ผลของเจนนิสตีอินต่อการเจริญเติบโตของเชื้อเฮลิโคแบคเตอร์ ไพโลไร

และการอักเสบของกระเพาะอาหารในหนูแรท

นางสาวนิสารัตน์ เพ็ชรหนู

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

EFFECT OF GENISTEIN ON *HELICOBACTER PYLORI* GROWTH AND GASTRIC INFLAMMATION IN RATS

Miss Nisarat Phetnoo

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Science

Faculty of Medicine

Chulalongkorn University

Academic Year 2015

Thesis Title	EFFECT OF GENISTEIN ON *HELICOBACTER PYLORI* GROWTH AND GASTRIC INFLAMMATION IN RATS
By	Miss Nisarat Phetnoo
Field of Study	Medical Science
Thesis Advisor	Professor Duangporn Werawatganon, M.D.
Thesis Co-Advisor	Associate Professor Prasong Siriviriyakul, M.D.

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Medicine

(Professor Suttipong Wacharasindhu, M.D.)

THESIS COMMITTEE

.....Chairman

(Professor Vilai Chentanez, M.D.,Ph.D)

.....Thesis Advisor

(Professor Duangporn Werawatganon, M.D.)

.....Thesis Co-Advisor

(Associate Professor Prasong Siriviriyakul, M.D.)

.....Examiner

(Professor Pisit Tangkijvanich, M.D.)

.....Examiner

(Assistant Professor Wacharee Limpanasithikul, Ph.D.)

.....External Examiner

(Bubpha Pornthisarn, M.D.)

นิสารัตน์ เพ็ชรหนู : ผลของเจนนิสติอินต่อการเจริญเติบโตของเชื้อเฮลิโคแบคเตอร์ ไพโลไร และการอักเสบของกระเพาะอาหารในหนูแรท (EFFECT OF GENISTEIN ON *HELICOBACTER PYLORI* GROWTH AND GASTRIC INFLAMMATION IN RATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ พญ.ดวงพร วีระวัฒกานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. นพ.ประสงค์ ศิริวิริยะกุล, 97 หน้า.

การทดลองนี้มีวัตถุประสงค์เพื่อศึกษาผลของเจนนิสติอินต่อการยับยั้งการเจริญเติบโตของเชื้อเฮลิโคแบคเตอร์ ไพโลไร และการอักเสบของกระเพาะอาหารในหนูแรท โดยแบ่งหนูทดลอง เพศผู้ ออกเป็น 3 กลุ่ม กลุ่มละ7 ตัว ได้แก่กลุ่มควบคุม, กลุ่มเฮลิโคแบคเตอร์ ไพโลไร และกลุ่มเจนนิสติอิน หนูในสองกลุ่มหลังทำให้ติดเชื้อเฮลิโคแบคเตอร์ ไพโลไร โดยให้ยาปฏิชีวนะสเตรปโตมัยซิน (5 มก./มล.) ผสมในน้ำดื่มให้กินติดต่อกัน 3 วัน ก่อนที่จะให้เชื้อเฮลิโคแบคเตอร์ ไพโลไร ความเข้มข้น 108-10 CFUs/ml วันละ 2 ครั้งติดต่อกัน 3 วัน และในกลุ่มเจนนิสติอิน ให้เจนนิสติอิน (16 มก./กก.) วันละ 2 ครั้ง เป็นเวลา 14 วัน เมื่อสิ้นสุดการทดลอง เก็บตัวอย่างซีรัมเพื่อวัดระดับทีเอ็นเอฟแอลฟาและซีไอเอ็นซีวัน เก็บตัวอย่างกระเพาะอาหารส่วนแอนทรัม เพื่อทดสอบหาเชื้อเฮลิโคแบคเตอร์ ไพโลไร โดยวิธี Urease test และทดสอบหาสารเอ็มดีเอ กระเพาะอาหารส่วนที่เหลือส่งตรวจทางพยาธิวิทยา เพื่อประเมินจำนวนของเชื้อและการอักเสบโดยแพทย์พยาธิ นอกจากนั้นได้ทดสอบคุณสมบัติของเจนนิสติอินในการยับยั้งการเจริญเติบโตของเชื้อเฮลิโคแบคเตอร์ ไพโลไร โดยใช้เจนนิสติอินในความเข้มข้น 0.25, 0.5, 1, 1.5, 2 และ 4 โมลาร์ ทดสอบด้วยวิธี Disc diffusion method ผลการศึกษาในหนูทดลองพบว่ากลุ่มเฮลิโคแบคเตอร์ ไพโลไร มีระดับทีเอ็นเอฟแอลฟาและซีไอเอ็นซีวันในซีรัมเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม (43.50 ± 16.51 pg/ml vs. 20.89 ± 8.90 pg/ml, 138.10 ±43.56 pg/ml vs. 81.27±19.89,$P<0.05$ ตามลำดับ) กลุ่มเจนนิสติอิน มีระดับทีเอ็นเอฟแอลฟาและซีไอเอ็นซีวันในซีรัมลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับ กลุ่มเฮลิโคแบคเตอร์ ไพโลไร (29.33 ± 10.77 pg/ml vs. 43.50 ± 16.51, 103.25± 23.76 pg/ml vs. 138.10 ±43.56 pg/ml, $P<0.05$ ตามลำดับ) ผลการประเมินพยาธิสภาพของกระเพาะอาหารพบว่า ในกลุ่มควบคุมไม่มีการเปลี่ยนแปลงทางพยาธิสภาพ ส่วนกลุ่มที่ให้เจนนิสติอินพบพยาธิสภาพจากการติดเชื้อลดลงเมื่อเทียบกับกลุ่มเฮลิโคแบคเตอร์ ไพโลไร และการทดลองนี้ไม่พบการเปลี่ยนแปลงของสารเอ็มดีเอ ส่วนผลการศึกษา In vitro พบว่าเจนนิสติอินไม่มีคุณสมบัติในการยับยั้งการเจริญเติบโตของเชื้อเฮลิโคแบคเตอร์ ไพโลไร สรุปผลการทดลองเจนนิสติอินสามารถลดระดับทีเอ็นเอฟแอลฟาและซีไอเอ็นซีวันในซีรัม และปรับปรุงพยาธิสภาพในกระเพาะอาหารของหนูแรทที่ติดเชื้อเฮลิโคแบคเตอร์ ไพโลไร ให้เป็นไปในทางที่ดีขึ้น เจนนิสติอินจึงสามารถบรรเทาภาวะกระเพาะอาหารอักเสบในหนูแรทที่ติดเชื้อเฮลิโคแบคเตอร์ ไพโลไรได้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5574210530 : MAJOR MEDICAL SCIENCE

KEYWORDS: HELICOBACTER PYLORI / GENISTEIN / GASTRIC INFLAMMATION

NISARAT PHETNOO: EFFECT OF GENISTEIN ON *HELICOBACTER PYLORI* GROWTH AND GASTRIC INFLAMMATION IN RATS. ADVISOR: PROF. DUANGPORN WERAWATGANON, M.D., CO-ADVISOR: ASSOC. PROF. PRASONG SIRIVIRIYAKUL, M.D., 97 pp.

The present study aims to determine the effects of genistein on *H. pylori* growth and gastritis in rats. Male Sprague-Dawley rats were randomly divided into three groups including control group, *H. pylori* infection group and genistein treatment group. *H. pylori* infection group and genistein treatment groups were 3 day pre-treatment with streptomycin (5 mg/mL) and inoculated with *H. pylori* suspension (10^{8-10} CFU/ml; 1 ml/rat, b.i.d.) for 3 consecutive days. Then, in the rats of genistein treatment group were treated with genistein (16 mg/kg BW b.i.d.) for 14 days. On the last day of experimental protocol, serum samples were collected to measure TNF-α level and CINC-1 level. The stomach were removed for *H. pylori* detection by urease test, gastric MDA level and pathological examination. Furthermore, the anti-*H. pylori* activity of genistein were investigated at concentrations of 0.25, 0.5, 1, 1.5, 2 and 4 M by using disc diffusion method. *In vivo* showed *H. pylori* infection group had significantly higher levels of serum TNF-α and CINC-1 than control group (43.50 ± 16.51 pg/ml vs. 20.89 ± 8.90 pg/ml, 138.10 ±43.56 pg/ml vs. 81.27 ± 19.89 pg/ml, $P<0.05$, respectively). In genistein treatment group had significantly lower levels of serum TNF-α and CINC-1 than in *H. pylori* infection group (29.33 ± 10.77 pg/ml vs. 43.50 ± 16.51 pg/ml, 103.25 ± 23.76 pg/ml vs. 138.10 ± 43.56 pg/ml, $P<0.05$, respectively). This study showed no significant change in gastric MDA levels in each group. *In vitro* study showed the growth of *H. pylori* was not inhibited by the various concentrations of genistein. In conclusion, these observations suggested that administration of genistein could attenuate *H. pylori*-induced gastritis, possibly by reducing inflammatory mediators and improve gastric pathology.

Field of Study: Medical Science
Academic Year: 2015

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

The success of this study required the supports from many people. I would like to offer my special thanks to the following for their priceless helps.

First of all, I would like to express my sincere gratitude to my advisor Professor Duangporn Werawatganon, M.D. and my co-advisor Associate Professor Prasong Siriviriyakul, M.D. for the continuous support of my Master degree study and related research, for the great patience, motivation, and immense knowledge. Their guidance helped me in all the time of research and writing of this thesis. Moreover, I would like to thank the rest of my thesis committee: Professor Vilai Chentanez, M.D., Ph.D., Professor Pisit Tangkijvanich, M.D., Assistant Professor Wacharee Limpanasithikul, Ph.D, and Bubpha Pornthisarn, M.D. for their insightful comments and encouragement, but also for the hard question which incented me to widen my research from various perspectives.

My sincere thanks also go to Dr. Tanittha Chatsuwan and Miss Panjaporn Prasurthsin, Department of Microbiology, Faculty of Medicine, Chulalongkorn University offers their help in preparing H. pylori and suggestion for microbiological technique. Especially, Assoc. Prof. Naruemon Klaikaew, M.D., Department of Pathology, Faculty of Medicine, Chulalongkorn University for her helps in histopathology of H. pylori infection. Additionally, I am exceptionally grateful for the assistance provided by all staff of Department of Physiology, Faculty of Medicine, Chulalongkorn University.

Most importantly, I must express my very profound gratitude to my parents and to my friend for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

CONTENTS

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

H. pylori	=	*Helicobacter pylori*
TNF-α	=	Tumor necrosis factor alpha
IL-1	=	Interleukin-1
IL-6	=	Interleukin-6
IL-12	=	Interleukin-12
mRNA	=	Messenger RNA
CINC-1	=	Cytokine-induced neutrophil chemoattractant 1
GRO	=	Growth-regulated gene product
IL-1β	=	Interleukin 1 beta
LPS	=	Lipopolysaccharide
kDa	=	Kilodalton
MALT	=	Mucosa-associated lymphoid tissue
μm	=	Micrometer
PCR	=	Polymerase chain reaction
DNA	=	Deoxyribonucleic acid
TH17	=	T helper 17
CagA	=	Cytotoxin-associated gene A
SabA	=	Sialic acid–binding adhesion
BabA	=	Blood group Ag-binding adhesin
CagL	=	Cytotoxin-associated gene L
Vac A	=	Vacuolating cytotoxin A
PS	=	Phosphatidylserine
T4SS	=	Type IV secretion system
ROS	=	Reactive oxygen species
PG	=	Peptidoglycan
cagA-P	=	The phosphorylated form of cytotoxin-associated gene A

PMN	=	Polymorphonuclear cell
mØ	=	Macrophage
PAI	=	Cytotoxin-associated gene pathogenicity island
kb	=	Kilobase
TLR	=	Toll-like receptor
OipA	=	Outer membrane protein A
NAP-A	=	Neutrophil activating protein A
PRRs	=	Pattern recognition receptors
PAMPs	=	Pathogen-associated molecular patterns
NOD	=	Nucleotide oligomerization domain
NF-**κ**B	=	Nuclear factor- kappaB
AP 1	=	Activator protein 1
RANTES	=	Regulated on activation normal t cell expressed and secreted
MIP	=	Macrophage inflammatory protein
NADPH oxidase	=	Nicotinamide adenine dinucleotide phosphate oxidase
COX2	=	Cyclooxygenase 2
VEGF	=	Vascular endothelial growth factor
ICAM-1	=	Intercellular adhesion molecule-1
G cell	=	Gastrin cells
D cell	=	Somatostatin cells
Th1	=	Type 1 T helper
Mac-1	=	Macrophage 1 antigen
$O_2^{\bullet-}$	=	Superoxide radical anion
MDA	=	Malondialdehyde
4-HNE	=	4-hydroxynonenal
SOD	=	Superoxide dismutase
Treg	=	T regulatory

IFNγ	=	Interferon gamma
IgG	=	Immunoglobulin G
IgA	=	Immunoglobulin A
HpaA	=	*H. pylori* adhesin A
TSLP	=	Thymic stromal lymphopoietin
MIC	=	Minimum inhibitory concentration
H&E stain	=	Hematoxylin and eosin stain
IHC	=	Immunohistochemical
µg/l	=	Microgram per liter
ER	=	Estrogen receptors
Nrf1	=	Nuclear respiratory factor 1
Nrf2	=	Nuclear respiratory factor 2
CFU	=	Colony forming unit
ERK	=	Extracellular signal-regulated kinass
MPO	=	Myeloperoxidase
TBARS	=	Thio-barbituric acid reactive substances
CGRP	=	Calcitonin gene-related peptide
Bcl-2	=	B-cell lymphoma 2
Bax	=	Bcl2-associared X protein
TGF-β1	=	Transforming growth factor beta 1
MAPKs	=	The mitogen-activated protein kinases
UDP	=	Undegraded dietary protein
ALT	=	Alanine aminotransferase
AST	=	Aspartate aminotransferase
LDH	=	Lactate dehydrogenase
UGTs	=	Glucuronosyltransferases

CYP450	=	Cytochrome P450
ng/ml	=	Nanogram/milliliter
PBS	=	Phosphate buffer saline
DMSO	=	Dimethyl sulfoxide
CO_2	=	Carbon dioxide
O_2	=	Oxygen
N_2	=	Nitrogen
v/v %	=	Volume/volume percent
b.i.d	=	Bis in die
mg/ml	=	Milligram/milliliter
ELISA	=	Enzyme-linked immunosorbent assay
O.D	=	Optical density
4-PL	=	Four parameter logistic
pg/mL	=	Picogram/milliliter
mm^2	=	Square millimeter
BCA	=	Bicinchoninic Assay
M	=	Molar
SD	=	Standard deviation
ANOVA	=	One way analysis of variance
LSD post hoc test	=	Least significant difference post hoc test

CHAPTER I INTRODUCTION

Background and rationale

Helicobacter pylori (*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, with oral – oral and fecal – oral transmission (1, 2). Infection with *H. pylori* causes chronic active gastritis and significantly increases the risk factor for the development of duodenal ulcer and gastric ulcer, 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 (3). A nation-wide study of gastric biopsy in 3776 dyspeptic patients in Thai patients from six different geographic regions for incidence of gastritis was found 58.7% of dyspeptic patients had histological gastritis and was found *H. pylori* infection in 48.2% of dyspeptic patients (4).

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 gastric mucosa, that is produced in the gastric mucosa in response to *H. pylori* infection. Moreover, TNF- α stimulates transcription factors, induces the synthesis of various inflammatory cytokines, including Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Interleukin-8 (IL-8) (5, 6). IL-8, a prototypic human chemokine belongs to the CXC family, which exerts chemotactic effects on polymorphonuclear leukocytes to site of inflammation. It also has effects on cell proliferation, migration and tumor angiogenesis (7). *H. pylori* infection rapidly up-regulates the expression of IL-8 in human gastric epithelial cell. Therefore, IL-8 messenger RNA (mRNA) expression was up-regulated within 1 hour after *H. pylori* infection, reached a maximal increase of ~120-fold at 8 hours post-infection, and then decreased(8). Cytokine-induced neutrophil chemoattractant 1(CINC-1), a counterpart of the human growth-regulated gene product (GRO) of the IL-8 family, has a potent neutrophil chemotactic activity in rats, similar to the effect of IL-8 in human (9-11). Although various cells have been reported to produce CINC-1 in response to inflammatory mediators, such as TNF-α, Interleukin 1 beta (IL-1β), and lipopolysaccharide (LPS) (10, 12, 13).

Thus, the degree of gastritis induced by the expression of various inflammatory cytokines, reduction of them could be a promising target for prevention and 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 belongs to the flavonoids family. Soybeans and most soy products are the major foods containing nutritionally relevant amounts of it. One gram of powdered soybean chips contains nearly over 500 μg of genistein (primarily as glycosides) (14-16). In particular, genistein exerts various effects, including estrogen-like activity (17, 18), aniti-inflammatory effect, anti-oxidant effects (19-24) and anti-cancer effect (25, 26). Genistein as key a tyrosine-specific-protein kinase inhibitor has been demonstrated to be related anti-inflammatory property (27-29). Numerous studies have proposed that genistein is tyrosine kinase inhibitor to reduce inflammatory cytokine and may be useful in the prevention or cure of *H. pylori*-associated gastric diseases (29) such as it has been widely used as a protein tyrosine kinase inhibitor that blocks LPS-induced release of TNF-α *in vitro* (30). Genistein inhibited tyrosine phosphorylation of the host 145- Kilodalton (kDa), protein and induction of IL-8. Previous studies were evaluated *H. pylori* and TNF- α produced a dose-dependent increase in IL-8 production, this increase was 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 (6). Furthermore, genistein has been shown to down-regulate cytokine-induced signal transduction events in the inflammatory cells (29). This study explored the anti-inflammatory effect and the anti-oxidant effect of genistein on *H. pylori*-associated gastritis by the *H. pylori* infection in a rat model, following genistein continuous administration a period of 14 day. Therefore, this study observed following histological evaluation, a significant reduction in *H. pylori*-associated gastritis including reduced levels of serum TNF-α, CINC-1 and gastric malondialdehyde (MDA) level. Additionally, the anti-*H. pylori* growth of genistein were investigated at the various concentration *in vitro*.

Research question

In vivo study; Can genistein attenuate gastric inflammation by reduction of inflammatory mediators, reactive oxygen species and improvement of gastric pathology in rat with *H. pylori* infection?
In vitro study; Can genistein inhibit growth of *H. pylori* ?

Research objectives

In vivo study; To determine the attenuates effects of genistein on pathophysiology of *H. pylori* infection by measure serum TNF-α level, serum CINC-1 level, gastric MDA level and gastric histopathology.

In vitro study; To determine the inhibitory growth of *H. pylori* by genistein

Hypothesis

- Genistein attenuates gastric inflammation by reduction of inflammatory mediators, reduction of oxidative stress and improve gastric pathology in rat with *H. pylori* infection.

-Genistein inhibit growth of *H. pylori in vitro*.

Expected benefit and application

The findings will provide an understanding of attenuates effects of genistein on *H. pylori* in developing of gastritis, decreasing inflammatory response and improvement of gastric pathology. Moreover, this study will be useful for further studies and sufficient efforts were made to identify natural products for prevention or adjuvants of allopathic anti- *H. pylori* eradication therapy.

CHAPTER II LITERATURE REVIEW

General overview of *Helicobacter pylori*

Helicobacter pylori (H. pylori) is a member of bacteria adapted to life in the mucus of the digestive tract of humans. Its specific characteristics include its morphology, metabolism and pathogenesis.

Natural history *of Helicobacter pylori* infection

Even though, more than half of the world's population is infected with this bacterium but 90% of whom will remain asymptomatic. *H. pylori* infection is usually acquired during, whereas acute bacterial infection is rarely diagnosed. In preference, development of chronic gastritis in nearly all persistently colonized individuals. Patients with high acid concentrations in the corpus and increased acid secretion from stomach to duodenum are more likely to have antral-predominant gastritis. This pattern is found in patients with duodenal ulcers. Patients with low acid secretion will more likely gastritis development in the corpus and are thus more likely to develop gastric ulcer, leading to gastric atrophy, intestinal metaplasia, dysplasia and finally in rare cases that found gastric cancer. In people of advanced age, this sequence of events is more frequent. *H. pylori* infection induces the formation of lymphoid follicles within the stomach, leading to mucosa-associated lymphoid tissue in the gastric mucosa. This is another rare complication associated with *H. pylori* infection. The eventual clinical outcome of *H. pylori* infection is variable depending on bacterial (such as bacterial virulence factors), host (such as host genetic, lifestyle, environmental and epigenetic factors). Previous studies have reported the possible role of bone marrow-derived cells (such as gastric stem cells) in tumor progression(31). Natural history of *H. pylori* infection shown as figure 1. Furthermore, a nationwide study of gastric biopsy in 3776 dyspeptic patients in Thai population from six different geographic regions for incidence of gastritis was founded 58.7% of dyspeptic patients had histological gastritis and *H. pylori* infection in 48.2% of dyspeptic patients with high incidence in the age-group 31-60 years (63.7%) and 98.2% of *H. pylori* infection was found to be associated with gastritis. Moreover, that founded semi-arid plateau, mountain, jungle and fertile plain communities had high incidences of *H. pylori* infection while the coastal and peninsular communities had low incidences and oral to oral spread is proposed to be the mode of bacterial transmission (4).

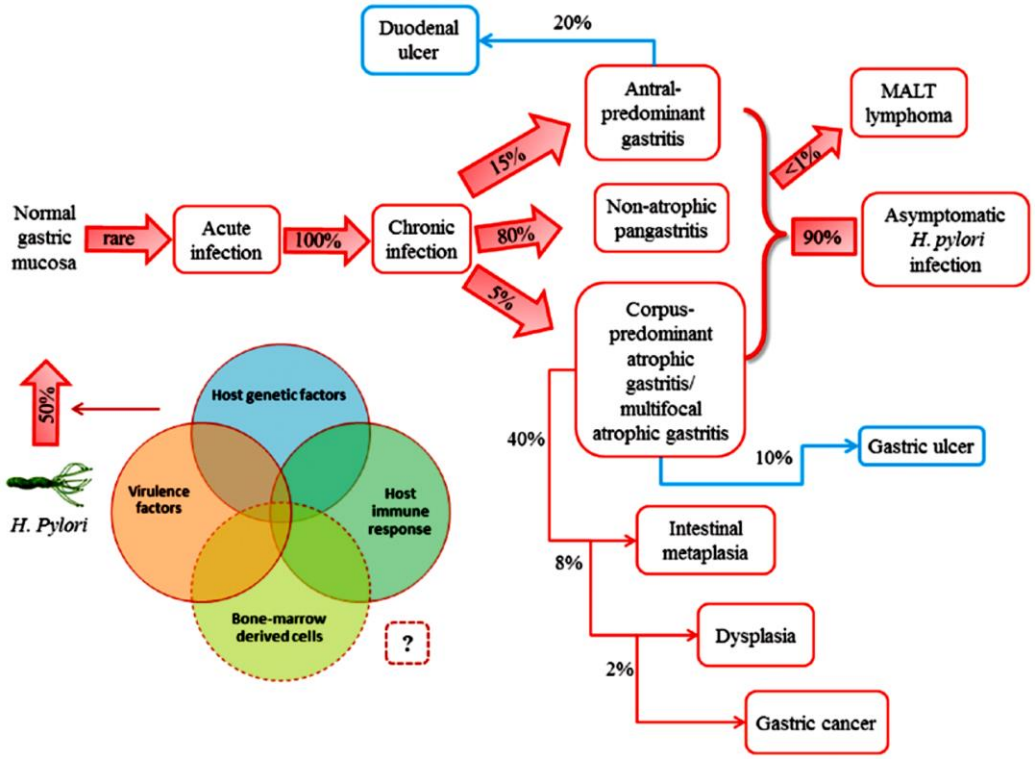


Figure 1 Natural history of *H. pylori* infection (31)

Morphology

H pylori differs genetically from members of the genus *Helicobacter*. This organisms are microaerophilic, nonsporulating, gram-negative bacterium, measuring 2 to 4 micrometre (μm) in length and 0.5 to 1μm in width. Whereas usually helical-shaped, possessing 4 to 6 polar sheathed flagella with a membraneous terminal bulb protected by a lipid structure, facilitating its penetration of the thick mucous layer in the stomach. *H. pylori* can appear as a helical rod-shaped while, the conversion of the helical to coccoidal form occurs under adverse conditions such as exposure to antibiotics, nutrient deficiency or prolonged in vitro culture (32). Difference for helical form and coccoid form as shown in figure 2.

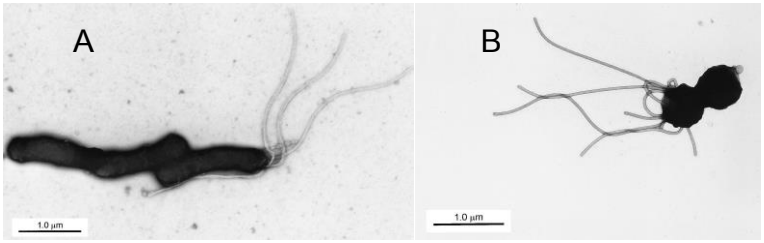


Figure 2 A; Electron micrograph of *H. pylori* (helical form), B; Electron micrograph of *H. pylori* (coccoid form) (33)

Transmission routes

In spite of the worldwide spread of *H. pylori* infection, the route of transmission appears to be person to person with evidence of waterborne transmission. The animals do not appear to be a cause of infection,since *H. pylori* is strictly a human pathogen. Poor hygiene and crowded conditions may facilitate transmission of infection with oral – oral route and fecal – oral route as the most probable modes of transmission. The fecal – oral transmission could be a result of direct contact with the infected person or through contaminated water or food. The oral – oral route has been supported by the polymerase chain reaction (PCR) detection of *H. pylori* deoxyribonucleic acid (DNA) in saliva and dental plaque. However, the presence of DNA does not indicate viability of the bacteria. Possible oral–oral transmission has been investigated in the eating of premasticated foods among some ethnic groups, the use of the same spoon by both mother and child, intimate oral–oral contact, and aspiration from vomit. In addition, Iatrogenic is one of mode of transmission, in which tubes or endoscopes that have been in contact with the gastric mucosa of one individual are used for another patient. Infection is transmitted from a patient to staff member have also been reported, especially among endoscopists and gastroenterologists. Nevertheless, in quantitative terms the iatrogenic route is considered to be margina (34, 35).

Pathogenesis of *Helicobacter pylori* infection

H. pylori pathogenesis and disease outcomes are mediated by a complex interplay between bacterial virulence factors, host, and environmental factors. After *H. pylori* enters the host stomach, four steps are critical for bacteria to establish successful colonization, persistent infection, and disease pathogenesis: (1) Survival in the acidic stomach; (2) movement toward epithelium cells by flagella-mediated motility; (3) attachment to host cells by adhesins/receptors interaction;

(4) causing tissue damage by toxin release (36). *H pylori* is highly adapted to colonize the human stomach, whereas most other bacteria cannot persistently. The major factors that limit bacterial colonization of the human stomach are (1) acidity, (2) peristalsis, (3) nutrient availability, (4) host innate and adaptive immunity, and (5) competing microbes. Specific features of *H. pylori* allow it to resist each of these stresses (14).

Escape from the acidic lumen

The gastric lumen carries gastric juice of pH 1-2 during fasting periods, which prevents bacterial growth. Gastric acid is secreted by parietal cells under regulation accomplished by the coordination of neural, hormonal, and paracrine pathways. *H. pylori* can survive for minutes in the stomach lumen and must quickly migrate to the gastric epithelial surface. Bacterial urease production is required for acid resistance through the localized production of ammonium ions, and flagellar motility allows penetration of the mucus. Moreover, urease activity facilitates flagellar motility through the mucous layer by changing the viscoelasticity properties of gastric mucins. At low pH, gastric mucins form a gel that effectively traps the bacteria, but urease-catalysed production of ammonium ions raises the pH to near neutral and the mucous gel transitions to a viscoelastic solution through which *H. pylori* can swim. Regulators of motility, including chemotaxis and cell shape have been probed to discover additional colonization factors and to better define the optimal niche for *H. pylori*. Helical cell shape is thought to enhance motility through viscous media by a corkscrew mechanism, and cell shape mutants that have lost helical twist and/or curvature exhibit attenuated colonization. Chemotaxis mutants have an altered localization, including lower numbers of bacteria that are in close association with gastric epithelial cells and that are deeply penetrating the gastric glands. Additionally, to promoting clearance, the altered localization of chemotaxis mutants correlates with lower inflammation, impaired recruitment of CD^{4+} T cells and the absence of a T helper 17 (TH17) response. Accordingly, the intimate association with the gastric epithelium promotes stable infection while simultaneously provoking more inflammation. Higher inflammation correlates with lower bacterial loads, which suggests that *H. pylori* must actively manage its interaction with the host epithelium to avoid clearance and to persist at this site (37).

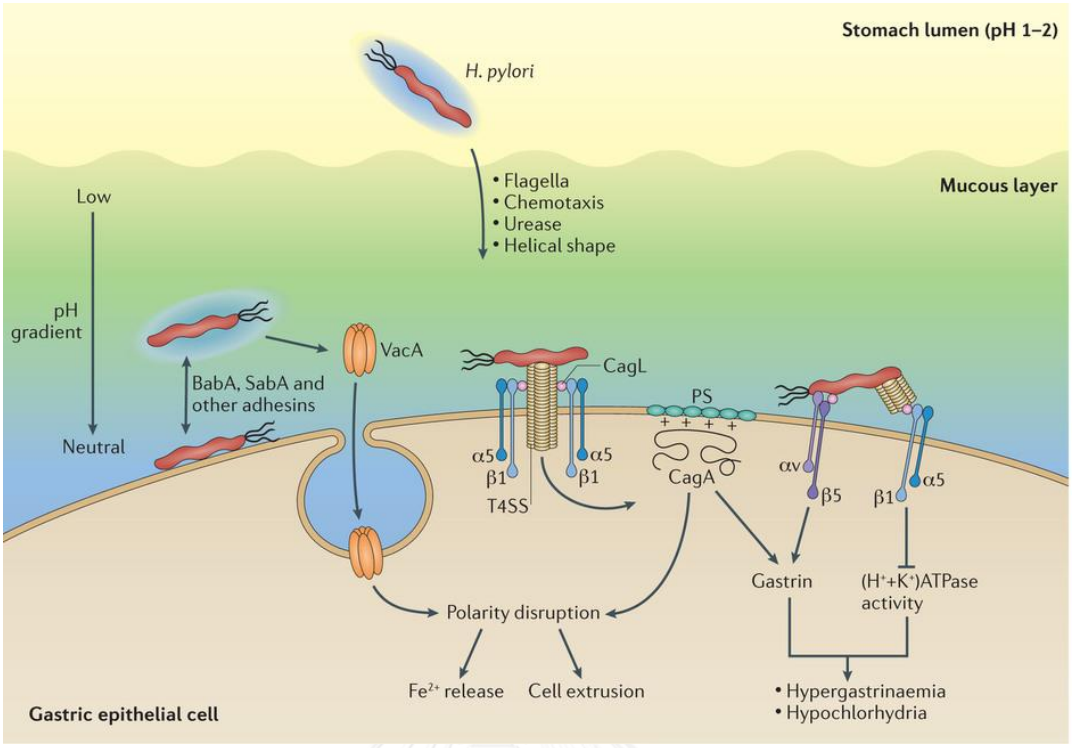


Figure 3 **Escape from the acidic lumen of *H. pylori*:** During initial infection of the stomach lumen, urease-dependent ammonia production locally raises the pH, which promotes bacterial survival and solubilizes the mucous gel to facilitate bacterial motility. Chemotaxis and helical rod shape promote flagellar motility away from the acidic lumen to the preferred niche of *H. pylori*, which is on and adjacent to gastric epithelial cells. SabA, BabA and other variably expressed adhesins might shift the balance from mucus-associated to cell-associated bacteria. Cell-associated bacteria alter gastric epithelial cell behaviour through VacA, CagA and CagL, which all have multiple cellular targets. CagL interactions with the $\alpha_5\beta_1$ cell surface receptor are mediated through the RGD motif of the protein. While, interactions with the other cell surface receptor ($\alpha_v\beta_5$ integrin) are RGD-independent. The combined action of these three effectors leads to a number of changes in the gastric epithelial cell, including CagA- and VacA-dependent disruption of cell polarity, which can promote iron acquisition and cell extrusion; CagA- and CagL-dependent induction of chemokines and/or the gastric hormone gastrin; CagL-dependent inhibition of acid secretion by the (H^++K^+) ATPase and cellular proliferation, apoptosis and differentiation, which are mediated by all three effectors. (SabA, sialic acid–binding adhesion; BabA ,blood group Ag-binding adhesin, VacA, vacuolating cytotoxin A; CagA, cytotoxin-associated gene A; CagL, cytotoxin-associated gene L; PS, phosphatidylserine; T4SS, type IV secretion system (37).

Main bacterial virulence factors associated with inflammation

Several virulence factors are more commonly present in disease of *H. pylori* infection. Most, but not all, of these are thought to enhance disease risk principally by causing increased pro-inflammatory cytokine release from epithelial cells, as a result of increasing local inflammatory cell infiltration into the gastric mucosa. This local inflammation may be the initiator of the more complex final inflammatory immune response to infection. In chronic infection, pro-inflammatory cytokine expression by epithelial cells continues and is thought to be a major determinant of both peptic ulceration and gastric malignancy (38). Interactions between bacterial products and host cell receptors could enhance bacterial binding, even though secreted bacterial products may also engage the receptors. These interactions allow the epithelial cell to transduce a signal to the host, indicating that a luminal infection is present that may represent some danger. One aspect that is evident is that few bacterial/epithelial cell interactions have been validated *in vivo* or linked to specific signaling pathways that result in a known epithelial cell response. It is also evident that no single pathway is responsible for all changes in epithelial cell responses. Moreover, it is clear that the bacterial effects on epithelial cell signaling are insufficient to explain the magnitude of gastritis and the local or systemic consequences that are attributed to *H pylori*. Thus, these interactions complement the effects of cytokines, neuroendocrine influences, and other signals emanating from the lamina propria. Summary of the best-known interactions between bacterial products and host cell receptors that have been described figure 4 (32).

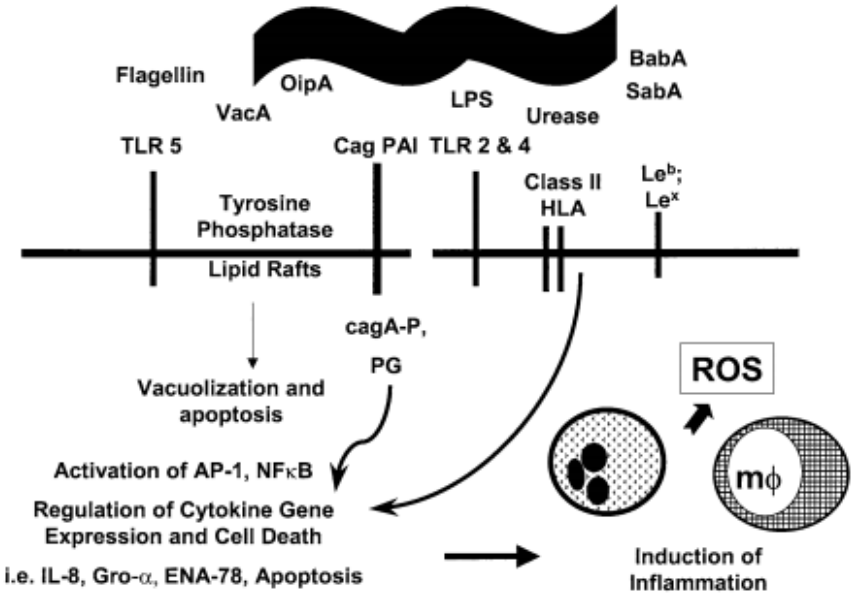


Figure 4 Potential interactions between *H pylori* and gastric epithelial cells: (ROS, reactive oxygen species; PG, peptidoglycan; cagA-P, the phosphorylated form of cagA; PMN, polymorphonuclear cell; mØ , macrophage) (32).

The cag pathogenicity island and relationship with cytotoxin-associated gene A

Interaction between the bacteria and gastric epithelium involves a segment of bacterial DNA referred to as the cytotoxin-associated gene pathogenicity island (cag PAI), a 40 kilobase (kb) region of chromosomal DNA encoding approximately 31 genes that forms a type IV secretion system (T4SS), a sort of 'molecular syringe', through which a protein encoded on the cag PaI. CagA is a protein with a molecular mass of approximately 140 kDa and it injected into the epithelial cytosol by a type IV secretion system. The CagA protein is a highly immunogenic protein encoded by the CagA gene. This gene is present in approximately 50% - 70% of *H. pylori* strains. Strains carrying the cag PAI are referred to as $CagA^+$ strains, as they are commonly identified in patients by their potential to induce significant antibody titers against the CagA marker protein. Patients infected with $CagA^+$ strains usually have a higher inflammatory response and are significantly more at risk for developing a symptomatic outcome (peptic ulcer or gastric cancer) in Western populations, though not in Asian populations. *H. pylori* strains possessing the cag PAI stimulate epithelial cell lines to express large amounts of the IL-8 and $CagA^+$ strains are associated with high levels of epithelial cell IL-8 expression *in vivo*. Thus, *H. pylori* strains have been divided into two broad categories, type I and type II, based on whether or not they possess the cag PAI. Type I strains are those that contain the cag PAI or Cag^+ and $VacA^+$, whereas type II strains lack functional cag PAI or $CagA^-$ and fail to produce a functional VacA toxin (39-41).

In addition to date a number of other virulence factors have been identified and characterized from the gastric pathogen *H. pylori*. Example of *H. pylori* adaptations and virulence factor shown as table1.

Table 1 Example of *H. pylori* adaptations and virulence factor (14, 36, 42, 43)

Virulence factor	Predicted role
Spiral shape	Hydrodynamic movement
Polar flagella	Motility in the gastric niche
Flagellin structure	Modification of Toll-like receptor (TLR) recognition site
Urease	Neutralization of acid and toxic effect on epithelial cells, disrupting cell tight junctions
Adhesins	Anchoring *H. pylori* to epithelium
Proteolytic enzymes	Glucosulfatase degrades mucin
Vacuolating cytotoxin A (Vac A)	Damage of the epithelium, membrane channel formation, disruption of endosomal and lysosomal activity, effects on integrin receptor-induced cell signaling, interference with cytoskeleton-dependent cell functions, induction of apoptosis, and immune modulation
Phospholipase A	Digest phospholipids in cell membranes
Alcohol dehydrogenase	Gastric mucosal injury

Virulence factor	Predicted role
Blood group antigen binding adhesin (BabA)	Binds to fucosylated Leb blood group antigen on cells
Sialic acid–binding adhesion (SabA)	Binds to sialyl-Lex and sialyl-Lea antigens and is involved in activation of neutrophils
Sialic acid–binding adhesion (SabB)	Binding specificity is unknown
Outer membrane protein A (OipA)	Assist in IL-8 induction, but this association is not universal
AlpA and AlpB	Inactivation of the AlpA and AlpB genes results in decreased adherence to gastric epithelial cells
Neutrophil activating protein A (NAP-A)	First identified to stimulate high production of oxygen radicals from neutrophils, leading to damage of local tissues, and promote neutrophil adhesion to endothelial cells; possible function in protection of *H. pylori* DNA or iron storage
IceA	The IceA1 allele encodes a CATG-recognizing restriction endonuclease
The duodenal ulcer promoting (DupA)	The DupA gene encodes a VirB4 ATPase homolog
Lipopolysaccharide	Lipid A with low biological activity
LPS Lewis antigens	Mimicry of host cell molecules
Lewis X/Y blood group homology	Autoimmunity

Virulence factor	Predicted role
Heat shock protein 60 (Hsp60)	Potential immunogens of the bacterium that induces IL-6, IL-8, TNF-α, and GRO production from monocytes or gastric epithelial cells, activation of NF-kβ via TLR2 and the mitogen-activated protein kinase pathway and thereby induces human monocytes to secrete IL-8

The immune response to *Helicobacter pylori*

The innate immune response

The first line of defense results in gastritis found that *H. pylori* stimulates innate immune responses from these infiltrating cells, which may subsequently influence amount of bacterial colonization, the level of inflammation and also the generation of adaptive immune responses. The innate response is therefore a central determinant of disease severity and is thought to be a main mediator in gastric carcinogenesis (Table 1). Innate immune mechanisms are largely dependent upon the engagement of pattern recognition receptors (PRRs) however are the TLRs, by pathogen-associated molecular patterns (PAMPs) present on infectious organisms. Intracellular Nod-like receptors, the intracellular receptor stimulated by $CagA^+$ strains, is a PRR and the PAMP it recognizes is a component of gram-negative peptidoglycan. The acute inflammatory immune response is initiated following contact between *H. pylori* and gastric epithelial cells. *H. pylori* urease, CagA, and the cagPAI stimulate rapid activation of the nuclear factor- kappaB (NF-κB) , activator protein (AP) 1 and induction of IL-8, growth-regulated oncogene-α (GRO-α), regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, and MIP-3α. Neutrophils, monocytes, macrophages, and dendritic cells, which are recruited to the gastric mucosa, then escalate inflammation through secretion of the proinflammatory cytokines IL-α, IL-8, and TNF-α. In a mouse model, neutrophils and eosinophils accounted for the first wave of infiltrating innate immune effector cells, with increased numbers at 8 week and then at 26 week after experimental infection, while a second wave of infiltrating macrophages was recorded at 26 week

after infection. In a challenge model utilizing human volunteers, the number of lymphocytes and monocytes in *H. pylori*-infected gastric mucosa increased 2 week after infection. This was followed 4 week after infection by increased numbers of $CD4^+$ and $CD8^+$ T cells, signifying the start of an adaptive immune response. The importance of T cells in the immune response to *H. pylori* was shown in another human trial, in which the apparent clearance of acute *H. pylori* infection in some individuals was associated with circulating T cells (38, 44).

The role of infiltrating leukocytes; Inflammatory mediators were secreted by gastric epithelium cell and they stimulate the migration of granulocytes, monocytes and lymphocytes into the inflamed mucosa. It is known that high densities of infiltrating cells are associated with more severe inflammatory response. Where the innate response to *H. pylori* results in tissue damage, these infiltrating cells with their own array of PRRs are exposed to bacteria or bacterial components, to induce further pro-inflammatory gene expression. A lot of groups have shown that human dendritic cells are activated and secrete cytokines, including IL-6, IL-8, IL-10, IL-12, IL-1β and TNF-α when cultured in the presence of *H. pylori*. Data conflict on whether cag PaI activity determines the capacity of *H. pylori* strains to stimulate. Different clinical $CagA^+$ *H. pylori* isolates vary enormously in their capacity to induce pro-inflammatory cytokine expression, thus other bacterial factors must be involved. Phagocytosis is an essential anti-bacterial innate immune mechanism, and there is a substantial phagocytic cell infiltrate in the infected gastric mucosa. Macrophage IL-6 expression is dependent upon phagocytosis. *H. pylori* partially evades phagocyte-mediated killing, but mechanisms are controversial. A large proportion of engulfed bacteria appear to survive inside phagosomes which fuse to become megasomes and may provide a protected intracellular niche contributing to the persistence of infection. The bacteria also evade phagocyte-mediated killing by disrupting nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) activity. NADPH oxidase catalyzes the conversion of molecular oxygen into superoxide anions, as a result of the extracellular release of toxic ROS rather than their accumulation within phagosomes. However, considerable ROS release occurs, but the bacteria survive by neutralizing this, for example through their catalase activity. Mast cells have been described to be present at higher frequencies in the human gastric mucosa infection and to decline after *H. pylori* eradication therapy. The role of these cells during infection has not been widely studied, but they may be involved in tissue repair or

increased inflammatory responses. VacA can attract bone marrow-derived murine mast cells, and induce these cells to secrete inflammatory mediators(38).

Table 2 The role of major *H. pylori*-induced inflammatory factors in cancer development (38)

Factor	Role in inflammation	Role in tumorigenesis
IL-8	Recruitment of lymphocytes and neutrophils	- Potentiates gastrin release, leading to the proliferation of epithelial cells, NF-kB activation and Cyclooxygenase (COX) 2, expression. - Pro-angiogenic activity
IL-1β	- Activation of macrophages and polymorphonuclear leukocytes - Stimulation of IL-6 release, COX-2 expression	- Stimulates hypergastrinaemia, leading to the proliferation of epithelial cells. - Pro-angiogenic activity induces matrix metalloproteinase secretion and activation.
IL-6	- Activation and differentiation of macrophages - Increases the phagocytic activity of neutrophils - Intercellular adhesion molecule-1 (ICAM-1) expression on endothelial cells - B-cell differentiation	- Increases angiogenesis via expression of vascular endothelial growth factor (VEGF)
TNF-α	- Activation and differentiation of macrophages - Apoptosis of epithelial cells and disruption of the epithelial barrier - Inhibition of microvascular epithelial cell proliferation and wound healing	- Stimulates hypergastrinaemia, leading to the proliferation of epithelial cells, NF-kB activation and COX-2 expression

Factor	Role in inflammation	Role in tumorigenesis
COX-2	- Catalyses the production of prostaglandins which increase vascular permeability and promote cellular migration to inflamed tissue - Wound healing	- Anti-apoptotic activity - Modulates immunity and tumour immunosurveillance via IL-10
ROS	- DNA damage and killing of bacteria	- Tissue DNA damage, mutation - Activation of host signaling pathways and angiogenesis
Nitric oxide	DNA damage and killing of bacteria	- Tissue DNA damage, mutation - Inhibits DNA repair mechanisms, inhibits apoptosis - Angiogenic activity

Role of tumor necrosis factor alpha in *Helicobacter pylori* infection

TNF-α is a major proinflammatory cytokine and plays an important role in the development of acute inflammation, including neutrophil infiltration of gastric mucosa, that is produced in the gastric mucosa in response to *H. pylori* infection. Moreover, TNF- α stimulates transcription factors, such as NF-kB, induces the synthesis of various inflammatory cytokines, including IL-1, IL-6 and IL-8. Increased plasma basal and postprandial gastrin levels and decreased mucosal concentrations of somatostatin are evident in *H. pylori*-infected subjects. TNF- α is potently inhibit acid secretion by *in vitro* cultured parietal cells and likely to affect local acid secretion, accordingly the distribution of *H. pylori* colonization and gastritis. Whereas pro-inflammatory cytokines have a negative effect on parietal cells. Although, gastrin secretion from cultured gastrin (G) cells is stimulated by a range of cytokines, including TNF-α and IL-8. Gastrin secretion is negatively regulated by somatostatin that is released from somatostatin (D) cells in the gastric mucosa. In *H. pylori* infection this negative

feedback mechanism is disrupted. The type 1 T helper (Th1) cell immunoregulatory cytokine TNF-α inhibit somatostatin secretion by D cell(45). Additionally, positive associations between genotypes with increased expression of IL-1β or TNF-α and increased risk of gastric adenocarcinoma have been found (38).

Role of interleukin-8 in *Helicobacter pylori* infection

IL-8 is chemokine belongs to the CXC family, which play an important role in the chemotactic activity for neutrophils to site of inflammation, induces the expression of adhesion molecules, Macrophage 1 antigen (Mac-1), and the production of ROS. It also has effects on cell proliferation, migration and tumor angiogenesis. (Figure 5). Attachment of *H. pylori* to gastric epithelial cells can induce host cellular responses, including the reorganization of actin cytoskeletons, the tyrosine phosphorylation of a 145-kD protein , and release of IL-8(46). Neutrophils are then activated by *H. pylori* or its soluble products, and proceed to release ROS and more IL-8(47). *H. pylori* infection rapidly up-regulates the expression of IL-8 in human gastric epithelial cell. Additionally, although the IL-8 gene is up-regulated in gastric epithelial cells or neutrophils infected with *H. pylori*, this up-regulation is relatively limited. Therefore, IL-8 mRNA expression was up-regulated within 1 hour after *H. pylori* infection, reached a maximal increase of ~120-fold at 8 hours post-infection, and then decreased(8). The CINC-1 has a potent neutrophil chemotactic activity in rats, similar to the effect of IL-8 in human neutrophils. Yagihashi *et al.* reported that pretreatment with anti-CINC-1 antibody significantly reduced tissue myeloperoxidase activity and injury after small intestinal I/R treatment in rats (9).

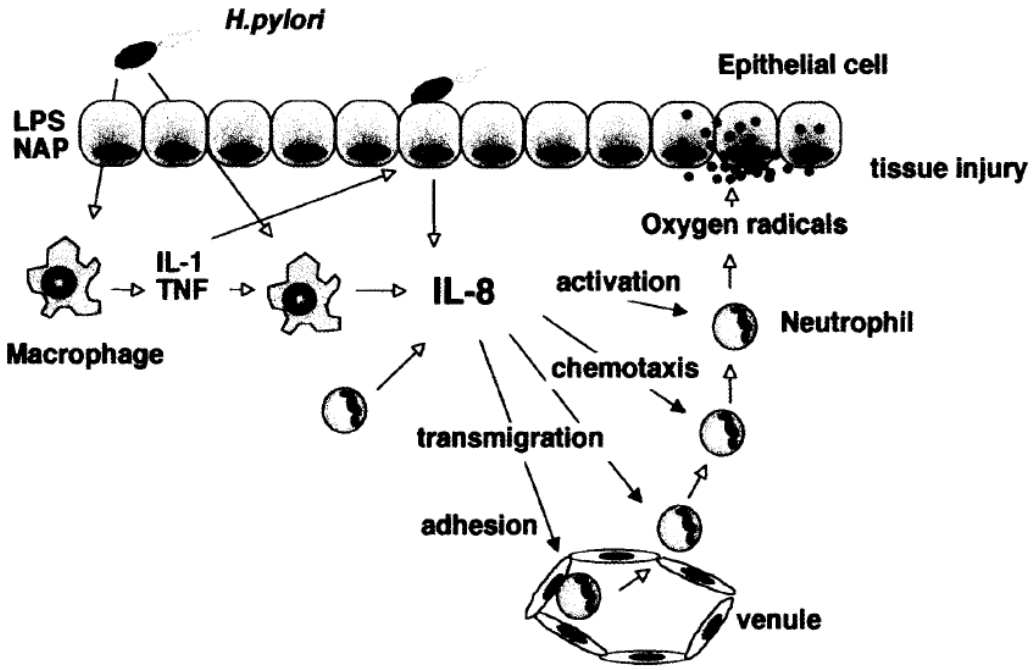


Figure 5 *H. pylori*-induced inflammation and inflammatory cytokine IL-8 (9)

The gastric epithelial cells and macrophages are major sources of IL-8 in the case of *H. pylori*-infected gastric mucosa. Epithelial IL-8 can be induced, not only by direct bacterial stimulation, but also following exposure to the endogenous proinflammatory mediators IL-1 and TNF-α. Macrophages in the lamina propria are the main sources of IL-1and TNF-α. The bacterial components inducing the proinflammatory cytokines from macrophages are likely to be multiple. *H. pylori* LPS and NAP will induce cytokine secretion. Neutrophils once accumulated at the site of infection may secrete chemokines, thus further amplifying the cellular response to infection (48).

Helicobacter pylori and reactive oxygen species

H. pylori induces a strong inflammatory response, generating large amounts of ROS. Many clinical data suggest that other factors inherent to host conditions such as diet, stress, tobacco, genetics and hygiene were contributed the pathogenesis of this infection. It should be noted that some of these factors directly influence mucosal oxidative status, as they expose the gastric epithelium to the ROS they generate within the gastric lumen in a sustained manner. Besides these ROS other potential sources associated with infection include: (1) Inflammatory cells (neutrophils, macrophages) infiltrating the mucosa. The imbalance between gastrotoxic agents and protective

mechanisms results in an acute inflammation. The IL-1β and TNF-α are major proinflammatory cytokines, playing important role in production of acute inflammation. This acute inflammation is accompanied by neutrophils infiltration of gastric mucosa. Neutrophils produce superoxide radical anion ($O_2^{\bullet-}$), which belongs to group of ROS. Superoxide radical anion reacts with cellular lipids, leading to the formation of lipid peroxides, that are metabolized to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Two parameters are usually useful for assessment of biological effects of ROS namely the tissue levels of MDA plus 4-HNE and the activity of superoxide dismutase (SOD). Tissue levels of MDA and 4-HNE are used as indicators of lipid peroxidation. SOD activity reflects the antioxidative properties of various tissues including gastric mucosa (Figure 6), (2) gastric epithelial cell and (3) the bacterium itself, which generates a great amount of superoxide anion to inhibit the bactericidal effects of nitric oxide as synthesized by inflammatory cells. ROS are highly reactive. When they are generated close to cell membranes, possibly by gastric mucosal cells, they induce oxidative stress and oxidize membrane phospholipids (lipid peroxidation), which may continue in a form of a chain reaction. This process is mediated by the interaction of hydroxyl radicals with the cell membrane; subsequently producing lipid-derived free radicals such as conjugated dienes and lipid hydroperoxides. These radicals are known to be extremely reactive products that cause oxidative damage (5, 49, 50).

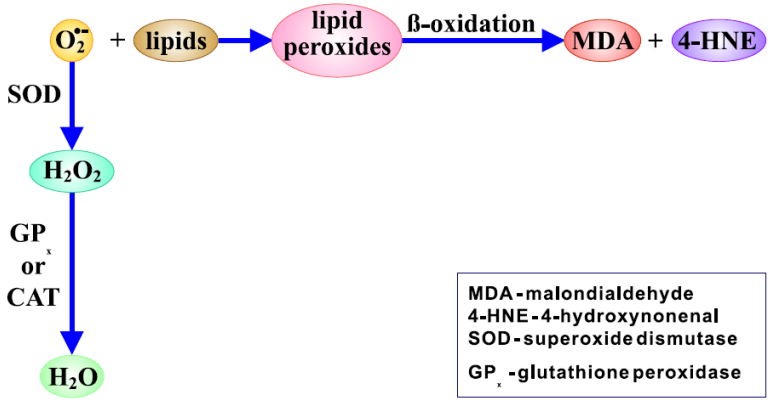


Figure 6 Transformation pathways of a superoxide radical anion in the organ tissue: Superoxide is metabolized via the process of lipid peroxidation or neutralization undergo to form Hydrogen peroxide (H_2O_2) and H_2O (50).

Adaptive immune response

Macrophages and dendritic cells are the major antigen presenting cells located in the lamina propria of the gastric mucosa with initiation of the adaptive immune response to *H. pylori* infection. *H. pylori* was showed to stimulate the release of IL-6 from macrophages in of phagocytosis. IL-6 is one of the principal drivers of the adaptive immune response, regulating the ratio of T helper type 17 (Th17) cells to T regulatory (Treg) cells. It can stimulate the differentiation of B cells into antibody-producing plasma cells. Development of inflammatory and adaptive immune responses shown as Figure 7 (38, 44).

Cell-mediated immunity (CMI); Include of a complex mixture of T helper and Treg cells infiltrating the inflamed gastric mucosa. The potent T cell type in the *H. pylori*-infected gastric mucosa is the proinflammatory Th1 cell. Th1 cells promote inflammation and tissue damage through Interferon gamma (IFNγ) and IL-12. Th1 can be modulated by the characteristic Th2 cytokine IL-4, which appears to downregulate IL-12 production, and *H. pylori* directly stimulates gastric epithelial cells to produce thymic stromal lymphopoietin, inducing response of a dendritic cell-mediated Th2. The main of Th17 cells and the Th17-to-Treg ratio in mucosal damage and immune escape has been established in recent times. (Figure. 7) Skewing the Th17-to-Treg ratio toward a Th17-based response is serious to the development of a vaccine-mediated clearance but also increases the amount of inflammation-based damage, while skewing the ratio toward a Treg response is required for *H. pylori* immune escape and tolerance (38, 44).

Humoral immunity (HI); Most individuals infected with the *H. pylori* develop specific antibodies, which are found in serum and in gastric aspirates or extracts of stomach. The B-cell response plays an essential role in pathogenesis through participating in *H. pylori*-precipitated autoimmune process with antibodies cross-react via host antigens such as those on gastric epithelial cells and the parietal cell H^+,K^+- ATPase, likely inducing damage and local inflammation. The high titers of IgG and IgA antibodies against membrane preparation, flagellin, urease, LPS, and *H. pylori* adhesin A (HpaA) have been founded in patients infected with *H. pylori*. Nevertheless, those titers do not differ between asymptomatic patients and patients with duodenal. As *H. pylori* infection is not cleared in humans without antibiotic intervention. Previous studies performed with biopsies from the

antral of stomach in non-infected and *H. pylori* –infected patients. IgM- and IgA-producing cells showed frequencies of 40- to 50-fold higher in infected subjects. Nevertheless, amount of IgG-producing cell are the identical for non-infected and infected *H. pylori* subjects. Those results suggest that the infection induces a large recruitment of immune cells into the gastric mucosa, particularly IgA-producing cells. Therefore, the humoral immune response has been considered to be an indication of infection, rather than a marker of protection. Additionally, on the basis of evidence obtained using a B cell-deficient mouse model, *H. pylori* specific antibodies may actually enhance colonization (38, 44, 51).

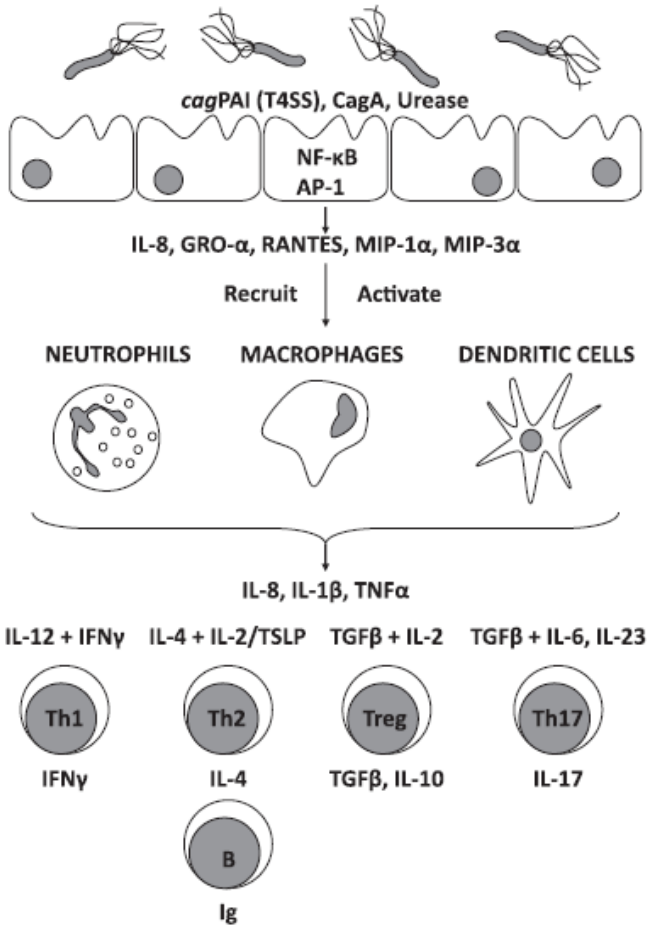


Figure 7 Development of inflammatory and adaptive immune responses to *H. pylori* infection: *H. pylori* stimulates gastric epithelial cells to release the proinflammatory chemokines IL-8, growth-regulated oncogene- (GRO-α), regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, and MIP-3α by activating NF-κB and/or AP-1 through attachment or by CagA-dependent and -independent means. These chemokines recruit

and activate neutrophils, macrophages, and dendritic cells, which further enhance the inflammatory immune response by releasing proinflammatory cytokines, including IL-8, IL-1, and TNF-α. Macrophages and dendritic cells are antigen presentating cell, they are initiate the adaptive immune response and release of cytokines that drive differentiation of T helper cell subsets (Th1, Th2, Th17, and Treg). IL-12 and IFNγ induce a Th1-polarized response with concomitant production of IFNγ. TGFβ, IL-23, and IL-6/IL-2 regulate the Th17-to-Treg ratio, resulting in release of IL-17 or TGFβ/IL-10 by Th17 or Treg cells, respectively; IL-4 and IL-2/thymic stromal lymphopoietin (TSLP) provide the stimulus for a Th2 (IL-4)-mediated response and downstream production of *H. pylori*-specific antibodies by B cells. T4SS, type IV secretion system; cagPAI, cag pathogenicity island (44).

Disease type

Though colonization with *H. pylori* induces gastritis in all infected individuals. The risk of development of these disorders in the presence of *H. pylori* infection depends on a variety of bacterial, host and environmental factors that mostly relate to the pattern and severity of gastritis (34). Three main gastric phenotypes have been identified, and each is associated with a set of pathophysiologic abnormalities that could explain why a certain outcome occurs (Figure 8) (37).

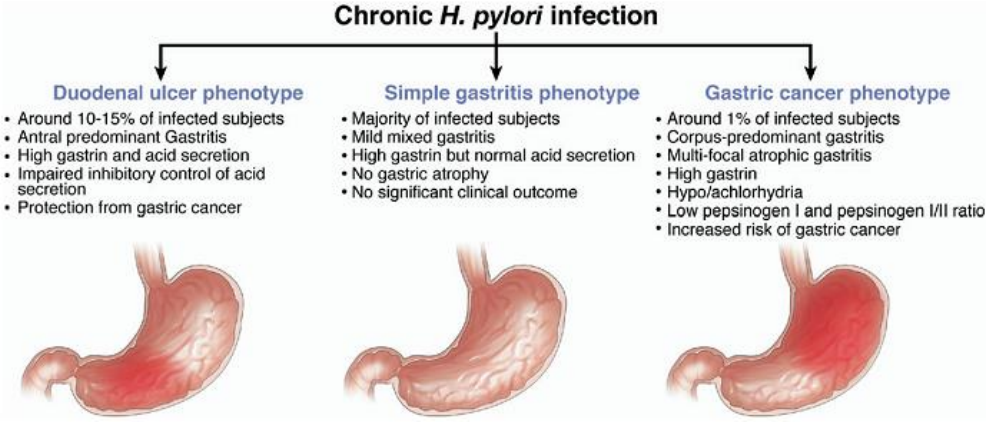


Figure 8 Pathophysiologic and clinical outcomes of chronic *H. pylori* infection: *H. pylori* infection associated with 3 potential outcomes, and the figure illustrates the histologic, physiologic, and clinical characteristics of each (37).

Diagnosis

A diagnostic test are divided into invasive tests and noninvasive tests. Histology, urea breath testing and culture are routine diagnostic purposes, while the use of serology is most appropriate for several epidemiological studies (52). Antimicrobial susceptibility testing of *H. pylori* infection can be performed by phenotypic test such as disk diffusion (standard antibiogram), agar or broth dilution and break-point testing. These methods have the advantage of evaluating all of the drugs at the same time, and, for most of them, an exact minimum inhibitory concentration (MIC) can be determined for each isolate (53).

Table 3 Diagnostic tests for the detection of *H. pylori* infection (43, 44)

Test	Sensitivity	Specificity	Advantages	Disadvantages
Noninvasive				
Serology	76-84%	79-90%	-Mainly used for epidemiological studies and inexpensive	- Insufficient reliability for routine screening - Cannot prove ongoing infection due to immunological memory because, positive result may reflect previous rather than current infection, not useful after treatment
Urea breath test	>95%	>95%	High negative and positive predictive values, useful before and after treatment	- False-negative results possible in the presence of PPIs or with recent use of antibiotics of bismuth preparations, considerable resources and personnel required to perform test
Stool antigen test	96%	97%	High negative and positive predictive values, useful before and after treatment	- False-negative results possible in the presence of PPIs or with recent use of antibiotics

Test	Sensitivity	Specificity	Advantages	Disadvantages
Invasive				
Histology	69-93%	87-99%	- Gold standard" in routine hospital diagnostics - Excellent sensitivity and specificity, especially with special and immune stains, provides additional information about gastric mucosa	- Requires expert pathologist; also provides histological data on inflammation and atrophy - Expensive (endoscopy and histopathology costs), inter observer variability, accuracy affected by PPI and antibiotics use
Rapid urease test	90%	93%	- Rapid results - Accurate in patients not using PPIs or antibiotics, no added histopathology cost	- Requires an additional test for confirmation of *H. pylori* infection - Requires endoscopy, less accurate after treatment or in patients using PPIs
Culture	58.1%	100%	- Alternative gold standard -Specificity 100%, allows antibiotics sensitivity testing	- Allows for testing of antimicrobial sensitivity - Requires specific microbiological expertise and properly equipped facilities

Role of histologic staining

H. pylori can stain by hematoxylin and eosin (H&E), the previous study showed 69-93% of sensitivity and 87-90% of specificity for *H. pylori* staining. Nevertheless, the special stains such as Warthin-starry silver stain, modified Giemsa stain, Genta stain and immunohistochemical (IHC) stain could enhance the specificity (90-100%)(54). *H. pylori* can directly identify by H&E stain in a measure of inflammation degree (Figure 9). When a low amount of *H. pylori* and change of atrophic mucosal are combined, this event is difficult to see the organism. Since Giemsa staining is easy method, provides consistent results and inexpensive, so it is the recommended for using in laboratories (54, 55).

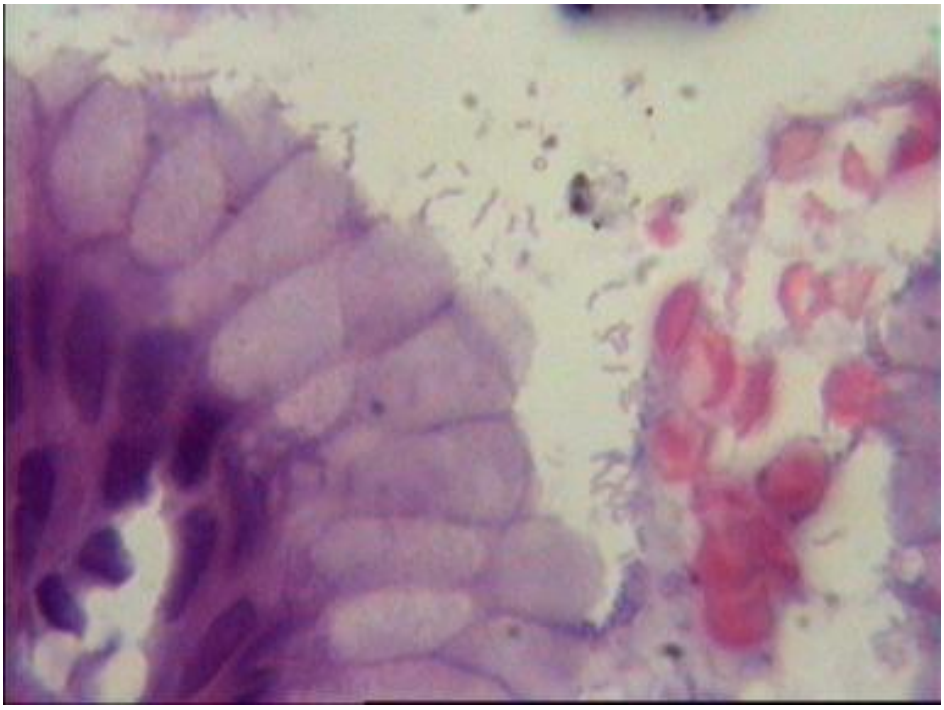


Figure *9* *H. pylori* of antral biopsy specimen from gastric patients stained by H & E (100xs) (55)

General overview of genistein

Genistein is a basic precursor in the antimicrobial phytoalexins biosynthesis and phytoanticipins in legumes. It is the important molecule in soybean seeds (15) and one of the naturally isoflavones belongs to the flavonoids family with three or more phenol hydroxyl residues, so it is called a soybean polyphenol. C6-C3-C6 is the basic structural skeleton of flavonoids and it consists of two aromatic C6 rings (A and B) and a heterocyclic ring (C) that contains one oxygen atom (Figure 10) (56, 57).

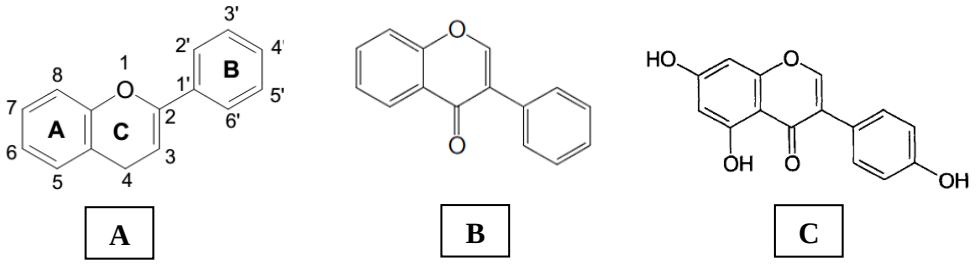


Figure 10 A. Phenol structure (The basic structure of flavonoids), B. Isoflavone structure, C. Genistein structure (56)

Pharmacokinetic of genistein

Glycosides Isoflavones is inactive form that found in plants. There are 2 main different chemical forms of isoflavone; 1.unconjugated form (Isoflavone aglycone): daidzein, genistein and glycitein, 2.Sugar-conjugated (Isoflavone glucoside): daidzin, genistin and glycitin (58). Once ingested, bacterial β-glucosidases in intestine can hydrolyzed glycosides and converted to corresponding bioactive aglycones. Further fermentation proceeds in the distal intestine with the formation of specific metabolites. The aglycones are absorbed from the intestinal tract and conjugated mainly in the liver to glucuronides, which are either re-excreted through the bile and reabsorbed by enterohepatic recycling or excretion in the urine (59).

The medical tests for genistein exposure

Blood Tests; The plasma half-life of genistein measured from their plasma appearance and disappearance curves to be 7.9 hours in adults (60). Peak blood concentrations occur approximately 4-8 hours after ingestion of single dose (61). 50-800 ng/mL of plasma concentrations are achieved for genistein and equol in adults consuming of soy-foods containing in the region of 50 mg/day of total isoflavones. These values are similar to consuming their traditional diet in Japan's population (62).

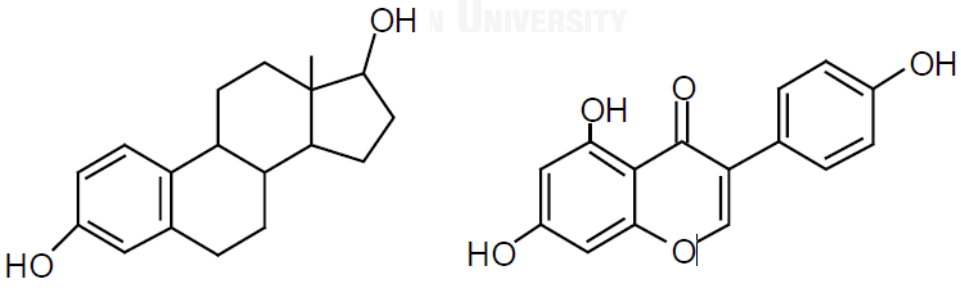
Urine Tests; The half-lives of the isoflavones are short, so the majority of isoflavones dose is excreted in urine and feces within 24 hours. There is considerable variation of interindividual in gut bacterial metabolism leads to markedly different urinary concentrations and its metabolites in different individuals(63).

Soy-food sources and genistein composition

Soybeans and soy products are the major foods containing nutritionally relevant amounts of genistein. Ingestion is the source of human exposure to genistein and occurs principally food, infant formulas, and dietary supplements made with soybeans products. Human consume contain significant amounts of the isoflavones genistein and daidzein in soybean foods(64). Volumes containing of powdered soybean chips contains daidzein about 800 µg and genistein (primarily as glycosides) about over 500 µg. Tofu is the major source of isoflavones in the Asian diet with contain higher levels of genistein than other soy products such as miso and soy sauce (56).

Genistein as a phytoestrogen

The basic structural of genistein was originally identified as having a close similarity in structure to 17β –estradiol (female endogenous estrogen), particularly the phenolic ring and the distance between its 40- and 7- hydroxyl groups such, was labeled as a phytoestrogen (Figure 11). Because of its structural similarity to 17β -estradiol, genistein has been shown to compete with 17β-estradiol in estrogen receptor binding assays , it is able to bind to estrogen receptors (ER) and elicit either a weak estrogenic (agonistic) or anti-estrogenic (antagonistic) effect, depending on the levels of endogenous estrogens present and the tissue and the estrogen receptor (ER) subtype(26). Genistein is capable of binding to the ER, with a preference for ERβ, the predominantly expressed ER subtype in the gastrointestinal tract (65, 66). The estrogen receptor activity of genistein may play a major role in their effects against cancers of tissues that express estrogen receptors (26).



17β –estradiol

Genistein

Figure 11 Structures of genistein in relation to 17β –estradiol (67)

Health effects of genistein

Previous animal studies and *in vitro* research with genistein have identified effects in a multitude of disorders including chemoprevention (68).cardioprotective (69) and antiosteoporosis activity (70). Some of the observed pharmacological effects of genistein can be associated with its estrogenic activity (17) with treatment for acute menopausal symptoms (18). A number of power health benefits associated with consuming a genistein-rich diet, as summarized in figure 12.

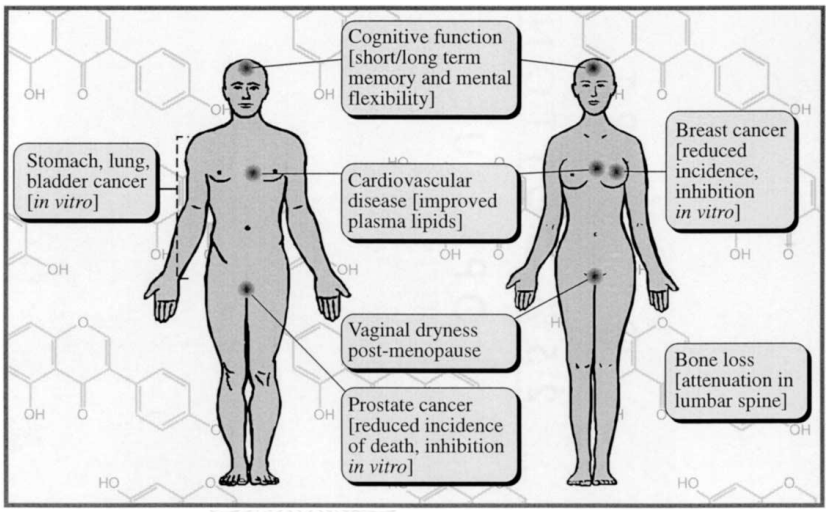


Figure 12 Proposed targets for beneficial effects of dietary genistein or a high soy diet on human health (15)

Genistein is natural antioxidant

Previous studies have demonstrated that genistein inhibited DNA strand breaks in plasmid DNA mediated by reactive oxygen species (71). It also inhibits the enzyme xanthine oxidase which produces hydrogen peroxide and superoxide anion (72). Genistein was showed exhibits peroxyl radicals scavenging activity so, many studies have been showed the inhibitory properties of genistein on lipid peroxidation. Such studies indicate that function of genistein results in lengthening the lag time of lipid peroxidation in concentration-dependent manner with the peroxyl radical scavenging properties. Genistein should also scavenge hydroxyl radical, the radical associated the primary damage to lipid systems(73). Additionally, genistein in animal experiments, has been found to increase the activities of the antioxidant enzyme superoxide dismutase (74), gluththionine peroxidase and glutathione reductase (75). The protective effect of genistein against oxidative

stress-induced injury depends on the modulation of transcription factors. The activation of the transcription factors nuclear respiratory factor 1(Nrf1) and Nrf2, which regulate genes involved in oxidative stress (76). Moreover, some data showed that genistein is an antioxidant by virtue of its inhibition of the activation of NF-kB stimulated by oxidative stress (77).

Antibacterial activity

Type II topoisomerases of prokaryote (DNA gyrase and topoisomerase IV) are targets for broad-spectrum antibiotics. These are the ubiquitous enzymes that play a crucial role for control of replicative DNA synthesis share structural and functional homology among different prokaryotic and eukaryotic organisms (78). Genistein is a topoisomerase II inhibitor that it inhibits the catalytic activity of DNA topoisomerase II and leads to the formation of cleavable complexes *in vitro* (79) Margareta Verdrengh *et al*. (80) found that exposure to genistein exhibited an inhibitory effect on all *Staphylococcal* strains tested, including methicillin-resistant strains and inhibition the growth of *Streptococcus pasteurianus*, *Bacillus cereus*, and *H. pylori* as well as the recent study In the colony forming unit (CFU) assays, significant reductions in CFUs were found for *S. aureus and B. anthracis* when cultured in the presence of 100 μM genistein (81). In addition, genistein (50 or 100 mg/L) effectively minimized the morphologic damages and inhibited acute death of HeLa cells by *Vibrio vulnificus* (82).

Inhibition the activity of *Helicobacter pylori* by genistein

In previous study, Tetsu Akiyama *et al. (27)* demonstrated that genistein inhibit the activities of tyrosine-specific protein kinases, that are molecularly targeted therapeutic agents which inhibit signal transduction pathway. Ian L.P. *et al* (83) studied the early signal transduction mechanisms in gastric epithelial cell which lead to IL-8 production. They found *H. pylori*, TNF-α and IL-1β produced a dose-dependent increase in IL-8 production. The increase with all three was significantly reduced by the tyrosine kinase inhibitors genistein. In the same way, Song-Ze Ding *e. al*. (29) found that tyrosine kinase inhibitors genistein at various concentration dose-dependently reduced LPS, TNF-α, *H. pylori* and its toxin-induced IL-8 expression. Additionally, Yoshihisa Nozawa *et al.* demonstrated the IL-8 production pathway *H. pylori*-induced IL-8 production is dominantly regulated by $Ca^{2+/}$calmodulin signaling, and extracellular signal-regulated kinas (ERK) plays an important role in signal transmission for the efficient activation of *H. pylori*-induced NF-kβ activity, resulting in IL-8 production. They found that genistein completely blocked IL-8 release and suppressed the ERK activation induced by *H. pylori* infection (84).

Table 4 The studies of genistein attenuated inflammation in gastrointestinal disease

Topics	Protocol
The protective effect of the soybean polyphenol genistein against stress-induced gastric mucosal lesion in rats and its hormonal mechanisms (74)	**Dosage**: 50 or 100 mg/kg/day, IG, b.i.d.,2 wk **Marker**: The ratio of the mucosal hemorrhagic erosion area to the whole stomach body area, Myeloperoxidase (MPO), Superoxide dismutases (SOD), Thio-barbituric acid reactive substances (TBARS) level, TNF-α and CINC-1
The protective effect of genistein postconditioning on hypoxia/reoxygenation-induced injury in human gastric epithelial cells (85)	**Dosage**: 50 or 100 mg/kg/day, IG, b.i.d.,2 wk **Marker** The protein expression of calcitonin gene-related peptide (CGRP), B-cell lymphoma 2 (Bcl-2), and Bcl2-associared X protein (Bax)
The protective effect of capsaicin receptor-mediated genistein postconditioning on gastric ischemia–reperfusion Injury in rats (86)	**Dosage**:100, 500 or 1,000 μg/kg ,IV , single treat **Marker**: Gastric mucosal cell apoptosis, calcitonin gene-related peptide, protein kinase B phosphorylation
Protective effect of soy isoflavone genistein on ischemia-reperfusion in the rat small intestine (87)	**Dosage**: 500 microliters of genistein solution (1 mM), direct jejunum, single treat **Marker**: Superoxide anion scavenging activity, Hydroxyl radical scavenging activity, Radical chain-breaking activity, Histological examination, Evaluation of changes in vascular permeability, Measurement of lipid peroxide
Oral treatment with genistein reduces the expression of molecular and biochemical markers of inflammation in a rat model of chronic TNBS- (88)	**Dosage**: 100 mg/kg BW .Application volume was 5 ml/kg BW, IG, QD, 14 day **Marker**: COX-2 and MPO, the efficacy of genistein treatment, relative weights of the accessory sexual organs, specifically prostate and the seminal vesicle

Topics	Protocol
Anti-inflammatory effect of genistein on non-alcoholic steatohepatitis rats induced by high fat diet and its potential mechanisms (89)	**Dosage**: 4 mg/kg BW and 8 mg/kg BW, IG, QD, 12 wk **Marker**: serum TBARS, TNF-α, IL-6 and transforming growth factor beta 1(TGF-β1), liver TBARS, proteins involved in the mitogen-activated protein kinases (MAPKs) and NF-κβ pathways .
Genistein protection against acetaminophen-induced liver injury via its potential impact on the activation of undegraded dietary protein (UDP) glucuronosyltransferase and antioxidant enzymes (90)	**Dosage**: 50, 100 or 200 mg/kg BW, IG **Marker**: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), lactate dehydrogenase (LDH), MDA, the activities of glucuronosyltransferases (UGTs) and cytochrome P450 (CYP450)

IG, intragastric; IV, intravenous; BW, body weight; b.i.d., Bis in die; QD = Once a day

CHAPTER III MATERIALS AND METHODS

Chemical substances

Absolute alcohol

95% alcohol

Distilled water

10% formaldehyde in 0.2 M sodium phosphate buffer, pH 7.4

0.85% saline

Phosphate buffer saline (PBS)

Normal saline

Urea agar base

Agar bacteriological

Urea

10% neutral buffered formalin

Ethanol

Xylene

Paraffin

H&E stain

Giemsa stain

Methanol

Buffered water

Sterile water for injection (A.N.B. laboratories co.,LTD.,Thailand)

Thiopental sodium for injection BP (Jagsonpal Pharmaceuticais Ltd.,Haryana, India)

Streptomycin (General Drug House Co.,LTD.,Thailand)

Genistein Purity ≥98% (Cayman Chemical company, Michigan,USA)

Dimethyl sulfoxide, DMSO (Sigma Chemical CO.,USA)

Quantikine® ELISA Rat TNF-α (R&D Systems, Minneapolis, MN, USA)

Quantikine® ELISA Rat CXCL1/CINC-1 (R&D Systems, Minneapolis, MN, USA)

TBARS Assay Kit (Cayman Chemical company, Michigan,USA)

Brucella broth

Columbia agar (Oxoid, Basingstoke, United Kingdom)

Anaero-pack (MGC,Japan)

An anaerobic jar (Oxoid, Basingstoke, United Kingdom).

The experiment was divided into two major parts as follows:

1. ***In vivo* study**: To study the effects of genistein on *H .pylori*-induced change of the followings:

-Serum TNF-α level

- Serum CINC-1 level

-Gastric MDA level

-Gastric histopathology

2. ***In vitro* study**: To study the inhibitory growth of *H. pylori* by genistein

In vivo study

Genistein preparation

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, ≥98%) was purchased from Cayman Chemical Company and was dissolved using the universal sovent, ≥ 99.5% dimethyl sulfoxide (DMSO). Genistein was prepared by dissolving in 0.1 % DMSO before administered by gastric gavage in the rat.

Bacteria preparation

H.pylori strains was originally obtained from peptic ulcer patients (clinical isolate) who visited the King Chulalongkorn Memorial Hospital. The bacteria were grown in brucella broth (pH 7.0) supplemented with 10% goat serum for 24 hours at 37℃ 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

21 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 were approved by the Ethical Committee of Medicine Faculty, Chulalongkorn University, Thailand. The rats were housed in macrolon cages (4 and 3 animals per cage of each group), in a standard animal care room (at room temperature 18 ℃-22 ℃, humidity 55%) and were allowed free access to food and water.

Experimental protocol

All 21 rats were randomly divided into three groups including group 1 (control group, n=7), group 2 (*H. pylori* infection group, n=7) and group 3 (genistein treatment group, n=7)

Group 1 (Control group): The rat were received normal saline 1 ml/rat by gastric gavage twice a day at an interval of four hours for three sequential days. Later, they were treated with 0.1% (v/v%) DMSO (1 ml/rat, b.i.d by gastric gavage) for 14 day.

Group 2 (*H. pylori* infection group): Rats were inoculated with *H. pylori* by using model of Thong-Ngam *et al*. (91) Briefly, after pre-treatment with streptomycin suspended in drinking water (at dose 5 mg/ml) for three days; the rat were inoculated with *H. pylori* suspension about 10^{8-10} CFU/ml in normal saline (1 ml/rat, b.i.d. by gastric gavage) at an interval of four hours for 3 consecutive days. 24 hours after the inoculation, the rats were treated with 0.1% (v/v) DMSO (1 ml/rat, b.i.d by gastric gavage) for 14 day.

Group 3 (Genistein treatment group): Twenty four hours after *H. pylori* were inoculated in rats same as those in group 2, the rats were treated with genistein (16 mg/kg BW, b.i.d.) dissolved in 0.1% DMSO by gastric gavage for 14 days.

The amounts of genistein treatment were based on the safe doses ranging from 1 to 16 mg/kg body weight according to the pharmacokinetic study of isoflavones (92) and it was attenuatesd nonalcoholic steatohepatitis in a rat model (93). During the body weight of each rat was measured every two day. At the end of experimental protocol. The rats were sacrificed. Serum samples were collected to measure TNF-α level and CINC-1 level. The stomach were removed for detection of *H. pylori* infection by urease test, measuring of gastric tissue MDA level and pathological examination by a pathologist. The procedures of animal preparation were concluded in the diagram as shown in figure 13 and figure 14.

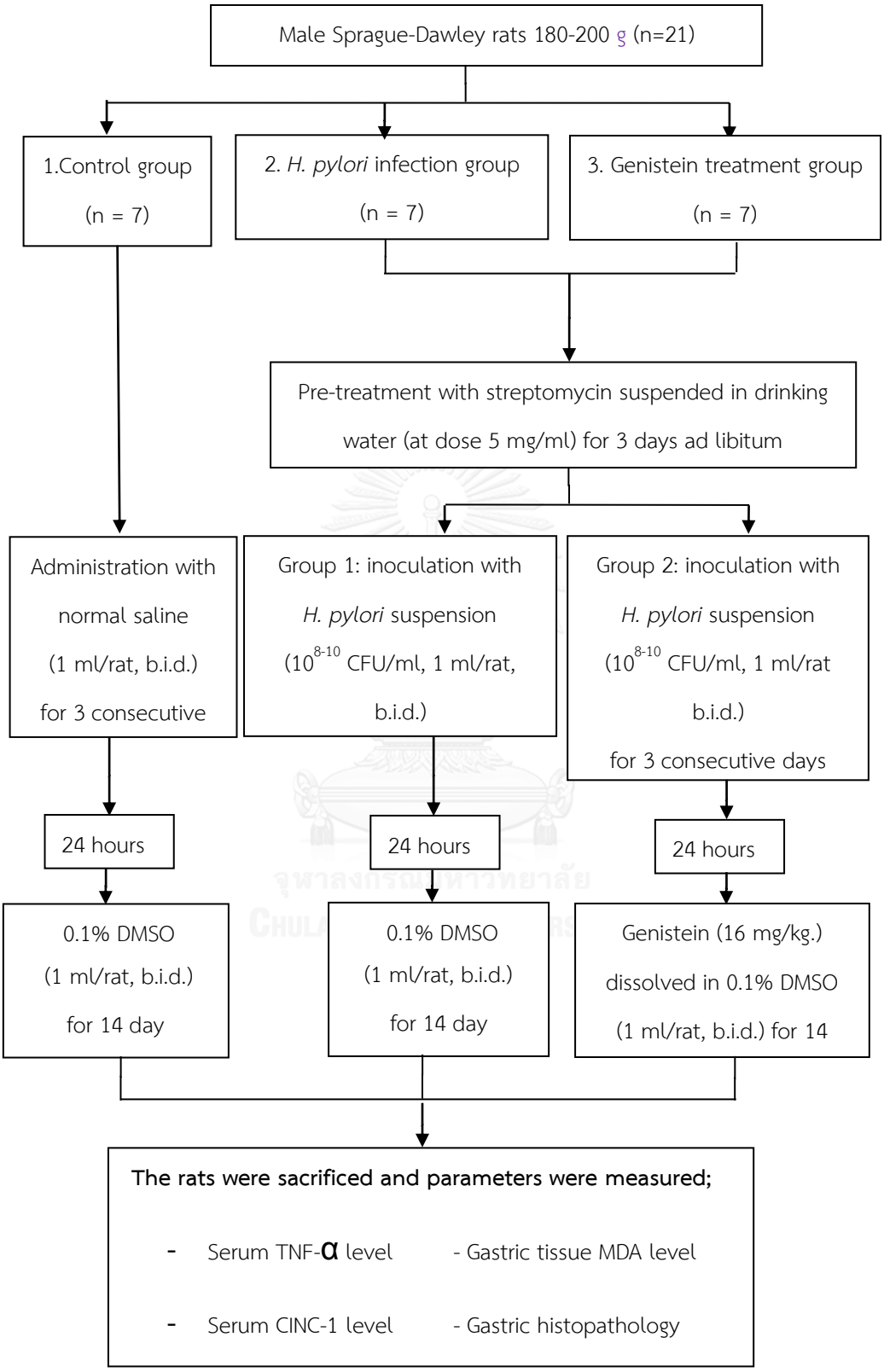


Figure 13 Schematic diagram of experimental protocol

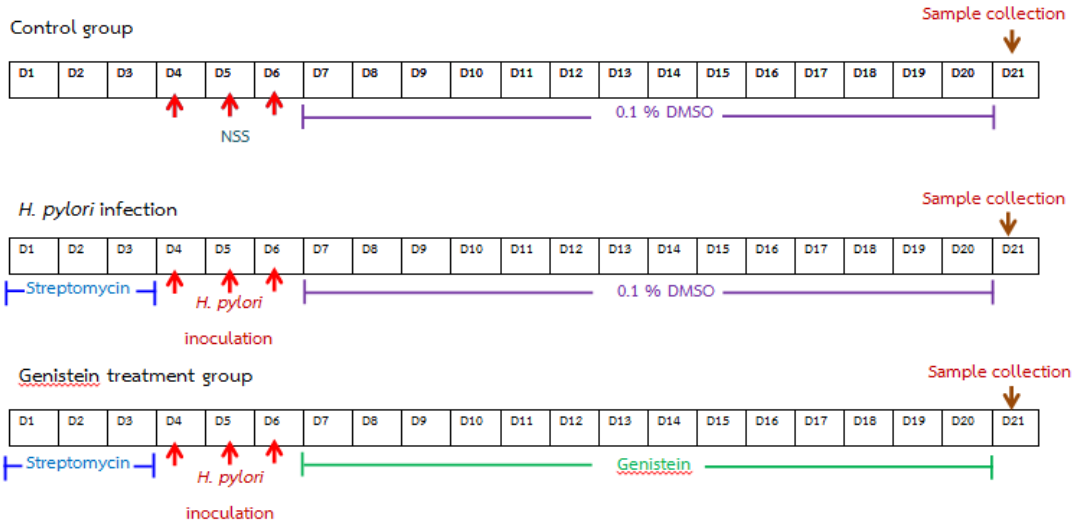


Figure 14 Diagram showed time line of experimental groups

Measurement of serum cytokine levels

Sample collection and storage

Blood samples were taken by cardiac puncture (as shown in figures 15) and allowed to clot for 2 hours at room temperature before centrifuging for 20 minutes at 1000 x g. Remove serum and store samples at -80°C for determination of TNF-α and CINC-1 level by enzyme-linked immunosorbent assay (ELISA), ELISA kit. This assay employs the quantitative sandwich enzyme immunoassay technique.

Figure 15 Drawing blood from rat by cardiac puncture

Principle of the quantitative sandwich enzyme immunoassay technique

ELISA is a useful method in evaluating the concentration of proteins in a sample such as serum, plasma, or cell culture supernatant. ELISA kit is performed in 96 well plates which allow high throughput results. In principle, a monoclonal antibody specific for rat TNF- α and rat CINC-1 have been pre-coated onto a microplate. standards, control, and samples are pipetted into the wells and any antigen (rat TNF- α and rat CINC-1) present are bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat TNF-α and rat CINC-1 are added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of antigen bound in the initial step. The sample values are then read off the standard curve. Diagram of the quantitative sandwich enzyme immunoassay as shown in figures 16. The assay procedures were performed as protocol descriptions from the company and explained below.

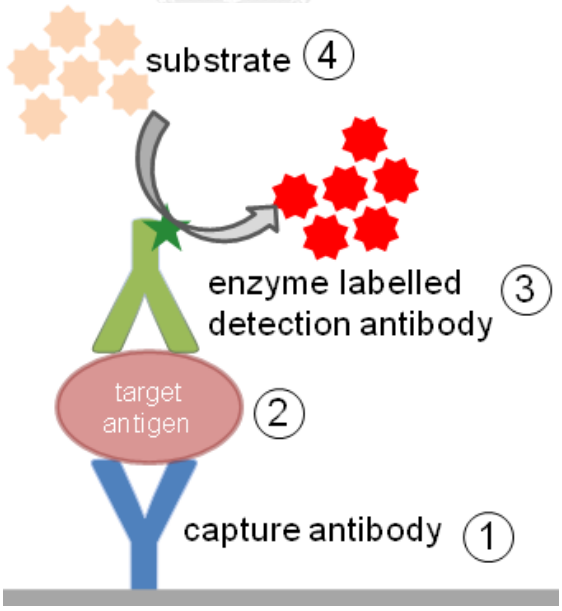


Figure 16 Diagram of the quantitative sandwich enzyme immunoassay: The addition of the enzyme's substrate leads to color development. The amount of color (absorbance) is directly proportional to the analyte concentration (94).

Measurement of tumor necrosis factor alpha in serum

The serum concentrations of TNF-α was measured by a rat TNF-α enzyme-linked immunosorbent assay (ELISA) using a colorimetric commercial kit from R&D Systems (Minneapolis, MN, USA).

Procedure of Rat TNF-α Immunoassay

1. Sample preparation, serum require a 2-fold dilution into calibrator diluent RD5-17 prior to assay. A suggested 2-fold dilution is 75 µL sample + 75 µL calibrator diluent RD5-17.

2. Standard curve preparation, the standard was diluted with 2 mL of calibrator diluent to obtain a stock solution of 800 and 2,000 pg/mL of TNF-α. After producing the stock solution, the standard was gently mixed at least 5 minutes prior to making dilutions. The polypropylene tubes were labeled and added the stock solution and calibrator diluent to each tube as described in table below.

Table 5 A preparation of TNF-α concentration (µM)

Tube	Stock Solution Volume (µL)	Calibrator Diluent Volume (µL)	TNF-α Concentration (pg/mL)
A	200 of stock	0	800
B	200 of vial A	200	400
C	200 of vial B	200	200
D	200 of vial C	200	100
E	200 of vial D	200	50
F	200 of vial E	200	25
G	200 of vial F	200	12.5
H	0	200	0

3. Performing the assay, all standards, control and samples were prepared as in the reagent preparation part of the protocol and assayed in duplicate. Next, 50 µL of assay diluent was added to each well and followed by 50 µL of standards, control, or samples to each well. Plate was gently tapped, covered with the adhesive strip and incubated for 2 hour at room temperature. Next, each well was aspirated and washed for five times. Then, 100 µL of TNF-α conjugate were added to each well. Plate was covered and incubated again for 2 hours at room temperature. After 2 hours, each well was aspirated and washed for five times. Then, 100 µL of substrate solution was added to each well. Incubation was performed for 30 minutes and must protect from light. After that, 100 µL of stop solution was added to each well and gently tapped the plate to ensure through mixing. The last step, the optical density (O.D.) was determined within 30 minutes by using a microplate reader set to 450 nm with wavelength correction set at 540 or 570 nm.

Calculations of results for rat TNF-α Immunoassay

Average the duplicate readings for each standard, control, and sample were average zero standard optical density (O.D). The standard curve was created by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit or a linear polynominal. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the rat TNF-α concentrations (pictogram per milliter; pg/mL.) versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. Because samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor. Example of a standard curve of TNF-α was showed as figure 17.

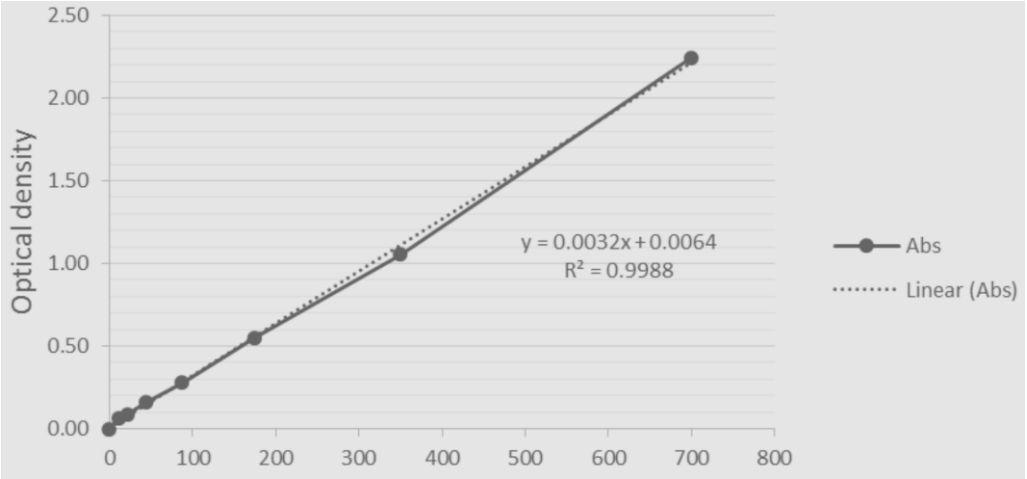


Figure 17 Example of TNF-α standard curve: The x-axis and y-axis of the standard curve represented standard TNF-α concentration (pg/ml) and O.D., respectively. Strong correlation (R^2 = 0.9988) was able to present by the linear equation of y = 0.0032x.

Measurement of Cytokine-induced neutrophil chemoattractant 1 in serum

The level of serum CINC-1 or CXCL1 was measured by enzyme-linked immunosorbent assay using a colorimetric commercial kit from R&D Systems (Minneapolis, MN, USA). This assay employs the quantitative sandwich enzyme immunoassay technique, the diagram of this as shown in figure 16 and the assay procedures were performed as protocol descriptions from the company and explained below.

1. Sample preparation, serum require a 2-fold dilution prior to assay. A suggested 2-fold dilution is 70 µL of sample + 70 µL calibrator diluent RD5-4.

2. Standard curve preparation, the standard was diluted with 2 mL of calibrator diluent to obtain a stock solution of 800 and 2,000 pg/mL of TNF-α. After producing the stock solution, the standard was gently mixed at least 5 minutes prior to making dilutions. The polypropylene tubes were labeled and added the stock solution and calibrator diluent to each tube as described in table below.

Table 6 A preparation of CINC-1 concentration (μM)

Tube	Stock Solution Volume (μL)	Calibrator Diluent Volume (μL)	CINC-1 Concentration (pg/mL)
A	200 of stock	0	500
B	200 of vial A	200	250
C	200 of vial B	200	125
D	200 of vial C	200	62.5
E	200 of vial D	200	31.2
F	200 of vial E	200	15.6
G	200 of vial F	200	7.8
H	0	200	0

3. Performing the assay, all standard dilutions, control, and samples were prepared as in the reagent preparation part of the protocol and assayed in duplicate. Next, 50 μL of assay diluent was added to each well and followed by 50 μL of standard, control, or sample per well. Plate was gently tapped for 1 minute, covered with the adhesive strip and incubated for 2 hours at room temperature. A plate layout was provided to record standards and samples assayed. Next, each well was aspirated and washed, repeating the process four times for a total of five washes. Then, 100 μL of rat CINC-1 conjugate were added to each well. Plate was covered and incubated again for 2 hours at room temperature. After 2 hours, each well was aspirated and washed for five times. Then, 100 μL of substrate solution was added to each well. Incubation was performed for 30 minutes at room temperature and must protect from light. After that, 100 μL of stop solution was added to each well and gently tapped the plate to ensure thorough mixing. The last step, the optical density of each well was determined within 30 minutes by using a microplate reader set to 450 nm with wavelength correction set at 540 or 570 nm.

Calculations of results for rat CXCL1/CINC-1 Immunoassay

The duplicate readings for each standard, control, and sample were averaged and subtracted the average zero standard optical density. Next, the standard curve was created by reducing the data and using computer software capable of generating a four parameter logistic (4-PL) curve-fit or a linear polynominal. The standard curve was made by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. The data were linearized by plotting the log of the rat CINC-1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. The sample concentration were calculated from the linear equation of standard curve and were expressed in pg/mL. Example of a standard curve of CINC-1 was showed as figure 18.

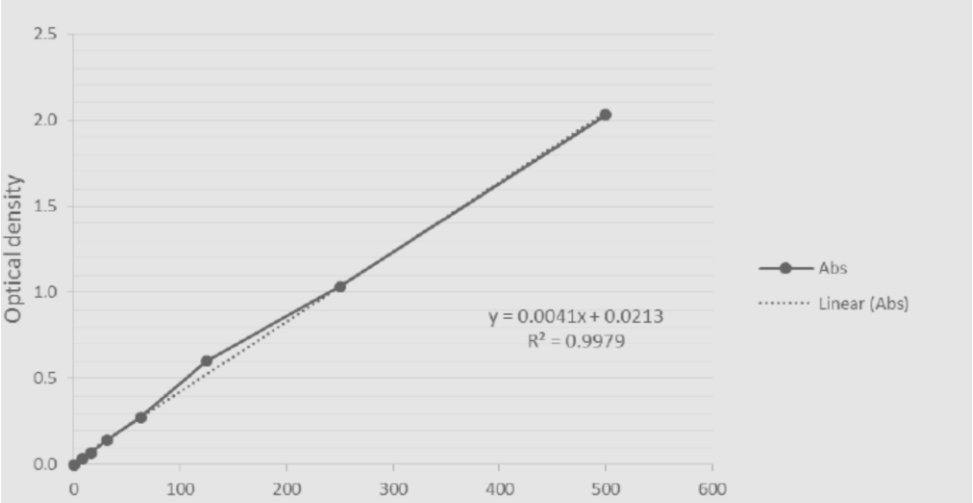


Figure 18 Example of CINC-1 standard curve: The x-axis and y-axis of the standard curve represented standard CINC-1 concentration (pg/ml) and O.D., respectively. Strong correlation (R^2 = 0.9979) was able to present by the linear equation of y = 0.9979X.

Detection of *Helicobacter pylori* infection in gastric tissues

The presence of *H. pylori* infection in individual rat were determined by urease test and pathological examination. Enzymatic test by using urease test, after all rats were terminated 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 *H. pylori* organism in tissue.

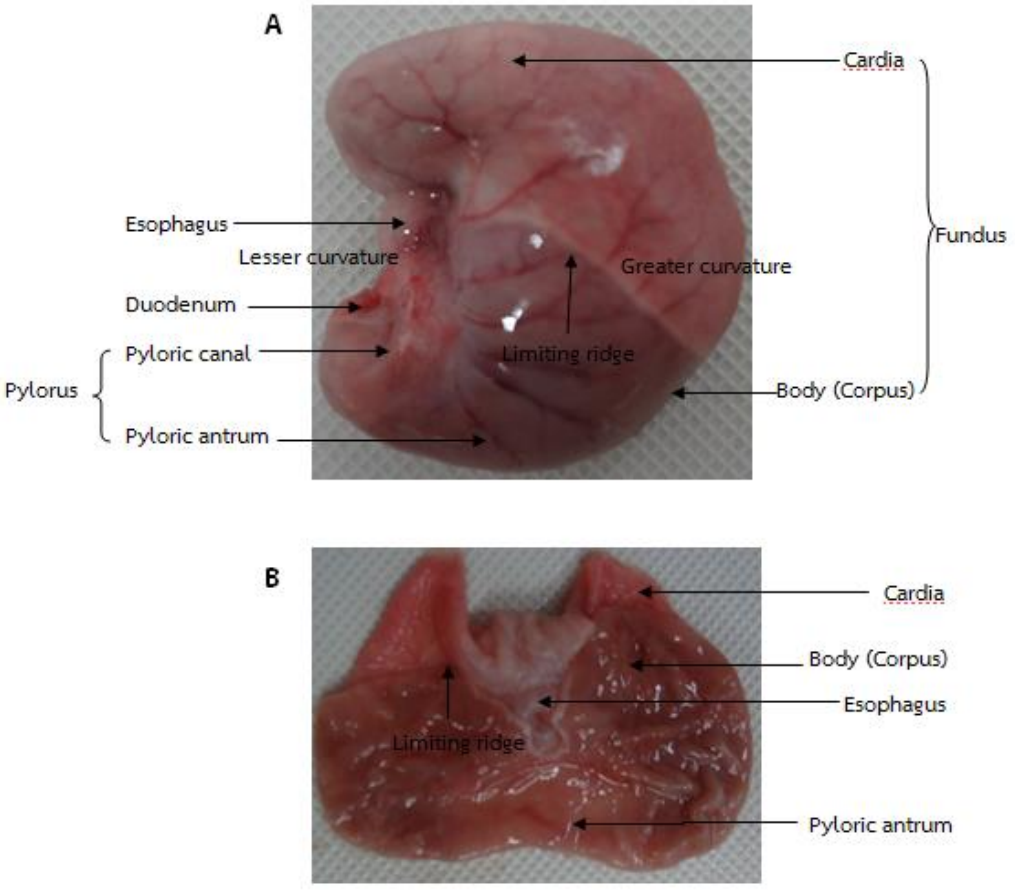


Figure 19 Anatomy of the rat stomach: A; Anatomy of the rat stomach before dissection, B The rat stomach was longitudinally dissected along the greater curvature.

Enzymatic test by using urease test

Urease test was conducted on 2 mm^2 gastric antral biopsy specimen, the area that *H. pylori* most colonized (95) was cut and examined the urease enzyme activity. The tissue were put in a gel tube that contained urea and phenol red, a pH indicator. In case of *H. pylori* infection, the *H. pylori* urease enzyme activity converting urea into ammonia. As a result of this, the pH indicator in urease test tube was increase, so that the pH indicator was changed from the yellow color to pink color within 24 hours. The urease test has 86% to 97% sensitivity and 86% to 98% specificity (96).

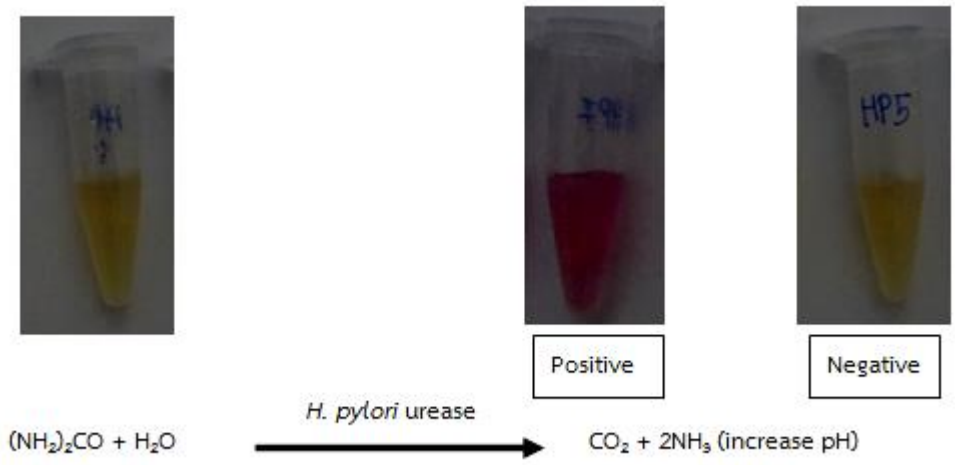


Figure 20 Rapid urease test: The presence of *H. pylori* urease is indicated by ammonia production

Gastric histopathology

Histological examination was diagnosed under supervision of the histopathologist. The gastric specimen was fixed in 10% formaldehyde in 0.2 M sodium phosphate buffer at pH 7.4 at room temperature. Then, gastric tissue was processed by routine technique before paraffin embedding and sections were cut at 5 μm thickness. Sections were stained with H&E. One experienced histopathologist examined all blinded samples by using light microscope with magnification X10, X40 following the updated Sydney System (97). All histopathological findings were recorded and graded by using the bacterial colonization score and gastric inflammation score as follows, score 0: no bacteria detected, score 1: mild colonization, score 2: moderate colonization,

score 3: marked colonization. For gastric inflammation score was scored following the infiltration of polymorphonuclear and mononuclear in the gastric mucosa, score 0 normal, score 1 mild, score 2 moderate and score 3: marked. Using the visual analogue scales of the updated Sydney System was showed as figure 21. Additionally, presentation of *H. pylori* was detected by giemsa staining in the unclear cases.

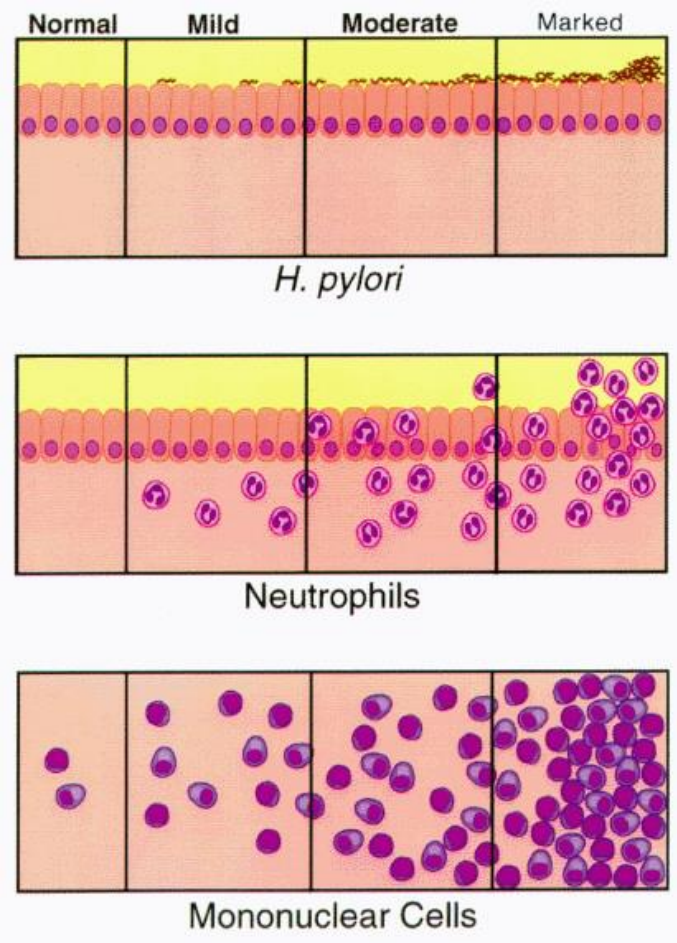


Figure 21 Using the visual analogue scales: The updated Sydney System (97)

The percentage of *H. pylori*-infected rate was calculated from following equation: 100 X (number of infected-rats / number of inoculated-rats). Number of infected rats was identified by positive test either urease test or histopathology. Positive urease test must detect the color change of pH indicator in 24 hours. Gastric antral sample that give pink in the urease test tube were positive to *H. pylor*i detection. Furthermore, the remained stomach sample were detected *H. pylori* colonization by the pathologist as score o means no *H. pylori* detection. The stomach samples that were given score 1 or more were *H. pylori* positive.

Gastric tissue malondialdehyde measurement

Principle of gastric tissue MDA measurement

Gastric MDA level was measured by the thiobarbituric acid -reactive substances (TBRAS)- an index of lipid peroxidation- assay kit (Cayman, United States) in the principle is that the reaction of one molecule of MDA and two molecules of TBA from a red MDA-TBA complex under high temperature (90°C-100°C) and acidic conditions is measured colorimetrically at 530-540 nm or fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Although this reaction has a much higher sensitivity when measured fluormetrically, protocols for both methods are provided see figure 1 below and the assay procedures were performed as protocol descriptions from the company and explained below

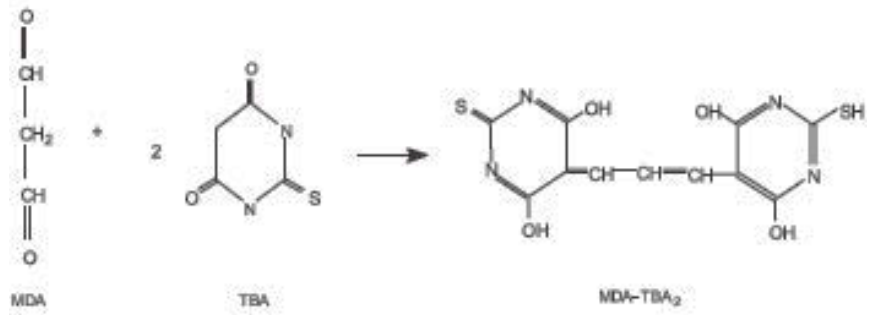


Figure 22 The principle reaction of the thiobarbituric acid -reactive substances (98)

Procedure of gastric tissue MDA measurement

1. Sample preparation (gastric tissue homogenates), 50 mg of gastric tissue was placed into a 1.5 ml centrifuge tube containing 500 µL of RIPA buffer concentrate with protease inhibitors of choice and sonicated for 15 seconds at 40V over ice. Secondly, the supernatant was collected by centrifugation at 1,600 x g for 10 minutes at 4º C, aliquot into sterile microcentrifuge tubes for detecting MDA and protein assay. And lastly, stored supernatant on ice. If not assaying the same day, freeze at -80º C. (Tissue homogenates do not need to be diluted before.)

2. Standard curve preparation, 250 µL of MDA standard was diluted with 750 µL of water to obtain a stock solution of 125 µM. The test tubes were labeled and added the amount of 125 µM. MDA stock solution and water to each tube as described in table below.

Table 7 A preparation of MDA concentration (μM)

Tube	MDA (μL)	Water (μL)	MDA concentration (μM)
A	0	1,000	0
B	5	995	0.625
C	10	990	1.25
D	20	980	2.5
E	40	960	5
F	80	920	10
G	200	800	25
H	400	600	50

3. Performing the assay, after label vial caps with standard number or sample identification number. First, added 100 μL of sample or standard to appropriately labeled 5 ml vial. Second, added 100 μL of SDS and 4 mL of the color reagent to each vial. Later, the tubes were capped and heated in the water-bath at 95°C for one hour. After cooling the tubes by immersion in ice bath and incubation on ice for 10 minutes, they were centrifuged at 1,600 x g for 10 minutes at 4°C. and then placed at room temperature for 30 minutes. After loading 150 μL (in duplicate) of each vial to either the clear plate, the absorbance was measured at 530-540 nm.

Calculations of results for MDA measurement

By plotting the mean absorbance for each standard on the y-axis against the concentration of MDA (μM) on the x-axis, a standard curve was created to calculate the results. MAD levels of the samples were examined from the linear regression equation of a standard curve. The content of lipid peroxide was expressed in term of nmol of MDA/gram of wet weight and the total protein was determined by

the BCA protein assay kit to correct the MDA level which was expressed in terms of nmol/mg protein. An example of MDA standard curve was showed below in Figure 23.

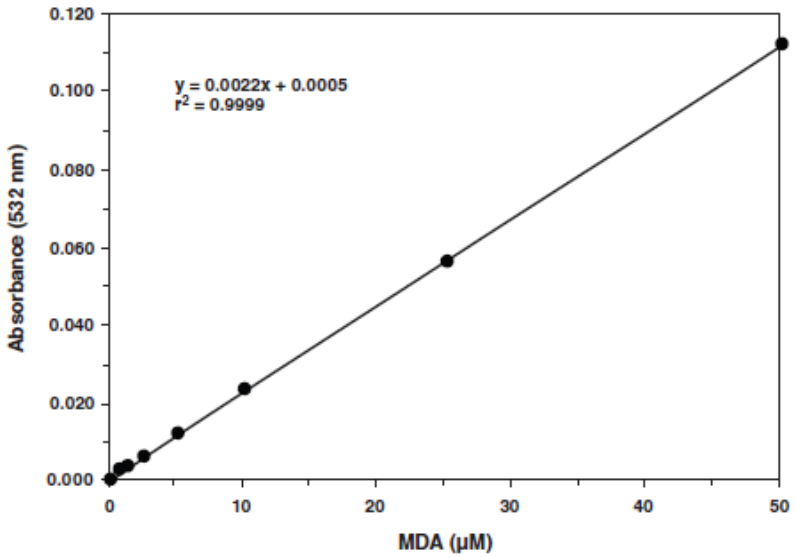


Figure 23 An example of the MDA standard curve; The x-axis and y-axis of the standard curve represented standard MDA concentration (µM) and absorbance (O.D.), respectively

The content of lipid peroxide is expressed in term of nmol of MDA/gram of wet weight and the total protein was determined by the BCA protein assay kit to correct the MDA level which is expressed in term of nmol/ mg protein

Bicinchoninic assay protein measurement

Bicinchoninic assay (BCA) was measured from the homogenized tissue by using BCA protein. In principle, the amino acid (cysteine, cysteine, tryptophan and tyrosine) and the peptide bonds of protein react with the cuprous cation -protein complex (Cu^{2+} -protein complex) under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. Interestingly, the amount of reduction is proportional to the protein present. Thus, the cuprous cation is colorimetrically detected by reaction with BCA. The Cu^{1+}-BCA complex results in the purple-colored reaction product which is analyzed by visible spectrophotometer at 562 nm.

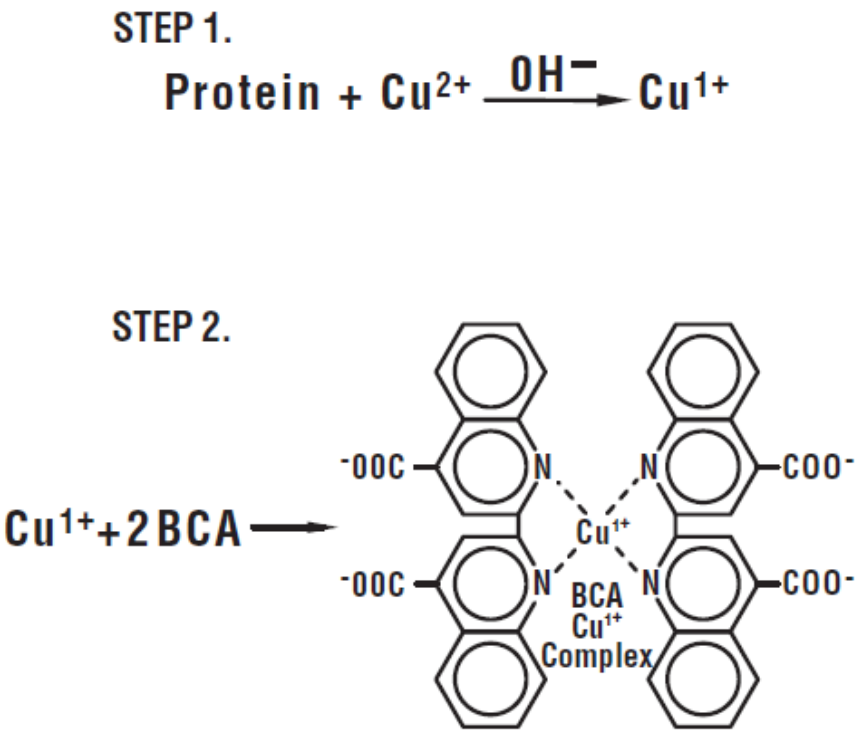


Figure 24 An overview of the reaction scheme in the BCA assay (99)

The BCA protein assay kit ((Thermo Scientific, USA) was used to assess the total protein of gastric tissue homogenates in terms of mg protein. The assay procedures were performed as protocol descriptions from the company and explained below

Procedure of BCA protein measurement

1. **Sample preparation**, the same sample, aliquot into microcentrifuge tubes and stored at – 80° C until the day of analysis, as the MDA detection was used.

2. **Standard curve preparation**, Albumin Standard (BSA) was diluted with RIPA buffer. Use the following table as a guide to prepare a set of standards (assay range = 125-2,000 µg/mL).

Table 8 A preparation of BCA concentration (μg/mL)

Vial	RIPA buffer Volume (μL)	BSA Source and Volume (μL)	Concentration (μg/mL)
A	0	200 of stock	2,000
B	66	200 of stock	1,500
C	100	100 of vial A	1,000
D	100	100 of vial B	750
E	100	100 of vial C	500
F	100	100 of vial E	250
G	100	100 of vial F	125

3. Performing the assay, 9 μL of each replicate of control, standard and unknown sample was pipetted to the center of the microplate well. Next 4 μL of compatibility reagent solution was added to each well. The plate was covered, mixed on a plate shaker at medium speed for one minute and incubated at 37° C for 15 minutes. Then, 260 μL of the BCA working reagent was added to each well. The plate was covered and mixed on a plate shaker for one minute again. After cooling the plate at room temperature for 5 minutes, the absorbance of the standards, controls and unknown samples were measured at 562 nm on a plate reader.

Calculations of results for BCA measurement

By using the control replicates from the 562 nm absorbance value as the blank, they were subtracted from the 562 nm absorbance value of all standard and unknown sample replicates. The standard curve was prepared by plotting the average blank-corrected 562 nm value for each BCA standard on the y-axis against its concentration (μg/mL) on the x-axis. To determine the protein concentration of each unknown sample, the linear regression equation of the standard curve was used.

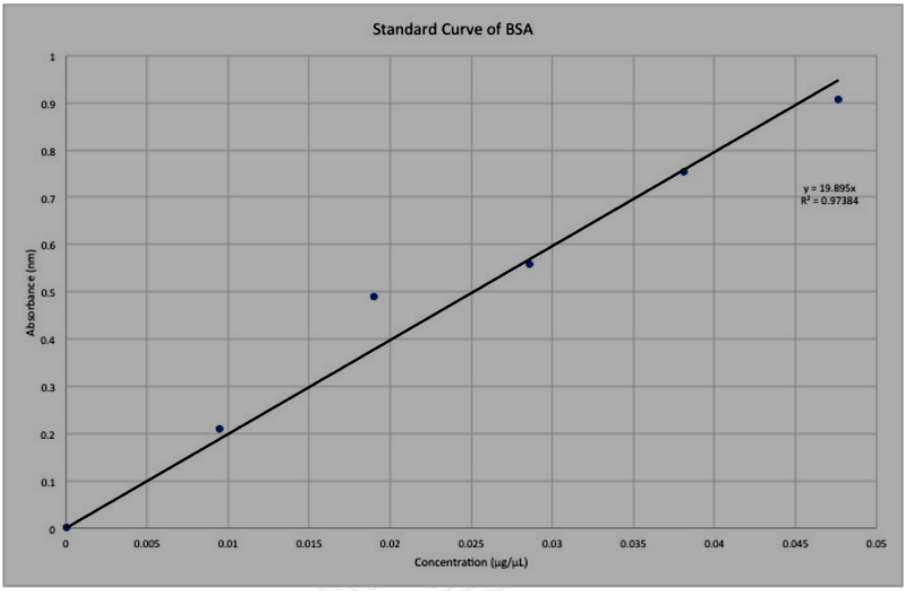


Figure 25 An example of a standard curve of BCA: The x-axis and y-axis of the standard curve represented standard BCA concentration (µg/mL) and O.D., respectively. Strong correlation (R^2 = 0.97384) was able to present by the linear equation of y = 19.895x

In vitro study

The disk diffusion method was used to assess the anti-*H. pylori* activity of genistein at the various concentrations.

Bacterial strains and growth conditions

H. pylori was isolated from peptic ulcer patients who visited the King Chulalongkorn Memorial Hospital and was grown on Columbia agar (Oxoid, Basingstoke, United Kingdom) containing 7% sheep blood and 7% horse serum. Plates were incubated at 37° C under microaerophilic conditions (10% CO_2 5% O_2 and 85% N_2) produced by a gas generating system, Anaero-Pack (MGC,Japan) , for 72 h in an anaerobic jar (Oxoid, Basingstoke, United Kingdom).

Genistein preparation

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, ≥ 98%) was obtained from Cayman Chemical Company and was dissolved using the universal solvent, DMSO. For the disk diffusion method determinations, genistein were diluted to establish the concentration of 0.25, 0.5, 1, 1.5, 2 and 4 M with ≥ 99.5% DMSO (in control experiments, ≥ 99.5% DMSO was added to disks). These concentrations were chosen based on earlier studies for testing the anti-*H. pylori* activity of genistein (80, 81) and adjusted to a higher concentration on the advice of experts. A stock

of 4 M genistein was prepared by dissolving genistein in sterilized ≥ 99.5% DMSO and storing it at –70 °C.

Disk diffusion method

H. pylori of the inoculated agar was spreader by sterile cotton swab on Columbia agar plates supplemented in three different planes (by rotating the plate approximately 60° each time to obtain an even distribution of the inoculums). The inoculated plates were then placed at room temperature for 10-15 minutes to allow absorption of excess moisture. With a sterile forceps, the genistein disks were placed on the inoculated plates and incubated at 37°C under microaerophilic conditions for 72 hour. After incubation, the diameters of inhibition zones were measured by a ruler in millimeters. The experiments were carried out in duplicate and mean values of the growth inhibition zones measured.

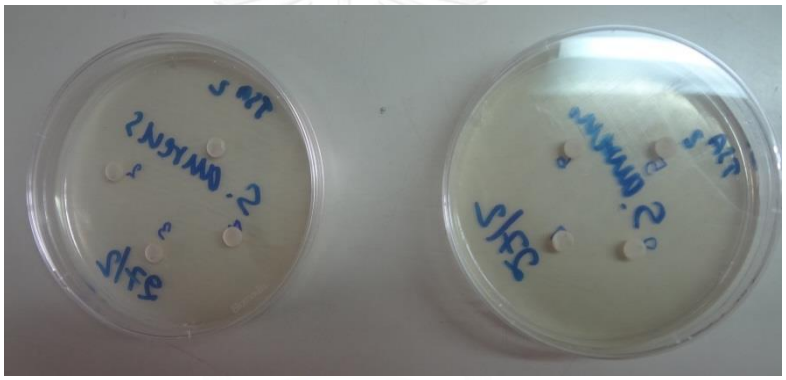


Figure 26 Preparation of paper disks: Various concentration of genistein and negative control were dipped into the sterile 6 millimeter -membrane disks.

Statistical analysis

Results are presented as mean ± standard deviation (SD) for the *in vivo* measurements. For comparison among all groups of animals were evaluated by one way analysis of variance (ANOVA), followed by the least significant different least significant difference (LSD) post hoc test. Results with $p<0.05$ were considered significant. The data will be analyzed using the SPSS software version 20.0 for windows. In addition, descriptive statistics were used for histological examination of the stomach.

CHAPTER IV RESULTS

In vivo study

Changes in level of serum tumor necrosis factor alpha

This study hypothesized that treatment with genistein was attenuated gastric inflammation by reduction of inflammatory mediators TNF-α. As shown in figures 26, *H. pylori* infection group was significantly higher levels of serum TNF- α than control group (43.50 ± 16.51 pg/ml versus 20.89 ± 8.90 pg/ml, P<0.05). In genistein treatment group was significantly lower levels of serum TNF- α than in *H. pylori* infection group (29.33 ± 10.77 pg/ml versus 43.50 ± 16.51 pg/ml, P< 0.05).

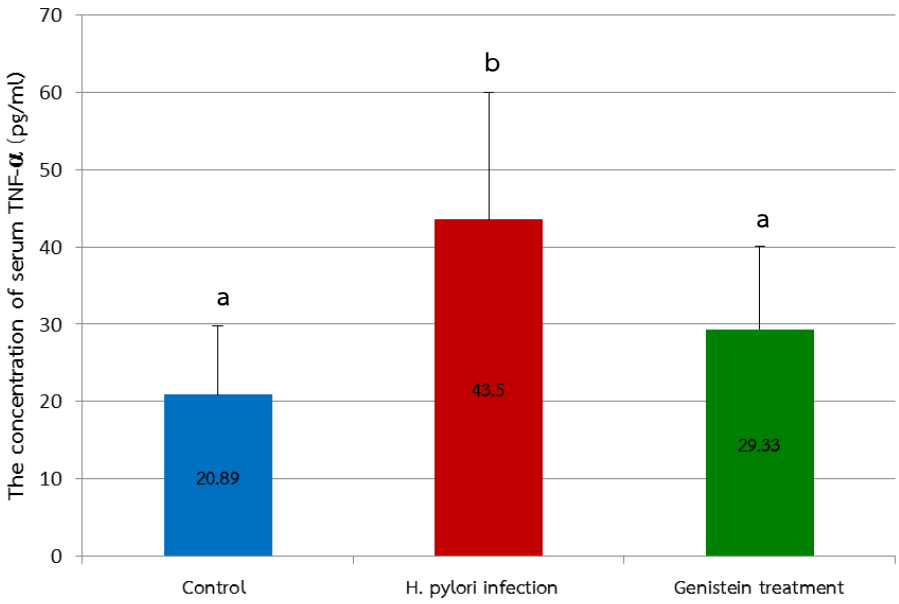


Figure 27 **Bar graphs showed the concentration of serum TNF- α (pg/mL) in all groups (mean ± SD):** [ab] Different superscript letters indicate significant differences ($p < 0.05$). **Control group** (n=7): rats were treated with 0.1% DMSO (1 ml/rat, b.i.d.); ***H. pylori* infection group** (n=7): rats were inoculated with *H. pylori* suspension and treated with 0.1% DMSO (1 ml/rat, b.i.d.); **Genistein treatment group** (n=7): rats were inoculated with *H. pylori* suspension and treated with genistein (16 mg/kg BW, b.i.d.).

Changes in level of serum cytokine-induced neutrophil chemoattractant 1

As shown in figures 27, *H. pylori* infection group was significantly higher levels of serum CINC-1 than control group (138.10 ± 43.56 pg/ml versus 81.27 ± 19.89 pg/ml, $P < 0.05$). In genistein treatment group was significantly lower levels of serum CINC-1 than in *H. pylori* infection group (103.25 ± 23.76 pg/ml versus 138.10 ±43.56 pg/ml, $P < 0.05$).

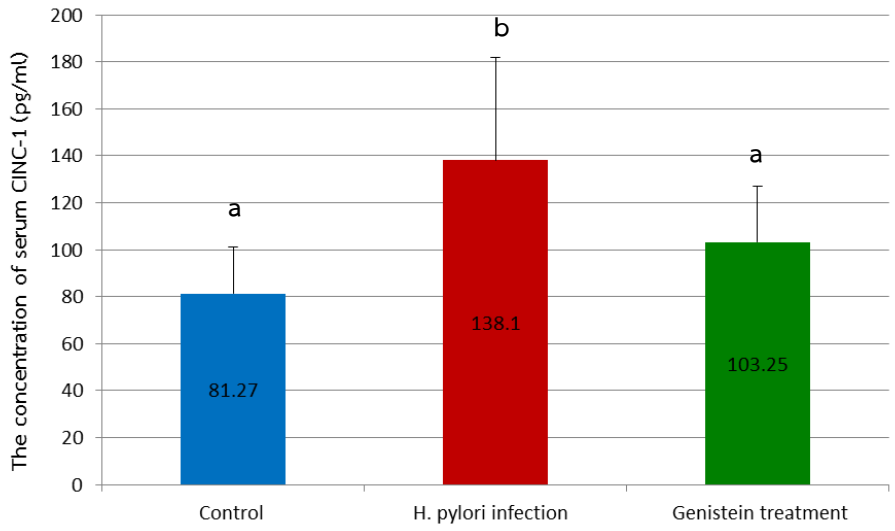


Figure 28 **Bar graphs showed the concentration of serum CINC-1 (pg/mL) in all groups (mean ± SD):** [ab] Different superscript letters indicate significant differences ($p < 0.05$). **Control group** (n=7): rats were treated with 0.1% DMSO (1 ml/rat, b.i.d.); ***H. pylori* infection group** (n=7): rats were inoculated with *H. pylori* suspension and treated with 0.1% DMSO (1 ml/rat, b.i.d.); **Genistein treatment group** (n=7): rats were inoculated with *H. pylori* suspension and treated with genistein (16 mg/kg BW, b.i.d.).

Detection of *Helicobacter pylori* infection in gastric tissues

In this study, the method of *H. pylori* inoculation was using according to the method described by Thong-Ngam *et al* (91). It was performed that *H. pylori* was detected by positive either rapid urease test or histopathology examination. For overall results, there were 14 of 14 rats (100%) that were infected by *H. pylori*

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). (Figures 28-31)In the same way, *H. pylori* colonization score was reduced. The histopathological scores for gastric inflammation and *H. pylori* colonization are summarized in Table 9

Table 9 Summary of the bacterial colonization and gastric inflammation score (n=7, each group)

Group	N	Urease Test		Gastric inflammation score[a]				*H.pylori* colonization score[b]			
		Positive	Negative	0	1	2	3	0	1	2	3
Control group	7	0	7	7	-	-	-	7	-	-	-
H. pylori infection group	7	6	1	1	-	5	1	1	2	2	2
Genistein treatment group	7	6	1	3	4	-	-	2	4	1	-

[a] Gastric inflammation score: 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.

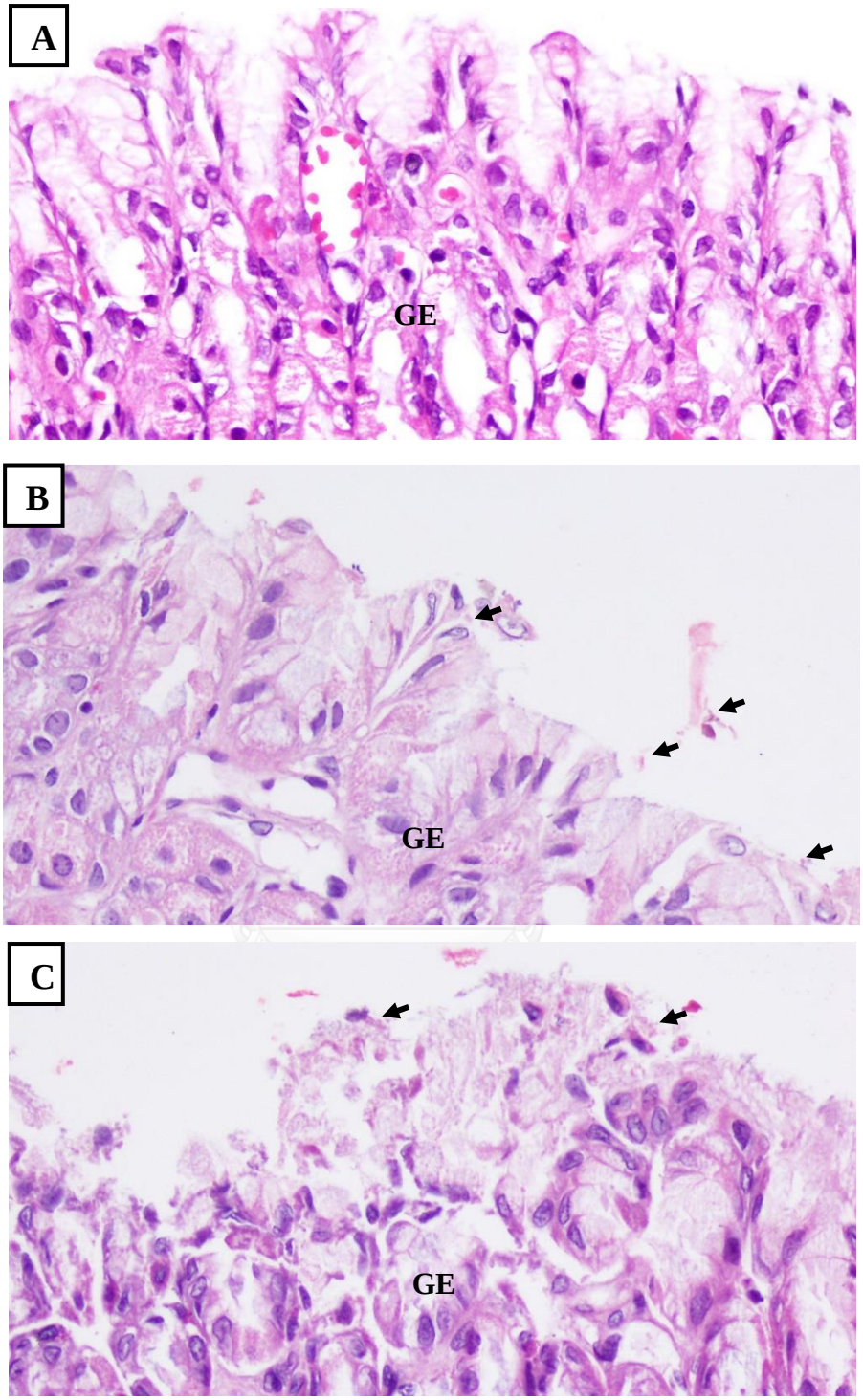


Figure 29 H&E stained gastric tissue (X40): A; Control group showed no *H. pylori*, B; *H. pylori* infection group showed colonization (arrows) of *H. pylori*, C; Genistein treatment group showed decrease *H. pylori* colonization. (GE: Gastric epithelium)

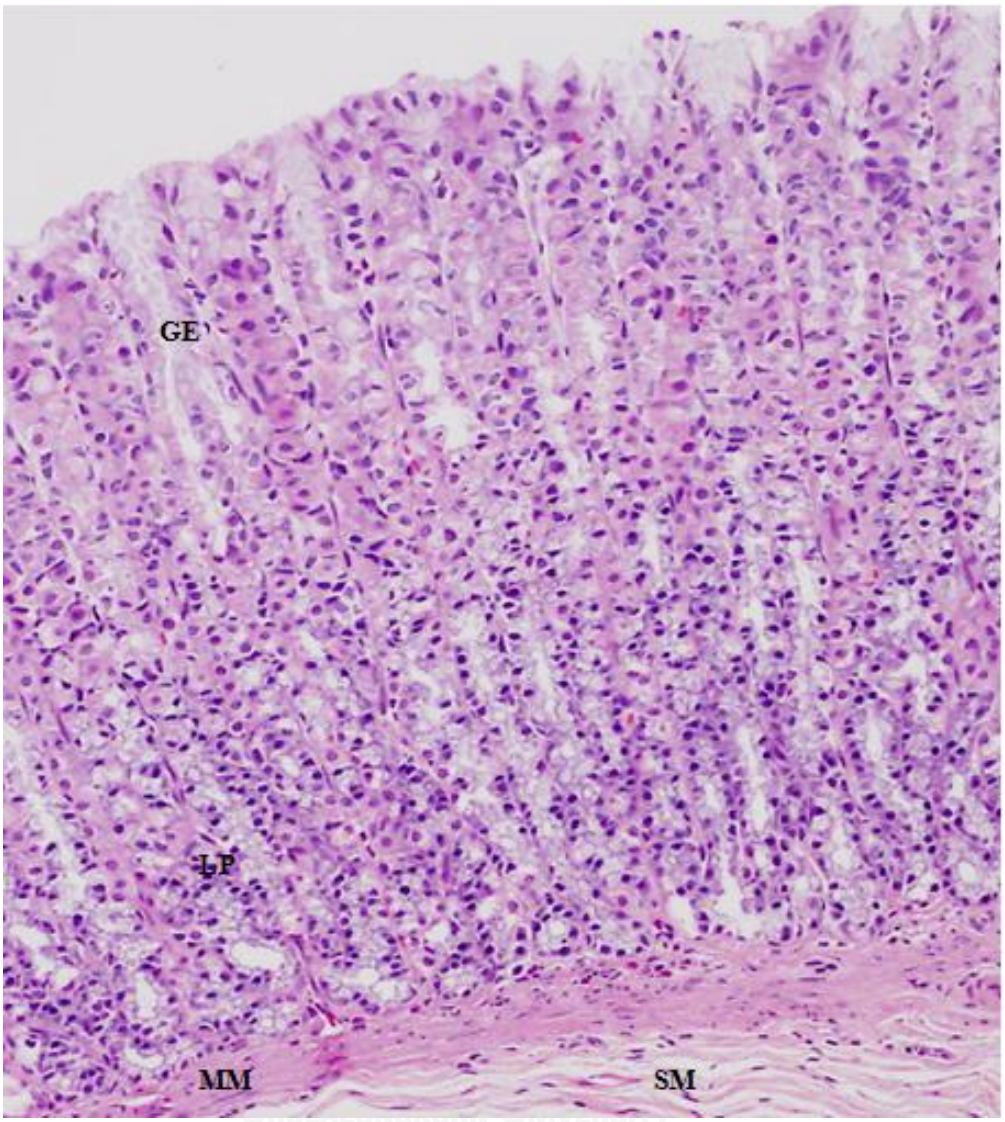


Figure 30 H & E-stained gastric tissue (X10): Control group showed normal gastric histopathology. GE: Gastric epithelium; LP: Lamina propria; MM: Muscularis mucosae; SM: Submucosa

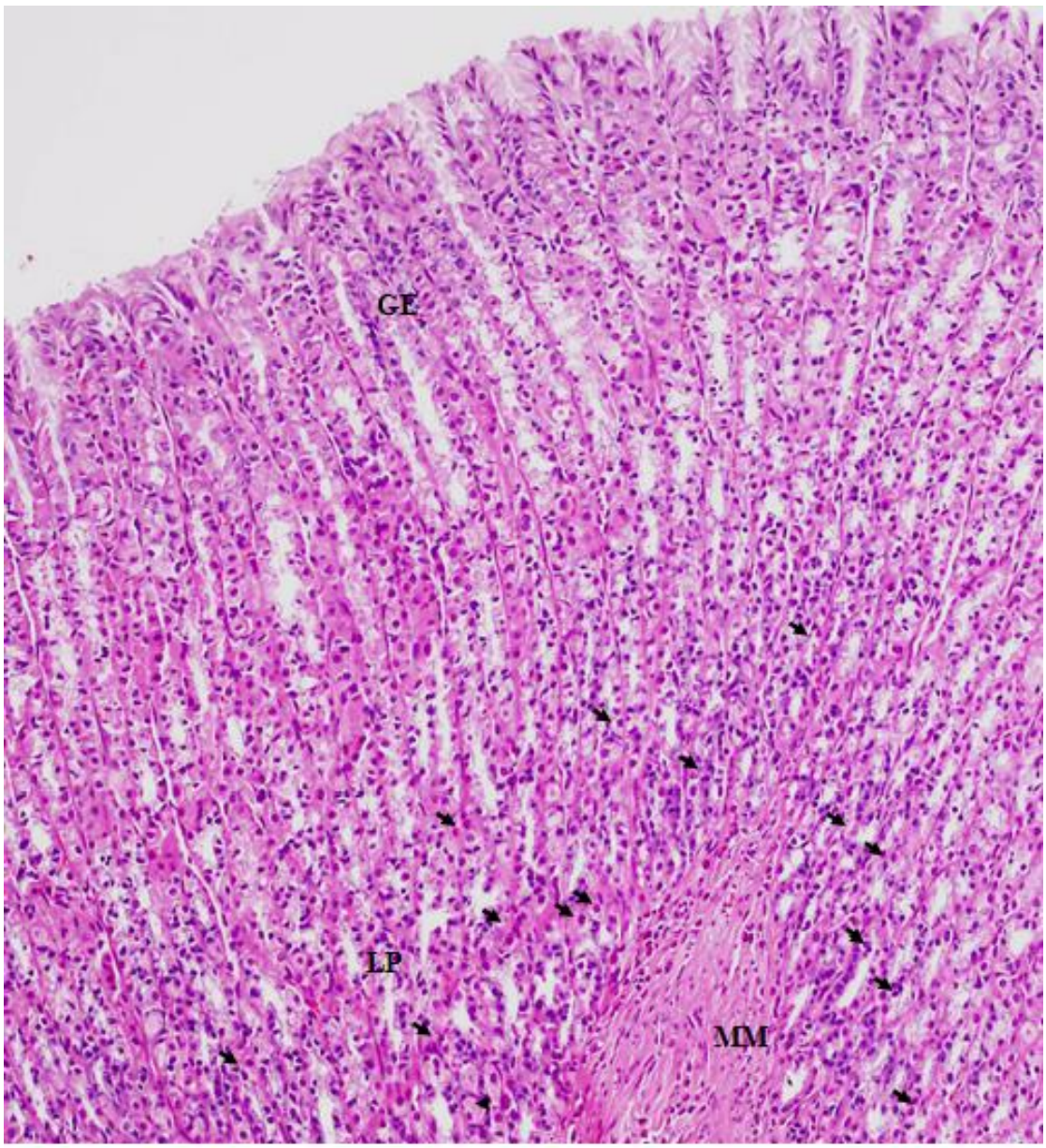


Figure 31 H & E-stained gastric tissue (X10): *H. pylori* infection group showed polymorphonuclear inflammatory cells infiltrating the lamina propria. GE: Gastric epithelium; LP: Lamina propria; MM: Muscularis mucosae; SM: Submucosa.

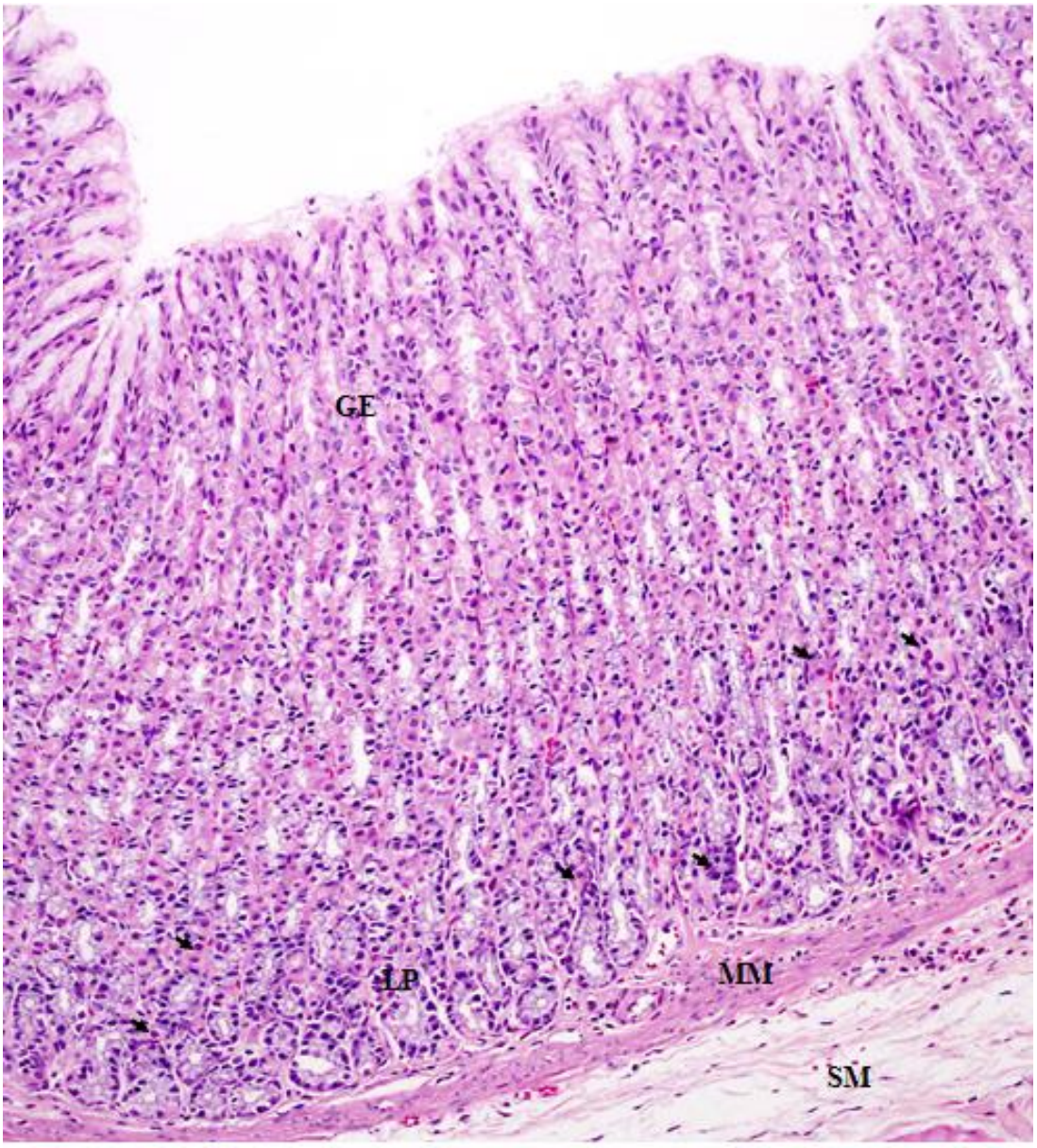


Figure 32 Hematoxylin-eosin stained gastric tissue (X10): Genistein treatment group showed improvements in gastric inflammation. GE: Gastric epithelium, LP: Laminar propria, MM: Muscularis mucosae, SM: Supmucosa, ML: Muscularis).

Changes in level of gastric tissue malondialdehyde

As shown in figures 32, *H. pylori* infection group was no significantly lower levels of gastric MDA than control group (0.49 ± 0.38 nmol/mg protein versus 0.67 ± 0.69 nmol/mg protein, P=0.51). Additionally, in genistein treatment group was no significantly higher levels of gastric MDA than in *H. pylori* infection group (0.56 ± 0.29 nmol/mg protein versus 0.49 ± 0.38 nmol/mg protein, P=0.79).

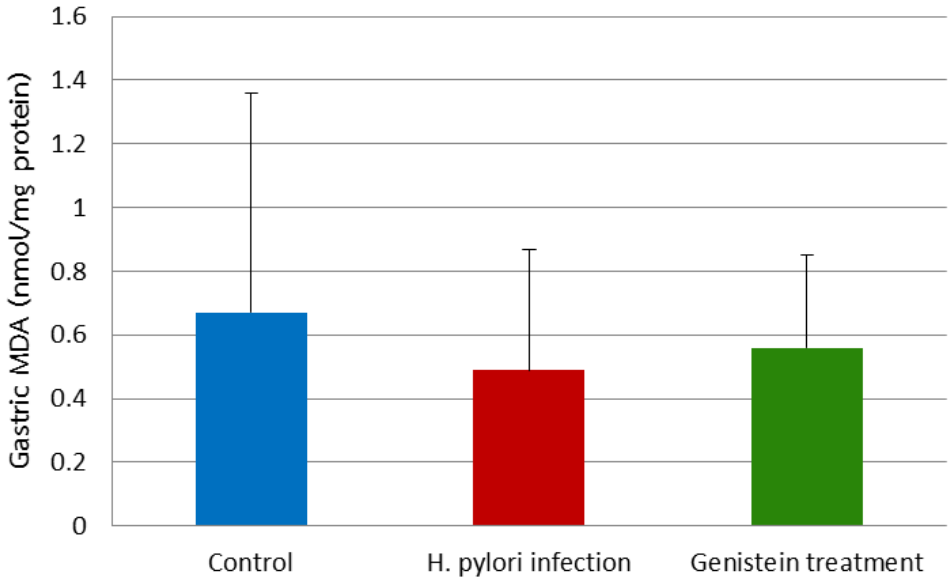


Figure 33 Bar graphs showed the concentration of gastric MDA in all groups (mean ± SD): Control group (n=7); rats were treated with 0.1% DMSO (1 ml/rat, b.i.d.), ***H. pylori* infection group** (n=7); rats were inoculated with *H. pylori* suspension and treated with 0.1% DMSO (1 ml/rat, b.i.d.), **Genistein treatment group** (n=7) ;rats were inoculated with *H. pylori* suspension and treated with genistein (16 mg/kg BW, b.i.d.).

Change of body weight

Body weight of the rats were measured and recorded every two days. As shown in figure 34, there was no significant difference ($p < 0.05$) of the body weight among groups.

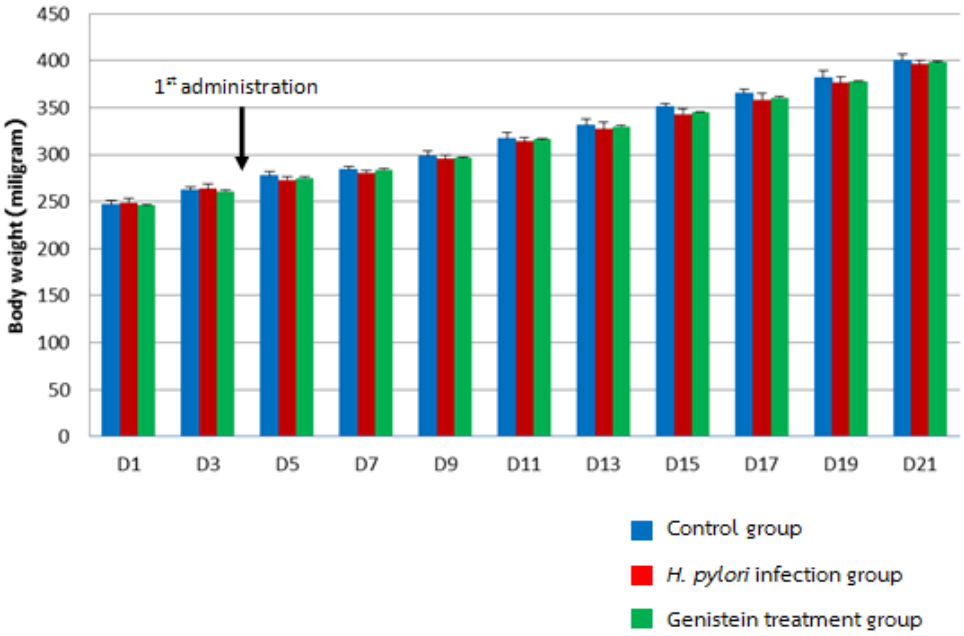


Figure 34 **Bar graphs showed the body weight in all groups (mean ± SD): Control group** (n=7); rats were treated with 0.1% DMSO (1 ml/rat, b.i.d.), ***H. pylori* infection group** (n=7); rats were inoculated with *H. pylori* suspension and treated with 0.1% DMSO (1 ml/rat, b.i.d.), **Genistein treatment group** (n=7); rats were inoculated with *H. pylori* suspension and treated with genistein (16 mg/kg BW, b.i.d.).

In vitro study

Experiments were performed using genistein at concentrations of 0.25, 0.5, 1, 1.5, 2 and 4 M. Interestingly, the growth of *H. pylori* was not inhibited by the various concentrations of genistein ,as shown in figures 35.

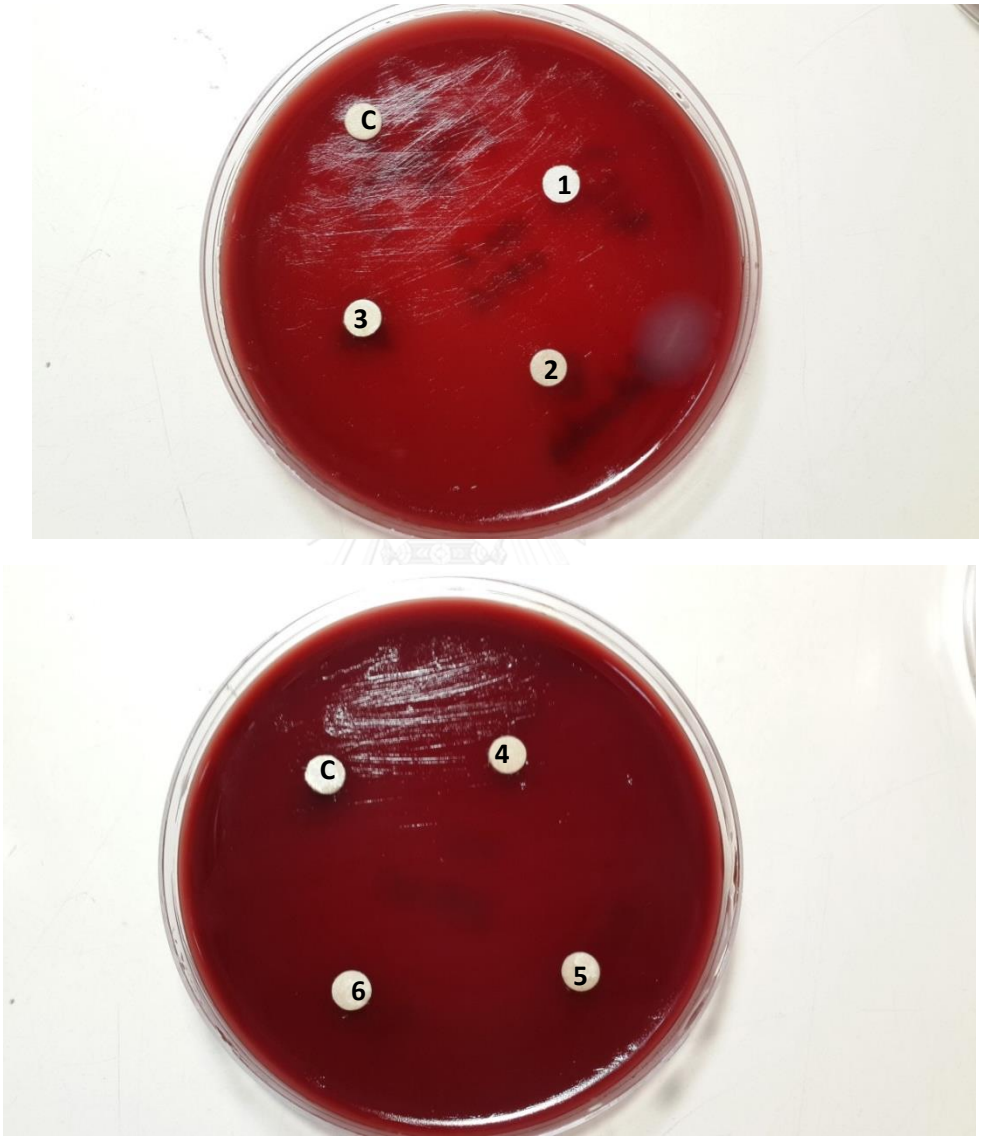


Figure 35 The growth of *H. pylori* was not inhibited by the various concentrations of genistein: 1; concentrations 0.25 M, 2; concentrations 0.5 M, 3; concentrations 1 M, 4; concentrations 1.5 M, 5; concentrations 2 M and 6; concentrations 4 M

CHAPTER V DISCUSSION

The presence of *H. pylori* in the gastric antrum is always associated with a mucosal inflammatory reaction involving the infiltration of a large number of polymorphonuclear and mononuclear cells. 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 was induced by the contact of *H. pylori* with gastric cells and is followed by the stimulation of proinflammatory cytokine production. Several of *in vivo* and *in vitro* studies have shown that *H. pylori* can induce cytokine expression in epithelial cells characterized by the up-regulation of several genes such as IL-1β, IL-6, IL-8, IL-10, and TNF-α (46, 100, 101). *H. pylori* adherence to *in vitro* cultured gastric epithelial cells induces several cellular responses, including the 145-kDa host protein tyrosine phosphorylation, host cell actin reorganization, like vasodilator-stimulated phosphoprotein, adjacent to the attached bacterial cell, and the subsequent release of the cytokine. These effects of *H. pylori* attachment to cells suggests that alteration of host cell signal transduction might lead to chronic inflammation and perhaps may lead to the oncogenic transformation that are the hallmarks of symptomatic.(1, 6). There is no effective therapy for eradicating *H. pylori* infection, otherwise combination therapies employing one proton pump inhibitor and two or three antibiotics have been preferred for treatment. The multiple therapies are not very effective in a clinical setting as this may develop resistance. Furthermore, this may disrupt the natural population of commensal microorganisms in the gastrointestinal tract, potentially leading to undesired side effects. Therefore, there is a need to search for an indigenous herbal based modified drugs with minimal side effects for the elimination which would have a major impact on present and future of the world population (101-104). Genistein is a naturally occurring the major isoflavone, a flavonoid component, in soybean with three phenol hydroxyl residues. Recent studies reported that gensitein has numerous anti-inflammation, anti-oxidative and anti-cancer effects (23, 24, 105). In 1987 genistein was identified as a protein-tyrosine kinase inhibitor because it inhibited the EGF receptor PTK activity *in vitro* (27), apart from its PTK-inhibitory activity, genistein has also been shown to inhibit DNA topoisomerase II and regulation of cell cycle checkpoints (79). Numerous

studies have proposed that genistein is tyrosine kinase inhibitor to reduce inflammatory cytokine and may be useful in the prevention or cure of *H. pylori*-associated gastric diseases (83, 106-108). Most of these activities have been shown only *in vitro* and at genistein concentrations in excess of those attainable physiologically in humans (10 μmol/L) (109). Thus, the activities of genistein *in vivo* remains to be ascertained and the effect of genistein on *H. pylori* induced gastritis in rat model has not yet been revealed. Therefore, this study explored the potential anti-inflammatory effect of genistein on *H. pylori*-associated gastritis in a rat model.

Rat model of *Helicobacter pylori* infection

The rat model of *H. pylori* infection in this study followed previous study (91) that has 69.84% successful infected rate. The first step of this model was pre-treatment of streptomycin (5 mg/mL in drinking water) led to a transient clearance and increased a susceptibility in the normal flora. This was a great advantage for induction of infected *H .pylori*. By doing this, it affected a decrease of the pathogen in gastro-intestinal tract as well as highly increased a susceptibility to *H. pylori* infection. It can also induce gastritis in rats as well. The results of studies demonstrated that detection of *H. pylori* infection in gastric tissues by urease test were showed 85.71% positive in both *H. pylori* infection group and genistein treatment group. In contrast, all rat negative result was showed in control group. In the same way, the colonization of *H. pylori* in gastric tissue were not detected in control group, whereas in *H. pylori* infection group, in gastric tissue were detected 85.71% for *H. pylori* colonization and almost all of the gastric tissues showed moderate infiltration of polymorphonuclear cells. In genistein treatment group, gastric tissue were detected 71.43% for *H. pylori* colonization and almost all of the gastric tissues showed mild infiltration of polymorphonuclear cells. The organisms in experiment used were originally obtained from peptic ulcer patient. Because the strain of *H. pylori* is importantly for the pathogenesis; as a result of this, *H. pylori* organisms that originally obtained from peptic ulcer patient or pathogenic strain could increase rate of infection and could develop to pathogenesis in animal stomach (91). Although, this study found that some rats were uninfected with *H. pylori*, may be involved to the host's immune response or strain of *H. pylori*. In fact, *H. pylori* strains have been divided into two broad categories, type I and type II, based on whether or not they possess the cag pathogenicity island (PAI), called cag1. Type I strains are those that contain the cag PAI or Cag^+ and $VacA^+$, whereas type II strains lack functional cag PAI or $CagA^-$ and fail to produce a functional VacA toxin (1, 6). Several cag genes encode products that form a type IV bacterial secretion system, which translocates the product of the terminal gene in the island (CagA) into host epithelial cells after bacterial attachment

(110). Most of the clinical isolates of *H. pylori* isolated from duodenitis, peptic ulcer disease or patients suffering from malignant disease express CagA or type I strains even though type II strains more often associated with asymptomatic gastritis do not exclusively in type I (6). In the mouse model, type I *H. pylori* strains can induce visible gastric damage, whereas type II strains do not induce dramatic changes (111). In addition, IL-8 has been shown to be increased in *H. pylori* infected patients with active gastritis. Type I strains to induce tyrosine phosphorylation of a host cell 145-kDa protein and induce IL-8 secretion (6, 101, 112). IL-8 is synthesized by many cells after stimulation by IL-1, TNF-α, or endotoxin. It may induce release of proteases from neutrophils, and some evidence exists that this cytokine is also involved in the production of oxygen radicals (113). This *in vivo* study found that a significant increase of CINC-1 level in *H. pylori* infection group as compared with control group. However, in this result of *H. pylori* infection showed gastric inflammation with not ulceration in rat gastric mucosa. Moreover, they found that mild to moderate gastritis with polymorphonuclear inflammatory cells infiltration and *H. pylori* colonization in histopathology examination as shown in figure 31. Similar to other models, *H. pylori*-infected rats induce only mild and moderate gastritis (95, 114).

Effect of genistein on body weight

During the experimental process, the body weight of each rat was measured every two days. Throughout the experiment, no statistically significant difference in the body weights was found in both *H. pylori*-infected groups and uninfected groups. In accordance with previous study, that showed the same result when this study was to investigate the inhibit *H. pylori* growth in both in *mongolian gerbils* and mouse models (115).

Effect of genistein on serum tumor necrosis factor alpha level and serum cytokine-induced neutrophil chemoattractant 1 level

TNF-α is a key mediator in a host's response against gram-negative bacteria and in the septic shock syndrome induced by either LPS or bacterial superantigens (116). It is a major proinflammatory cytokine and plays an important role in the development of acute inflammation, including neutrophil infiltration of gastric mucosa. Expression of TNF-α increase in *H. pylori* induced gastric damage. In the same way, the results of this study showed that a significant increase of TNF-α level in *H. pylori* infection group as compared with control group. Furthermore, TNF-α stimulates transcription factors, such as NF-kB, induces the synthesis of various inflammatory cytokines including IL-8 (6,113,117,118). 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 (119). 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 (46, 120-122). Therefore, IL-8 mRNA expression was up-regulated within 1 hour after *H. pylori* infection, reached a maximal increase of ~120-fold at 8 hours post-infection, and then decreased (123, 124). Attachment of *H. pylori* to gastric epithelial cells can induce host cellular responses, including the reorganization of actin cytoskeletons, the tyrosine phosphorylation of a 145-kD protein, and release of IL-8 (100, 119, 125). Several studies have shown that *H. pylori* produced a dose-dependent increase in IL-8 production (78, 123, 126). 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 (127). Although various cells have been reported to produce CINC-1 in response to inflammatory mediators, such as TNF-α, IL-1β, and LPSs (128). This study findings support a significant increase of CINC-1 level in *H. pylori* infection group as compared with control. In this experiment hypothesized that treatment with genistein was attenuated gastric inflammation by reduction of inflammatory mediators. This study explored anti-inflammatory effect of genistein on *H. pylori*-associated gastritis can be related in two ways. Firstly, genistein inhibited TNF- α production. Previous reports demonstrated that genistein could inhibit lipopolysaccharide (LPS)-induced alveolar macrophage TNF- α production and thus reduce the alveolar neutrophil influx following LPS (30, 102, 129) , as well as LPS-induced nuclear factor kappa B (NF-kB) activation (102) and secondly, 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 (6, 29, 126). In accordance with this study that showed a significant decrease of TNF-α level and CINC-1 in genistein treatment group as compared with *H. pylori* infection group.

Effect of genistein on histopathology

At histologic examinations revealed that *H. pylori* infection caused mucosal inflammation characterized, and intense polymorphonuclear leukocyte infiltration (neutrophils and some eosinophils) in the lamina propria, mucus layer, and inside the glands. Thus, neutrophil infiltration is an almost invariable finding in *H. pylori*-associated gastritis and is topographically related to *H. pylori* colonization (130, 131). In this results demonstrated that in *H. pylori* infection group, the stomach tissues showed polymorphonuclear inflammatory cells infiltrating the lamina propria with increase

score of gastric inflammation and score of *H. pylori* colonization. On the other hand, in genistein treatment group, stomach histopathology was improved when compared to *H. pylori* infection group, especially in the reduction of polymorphonuclear inflammatory cells infiltration and *H. pylori* colonization score was reduced.

The changes of polymorphonuclear inflammatory cells in histologic examinations

The results of histologic examinations were shown polymorphonuclear inflammatory cells may be decreased by genistein administration. Previous studies demonstrated that the eradication of *H. pylori* results in a reduction in neutrophil numbers, which is followed by the gradual disappearance of mononuclear cells (33, 65, 132). These 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 activity).

The changes of *Helicobacter pylori* colonization in histologic examinations *in vivo* and the effects of genistein on anti-*Helicobacter pylori* activity *in vitro*

The results of histologic examinations were shown *H. pylori* colonization may be decreased by genistein administration. Genistein was reported to show *in vitro* antibacterial activities against *Staphylococcus aureus* strains including Methicillin-resistant *S. aureus* strains and *H. pylori* (80). Published reports also mentioned its ability to inhibit the *in vitro* growth of *Bacillus anthracis*, but did not affect the growth of *Escherichia coli* (81). Previous study found genistein is a topoisomerase II inhibitor that mediated stabilization of the covalent topoisomerase II–DNA cleavage complex may be responsible for moderate inhibition of bacterial growth (79). Type II topoisomerases are ubiquitous enzymes that play an essential role in the control of replicative DNA synthesis share structural and functional homology among different prokaryotic and eukaryotic organisms (78). As inhibition of topoisomerase II were demonstrated that genistein is bacteriostatic activity via action of limit the growth of bacteria by interfering with bacterial protein production, DNA replication (133). Moreover, studies on cell survival suggested that genistein is a bacteriostatic agent rather than a bactericidal compound. Ulanowska *et al*. found that no decrease in the number of colony forming units was observed in the culture of *Vibrio harveyi*, whose growth was severely inhibited by genistein. This suggests that even if cell growth was impaired, after removing genistein most of the bacterial cells were able to recover their metabolic functions and produce progeny on agar plates (134). Also, Dool-Ri Oh *et al*. found that genistein has bacteriostatic activity, but does not possess bactericidal activity, these results suggest that genistein might inhibit *Vibrio vulnificus* cytotoxicity through unknown mechanisms that do not involve the inhibition of bacterial survival (82). Bactericidal drugs have more powerful antibacterial action and are able to kill bacteria. In contrast, bacteriostatic

antibiotics are assumed to require phagocytic cells to definitely clear bacteria and are therefore thought to be less effective without an efficient immune response (133). Phagocytosis is an important anti-bacterial innate immune mechanism and there is a substantial phagocytic cell infiltrate in the infected gastric mucosa. Macrophage IL-6 expression is dependent upon phagocytosis. *H. pylori* partially evades phagocyte-mediated killing. A main proportion of engulfed bacteria appear to survive inside phagosomes which fuse to become megasomes and may provide a protected intracellular niche contributing to the persistence of infection. The bacteria also evade phagocyte-mediated killing by disrupting nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) activity. NADPH oxidase catalyzes the conversion of molecular oxygen into superoxide anions, resulting in the extracellular release of toxic ROS rather than their accumulation within phagosomes (38). In accordance with histologic examinations of this *in vivo* study that showed in the group of *H. pylori* inoculation, almost all of the stomach tissues showed increase infiltration of phagocytic cells and *H. pylori* colonization may be decreased by genistein administration. On the other hand, *in vitro* study demonstrated that genistein (0.25, 0.5, 1, 1.5, 2 and 4 M) showed no antimicrobial activity against *H. pylori* using disk diffusion method. Although, the concentration of genistein was too high to eradicate when we compared with the concentration that we used *in vivo* (Converting *in vivo* dose 16 mg/kg, into the concentration that we used in in vitro assays, the resultant concentration (molarity) will be 0.6 molar.) As a consequence, these difference results between *in vivo* study and *in vitro* study might occur from genistein has a bacteriostatic effect on *H. pylori* rather than a bactericidal compound.

Effect of genistein on gastric malondialdehyde level

H. pylori infection was generated large amounts of ROS and potential sources associated with infection include inflammatory cells (neutrophils, macrophages), gastric epithelial cells and the bacterium itself (135). This study hypothesized that treatment with genistein was attenuated gastric inflammation by reduction of reactive oxygen species. Conversely, in this results demonstrated that both *H.pylori* infection group and genistein treatment group no significantly changes in levels of gastric MDA than control group. Regarding *H. pylori*-induced gastritis, the histological examinations in this study showed that *H. pylori* colonization with a degree between mild to moderate infiltration of polymorphonuclear cells in lamina propria. However, this study showed that less mucosal erosion and but not found ulceration in rat gastric mucosa. The results obtained on detection of gastric MDA level might occur from the less mucosal damage and less progression of gastritis.

CHAPTER V CONCLUSION

The study demonstrated whether *H. pylori* infection associated with elevated levels of serum TNF-α serum CINC-1 and increased polymorphonuclear inflammatory cells infiltration in gastric mucosa with *H. pylori* colonization. Additionally, this study 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, polymorphonuclear inflammatory cells infiltration and *H .pylori* colonization in gastric epithelium. This is the first study suggest that genistein attenuate gastritis, likely via its ability to inhibit inflammation and improve gastric pathology in a rat model of *H. pylori* infection. Moreover, this study is will be useful for further studies and sufficient efforts were made to identify natural products for prevention and adjuvants of allopathic anti-*H. pylori* eradication therapy. The summary effect of genistein on *H. pylori*-associated gastritis in our present were concluded in the diagram as shown in figure 36.

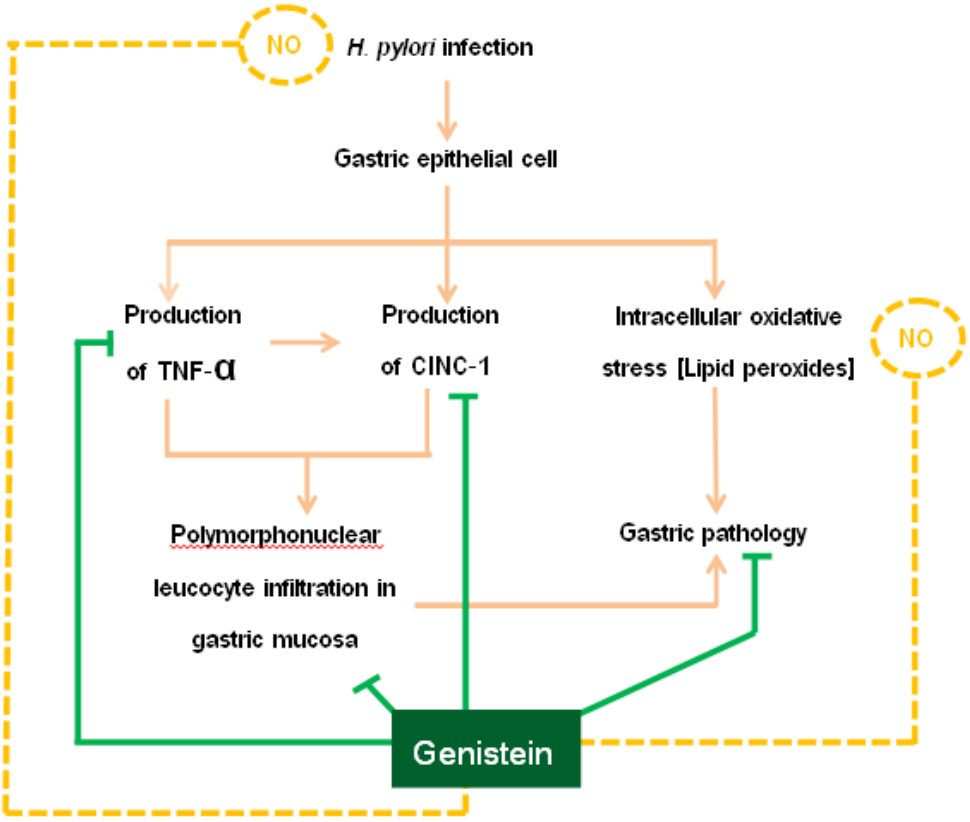


Figure 36 The summary effect of genistein on *H. pylori*-associated gastritis

REFERENCES

1.Asahi M, Azuma T, Ito S, Ito Y, Suto H, Nagai Y, et al. *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. The Journal of experimental medicine. 2000;191(4):593-602.

2.Suzuki H, Hibi T, Marshall BJ. *Helicobacter pylori*: present status and future prospects in Japan. Journal of gastroenterology. 2007;42(1):1-15.

3.Das JC, Paul N. Epidemiology and pathophysiology of *Helicobacter pylori* infection in children. Indian journal of pediatrics. 2007;74(3):287-90.

4.Atisook K, Kachinthorn U, Luengrojanakul P, Tanwandee T, Pakdirat P, Puapairoj A. Histology of gastritis and *Helicobacter pylori* infection in Thailand: a nationwide study of 3776 cases. *Helicobacter*. 2003;8(2):132-41.

5.Kwiecien S, Brzozowski T, Konturek SJ. Effects of reactive oxygen species action on gastric mucosa in various models of mucosal injury. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society. 2002;53(1):39-50.

6.Segal ED, Lange C, Covacci A, Tompkins LS, Falkow S. Induction of host signal transduction pathways by *Helicobacter pylori*. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(14):7595-9.

7.Ohyauchi M, Imatani A, Yonechi M, Asano N, Miura A, Iijima K, *et al*. The polymorphism interleukin 8 -251 A/T influences the susceptibility of *Helicobacter pylori* related gastric diseases in the Japanese population. Gut. 2005;54(3):330-5.

8.Kim JM, Kim JS, Jung HC, Oh YK, Kim N, Song IS. Inhibition of *Helicobacter pylori*-induced nuclear factor-kappa B activation and interleukin-8 gene expression by ecabet sodium in gastric epithelial cells. *Helicobacter*. 2003;8(5):542-53.

9.Yagihashi A, Tsuruma T, Tarumi K, Kameshima T, Yajima T, Yanai Y, et al. Prevention

of small intestinal ischemia-reperfusion injury in rat by anti-cytokine-induced neutrophil chemoattractant monoclonal antibody. The Journal of surgical research. 1998;78(2):92-6.

10.Watanabe K, Kinoshita S, Nakagawa H. Purification and characterization of cytokine-induced neutrophil chemoattractant produced by epithelioid cell line of normal rat kidney (NRK-52E cell). Biochemical and biophysical research communications. 1989;161(3):1093-9.

11.Anisowicz A, Bardwell L, Sager R. Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. Proceedings of the National Academy of Sciences of the United States of America. 1987;84(20):7188-92.

12.Seino Y, Ikeda U, Minezaki KK, Funayama H, Kasahara T, Konishi K, et al. Expression of cytokine-induced neutrophil chemoattractant in rat cardiac myocytes. Journal of molecular and cellular cardiology. 1995;27(9):2043-51.

13.Yoshida H, Miura S, Kishikawa H, Hirokawa M, Nakamizo H, Nakatsumi RC, *et al*. Fatty acids enhance GRO/CINC-1 and interleukin-6 production in rat intestinal epithelial cells. The Journal of nutrition. 2001;131(11):2943-50.

14.Cover TL, Blaser MJ. *Helicobacter pylori* in health and disease. Gastroenterology. 2009;136(6):1863-73.

15.Dixon RA, Ferreira D. Genistein. Phytochemistry. 2002;60(3):205-11.

16.Middleton E, Jr., Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacological reviews. 2000;52(4):673-751.

17.Cassidy A. Physiological effects of phyto-oestrogens in relation to cancer and other human health risks. The Proceedings of the Nutrition Society. 1996;55(1b):399-417.

18.Cheng G, Wilczek B, Warner M, Gustafsson JA, Landgren BM. Isoflavone treatment for acute menopausal symptoms. Menopause (New York, NY). 2007;14(3 Pt 1):468-73.

19.Hamalainen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators of inflammation. 2007;2007:45673.

20.Rimbach G, Weinberg PD, de Pascual-Teresa S, Alonso MG, Ewins BA, Turner R, *et al*. Sulfation of genistein alters its antioxidant properties and its effect on platelet aggregation and monocyte and endothelial function. Biochimica et biophysica acta. 2004;1670(3):229-37.

21.Zielonka J, Gebicki J, Grynkiewicz G. Radical scavenging properties of genistein. Free radical biology & medicine. 2003;35(8):958-65.

22.Kruk I, Aboul-Enein HY, Michalska T, Lichszteld K, Kladna A. Scavenging of reactive oxygen species by the plant phenols genistein and oleuropein. Luminescence : the journal of biological and chemical luminescence. 2005;20(2):81-9.

23.Choi C, Cho H, Park J, Cho C, Song Y. Suppressive effects of genistein on oxidative stress and NFkappaB activation in RAW 264.7 macrophages. Bioscience, biotechnology, and biochemistry. 2003;67(9):1916-22.

24.Ruiz-Larrea MB, Mohan AR, Paganga G, Miller NJ, Bolwell GP, Rice-Evans CA. Antioxidant activity of phytoestrogenic isoflavones. Free radical research. 1997;26(1):63-70.

25.Banerjee S, Li Y, Wang Z, Sarkar FH. Multi-targeted therapy of cancer by genistein. Cancer letters. 2008;269(2):226-42.

26.Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, *et al*. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology. 1998;139(10):4252-63.

27.Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, et al. Genistein, a specific inhibitor of tyrosine-specific protein kinases. The Journal of biological chemistry. 1987;262(12):5592-5.

28.Xagorari A, Papapetropoulos A, Mauromatis A, Economou M, Fotsis T, Roussos C. Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. The Journal of pharmacology and experimental therapeutics. 2001;296(1):181-7.

29.Ding SZ, Cho CH, Lam SK. *Helicobacter pylori* induces interleukin-8 expression in endothelial cells and the signal pathway is protein tyrosine kinase dependent. Biochemical and biophysical research communications. 1997;240(3):561-5.

30.Morris PE, Olmstead LE, Howard-Carroll AE, Dickens GR, Goltz ML, Courtney-Shapiro C, et al. In vitro and in vivo effects of genistein on murine alveolar macrophage TNF alpha production. Inflammation. 1999;23(3):231-9.

31.Conteduca V, Sansonno D, Lauletta G, Russi S, Ingravallo G, Dammacco F. *H. pylori* infection and gastric cancer: state of the art (review). International journal of oncology. 2013;42(1):5-18.

32.Graham DY, Fischbach L. *Helicobacter pylori* infection. The New England journal of medicine. 2010;363(6):595-6; author reply 6.

33.Berry V, Jennings K, Woodnutt G. Bactericidal and morphological effects of amoxicillin on *Helicobacter pylori*. Antimicrobial agents and chemotherapy. 1995;39(8):1859-61.

34.Ernst PB, Gold BD. The disease spectrum of Helicobacter pylori: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. Annual review of microbiology. 2000;54:615-40.

35.van Duynhoven YT, de Jonge R. Transmission of *Helicobacter pylori*: a role for food? Bulletin of the World Health Organization. 2001;79(5):455-60.

36.Kao CY, Sheu BS, Wu JJ. *Helicobacter pylori* infection: An overview of bacterial virulence factors and pathogenesis. Biomedical journal. 2016;39(1):14-23.

37.Amieva MR, El-Omar EM. Host-bacterial interactions in *Helicobacter pylori* infection. Gastroenterology. 2008;134(1):306-23.

38.Robinson K, Argent RH, Atherton JC. The inflammatory and immune response to *Helicobacter pylori* infection. Best practice & research Clinical gastroenterology.

2007;21(2):237-59.

39.Odenbreit S, Puls J, Sedlmaier B, Gerland E, Fischer W, Haas R. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. Science (New York, NY). 2000;287(5457):1497-500.

40.Higashi H, Tsutsumi R, Fujita A, Yamazaki S, Asaka M, Azuma T, et al. Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(22):14428-33.

41. Ahmadzadeh A, Ghalehnoei H, Farzi N, Yadegar A, Alebouyeh M, Aghdaei HA, et al. Association of CagPAI integrity with severeness of *Helicobacter pylori* infection in patients with gastritis. Pathologie-biologie. 2015;63(6):252-7.

42. Andersen LP, Wadstrom T. Basic Bacteriology and Culture. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics. Washington (DC): ASM Press ASM Press.; 2001.

43.Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of *Helicobacter pylori* infection. Clinical microbiology reviews. 2006;19(3):449-90.

44.Borlace GN, Keep SJ, Prodoehl MJ, Jones HF, Butler RN, Brooks DA. A role for altered phagosome maturation in the long-term persistence of *Helicobacter pylori* infection. American journal of physiology Gastrointestinal and liver physiology. 2012;303(2):G169-79.

45.Wilson KT, Crabtree JE. Immunology of Helicobacter pylori: insights into the failure of the immune response and perspectives on vaccine studies. Gastroenterology. 2007;133(1):288-308.

46.Crabtree JE, Covacci A, Farmery SM, Xiang Z, Tompkins DS, Perry S, et al. *Helicobacter pylori* induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. Journal of clinical pathology. 1995;48(1):41-5.

47.Oikawa T, Asano N, Imatani A, Ohyauchi M, Fushiya J, Kondo Y, et al. Gene

polymorphisms of NOD1 and interleukin-8 influence the susceptibility to erosive esophagitis in *Helicobacter pylori* infected Japanese population. Human immunology. 2012;73(11):1184-9.

48.Yoshikawa T, Naito Y. The role of neutrophils and inflammation in gastric mucosal injury. Free radical research. 2000;33(6):785-94.

49.Calvino Fernandez M, Parra Cid T. H. pylori and mitochondrial changes in epithelial cells. The role of oxidative stress. Revista espanola de enfermedades digestivas : organo oficial de la Sociedad Espanola de Patologia Digestiva. 2010;102(1):41-50.

50.Kwiecien S, Brzozowski T, Konturek P, Konturek SJ. The role of reactive oxygen species in action of nitric oxide-donors on stress-induced gastric mucosal lesions. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society. 2002;53(4 Pt 2):761-73.

51.Ihan A, Pinchuk IV, Beswick EJ. Inflammation, immunity, and vaccines for *Helicobacter pylori* infection. Helicobacter. 2012;17 Suppl 1:16-21.

52.Vaira D, Malfertheiner P, Megraud F, Axon AT, Deltenre M, Hirschl AM, *et al.* Diagnosis of *Helicobacter pylori* infection with a new non-invasive antigen-based assay. HpSA European study group. Lancet (London, England). 1999;354(9172):30-3.

53.Vakil N, Megraud F. Eradication therapy for *Helicobacter pylori*. Gastroenterology. 2007;133(3):985-1001.

54.Lee JY, Kim N. Diagnosis of Helicobacter pylori by invasive test: histology. Annals of translational medicine. 2015;3(1):10.

55.Laine L, Lewin DN, Naritoku W, Cohen H. Prospective comparison of H&E, Giemsa, and Genta stains for the diagnosis of *Helicobacter pylori*. Gastrointestinal endoscopy. 1997;45(6):463-7.

56.Moon YJ, Wang X, Morris ME. Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicology in vitro : an international journal published in association with BIBRA. 2006;20(2):187-210.

57.The role of soy isoflavones in menopausal health: report of The North American Menopause Society/Wulf H. Utian Translational Science Symposium in Chicago, IL (October 2010). Menopause (New York, NY). 2011;18(7):732-53.

58.Sepehr E, Cooke GM, Robertson P, Gilani GS. Effect of glycosidation of isoflavones on their bioavailability and pharmacokinetics in aged male rats. Molecular nutrition & food research. 2009;53 Suppl 1:S16-26.

59.Bhathena SJ, Velasquez MT. Beneficial role of dietary phytoestrogens in obesity and diabetes. The American journal of clinical nutrition. 2002;76(6):1191-201.

60. Janice Barlow, Jo Ann P. Johnson, Lacie Scofield. Early life exposure to the phytoestrogen genistein and breast cancer risk in later years. Breast cancer & the environment research centers. 2007:47-55.

61.Faughnan MS, Hawdon A, Ah-Singh E, Brown J, Millward DJ, Cassidy A. Urinary isoflavone kinetics: the effect of age, gender, food matrix and chemical composition. The British journal of nutrition. 2004;91(4):567-74.

62.Adlercreutz H, Markkanen H, Watanabe S. Plasma concentrations of phyto-oestrogens in Japanese men. Lancet (London, England). 1993;342(8881):1209-10.

63.Messina M, McCaskill-Stevens W, Lampe JW. Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings. Journal of the National Cancer Institute. 2006;98(18):1275-84.

64.Sakai T, Kogiso M. Soy isoflavones and immunity. The journal of medical investigation : JMI. 2008;55(3-4):167-73.

65.Larkin T, Price WE, Astheimer L. The key importance of soy isoflavone bioavailability to understanding health benefits. Critical reviews in food science and nutrition. 2008;48(6):538-52.

66.Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, *et al*. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology. 1997;138(3):863-70.

67.Farmakalidis E, Hathcock JN, Murphy PA. Oestrogenic potency of genistin and

daidzin in mice. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 1985;23(8):741-5.

68.Lamartiniere CA, Cotroneo MS, Fritz WA, Wang J, Mentor-Marcel R, Elgavish A. Genistein chemoprevention: timing and mechanisms of action in murine mammary and prostate. The Journal of nutrition. 2002;132(3):552s-8s.

69.Honore EK, Williams JK, Anthony MS, Clarkson TB. Soy isoflavones enhance coronary vascular reactivity in atherosclerotic female macaques. Fertility and sterility. 1997;67(1):148-54.

70.Kenny AM, Mangano KM, Abourizk RH, Bruno RS, Anamani DE, Kleppinger A, *et al*. Soy proteins and isoflavones affect bone mineral density in older women: a randomized controlled trial. The American journal of clinical nutrition. 2009;90(1):234-42.

71.Win W, Cao Z, Peng X, Trush MA, Li Y. Different effects of genistein and resveratrol on oxidative DNA damage in vitro. Mutation research. 2002;513(1-2):113-20.

72.Umamaheswari M, Madeswaran A, Asokkumar K. Virtual Screening analysis and *in vitro* xanthine oxidase inhibitory activity of some commercially available flavonoids. Iranian journal of pharmaceutical research : IJPR. 2013;12(3):317-23.

73.Rufer CE, Kulling SE. Antioxidant activity of isoflavones and their major metabolites using different in vitro assays. Journal of agricultural and food chemistry. 2006;54(8):2926-31.

74.Takekawa S, Matsui T, Arakawa Y. The protective effect of the soybean polyphenol genistein against stress-induced gastric mucosal lesions in rats, and its hormonal mechanisms. Journal of nutritional science and vitaminology. 2006;52(4):274-80.

75.Kano M, Ishikawa F, Matsubara S, Kikuchi-Hayakawa H, Shimakawa Y. Soymilk products affect ethanol absorption and metabolism in rats during acute and chronic ethanol intake. The Journal of nutrition. 2002;132(2):238-44.

76.Hernandez-Montes E, Pollard SE, Vauzour D, Jofre-Montseny L, Rota C, Rimbach G,

et al. Activation of glutathione peroxidase via Nrf1 mediates genistein's protection against oxidative endothelial cell injury. Biochemical and biophysical research communications. 2006;346(3):851-9.

77.Davis JN, Kucuk O, Djuric Z, Sarkar FH. Soy isoflavone supplementation in healthy men prevents NF-kappa B activation by TNF-alpha in blood lymphocytes. Free radical biology & medicine. 2001;30(11):1293-302.

78.Heisig P. Inhibitors of bacterial topoisomerases: mechanisms of action and resistance and clinical aspects. Planta medica. 2001;67(1):3-12.

79.Markovits J, Linassier C, Fosse P, Couprie J, Pierre J, Jacquemin-Sablon A, *et al.* Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. Cancer research. 1989;49(18):5111-7.

80.Verdrengh M, Collins LV, Bergin P, Tarkowski A. Phytoestrogen genistein as an anti-staphylococcal agent. Microbes and infection / Institut Pasteur. 2004;6(1):86-92.

81.Hong H, Landauer MR, Foriska MA, Ledney GD. Antibacterial activity of the soy isoflavone genistein. Journal of basic microbiology. 2006;46(4):329-35.

82.Oh DR, Kim JR, Kim YR. Genistein inhibits Vibrio vulnificus adhesion and cytotoxicity to HeLa cells. Archives of pharmacal research. 2010;33(5):787-92.

83.Beales IL, Calam J. Stimulation of IL-8 production in human gastric epithelial cells by *Helicobacter pylori*, IL-1beta and TNF-alpha requires tyrosine kinase activity, but not protein kinase C. Cytokine. 1997;9(7):514-20.

84.Nozawa Y, Nishihara K, Peek RM, Nakano M, Uji T, Ajioka H, *et al*. Identification of a signaling cascade for interleukin-8 production by Helicobacter pylori in human gastric epithelial cells. Biochemical pharmacology. 2002;64(1):21-30.

85.Li Y, Zhang JF, Zhang YM, Ma XB. The protective effect of genistein postconditioning on hypoxia/reoxygenation-induced injury in human gastric epithelial cells. Acta pharmacologica Sinica. 2009;30(5):576-81.

86.Du DS, Ma XB, Zhang JF, Zhou XY, Li Y, Zhang YM, *et al*. The protective effect of capsaicin receptor-mediated genistein postconditioning on gastric ischemia-reperfusion injury in rats. Digestive diseases and sciences. 2010;55(11):3070-7.

87.Sato Y, Itagaki S, Oikawa S, Ogura J, Kobayashi M, Hirano T, *et al*. Protective effect of soy isoflavone genistein on ischemia-reperfusion in the rat small intestine. Biological & pharmaceutical bulletin. 2011;34(9):1448-54.

88.Seibel J, Molzberger AF, Hertrampf T, Laudenbach-Leschowski U, Diel P. Oral treatment with genistein reduces the expression of molecular and biochemical markers of inflammation in a rat model of chronic TNBS-induced colitis. European journal of nutrition. 2009;48(4):213-20.

89.Ji G, Yang Q, Hao J, Guo L, Chen X, Hu J, *et al*. Anti-inflammatory effect of genistein on non-alcoholic steatohepatitis rats induced by high fat diet and its potential mechanisms. International immunopharmacology. 2011;11(6):762-8.

90.Fan YJ, Rong Y, Li PF, Dong WL, Zhang DY, Zhang L, *et al*. Genistein protection against acetaminophen-induced liver injury via its potential impact on the activation of UDP-glucuronosyltransferase and antioxidant enzymes. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2013;55:172-81.

91.Werawatganon D. Simple animal model of *Helicobacter pylori* infection. World journal of gastroenterology. 2014;20(21):6420-4.

92.Busby MG, Jeffcoat AR, Bloedon LT, Koch MA, Black T, Dix KJ, et al. Clinical characteristics and pharmacokinetics of purified soy isoflavones: single-dose administration to healthy men. The American journal of clinical nutrition. 2002;75(1):126-36.

93.Susutlertpanya W, Werawatganon D, Siriviriyakul P, Klaikeaw N. Genistein Attenuates Nonalcoholic Steatohepatitis and Increases Hepatic PPARgamma in a Rat Model. Evidence-based complementary and alternative medicine : eCAM. 2015;2015:509057.

94. Claire Horlock. Enzyme-linked immunosorbent assay (ELISA). Bitesized immunology. 2016.

95. Li H, Andersson EM, Helander HF. Reactions from rat gastric mucosa during one

year of *Helicobacter pylori* infection. Digestive diseases and sciences. 1999;44(1):116-24.

96. Nakamura RM. Laboratory tests for the evaluation of *Helicobacter pylori* infections. Journal of clinical laboratory analysis. 2001;15(6):301-7.

97. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. The American journal of surgical pathology. 1996;20(10):1161-81.

98. Denise Grotto, Lucas Santa Maria, Juliana Valentini, Clovis Paniz, Gabriela Schmitt e Solange Cristina Garcia. Importance of the lipid peroxidation biomarkers and methodological aspects FOR malondialdehyde quantification. Quim. Nova. 2009;32(1):169-174

99. Coutant, F., Goyer, A., Requena, J. Protein Assay Technical Handbook. Pierce Biotechnology. 2005;14-24.

100. Yamaoka Y, Kita M, Kodama T, Sawai N, Imanishi J. *Helicobacter pylori* cagA gene and expression of cytokine messenger RNA in gastric mucosa. Gastroenterology. 1996;110(6):1744-52.

101. Crabtree JE, Wyatt JI, Trejdosiewicz LK, Peichl P, Nichols PH, Ramsay N, *et al.* Interleukin-8 expression in *Helicobacter pylori* infected, normal, and neoplastic gastroduodenal mucosa. Journal of clinical pathology. 1994;47(1):61-6.

102. Ji G, Zhang Y, Yang Q, Cheng S, Hao J, Zhao X, et al. Genistein suppresses LPS-induced inflammatory response through inhibiting NF-kappaB following AMP kinase activation in RAW 264.7 macrophages. PloS one. 2012;7(12):e53101.

103. Storskrubb T, Aro P, Ronkainen J, Wreiber K, Nyhlin H, Bolling-Sternevald E, *et al*. Antimicrobial susceptibility of *Helicobacter pylori* strains in a random adult Swedish population. Helicobacter. 2006;11(4):224-30.

104. Ford A, Delaney B, Forman D, Moayyedi P. Eradication therapy for peptic ulcer disease in *Helicobacter pylori* positive patients. The Cochrane database of systematic reviews. 2004(4):Cd003840.

105.Verdrengh M, Jonsson IM, Holmdahl R, Tarkowski A. Genistein as an anti-inflammatory agent. Inflammation research : official journal of the European Histamine Research Society. 2003;52(8):341-6.

106.Su B, Johansson S, Fallman M, Patarroyo M, Granstrom M, Normark S. Signal transduction-mediated adherence and entry of *Helicobacter pylori* into cultured cells. Gastroenterology. 1999;117(3):595-604.

107.Tombola F, Campello S, De Luca L, Ruggiero P, Del Giudice G, Papini E, *et al*. Plant polyphenols inhibit VacA, a toxin secreted by the gastric pathogen *Helicobacter pylori*. FEBS letters. 2003;543(1-3):184-9.

108.Kwok T, Backert S, Schwarz H, Berger J, Meyer TF. Specific entry of *Helicobacter pylori* into cultured gastric epithelial cells via a zipper-like mechanism. Infection and immunity. 2002;70(4):2108-20.

109.Mahmoud AM, Zhu T, Parray A, Siddique HR, Yang W, Saleem M, *et al*. Differential effects of genistein on prostate cancer cells depend on mutational status of the androgen receptor. PloS one. 2013;8(10):e78479.

110.Backert S, Tegtmeyer N, Fischer W. Composition, structure and function of the *Helicobacter pylori* cag pathogenicity island encoded type IV secretion system. Future microbiology. 2015;10(6):955-65.

111.Peek RM. *Helicobacter pylori* infection and disease: from humans to animal models. Disease models & mechanisms. 2008;1(1):50-5.

112.Marchetti M, Arico B, Burroni D, Figura N, Rappuoli R, Ghiara P. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. Science (New York, NY). 1995;267(5204):1655-8.

113.Crowe SE, Alvarez L, Dytoc M, Hunt RH, Muller M, Sherman P, *et al*. Expression of interleukin 8 and CD54 by human gastric epithelium after *Helicobacter pylori* infection *in vitro*. Gastroenterology. 1995;108(1):65-74.

114.Thornton AJ, Strieter RM, Lindley I, Baggiolini M, Kunkel SL. Cytokine-induced gene expression of a neutrophil chemotactic factor/IL-8 in human hepatocytes. Journal of immunology (Baltimore, Md : 1950). 1990;144(7):2609-13.

115.Li H, Kalies I, Mellgard B, Helander HF. A rat model of chronic *Helicobacter pylori* infection. Studies of epithelial cell turnover and gastric ulcer healing. Scandinavian journal of gastroenterology. 1998;33(4):370-8.

116.Kim JM, Zheng HM, Lee BY, Lee WK, Lee DH. Anti-*Helicobacter pylori* Properties of GutGard. Preventive nutrition and food science. 2013;18(2):104-10.

117.Beutler B, Cerami A. Tumor necrosis, cachexia, shock, and inflammation: a common mediator. Annual review of biochemistry. 1988;57:505-18.

118.Santos JC, Ladeira MS, Pedrazzoli J, Jr., Ribeiro ML. Relationship of IL-1 and TNF-alpha polymorphisms with *Helicobacter pylori* in gastric diseases in a Brazilian population. Brazilian journal of medical and biological research. 2012;45(9):811-7.

119.Hoffmann E, Dittrich-Breiholz O, Holtmann H, Kracht M. Multiple control of interleukin-8 gene expression. Journal of leukocyte biology. 2002;72(5):847-55.

120.Crabtree JE, Farmery SM, Lindley IJ, Figura N, Peichl P, Tompkins DS. CagA/cytotoxic strains of *Helicobacter pylori* and interleukin-8 in gastric epithelial cell lines. Journal of clinical pathology. 1994;47(10):945-50.

121.Mahalingam S, Karupiah G. Chemokines and chemokine receptors in infectious diseases. Immunology and cell biology. 1999;77(6):469-75.

122.Rieder G, Hatz RA, Moran AP, Walz A, Stolte M, Enders G. Role of adherence in interleukin-8 induction in *Helicobacter pylori*-associated gastritis. Infection and immunity. 1997;65(9):3622-30.

123.Aihara M, Tsuchimoto D, Takizawa H, Azuma A, Wakebe H, Ohmoto Y, *et al.* Mechanisms involved in *Helicobacter pylori*-induced interleukin-8 production by a gastric cancer cell line, MKN45. Infection and immunity. 1997;65(8):3218-24.

124.Noach LA, Bosma NB, Jansen J, Hoek FJ, van Deventer SJ, Tytgat GN. Mucosal tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 production in patients with *Helicobacter pylori* infection. Scandinavian journal of gastroenterology. 1994;29(5):425-9.

125.Crabtree JE, Shallcross TM, Heatley RV, Wyatt JI. Mucosal tumour necrosis factor

alpha and interleukin-6 in patients with *Helicobacter pylori* associated gastritis. Gut. 1991;32(12):1473-7.

126.Beales I, Blaser MJ, Srinivasan S, Calam J, Perez-Perez GI, Yamada T, et al. Effect of *Helicobacter pylori* products and recombinant cytokines on gastrin release from cultured canine G cells. Gastroenterology. 1997;113(2):465-71.

127.Watanabe K, Koizumi F, Kurashige Y, Tsurufuji S, Nakagawa H. Rat CINC, a member of the interleukin-8 family, is a neutrophil-specific chemoattractant i*n vivo*. Experimental and molecular pathology. 1991;55(1):30-7.

128.Handa O, Naito Y, Takagi T, Shimozawa M, Kokura S, Yoshida N, *et al*. Tumor necrosis factor-alpha-induced cytokine-induced neutrophil chemoattractant-1 (CINC-1) production by rat gastric epithelial cells: role of reactive oxygen species and nuclear factor-kappaB. The Journal of pharmacology and experimental therapeutics. 2004;309(2):670-6.

129.Fiorucci S, Antonelli E, Migliorati G, Santucci L, Morelli O, Federici B, *et al*. TNFalpha processing enzyme inhibitors prevent aspirin-induced TNFalpha release and protect against gastric mucosal injury in rats. Alimentary pharmacology & therapeutics. 1998;12(11):1139-53.

130.Gottstein N, Ewins BA, Eccleston C, Hubbard GP, Kavanagh IC, Minihane AM, *et al*. Effect of genistein and daidzein on platelet aggregation and monocyte and endothelial function. The British journal of nutrition. 2003;89(5):607-16.

131.Rocha GA, Queiroz DM, Mendes EN, Barbosa AJ, Lima Junior GF, Oliveira CA. *Helicobacter pylori* acute gastritis: histological, endoscopical, clinical, and therapeutic features. The American journal of gastroenterology. 1991;86(11):1592-5.

132.Kondo Y, Joh T, Sasaki M, Oshima T, Itoh K, Tanida S, *et al*. *Helicobacter pylori* eradication decreases blood neutrophil and monocyte counts. Alimentary pharmacology & therapeutics. 2004;20 Suppl 1:74-9.

133.Nemeth J, Oesch G, Kuster SP. Bacteriostatic versus bactericidal antibiotics for

patients with serious bacterial infections: systematic review and meta-analysis. The Journal of antimicrobial chemotherapy. 2015;70(2):382-95.

134.Ulanowska K, Tkaczyk A, Konopa G, Wegrzyn G. Differential antibacterial activity of genistein arising from global inhibition of DNA, RNA and protein synthesis in some bacterial strains. Archives of microbiology. 2006;184(5):271-8.

135.Ding SZ, Minohara Y, Fan XJ, Wang J, Reyes VE, Patel J, *et al*. *Helicobacter pylori* infection induces oxidative stress and programmed cell death in human gastric epithelial cells. Infection and immunity. 2007;75(8):4030-9.

APPENDIX

TNF-α and CINC-1

Oneway- Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
TNF-α	Control	7	20.8871	8.90036	3.36402	12.6557	29.1186	7.41	32.50
	SH	7	43.5000	16.51313	6.24138	28.2279	58.7721	19.70	62.60
	GH	7	29.3314	10.77399	4.07219	19.3671	39.2957	9.82	45.90
	Total	21	31.2395	15.22464	3.32229	24.3093	38.1697	7.41	62.60
CINC1	Control	7	81.2714	19.89319	7.51892	62.8733	99.6696	64.30	121.00
	SH	7	138.1000	43.56715	16.46684	97.8071	178.3929	86.30	204.00
	GH	7	103.2571	23.76740	8.98323	81.2760	125.2383	75.90	139.00
	Total	21	107.5429	37.84733	8.25897	90.3150	124.7708	64.30	204.00

Post Hoc Tests - Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
TNF-α	Tukey HSD	Control	SH	-22.61286*	6.67603	.009	-39.6512	-5.5745
			GH	-8.44429	6.67603	.432	-25.4826	8.5940
		SH	Control	22.61286*	6.67603	.009	5.5745	39.6512
			GH	14.16857	6.67603	.113	-2.8698	31.2069
		GH	Control	8.44429	6.67603	.432	-8.5940	25.4826
			SH	-14.16857	6.67603	.113	-31.2069	2.8698
	LSD	Control	SH	-22.61286*	6.67603	.003	-36.6387	-8.5870
			GH	-8.44429	6.67603	.222	-22.4701	5.5815
		SH	Control	22.61286*	6.67603	.003	8.5870	36.6387
			GH	14.16857*	6.67603	.048	.1427	28.1944
		GH	Control	8.44429	6.67603	.222	-5.5815	22.4701
			SH	-14.16857*	6.67603	.048	-28.1944	-.1427
CINC1	Tukey HSD	Control	SH	-56.82857*	16.50029	.008	-98.9400	-14.7171
			GH	-21.98571	16.50029	.396	-64.0971	20.1257
		SH	Control	56.82857*	16.50029	.008	14.7171	98.9400
			GH	34.84286	16.50029	.116	-7.2686	76.9543
		GH	Control	21.98571	16.50029	.396	-20.1257	64.0971
			SH	-34.84286	16.50029	.116	-76.9543	7.2686
	LSD	Control	SH	-56.82857*	16.50029	.003	-91.4944	-22.1627
			GH	-21.98571	16.50029	.199	-56.6515	12.6801
		SH	Control	56.82857*	16.50029	.003	22.1627	91.4944
			GH	34.84286*	16.50029	.049	.1770	69.5087

	GH	Control	21.98571	16.50029	.199	-12.6801	56.6515
		SH	-34.84286*	16.50029	.049	-69.5087	-.1770

*. The mean difference is significant at the 0.05 level.

MDA

Oneway- Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	7	.6720544	.69083481	.26111101	.0331388	1.3109700	.08377	1.92214
SH	7	.4993720	.38297486	.14475089	.1451794	.8535647	.09714	1.22581
GH	7	.5679769	.29128048	.11009367	.2985874	.8373664	.17803	.89706
Total	21	.5798011	.46682095	.10186868	.3673068	.7922955	.08377	1.92214

Post Hoc Tests- Multiple Comparisons

Dependent Variable: MDA

			Mean			95% Confidence Interval	
(I) Group			Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Tukey	Control	SH	.17268236	.25981070	.787	-.4903970	.8357617
HSD		GH	.10407750	.25981070	.916	-.5590018	.7671568
	SH	Control	-.17268236	.25981070	.787	-.8357617	.4903970
		GH	-.06860486	.25981070	.962	-.7316842	.5944744
	GH	Control	-.10407750	.25981070	.916	-.7671568	.5590018
		SH	.06860486	.25981070	.962	-.5944744	.7316842
LSD	Control	SH	.17268236	.25981070	.515	-.3731597	.7185244
		GH	.10407750	.25981070	.693	-.4417645	.6499195
	SH	Control	-.17268236	.25981070	.515	-.7185244	.3731597
		GH	-.06860486	.25981070	.795	-.6144469	.4772372
	GH	Control	-.10407750	.25981070	.693	-.6499195	.4417645
		SH	.06860486	.25981070	.795	-.4772372	.6144469

Body weight

Oneway- Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
BW_D1	Control	7	246.71	4.572	1.728	242.49	250.94	239	253
	H.pylori infection	7	248.71	4.990	1.886	244.10	253.33	239	254
	Genistein treatment	7	245.86	3.436	1.299	242.68	249.04	239	249
	Total	21	247.10	4.335	.946	245.12	249.07	239	254
BW_D3	Control	7	262.00	3.830	1.447	258.46	265.54	256	269
	H.pylori infection	7	263.71	4.751	1.796	259.32	268.11	258	269
	Genistein treatment	7	261.00	4.123	1.558	257.19	264.81	256	267
	Total	21	262.24	4.194	.915	260.33	264.15	256	269
BW_D5	Control	7	277.00	4.619	1.746	272.73	281.27	272	285
	H.pylori infection	7	272.86	3.288	1.243	269.82	275.90	269	278
	Genistein treatment	7	275.14	6.094	2.304	269.51	280.78	268	283
	Total	21	275.00	4.879	1.065	272.78	277.22	268	285
BW_D7	Control	7	283.86	3.288	1.243	280.82	286.90	279	287
	H.pylori infection	7	280.29	2.215	.837	278.24	282.33	278	284
	Genistein treatment	7	283.71	6.550	2.476	277.66	289.77	276	292
	Total	21	282.62	4.522	.987	280.56	284.68	276	292
BW_D9	Control	7	298.57	5.192	1.962	293.77	303.37	291	305
	H.pylori infection	7	295.14	4.298	1.625	291.17	299.12	290	301
	Genistein treatment	7	296.71	6.775	2.561	290.45	302.98	289	310
	Total	21	296.81	5.428	1.184	294.34	299.28	289	310
BW_D11	Control	7	316.86	7.198	2.721	310.20	323.51	309	326
	H.pylori infection	7	313.71	4.348	1.643	309.69	317.74	306	319
	Genistein treatment	7	316.43	2.992	1.131	313.66	319.20	312	321
	Total	21	315.67	5.092	1.111	313.35	317.98	306	326
BW_D13	Control	7	330.71	6.873	2.598	324.36	337.07	323	339
	H.pylori infection	7	327.14	6.866	2.595	320.79	333.49	318	340
	Genistein treatment	7	329.86	2.478	.937	327.56	332.15	327	334
	Total	21	329.24	5.709	1.246	326.64	331.84	318	340
BW_D15	Control	7	350.57	4.036	1.525	346.84	354.30	345	356
	H.pylori infection	7	343.00	6.298	2.380	337.18	348.82	337	356
	Genistein treatment	7	345.00	7.234	2.734	338.31	351.69	332	356
	Total	21	346.19	6.577	1.435	343.20	349.18	332	356
BW_D17	Control	7	366.00	4.865	1.839	361.50	370.50	356	370
	H.pylori infection	7	358.29	6.601	2.495	352.18	364.39	351	369

	Genistein treatment	7	360.43	5.827	2.202	355.04	365.82	355	369
	Total	21	361.57	6.439	1.405	358.64	364.50	351	370
	Control	7	382.14	6.669	2.521	375.98	388.31	370	390
BW_D1	*H.pylori* infection	7	376.29	6.343	2.398	370.42	382.15	368	387
9	Genistein treatment	7	377.86	2.545	.962	375.50	380.21	373	380
	Total	21	378.76	5.813	1.268	376.12	381.41	368	390
	Control	7	400.86	7.647	2.890	393.78	407.93	389	410
BW_D2	*H.pylori* infection	7	396.14	4.488	1.696	391.99	400.29	389	401
1	Genistein treatment	7	398.14	3.625	1.370	394.79	401.50	394	405
	Total	21	398.38	5.608	1.224	395.83	400.93	389	410

Post Hoc Tests-Multiple Comparisons

Dependent Variable		(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
BW_D1	Tukey HSD	Control	*H.pylori* infection	-2.000	2.343	.675	-7.98	3.98
			Genistein treatment	.857	2.343	.929	-5.12	6.84
		H.pylori infection	Control	2.000	2.343	.675	-3.98	7.98
			Genistein treatment	2.857	2.343	.457	-3.12	8.84
		Genistein treatment	Control	-.857	2.343	.929	-6.84	5.12
			H.pylori infection	-2.857	2.343	.457	-8.84	3.12
	LSD	Control	*H.pylori* infection	-2.000	2.343	.404	-6.92	2.92
			Genistein treatment	.857	2.343	.719	-4.06	5.78
		H.pylori infection	Control	2.000	2.343	.404	-2.92	6.92
			Genistein treatment	2.857	2.343	.238	-2.06	7.78
		Genistein treatment	Control	-.857	2.343	.719	-5.78	4.06
			H.pylori infection	-2.857	2.343	.238	-7.78	2.06

BW_D3	Tukey HSD	Control	*H.pylori* infection	-1.714	2.273	.735	-7.51	4.09
			Genistein treatment	1.000	2.273	.899	-4.80	6.80
		H.pylori infection	Control	1.714	2.273	.735	-4.09	7.51
			Genistein treatment	2.714	2.273	.472	-3.09	8.51
		Genistein treatment	Control	-1.000	2.273	.899	-6.80	4.80
			H.pylori infection	-2.714	2.273	.472	-8.51	3.09
	LSD	Control	*H.pylori* infection	-1.714	2.273	.460	-6.49	3.06
			Genistein treatment	1.000	2.273	.665	-3.77	5.77
		H.pylori infection	Control	1.714	2.273	.460	-3.06	6.49
			Genistein treatment	2.714	2.273	.248	-2.06	7.49
		Genistein treatment	Control	-1.000	2.273	.665	-5.77	3.77
			H.pylori infection	-2.714	2.273	.248	-7.49	2.06
BW_D5	Tukey HSD	Control	*H.pylori* infection	4.143	2.569	.266	-2.41	10.70
			Genistein treatment	1.857	2.569	.753	-4.70	8.41
		H.pylori infection	Control	-4.143	2.569	.266	-10.70	2.41
			Genistein treatment	-2.286	2.569	.653	-8.84	4.27
		Genistein treatment	Control	-1.857	2.569	.753	-8.41	4.70
			H.pylori infection	2.286	2.569	.653	-4.27	8.84
	LSD	Control	*H.pylori* infection	4.143	2.569	.124	-1.25	9.54
			Genistein treatment	1.857	2.569	.479	-3.54	7.25
		H.pylori infection	Control	-4.143	2.569	.124	-9.54	1.25
			Genistein treatment	-2.286	2.569	.385	-7.68	3.11
		Genistein treatment	Control	-1.857	2.569	.479	-7.25	3.54
			H.pylori infection	2.286	2.569	.385	-3.11	7.68

BW_D7	Tukey HSD	Control	*H.pylori* infection	3.571	2.363	.309	-2.46	9.60
			Genistein treatment	.143	2.363	.998	-5.89	6.17
		H.pylori infection	Control	-3.571	2.363	.309	-9.60	2.46
			Genistein treatment	-3.429	2.363	.337	-9.46	2.60
		Genistein treatment	Control	-.143	2.363	.998	-6.17	5.89
			H.pylori infection	3.429	2.363	.337	-2.60	9.46
	LSD	Control	*H.pylori* infection	3.571	2.363	.148	-1.39	8.54
			Genistein treatment	.143	2.363	.952	-4.82	5.11
		H.pylori infection	Control	-3.571	2.363	.148	-8.54	1.39
			Genistein treatment	-3.429	2.363	.164	-8.39	1.54
		Genistein treatment	Control	-.143	2.363	.952	-5.11	4.82
			H.pylori infection	3.429	2.363	.164	-1.54	8.39
BW_D9	Tukey HSD	Control	*H.pylori* infection	3.429	2.949	.490	-4.10	10.96
			Genistein treatment	1.857	2.949	.806	-5.67	9.38
		H.pylori infection	Control	-3.429	2.949	.490	-10.96	4.10
			Genistein treatment	-1.571	2.949	.856	-9.10	5.96
		Genistein treatment	Control	-1.857	2.949	.806	-9.38	5.67
			H.pylori infection	1.571	2.949	.856	-5.96	9.10
	LSD	Control	*H.pylori* infection	3.429	2.949	.260	-2.77	9.62
			Genistein treatment	1.857	2.949	.537	-4.34	8.05
		H.pylori infection	Control	-3.429	2.949	.260	-9.62	2.77
			Genistein treatment	-1.571	2.949	.601	-7.77	4.62
		Genistein treatment	Control	-1.857	2.949	.537	-8.05	4.34
			H.pylori infection	1.571	2.949	.601	-4.62	7.77

BW_D11	Tukey HSD	Control	*H.pylori* infection	3.143	2.755	.502	-3.89	10.17
			Genistein treatment	.429	2.755	.987	-6.60	7.46
		H.pylori infection	Control	-3.143	2.755	.502	-10.17	3.89
			Genistein treatment	-2.714	2.755	.595	-9.74	4.32
		Genistein treatment	Control	-.429	2.755	.987	-7.46	6.60
			H.pylori infection	2.714	2.755	.595	-4.32	9.74
	LSD	Control	*H.pylori* infection	3.143	2.755	.269	-2.64	8.93
			Genistein treatment	.429	2.755	.878	-5.36	6.22
		H.pylori infection	Control	-3.143	2.755	.269	-8.93	2.64
			Genistein treatment	-2.714	2.755	.337	-8.50	3.07
		Genistein treatment	Control	-.429	2.755	.878	-6.22	5.36
			H.pylori infection	2.714	2.755	.337	-3.07	8.50
BW_D13	Tukey HSD	Control	*H.pylori* infection	3.571	3.094	.495	-4.33	11.47
			Genistein treatment	.857	3.094	.959	-7.04	8.75
		H.pylori infection	Control	-3.571	3.094	.495	-11.47	4.33
			Genistein treatment	-2.714	3.094	.661	-10.61	5.18
		Genistein treatment	Control	-.857	3.094	.959	-8.75	7.04
			H.pylori infection	2.714	3.094	.661	-5.18	10.61
	LSD	Control	*H.pylori* infection	3.571	3.094	.263	-2.93	10.07
			Genistein treatment	.857	3.094	.785	-5.64	7.36
		H.pylori infection	Control	-3.571	3.094	.263	-10.07	2.93
			Genistein treatment	-2.714	3.094	.392	-9.21	3.79
		Genistein treatment	Control	-.857	3.094	.785	-7.36	5.64
			H.pylori infection	2.714	3.094	.392	-3.79	9.21

BW_D15	Tukey HSD	Control	*H.pylori* infection	7.571	3.211	.073	-.62	15.77
			Genistein treatment	5.571	3.211	.220	-2.62	13.77
		H.pylori infection	Control	-7.571	3.211	.073	-15.77	.62
			Genistein treatment	-2.000	3.211	.810	-10.20	6.20
		Genistein treatment	Control	-5.571	3.211	.220	-13.77	2.62
			H.pylori infection	2.000	3.211	.810	-6.20	10.20
	LSD	Control	*H.pylori* infection	7.571*	3.211	.030	.82	14.32
			Genistein treatment	5.571	3.211	.100	-1.18	12.32
		H.pylori infection	Control	-7.571*	3.211	.030	-14.32	-.82
			Genistein treatment	-2.000	3.211	.541	-8.75	4.75
		Genistein treatment	Control	-5.571	3.211	.100	-12.32	1.18
			H.pylori infection	2.000	3.211	.541	-4.75	8.75
BW_D17	Tukey HSD	Control	*H.pylori* infection	7.714	3.104	.057	-.21	15.64
			Genistein treatment	5.571	3.104	.200	-2.35	13.49
		H.pylori infection	Control	-7.714	3.104	.057	-15.64	.21
			Genistein treatment	-2.143	3.104	.772	-10.07	5.78
		Genistein treatment	Control	-5.571	3.104	.200	-13.49	2.35
			H.pylori infection	2.143	3.104	.772	-5.78	10.07
	LSD	Control	*H.pylori* infection	7.714*	3.104	.023	1.19	14.24
			Genistein treatment	5.571	3.104	.090	-.95	12.09
		H.pylori infection	Control	-7.714*	3.104	.023	-14.24	-1.19
			Genistein treatment	-2.143	3.104	.499	-8.66	4.38
		Genistein treatment	Control	-5.571	3.104	.090	-12.09	.95
			H.pylori infection	2.143	3.104	.499	-4.38	8.66

BW_D19	Tukey HSD	Control	*H.pylori* infection	5.857	2.947	.144	-1.66	13.38
			Genistein treatment	4.286	2.947	.336	-3.24	11.81
		H.pylori infection	Control	-5.857	2.947	.144	-13.38	1.66
			Genistein treatment	-1.571	2.947	.856	-9.09	5.95
		Genistein treatment	Control	-4.286	2.947	.336	-11.81	3.24
			H.pylori infection	1.571	2.947	.856	-5.95	9.09
	LSD	Control	*H.pylori* infection	5.857	2.947	.062	-.33	12.05
			Genistein treatment	4.286	2.947	.163	-1.91	10.48
		H.pylori infection	Control	-5.857	2.947	.062	-12.05	.33
			Genistein treatment	-1.571	2.947	.600	-7.76	4.62
		Genistein treatment	Control	-4.286	2.947	.163	-10.48	1.91
			H.pylori infection	1.571	2.947	.600	-4.62	7.76
BW_D21	Tukey HSD	Control	*H.pylori* infection	4.714	2.956	.273	-2.83	12.26
			Genistein treatment	2.714	2.956	.636	-4.83	10.26
		H.pylori infection	Control	-4.714	2.956	.273	-12.26	2.83
			Genistein treatment	-2.000	2.956	.780	-9.54	5.54
		Genistein treatment	Control	-2.714	2.956	.636	-10.26	4.83
			H.pylori infection	2.000	2.956	.780	-5.54	9.54
	LSD	Control	*H.pylori* infection	4.714	2.956	.128	-1.50	10.93
			Genistein treatment	2.714	2.956	.371	-3.50	8.93
		H.pylori infection	Control	-4.714	2.956	.128	-10.93	1.50
			Genistein treatment	-2.000	2.956	.507	-8.21	4.21
		Genistein	Control	-2.714	2.956	.371	-8.93	3.50

	treatment	*H.pylori* infection	2.000	2.956	.507	-4.21	8.21

*. The mean difference is significant at the 0.05 level.

VITA

NAME Miss	Nisarat Phetnoo
DATE OF BIRTH	06 August 1985
PLACE OF BIRTH	House No. 65/6 Village No.6, Dontago Sub-distric, Tasala District, Nakhon si thammarat Province 80160
EDUCATION	Doctor of Veterinary Medicine (D.V.M.), Mahanakorn University of Technologt, 2010