vitro[36]. Genistein inhibits tyrosine phosphorylation of the host 145-kDa protein and induction of IL-8. Previous studies showed that TNF-α caused a dose-dependent increase in IL-8 production. Genistein, on the other hand, significantly reduce both TNF-α and IL-8. In order to determine which kinase was involved, that found genistein (protein tyrosine kinase inhibitor) showed dose-dependently reduced IL-8 expression[7,15,34]. Furthermore, genistein has been shown to down-regulate cytokine-induced signal transduction events in the inflammatory cells[29]. In the present study, we explored the potential anti-inflammatory effect of 14-day continuous administration of genistein on *H. pylori*-associated gastritis using the rat model of H. pylori infection. We observed a significant reduction in *H. pylori*-associated gastritis as well as reduced levels of serum TNF-α and CINC-1. Furthermore, we investigated the in vitro anti-*H. pylori* activity of genistein at the various concentrations.

MATERIAL AND METHODS

Bacteria preparations: *H. pylori* strains used for all experiments were originally obtained from peptic ulcer patients at King Chulalongkorn Memorial Hospital. The bacteria were grown in Brucella broth (pH 7.0) supplemented with 10% goat serum for 24 hours at 37˚C in an automatic CO_2-O_2 incubator under microaerophillic conditions (85%N_2, 10%CO_2, and 5%O_2). The same conditions were used in the following culture.

Animal preparation: Male Sprague-Dawley rats (Salaya research animal center, Mahidol university, Bangkok, Thailand) weighing 180-200 grams at the beginning of the experiment were used. The experimental protocol was approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn university, Thailand. The rats were housed in macrolon cages (5 animals per cage), in a standard animal care room (at room temperature 18˚C-22˚C, 55% humidity) and were allowed free access to food and water.

Experimental protocol: All rats were randomLy divided into three groups. Group 1 (Control group, n=7) were treated with 0.1% dimethyl sulfoxide (DMSO) (1 mL/rat, b.i.d. by gastric gavage) for 17 day. Group 2 (*H. pylori* infection group, n=7). After pre-treatment with streptomycin suspended in drinking water (5 mg/mL) for 3 days, each rat was inoculated with *H. pylori* 1 mL saline suspension (about 108-10 colony-forming unit (CFU) /mL per rat b.i.d. by gastric gavage for 3 consecutive days, using the method of Thong-Ngam et al, 2005[37]. Group 3 (Genistein treatment group, n=7), The rats were similarly inoculated with *H. pylori* as in Group 2, and thereafter treated with genistein dissolved in DMSO (16 mg/kg BW, b.i.d. by gastric gavage) for 14 days. The amounts of genistein giver were based on the safety dosages ranging from 1 to 16 mg/kg body weight, in accordance with the pharmacokinetic study of isoflavones[38] and attenuated nonalcoholic steatohepatitis study in a rat model[39]. The rats were sacrificed at the end of the experimental protocol. Serum samples were collected to measure TNF-α level and CINC-1 level. The stomach was removed for detection of *H. pylori* infection by urease test and for a pathological examination by the pathologist.

Measurement of TNF-α and CINC-1 in serum: Blood samples were taken by cardiac puncture. The samples were centrifuged (1,500 g for 10 min) and the supernatant stored at -70˚C until measurement. The serum concentrations of TNF-α and CINC-1 were measured by a rat TNF-α enzyme-linked immunosorbent assay and a rat CXCL1/CINC-1 Immunoassay (R and D systems, USA) respectively, according to the manufacturer's protocols. The quantitative sandwich enzyme immunoassay technique was employed for the assay. A polyclonal antibody specific for rat cytokines was pre-coated onto a microplate. Standards, control, and samples were pipetted into the wells, and any rat cytokines present were bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat cytokines was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the stop solution was added. The intensity of the color measured was proportional to the amount of rat cytokine bound in the initial step. The sample values were then read off the standard curve.

Detection of *H. pylori* infection in gastric tissues: The presence of *H. pylori* infection in the individual rats were determined by urease test and pathological examination.

Enzymatic test by using urease test. After all rats were sacrificed by intra-peritoneum injection of an overdose (60 mg/kg BW) of thiopental sodium, the stomach was removed and longitudinally dissected along the greater curvature for detection of *H. pylori* organism in tissue. The urease test was conducted on 2 mm^2 of gastric antral biopsy specimen. In case of *H. pylori* infection, the yellow color in the urease tube was converted to pink within 24 hours.

Gastric histopathology, the remaining tissue from the gastric antral biopsy was fixed in 10% formaldehyde in 0.2 M sodium phosphate buffer at pH 7.4 and was processed by routine technique before paraffin embedding. Sections were cut at 5 μm thickness and stained with Hematoxylin-eosin (H&E) and Giemsa staining methods. An experienced gastrointestinal pathologist examined all blinded samples using light microscope with a magnification X10 and X40, following the updated Sydney System[40]. All histopathological findings were recorded and graded by using the bacterial colonization score as follows: score 0=no bacteria detected, score 1=mild colonization, score 2=moderate colonization, score 3=marked colonization. Gastric inflammation was scored according to the degree of polymorphonuclear and mononuclear infiltration in the gastric mucosa as follow: score 0=normal, score 1=mild, score 2=moderate and score 3=marked.

Statistical analysis

Results were presented as mean ± standard deviation (SD) for the in vivo measurements. Comparison among all groups of animals was evaluated by one-way analysis of variance (ANOVA), followed by the least significant different post hoc test. Results with $p<0.05$ were considered significant. The

data were analyzed using the SPSS software version 20.0 for windows. In addition, descriptive statistics were used for histological examination of the stomach.

RESULTS

Changes in the level of serum TNF-α

We hypothesized that treatment with genistein would attenuate gastric inflammation by reduction of inflammatory mediators. As shown in Figure 1, the level of TNF-α in *H. pylori* infection group was significantly higher than in the control group (43.50 ± 16.51 vs. 20.89 ± 8.90 pg/mL, $p < 0.05$). As expected, the level of serum TNF-α in the genistein treatment group was significantly lower than in the *H. pylori* infection group (29.33 ± 10.77 vs. 43.50 ± 16.51 pg/mL, $p < 0.05$).

Changes in the level of serum CINC-1

CINC has been shown to be a most potent inducer of neutrophil chemotaxis in rat[15], and may therefore be involved in the evolution of acute neutrophilic inflammation. As shown in Figure 2, the level of serum CINC-1 in *H. pylori* infection group was significantly higher than in the control group (138.10 ± 43.56 vs.

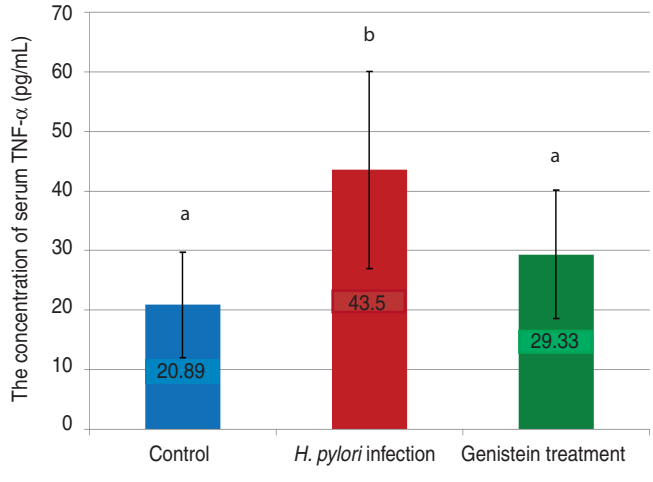

Figure 1. Bar graphs show the concentration of serum TNF- α (pg/mL) in all groups (mean ± SD). [ab]indicate significant differences ($p < 0.05$). Control group (n=7): rats treated with 0.1% DMSO (1 mL/rat, PO, b.i.d.); *H. pylori* infection group (n=7): rats inoculated with *H. pylori* suspension; the genistein treatment group (n=7) : rats inoculated with *H. pylori* suspension and treated with genistein (50 mg/kg BW, 1 mL/rat, PO, b.i.d.).

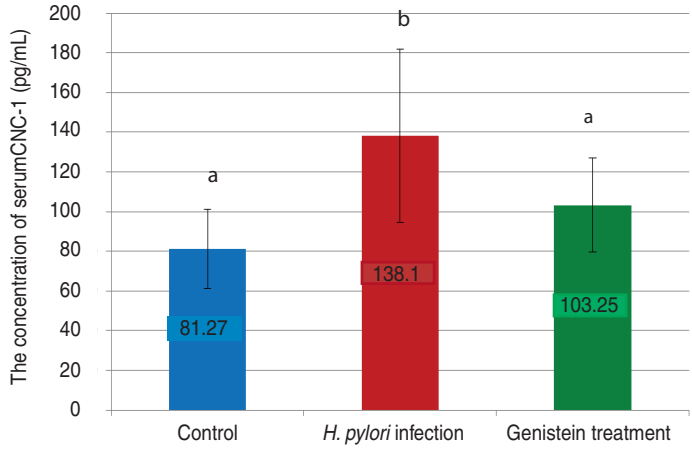

Figure 2. Bar graphs show the concentration of serum CINC-1 (pg/mL) in all groups (mean ± SD). [ab]indicate significant differences ($p < 0.05$). Control group (n=7): rats were treated with 0.1% DMSO (1 mL/rat, PO, b.i.d.); *H. pylori* infection group (n=7): rats were inoculated with *H. pylori* suspension; the genistein treatment group (n=7): rats were inoculated with *H. pylori* suspension and treated with genistein (50 mg/kg BW, 1 mL/rat, PO, b.i.d.).

81.27 ± 19.89 pg/mL, $p < 0.05$). As expected, the level of serum CINC-1 in the genistein treatment group was significantly lower than in the *H. pylori* infection group (103.26 ± 23.76 vs. 138.10 ± 43.56 pg/mL, $p < 0.05$).

Histological changes

H. pylori infection in rat was determined by the urease test and histopathological examination. The stomach histology was normal in the control group, whereas in *H. pylori* infection group, almost all of the stomach tissues showed moderate gastric inflammation (Score 2: n=5). *H. pylori* colonization score, almost all the stomach tissues showed mild to marked colonization. In genistein treatment group, stomach histopathology was improved when compared to *H. pylori* infection group, especially in the reduction of inflammatory cells infiltration) (Score 0: n=3, Score 1: n=4) (Figure 3 and 4). In the same way, *H. pylori* colonization score was reduced. The histopathological scores for gastric inflammation and *H. pylori* coloni-

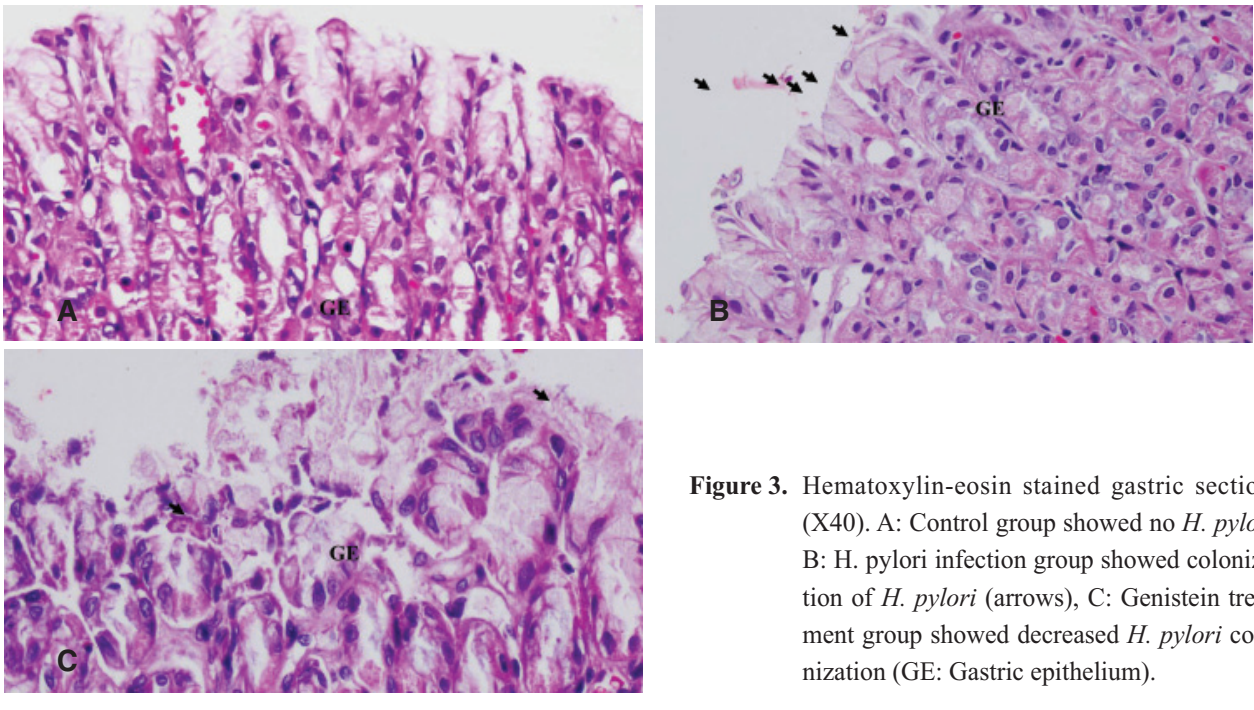

Figure 3. Hematoxylin-eosin stained gastric sections (X40). A: Control group showed no *H. pylori*, B: H. pylori infection group showed colonization of *H. pylori* (arrows), C: Genistein treatment group showed decreased *H. pylori* colonization (GE: Gastric epithelium).

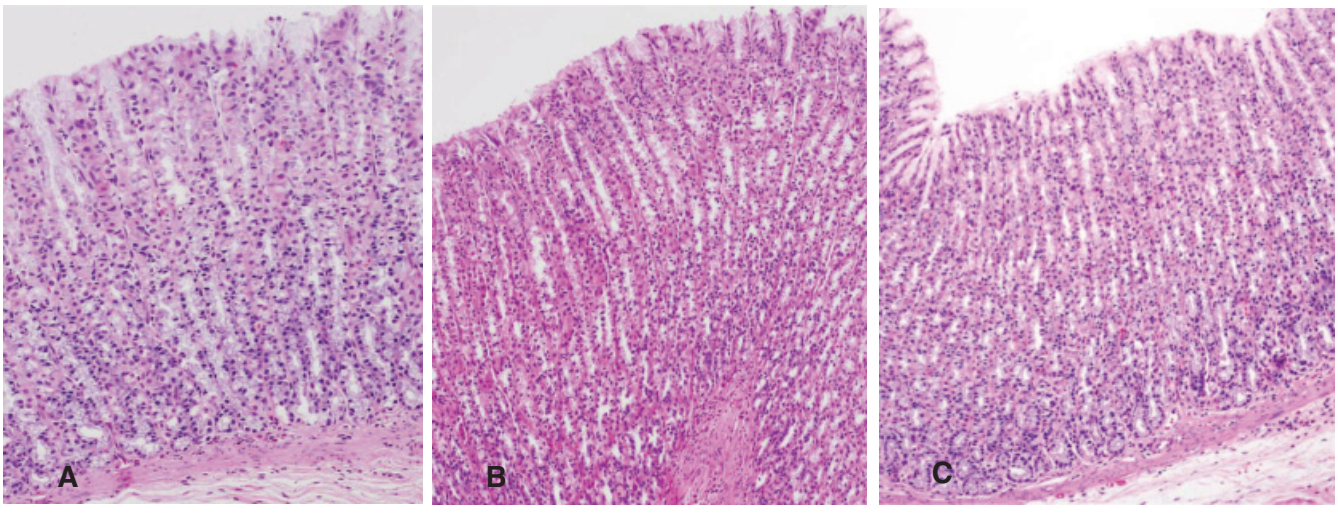

Figure 4. H & E-stained gastric tissue (X10) from Sprague-Dawley rats, A: Control group showed normal gastric histopathology. B: *H. pylori* infection group showed polymorphonuclear inflammatory cells infiltrating the lamina propria. C: Genistein treatment group showed improvements in gastric inflammation.GE: Gastric epithelium; LP: Lamina propria; MM: Muscularis mucosae; SM: Submucosa

Table 1. Summary of the bacterial colonization and gastric inflammation score.

Group	N	Gastric inflammation score[a]				*H. pylori* colonization score[b]			
		0	1	2	3	0	1	2	3
Control group	7	7	-	-	-	7	-	-	-
H. pylori infection group	7	1	-	5	1	1	2	2	2
Genistein treatment group	7	3	4	-	-	2	4	1	-

[a]Gastric inflammation; score 0: normal infiltration of polymorphonuclear and mononuclear, score 1: mild infiltration of polymorphonuclear and mononuclear, score 2: moderate infiltration of polymorphonuclear and mononuclear, score 3: marked infiltration of polymorphonuclear and mononuclear.
[b]*H. pylori* colonization score; score 0: no bacteria detected, score 1: mild colonization, score 2: moderate colonization, score 3: marked colonization.

zation are summarized in Table 1.

Discussion

The presence of *H. pylori* in the gastric antrum is always associated with a mucosal inflammatory reaction involving infiltration by a large number of polymorphonuclear and mononuclear cells. Neutrophils infiltration of neutrophils into the mucosa is known to occur in *H. pylori*-induced chronic active gastritis. Activated neutrophils release proteases and reactive oxygen metabolites that cause gastric mucosal injury. The immune response of the host is considered to be a key event in the development of gastritis. This reaction is induced by the contact of *H. pylori* with gastric cells and is followed by stimulation of proinflammatory cytokine production. Several of in vivo and in vitro studies have shown that *H. pylori* can induce cytokine expression in the epithelial cells, characterized by up-regulation of several genes such as IL-1β, IL-6, IL-8, IL-10, and TNF-α[7,34,43-49]. Adherence of *H. pylori* to culture gastric epithelial cells induces several cellular responses, including the tyrosine phosphorylation of a 145-kDa host protein, the reorganization of the host cell actin and associated cellular proteins, such as vasodilator-stimulated phosphoprotein adjacent to the attached bacterial cells, and subsequent release of cytokines. These effects following *H. pylori* attachment to cells suggests that alteration of host cellular signal transduction may lead to chronic inflammation and oncogenic transformation that are the hallmarks of symptomatic *H. pylori* infection[2,7,44]. There is no effective therapy for eradicating *H. pylori* infection, although combination therapies employing one proton pump inhibitor plus two or three antibiotics have been standard treatment. Such therapies are not very effective as bacterial resistance often develops. Furthermore, therapy may disrupt the natural population of commensal microorganisms in the gastrointestinal tract, potentially leading to undesirable side effects. Thus, there is a need to search an indigenous herbal based modified drug with minimal side effects for eliminating the bacteria. This would have a major impact on the present and the future health of the world population[3,45,51-54]. Genistein is a naturally occurring isoflavone, a flavonoid component of soybean with three phenol hydroxyl residues. Genistein has been reported to have many anti-inflammatory anti-oxidative and anti-cancer effects[18,19,26-29]. In 1987, genistein was identified as a protein-tyrosine kinase (PTK) inhibitor, because it inhibited the epidermal growth factor (EGF) receptor PTK activity in vitro, apart from its PTK-inhibitory activity. Genistein has also been shown to inhibit DNA topoisomerase II and regulation of cell cycle checkpoints[32,56]. Several studies have proposed that genistein act as a tyrosine kinase inhibitor to reduce inflammatory cytokines, and may be useful in the prevention or cure of *H. pylori*-associated gastric diseases[57-60]. Most of these properties have been shown only in vitro and at genistein concentrations in excess of those attainable physiologically in human (10 μmol/L)[61]. Thus, the in vitro activities of genistein remains to be ascertained, while the effect of genistein on *H. pylori* induced gastritis in rat model has not yet been shown. We therefore explored the potential anti-inflammatory effect of genistein on *H. pylori*-associated gastritis in a rat model. TNF-α is a key mediator in the host response against gram-negative bacteria as well as in the septic shock syndrome induced by either LPS or bacterial superantigens[62]. It was a major

proinflammatory cytokine that plays an important role in the development of acute inflammation, including neutrophil infiltration of the gastric mucosa. Expression of TNF-α increase in *H. pylori* induced gastric damage. Our result showed a significant increase of TNF-α level in the *H. pylori* infection group as compared with the control group. Furthermore, TNF-α stimulates transcription factors such as nuclear factor kappa B (NF-κB) and induces the synthesis of various inflammatory cytokines including IL-8[7,49,51,58,59,63]. One of the most remarkable properties of IL-8 is the variation of its expression levels. In healthy tissues, IL-8 is barely detectable, but it is rapidly induced by ten- to 100-fold in response to proinflammatory cytokines including TNF-α, IL-1, bacterial or viral products, and cellular stress[64]. Production of IL-8 by the gastric mucosa is viewed as a very important stimulus to the influx and activation of neutrophils in *H. pylori* gastritis[43,45,65-70]. Therefore, IL-8 mRNA expression was up-regulated within one hour after H. pylori infection, reaching a maximal increase of ~120-fold at 8 hours post-infection, and then decreases[71,72]. Attachment of *H. pylori* to gastric epithelial cells can induce host cellular responses, including reorganization of actin cytoskeletons, tyrosine phosphorylation of a 145-kD protein, and release of IL-8[43,46,64,69,73,74]. Several studies have shown that *H. pylori* produced a dose-dependent increase in IL-8 production[34,48,50]. CINC-1, a counterpart of the human GRO of the interleukin-8 family, has a potent neutrophil chemotactic activity in rats, similar to the effect of IL-8 in human[13,60,76]. Various cells have been reported to produce CINC-1 in response to inflammatory mediators such as TNF-α, IL-1β, and LPSs[48,71,72]. Our findings showed a significant increase of CINC-1 level in the *H. pylori* infection group as compared with controls. This study explored the anti-inflammatory effect of genistein on *H. pylori*-associated gastritis, which could be related in three ways. Firstly, genistein acted as an inhibitor of tyrosine kinases, resulting in a decreased infection of *H. pylori* into the gastric epithelial cells and indicating that tyrosine kinases play a significant role in the intracellular uptake of *H. pylori*[58,59]. Secondly, genistein inhibited TNF-α production. Previous reports had demonstrated that genistein could inhibit LPS-induced alveolar macrophage TNF-α production thereby reducing the alveolar neutrophil influx following LPS[36,51,77] as well as LPS-induced NF-κB activation[51]. And lastly, genistein inhibited tyrosine phosphorylation of the host 145-kDa protein and induction of IL-8. Previous studies were evaluated *H. pylori*, TNF-α produced a dose-dependent increase in IL-8 production, the increase with all two was significantly reduced by genistein. In order to determine which kinase was involved, that found genistein (protein tyrosine kinase inhibitor) showed dose-dependently reduced IL-8 expression[7,34,50]. Our result showed a significant decrease of TNF-α level and CINC-1 in genistein treatment group as compared with *H. pylori* infection group. Histological examination showed that *H. pylori* infection was associated with mucosal inflammation and intense polymorphonuclear leukocytic infiltration (neutrophils and some eosinophils) in the lamina propria, in the mucus layer, as well as inside the glands. Thus, neutrophilic infiltration is an almost invariable finding in *H. pylori*-associated gastritis and is topographically related to *H. pylori* colonization[78,79]. Our results demonstrated that in the *H. pylori* infection group, the stomach tissues exhibited polymorphonuclear inflammatory cells infiltration of the lamina propria, with an increased score of gastric inflammation and *H. pylori* colonization. On the other hand, In the genistein treatment group, gastric histopathology was improved when compared with the *H. pylori* infection group, especially regarding the reduction of polymorphonuclear inflammatory infiltration. Similarly, the *H. pylori* colonization score was reduced. The results indicated that polymorphonuclear inflammatory cells infiltration may be decreased by genistein administration. Previous studies had demonstrated that the eradication of *H. pylori* resulted in a reduction in neutrophil numbers, which was followed by gradual disappearance of mononuclear cells[80,81]. Such findings indicate that genistein likely inhibits neutrophil infiltration into the gastric mucosa by suppressing the production of TNF-α and CINC-1 (a potent neutrophil chemotactic agent).

In conclusion, our study showed that *H. pylori* infection was associated with elevated levels of serum TNF-α and serum CINC-1, and an increased polymorphonuclear inflammatory cell infiltration in the gastric mucosa with *H. pylori* colonization. We also demonstrated that genistein administration (16 mg/kg) resulted in significant suppression of *H. pylori*-induced gastritis via reduction of serum TNF-α, serum CINC-1 and polymorphonuclear inflammatory cell infiltration. Our data suggested that genistein attenuated gastritis, likely via its ability to inhibit inflammation and

improve gastric pathology. Our study could be useful for further research to identify natural products for prevention and eradication of *H. pylori*.

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