Effectiveness of Chitosan-Curcumin Preparation in Management of Oral and Gastric Ulcers in Experimental Animals

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ชื่อวิทยานิพนธ์ ประสิทธิภาพของยาเตรียมไคโตแซน-เคอร์คูมินต่อการจัดการโรคแผลในปากและแผลกระเพาะอาหารในสัตว์ทดลอง

ผู้เขียน นางสาว สินีนาฏ ขวดแก้ว

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บทคัดย่อ

Curcumin เป็นสาร lipophilic polyphenol ที่สกัดจากเหง้าของสมุนไพรขมิ้นชัน (*Curcuma longa* Linn) มีรายงานถึงคุณสมบัติการรักษาโรคที่หลากหลายซึ่งรวมถึงฤทธิ์ต้านอนุมูลอิสระ ต้านการอักเสบ ต้านการเกิดแผลเปื่อย และกระตุ้นการหายของแผล อย่างไรก็ตาม สาร curcumin มีข้อจำกัดสำหรับการนำไปใช้ทางคลินิกเนื่องจากมีความสามารถในการละลายน้ำน้อย มีค่าชีวปริมาณออกฤทธิ์ (bioavailability) ต่ำ ขนาดยารับประทานต่อวันสูงและต้องบริหารยาบ่อยครั้ง สาร chitosan เป็น polysaccharide biopolymer ที่รู้จักกันดีในคุณสมบัติด้านการยึดเกาะกับเยื่อบุผิวและการเพิ่มการซึมผ่านเข้าเซลล์ของยา ซึ่งมีประโยชน์ในการคงระดับความเข้มข้นของสาร curcumin ในเยื่อบุผิวช่องปากและกระเพาะอาหารรวมทั้งเพิ่มค่าชีวปริมาณออกฤทธิ์ ฤทธิ์ต้านอนุมูลอิสระ ต้านการอักเสบและกระตุ้นการหายของแผล สาร chitosan ยังช่วยเสริมประสิทธิภาพในการป้องกันและรักษาแผลของสาร curcumin ดังนั้น ยาเตรียม chitosan-curcumin จึงได้ถูกพัฒนาขึ้นและทำการประเมินประสิทธิภาพและกลไกการออกฤทธิ์ในการจัดการแผลในช่องปากและกระเพาะอาหารในสัตว์ทดลอง

ยาน้ำรับประทานรูปแบบยาผสม chitosan-curcumin (chitosan-curcumin mixture) สำหรับแผลกระเพาะอาหาร เตรียมโดยใช้ 0.1 โมลาร์ acetic acid เป็นตัวทำละลาย ยาน้ำผสมที่ได้มีลักษณะสีเหลืองสม่ำเสมอ มีค่าพีเอช (pH) อยู่ในช่วงค่าพีเอชของกระเพาะอาหาร การศึกษาในหนูแร็ท (rat) พบว่า ยาน้ำรับประทานรูปแบบยาผสม chitosan-curcumin ที่มีสัดส่วนการผสมของสาร curcumin (20 มิลลิกรัม) และสาร chitosan (150 มิลลิกรัม) ให้ประสิทธิภาพเหนือกว่ายารักษาแผลมาตรฐาน lansoprazole ในการป้องกันและรักษาแผลกระเพาะอาหารเฉียบพลันที่ถูกชักนำโดยยา

ต้านอักเสบที่ไม่ใช่สเตียรอยด์ indomethacin เป็นที่น่าสนใจอย่างยิ่งว่า การบริหารยาน้ำรับประทานรูปแบบยาผสม chitosan-curcumin เพียงวันละครั้ง มีประสิทธิภาพในการกระตุ้นการหายของแผลเทียบเท่ากับการบริหารสาร curcumin สาร chitosan หรือ ยามาตรฐาน lansoprazole วันละ 2 ครั้ง ในการรักษาแผลเปื่อยกระเพาะอาหารที่เกิดจากการชักนำด้วย acetic acid ซึ่งมีลักษณะของการเกิดพยาธิวิทยาและการหายของแผลคล้ายคลึงกับแผลเปื่อยกระเพาะอาหารในมนุษย์ การตรวจสอบฤทธิ์ทางเภสัชวิทยาในฤทธิ์ต้านอนุมูลอิสระและต้านการอักเสบนอกร่างกาย (*in vitro*) รวมไปถึงฤทธิ์ต้านอนุมูลอิสระ ต้านการอักเสบ ยับยั้งการหลั่งกรดและกระตุ้นการสร้างเมือกในหนูแร็ท (*in vivo*) บ่งชี้ว่ายาน้ำรับประทานรูปแบบยาผสม chitosan-curcumin มีฤทธิ์ยับยั้งการหลั่งกรดต่ำกว่ายามาตรฐาน lansoprazole แต่มีฤทธิ์กระตุ้นการสร้างเมือก ต้านอนุมูลอิสระ และต้านการอักเสบ สูงกว่าสาร curcumin สาร chitosan และยามาตรฐาน lansoprazole การวิเคราะห์ด้วยวิธี RT-PCR และ qRT-PCR ต่อการแสดงออก (expression) ของเอนไซม์ COX และ NOS ในเนื้อเยื่อแผลกระเพาะอาหาร แสดงให้เห็นว่า ยาน้ำรับประทานรูปแบบยาผสม chitosan-curcumin มีฤทธิ์ต้านการอักเสบโดยยับยั้งการแสดงออกของเอนไซม์ที่ส่งเสริมการอักเสบ (pro-inflammatory enzymes) COX-2 และ iNOS รวมไปถึงกระตุ้นการแสดงออกของเอนไซม์ที่ช่วยปกป้องเยื่อบุกระเพาะอาหาร (cytoprotective enzymes) eNOS มีฤทธิ์ยับยั้งการหลั่งกรดโดยกระตุ้นการแสดงออกของเอนไซม์ที่ช่วยปกป้องเยื่อบุกระเพาะอาหาร COX-1 และ nNOS และมีฤทธิ์กระตุ้นการสร้างเมือกในกระเพาะอาหารโดยกระตุ้นการแสดงออกของเอนไซม์ที่ช่วยปกป้องเยื่อบุกระเพาะอาหาร COX-1 และ nNOS

ยาเตรียมเฉพาะที่น้ำยาบ้วนปาก chitosan-curcumin (chitosan-curcumin mouthwash) สำหรับแผลในช่องปาก เตรียมโดยการละลายสาร curcumin 0.1 กรัม ในระบบตัวทำละลายร่วมที่ประกอบด้วยสาร chitosan 0.5 กรัม ในสารละลาย acetic acid ความเข้มข้น 1% และ สารละลาย polyethylene glycol 400 40 มิลลิลิตร น้ำยาบ้วนปากที่พัฒนาได้มีลักษณะสีเหลืองใสและมีค่าพีเอชอยู่ในช่วงค่าพีเอชที่เหมาะกับการใช้ในช่องปาก ทำการประเมินประสิทธิภาพการรักษาแผลในช่องปากต่อแผลเยื่อเมือกกระพุ้งแก้มที่เกิดจากการเหนี่ยวนำด้วย acetic acid ในหนูแฮมสเตอร์ซึ่งมีระยะการเกิดพยาธิสรีรวิทยาของแผลคล้ายกับการเกิดแผลในช่องปากที่ถูกชักนำโดยรังสีบำบัดหรือเคมี

บำบัด การบริหารน้ำยาบ้วนปาก chitosan-curcumin วันละสองครั้งเป็นเวลา 7 วันติดต่อกัน สามารถลดความรุนแรงของการเกิดแผลได้อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) มีประสิทธิภาพในการกระตุ้นการหายของแผลสูงกว่าการบริหารด้วยน้ำยาบ้วนปากมาตรฐาน benzydamine โดยผลทางเนื้อเยื่อวิทยาแสดงถึงการเกิดการสร้างเนื้อเยื่อบุผิว (epithelization) ของแผล การหดรั้งของเนื้อเยื่อแผลที่ซ่อมแซมแล้ว (wound contraction) และการปรับเปลี่ยนโครงสร้างเนื้อเยื่อที่สร้างขึ้นใหม่ (tissue remodelling) อย่างสมบูรณ์ ยาน้ำรับประทานในรูปแบบยาผสม chitosan-curcumin มีประสิทธิภาพในการรักษาแผลเช่นเดียวกันโดยมีค่า histological healing score ใกล้เคียงกับกลุ่มที่ได้รับน้ำยาบ้วนปาก chitosan-curcumin

นอกจากนี้ การทดสอบฤทธิ์กระตุ้นการหายของแผลด้วยวิธี scratch แสดงให้เห็นว่า ยาน้ำผสม chitosan-curcumin สาร curcumin และสาร chitosan มีประสิทธิภาพในการกระตุ้นการเพิ่มจำนวน (proliferation) และการเคลื่อนตัว (migration) ของเซลล์ HGF human gingival fibroblast และ เซลล์ AGS human gastric epithelial cell เทียบเท่ากัน

ผลการสืบค้นศักยภาพทางเภสัชวิทยาที่ได้จากการศึกษาในปัจจุบัน บ่งชี้ถึงประโยชน์ของยาเตรียม chitosan-curcumin ที่มีขนาดยาที่ต่ำของสาร curcumin และมีความถี่ในการบริหารยาต่ำ ในการใช้เป็นยาทางเลือกที่มีศักยภาพในการจัดการโรคแผลกระเพาะอาหารและโรคแผลในปาก สิ่งสำคัญที่ต้องตระหนักถึงคือ ทั้งสาร curcumin และสาร chitosan มีฤทธิ์แรงในการยับยั้งการแสดงออกของเอนไซม์ iNOS และ COX ดังนั้น ยาเตรียม chitosan-curcumin สามารถออกฤทธิ์ที่ขัดแย้งกันระหว่างฤทธิ์ป้องกันการเกิดแผลและฤทธิ์กระตุ้นการกำเริบของแผล หรือฤทธิ์รักษาแผลและฤทธิ์กระตุ้นการกลับเป็นซ้ำของแผลขึ้นอยู่ความสัมพันธ์ของขนาดที่ใช้และผลการรักษา

Thesis Title Effectiveness of Chitosan-Curcumin Preparation in Management of Oral and Gastric Ulcers in Experimental Animals

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ABSTRACT

Curcumin, a lipophilic polyphenol extracted from the rhizomes of *Curcuma longa* Linn, has been reported to exert a variety of therapeutic properties including antioxidant, anti-inflammatory, antiulcer and wound healing. However, its limitations for further clinical use include its poor aqueous solubility, low bioavailability, large oral daily dosing and frequent drug administration. Chitosan is a well-known polysaccharide biopolymer with bioadhesive and drug penetration enhancing properties that could be beneficial in enhancing the substantivity in the oral and gastric mucosa, including the bioavailability through the gastric mucosa of curcumin. Its anti-oxidant, anti-inflammatory and ulcer-healing properties could also enhance the antiulcer efficacy of curcumin. Accordingly, a chitosan-curcumin preparation was developed and evaluated for its efficacy and mechanisms of antiulcer action in management of oral and gastric ulcers in experimental animals.

An oral chitosan-curcumin mixture used for gastric ulcers was prepared using 0.1 M acetic acid as a solvent. The developed mixture was a uniform yellowed-color mixture with an optimal gastric pH range. Using a rat model, a chitosan-curcumin mixture with a combination ratio of curcumin (20 mg) and chitosan (150 mg) was

superior to curcumin, chitosan or a standard antiulcer agent (lansoprazole) in prevention and treatment of acute gastric ulcers induced by non-steroidal anti-inflammatory drug (indomethacin). Interestingly, once-daily administration of an oral chitosan-curcumin mixture exerted comparable ulcer healing efficacy to twice-daily administration of curcumin, chitosan or a standard lansoprazole in treating acetic acid-induced chronic gastric ulcer in which the pathological aspects and healing process highly resembles a human chronic gastric ulcer. The pharmacological investigation on *in vitro* antioxidant and anti-inflammatory activities including *in vivo* antioxidant, anti-inflammatory, antisecretory and gastric mucus producing activities in rats indicated that an oral chitosan-curcumin mixture possessed a lower potent antisecretory activity than lansoprazole but exerted the highest gastric mucus producing, anti-oxidant and anti-inflammatory activities among curcumin, chitosan and lansoprazole. RT-PCR and real time RT-PCR (qRT-PCR) analysis on the expression of COX and NOS in the gastric ulcerated tissue revealed that a chitosan-curcumin mixture exerted anti-inflammatory activity through a down-regulation of pro-inflammatory COX-2 and iNOS expression including an up-regulation of cytoprotective eNOS expression, antisecretory activity through an up-regulation of cytoprotective COX-1 and nNOS expression and gastric mucus producing activity through an up-regulation of cytoprotective COX-1 and nNOS expression.

A topical chitosan-curcumin mouthwash used for oral ulcers was prepared by dissolving 0.1 g of curcumin in a co-solvent system composed of 0.5 g of chitosan in 1% acetic acid solution and 40 ml of polyethylene glycol 400. The developed mouthwash was a clear solution with an optimum pH range to use in the human oral cavity. Its oral ulcer healing efficacy was evaluated on acetic acid-induced buccal

mucosal ulcer in hamsters of which pathophysiologic ulcer phase is similar to that of radiation or chemotherapy-induced oral ulcers. The application of the mouthwash twice a day for 7 consecutive days significantly decreased the ulcer severity ($p<0.05$) with a better ulcer healing efficacy than those of a standard 0.15% benzydamine mouthwash with histological evidences on its beneficial effects of complete epithelization, wound contraction and tissue remodelling. An oral chitosan-curcumin mixture also exerted a comparable histological healing score to that of a topical chitosan-curcumin mouthwash.

Additionally, the scratch wound healing assay demonstrated that a chitosan-curcumin mixture, curcumin and chitosan had comparable efficacy in stimulating HGF human gingival fibroblast cell and AGS human gastric epithelial cell proliferation and migration.

The potential pharmacological findings obtained from the present studies indicated the benefit of a chitosan-curcumin preparation with a low dose of curcumin and low frequent drug administration as a potential alternative in management of gastric ulcer and oral ulcer. It is important to recognize that both curcumin and chitosan possess a potent down-regulation of iNOS and COX expression, therefore, a chitosan-curcumin preparation can exert the opposing effects of prevention and exacerbation of ulcer or ulcer healing and ulcer relapse depending on the dose-effect relationship.

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LIST OF ABBREVIATIONS AND SYMBOLS

5-HT	serotonin
AA	arachidonic acid
Ach	acetylcholine
AUC	area under the plasma concentration-time
bid	bis in die
BH_4	tetrahydrobiopterin
CaM	calmodulin
CAPE	caffeic acid phenethylester
CAT	catalase
CCK_2	cholecystokinin receptor 2
cDNA	complementary DNA
CGRP	calcitonin gene related peptide
CMC	carboxylmethycellulose sodium salt
cNOS	constitutive nitric oxide synthase
CO_2	cabon dioxide
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
CSE	cystathione-γ-lyase
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
ECE-1	endothelin-converting enzyme-1

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

ECL	enterochromaffin-like
e.g.	for example
EGF	epithelial growth factor
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ENS	enteric nervous system
et al	et alibi
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FDA	Food and Drug Administration
FFA	free fatty acid
FGF	fibroblast growth factor
FMN	flavin mononucleotide
g	gram
G	gastrin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GMBF	gastric mucosal blood flow
GPCR	G-protein coupled receptors
GPx	glutathione peroxidase
GRAS	generally recognized as safe
GST	glutathione *S*-transferases
GU	gastric ulcer
h	hour

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

H	histamine receptor
H&E	hematoxylin and eosin
H^+, K^+-APTase	hydrogen-potassium adenosine triphosphate
HCl	hydrochloric acid
HCO_3^-	bicarbonate
HGF	human gingival fibroblast
HI	healing index
HMW	high molecular weight
HTAB	hexadecyltrimethylammonium bromide
H_2O_2	hydrogen peroxide
HOCl	hypochlorous
H_2RAs	histamine-2 receptor antagonists
H_2S	hydrogen sulphide
IC_{50}	the half maximal inhibitory concentration
ICAM-1	intercellular adhesion molecule-1
i.d.	intraduodenal
IL	interleukin
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
kg	kilogram
KH_2PO_4	potassium dihydrogen ortho-phosphate
LMW	low molecular weight
L-NA	L-Nitroarginine

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

LOX	lipoxygenase
LPs	phospholipids
LPS	lipopolysaccharide
LTB_4	leukotriene B4
MAPK	mitogen-activated protein kinase
mcg	microgram
mg	milligram
min	minute (time)
MIC	minimum inhibitory concentration
ml	milliliter
mm^2	square millimeter
mm	millimeter
M	muscarinic receptor
MDA	malondialdehyde
MMPs	metalloproteinases
MPO	myeloperoxidase
MRI	mucosal regeneration index
MT	melatonin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
n	number of observations (sample size)
N	nicotinic receptor
n.d.	no data, not determined

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

NADPH	nicotinamide adenine dinucleotide phosphate
NAG-1	nonsteroidal anti-infalmmatory drug activated gene-1
NaOH	sodium hydroxide
Na_2CO_3	sodium carbonate
NF-κB	nuclear factor kappa B
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NSAID	non steroidal anti-inflammatory drugs
$O_2^{-\cdot}$	superoxide anion
od	once daily
ODC	ornithine decarboxylase
$^{\bullet}OH$	hydroxyl radical
$OONO^-$	peroxynitrite
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PEG 400	polyethylene glycol 400
PGs	prostaglandins
PGE_2	prostaglandin E2
PGG_2	prostaglandin endoperoxide
PGI_2	prostacyclin
pH	potential of hydrogen
pKa	negative log of Ka (acid dissociation constant)
PLA_2	phospholipase A_2

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

PMN	polymorphonuclear
PPAR	peroxisome proliferator-activated receptors
PPIs	proton pump inhibitors
p.o.	per oral
qRT-PCR	quantitative real-time polymerase chain reaction
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
s	second (time)
SD	standard deviation
SEM	standard error of mean
SOD	superoxide dismutase
SPs	stress protein
SST	somatostatin
std	standard
TAE	tris-acetate-EDTA
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TFFs	trefoil factors
TGF	transforming growth factor
TMB	3,3′,5,5′-tetramethylbenzidine

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
w/v	weight per volume
w/w	weight per weight
α	Alpha
β	Beta
°C	degree Celsius
µ	micro
=	equal to
>	greater than
±	plus or minus
%	percent
®	trade name
:	ratio

CHAPTER 1

INTRODUCTION

1.1. Background and rationale

Gastric ulcer (GU) remains one of the most common chronic upper gastrointestinal (UGI) tract diseases that result in impaired quality of life, work loss and high-cost medical care especially in whom with severe complications including hemorrhages, perforation, GI obstruction and malignancy. It is generally caused by the disruption of the normal balance between aggressive factors [e.g. gastric acid, non-steroidal anti-inflammatory drugs (NSAIDs), and *H. pylori*] and defense factors [e.g. gastric bicarbonate, prostaglandins (PGs), mucus and mucosal blood circulation]. In 80% of the cases, GU is caused primarily due to the long-term use of NSAIDs which is associated with the inhibition on the synthesis of endogenous cytoprotective PGs through cyclooxygenase (COX)-1 and PGs responsible for both inflammation and mucosal repair through COX-2 (1). The remaining about 20% of the cases is caused by various factors including *H. pylori* infection and excessive amount of gastric acid secreted by parietal cells (2,3). Current standard pharmacotherapy in prevention and treatment of NSAIDs induced GU is proton pump inhibitors (PPIs). However, many GU patients still suffered from ulcer recurrence or refractory GU (nonhealing ulcer). Moreover, the long-term use of PPIs especially in elderly patients may increase a risk of fundic gland polyps (through an increased levels of gastrin or hypergastrinaemia resulting from gastric acid suppression and its trophic effects on stomach mucosa), community–acquired pneumonias and infective

diarrhea (through induction of hypochlorhydria which leading to an alteration of the bacterial content of the gut), and hip fractures (through interfering in insoluble calcium absorption or inhibition of osteoclastic vacuola proton pumps that leading to a reduced bone resorption) (4–6). In recent year, several studies have shown that the presence of reactive oxygen and nitrogen species; pro–inflammatory cytokines (such as TNF-α, IL-1β and IL–6) and pro–inflammatory mediators [such as PGE_2 and nitric oxide (NO) generated from the induction of inducible nitric oxide synthase (iNOS)]; all may play some part in the pathophysiology of NSAIDs induced GU (7). Hence, NSAIDs induced GU remains to be further investigated to develop more effective treatment regimens.

Oral or mouth ulcers are painful ulcerative lesions usually occur on the oral mucosal epithelium inside the mouth, on the cheek, lips and sides of the tongue. Oral ulcer can be caused by a number of factors including stomatotoxic agents especially chemotherapeutic agents and radiation, immune-mediated conditions (such as aphthous ulcer and lichen planus), drug hypersensitivity reactions and trauma. It has been found that about 40% of patients treated with standard chemotherapeutic regimens, 30-60% of patients receiving radiation therapy for cancer of the head and neck and more than 90% of patients receiving combination chemotherapy and localized radiation therapy will be affected (8). These oral ulcers frequently contribute to the interference of the therapy leading to both tumor recurrence and worsening the patient’s prognosis. Severe cases of oral ulcer with infection can lead to life-threatening septicemia and contribute indirectly to increased hospital stays and overall cost of treatment which is a significant economic burden (9,10). The activation of reactive oxygen species (ROS) and their subsequent ability to stimulate a number of

transcription factors such as nuclear factor kappa B (NF-κB) which regulate inflammatory responses and the expression of various pro-inflammatory genes seem to characterize the acute tissue response to a stomatotoxic challenge and are considered the hallmark of the initiation phase of oral ulcers leading to other biologic events. An increase of pro-inflammatory cytokines (such as tumor necrosis factor-alpha (TNF-α), interleukins (IL)-1β, IL-6 and IL-8) production and leukocyte recruitment has also been found to correlate with the extent of non-hematologic toxicities in patients following chemotherapy. Recently, it has been found that intervention with antimicrobial, analgesic, anesthetic, antioxidant and anti-inflammatory agents is benefit for patients receiving radiation and/or chemotherapy regimens (11). Although, topical oral viscous lidocaine has been widely used for palliation of oral ulcer pain, there is no much effort for scientific and the limitations include local discomfort and insensitive affecting the sensation of taste and the gag reflex. The toxic reactions associated with an accidental overdose have also been reported in pediatric cases and in adult case of frequent viscous lidocaine use when the amount of oral viscous lidocaine exceeded 240 ml per day (12). A standard benzydamine mouthwash (Difflam®) with analgesic, anesthetic, anti-inflammatory and antimicrobial properties is potentially beneficial in reducing the incidence of ulceration and erythema. Nevertheless, it is expensive and has some adverse effects as causing a burning sensation and discomfort. The alcohol content in the mouthwash formulation may also often cause irritation to the inflamed mucosa. Hence, effective medication with alcohol free formulation remains to be investigated for more successful outcomes.

At present, medicinal plants are becoming important sources of biologically active compounds with a variety of pharmacological activities especially antioxidant, anti-inflammatory, analgesic, antimicrobial and wound healing activities. Medicines derived from plants are becoming popular in the healthcare of many cultures, both ancient and modern, due to their broad pharmacological activities, low toxicity and fewer side-effects. Some medicinal plants with antioxidant, anti-inflammatory, wound healing and antimicrobial activities seem to be useful in reducing the severity and treatment of oral ulcer and GU.

Clinacanthus nutans (Burm. F.) Lindau or payayor, a Thai medicinal plant containing flavonoids and glycosides, has been found to be superior to benzydamine in relieving radiation-induced oral ulcers in patients with head and neck cancer through its analgesic, antiviral and anti-inflammatory properties. It was also found that an application of glycerine payayor oral solution upon the mucosal lesion after 24 h of chemotherapy session, significantly reduced the incidence, duration and severity of oral ulcers while the onset was later than in the control period (13). However, the solution is not yet capable of completely preventing oral ulcers as the grade 1 mucositis still occur. In addition, glycerin containing in the preparation increase the sensation of a dry mouth. It is also generally recommended to avoid mouthwash containing alcohol or glycerin in cancer patient with bad mouth sores from chemotherapy or radiotherapy.

A number of herbal medicines including *Curcuma longa, Piper betel, Phyllanthus emblica, Nyctanthes arbortristis* and *Centella asiatica* have been claimed to have antiulcerogenic effect in treating GU with a comparable or superior efficacy to standard antiulcer drugs such as PPIs in various animal models. The mechanisms

by which herbal medicines benefit GU include stimulation of cell proliferation or wound healing, stimulation of mucus production, inhibition of gastric acid secretion and anti-oxidation and anti-inflammatory activities. In Thailand, there is only one limited clinical trial publication of *Curcuma longa* rhizome powder or turmeric in 54 Thai patients with symptoms indicating GU. It was found that the percentages of ulcer healing in patient receiving 2 capsule-filled with turmeric (300 mg/capsule) five times daily at 4, 8 and 12 weeks after treatment were 48, 72 and 76% respectively. No serious adverse effects were found in all patients (14). However, an administration regimen of the rhizome powder with two capsules five times daily may decrease the patient compliance or cause poor patient medication adherence that may lead to the therapeutic compliance. The main antiulcer active ingredient in turmeric has been claimed to be curcumin (diferuloylmethane), a main yellow oil-soluble pigment that belongs to curcuminoid, a group of polyphenolic pigment.

Curcumin has been reported to exert variety of biological and pharmacological activity including antioxidant activity in scavenging a variety of ROS including superoxide anion radicals, hydroxyl radicals and nitrogen dioxide radicals (such as peroxynitrite ($ONOO^-$) (15,16); anti-inflammatory activity in suppressing the synthesis of PGs and pro-inflammatory leukotrienes through cyclooxygenase and lipoxygenase (LOX) pathway, respectively (17); an inhibition on the release of pro-inflammatory cytokines from the activated macrophage such as TNF-α, interleukins (IL-1-2, -6, -8 and IL-12) and NO derived from iNOS; antibacterial against oropharyngeal bacterial pathogens and *H. pylori* (18–21); antifungal against *Candida albican*s (22,23); analgesic (24) and wound healing activities (25,26). Curcumin has also been found to be an effective gastroprotective agent by stimulating secretion of

gastric mucus (at doses lower than 50 mg/kg) (27), Furthermore, curcumin fed at 20-80 mg/kg effectively inhibited gastric acid secretion in pylorus-ligated rats, prevented the development of acute gastritis induced by various ulcerogenic agents including ethanol, serotonin (5-HT) and compound 48/80 and also accelerated the healing of chronic GU induced by acetic acid in rats (28). This makes curcumin an agent of potential interest for treating oral ulcers and NSAIDs induced GU. However, the poor aqueous solubility to the basic pH range and the low bioavailability of curcumin limit its therapeutic efficacy (29). In addition, there is a limitation in the antiulcer prophylactic or therapeutic dosage level of curcumin that the prophylactic dosage should not be more than 40 mg/kg/day whereas the therapeutic dosage level should not be more than 160 mg/kg/day since curcumin can exert opposite effects to either delay ulcer healing or to exacerbate ulcer inflammation through some curative mechanisms differently modified by curcumin dosage (28). Recently, various formulation strategies have been carried out to improve the stability, bioavailability and pharmacokinetic properties of curcumin in treatment of GU using self-microemulsoifying liquid and pellet technology (30) or the floating drug delivery technology such as a raft forming system (31). However, there is no pharmacological evidence on the exact interaction mechanism in healing GU between curcumin and the constituents of these novel drug delivery systems. Moreover, these novel drug delivery systems have high cost expenditure.

Chitosan is a linear polysaccharide biopolymer derived from chitin shells of crustaceans. The amino and hydroxyl functional groups in chitosan, leads to unique polycationic, chelating and film-forming properties. It is soluble in dilute aqueous acid solution, but is insoluble in water and common organic solvents (32). The natural

biocompatible, biodegradable, low-toxic and non-allergenic properties of chitosan make it received much intention in biomedical, food, pharmaceutical, agricultural and environmental industries. Chitosan is also used to enhance the stability of the drug in which the drugs are loaded in chitosan film or chitosan nanoparticles, resulting in enhancement of drug accumulation and the delivery of drugs to the target site (33). In addition, chitosan has been claimed to have various biological effects including anti-oxidant, anti-inflammatory, anti-allergic, anti-microbial, anti-cancer effects and antiulcer against ethanol-induced gastric mucosal lesions (34,35). Nevertheless, there is still no study on its antiulcer efficacy against NSAIDs-induce GU. Moreover, chitosan has been used to prevent or treat wound and burn infections due to its intrinsic antimicrobial, antioxidant and anti-inflammatory properties, its ability to deliver extrinsic antimicrobial agents to wounds or burns (36) and its efficacy to accelerate the functions of inflammatory cells and fibroblasts (37,38).

Considering the safety profile, bioadhesive nature, an ability to increase the oral bioavailability of the highly variable oral absorption and low bioavailability drugs, and the beneficial pharmacological properties of chitosan; the preparation of chitosan-curcumin combination can be extremely beneficial to maintain the concentration of curcumin in oral and gastric cavity and enhance the ulcers healing efficacy of curcumin in NSAIDs-induced GU and chemical induced oral ulcer. Thus, a simple preparation method of chitosan-curcumin combination is developed and being investigated for the pharmacological interaction mechanism between curcumin and chitosan in management of NSAIDs-induced GU and chemical induced oral ulcer *in vitro* and *in vivo* models. The potential pharmacological results obtain from the animal studies will be useful for further clinical study on the treatment of NSAIDs

induced GU and oral inflammatory ulcer. In addition, low oral daily dosing and low dosing frequency of a chitosan-curcumin preparation may increase patient compliance or medication adherence and decrease adverse drug reaction of curcumin. As gastrointestinal mucositis is increasingly becoming recognized as a toxicity associated with high-dose of standard chemotherapy regimens commonly used for treatment of cancer, a preparation of chitosan-curcumin combination by a simple method will also be beneficial as a safe, cheap and effective adjunct intervention in treatment of chemotherapy induced gastrointestinal mucositis especially in primary care unit.

1.2. Objective

1. To evaluate the effect of chitosan-curcumin preparation on NSAID-induced GU and chemical induced oral ulcer in experimental animals.

2. To investigate the mechanism of action if a chitosan-curcumin preparation was experimentally effective against any of experimental animals for GU and oral inflammatory ulcer.

CHAPTER 2

REVIEW OF LITERATURE

NSAIDs-induced GU

Physiology of the stomach

The stomach is a digestive part of alimentary canal, having a capacity of about 1,000-1,500 ml. It is situated between the lower esophageal sphincter and pyloric sphincter. The stomach can be separated into five regions. The first part is *cardia* region that helps to prevent stomach contents from going back up into the esophagus. The *fundus* region is the dome-shaped located to the left of cardia in the stomach. The *corpus* and the *antrum* region are the largest and main part of the stomach. The *pyloric sphincter* region is a small point that regulates stomach emptying and the movement of material between the stomach and the duodenum (Figure 2-1A) (39).

Figure 2-1 panels B and C illustrate the microscopic invaginations of glands. 75% of the total numbers of gastric glands are oxyntic glands which secrete hydrochloric acid (HCl), intrinsic factor, mucus and pepsinogen. The remaining of 25% are pyloric glands (covering both the antrum and pylorus part of the stomach) which secrete gastrin into the circulation by G-cells. Specially, the parietal cells are located primarily in the oxyntic gland and secrete both HCl and intrinsic factor. HCl is responsible for the acid environment (pH 1.5-3.5) of the stomach to activate pepsinogen into pepsin (a protein-digesting enzyme) and to prevent the overgrowth of microbiota (40). Intrinsic factor is an important glycoprotein for the absorption of calcium and vitamin B_{12} in the small intestine. Surface epithelial cells, especially

mucus neck cells secrete mucin which is a high molecular weight glycoprotein that is capable to form a hydrated gel to protect the epithelium from the corrosive/poisonous effects of the acid and from the physical erosion of the stomach contents (41). The bicarbonate (HCO_3^-) secreted from the mucus neck cells will neutralize acid in the stomach as well. Thus, there is a gradient in pH from acidic in the lumen to near neutral pH adjacent to the cells that are covered with mucus. The chief cells are located primarily in the gastric glands and synthesize pepsinogen as a heterogeneous mixture of isozymes which contact with acid conversion of pepsinogen to pepsin (39).

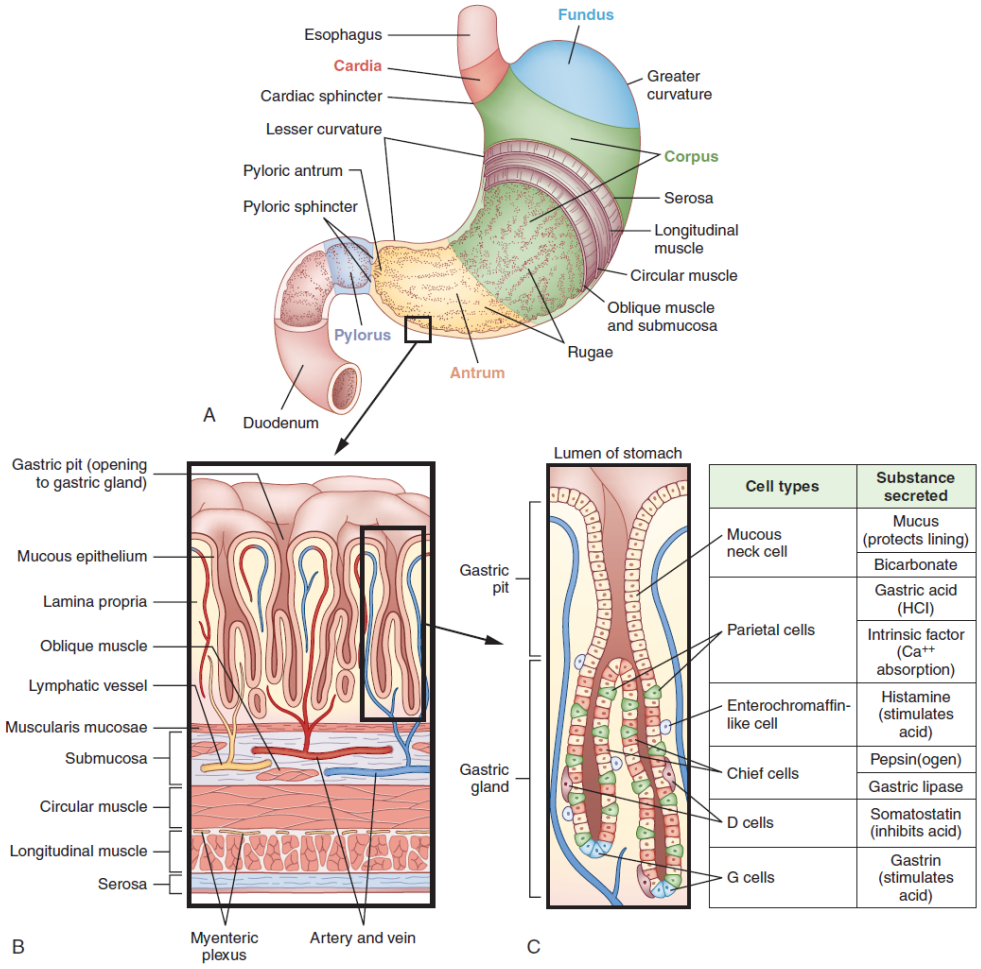


Figure 2-1 Structure of the stomach (40)

Gastric acid secretion is a complex, continuous process in which multiple central and peripheral factors contribute to a common end point: the secretion of H^+ by parietal cells. The parietal cell is stimulated by neuronal (acetylcholine, ACh), endocrine (gastrin, G) and paracrine (histamine, H) mediators as summarized in Table 2-1. Initiated by the sight, smell and taste of food; neurologic impulses from the CNS along cholinergic pathways stimulate the release of Ach which exerts its direct effect on the parietal cells via a muscarinic (M_3) receptor. Vagal stimulation also releases gastrin (polypeptide hormone) from G-cells in the antrum that can stimulate parietal cell to secret acid directly through binding with cholecystokinin (CCK_2) receptor located in the parietal cell. Both Ach and gastrin also promote the release of histamine from enterochromaffin-like (ECL) cells residing in close poximility to parietal cells and from the mast cell in the lamina propia, further increasing acid secretion by binding with H_2 receptor located on the basolateral membrane of parietal cells (39). These H_2 receptors are blocked by H_2 receptor antagonists (ranitidine, cimetidine and famotidine) (42). The H_2 receptor is the G-protein coupled receptors (GPCR) that activates the G_s–adenylyl cyclase–cyclic AMP–PKA pathway whereas ACh and gastrin signal through GPCRs that couple to the G_q–PLC-IP_3–Ca^{2+} pathway in parietal cells. Both the cyclic AMP and the Ca^{2+}-dependent pathways activate the hydrogen-potassium adenosine triphosphate (H^+, K^+-ATPase) or proton pump), which transports H^+ out of the cytoplasm into the secretory cancliculus where they exchanged for K^+ ions that across the parietal cell membrane via the ion channel. Thus, inhibition of the H^+, K^+-ATPase by proton pump inhibitors such as omeprazole and lansprazole inhibits the final step of HCl or gastric acid production and release by parietal cells.

Table 2-1 The production site, stimulus and action of mediators secreted in the antral part of the stomach

Mediators	Production site	Stimulus	Action
Acetylcholine (ACh)	Ending cholinergic nerve	Vagal efferent through a muscarinic receptor	Stimulate parietal cell to secret HCl
Gastrin (G)	G-cells	Presence of peptide and amino acid in the pyloric antrum	Stimulate parietal cell to secret HCl Promote gastric emptying
Histamine (H)	Enterochromaffin like (ECL) cell	Presence of food in the stomach	Stimulate parietal cell to secret HCl
Somatostatin (SST)	D cell Duodenum	Present of food in the stomach Sympathetic axon Stimulation	Inhibit gastric secretion, gastric emptying and motility

In the circumstance of overproduction of gastric acid to a low gastric pH <2, somatostatin (a potent acid secretion inhibitor) will be released from D-cells in the body and antral part to suppress further secretion of gastrin (39,43,44).

Pathogenesis of NSAIDs induced GU

Normally, gastric mucosa is prevented by the process of mucus layer, secretion of HCO_3^- for acid-neutralizing and replacement of epithelial cells by new cells produced. GU is occured when there is an overproduction of aggressive factors (e.g gastric acid, pepsin, NSAIDs and *H. pylori*) or the destruction of protective factors (e.g bicarbonate, PGs, mucus and mucosal blood flow). Nowadays, it has been found that about 80% of the cases, GU is caused primarily due to the use of non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, aspirin, ibuprofen,

etc. whereas about 10% of the cases is caused by *H. pylori* (45). The mechanisms of NSAIDs-induced GU can be divided into topical (local) actions and systemic actions (Figure 2-2).

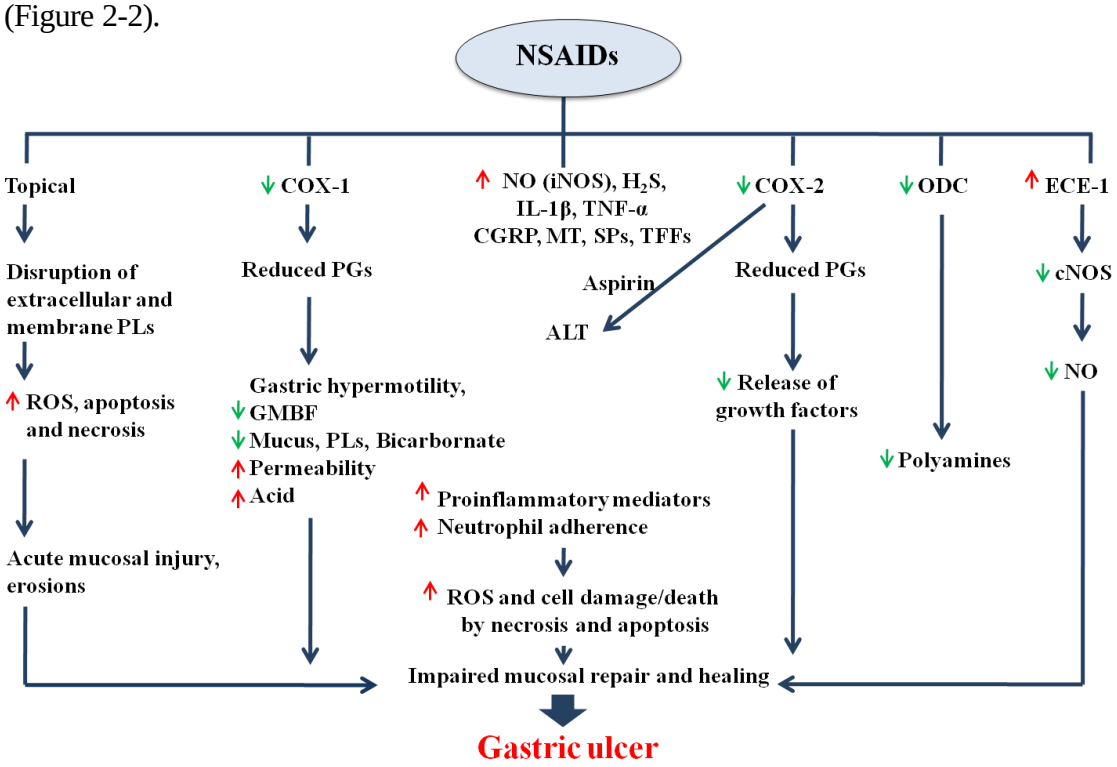


Figure 2-2 Cellular and molecular pathogenesis of NSAID-induced ulcers (46)

ATL: aspirin triggered lipoxin; COX-1: cyclooxygenase-1; COX-2: cyclooxygenase-1; LPs: phospholipids; CSE: cystathione-γ-lyase; ECE-1: endothelin-converting enzyme-1; cNOS: constitutive nitric oxide synthase; ODC: ornithine decarboxylase; NO: nitric oxide; H_2S: hydrogen sulphide; IL-1β: interlukin-1β; CGRP: calcitonin gene related peptide; MT: melatonin; SPs: stress protein; TFFs: trefoil factors; GMBF: gastric mucosal blood flow; ROS: reactive oxygen species

Topical actions The topical action of NSAIDs on the gastric epithelium might be involved various mechanisms. NSAIDs, particularly those of weak acidic nature (pKa 3-5) lead to initial mucosal erosions by disrupting the gastric epithelial cell

barrier and the carboxylic group of NSAIDs also contributes significantly to their water solubility and confers their detergent properties into the gastric cell by induction of osmotic lysis via trapping of charged NSAIDs (46,47). It has been suggested that NSAIDs accumulate in gastric epithelial cells results in the generation of reactive oxygen species (ROS) like superoxide (O_2^-) and hydroxyl radicals ($^{\bullet}OH$) which directly oxidize cellular proteins, lipids or nucleic acids leading to cell necrosis and apoptosis and finally the development of GU (48). NSAIDs can also disrupt the layer of surface-active phospholipids on the mucosal surface (independent of effects on PGs synthesis) that allows acid back diffusion, leading to the cell death (49,50).

Systemic action Mechanistically, inflammatory pathways can be divided into arachidonic acid (AA)-dependent and AA-independent pathways. The phospholipase A_2 (PLA_2), lipoxygenase (LOX) and cyclooxygenase (COX) pathways involve the metabolism of AA-dependent. In contrast, NF-κB, nitric oxide synthase (NOS), peroxisome proliferator activated receptors (PPAR) and NSAIDs- activated gene-1 (NAG-1) are classified as AA-independent (Figure 2-3) (51).

AA-dependent pathway (Figure 2-2, 2-3, 2-4)

The integrity of gastric mucosal defence depends on continuous generation of PGE_2 and prostacyclin (PGI_2), mediated by COX-1 and COX-2 which catalyse the rate-limiting step in the convension of AA to prostaglandin endoperoxide (PGG_2) and prostanoids. The prostaglandin type, receptors that mediate effects on mucosal defense and healing by each EP subtype and the role in various physiological and pharmacological responses were shown in Table 2-2 (46,52).

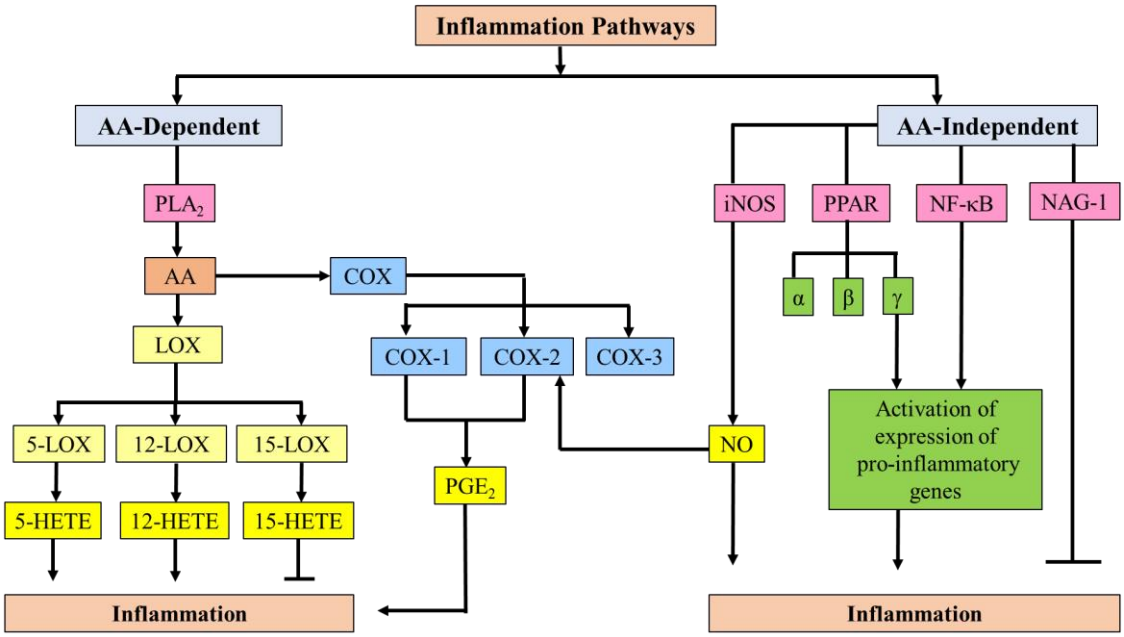


Figure 2-3 The inflammation pathways (51)

AA = arachidonin acid; PLA_2 = phospholipase A_2; LOX = lipoxygenase; COX = cyclooxygenase; PGE_2 = prostaglandin E_2; HETE = hydroxyeicosatetraenoic acid; NO = nitric oxide; iNOS = inducible nitric oxide synthase;

NF-κB = nuclear factor kappa B;

NAG-1= nonsteroidal anti-infalmmatory drug activated gene-1

PPAR = peroxisome proliferator activated receptors

(↓ = stimulation, ⊥ = inhibition)

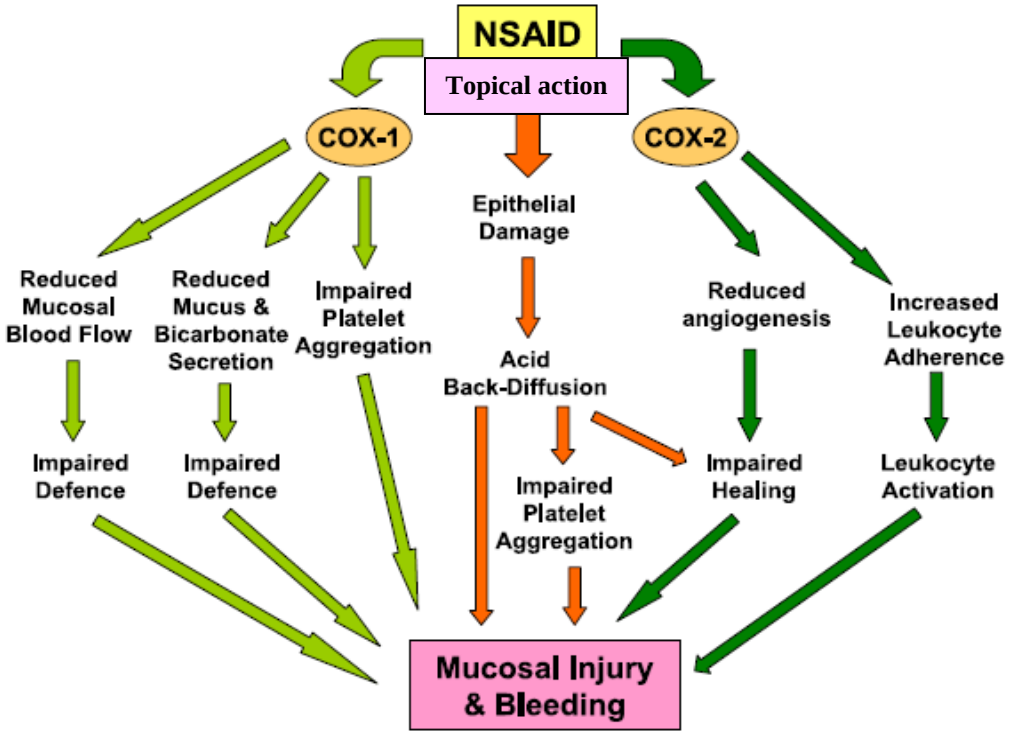


Figure 2-4 Pathogenesis of NSAIDs-induced gastric injury and bleeding through topical action and systemic action: Arachidonic (AA)-dependent pathway (52)

COX-1-derived prostaglandins (PGE_2) can inhibit the release of gastric acid via EP_3 receptor. This property is important for its gastric cytoprotective effect against various noxious stimuli (such as NSAIDs, indomethacinand, ethanol, etc) and its maintenance of a pH gradient that reduces acid back-diffusion and enhances the gastric mucosal integrity. The stimulation of epithelial cells also leads to the increase secretion of bicarbonate ion and mucus. Moreover, PGE_2 derived from COX-1 are potent vasodilators, producing effect in the stomach via EP2/EP4 receptors. The increase of gastric blood flow through vasodilatory effect of PGE_2 accelerates epithelial restoration and reduces acid back diffusion (53). It has been reported that an administration of COX-1 inhibitor leads to a significant release of endothelin-

converting enzyme 1 (ECE-1): a potent vasoconstrictor when the gastric mucosa is exposed to acid, resulting to a decrease of gastric mucosal blood flow that lead to an acid back diffusion (54). The ability of PGs to reduce gastric acid secretion, increase blood flow and enhance the regeneration of epithelial cells that promote of ulcer healing is likely attributed through PGs of the E and I series. In addition, PGE_2 has been shown to be a potent inhibitor of leukocyte adherence to the vascular endothelium including the release of histamine, TNF-α and IL-1β from macrophages; IL-B4 and IL-8 from neutrophils; and platelet-activating factor from mast cells (55). Furthermore, PGE_2 is found to activate the release of vascular endothelial growth factor (VEGF) via COX-2 induction in gastric fibroblasts stimulated by IL-1 (56,57). This beneficial effects of PGE_2 on gastric ulcer healing is mediated via the EP_4 receptor (58). Consequently, the underlying mechanism of PGs in inflammatory reaction differs depending on the period after the irritation. The early phase is mediated mainly by PGs derived from COX-1 for maintaining gastric mucosal integrity and by PGs derived from COX-2 for initiating inflammation, while the later phase is mediated by PGs derived from both COX-1 and COX-2 for promoting gastric ulcer healing.

Table 2-2 Prostaglandin receptor and COX isoforms in gastric mucosal defense in rat
Modified from references (46,52)

Type of PGs	**Gastroprotective Effect**	**Receptor(s)**	**COX isoform**
Prostaglandin E_2 (PGE_2)	- Protection against gastric injury induced by ethanol or indomethacin	EP1	COX-1
	- Stimulation bicarbonate secretion	EP1	
	- Decrease gastric hypermotility	EP1	
	- Mucosal blood flow	EP2, EP4	
	- Inhibition of gastric acid secretion	EP3	
	- Stimulation of gastric acid secretion	EP3	
	- Stimulation mucus secretion	EP4	
	- Ulcer healing	EP4	COX-2

AA-independent pathway

NF-κB is one of transcription factors that are activated in the initiation phase of inflammation. It appears to be a regulator of the inducible expression of many cytokine genes in lymphocytes, monocytes and epithelial cell in the GI tract. Following cytokine stimulation, the endogenous inhibitor of NF-κB is phosphorylated and NF-κB is released, translocates to the nucleus and activates transcription of multiple κB-dependent gene including TNF-α, IL-6, IL-8 and other chemokines (59).

TNF-α and IL-1β are two main cytokines released from activated monocytes and macrophages at an early inflammatory phase and play as important role in the regulation of inflammatory responses (60). TNF-α can activate resident macrophage

and promote the release of other pro-inflammatory mediators such as NO, PGs and platelet-activating factor. Additionally, TNF-α induces expression of adhesion molecules on vascular endothelium and the influx of new inflammatory cells into the mucosa. IL-1β stimulates the production of inflammatory eicosanoids (PGE_2 and LTB_4) (61) and the production of IL-8 (62). TNF-α and IL-1β potentiate the production of each other and also act together to mediate both inflammation in activating endothelial cells and neutrophil and stimulating the release of lipid mediators and wound or ulcer healing in synthesis and proliferation of epithelial cells and fibroblasts (63,64). Moreover, it has been found that IL-1β increases the resistance of gastric mucosa against injury and reduces the severity of ulcerative damage through its action as a potent inhibitor of gastric acid secretion, stimulator of PGE_2 release and inhibitor of ulcer-promoting mediators such as platelet-activating factor released from mast cells (65). Nevertheless, at high concentrations, TNF-α causes disadvantageous effects in inducing tissue injury and potentiating septic shock (66). Likewise, overproduction of IL-1β is implicated in the pathophysiological changes that occur during different disease states, such as rheumatoid arthritis, neuropathic pain, inflammatory bowel disease and osteoarthritis.

NO is a soluble gas that synthesized from L-arginine, molecular oxygen, NADPH, and five cofactors [flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), heme and calmodulin (CaM)] via the catalytic action of three isoforms of NOS enzymes. At the gastrointestinal level, the two constitutive NOS (cNOS) expressed isoforms, namely endothelial NOS (eNOS) and neuronal NOS (nNOS); are expressed basally at the vascular endothelium and the enteric nervous system of the GI, respectively. The iNOS is expressed in

activated macrophages and neutrophils that are up-regulated in response to inflammation and other stimuli. Comparing with the other isoforms, once iNOS is activated it remains active for longer periods of time thereby generating a sustained increase in the amount of NO. NO produced by neuronal NOS (nNOS) detected in the gastric mucosa and in the gastric cells including chief, endocrine, and parietal cells; is involved in the regulation of gastric acid and mucus secretion whereas NO produced by endothelial NOS (eNOS) in endothelial cells and platelets plays a role in the angiogenesis, the regulation of gastric mucosal blood flow and the repair of the ulcerated gastric mucosa (67). In contrast to cNOS-derived NO, NO produced by iNOS in activated macrophages acts immunologically as a pro-inflammatory mediator and cytotoxic agent on invading microorganisms or on tumor cells (68). Sustained overproduction of NO generated from iNOS can lead to a modulation of leukocyte infiltration, a COX-2-dependent production of inflammatory PGE_2, and an interaction of NO with leukocyte-derived O_2^- that forms other potent cytotoxic reactive nitrogen species (69). In circumstances in which the production of one of these mediators is suppressed, there are compensatory increases in the production of others. COX-1 and cNOS/NO catalyze the synthesis of PGI_2, whereas COX-2 and iNOS/NO catalyze the synthesis of PGE_2. Therefore, NO is likely to have multifaceted role in inflammatory reactions, ranging from the enhancement of vasodilation and the formation of edema, through modulation of sensory nerve endings and leukocyte activity, to tissue cytotoxicity. These actions greatly depend on the type of isozyme involved, the source NO derived, the total amount and duration of NO produced, and the circumstances where NO is acting.

In the setting of inflammatory process, inflammatory cells (neutrophils, macrophages and lymphocytes) migrate into the injured area where they are activated for their phagocytotic function to clear the injured area in preparation for wound healing. The ultimate steps during phagocytosis by these inflammatory cells involve killing and degradation by oxygen-independent or oxygen dependent mechanisms. The oxygen-dependent mechanisms stimulate a burst in oxygen consumption, glycogenolysis, glucose oxidation, and production of reactive oxygen species (ROS): O_2^- and $^{\bullet}OH$ (Figure 2-2). Myeloperoxidase (MPO), a heme-containing enzyme stored in azurophilic granules of neutrophils and macrophages, is also released into extracellular fluid and catalyzes the conversion of chloride and hydrogen peroxide (H_2O_2) to more potent ROS: hypochlorite which is 100-1,000 times more cytotoxic than either O_2^-, H_2O_2 or $^{\bullet}OH$. Moreover, MPO consumes endothelial-derived NO, thereby reducing NO bioavailability and impairing its vasodilating and anti-inflammatory properties. Due to its importance during inflammatory processes and for being an indicator of polymorphonuclear leukocytes (PMN) presence in tissues, MPO is mainly produced by neutrophils and widely used as an index for the evaluation of neutorphil infiltration and inflammation of both acute and chronic conditions (70,71). Increased MPO activity is a useful risk marker of inflammation and oxidative stress even under clinical condition (71). In addition, a positive correspondence between MPO activity and neutorphil infiltration in intestinal inflammation model has been reported (72).

Although, NSAIDs formulated as enteric-coated or as a prodrug that is inactive until metabolized in the liver and prevents its topical direct contact of the NSAID molecule with the gastric mucosa, the incidence of significant gastric

ulceration and bleeding is not appreciably reduced (73). The ability of NSAIDs to induce GU by systemic effect has been shown to correlate well with their ability to suppress gastric cytoprotective PGs synthesis (increase mucus and bicarbonate secretion and mucosal blood flow) through COX-1 inhibition and their ability to suppress angiogenesis and promote leukocyte adherence through COX-2-inhibiton. Gastric ulcerogenic effects are not only mediated by the inhibition of COX-1 but also require the inhibition of COX-2. Selective inhibition of COX-1 or of COX-2 is unlikely to produce significant gastric damage (46,52). COX-2 and iNOS can modulate the expression of enzymes responsible for the synthesis of one another. In circumstances in which the production of one of these mediators is suppressed, there are compensatory increases in the production of the other. Thus, iNOS will compensate the temporary loss of COX-2 leading to an increase of PGE_2 level (74). NSAIDs can also diminish the ability of epithelial growth factor (EGF) which promote epithelial repair through a reduction of EGF binding to its receptor (75) and inhibition of EGF signaling pathways (76). In addition, experimental and clinical studies have demonstrated that NSAIDs can increase expression of NF-κB, pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 including pro-inflammatory mediator: NO generated from the induction of iNOS in activated macrophages located in the gastric mucosa.

Management of NSAIDs-induced GU

Current standard pharmacotherapy in prevention and treatment of NSAIDs-induced GU is proton pump inhibitors (PPIs) which are the most potent inhibitors of gastric acid secretion. Available PPIs include omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeoprazole. These drugs are substituted benzimidazoles that

irreversibly bind and inactivate the H^+, K^+-ATPase enzyme located on the apical membrane of the gastric parietal cell (about 70% of active pumps), thereby inhibit the terminal step in the acid production cycle (Figure 2-5) (77). They are prodrugs which are absorbed from the small intestine, transported via the bloodstream to the gastric mucosa, and ultimately secreted into the parietal cell secretory canaliculus, PPIs are weak bases with a pKa of 3.8 to 4.9. This weak base pKa enable PPIs to accumulate selectively in the acid space of the secretory canaliculus of the stimulated parietal cell (pH~ 0.8 to 1.0). PPIs inhibit both basal and stimulated gastric acid secretion in a dose dependent and sustained fashion. This class of drugs markedly diminishes gastric acid secretion over a 24 h period (78). It is important to recognize that PPIs bind only to activated proton pumps; thus, the optimal time to administer PPIs is prior to a meal to ensure that drug is circulating during a period of parietal cell activation. The drugs are most efficacious when take 30 to 60 min before meal. Their superior efficacy compared to histamine H_2 receptor antagonists is based on their ability to maintain an intragastric pH<4 longer between 15 h and 21 h, compared to approximately 8 h daily with that of H_2RAs. All PPIs have equivalent efficacy at comparable doses (77).

For the pharmacokinetic property of PPIs in healthy humans, the half-life is about 1 h but the duration of acid inhibition is 48 h because of their irreversible binding to the H^+, K^+-ATPase. The maximal plasma drug concentration (Cmax) and the degree of acid suppression are poorly correlated, but the area under the plasma concentration-time (AUC) correlates well with acid suppression. The oral bioavailability of PPIs is high about 80% to 90% for lansoprazole, 77% for pantoprazole and 89% for esomeprazole (77).

Studies in animal models have been demonstrated that PPIs also exhibit anti-oxidant and anti-inflammatory effect. Lansoprazole at a dose of 1 mg/kg has been found to inhibit lipid peroxidation (78,79); inhibit the generation of OH (80); inhibit the expression of many adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial-dependent neutrophil adhesion (81); and decrease the production of pro-inflammatory cytokine (IL-8) (82). Moreover, the preventive efficacy of lansoprazole against indomethacin-induced small intestinal ulceration in rats has been found to be associated with its inhibition of iNOS expression, through up-regulation of heme oxygenase-1/carbon monoxide production in the mucosa (83).

Suppression of gastric acid secretion enhances the healing of acid-related diseases. The optimal healing of reflux esophagitis is achieved when the intragastric pH is greater than 4 for 16 h per day whereas peptic ulcer is optimally healed when the intragastric pH is greater than 3 for 16 h per day (84). In comparative the antiulcer efficacy of lansoprazole with omeprazole, rabeprazole and pataprazole; all show equivalent efficacy in the healing of reflux esophagitis or duodenal ulcer.

PPIs are the agent with the longest document safety record with common side effects (headache, abdominal pain, diarrhea, nausea and constipation) (85); however; an increase level of gastrin (hypergastrineamia) from gastric acid suppression and its trophic effects on stomach mucosa have also been a concern particularly for chronic use though initial concerns in causing gastric malignancies in rats have not been substantiated in long-term patient studies. Long-term proton pump inhibitor use has been found to be associated with up to a 4-fold increase in the risk of fundic gland polyps which seem to arise because of parietal cell hyperplasia and parietal cell

protrusions resulting from acid suppression (4). Chronic acid suppression of PPIs has also been reported to be associated with an increased risk of community-acquired pneumonias and enteric infection. Moreover, long-term PPIs use may alter blood calcium level either through induction of hypochlorhydria interfering in insoluble calcium absorption or reduced bone resorption through the inhibition of osteoclastic vacuolar proton pumps (5). In a recent nested case-control study, the risk of hip fractures was significantly increased among patients prescribed more than one year of PPIs therapy and among those on long-term high dose PPIs. The strength of this association increased with increasing duration of PPI therapy. For elderly patients requiring long-term PPIs, it may be prudent to re-emphasize increased calcium intake, preferably from a dairy source, and congestion of a meal when taking insolution calcium supplements (6). A decrease in vitamin B_{12} (cyanocobalamin) has also been described in patients with long-term PPIs use and may be problematic in the elderly, vegetarians and patients with chronic alcohol ingestion (44,86).

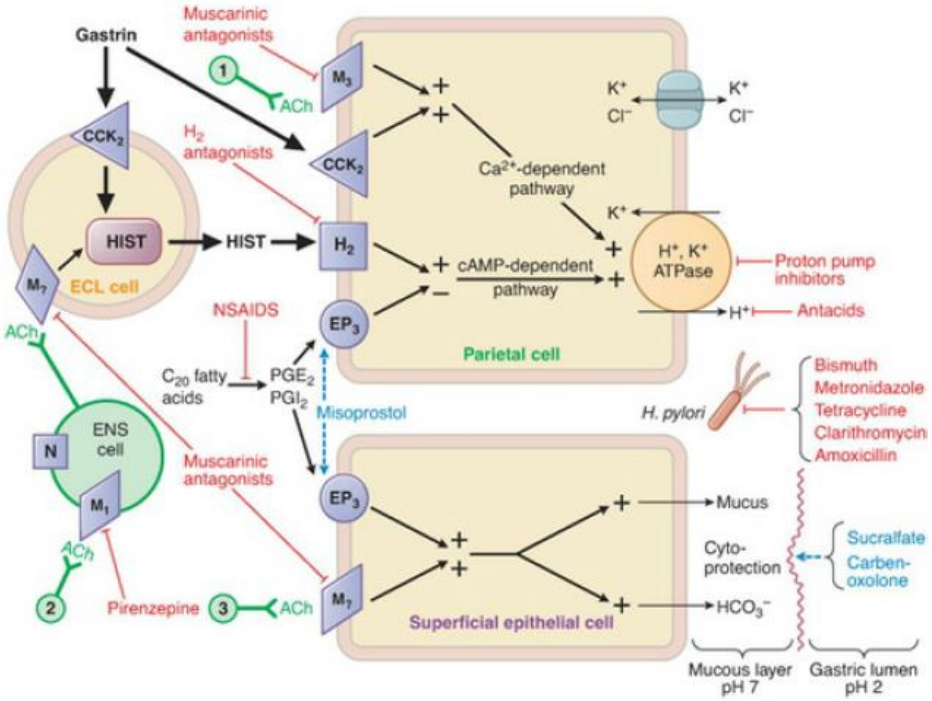


Figure 2-5 Physiological and pharmacological regulation of gastric secretion: the basis for therapy of acid-peptic disorders (44). [Shown are the interaction among an enterochromaffin-like (ECL) cell that secretes histamine, a ganglion cell of the enteric nervous system (ENS), a parietal cell that secretes acid, and a superficial epithelial cell that secretes mucus and bicarbonate. Physiological pathways, shown in solid black, may be stimulatory (+) or inhibitory (-). 1 and 3 indicate possible inputs from postganglionic cholinergic fibers; 2 shown neural input from the vagus nerve. Physiological agonists and their respective membrane receptors include acethylcholine (Ach), muscarinic (M) and nicotinic (N) receptors; gastrin, cholecystokinin receptor 2 (CCK_2); histamine (HIST), H_2 receptor; and prostaglandin E_2 (PGE_2), EP_3 receptor. A red line —| indicates targets of pharmacological antagonism. A light blue dashed arrow indicates a drug action that mimic or enhances a physiological pathway. Shown in red are drugs used to treat acid-peptic disorders

Oral ulcer

Physiology of oral mucosal

Oral or mouth ulcer is defined as an inflammatory and ulcerative condition of the oral mucosa (mucositis) leading to the mucosal atrophy and ulceration. It may occur due to a number of causes including high dose of stomatotoxic agents especially chemotherapeutic agents and radiation, immune-mediated conditions (such as aphthous ulcer and lichen planus), drug hypersensitivity reactions, infection and trauma (87). The oral mucosa is a squamous cell epithelium consists of keratinocytes or nonkeratinocytes. The oral cavity has been used as a site for local and systemic drug delivery. Drugs and therapeutic agents applied by extracellular route can cross the oral mucosa and act upon keratinocytes cell surface receptors or have an action in the connective tissue (Figure 2-6). Drugs and therapeutic agents which act upon intracellular targets (intracellular route) within the epithelium should be easily internalised by cells and retained within the epithelium (88).

Several types of topical oral formulations including mouthwashes, gels, sprays, patches are presently used for delivery drug into the oral mucosa. Among various formulations mentioned above, mouthwash is the most commonly used for delivery drugs with antimicrobial or anti-inflammatory propertied as it can be easily self-administrable for large surface area of oral cavity and the administered drug can reach the all the target sites.

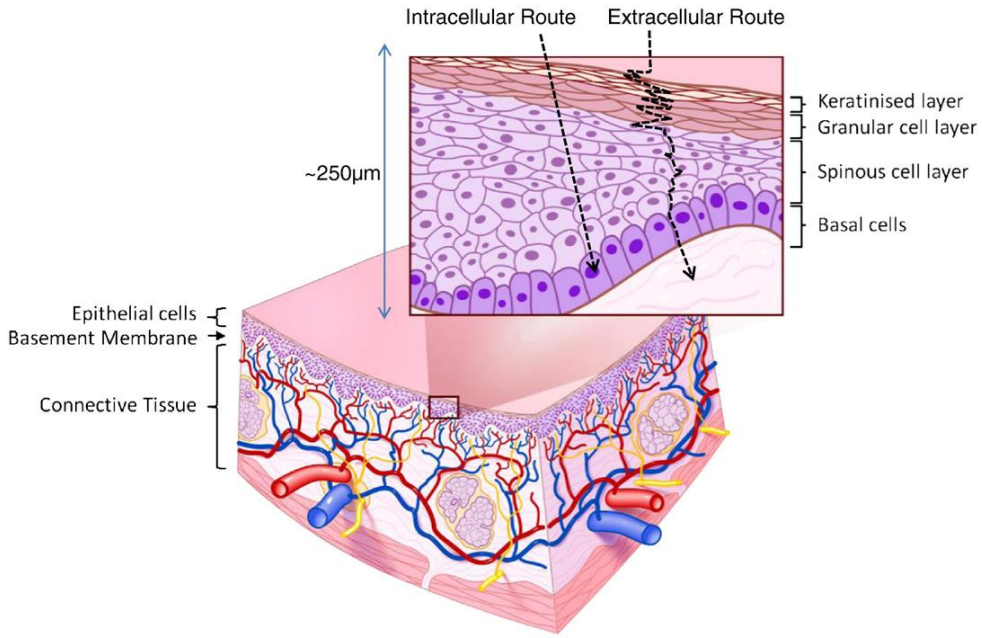


Figure 2-6 Structure of the oral mucosa and oral mucosal drug delivery (88)

Pathogenesis of oral ulcer

The pathobiology of oral ulcer is characterized in a five interrelated phases (Figure 2-7) including *an initiation phase* (the generation of free radicals and DNA damage); *a message generation phase* (the activation of transcription factors such as NF-κB which then up-regulation a number of pro-inflammatory cytokines such as TNF-α, IL–1β and IL-8); *a signaling and amplification phase* (the activation and release of inflammatory modulators that causing erythema from increased vascularity and epithelial atrophy); *an ulceration phase* (the occurrence of putative bacterial colonization of ulcerations and the stimulation of inflammatory cytokines release that further causing severe pain and limiting the patients' daily activities); and *a healing phase* (the occurrence of cell proliferation with re-epithelialization of ulcers and mesenchymal cells and extracellular matrix signals) (87). In the case of oral ulcer induced by chemotherapy, vascular endothelium and platelets have also been found to

play important roles in the pathogenesis process as an administration of platelet aggregation inhibitors can reduce the mucosal toxicity (89). An increased risk of microbial infection has been found to be associated with the atrophy and ulceration of the oral mucosa, particularly in the immunosuppressed or compromised patient. The most common pathogenic agent is *Candida albicans* which can rapidly invade the oral tissue and spread to the esophagus or lung (90). Severe cases of oral ulcer with infection can lead to life-threatening septicemia and contribute indirectly to increased hospital stays and overall cost of treatment which is a significant economic burden.

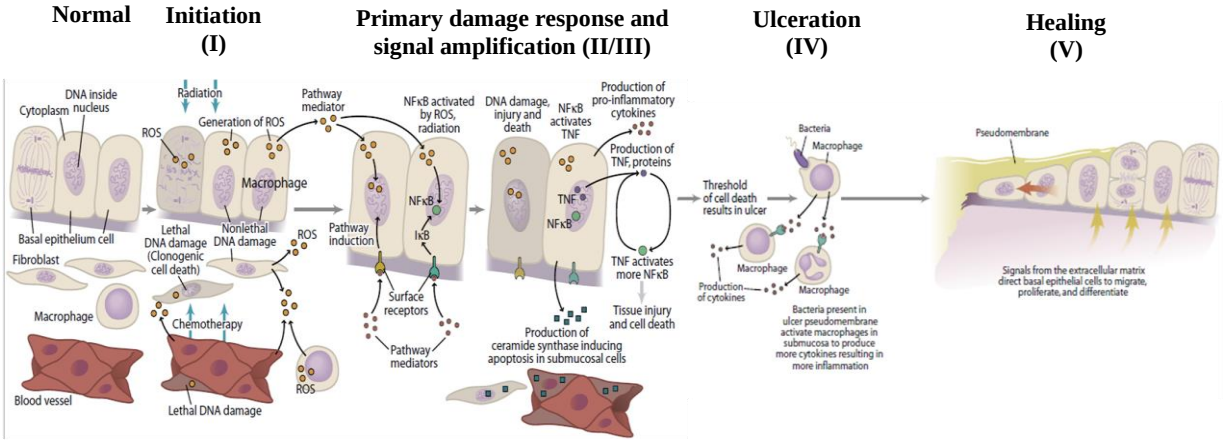


Figure 2-7 Pathobiology of the oral ulcer (87)

Management of oral ulcer

Topical oral viscous lidocaine is an obvious choice for palliation of oral ulcer pain. This therapeutic intervention has been widely used despite there is no much effort for scientific. The limitations of this intervention include local discomfort and insensitive affecting the sensation of taste and the gag reflex. The toxic reactions associated with an accidental overdose have also been reported in pediatric cases and

in adult case of frequent viscous lidocaine use when the amount of oral viscous lidocaine exceeded 240 ml per day (12).

A standard benzydamine mouthwash (Difflam®) with analgesic, anesthetic, anti-inflammatory and antimicrobial properties is potentially beneficial in reducing the incidence of ulceration and erythema and is recommended to be used in patient with oral inflammatory ulcer induced by radiation or chemotherapy. The anti-inflam -matory activity of benzydamine has been recently found to be relate to its capacity to inhibit the production of proinflammatory cytokines (TNF-α, IL-1β). It has also been reported that benzydamine inhibits the migration of inflammatory leukocytes through an inhibition of the mitogen-activated protein kinase (MAPK) pathway (91). In addition, it has been proved that benzydamine has antimicrobial property with a rapid biocidal activity against a variety of organisms at concentrations less than those advocated for treatment of inflammatory conditions (92). Moreover, it has been hypothesized that benzydamine produces local analgesia by stabilization of the cellular membrane and inhibition of prostaglandin synthesis (93). Nevertheless, it is expensive and has some adverse effects as causing a burning sensation and discomfort. The alcohol content in the mouthwash formulation may also often cause irritation to the inflamed mucosa.

Wound or ulcer healing

The wound or ulcer can occur from pathological process that begins in externally or internally conditions leading to the tissue damage and disrupts the function of tissue. It is a complex physiological response process including bleeding; coagulation; initiation of an acute inflammatory response to the initial injury; and the regeneration,

migration and proliferation of fibroblast cells which resume the function and restoration of tissue integrity (94,95). Wound or ulcer healing has been divided into three overlapping phases: inflammatory, proliferative and remodeling phases as illustrated in Figure 2-8 with a few modifications (96).

Inflammatory phase (Latent)

Immediately after injury, tissue begins to leak blood that fills the injured area with plasma and cellular elements, mainly platelets. Platelets are essential to the formation of a hemostatic reaction or giving rise a blood-clotting that slows or prevents further bleeding and provides temporary protection for the wound area. They also secrete multiple growth factors, cytokines and other agents into the injured area such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, fibroblast growth factor (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), which attract inflammatory cells such as, neutrophils, macrophages and leukocytes for their phagocytosis activity at the injured site (97). In addition, these inflammatory cells also produce growth factors that prepare the wound for the proliferative phase, at which the fibroblasts and endothelial cells will continue to be recruited (98).

Proliferative phase

This stage involves fibroblast migration, granulation tissue formation, angiogenesis, collagen synthesis (the formation of the extracellular protein matrix), and epithelialization (98). Following injury, fibroblasts in the environment tissue are stimulated to proliferate into the wound which being attracted by factors such as PDGF and TGF-β that are released by inflammatory cells and platelets. The growth of new blood vessels or angiogenesis is necessary in wound healing and is promoted by

numerous angiogenic factors (FGF, VEGF, PDGF and TGF). Collagens are an important factor in the proliferative and remodeling phase of repair. Migration of epithelial cells is occured around the wound edges from a single layer of cells initially to increase in epithelial cell mitotic activity around the wound edges. A hallmark of healing or repair by connective tissue is granulation tissue consists of new capillaries, myofibroblasts, inflammatory cells and cellular debris (94,97,99).

Remodeling phase (Repair)

At the final phase of healing, the normal tissue structure is recovered by the development of new epithelium and scar tissue formation. The synthesis of the extracellular matrix results in the granulation tissue development and deposition of collagen and proteoglycans. In a later stage of the remodeling phase, the fibroblasts of the granulation tissue are transformed into myofibroblasts that interact with extracellular matrix regulated by PDGF, TGF-β and FGF. As this occurs, a reorganization of the extracellular matrix takes place, making by regulatory machanisms on a balance between regeneration and scarring (97,100).

Successful wound or ulcer healing depends on the time and the functions of many process including maturation and remodeling, angiogenesis, fibroblasts migration and the removement of inflammatory cells influxed from the wound site, apoptosis or other mechanisms of cell death. This leads to the formation of scar with a small number of cells. On the other hand, if the cells persist at the site, the formation of hypertrophic scars or keloids will occur (101).

The main cytokines involved in this phase are TNF-α, IL-1β, PDGF and TGF-β produced by fibroblasts, and those produced by epithelial cells such as EGF and TGF-β (102).

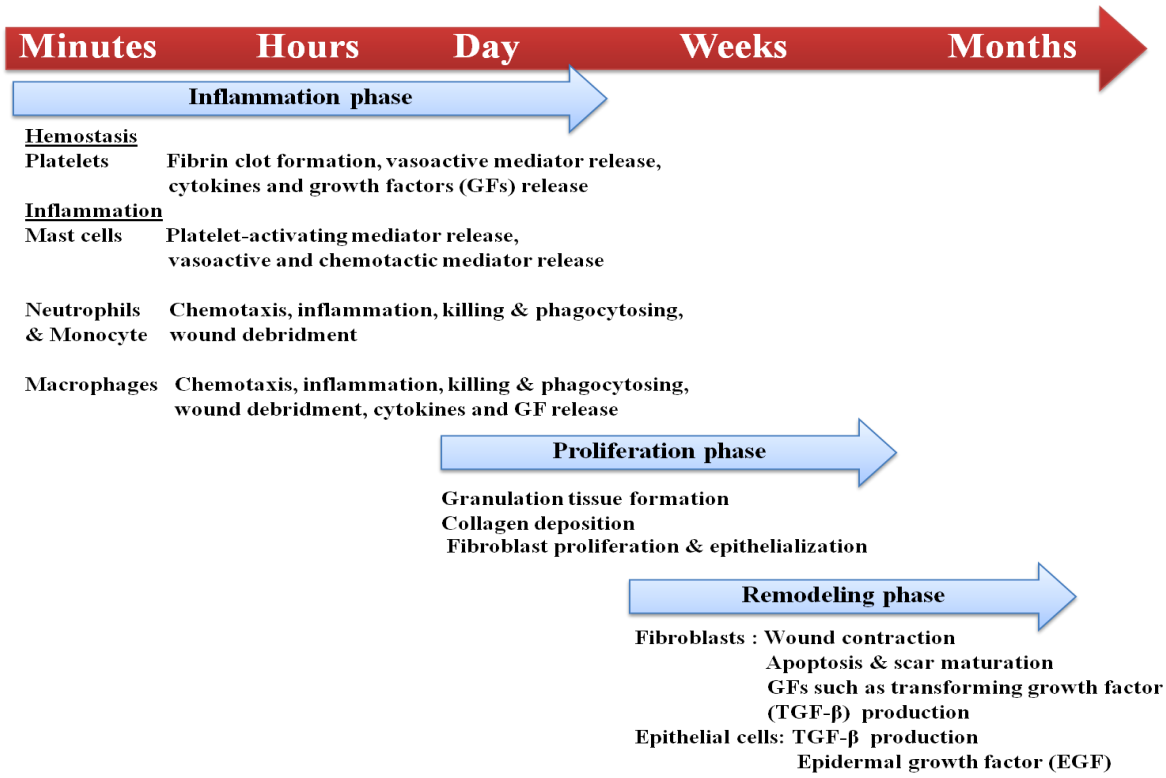


Figure 2-8 Phase of wound or ulcer healing. Modified from reference (96)

Chitosan

Chitosan is a biologically polysaccharide obtained from naturally sources which is the shells of crustaceans such as shrimp, crab and other sea crustaceans, the exoskeleton of insects and cell walls of the fungi (103). The chemical structure is composed of *β*-(1-4) linked D-glucosamine and *N*-acetyl-D-glucosamine (a) and physical appearance is off-white to light tan powder (b) (Figure 2-9). It is soluble in dilute aqueous acid solution such as acetic acid, citric acid and formic acid, but is insoluble in water and common organic solvents. Chitosan can increase the drugs oral bioavailability by retaining a dosage form within the GI tract and its bioadhesive polymer property to cover a wide area of mucosa for both drug delivery and physical protection (104). It has been now received a considerable attention in both

pharmaceutical excipient and drug carrier especially for the drugs with highly variable oral absorption and low bioavailability due to its excellent biocompatibility, biodegradability, low allergenicity and low toxicity properties (34). The generally recognized as safe (GRAS) are provided potential notifies for its certain uses in food products (105).

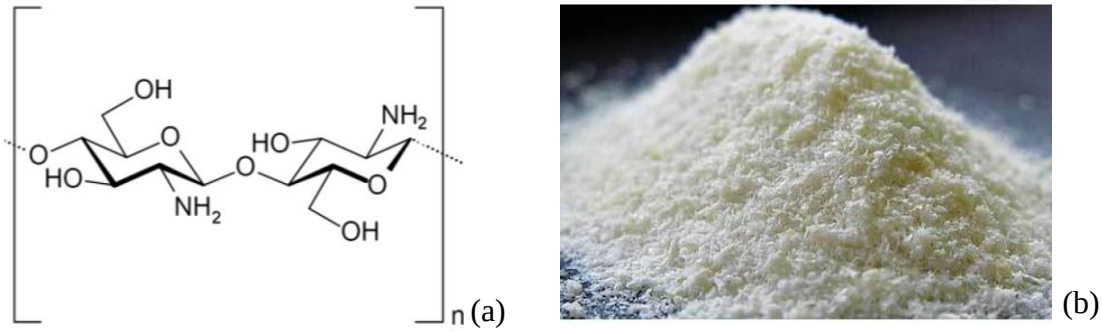


Figure 2-9 The chemical structure (a) and physical appearance (b) of chitosan

Pharmacological properties of chitosan related to oral and gastric ulcer

Chitosan and its derivatives have been reported to have other pharmacological actions, including antioxidant, anti-inflammatory, anti-microbial against oral pathogens (*Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*), anti-fungal (*Candida albicans*), wound healing and antiulcer activities.

Anti-oxidant activities

Both chitosan and its oligosaccharides have been shown antioxidant effects. Chitosan at concentration of 0.02% (w/v) (0.02 g) has been found to reduce lipid peroxidation by decreasing serum marker of oxidative stress and lipid peroxidation including free fatty acid (FFA) and malondialdehyde (MDA) concentrations and elevate antioxidant enzymes including antiperoxidative enzyme [superoxide

dismutase (SOD) and catalase (CAT)] and glutathione-dependent antioxidant enzyme [glutathione peroxidase (GPx) and glutathione *S*-transferases (GST)] activities in the body (106). The hetero-chitosan of three kinds (90% deacetylated chitosan; 75% deacetylated chitosan and 50% deacetylated chitosan) has also been shown to act against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, alkyl radical, hydroxyl radical and superoxide radical using electron spin resonance spectrometer (107). In addition, chitosan at oral dose of 2.0 g was found to increase the activity of antiperoxidative enzyme (SOD and CAT) and glutathione-dependent antioxidant enzyme (GPx and GST) in HCl-ethanol induced a significant increase in lipid peroxidation in the rat gastric mucosa (108).

Anti-inflammatory activities

Chitosan (MW = 50,000, 150,000 or 300,000 unit), at a dose of 2.5–62.5 μg/ml was found to exert anti-inflammatory properties by inhibiting the production of PGE_2 and pro-inflammatory cytokines (such as TNF-α and IL-1β) including the expression of COX-2 but increasing the anti-inflammatory cytokine: IL-10 formation in lipopolysaccharide (LPS)-treated RAW 264.7 macrophage cells (109).

Antimicrobial activities

Many previous reports have been reported that chitosan exhibits broad-spectrum antimicrobial activity against Gram-positive (*Staphylococcus sp., Streptococcus sp*) and negative bacteria (*E. coli, Pseudomonas sp.*), and fungi (*C. albicans*) (103,110). It has been suggested that a cationic nature (pH<6.3) of low molecular weight chitosan can insert to negative charged bacterial cell walls and bind with DNA leading to inhibit DNA transcription and mRNA synthesis (111). It has also been demonstrated that the hydrophilicity and negative charge on the cell

membrane are higher on gram-negative bacteria cell walls than those of gram-positive bacteria. Therefore, chitosan shows a stronger antibacterial activity against gram-negative bacteria (103). In addition, 0.5 g of low molecular weight chitosan was found to exhibit anti-fungal activity against oropharyngeal organisms, including *C. albicans* in biofilm method (112).

Wound healing activities

Apart from its biodegradable, biocompatibility, low-allergenicity and low-toxicity properties, chitosan also has a superior physical properties such as porosity, tensile strength, high surface and conductivity which are advantageous for development as drug delivery and biomaterial for wound healing (103,113). Several studies have reported that chitosan promotes tissue growth matrix and fibroblasts activity which stimulate cell proliferation for repaired tissues (114,115). In addition, chitosan has local biological activity in the form of haemostatic action and, together with its ability to activated macrophages and cause cytokine stimulation which has resulted in interest in medical device and wound healing applications (116). Wound dressing is one of the most promising medical applications of chitosan. The adhesive nature of chitosan, together with their antifungal and bactericidal character, and their permeability to oxygen, are very important properties associated with the treatment of wounds and burns. Additionally, chitosan's positive charge allows for electrostatic interactions with glycosaminoglycan, which attracts growth factors that enhance cell growth and proliferation (117).

Anti-ulcer activities

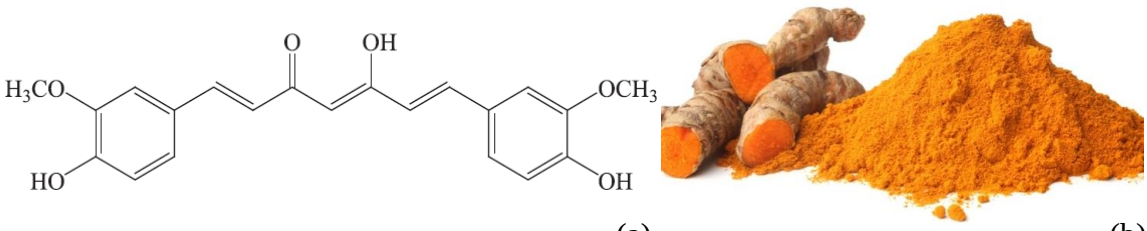
Oral administration of low molecular weight (LMW) chitosan (MW: 25,000-50,000 unit) at the dose of 250, 500 and 1000 mg/kg was been found to prevent

ethanol-induced gastric ulcer in rat model by 87%, 95% and 99% inhibitory efficacy, respectively whereas oral administration of high molecular weight (HMW) chitosan (MW: 50,000-100,000 unit) at the dose of 250 and 500 mg/kg was been found to prevent ethanol-induced gastric mucosal injury by 64% and 83% inhibitory efficacy, respectively. This ulcer inhibitory of chitosan was comparable to those of sucralfate and cimetidine. In addition, oral administration of LMW chitosan at the dose of 100, 200 and 400 mg/kg twice daily for 14 consecutive days in rat treated with acetic acid was been found to decrease the gastric ulcer index by 21%, 49% and 60%, respectively which was more effective than HMW chitosan and chitin. This ulcer healing capacity of LMW chitosan (200 and 400 mg/kg) is as potent as that of sucralfate (250 and 500 mg/kg) but is more potent than that of cimetidine (100 mg/kg) (118). Chitosan at oral dose of 2.0 g has also been found to decrease acidic output with mechanism of action that might be due to its acid-neutralizing capability by the gradual release of glucosamine residues into the gastric mucosa (108). Furthermore, LMW of chtitosan at oral doses of 250-1000 mg/kg is found to increase gastric mucus content in a dose-dependently manner (118). Nevertheless, there is still no study on its antiulcer efficacy against NSAIDs-induce GU.

Curcumin

Curcumin is diferuloylmethane (1,7-bis-(4-hydroxyl-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione) with chemical formulation as $C_{12}H_{20}O_6$ (Figure 2-10). the rhizome of *Curcuma longa* Linn (119). Among of three yellowish curcuminoids [curcumin (80%), demethoxycurcumin (15%) and bisdemethoxycurcumin (5%)] containning in turmeric isolated from the rhizome of *Curcuma longa* Linn, curcumin

is an important main active polyphenolic constituent. It is nearly insoluble in water but is quite stable in the acidic pH of the stomach (120,121). Its pKa value is 8.54 so that curcumin degrades rapidly at basis or neutral pH solution between 7-10 (122). Even though curcumin is not soluble in water but water based formulation of curcumin can be prepared using different surfactants like PEG 400, tween 80, and span 80. However, the percentage solubility of curcumin in PEG 400 is shown higher than others (123).



(a) (b)

Figure 2-10 The chemical structure (a) and physical appearance (b) of curcumin

Curcumin has been characterized as safe by health authorities such as the Food and Drug Administration (FDA) of the United States of America and Food and Agriculture Organization/World Health Organization. In human phase 1 clinical trial with 25 volunteers, administration of up to 8 g of curcumin per day for 3 months induced no apparent toxic sign (124). In the other clinical trial on the safety of using topical curcumin preparation to treat skin and mucous membrane cancers for 18 month in 62 patients, only one patient reported an adverse effect of scalp itching (125). Recently, curcumin is classified as generally recognized as safe (GRAS) for certain uses in food products (105).

Data on the pharmacokinetics, metabolites, and systemic bioavailability of curcumin in animal models and in humans mainly conducted on cancer patients, have shown that curcumin is poorly absorbed and has limited systemic bioavailability. Recently, various formulation strategies have been carried out to improve the stability, bioavailability and pharmacokinetic properties of curcumin in treatment of GU using self-microemulsoifying liquid and pellet technology (30) or the floating drug delivery technology such as a raft forming system (31).

Pharmacological properties of curcumin related to oral and gastric ulcer

Curcumin has been variedly used in ayurvedic medicine for centuries as it has many of therapeutic properties such as anti-inflammatory, antioxidant, analgesic, antibacterial, antifungal, wound healing and antiulcer properties.

Antioxidant property

Curcumin acted as a scavenger of oxygen free radical (126) and was found to protect haemoglobin from oxidation (127). *In vitro* study, an IC_{50} value of DPPH radical scavenging activity of curcumin is about 21.0 µM (128). Curcumin (10 µM) was found to inhibit the generation of ROS like O_2^-, H_2O_2 and nitrite radical generated by activated macrophages (129). Curcumin was also found to prevent oxidative damage during indomethacin-induced gastric lesion by an inactivation of gastric peroxidase and a direct scavenging of H_2O_2 and $^{\bullet}OH$ (130). Additionally, curcumin was shown to enhance the activities of natural anti-oxidant enzymes, such as SOD, catalase and GPx in liver homogenates (131). Moreover, it has been reported that curcumin can protect tissues from the effects of oxidative stress induced by radiation, metals and severe injury to skeletal muscles (132). These antioxidative results of

curcumin have been implicated with the methoxy groups in the phenyl ring of its structure (133).

Anti-inflammatory property

It has been shown from *in vitro* anti-inflammatory studies that curcumin inhibits lipopolysaccharide-induced production of IL-1 and TNF-α in human monocyte protein macrophage cell line (134). An *in vivo* study in mice injected intraperitoneally (i.p.) with 500 μg of curcumin (body weight) showed that curcumin-treated mice produced much lower amounts of IL-12 in response to either LPS or head-killed *Listeria monocytogenes* (HKL) than that of the control group (135). Curcumin at the dose of 20 μM was also found to inhibit NF-κB activation pathway through induction of COX-2 by inflammatory cytokines or hypoxia-induced oxidative stress (136). In addition, curcumin inhibited the cellular uptake of AA and the activities of COX, lipo-oxygenases (LOX) and several phospholipases involved in the release of AA from membranes. Moreover, it has been shown to inhibit the upregulation of metalloproteinases (MMPs) possibly by inhibiting on protein kinase C (137) and collagen synthesis including decreasing the release of many proteolytic enzymes, such as elastase, collagenase and hyaluronidase from activated macrophages (138).

Antiulcer property

Oral administration of curcumin at 50 mg/kg dose was shown to protect the stomach from ulcerogenic effects such as phenylbutazone (139) and indomethacin (140,141) in animals. Curcumin was found to prevent indomethacin-induced gastric lesion through antioxidant property in blocking inactivation of gastric peroxidase; direct scavenging of H_2O_2, and $^{\bullet}OH$; enhancing the activity of natural antioxidant

enzyme such as SOD, catalase and GPx and reducing the level of MDA (a marker of tissue lipid peroxidation); anti-inflammatory property in suppressing the expression of iNOS, nuclear factor-κB and caspase-3; antisecretory property and gastric mucus producing property (141,142). In addition, it was found that curcumin at oral dose of 20-80 mg/kg effectively inhibited gastric acid secretion in pylorus-ligated rats, prevented the development of acute gastritis induced by various ulcerogenic agents like ethanol, 5-HT and compound 48/80 in rat models, and also accelerated the healing of chronic GU induced by acetic acid in rats (28). Its antiulcer effect might be due to its properties of decreasing gastric acid secretion and enhancing the mucosal defensive mechanism through suppression of iNOS and TNF-mediated inflammation (143). However, at higher doses of 50 mg/kg and 100 mg/kg curcumin, curcumin was found to produce gastric lesions in rats that might be involved its increase in gastric acid and/or pepsin secretion and its decrease in gastric mucin content. Consequently, the antiulcer prophylactic dosage level of curcumin should not be more than 40 mg/kg/day whereas the antiulcer therapeutic dosage level of curcumin should not be more than 160 mg/kg/day since curcumin can exert opposite effects to either delay ulcer healing or to exacerbate ulcer inflammation through some mechanisms differently modified by curcumin dosage (28). Recently, it has been reported that curcumin exerts gastroprotective in decreasing of gastric acid secretion and increasing of gastric microcirculation through endogenous PGs and NO including vasoactive neuropeptides against experimental stress-induced gastric lesions (140).

In animals with reflux esophagitis, curcumin (20 mg/kg, i.d) and lansoprazole (1 mg/kg, i.d) was been found to inhibit the formation of acute acid reflux esophagitis by 52.5 and 70.9%, respectively. Curcumin alone was found not to

be effective in preventing chronic acid reflux esophagitis, but the combination of curcumin and DMSO could reduce the ulcer index to the same extent (56.5%). Although, curcumin was less potent than lansoprazole in inhibiting acid reflux esophagitis, it was superior to lansoprazole in innibiting mixed reflux esophagitis (144). Curcumin was also reported to exert some beneficial effects on the intestine such as antispasmodic activity in isolated guinea pig ileum (145) and antiflatulent activity in both *in vivo* and *in vitro* experiments in rats (146).

Wound healing activity

The wound healing potential of curcumin is attributed to its biochemical effects such as its anti-inflammatory (147), anti-infectious (148) and antioxidant (149) activities. Various studies have shown that curcumin's application on wound enhances epithelial regeneration and increases fibroblast proliferation and vascular density (150). An animal study was conducted to determine the role of curcumin in wound healing process in rats. Topical preparation of curcumin was used on the full-thickeness excision wound made on the back of the rat. An enhanced cellular proliferation and a systhesis of collagen on wound were found on rats treated with curcumin. A decrease level of lipid peroxides and an increase levels and activities of SOD, catalase and GPx were observed indicating the antioxidant property of curcumin in accelerating wound healing. Wound treated by curcumin had also been found to heal much faster than that of the control as indicated by improving rates of epithelization and wound concentration including increased tensile strength confirmed by histopathological examination. The results of the study strongly indicated towards the wound healing activity of curcumin specifically when used topically (151).

A topically application of a curcumin-loaded oleic acid based polymeric bandage (COP) on the back of wounded rats was found to down-regulate the expression of various kinases in the PI3K/AKT/NF-κB pathway which in turn led to less activation of the NF-κB gene and an up-regulation of I-κB-(α) protein which involves in the inhibition of NF-κB pathway. Hence, this indicated that curcumin can reduce inflammation at wounded sites caused by the activation of the NF-κB pathway (152).

The efficacy of single curcumin or in a combination compound with α-tocopherol and sunflower oil (compound A) had been investigated in the treatment of radiation-induced oral mucositis in the mature (12 weeks old, 200–225 g) female Sprague–Dawley rats receiving 0.5 ml/day of either compound A, sunflower oil, α-tocopherol, curcumin or water containing 10% ethanol by oral gavage until the end of experiments. Either α-tocopherol or sunflower oil treatment was found to be resulted in dose-modification factor of 1.05. Curcumin treatment appeared to be effective in the prevention of radiation-induced oral mucositis with a dose-modification factor of 1.09. However, the mucositis preventive efficacy observed with the compound A appeared greater with a statistically significant dose-modification factor of 1.2±0.1 (153). In addition, other *in vivo* studies of curcumin on wound healing in rats and guinea pigs also observed the faster wound closure of punch wounds in curcumin-treated comparing with untreated controls. Biopsies of the wound showed the re-epithelialization of the epidermis and an increased migration of various cells including myofibroblasts, fibroblasts, and macrophages in the wound bed. Moreover, curcumin-treated group showed extensive neovascularization and greater collagen deposition in the multiple areas within the dermis and an increase TGF-1β in immunohistochemical localization. *In situ*

hybridization and polymerase chain reaction analysis also showed an increase in the mRNA transcripts of TGF-1β which is known to enhance wound healing and fibronectin (154).

Antimicrobial activity

Curcumin, in clinically relevant concentrations for topical use (100-200 μM), has been found to display strong antibacterial effect against a facultative upper respiratory tract pathogen by inhibiting bacterial growth, adherence and invasion including anti-inflammatory effect in decreasing pro-inflammatory IL-8 release from upper respiratory tract epithelial cells (155). An *in vitro* study published recently on the extent of its bactericidal activity and the kinetics of its anti-inflammatory effect on pharyngeal cells has also reported that curcumin exerts a concentration-dependent bactericidal effect on all 18 oropharyngeal species commonly associated with bacteremia in febrile neutropenia with complete suppression of the release of TNF-α, IL-6, IL-8, monocyte chemoattractant protein 1, granulocyte macrophage-colony stimulating factor, and vascular endothelial growth factor except fibroblast growth factor-2 and interferon-γ. Repetitive exposure to curcumin resulted in repetitive suppression of cytokine/ chemokine expression lasting from 4 to 6 h (156).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Drugs and chemicals

Carboxymethycellulose (CMC), chitosan (low molecular weight; 50,000-190,000 Da), curcumin (containing 71.73:17.38:7.19 curcumin:demethoxycurcumin :bisdemethoxycurcumin), gallic acid, hexadecyltrimethylammonium bromide (HTBA), indomethacin, lansoprazole, pentobarbital sodium, phosphate buffer saline (PBS), 3,3′,5,5′-tetramethylbenzidine (TMB), trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were purchased from Sigma–Aldrich, Missouri, USA. Acetic acid (99.7%) and formalin were purchased from RCI Lab–Scan, Thailand. All chemicals were of analytical grade.

AGS human gastric epithelial cell lines (CRL-1739™) and human gingival fibroblast (HGF-1) (CRL-2014) were purchased from American Type Culture Collection, USA. Mouse macrophage RAW 264.7 cell line was purchased from Cell Lines Services, Germany. Antitibiotics solution (penicillin and streptomycin), fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI) 1640 medium and trypsin-EDTA (0.25%) were purchased from Gibco, Life Technologies, USA. Caffeic acid phenethylester (CAPE), 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H- tetra-zoliumbromide (MTT), lipopolysaccharide (LPS) from *Escherichia coli* and L-nitroarginine (L-NA) were purchased from Sigma–Aldrich, Missouri, USA.

Tissue total RNA purification mini Kit (FavorPrep™) was purchased from Favorgen Biotech Corp., Taiwan.

SYBR® Safe DNA gel stain was purchased from Invitrogen (Thermo Fisher Scientific), USA.

5x FIREPol® Master mix (ready to load) and 5x HOT FIREPol® EvaGreen® qPCR Mix Plus were purchased from Solid BioDyne, Estonia.

Molecular biology agarose, 50x Tris-Acetate-EDTA (TAE) buffer, pH 8.0 (ultra puregrade), Viva cDNA synthesis kit and Vivantis 50 bp DNA ladder were purchased from Vivantis lnc., USA.

The forward and reverse primers used for rat COX-1, COX-2, eNOS GAPDH, iNOS and nNOS were purchased from Theera trading Co., LTD., Thailand.

Other reagents used in cell culture techniques including acetone, formaldehyde, hydrogen peroxide (H_2O_2), $NaH_2PO_4.2H_2O$ and $Na_2HPO_4.12H_2O$ were purchased from Sigma–Aldrich, Missouri, USA.

Curcumin used in *in vivo* study was suspended in 0.1 M acetic acid, while curcumin used in *in vitro* study was dissolved in DMSO (0.1%).

Chitosan used in *in vivo* and *in vitro* study was dissolved in 0.1 M acetic acid.

All drugs were prepared immediately before use and administered in a volume of 0.5 ml/100 g body weight.

3.1.2. Preparation of a chitosan-curcumin solution for topical use in oral ulcer

A chitosan-curcumin solution for topical use was prepared according to the method of Mustafa et al. (2019) (123). It was found from MIC testing that a required concentration of curcumin to be present in a formulation should be 0.1%. Considering

safety in comparison to ethanol and solubility of curcumin, PEG 400 was selected to be used as co-solvent with water in the formulation. In addition, chitosan was selected to enhance the substantivity of curcumin in the oral cavity and the antifungal efficacy of the formulation. The ratio of curcumin, chitosan and co-solvent for the preparation were prepared as following:

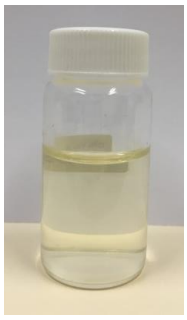
0.5 g of chitosan was dissolved in 1% acetic acid solution. 15 ml of sorbitol and 1 ml of paraben concentrate were added into the chitosan solution. A curcumin solution was prepared by dissolving 0.1 g of curcumin in 40 ml of PEG 400 and then added into the chitosan solution. The solution was adjusted to pH 5-6 by adding 100 µl of 10% NaOH solution. The sufficient quantity of distilled water was added to make a final volume of 100 ml. The obtained preparation was a clear yellow-colored solution with an optimum pH range to use in the human oral cavity (pH~5.5) (Figure 3-1).

Figure 3-1 An appearance of a topical 0.1% curcumin in 0.5% of chitosan solution

3.1.3. Preparation of a chitosan-curcumin mixture for oral use in oral and gastric ulcer

Base on several studies on the optimal dose of curcumin and chitosan in prevention or healing GU in animal models described in the literature review, it was found that the optimal prophylactic dose of curcumin against GU induction was 20 mg/kg whereas the therapeutic dosage level of curcumin for GU should not be more than 80 mg/kg/day since excess dose of curcumin can exert opposite effects in delay of ulcer healing or exacerbation of ulcer inflammation (28). In addition, it was found that chitosan at doses of 150-300 mg/kg exerted an optimal prophylactic efficacy against acute GU (118). Accordingly, curcumin at a dose of 20 mg/kg and chitosan at doses of 150-300 mg/kg would be the preferred dose for the development of an oral chitosan-curcumin mixture.

The clear colorless chitosan solution was prepared by dissolving 150 mg of chitosan in 10 ml of 0.1 M acetic acid solution (Figure 3-2a). The chitosan solution was slowly added into 20 mg of curcumin and the mixture was then triturated until a uniform yellowed-color mixture was obtained as shown in Figure 3-2b.


(a)


(b)

Figure 3-2 An appearance of a chitosan solution (a) and an oral chitosan-curcumin mixture (b)

3.1.4. Animals

Male and female Golden hamsters weighing 80-100 g and male Wistar rats weighing 180–200 g (5-weeks-of-age) were used for the study. They were procured from Nomura Siam international Co., Ltd. Thailand and were housed in cages under controlled conditions at 25±1°C with a 12–h light/dark cycle and maintained on standard rodent chow and free access to water *ad libitum*. The study was conducted in compliance with the guideline of the Animal Care and Use Committee of Prince of Songkla University and was approved by the Animal Experimental Ethics Committee of Prince of Songkla University (MOE 0521.11/382), Thailand. The rats were fasted, but with free access to water *ad libitum* 18 h before the experiments.

3.2. Methods

3.2.1. Preventive effect of a chitosan-curcumin mixture against indomethacin-induced acute gastric ulceration in rats (53)

Male Wistar rats were fasted 18 h with free access to water prior to experiments. Test preparations (Table 3-1) were administrated to the animals orally 1 h before an oral administration of indomethacin (30 mg/kg) dissolved in co-solvent (water : propylene glycol : tween 80 in the ratio of 5:4:1). Four hours later, the animal was sacrificed using pentobarbital sodium overdose (150 mg/kg) and the stomach was separated and opened along the greater curvature to evaluate the gastric damage and the sum of the length (mm) of all lesions (erosion and hemorrhagic ulceration) for each stomach was expressed as a gastric ulceration index.

$$\% \text{ Inhibition} = \frac{(\text{Total ulcerated area}_{(\text{control})} - \text{Total ulcerated area}_{(\text{treatment})})}{\text{Total ulcerated area}_{(\text{control})}} \times 100$$

The experimental groups were shown in Table 3-1 with each consisting of 6 rats. Each test preparation was given orally in a volume of 5 ml/kg.

The schematic plan for evaluation of the preventive efficacy of test preparations was shown in Figure 3-3.

Table 3-1 Groups of test preparations using in a preventive study against indomethacin induced acute GU in rats (n=6)

Experimental groups	**Test preparations**
1	Water control (5 ml/kg, *p.o.*)
2	0.1 M acetic acid (5 ml/kg, *p.o.*) (vehicle solution for curcumin, chitosan and chitosan-curcumin mixture)
3	0.5% CMC suspension (5 ml/kg, *p.o.*) (vehicle solution for lansoprazole)
4	Curcumin (20 mg/kg, *p.o.*)
5	Curcumin (40 mg/kg, *p.o.*)
6	Chitosan (75 mg/kg, *p.o.*)
7	Chitosan (150 mg/kg, *p.o.*)
8	Chitosan (300 mg/kg, *p.o.*)
9	Chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, *p.o.*]
10	Chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (300 mg) dissolved in 0.1 M acetic acid, *p.o.*]
11	Lansoprazole (1 mg/kg, *p.o.*)

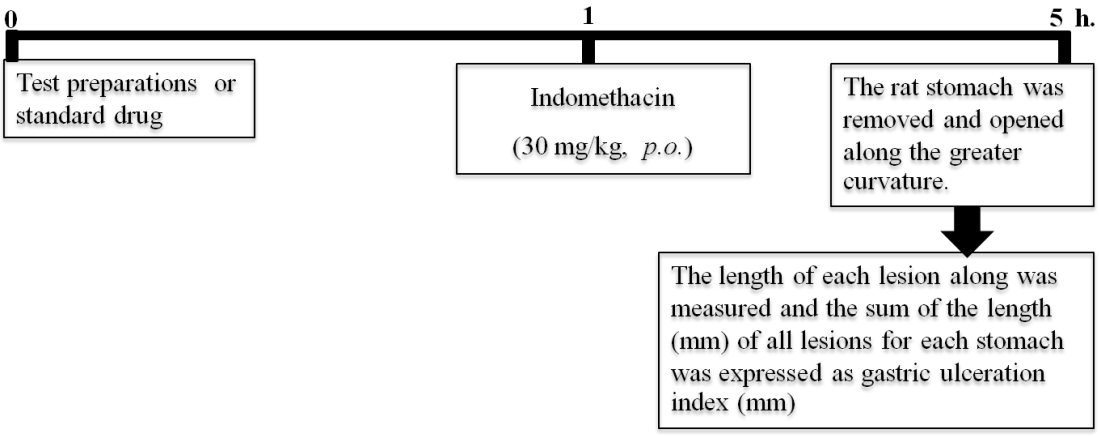


Figure 3-3 Schematic plan for evaluation of the preventive efficacy of test preparations against indomethacin-induced acute gastric ulceration in rats

3.2.2. Curative effect of a chitosan-curcumin mixture on acute GU induced by indomethacin in rats

Male Wistar rats were fasted for 18 h with free access to water prior to the experiment. Indomethacin dissolved in co-solvent (water : propylene glycol : tween 80 in the ratio of 5:4:1) at the dose of 30 mg/kg was given orally to the rats. Test preparations were administered to the rat at 5 and 24 h later (Table 3-2). After the last administration dose of test preparations, the animal was sacrificed using pentobarbital sodium overdose (150 mg/kg) and the stomach was then removed, opened along the greater curvature and washed with saline solution. The ulcerated areas (mm^2) were examined and measured using the computer program (imageJ). The sum of the area (mm^2) of all ulcerated areas for each stomach was used as ulcer index.

Ulcerated area (mm^2) = length (mm) x the width of the ulcer (mm)

$$\% \text{ Curation} = \frac{(\text{Total ulcerated area}_{(\text{control})} - \text{Total ulcerated area}_{(\text{treatment})})}{\text{Total ulcerated area}_{(\text{control})}} \times 100$$

The experimental groups were shown in Table 3-2 with each consisting of 6 rats. Each test preparation was given orally in a volume of 5 ml/kg.

Table 3-2 Groups of test preparations using in a curative study on indomethacin induced acute GU in rats (n=6)

Experimental groups	**Test preparations**
1	Water control (5 ml/kg, *p.o.*)
2	0.1 M acetic acid (5 ml/kg, *p.o.*) (vehicle solution for curcumin, chitosan and chitosan-curcumin mixture)
3	0.5% CMC suspension (5 ml/kg, *p.o.*) (vehicle solution for lansoprazole)
4	Curcumin (20 mg/kg, *p.o.*)
5	Chitosan (150 mg/kg, *p.o.*)
6	Chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, *p.o.*]
7	Lansoprazole (1 mg/kg, *p.o.*)

The schematic plan for evaluation of the curative efficacy of test preparations was shown in Figure 3-4.

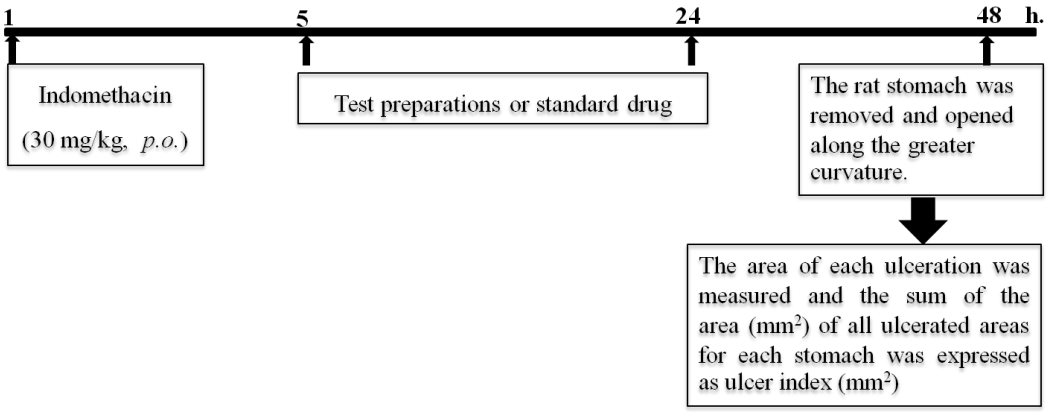


Figure 3-4 Schematic plan for evaluation of the curative efficacy of test preparations on acute GU induced by indomethacin in rats

3.2.3. Curative effect of a chitosan-curcumin mixture on chronic GU induced by acetic acid in rats

A chronic gastric ulcer was induced by a topical application of acetic acid according to the method of Okabe et al (1971) (157) with few modifications. Male Wistar rats were fasted 18 h with free access to water prior to an experiment. After the animal was anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*), the abdomen of rats were opened and a cylindrical plastic mold (6 mm diameter) was tightly placed upon the anterior serosal surface of the stomach (antrum) wall. Thereafter, 0.06 ml of 99.7% acetic acid was poured into the mold and allowed to remain for 1 min. The acetic acid remaining on the surface was sucked out and gently washed with normal saline solution. The opened abdomen was then closed and the rat was fed normally. Each test preparation (Table 3-3) was administered orally to the animals for 10 consecutive days, beginning on the 4^{th} day after ulcer induction. The animal was sacrificed using pentobarbital sodium overdose (150 mg/kg) on the 14^{th} day after

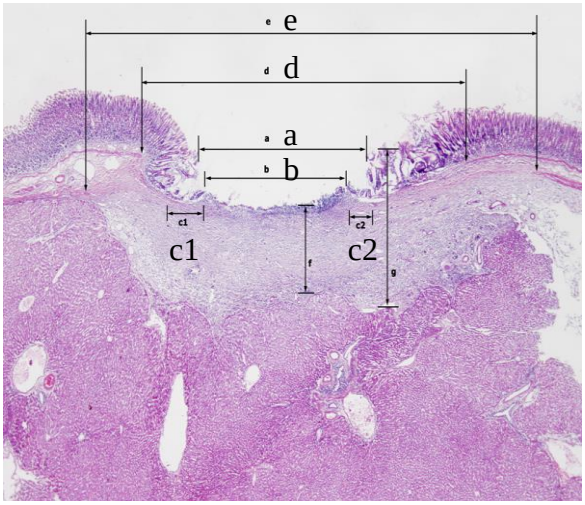
ulcer induction and the ulcer was examined macroscopically and histologically (Figure 3-5) for the following parameters:

Ulcer index (UI) (mm^2) = length (mm) x width of the ulcer (mm)

$$\% \text{ Curation} = \frac{(\text{UI control on } 4^{th} \text{ day} - \text{UI treatment on } 14^{th} \text{ day})}{\text{UI control on } 4^{th} \text{ day}} \times 100$$

% Mucosal regeneration index = [(c1+c2)/(b+c)] x100

% Healing index = [1-(a/e)] x100



a = apparent defect in mucosal layer

b = true defect in mucosa

c = regeneration of mucosal layer

d = distance ruptured of muscularis mucosa

e = distance ruptured of muscularis propria

Figure 3-5 Histological measurements of chronic GU induced by a topical application of acetic acid in rat

The experimental groups were shown in Table 3-3 with each consisting of 6 rats. Each test preparation was given orally in a volume of 5 ml/kg.

Table 3-3 Groups of test preparations using in curative study on acetic acid induced chronic GU (n=6)

Experimental groups	Test preparations
1	Water control (5 ml/kg, *p.o* bid)
2	0.1 M acetic acid (5 ml/kg, *p.o* bid) (vehicle solution for curcumin, chitosan and chitosan-curcumin mixture)
3	0.5% CMC suspension (5 ml/kg, *p.o* bid) (vehicle solution for lansoprazole)
4	Curcumin (20 mg/kg, *p.o* bid)
5	Chitosan (150 mg/kg, *p.o* bid)
6	Chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, *p.o* od]
7	Lansoprazole (1 mg/kg, *p.o* bid)

3.2.4. Qualitative analysis on the bioactivity of a chitosan-curcumin mixture

3.2.4.1. *In vitro* antioxidant activity determination

An organic free radical: DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay (158)

The DPPH solution was prepared at a concentration of 6×10^{5} M in 95% ethanol. Briefly, 100 µl of each test sample was mixed with 100 µl of the DPPH methanolic solution in a 96-well plate and incubated at 37°C for 30 min. When the violet solution of DPPH radical was scavenged by antioxidant substances, the violet

color was faded. If the reaction system contained high antioxidant activity, the violet solution color was largely conducted to be lighter and resulting in a lower absorbance value at 515 nm measured by a microplate reader. The antioxidant capacity of test samples was expressed as IC_{50} values (the concentration of sample that required to scavenging 50% of DPPH free radicals). Ascorbic acid was used as a positive standard antioxidant. The antioxidant capacity was calculated and expressed as % inhibition according to the following equation.

$$\% \text{ Inhibition} = [(A_{control} - A_{test\ sample}) / A_{control}] \times 100$$

$A_{control}$ = absorbance of control sample

A_{test} = absorbance of the samples or standard

Nitrite radical scavenging assay (159)

A volume of 0.5 ml of 20 mM sodium nitroprusside dissolved in phosphate buffered saline (pH 7.4) was added with 1 ml of various concentrations of the test sample (5-100 µg/ml) in the plate. The plate was mixed and incubated under light at room temperature for 2.5 h. The sample was then mixed with an equal volume of the Griess reagent (prepared by mixing 1% of sulphanilamide in 5% of phosphoric acid and 0.1% of naphthylethylene diamine dihydrochloride immediately before use). A volume of 200 µl of the sample mixture was transferred to a 96-well plate and the amount of nitric oxide radical was measured at 546 nm using a microplate reader. Gallic acid was used as the positive control. The percentage of nitric oxide radical scavenging activity of the sample and gallic acid was calculated according to the following equation:

Nitric oxide scavenged (%) = $[(A_{control} - A_{test})/A_{control}] \times 100$

$A_{control}$ = absorbance of control sample

A_{test} = absorbance of the samples or standard

3.2.4.2. *In vitro* anti-inflammatory activity determination: (inhibition of nitrite production) (160)

The macrophage RAW 264.7 cell was cultured in RPMI 1640 medium supplemented with 0.1% $NaHCO_3$, 1% of penicillin and streptomycin and 10% FBS in a humidified atmosphere containing 5% CO_2 incubator at 37°C. The cells were seeded with $1x10^5$ cells/well in a 96-well plate and incubated in a humidified atmosphere containing 5% CO_2 incubator at 37°C for 1 h to promote cell adherence at the bottom of the well. The medium in a 96-well plate was then replaced with a fresh medium containing LPS (1 µg/ml) and test samples and further incubated for 48 h. NO production was evaluated by measuring the amount of nitrite in the culture supernatant of samples using the Griess reagent. The density of the NO production was measured at 570 nm with a microplate reader.

Cytotoxicity was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) colorimetric method (161). After incubating the samples in a 96-well plate for 48 h, 10 µl of MTT solution (5 µg/ml of MTT in PBS) was added to the well plate and all the mixtures in a well plate were incubated for 4 h. The medium was then removed and the DMSO was added to dissolve the insoluble purple formazan production in the cells line. The absorbance of formazan solution was measured at 570 nm with a microplate reader. The test sample was observed to be cytotoxic if the amount of purple formazan produced of

the sample-treated group was less than 80% of the vehicle-treated group. L-NA (NOS inhibitor), CAPE (NF-κB inhibitor) and indomethacin (COX and iNOS inhibitor) were used as standard positive controls.

% inhibitory activity on NO production was determined according to the following equation:

% Inhibitory activity = (A - B)/(A - C) x 100

A – C : nitrite concentration (μg)

A : LPS (+), sample (-); B : LPS (+), sample (+); C : LPS (-)

3.2.4.3. *In vivo* determination on antioxidant and anti-inflammatory activities

The rat was treated with indomethacin as described in section 3.2.2. The rat stomach tissue was removed for biochemical analysis on malondialdehyde (MDA) content, anti-inflammatory enzymes [myeloperoxidase (MPO) activity and pro-inflammatory cytokines (PGE_2 and TNF-α) content using enzyme immunoassay kit (R&D System, Inc., MN, USA)]. The homogenates of animal tissue were prepared and evaluated according to the following method;

To prepare the tissue homogenate, the stomach was opened, and the glandular portion of the stomach tissue was collected, weighed and immediately rinsed in an ice phosphate buffered saline. The tissue was then fragmented and homogenized with 1.5 ml of ice-cold Ripa buffer (a tissue lysis) for 5 min (162). The supernatant was used for the further assays.

Determination on MDA level (a biomarker for lipid peroxidation)

The level of MDA in the homogenates was evaluated following the method of Sharma and Krishna (1968) (163) with few modifications. In brief, protein was precipitated by adding 500 µl 10% of trichloroacetic acid (TCA) to 500 µl of supernatant. The supernatant was filtered and centrifuged at 3,000 rpm for 10 min at 4°C. Then, 500 µl of supernatant was mixed with 500 µl of 0.67% thiobarbituric acid (TBA) (a substrate) and kept in a boiling water bath for 15 min. Finally, the reaction mixture was cool immediately in an ice water bath to stop the reaction. 200 µl of the supernatant was transferred into a 96 well plate and the absorbance was then read using a microplate reader at 531 nm. MDA concentration was expressed as MDA per gram wet of tissue.

Determination on MPO activity (a biomarker for neutrophils infiltration)

The MPO activity was assayed following a method of Schierwagen et al. (1990) (164) with few modifications. The sample was centrifuged at 9,000g for 10 min at 4°C. The supernatant was removed and the pellet was re-extracted with a phosphate buffer (80 mM, pH 6.6) and 0.05% of hexadecyltrimethylammonium bromide (HTBA) and then sonicated for 30 sec. Next, the sample was re-centrifuged at 11,000g for 20 min at 4°C. The reaction was performed in a polyethylene tube, 120 µl of the supernatant, 800 µl of solution containing a following mixture of 400 µl of 80 mM phosphate buffer, 340 µl of 22 mM phosphate buffer and 60 µl of 0.017% H_2O_2. The reaction was transferred to a 96 well plate and stared by adding 20 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) and the sample was then incubated for 3 min at 37°C. The reaction was stopped with 30 µl of 1 M H_2SO_4 and the sample absorbance

was read using a microplate reader at 450 nm. The enzyme activity was calculated and expressed as MPO per gram wet of tissue.

Determination on pro-inflammatory mediator: PGE_2

The PGE_2 level in the tissue supernatant was determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, USA). Briefly, PGE_2 was presented in a sample competed with horseradish peroxidase (H/RP)-labeled PGE_2 (PGE_2 conjugate). The tissue supernatant, standards and control were added to a 96 well plate to bind the antibody in the first incubation for 1 h. After incubation period, PGE_2 conjugate was added to each well to bind with the remaining antibody sites in the second incubation for 2 h. Following a wash to remove unbound materials for a total of four washes, a substrate solution was added to each well to determine the bound enzyme activity. The color development was stopped with a stop solution (the color in the wells changed from blue to yellow). The absorbance was determined using a microplate reader at 450 nm. The results were calculated compared with a standard curve and expressed as pg/ml of PGE_2/ g wet tissue.

Determination on pro-inflammatory cytokine: TNF-α

The concentration of TNF-α was determined by using an ELISA kit (R&D systems, USA). Briefly, a monoclonal antibody specific for rat TNF-α had been pre-coated onto microplate wells. The tissue supernatant, standards and control were pipetted into each well and incubated for 2 h. Any TNF-α present in the microplate well was bound by the immobilized antibody. After washing away any unbound substance for a total of five washes, an enzyme-linked polyclonal antibody specific for rat TNF-α conjugate was added into each well. Following a wash to remove any unbound antibody enzyme reagent, the substrate solution was added to

each well and incubated for 30 min. Finally, the stop solution was added and the enzyme reaction produced in blue color was changed to yellow. The intensity of the color measured was in proportion to the amount of TNF-α bound in the initial step. The absorbance was determined using a microplate reader at 450 nm. The results were calculated compared with a standard curve and expressed as pg/ml of TNF-α/ g wet tissue.

Determination on gastric nitric oxide synthase and cyclooxygenase activities

The rats were treated with oral indomethacin (30 mg/kg) as described in section 3.2.2 and each test drug was administered orally for 3 consecutive days. The rats were killed on the 3rd day, and the ulcerated area was then excised for qualitative analysis of COX–1, COX–2, eNOS, nNOS and iNOS mRNA expression by reverse transcription polymerase chain reaction (RT–PCR) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control since its activity was essential for the maintenance of cell function as a housekeeping gene.

Purification of total RNA from gastric tissues

To prepare the tissue homogenates, 40 mg of scraping stomach tissues was homogenized by adding 350 µl of FARB buffer and 3.5 µl of β-mercaptoethanol, passing the sample lysate through a 20-G needle syringe for 10 times and then incubating at room temperature for 5 min. Homogenates were transferred into a filter column and centrifuged at 18,000g for 2 min. The obtained supernatants were added with 350 µl of 70% RNase-free ethanol, mixed by vortexing, centrifuged at 18,000g for 1 min and then discarding the supernatant from the FARB mini column. The 500 µl of wash buffer 1 was added to the FARB mini column. The

column was centrifuged at 12,000g for 1 min and the supernatant was then discarded from the column. The 650 µl of wash buffer 2 was added to the FARB mini column. The column was centrifuged at 12,000g for 1 min and the supernatant was then discarded from the column. This last step was repeated for one more washing. After centrifugation, the FARB mini column was placed to an elution tube and 50 µl of RNase-free ddH_2O was added to the membrane center of the column. The column was allowed to stand for 1 min and then centrifuged at 12,000g for 1 min to elute RNA. The concentration of total RNA was checked at 260 nm, and the purity was measured at the absorbance 260/280 ratio. The complementary DNA (cDNA) was synthesized from 1 µg total RNA using reverse transcription reagent kit.

Procedure of reverse transcription (cDNA synthesis)

1. The RNA-primer mixture was prepared by adding 1 µg of total RNA template, 1 µl of Oligod $(T)_{18}$ primer, 1 µl of 10 mM dNTPs mix and nuclease-free water top up to 10 µl.

2. The mixture was incubated at 65°C for 5 min and transferred on ice for 2 min.

3. The cDNA synthesis mixture was prepared by adding 2 µl of 10X buffer M-MuLV, 0.5 µl of M-MuLV reverse transcriptase and nuclease-free water top up to 10 µl.

4. 10 µl of cDNA synthesis mixture was then added into each RNA-primer mixture.

5. The reaction was terminated by incubating the tube at 42°C for 60 min and following at 85°C for 5 min. The synthesized cDNA was stored at -20°C until used.

Determination of COX–1, COX–2, eNOS, nNOS and iNOS mRNA by RT-PCR

1. The synthesized cDNA (DNA template), forward and reverse primers for rat COX–1, COX–2, eNOS, nNOS, iNOS and GAPDH primers (Table 3-4), 5x FIREPol® Master Mix (Ready to Load) and RNase-free water were thawed and placed on ice.

2. Each PCR reaction was prepared according to Table 3-5 and then added to the individual PCR tube.

3. PCR conditions were carried out using BIOER TECHNOLOGY CO., LTD. Thermal Cycler. The PCR program was started while PCR tubes were still on ice and waited until the thermal cycler had reached 60°C. The PCR tubes were then placed in the thermal cycler. Thermal cycler conditions were shown in Table 3-7.

Agarose gel electrophoresis

Electrophoresis was performed using agarose gel and a buffering solution as described in the following procedure:

1. A 1.2% (%w/v) agarose gel was prepared by adding 1.2 g of agarose powder to 100 ml of 1X Tris-Acetate-EDTA (TAE) running buffer (40 mM Tris-Acetate and 1 mM; pH 8.0) in 250 ml erlenmeyer flask.

2. The gel solution was placed on the hot plate to boil and spin every 30 s until all of the agarose particles were well dissolved or completely melted.

3. The melten agarose was allowed to cool at 60°C and was then poured into a tray containing a sample comb and allowed to solidify at room temperature.

4. After the gel was solidified, the comb was carefully removed not to rip the bottom of the wells. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and covered with 1X TAE running buffer. Samples containing DNA mixed (15 µl of PCR product), the lid and power leads were placed on the apparatus, and a current was applied at 100V for 25 min.

5. The electrophoresis was stopped when the loading dye had migrated to three-fourths of the gel. The gel was then soaked with SYBR® Safe DNA gel stain in TAE buffer for 30 min in the dark gel tank. The gel tank was carried to the dark-room, and the DNA bands were observed under a UV light box (GeneDirex, BLooK LED transsilluminator). All bands were finally photographed.

Preparation of Quantitative Real-Time PCR (qRT-PCR) reaction

1. The synthesized cDNA (DNA template), forward and reverse primers for rat COX–1, COX–2, eNOS, nNOS, iNOS and GAPDH (Table 3-4), 5x HOT FIREPol®EvaGreen®qPCR Mix Plus and RNase-free water were thawed and placed on ice.

2. Each qRT-PCR reaction was prepared according to Table 3-6 and then added to the individual 0.1 ml 96 well qPCR plate.

3. The qRT-PCR condition was carried out using QuantStudio®3 Real-Time PCR system. The qRT-PCR program was started while a 96 well qPCR plate was still on ice and waited until the Real-Time PCR system had reached 95°C. The cycler step conditions were shown in Table 3-7.

Table 3-4 The sequences of forward and reverse primers for rat COX–1, COX–2, eNOS, nNOS, iNOS and GAPDH

Enzyme	Sequences of sense and antisense primers	PCR product
1. COX-1		447 bp
–forward	5'–AACCGTGTGTGTGACTTGCTGAA–3'	
–reverse	5'–GCATTTCTCGGGACTCCTTGATGA–3'	
2. COX-2		213 bp
–forward	5′–AGGTGTATCCTCCCACAGTCAAAG–3′	
–reverse	5′–TTTGGAACAGTCGCTCGTCATC –3′	
3. eNOS		349 bp
–forward	5′–ACCTGATCCTAACTTGCCTTGC–3′	
–reverse	5′–AGTGACATCACCGCAGACAAAC–3′	
4. nNOS		475 bp
–forward	5′–CGTCCTTTGAATACCAGCCTGATC –3′	
–reverse	TTCAGAGTCAACATGGGAGAGG–3′	
5. iNOS		348 bp
–forward	5'–CTTCAATGGTTGGTACATGGGCAC–3'	
–reverse	5'–ACGTAGTTCAACATCTCCTGGTGG–3'	
6. GAPDH		311 bp
–forward	5'–GAACGGGAAGCTCACTGGCATGGC–3'	
–reverse	5'–TGAGGTCCACCACCCTGTTGCTG–3'	

Table 3-5 Reaction components for PCR

Component	Volume/reaction
RNase-free water	13.0 µl
5x FIREPol® Master Mix (Ready to Load)	4.0 µl
Forward primers (from working primer 5 pmol/µl)	0.5 µl
Reverse primers (from working primer 5 pmol/µl)	0.5 µl
DNA template (5 ng/µl)	2.0 µl
Total volume	**20.0 µl**

Table 3-6 Reaction components for qRT-PCR

Component	Volume/reaction
RNase-free water	13.0 µl
5x HOT FIREPol®EvaGreen®qPCR Mix Plus	4.0 µl
Forward primers (from working primer 5 pmol/µl)	0.5 µl
Reverse primers (from working primer 5 pmol/µl)	0.5 µl
DNA template (5 ng/µl)	2.0 µl
Total volume	**20.0 µl**

Table 3-7 Thermal cycler conditions of PCR and qRT-PCR

Cycle step	PCR conditions		qRT-PCR conditions	
	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)
Initial denaturation for PCR	95	2.00		
Initial activation for qRT-PCR			95	12.00
Denaturation	95	0.30	95	0.20
Annealing				
- nNOS, COX-1, COX-2, GAPDH	55	0.30	55	0.30
- eNOS, iNOS	57	0.30	57	0.30
Elongation	72	1.00	72	0.50
Final elongation	72	5.00		
Number of cycles = 40 cycles				

3.2.4.4. Determination on gastric biochemical parameters

A pylorus ligation was carefully done in fasted rats under anesthesia with pentobarbital sodium (50 mg/kg, *i.p.*). The rats were divided in to 7 groups of 6 rats each including 1) water control (5 ml/kg, *i.d*), 2) 0.5% CMC (5 ml/kg, *i.d*), 3) 0.1 M acetic acid (5 ml/kg, *i.d*), 4) lansoprazole (1 mg/kg, *i.d*), 5) curcumin (20 mg/kg, *i.d*), 6) chitosan (150 mg/kg, *i.d*) and 7) chitosan (150 mg/kg)-curcumin (20 mg/kg) mixture *i.d*). All test samples were administered immediately after the pylorus ligation. Four hours later, the rat was sacrificed using pentobarbital sodium overdose

(150 mg/kg). The gastric juice was collected for measuring total acid output and pepsin activity while the gastric wall was collected for measuring the mucus content.

The collected gastric juice was transferred to polyethylene tubes and centrifuged at 1,300g for 10 min. After measuring the volume of the supernatant, the total gastric acidity was analyzed by titration with 0.01 N NaOH solution and using 2% phenolphthalein as an indicator. The pH of the samples solution was measured using pH meter and expressed as mEq (165).

Pepsin activity was determined by a few modification of previously described methods (166). Pepsin activity against acid-denatured using hemoglobin as the substrate was determined at pH 3, 37°C. First, 25 µl of gastric juice sample was added to 0.5 ml of bovine hemoglobin solution (0.25%) in 0.5 ml of 0.1 M solution acetate buffer-HCl (pH 3) to initiate the reaction. Second, the mixture was incubated in water bath to equilibration at 37°C for 30 min. Afterwards, the reaction was interrupted by addition of 0.5 ml of 12% ice cold trichloroacetic acid. The tube containing the mixture was kept in an ice bath at 4°C for 15 min. and then centrifuged at 20,800g at 4°C for 5 min to separate the precipitated proteins. Finally, 700 µl of the obtained supernatant was used to determine the concentration of release amino acids by measuring the absorbance using UV spectrophotometer at 280 nm. Individual values were interpolated on a pepsin standard curve (powder ≥250 units/mg solid; 0.05-5 mg/ml) and the results were expressed in terms of unit/ml gastric juice.

Gastric wall mucus content was determined by the methodology previously described (167) with few modifications. The stomach was open along the greater curvature, weighted and immersed in 10 ml of 0.1% alcian blue in 0.16 M sucrose/0.05 M sodium acetate, pH 5.8 for 2 h. The excessive of dye was rinse with

0.25 M sucrose solution (15 min/time) for two successive. The remaining of dye complex with the gastric mucus was extracted with 0.5 M $MgCl_2$ for 2 h and shaken intermittently for 1 min in every 30 min interval. The alcian blue extract was centrifuged at 3600 rpm for 10 min at 4°C. The absorbance of alcian blue was measured by UV spectrophotometer at 580 nm. Individual values were interpolated on alcian blue standard curve and the results were expressed as µg alcian blue/g wet tissue.

3.2.5. Wound healing activity determination on AGS human gastric epithelial cell line

AGS human gastric epithelial cell line (CRL-1739™, ATCC®) were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco®) supplemented with 10% FBS, 50 Unit/ml penicillin and 50 µg/ml streptomycin. The cells were grown in 75 cm^2 plastic cell culture flasks and incubated in a humidified atmosphere with 5% CO_2 at 37°C. AGS cells with less than 20 passage numbers were used. Culture media was changed every alternate day. After 80% confluence, culture cells were trypsinized using 0.25% trypsin-EDTA solution at 37°C. Cell suspensions were prepared at desired density before determining on wound healing effect and cytotoxicity of the test samples. The migration of AGS cells was investigated using a wound healing method as previously described by Kim et al. (2012) (168) and He et al. (2016) (169) with some modifications. AGS cells (5×10^5 cells/ml) in media containing 2% FBS were seeded into each well of a 6-well plate and incubated in a humidified atmosphere with 5% CO_2 at 37°C. After confluent monolayer of cells was formed, a wound was made by straight scratching the cells in a line with a sterile

pipette tip. After washing any cellular debris with PBS three times, the cells were cultured with 1 ml of fresh medium with or without the presence of test samples. The time of the scratching wound was defined as time 0. Images were taken at a 4x magnification using a microphotograph on time 0. Then the cells were allowed to migrate into the wound for the next 5 h (time 5) and 10 h (time 10) and subsequently taken the images at those determined time again. Relative wound closure (%) was measured from the images using computing software (imageJ) and compared with the value obtained before treatment (time 0). An increase of the relative size of wound closure (μm) indicated the cell migrations. The percentage of wound healing was calculated according to the following equation.

$$\% \text{ Wound healing} = \frac{[(\text{size of wound area}_{(\text{time 0})} - \text{size of wound area}_{(\text{time at 5 or 10 h})}]}{\text{size of wound area}_{(\text{time 0})}} \times 100$$

3.2.6. Curative effect of a chitosan-curcumin preparation on acetic acid-induce buccal mucosal ulcer in hamsters

The hamsters were deprived of food and water for 2 h before the procedure. Under sodium pentobarbital anesthesia (50 mg/kg, *i.p.*), a buccal ulcer was induced by a topical application of acetic acid according to the method of Karavana et al. (2011) (170), with few modifications. A round filter paper of 5 mm in diameter was soaked in 15 μl of 99.7% acetic acid and then pressed onto the left site buccal mucosa for 60 s. Each test topical preparation (Table 3-8) was applied twice daily whereas an oral chitosan-curcumin mixture containing 20 mg of curcumin and 150 mg of chitosan that exerted a maximum curative activity against acetic acid induced chronic GU was

administered orally once daily. All test preparations were administered beginning on the 5th day after the ulcer induction for 7 consecutive days. The animal was sacrificed using pentobarbital sodium overdose (150 mg/kg) on the 12th day after the ulcer induction and the buccal mucosa was removed and fixed in 10% formalin solution. The ulcer area (mm^2) was then measured macroscopically using the computer program (imageJ) and evaluated histologically using the scoring protocol according to the following criteria in Table 3-9 (170).

The experimental groups were shown in Table 3-8 with each consisting of 6 animals. The oral test preparation was given in a volume of 5 ml/kg.

Table 3-8 Groups of test preparation using in a curative study on acetic acid induced oral ulcer in hamsters (n=6)

Experimental groups	**Test preparations**
1	Water control
2	Topical 0.5% chitosan without curcumin solution (vehicle control for a topical 0.1% chitosan-curcumin preparation)
3	Topical 0.1% curcumin in 0.5% chitosan solution
4	Topical 0.15% benzydamine (Difflam®) solution (Standard solution)
5	Oral chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid,od]

Table 3-9 The scoring protocol to establish the histological level of the wound healing

Scores	Histological level of the wound healing
1	Presence of epithelial necrosis but no signs of inflammation
2	Presence of inflammatory reaction but no appearance of angiogenesis
3	Presence of inflammatory reaction with new capillary proliferations at the ulcer base, but no epithelization at the ulcer surface
4	Decrease of inflammatory reaction, presence of new capillary proliferation and the beginning of epithelization at the ulcer surface
5	Presence of complete epithelization at the ulcer surface

3.2.7. Wound healing activity determination on human gingival fibroblast (HGF) cell line (171)

The migration of HGF cell was investigated using a wound healing method as previously described by Liang et al. (171) with some modification. HGF cell line was cultured in DMEM supplement containing 10% FBS. The cells were seeded with $1.5x10^4$ cells/well in a 12-well plate and incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 incubator to allow cell adhesion. A straight line scratch was created in the native cell monolayer using a p200 pipetted tip. The debris was removed by gently washing the cells once with PBS (1 ml). The medium was then replaced with 1 ml of a fresh medium containing test sample and incubated at 37°C in a humidified atmosphere containing 5% CO_2 incubator. 3 day later, the closure of the wound size (μm) was observed and recorded using a phase-contrast inverted microscope. The percentage of wound healing was calculated according to the following equation.

$$\% \text{ Wound healing} = \frac{[(\text{size of wound area}_{(\text{Day } 0)} - \text{size of wound area}_{(\text{Day } 1,2 \text{ or } 3)}]}{\text{size of wound area}_{(\text{Day } 0)}} \times 100$$

Statistical analysis

All *in vitro* values were represented as mean ± SD. All *in vivo* parametric values were represented as mean ± SEM. Statistical analyses were performed by one way ANOVA followed by Dunnett's test. The results were considered statistically significant if p-values were less than 0.05.

Statistical analysis of histological healing scores for each test group was performed using Mann-Whitney U test. The results were considered statistically significant if p-values were less than 0.05.

CHAPTER 4

RESULTS

4.1. Preventive effect of a chitosan-curcumin mixture against indomethacin-induced acute gastric ulceration in rats

Indomethacin-induced acute gastric ulceration model is widely used for screening compounds with antiulcer activity. The severity of the gastric ulceration depended on the dose and time after administration of indomethacin. An oral administration of indomethacin (30 mg/kg) for 4 h schedule was suitable ulcer model for evaluation of potential antiulcer agents as linear band-like hemorrhagic ulcerations including erosion were produced in the glandular portion of the stomach (Figure 4-1). In the present study, it was found that pretreatment of curcumin at doses of 20-40 mg/kg significantly reduced the total gastric ulceration index from 68.33±1.89 mm into 28.83±0.54 and 36.67±2.52 mm, respectively ($p<0.05$) (Table 4-1). Curcumin at a dose of 20 mg/kg seemed to exert the most potent preventive efficacy against indomethacin induced acute gastric ulceration. Pretreatment of chitosan at doses of 75, 150 and 300 mg/kg also significantly reduced the gastric ulceration index in a dose dependent manner ($p<0.05$). Accordingly, a chitosan-curcumin mixture was prepared by suspending 20 mg of curcumin in 10 ml of 150 or 300 mg of chitosan dissolved in 0.1 M acetic acid. It was found that both concentrations of the mixture provided a comparable potent gastroprotective efficacy as shown in (Table 4-1) but exerted the superior gastroprotective efficacy than those of curcumin, chitosan and lansoprazole (a standard antisecretory agent). Due to a feeding difficulty through an

oral gavage needle of a high-viscosity liquid of a chitosan-curcumin mixture containing 300 mg of chitosan, a chitosan-curcumin mixture containing 20 mg of curcumin and 150 mg of chitosan was used for further ulcer curative efficacy study.

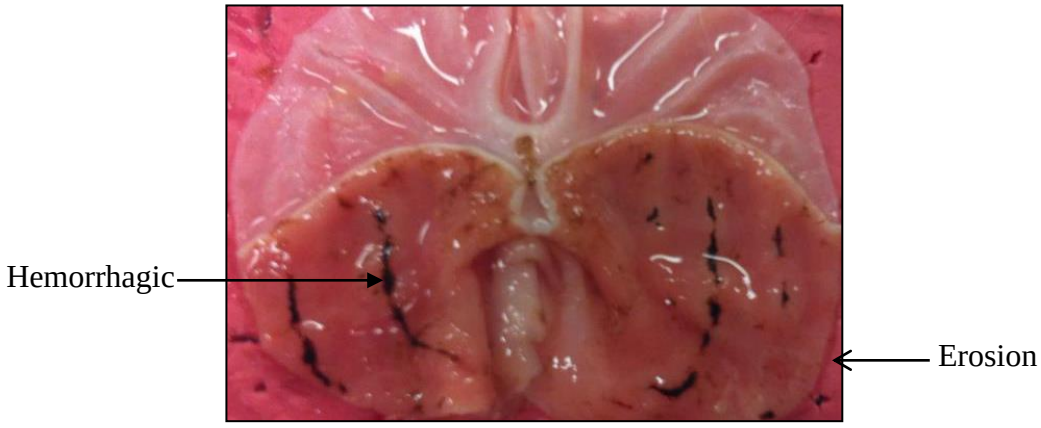


Figure 4-1 Gross appearance of acute gastric ulceration induced by an oral administration of indomethacin (30 mg/kg) in rats

Table 4-1 Preventive effect of a chitosan-curcumin mixture against indomethacin-induced acute gastric ulceration in rats

Treatment groups	Gastric ulceration index (mm)	Inhibition (%)
Water control (5 ml/kg, *p.o.*)	68.33±1.89	0.00±2.77
0.1 M Acetic acid (5 ml/kg, *p.o.*) (vehicle control for curcumin, chitosan and chitosan-curcumin mixture)	63.67±0.88	6.83±1.29
0.5% CMC suspension (5 ml/kg, *p.o.*) (vehicle control for lansoprazole)	58.33±1.21	14.63±1.84
Curcumin (20 mg/kg, *p.o.*)	28.83±0.54[a,b,e]	57.80±0.79[a,b,e]
Curcumin (40 mg/kg, *p.o.*)	36.67±2.52[a,b]	46.34±3.69[a,b]
Chitosan (75 mg/kg, *p.o.*)	40.50±1.20[a,b]	43.72±1.89[a,b]
Chitosan (150 mg/kg, *p.o.*)	25.50±1.20[a,b,e]	62.68±1.76[a,b,e]
Chitosan (300 mg/kg, *p.o.*)	16.83±1.01[a,b,d,e,g]	75.37±1.48[a,b,d,e,g]
Chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, *p.o.*]	15.33±1.31[a,b,d,e,f,g]	77.56±1.91[a,b,d,e,f,g]
Chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (300 mg) dissolved in 0.1 M acetic acid, *p.o.*]	13.17±1.01[a,b,d,e,f,g]	80.73±1.48[a,b,d,e,f,g]
Lansoprazole (1 mg/kg, *p.o.*)	23.33±1.82[a,c,e]	65.85±2.66[a,c,e]

Each value represents the mean ± S.E.M. (n=6)

Gastric ulceration index was determined as the sum of the length (mm) of all lesions (erosion and hemorrhagic ulceration) for each stomach

$$\% \text{ Inhibition} = \frac{(\text{Total ulcerated area}_{(\text{control})} - \text{Total ulcerated area}_{(\text{treatment})})}{\text{Total ulcerated area}_{(\text{control})}} \times 100$$

[a]$p<0.05$ when compared to the water control treated rats (Dunnett's test)

[b]$p<0.05$ when compared to the vehicle (0.1 M acetic acid) treated rats (Dunnett's test)

[c]$p<0.05$ when compared to the vehicle (0.5% CMC) treated rats (Dunnett's test)

[d]$p<0.05$ when compared to curcumin (20 mg/kg) and curcumin (40 mg/kg) treated rats (Dunnett's test)

[e]$p<0.05$ when compared to chitosan (75 mg/kg) treated rats (Dunnett's test)

[f]$p<0.05$ when compared to chitosan (150 mg/kg) treated rats (Dunnett's test)

[g]$p<0.05$ when compared to lansoprazole (1 mg/kg) treated rats (Dunnett's test)

4.2. Curative effect of a chitosan-curcumin mixture on acute GU induced by indomethacin in rats

An oral administration of indomethacin at a dose of 18 mg/kg following the method of Banerjee et al., (2008) studied in mice model (172) was tried out, but only few mild gastric ulcerations were found and healed within 3 day after an indomethacin administration. Therefore, this mice ulcer model was not suitable for evaluation of ulcer curative efficacy of the test compounds in rat model. It was found that an oral administration of indomethacin (30 mg/kg) for 5 h schedule was capable of producing GU in the glandular portion of the stomach with maximum ulceration occurred on the 3rd day after an indomethacin administration (Figure 4-2). However, if untreated, some of ulcerated rats had severe gastric hemorrhage and side effects of indomethacin that caused sedation, loss of weight and bleeding at paw and ear areas. An oral administration once daily of curcumin (20 mg/kg), chitosan (150 mg/kg), a chitosan-curcumin mixture (containing 20 mg of curcumin and 150 mg of chitosan) or a PPI: lansoprazole (a standard antisecretory agent) (1 mg/kg), beginning at 5 h after an indomethacin administration and then administered every 24 h for two consecutive days, showed a significant ulcer curative efficacy in term of ulcer index and % curation as compared to values in the water control group (Table 4-2). Only few side effects such as bleeding at paw and ear areas had been found in some treated animals. Chitosan was found to possess a higher ulcer curative efficacy than curcumin but less than lansoprazole. Similarly to its ulcer preventive efficacy, a chitosan-curcumin mixture exerted the most potent ulcer curative efficacy among the treatment groups including lansoprazole.

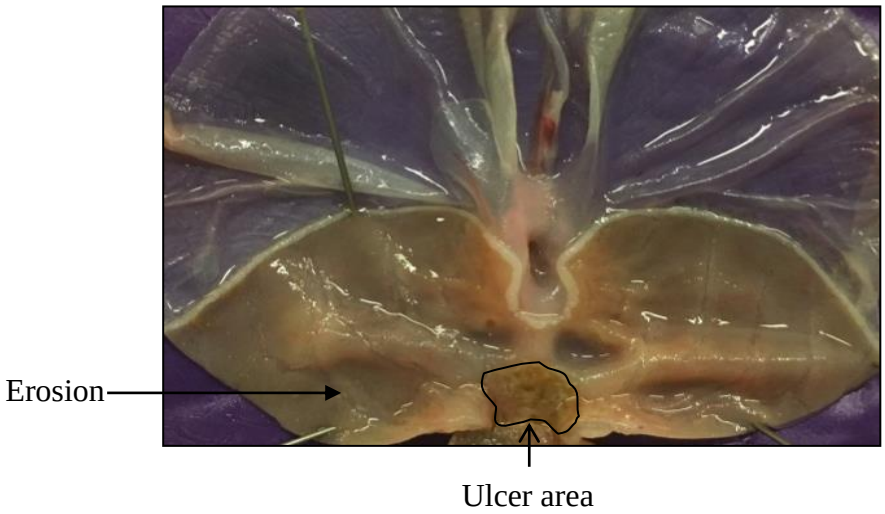


Figure 4-2 Gross appearance of acute gastric ulcer on day 3 after an oral administration of indomethacin (30 mg/kg) for 5 h

Table 4-2 Curative effect of a chitosan-curcumin mixture on acute GU induced by indomethacin in rats

Treatment groups	Ulcer index (mm^2)	% Curation
Water control (5 ml/kg, *p.o.*)	62.02±0.84	0.00±1.36
0.1 M Acetic acid (5 ml/kg, *p.o.*) (vehicle control for curcumin, chitosan and chitosan-curcumin mixture)	57.61±1.09	7.12±1.75
0.5% CMC suspension (5 ml/kg, *p.o.*) (vehicle control for lansoprazole)	54.20±1.63	12.67±2.62
Curcumin (20 mg/kg, *p.o.*)	36.81±2.47[a,b]	40.65±3.98[a,b]
Chitosan (150 mg/kg, *p.o.*)	28.53±1.12[a,b,d]	54.00±1.80[a,b,d]
Chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, *p.o.*]	9.02±1.37[a,b,d,e,f]	85.46±2.20[a,b,d,e,f]
Lansoprazole (1 mg/kg, *p.o.*)	19.30±1.48[a,c,d,e]	68.88±2.39[a,c,d,e]

Each value represents the mean ± S.E.M. (n=6)

Ulcer index determined as the length (mm) x the width of the ulcer (mm)

$$\% \text{ Curation} = \frac{[(\text{Total ulcerated area}_{(\text{control})} - \text{Total ulcerated area}_{(\text{treatment})})}{\text{Total ulcerated area}_{(\text{control})}] } \times 100$$

[a]$p<0.05$ when compared to the water control treated rats (Dunnett's test)

[b]$p<0.05$ when compared to the vehicle (0.1 M acetic acid) treated rats (Dunnett's test)

[c]$p<0.05$ when compared to the vehicle (0.5% CMC) treated rats (Dunnett's test)

[d]$p<0.05$ when compared to curcumin treated rats (Dunnett's test)

[e]$p<0.05$ when compared to chitosan treated rats (Dunnett's test)

[f]$p<0.05$ when compared to lansoprazole treated rats (Dunnett's test)

4.3. Curative effect of a chitosan-curcumin mixture on chronic GU induced by acetic acid treatment in rats

The present study also evaluated the curative efficacy of a chitosan-curcumin mixture in healing chronic gastric ulcer induced by a topical application of acetic acid. Immediately after an application of acetic acid onto the serosal surface of the gastric antral part, superficial blood vessels were damaged and leading to the gastric tissue ulceration due to the severe thrombosed blood vessels in the submucosal layer. Well-defined, deep, round or oval chronic ulcer with disrupted mucosal layer and muscularis mucosae was well established at the antral site 4 days after the acetic acid application. Some ulcers penetrated into the adjacent organs (mainly liver) but no ulcer perforation was observed during the course of study. The original ulcer undergoes a healing process of approximately 3 weeks after ulcer induction and is found macroscopically healed in 60% of the animals (157). The diameter of the produced ulcer on the 4^{th} day after ulcer induction in the present study was about 8.50±1.87 mm and the ulcer area was about 61.17±2.09 mm^2 (Figure 4-3). The area of the ulcer decreased with time but still remained up to the 14^{th} day after an ulcer induction with ulcer area about 10.33±0.67 mm^2. The data obtained showed that all test preparations (except a twice-daily oral administration of chitosan-curcumin mixture) given for 10 consecutive days beginning on the 4^{th} day after an acetic acid application significantly reduced the ulcer index (UI) and enhanced the percentage of ulcer curation, healing index (HI) and mucosal regeneration index (MRI) when compared to those of the control groups ($p<0.05$) (Table 4-3). Chitosan seemed to possess a lower UI and a higher percentage of ulcer curation than curcumin from macroscopically examination (UI and % curation). However, regarding to the

histological analysis, there was no significant difference of the healing index (HI) and mucosal regeneration index (MRI) between both treatment groups. It was also found that both curcumin and chitosan exerted a significant higher ulcerative curative efficacy than lansoprazole ($p<0.05$). Interestingly, an oral administration of a chitosan-curcumin mixture (containing 20 mg of curcumin and 150 mg of chitosan) only once a day was capable to exert a better curative efficacy to a twice-daily oral administration of curcumin, chitosan or lansoprazole (a standard antisecretory agent). The ulcer curative potency of a chitosan-curcumin mixture reached almost the maximum at the once-daily dosing frequency as the curative potency of a mixture tended to decrease at a twice-daily dosing.

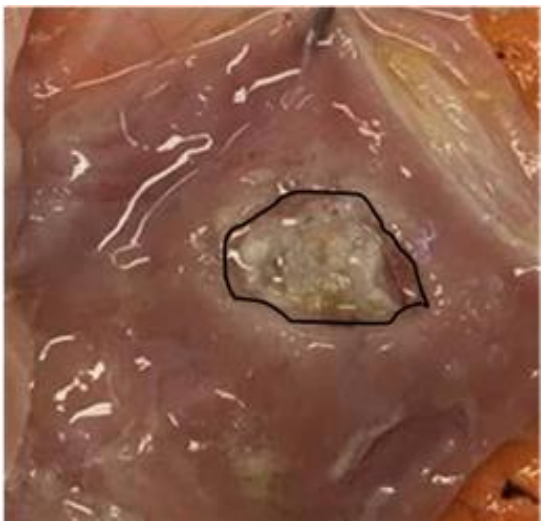


Figure 4-3 Gross and histopathological appearances of chronic GU induced by a topical application of acetic acid in rats on the 4th day after acetic acid application

Table 4-3 Curative effect of a chitosan-curcumin mixture on chronic GU induced by acetic acid treatment in rats

Groups	UI (mm^2)	% Curation	% HI	% MRI
Water control (5 ml/kg, *p.o.* bid)	33.83±1.33	44.69±1.31	40.52±1.32	45.31±1.12
0.1 M Acetic acid (5 ml/kg, *p.o.* bid)	26.67±0.80	56.40±1.02	42.25±1.42	48.94±1.25
0.5% CMC suspension (5 ml/kg, *p.o.* bid)	27.00±0.77	55.86±0.98	51.61±1.09	47.37±0.96
Curcumin (20 mg/kg, *p.o.* bid)	16.33±0.42[a,b,f]	73.30±0.89[a,b,f]	68.97±0.98[a,b,f]	69.23±1.02[a,b,f]
Chitosan (150 mg/kg, *p.o* bid)	12.17±0.48[a,b,d,f]	80.11±0.58[a,b,d,f]	66.94±1.08[a,b,f]	69.07±1.04[a,b,f]
Chitosan-curcumin mixture [Curcumin(20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, *p.o.* od]	10.33±0.67[a,b,d,f]	83.11±0.76[a,b,d,f]	70.00±0.77[a,b,f]	73.28±0.88[a,b,f]
Chitosan-curcumin mixture [Curcumin(20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, *p.o.* bid]	18.00±1.53[a,b]	70.57±1.46 [a,b]		
Lansoprazole (1 mg/kg, *p.o*. bid)	20.00±0.97[a,c]	67.30±1.58[a,c]	57.14±0.86[a,c]	61.11±0.96[a,c]

Each value represents the mean ± S.E.M. (n=6)

UI, Ulcer index; HI, Healing index; MRI, Mucosal regeneration index.

All measuring parameters were determined as described in Chapter 3.

[a]$p<0.05$ when compared to the water control treated rats (Dunnett's test)

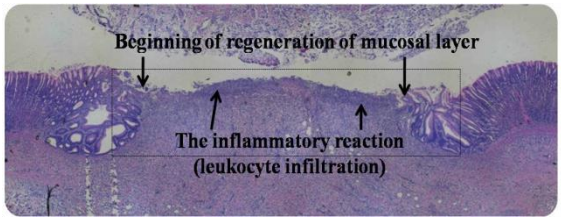
[b]$p<0.05$ when compared to the vehicle (0.1 M acetic acid) treated rats (Dunnett's test)

[c]$p<0.05$ when compared to the vehicle (0.5% CMC) treated rats (Dunnett's test)

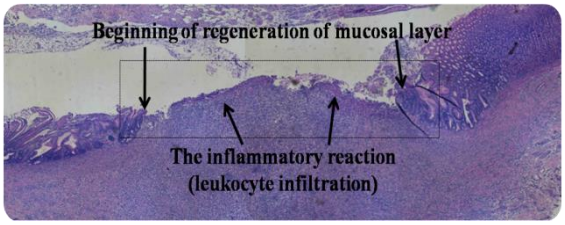
[d]$p<0.05$ when compared to curcumin treated rats (Dunnett's test)

[e]$p<0.05$ when compared to chitosan treated rats (Dunnett's test)

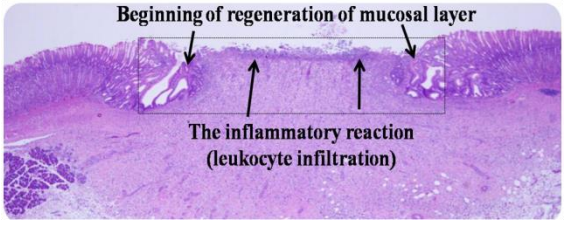
[f]$p<0.05$ when compared to lansoprazole treated rats (Dunnett's test)



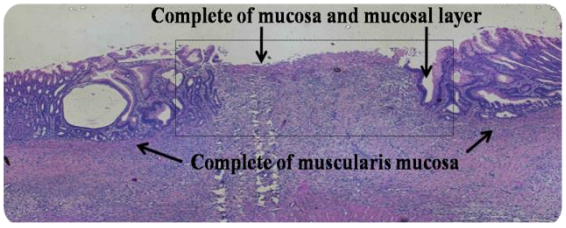
Water control (5 ml/kg, *p.o*. bid)



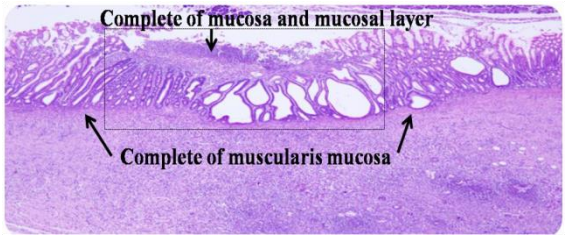
0.1 M acetic acid (5 ml/kg, *p.o*. bid)



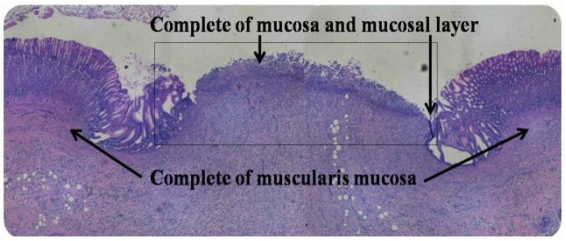
0.5% CMC (5 ml/kg, *p.o*. bid)



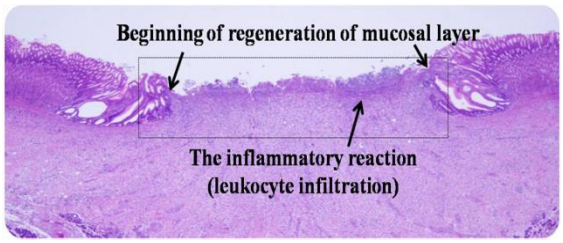
Curcumin (20 ml/kg, *p.o*. bid)



Chitosan (150 ml/kg, *p.o*. bid)



Chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, *p.o*. od]



Lansoprazole (1 ml/kg, *p.o*. bid)

Figure 4-4 Histological examination of the effect of a chitosan-curcumin mixture on chronic GU induced by a topical application of acetic acid in rats on the 14th day after the ulcer induction. Hematoxylin and eosin (H&E) strain, magnification 4x

Histopathological samples of each treatment group on the 14th day after ulcer induction were shown in Figure 4-4. In the group treated with water, 0.1 M acetic acid or 0.5% CMC, the infiltration of leukocytes had still been found indicating that the inflammatory reaction still happened along with the initiation of ulcer healing phase noted with the interruption of the lamina muscularis mucosae and the beginning of mucosal regeneration of the mucosal layer. In the group treated with lansoprazole (a standard antisecretory drug), no inflammation reaction was found but no complete mucosal generation and muscularis mucosa was evidented. On the contrary, in the group treated with curcumin, chitosan or a chitosan-curcumin mixture; no inflammation reaction was found and a mucosal regeneration and a complete of muscularis mucosa were well advanced.

4.4. Antioxidant and anti-inflammatory effect of a chitosan-curcumin preparation

4.4.1. *In vitro* antioxidant effect of a chitosan-curcumin preparation in scavenging an organic free DPPH radical and nitrite radical

The half maximal inhibitory concentration or IC_{50} values of curcumin, a chitosan-curcumin mixture and ascorbic acid in scavenging DPPH free radical were found to be 2.30, 3.61 and 2.38 µg/ml, respectively, as shown in Table 4-4. The DPPH free radical scavenging efficacy of curcumin was found to be comparable to that of the positive standard: ascorbic acid. In contrast, chitosan was not found to exert any effective antioxidant activity in scavenging DPPH free radical.

For nitrite radical scavenging study, NO was generated from an interaction of sodium nitroprusside with oxygen leading to the formation of nitrite and the nitrite

ions which further diazotized with sulphanilamide and coupled with napthyl ethylene diamine (Griess reagent) to form pink color. The obtained results showed that IC_{50} values of curcumin, a chitosan-curcumin mixture and gallic acid at various dose (5-100 µg/ml) were 17.02, 36.38 and 17.22 µg/ml, respectively ($p<0.05$) (Table 4-5). According to the cut-off point at 50 µg/ml for antioxidant activity against nitrite radicals, the test sample is considered to have high antioxidant efficacy with $IC_{50}<50$ µg/ml, moderate antioxidant efficacy with $50<IC_{50}<100$ µg/ml and low antioxidant efficacy with $IC_{50}>100$ µg/ml (173). Therefore, both curcumin and a chitosan-curcumin mixture were considered to have high antioxidant activity against nitrite radicals. On the contrary, chitosan showed too low NO scavenging efficacy to determine its IC_{50} value.

Table 4-4 The IC_{50} value of DPPH free radical scavenging effect of a chitosan-curcumin mixture

Test groups	**IC_{50} (µg/ml)**
Curcumin	2.30
Chitosan	>100
Chitosan-Curcumin mixture	3.61
Ascorbic acid	2.38

Each value represented the mean ± SD (n=4)

Ascorbic acid was used as standard positive control

Table 4-5 The IC_{50} value of nitrite radical scavenging effect of a chitosan-curcumin mixture

Test groups	% Inhibition at various concentrations (µg/ml)					
	5	10	25	50	100	**IC_{50}**
Curcumin	32.26±4.51	43.55±2.56	55.91±5.19	65.05±3.34*	77.69±2.78	**17.02**
Chitosan	42.55±3.96	38.30±5.33	31.91±4.42	21.28±5.32	17.02±4.91	**ND**
Chitosan-curcumin mixture	23.78±5.31	32.87±5.28	42.66±3.32	52.45±6.01	66.43±5.28	**36.38**
Gallic acid	43.50±3.77	45.50±2.18	54.50±0.50	47.50±2.78	45.50±0.50	**17.22**

Each value represents the mean ± SD (n=4), ND = not determined

Gallic acid was used as standard positive controls.

4.4.2. *In vitro* anti-inflammatory effect of a chitosan-curcumin preparation on LPS-induced NO production in macrophage cell line RAW 264.7

The obtained results indicated that curcumin exhibited the highest inhibitory activity on NO production in RAW 246.7 cell among the test compounds with an IC_{50} value of 5.04 µg/ml, followed by a chitosan-curcumin mixture (IC_{50} = 8.68 µg/ml). In addition to its *in vitro* nitrite radical scavenging property, chitosan exerted a low inhibitory activity on NO production (IC_{50} = 872.62 µg/ml) (Table 4-6). Curcumin as well as a chitosan-curcumin mixture exhibited higher inhibitory efficacy than indomethacin (COX and iNOS inhibitor, IC_{50} = 14.92 µg/ml) and L-NA (NOS inhibitor, IC_{50} = 10.96 µg/ml), but lower inhibitory efficacy than CAPE (NF-κB inhibitor, IC_{50} = 3.38 µg/ml). It was also found that curcumin, chitosan and a chitosan-curcumin mixture inhibited LPS induced NO production in a concentration-

dependent manner without obvious cytotoxic effect on macrophage RAW 264.7 cell line (cell survival > 80%) by MTT assay.

Table 4-6 The IC_{50} value of inhibitory effect on LPS-induced NO production in RAW 246.7 cell of a chitosan-curcumin mixture

Treatment groups	IC_{50} (μg/ml)
Curcumin	5.04
Chitosan	872.62
Chitosan-curcumin mixture	8.68
CAPE	3.38
L-NA	10.96
Indomethacin	14.92

Each value represented the mean±SD from four independent experiments

L-NA (NOS inhibitor), CAPE (NF-κB inhibitor) and indomethacin (COX and iNOS inhibitor) were used as standard positive controls

4.5. Effect of a chitosan-curcumin mixture on the production of biomarkers (MDA) and on the release of MPO and pro-inflammatory cytokines (PGE_2 and TNF-α) in gastric ulcerated tissue induced by indomethacin in rats

The data summarized in Table 4-7 and Figure 4-5 indicated that a chitosan-curcumin mixture (containing 20 mg of curcumin and 150 mg of chitosan) exerted the most potent antioxidant efficacy in significantly decreased the production of MDA: a biomarker of oxidative stress and lipid peroxidation in the rat gastric ulcerated tissue induced by indomethacin. The mixture also exerted the most potent anti-inflammatory efficacy in decrease the release of MPO (a biomarker of neutrophils infiltration) and

pro-inflammatory cytokines (PGE_2 and TNF-α) in the rat gastric ulcerated tissue Table 4-7 and Figure 4-6, 4-7 and 4-8. Furthermore, curcumin exerted a superior antioxidant and anti-inflammatory efficacy than lansoprazole whereas chitosan had the least antioxidant and anti-inflammatory efficacy in decrease the production of all test biomarkers. However, it showed that chitosan had endogenous anti-oxidant and anti-inflammatory property.

Table 4-7 Effect of a chitosan-curcumin mixture on the level of MDA, MPO, PGE_2 and TNF-α in the ulcerated stomach tissue of rats

Groups	**MDA (MDA/g wet tissue)**	**MPO (MPO/g wet tissue)**	**PGE_2 (pg/ml of PGE_2/ g wet tissue)**	**TNF-α (pg/ml of TNF-α/ g wet tissue)**
Water control (5 ml/kg, *p.o.*)	0.213±0.012	0.269±0.008	892±20.43	368.45±6.41
0.1 M aceti acid (5 ml/kg, *p.o.*)	0.160±0.007[a]	0.226±0.00[a]	795.82±16.54 [a]	324.63±3.15[a]
0.5% CMC (5 ml/kg, *p.o.*)	0.157±0.004[a]	0.235±0.00[a]	882.49±9.09	334.79±5.16
Curcumin (20 mg/kg, *p.o.*)	0.131±0.004[a,b,e]	0.152±0.007[a,b,e,f]	418.47±11.49[a,b,e,f]	165.48±1.13[a,b,e,f]
Chitosan (150 mg/kg, *p.o.*)	0.148±0.005[a,b]	0.186±0.006[a,b]	571.15±54.54[a,b]	235.27±4.34[a,b]
Chitosan-curcumin mixture [Curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, *p.o.*]	0.106±0.004[a,b,d,e,f]	0.107±0.005[a,b,d,e,f]	304.69±16.40[a,b,c,d,e,f]	152.01±8.34[a,b,c,d,e,f]
Lansoprazole (1 mg/kg, *p.o.*)	0.129±0.006[a,c,e]	0.175±0.009[a,c,e]	464.93±13.58[a,c]	176.89±5.84[a,c,e]

Each value represents the mean ± SEM (n=6).

[a]*P<0.05* when compared to water control group (Dunnett's test)

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group (Dunnett's test)

[c]*P<0.05* when compared to 0.5% CMC-treated group (Dunnett's test)

[d]*P<0.05* when compared to curcumin-treated group (Dunnett's test)

[e]*P<0.05* when compared to chitosan-treated group (Dunnett's test)

[f]*P<0.05* when compared to lansoprazole-treated group (Dunnett's test)

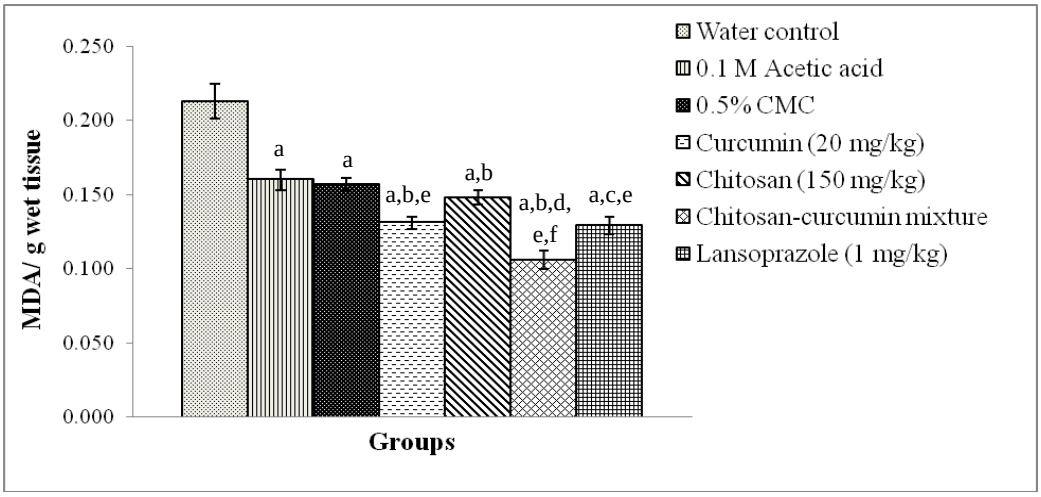


Figure 4-5 Effect of a chitosan-curcumin mixture on the level of MDA in the ulcerated stomach tissue of rats

Each value represents the mean ± SEM (n=6).

[a]*P<0.05* when compared to water control group (Dunnett's test)

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group (Dunnett's test)

[c]*P<0.05* when compared to 0.5% CMC-treated group (Dunnett's test)

[d]*P<0.05* when compared to curcumin-treated group (Dunnett's test)

[e]*P<0.05* when compared to chitosan-treated group (Dunnett's test)

[f]*P<0.05* when compared to lansoprazole-treated group (Dunnett's test)

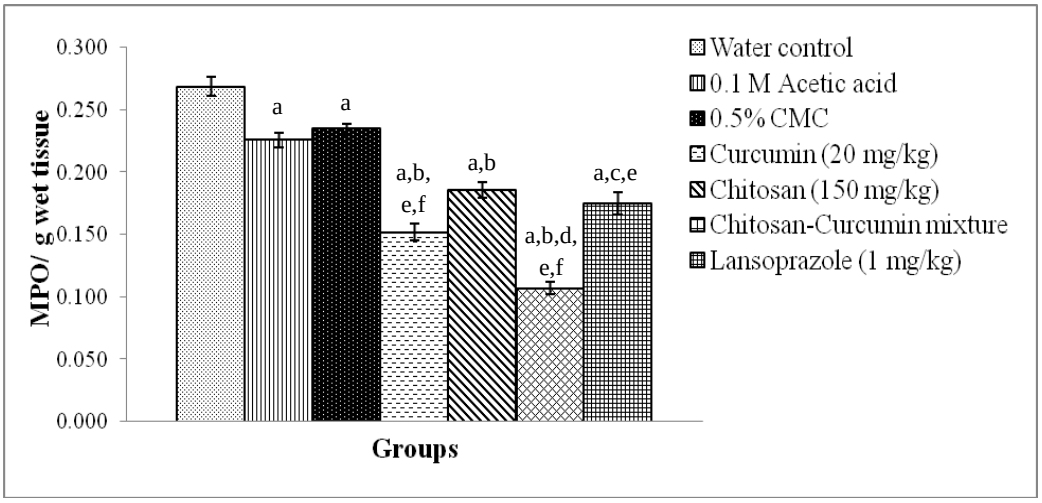


Figure 4-6 Effect of a chitosan-curcumin mixture on the level of MPO in the ulcerated stomach tissue of rats

Each value represents the mean ± SEM (n=6).

[a]*P<0.05* when compared to water control group (Dunnett's test)

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group (Dunnett's test)

[c]*P<0.05* when compared to 0.5% CMC-treated group (Dunnett's test)

[d]*P<0.05* when compared to curcumin-treated group (Dunnett's test)

[e]*P<0.05* when compared to chitosan-treated group (Dunnett's test)

[f]*P<0.05* when compared to lansoprazole-treated group (Dunnett's test)

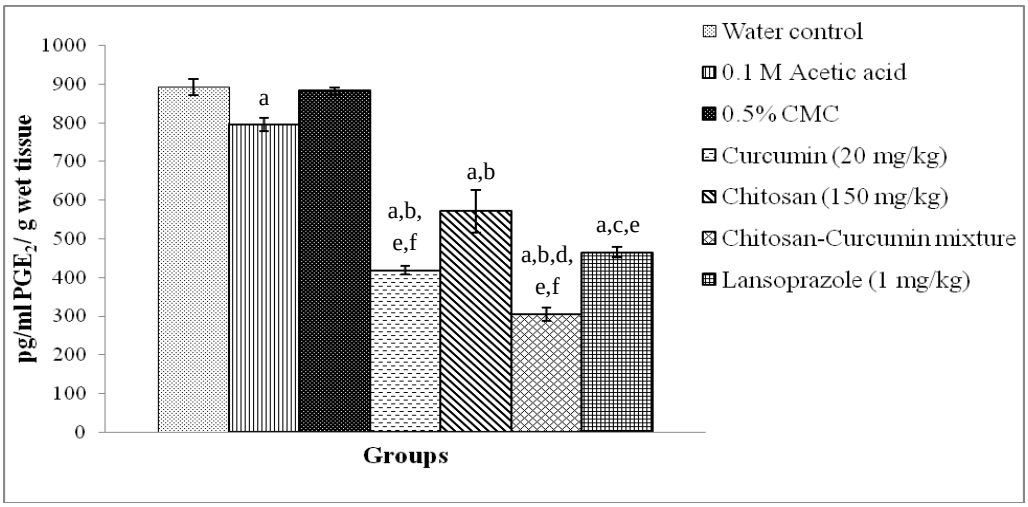


Figure 4-7 Effect of a chitosan-curcumin mixture on the level of PGE_2 in the ulcerated stomach tissue of rats

Each value represents the mean ± SEM (n=6).

[a]*P<0.05* when compared to water control group (Dunnett's test)

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group (Dunnett's test)

[c]*P<0.05* when compared to 0.5% CMC-treated group (Dunnett's test)

[d]*P<0.05* when compared to curcumin-treated group (Dunnett's test)

[e]*P<0.05* when compared to chitosan-treated group (Dunnett's test)

[f]*P<0.05* when compared to lansoprazole-treated group (Dunnett's test)

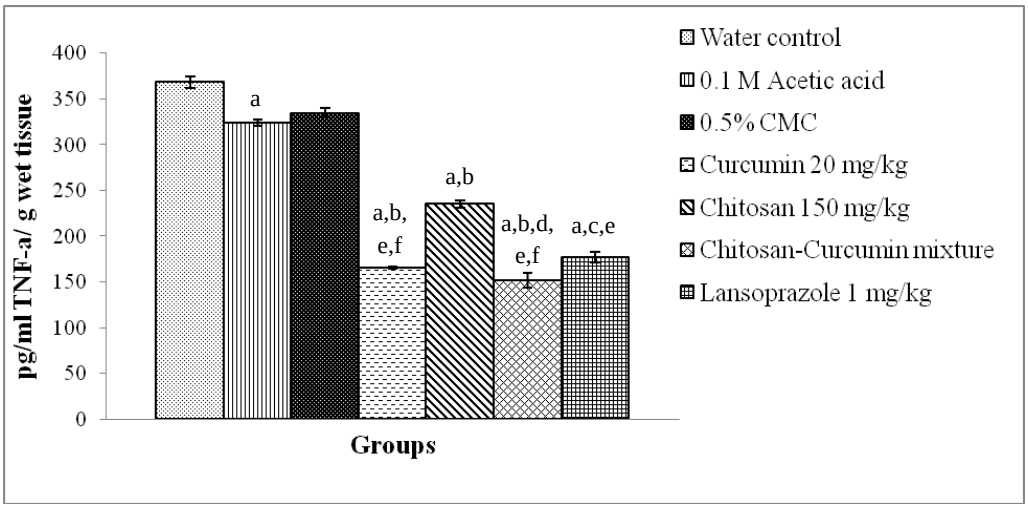


Figure 4-8 Effect of a chitosan-curcumin mixture on the level of TNF-α in the ulcerated stomach tissue of rats

Each value represents the mean ± SEM (n=6).

[a]*P<0.05* when compared to water control group (Dunnett's test)

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group (Dunnett's test)

[c]*P<0.05* when compared to 0.5% CMC-treated group (Dunnett's test)

[d]*P<0.05* when compared to curcumin-treated group (Dunnett's test)

[e]*P<0.05* when compared to chitosan-treated group (Dunnett's test)

[f]*P<0.05* when compared to lansoprazole-treated group (Dunnett's test)

4.6. Effect of a chitosan-curcumin mixture on mRNA expression of COX–1, COX–2, eNOS, nNOS and iNOS gene in gastric ulcerated tissue induced by indomethacin in rats

The effect of curcumin, chitosan, a chitosan-curcumin mixture and lansoprazole on mRNA expression of COX–1, COX–2, eNOS, nNOS and iNOS gene in gastric ulcerated tissue induced by indomethacin was detected by RT–PCR and agarose gel electrophoresis as shown in Figure 4-9. The results were also confirmed by quantitative real-time PCR reaction (qRT-PCR) especially, the expression of nNOS and iNOS gene which was vary low and could not be clearly identified using RT-PCR (Figure 4-10, 4-11 and 4-12). GAPDH was use as a housekeeping gene. It was found from the present study that indomethacin down-regulated the expression of constitutive enzymes: COX-1 and eNOS mRNA which maintains the integrity of gastric epithelium but up-regulated the expression of pro-inflammatory gastric enzymes: COX-2 and iNOS mRNA as found in the control rats treated with water or 0.1 M acetic acid. On the contrary, in the rats treated with curcumin, chitosan and a chitosan-curcumin mixture, a significant up-regulation on the expression of constitutive COX-1, eNOS and nNOS genes and a significant down-regulation on the expression of COX-2 and iNOS mRNA into the normal level were found ($p<0.05$) when compared to water control group. Chitosan seemed to exert the higher potency than curcumin in stimulation of nNOS gene expression and in suppression of COX-2 gene expression. Lansoprazole exerted a significant up-regulation on the expression of COX-1, eNOS and nNOS gene and a significant down-regulation on the expression of COX-2 and iNOS mRNA when compared to those of water control and its vehicle control group, however, it seemed to provide as a weak modulator on the expression

level of all test genes. Interestingly, it was found that a chitosan-curcumin mixture (containing 20 mg of curcumin and 150 mg of chitosan) provided the highest potency of the test drugs in up-regulation of constitutive enzymes responsible for gastric cytoprotection and anti-inflammation: COX-1 and eNOS expression and in down-regulation of pro-inflammatory enzyme: iNOS expression compared to curcumin, chitosan and lansoprazole.

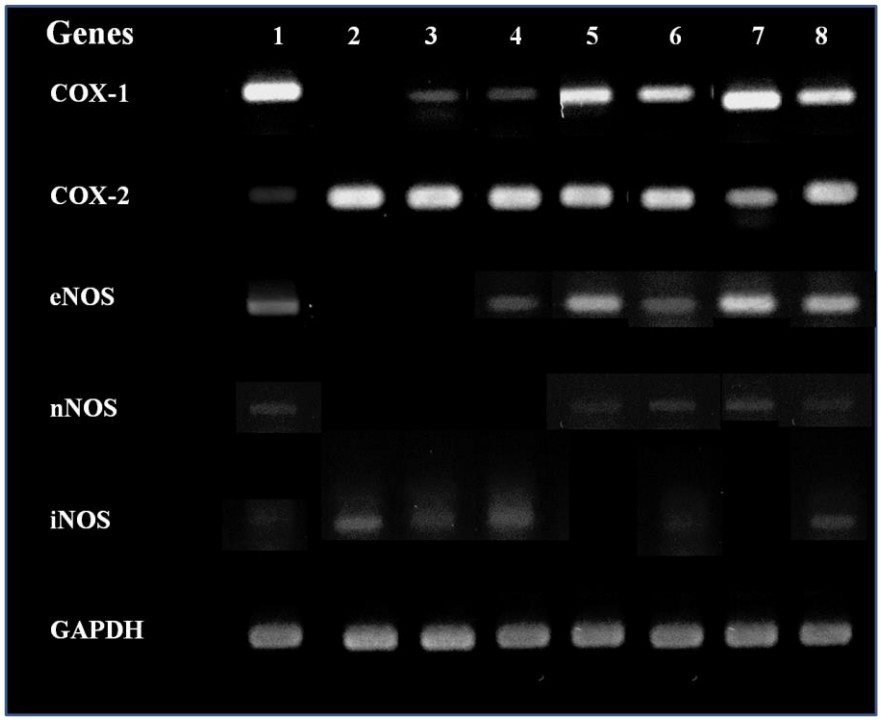


Figure 4-9 Gene expression of COX-1, COX-2, eNOS, nNOS and iNOS by reverse transcription polymerase chain reaction (RT–PCR) in the rat ulcerated gastric tissue induced by indomethacin on the 3rd day after ulcer induction

(GAPDH used as an internal control substance)

(1) Normal rat (2) Water control (5 ml/kg) (3) 0.1 M acetic acid (5 ml/kg)

(4) 0.5% CMC (5 ml/kg) (5) Curcumin (20 mg/kg) (6) Chitosan (150 mg/kg)

(7) Chitosan-curcumin mixture [Curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid]

(8) Lansoprazole (1 mg/kg)

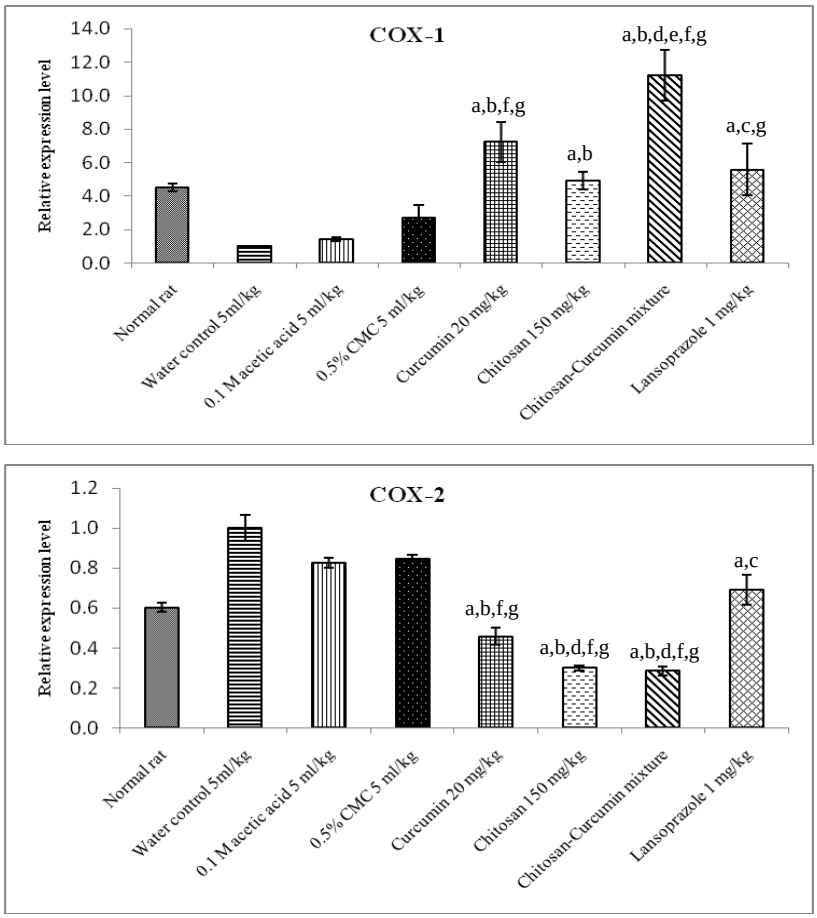


Figure 4-10 Expression of COX-1 and COX-2 mRNA by quantitative real-time PCR reaction in the rat gastric ulcerated tissue induced by indomethacin on the 3rd day after ulcer induction

Values were expressed as mean ± SEM (n=6); Dunnett's test

[a]*P<0.05* when compared to water control group

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group

[c]*P<0.05* when compared to 0.5% CMC-treated group

[d]*P<0.05* when compared to curcumin-treated group

[e]*P<0.05* when compared to chitosan-treated group

[f]*P<0.05* when compared to lansoprazole-treated group

[g]*P<0.05* when compared to normal rat group

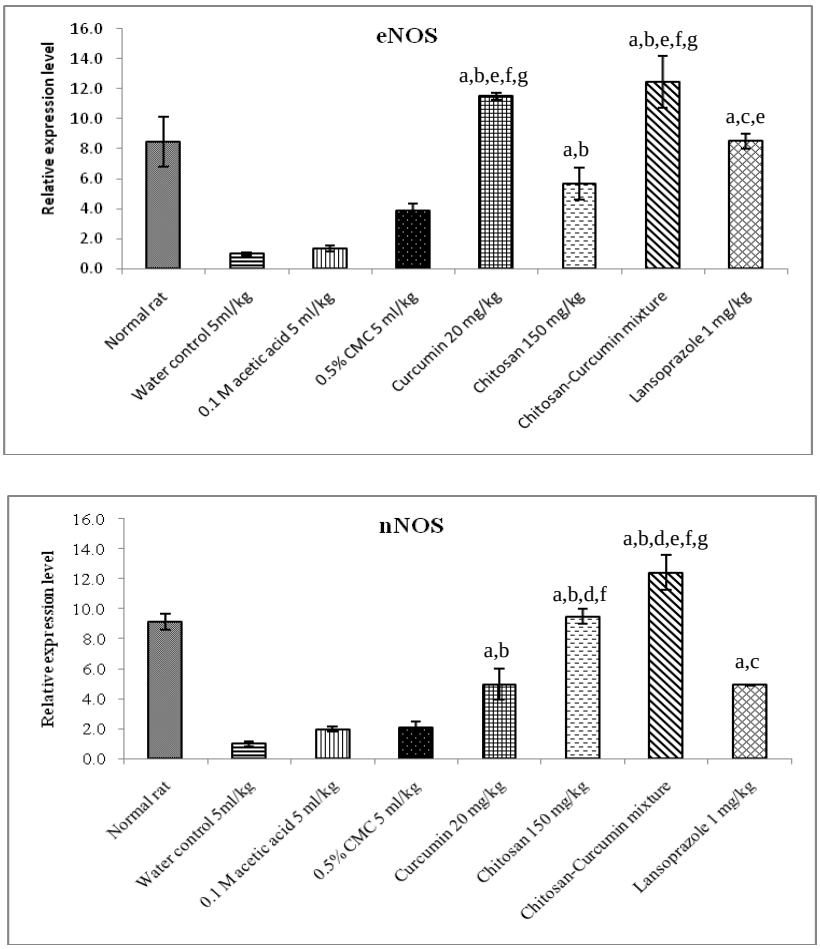


Figure 4-11 Expression of eNOS and nNOS mRNA by quantitative real-time PCR reaction in the rat gastric ulcerated tissue induced by indomethacin on the 3rd day after ulcer induction

Values were expressed as mean ± SEM (n=6); Dunnett's test

[a]*P<0.05* when compared to water control group

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group

[c]*P<0.05* when compared to 0.5% CMC-treated group

[d]*P<0.05* when compared to curcumin-treated group

[e]*P<0.05* when compared to chitosan-treated group

[f]*P<0.05* when compared to lansoprazole-treated group

[g]*P<0.05* when compared to normal rat group

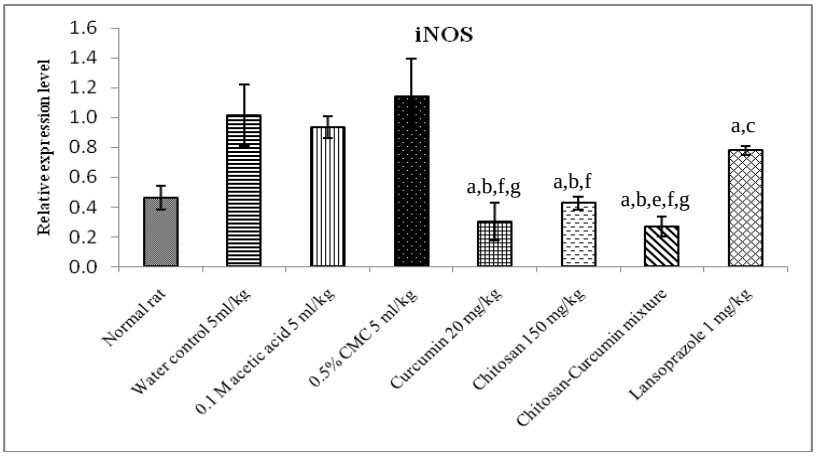


Figure 4-12 Expression of eNOS, nNOS and iNOS mRNA by quantitative real-time PCR reaction in the rat gastric ulcerated tissue induced by indomethacin on the 3rd day after ulcer induction

Values were expressed as mean ± SEM (n=6); Dunnett's test

[a]*P<0.05* when compared to water control group

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group

[c]*P<0.05* when compared to 0.5% CMC-treated group

[d]*P<0.05* when compared to curcumin-treated group

[e]*P<0.05* when compared to chitosan-treated group

[f]*P<0.05* when compared to lansoprazole-treated group

[g]*P<0.05* when compared to normal rat group

4.7. Effect of a chitosan-curcumin mixture on gastric biochemical parameters in rats

The effect of curcumin, chitosan and a chitosan-curcumin mixture (containing 20 mg of curcumin and 150 mg of chitosan) on gastric secretions and mucus content in rats was shown in Table 4-8 and Figure 4-13, 4-14 and 4-15. Curcumin, chitosan and a chitosan-curcumin mixture (containing 20 mg of curcumin and 150 mg of chitosan) showed not only a strong antisecretory activity in decrease total acidic output and pepsin activity but also a strong gastroprotective activity in stimulating gastric mucus production and secretion when compared with those of the control groups ($p<0.05$). Curcumin exerted the higher antisecretory potency than chitosan but less than lansoprazole (a standard antisecretory agent). On the other hand, chitosan exerted the more potent efficacy in stimulating gastric mucus production than curcumin. Although lansoprazole was found to exert a significant gastric mucus producing activity compared to the water control or no treatment group but this beneficial effect seemed to be result directly from the property of carboxy methyl cellulose (CMC) used for suspending the drug. Interestingly, a chitosan-curcumin mixture decreased gastric acid secretion and pepsin activity with a comparable potency to curcumin but with a superior potency than chitosan. However, its antisecretory efficacy was less than that of lansoprazole (a standard antisecretory agent). On the contrary, a chitosan-curcumin mixture exerted the highest gastroprotective efficacy in term of increase gastric mucus production and secretion than chitosan, curcumin and lansoprazole.

Table 4-8 Effect of a chitosan-curcumin mixture on total acid output, pepsin activity and mucus content

Treatment groups	Total acid output (mEq)	Pepsin activity (unit/ml)	Mucus content (µg alcian blue/g wet tissue)
Water control (5 ml/kg, *i.d.*)	0.062±0.003	116.63±5.58	21.08±1.25
0.1 M acetic acid (5 ml/kg, *i.d.*)	0.058±0.006	93.09±2.80	23.51±0.62
0.5% CMC (5 ml/kg, *i.d.*)	0.048±0.010	86.50±4.91[a]	26.58±0.96[a]
Curcumin (20 mg/kg, *i.d.*)	0.033±0.003[a,b,e]	45.73±2.67[a,b,e]	36.38±1.38[a,b,g]
Curcumin (40 mg/kg, *i.d.*)	0.040±0.009[a,b]	-	42.67±1.55[a,b,g]
Chitosan (150 mg/kg, *i.d.*)	0.049±0.008[a,b]	63.31±3.88[a,b]	42.67±1.55[a,b,d,g]
Chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, *i.d.*]	0.030±0.004[a,b,e]	43.22±5.07[a,b,e]	47.86±1.16[a,b,d,e,g]
Lansoprazole (1 mg/kg, *i.d.*)	0.018±0.006[a,c,d,e,f]	36.42±3.36[a,c,d,e]	26.90±2.02[a]

Each value represents the mean ± SEM (n=6); Dunnett's test

[a] *P<0.05* when compared to water control group

[b] *P<0.05* when compared to 0.1 M acetic acid-treated group

[c] *P<0.05* when compared to 0.5% CMC-treated group

[d] *P<0.05* when compared to curcumin-treated group

[e] *P<0.05* when compared to chitosan-treated group

[f] *P<0.05* when compared to chitosan-curcumin mixture-treated group

[g] *P<0.05* when compared to lansoprazole-treated group

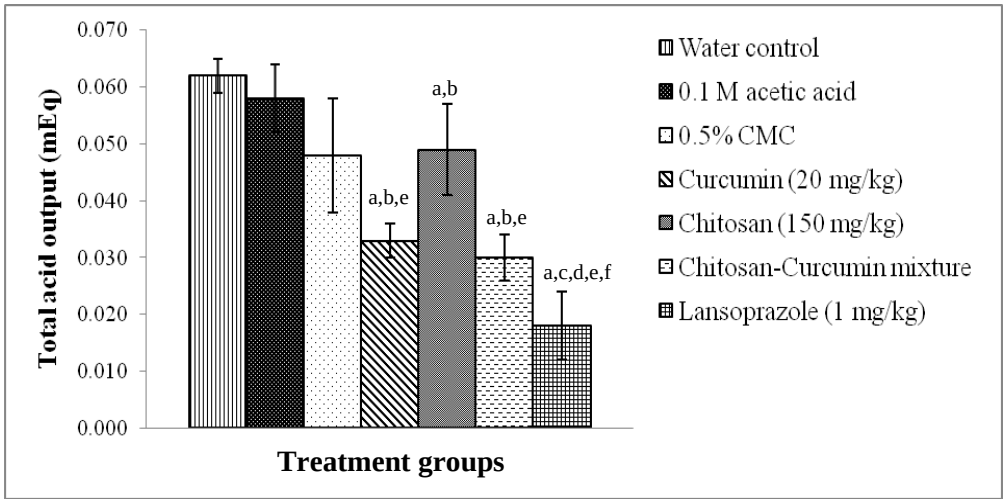


Figure 4-13 Effect of a chitosan-curcumin mixture on total acid output

Each value represents the mean ± SEM (n=6); Dunnett's test

[a]*P<0.05* when compared to water control group

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group

[c]*P<0.05* when compared to 0.5% CMC-treated group

[d]*P<0.05* when compared to curcumin-treated group

[e]*P<0.05* when compared to chitosan-treated group

[f]*P<0.05* when compared to chitosan-curcumin mixture-treated group

[g]*P<0.05* when compared to lansoprazole-treated group

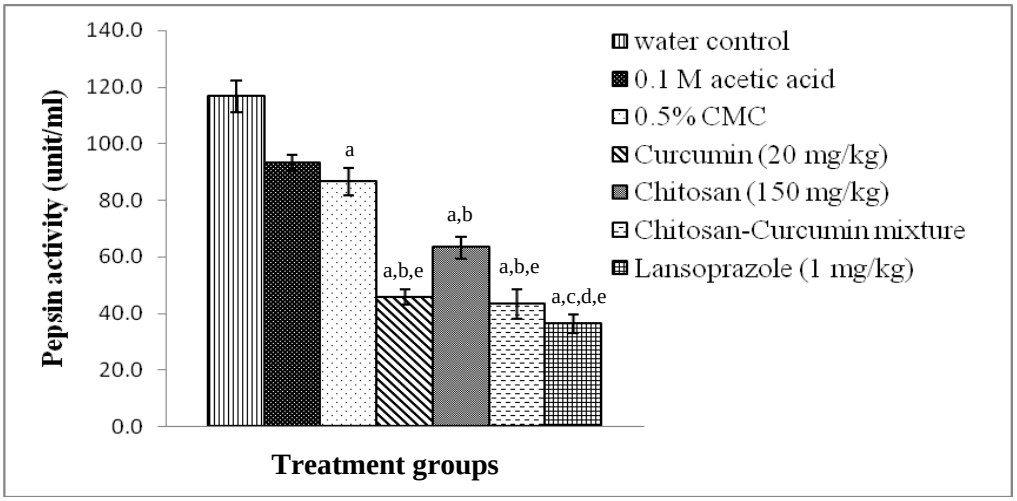


Figure 4-14 Effect of a chitosan-curcumin mixture on pepsin activity

Each value represents the mean ± SEM (n=6); Dunnett's test

[a]*P<0.05* when compared to water control group

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group

[c]*P<0.05* when compared to 0.5% CMC-treated group

[d]*P<0.05* when compared to curcumin-treated group

[e]*P<0.05* when compared to chitosan-treated group

[f]*P<0.05* when compared to chitosan-curcumin mixture-treated group

[g]*P<0.05* when compared to lansoprazole-treated group

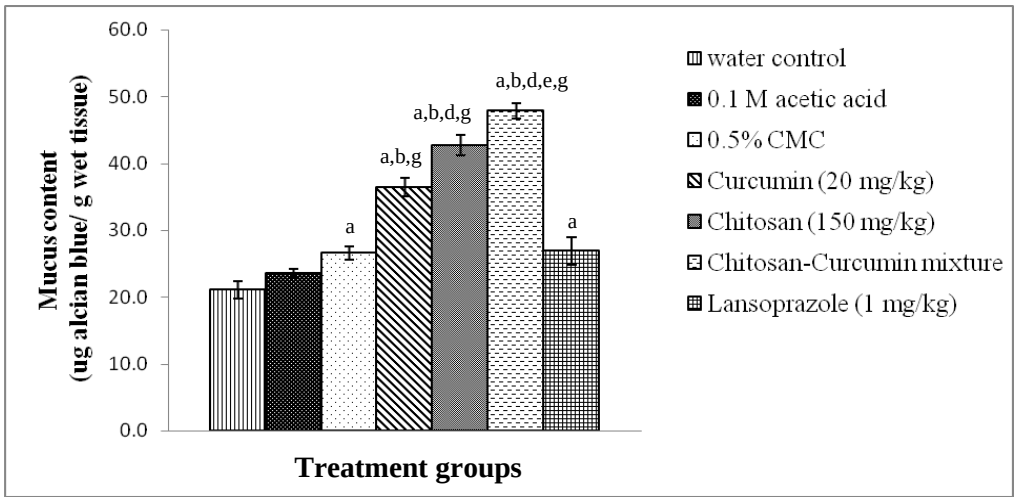


Figure 4-15 Effect of a chitosan-curcumin mixture on mucus content

Each value represents the mean ± SEM (n=6); Dunnett's test

[a]*P<0.05* when compared to water control group

[b]*P<0.05* when compared to 0.1 M acetic acid-treated group

[c]*P<0.05* when compared to 0.5% CMC-treated group

[d]*P<0.05* when compared to curcumin-treated group

[e]*P<0.05* when compared to chitosan-treated group

[f]*P<0.05* when compared to chitosan-curcumin mixture-treated group

[g]*P<0.05* when compared to lansoprazole-treated group

4.8. Wound healing effect of a chitosan-curcumin mixture on AGS human gastric epithelial cell line

The wound healing effect of a chitosan-curcumin mixture on AGS human gastric epithelial cell migration was evaluated in 10-hour timing course. The obtained result showed that curcumin (3.7 µg/ml), chitosan (100 µg/ml) and a chitosan-curcumin mixture (5 µg of curcumin and 37.5 µg of chitosan) exerted a significant increase in the migration of AGS human gastric epithelial cell at 5 to 10 hour after wound induction with the percentage of wound closure as 79.01±1.22%, 80.40±1.38%, and 76.32±2.02%, respectively ($p<0.05$) compared to that of the control or no treatment (21.32±1.45%) as shown in Table 4-9, Figure 4-16 and 4-17. All three preparations exerted a comparable potency in accelerating the migration of AGS human gastric epithelial cells in 10-hour timing. It was noted that the human gastric epithelial cell migration decreased at a higher dose of curcumin, chitosan and a chitosan-curcumin mixture.

Table 4-9 Effect of a chitosan-curcumin mixture on AGS human gastric epithelial cell migration

Treatment groups	Wound closure (%)		
	Time 0 h	**Time 5 h**	**Time 10 h**
Control (media+2%FBS)	0	13.67±2.69	21.32±1.45
Curcumin (1 µg/ml)	0	63.67±2.58[a]	74.77±2.92[a]
Curcumin (3.7 µg/ml)	0	68.73±2.59[a,b]	79.01±1.22[a]
Chitosan (100 µg/ml)	0	68.64±3.88[a]	80.40±1.38[a]
Chitosan (150 µg/ml)	0	59.42±3.40[a]	66.86±3.05[a]
Chitosan (300 µg/ml)	0	40.45±4.67	60.70±2.89[a]
Chitosan-Curcumin mixture (2.5 µg of curcumin and 18.75 µg of chitosan)	0	54.16±1.59[a]	68.98±2.07[a]
Chitosan-Curcumin mixture (5 µg of curcumin and 37.5 µg of chitosan)	0	54.56±3.14[a]	76.32±2.02[a]
Chitosan-Curcumin mixture (10 µg of curcumin and 75 µg of chitosan)	0	44.68±4.67[a]	61.48±1.90[a]

Each value represents the mean ± SD from four independent experiments

[a]*P<0.05* when compared to water control at 5 and 10 h

[b]*P<0.05* when compared to chitosan-curcumin mixture (5 µg of curcumin and 37.5 µg of chitosan) at 5 h

All measuring data were determined by Dunnett's test

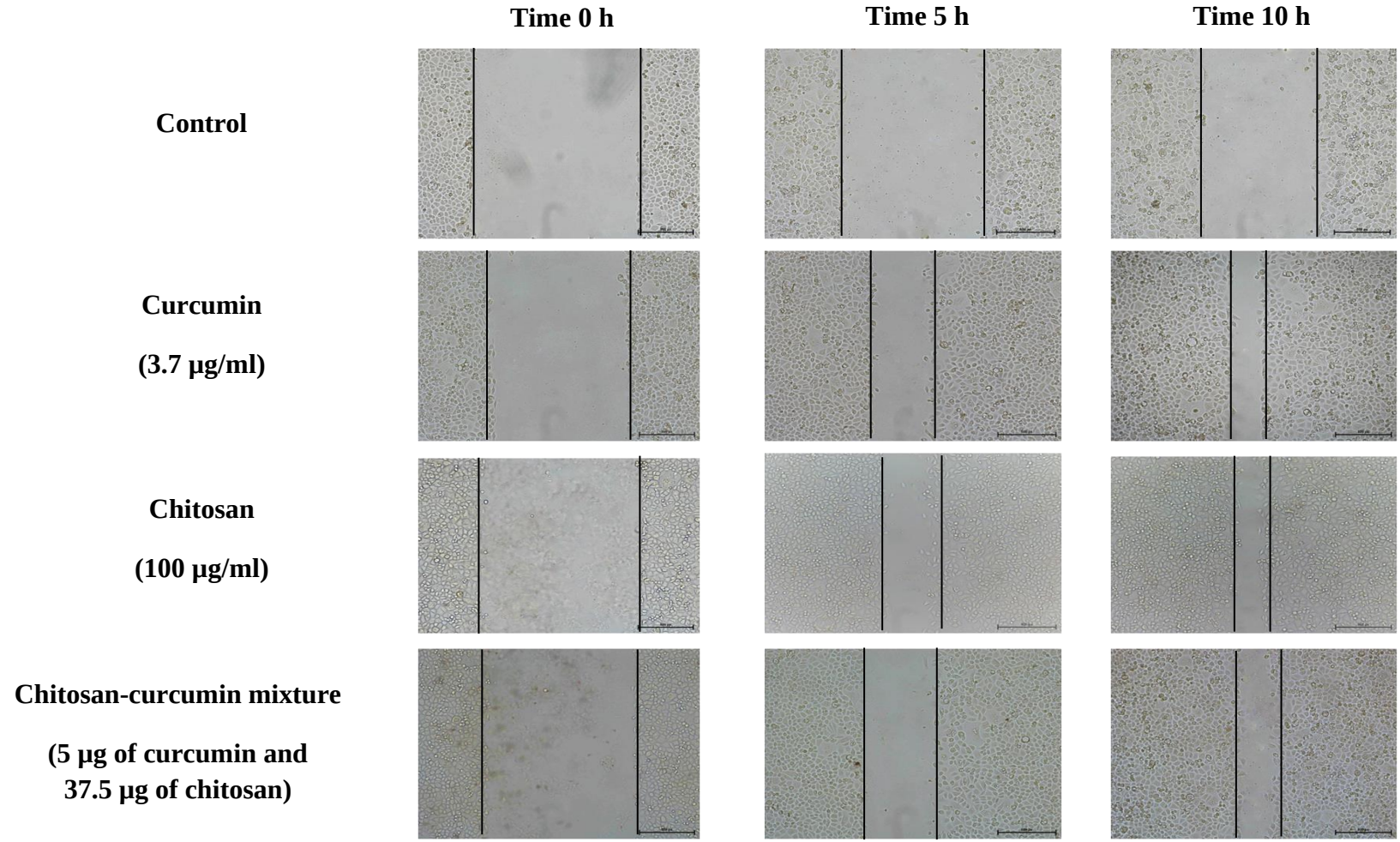


Figure 4-16 Effect of a chitosan-curcumin mixture on the migration of AGS human gastric epithelial cell

[The parallel lines show size (µm) of wound area]

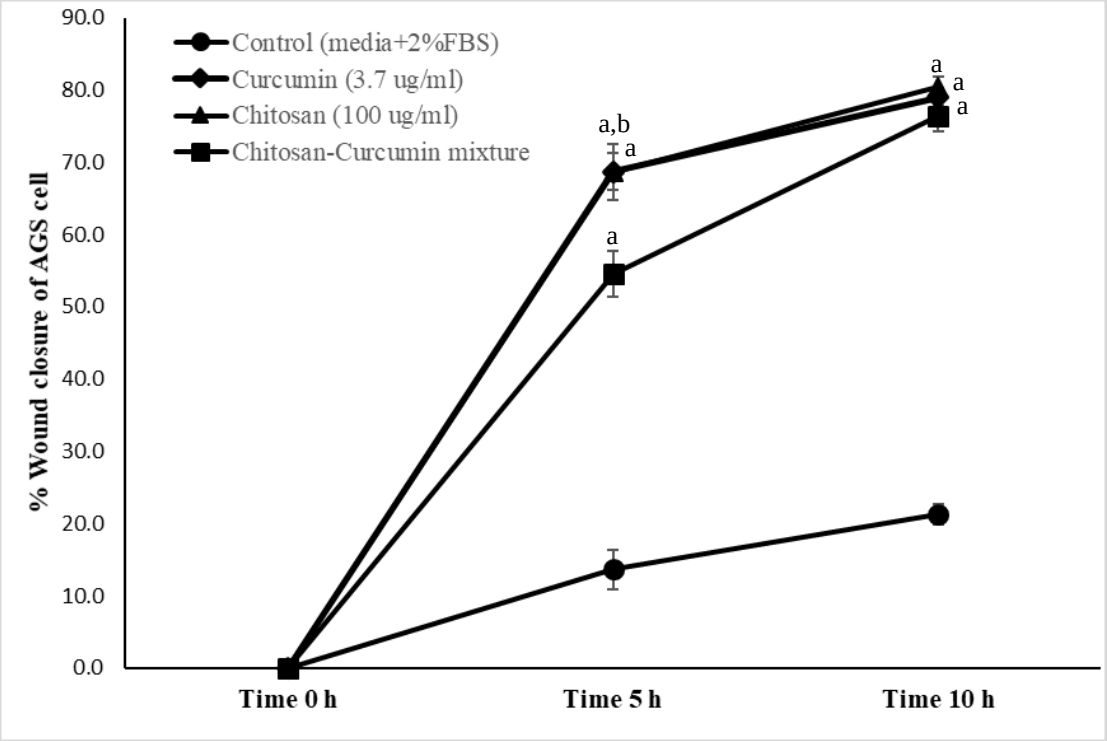


Figure 4-17 Effect of a chitosan-curcumin mixture on AGS human gastric epithelial cell migration

Each value represents the mean ± SD from four independent experiments

[a]*P<0.05* when compared to water control at 5 and 10 h

[b]*P<0.05* when compared to chitosan-curcumin mixture (5 µg of curcumin and 37.5 µg of chitosan) at 5 h

All measuring data were determined by Dunnett's test

4.9. Curative effect of a chitosan-curcumin solution on buccal mucosal ulcer induced by acetic acid treatment in hamsters

The maximum buccal mucosal ulcer was developed on the 4th day after the ulcer induction with a topical application of acetic acid. A thickening of the buccal epithelium around the ulcer, an infiltration of leukocytes and initial stages of granulation tissue formation were noted. The area of the ulcer decreased with time but still remained up to day 12. The ulcer area of each treatment group on day 4, 10 and 12 after the ulcer induction was presented in Figure 4-18. The macroscopic assessment from gross appearance showed that repeated application of a topical 0.1% cucumin in 0.5% chitosan for 7 consecutive day beginning on the 5th day after ulcer induction, exerted the superior ulcer healing efficacy than those of water control or 0.15% benzydamine (Difflam®) solution (a standard mouthwash) ($p<0.05$) (Table 4-10). It was also found that a topical 0.15% benzydamine (Difflam®) solution did not exert any significant ulcer healing efficacy on buccal mucosal ulcer induced by acetic acid treatment when compared to the no treatment or water control group. An oral chitosan-curcumin mixture also exerted a comparable histological healing score to that of a topical chitosan-curcumin mouthwash (Table 4-11).

The histological samples of each treatment group at the day 12 after ulcer induction was shown in Figure 4-19. In the group treated with water and 0.5% chitosan without curcumin (vehicle control for topical 0.1% chitosan-curcumin solution), the infiltration of leukocytes had still been found, indicated that the inflammatory reaction still happened along with the initiation of ulcer healing phase noted with the beginning of epithelization. In the group treated with 0.15% benzydamine or Difflam® solution (a standard mouthwash), no inflammation reaction

was found but still had no wound contraction or tissue remodeling. In contrast, in the group treatment with both of chitosan-curcumin preparations, complete of epithelization, wound contraction and tissue remodeling were noted.

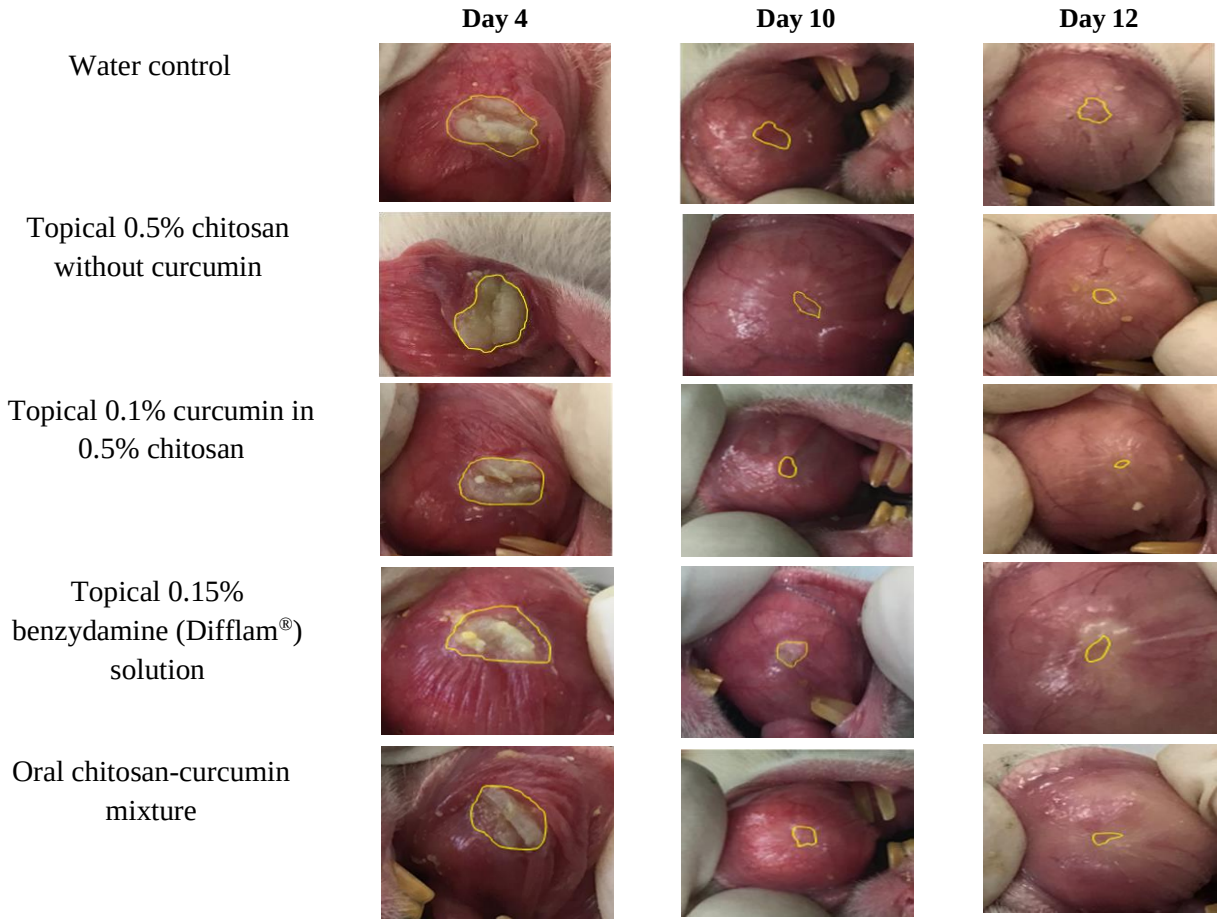


Figure 4-18 The representative buccal mucosal ulcer area from each treatment group on day 4, 10 and 12 after the topical application of acetic acid in hamsters

Table 4-10 Curative effect of a topical 0.1% curcumin in 0.5% chitosan solution on buccal mucosal ulcer induced by a topical application of acetic acid in hamsters

Treatment groups	**Ulcer area (mm^2)**	**% Healing**	**Histological healing scores**
Water control, bid	4.61±0.37	91.04±0.68	3.75±0.25
Topical 0.5% chitosan without curcumin solution (vehicle control for topical chitosan-curcumin solution), bid	2.71±1.21[a]	95.05±0.42[a]	4.00±0.17
Topical 0.1% curcumin in 0.5% chitosan solution, bid	1.20±0.05[a,b,c]	97.55±0.09[a,b,c]	4.80±0.2[a,b,c]
Topical (0.15% benzydamine) Difflam® solution, bid	4.52±0.54	91.71±1.10	3.67±0.33

Each value represents the mean ± S.E.M. (n=6)

Histological healing scores determined as described in Chapter 3

[a]*P<0.05* when compared to water control

[b]*P<0.05* when compared to topical 0.5% chitosan without curcumin solution (vehicle control)

[c]*P<0.05* when compared to topical 0.15% benzydamine (Difflam®) solution

Statistical analysis of parametric parameters (ulcer area and % healing) was determined by Dunnett's test

Statistical analysis of histological healing scores was derermined by Mann-Whiney U test

Table 4-11 Curative effect of an oral chitosan-curcumin mixture on buccal mucosal ulcer induced by a topical application of acetic acid in hamsters

Treatment groups	**Ulcer area (mm^2)**	**% Healing**	**Histological healing scores**
Water control, bid	4.61±0.37	91.04±0.68	3.75±0.25
Oral chitosan-curcumin mixture [curcumin (20 mg) suspended in 10 ml of chitosan (150 mg) dissolved in 0.1 M acetic acid, od]	2.20±0.16[a]	95.59±0.18[a]	4.50±0.22[a]

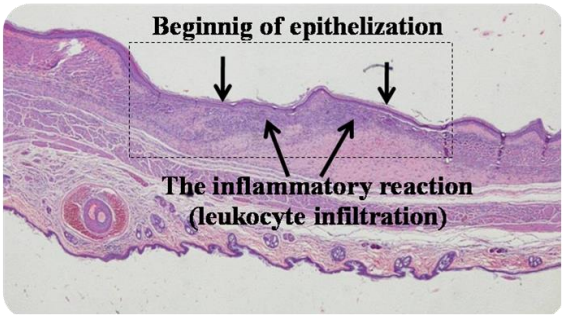
Each value represents the mean ± S.E.M. (n=6)

Histological healing scores determined as described in Chapter 3

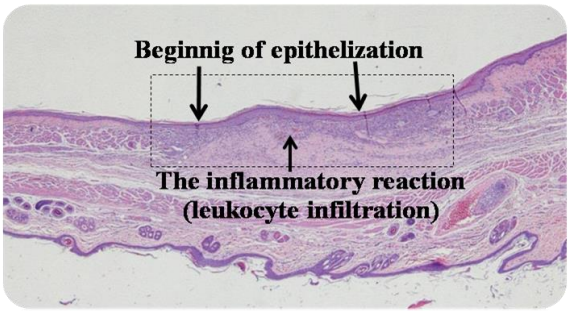
[a]*P<0.05* when compared to water control

Statistical analysis of parametric parameters (ulcer area and % healing) was determined by Dunnett's test

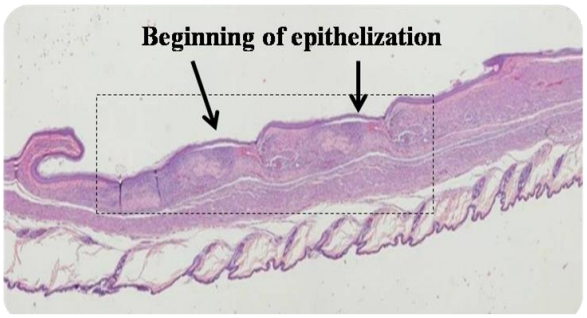
Statistical analysis of histological healing scores was derermined by Mann-Whiney U test



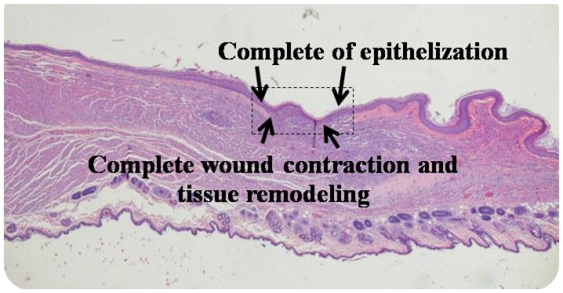
Water control



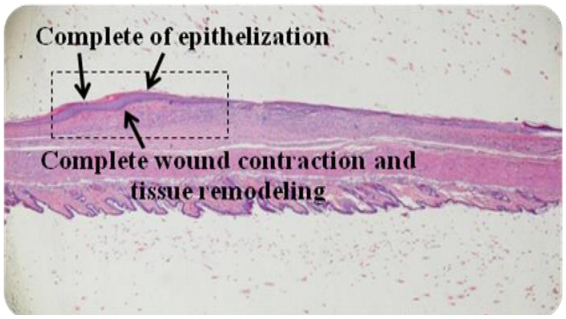
Topical 0.5% chitosan without curcumin solution
(vehicle control for topical chitosan-curcumin solution)



Topical (0.15% benzydamine) Difflam® solution



Topical 0.1% curcumin in 0.5% chitosan solution



Oral chitosan-curcumin mixture
[curcumin (20 mg) suspended in 10 ml of chitosan (150 mg)
dissolved in 0.1 M acetic acid]

Figure 4-19 Samples of the histological presentation of the buccal mucosal ulcer area induced by a topical application of acetic acid in each treatment group on day 12 after the ulcer induction. Hematoxylin and eosin (H&E) strain, maxnification 4x

4.10. Wound healing effect of a chitosan-curcumin mixture on HGF human gingival fibroblast cells

The wound healing effect of a chitosan-curcumin mixture on HGF human gingival fibroblast cells proliferation and migration was evaluated in 3-day timing course. It was found that curcumin (1 µg/ml), chitosan (100 µg/ml) and a chitosan-curcumin mixture (5 µg of curcumin and 37.5 µg of chitosan) exerted a significant increase in fibroblast migration on day 3 with the percentage of wound closure as 95.15±2.48%, 90.10±1.33% and 97.36±1.77%, respectively, when compared to that of control or no treatment (67.92±1.71%) ($p<0.05$) as shown in Table 4-12 , Figure 4-20 and 4-21. However, curcumin exerted the highest potency in accelerating wound healing with nearly 80% of the wound closure observed on day 2. Likewise, HGF human gingival fibroblast cells proliferation and migration decreased at a higher dose of curcumin, chitosan and a chitosan-curcumin mixture.

Table 4-12 Effect of a chitosan-curcumin mixture on HGF human gingival fibroblast cell proliferation and migration

Treatment groups	**Wound closure (%)**			
	Day 0	**Day 1**	**Day 2**	**Day 3**
Control (media+10%FBS)	0	8.26±0.83	24.44±1.88	67.92±1.71
Curcumin (1 µg/ml)	0	23.24±4.14[a,b]	76.07±4.83[a,b]	95.15±2.48[a]
Curcumin (3.7 µg/ml)	0	7.78±2.25	17.27±4.07[a]	51.56±2.75[a]
Chitosan (100 µg/ml)	0	6.38±1.56	40.93±6.59[a]	90.10±1.33[a]
Chitosan (150 µg/ml)	0	17.29±2.25	44.42±3.97[a]	75.13±3.77[a]
Chitosan-curcumin mixture (5 µg of curcumin and 37.5 µg of chitosan)	0	9.66+0.99	50.02±7.81[a,c]	97.36±1.77[a,b,c]
Chitosan-curcumin mixture (10 µg of curcumin and 75 µg of chitosan)	0	11.31±2.24	24.27±4.35	40.68±5.49

Each value represents the mean ± SD from four independent experiments

[a]*P<0.05* when compared to control

[b]*P<0.05* when compared to chitosan (100 µg/ml) and chitosan (150 µg/ml)

[c]*P<0.05* when compared to chitosan-curcumin mixture (10 µg of curcumin and 75 µg of chitosan)

All measuring data were determined by Dunnett's test

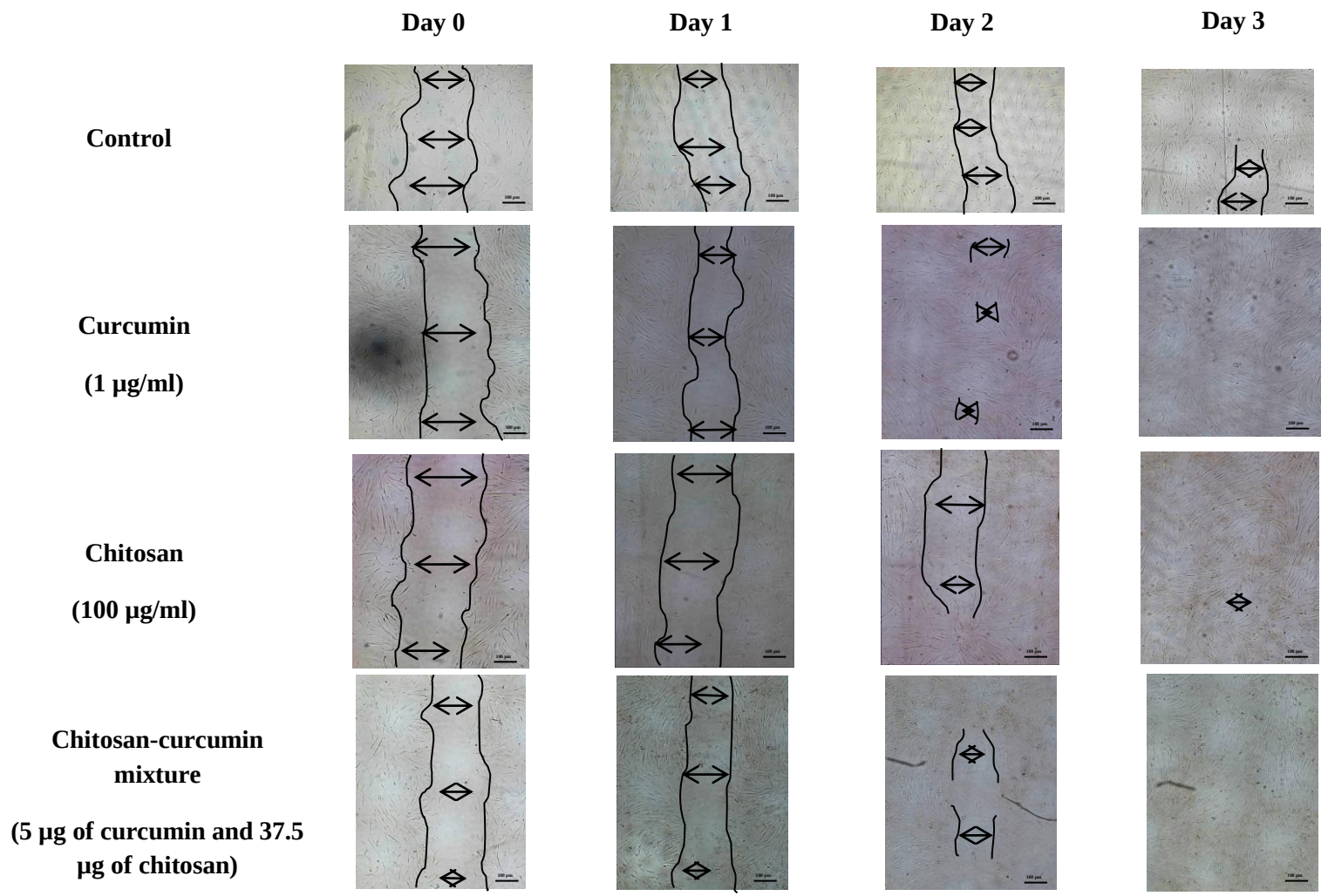


Figure 4-20 Effect of a chitosan-curcumin mixture on the proliferation and migration of human gingival fibroblast (HGF) cell

(The double arrow line show size (µm) of wound area)

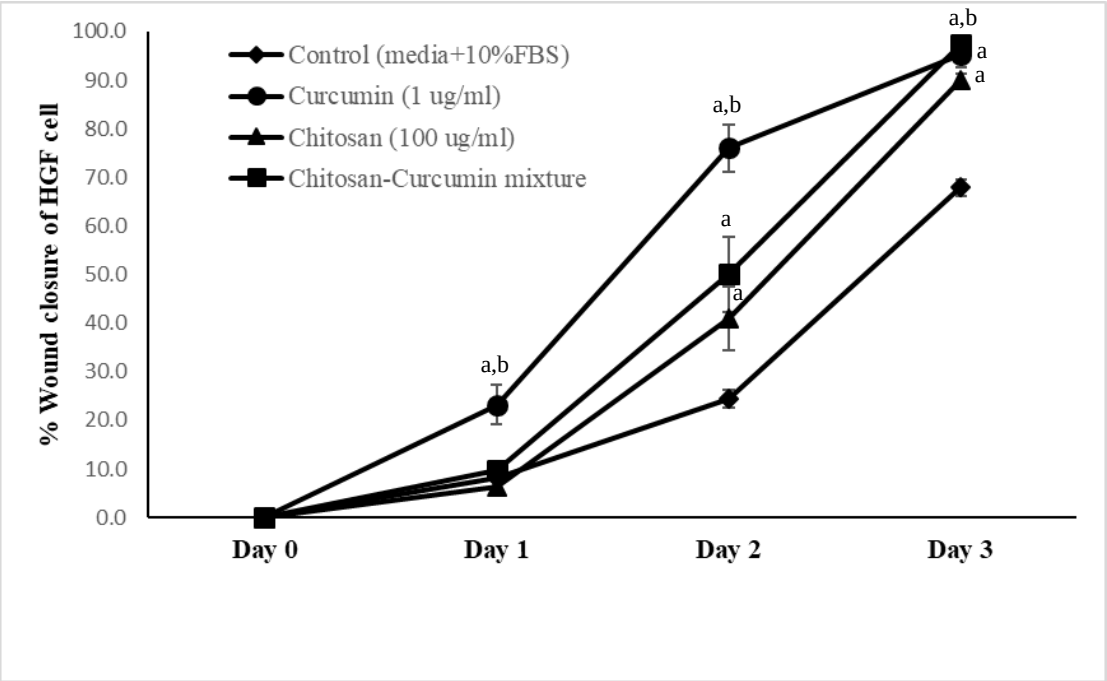


Figure 4-21 Effect of a chitosan-curcumin mixture on HGF human gingival fibroblast cell proliferation and migration

Each value represents the mean ± SD from four independent experiments

[a]*P<0.05* when compared to control

[b]*P<0.05* when compared to chitosan (100 µg/ml)

All measuring data were determined by Dunnett's test

CHAPTER 5

DISCUSSION

Curcumin has been claimed to have antiulcerogenic effect in treating acute and chronic GU with a comparable or superior efficacy to standard antiulcer drugs such as PPIs in various animal models (28,140,141). Nevertheless, its limitations for further clinical use include its poor aqueous solubility, low bioavailability (29) and its opposite effects to either delay ulcer healing or to exacerbate ulcer inflammation through some curative mechanisms differently modified by high dosage (28). Several previous studies have reported the prophylactic efficacy of turmeric or curcumin against aspirin or NSAIDs including indomethacin-induced gastric ulceration, however, there is still no study on the efficacy of curcumin in treatment GU induced by NSAIDs. Low molecular weight (LMW) chitosan has been reported not only exhibiting an ability to increase the oral bioavailability of the highly variable oral absorption and low bioavailability drugs but also having a potential gastric cytoprotective and ulcer healing promoting efficacy against ethanol-induced gastric mucosal injury in rats with an antiulcer potency comparable to those of sucralfate but superior than those of H_2 receptor antagonist: cimetidine (118,174). The generally recognized as safe (GRAS) is provided potential notifies for its certain uses in food products (105). There is still no study on the efficacy of LMW chitosan in treatment GU induced by NSAIDs as well. Curcumin is a lipophilic polyphenol that is nearly insoluble in water but is soluble in organic solvents such as methanol, DMSO, acetone and chloroform. In addition, it is quite stable in an acidic pH (8,9). On the other hand, chitosan is insoluble in both water and organic

solvents but is soluble in dilute aqueous acid solution (104). Accordingly, 0.1 M acetic acid was chosen as a solvent for preparing a chitosan-curcumin mixture with pH about 3.0 which is an optimal gastric pH range (pH 1.5-3.5) for activating the denaturation of pepsinogen into pepsin and control the acid environment of the stomach (40,41). The experiments in the first part of the present study were designed to validate the use of a chitosan-curcumin mixture in prevention and treatment of NSAIDs: indomethacin-induced acute GU and in treatment of acetic acid-induced chronic GU. Additionally, its potential underlying mechanisms of antiulcer action was evaluated in the latter part.

Among various representatives of NSAIDs that have been used to induce gastric ulceration in several animal model experiments, indomethacin has been documented to have a higher potential to cause gastric injury than other commonly-used NSAIDs (175–177). The degrees of GU depend on the ulcer-dose and time after administration of indomethacin. It has been found that the 50 mg/kg treated rat for 8 h is found to produce the highest degree of GU, however, it also causes a high degree of weakness, behavioral changes and reduce physical activity (178). In the present work, orally administration of indomethacin (30 mg/kg) for 4 h schedule significantly caused gastric erosion and hemorrhagic ulceration in the glandular area of the stomach and was a suitable ulcer model for evaluation the preventive efficacy against NSAIDs-induced GU of potential antiulcer agents. An orally administration of indomethacin at a dose of 30 mg/kg for 5 h schedule was also successful in induction acute GU that reached its peak on the 3rd day after an indomethacin administration. It has been reported that the drug can penetrate lipid membranes and accumulate in optimal concentration within cells to induce ulceration (179). The current study also investigated the ulcer curative efficacy of a chitosan-curcumin mixture on chronic GU induced by a topical application

of acetic acid in rats as the produced ulcer highly resembles to human chronic GU particularly in terms of pathological aspects and healing process.

Indomethacin has been found to cause GU by several mechanisms including an increase of aggressive factors through a disruption of the layer of surface-active phospholipids on the mucosal surface that allows back diffusion of acid into the gastric mucosa (180); an up-regulation of adhesion molecules which enhances polymorphonuclear leukocyte infiltration; an excessive production of aggressive factors [such as oxygen free radicals (O_2^-, H_2O_2), pro-inflammatory cytokines especially TNF-α and pro-inflammatory mediators especially NO derived from iNOS and inflammatory PGs derived from COX-2] (179); and a depletion of endogenous gastroprotective factors such as endogenous cytoprotective PGs through inhibition of COX-1 which maintains gastric mucosal integrity through inhibition of acid secretion stimulated by histamine and enhancement of gastric mucus production and gastic microcirculation, cytoprotective NO through inhibition of constitutive nNOS which regulates gastric acid and mucus secretion and constitutive eNOS which enhances angiogenesis and gastric mucosal blood flow, leading to a suppression of polymorphonuclear leukocyte infiltration (181,182). Additionally, the delay in GU healing caused by indomethacin is attributed to its capacity in inhibiting angiogenesis which maintains gastric mucosal blood flow to provide sufficient nutrients and oxygen to the damaged gastric mucosa or tissue to enhance restitution and re-epithelization of the ulcerated gastric mucosa (46,52). Furthermore, indomethacin has been reported to diminish the ability of epithelial growth factor (EGF) which promote epithelial repair through a reduction of EGF binding to its receptor (183) including an inhibition of EGF signaling pathways (184). Comparing on the severity of gastric damage, a topical application of acetic acid

causes more severe gastric tissue damage occurred as a chronic deep necrotic ulcer penetrating through the muscularis mucosae whereas indomethacin causes less gastric damage occurred as erosion or haemorrhagic ulceration or acute GU. Similarly to the pathogenesis of indomethacin-induced acute GU, both gastric acid and free radicals derived from infiltrated neutrophils can exert an inhibitory effect on the healing of ulcers induced by acetic acid in rats. In addition, an adequate gastric mucosal blood flow and growth factors such as TGF-α, EGF and VEGF have been implicated in acceleration of ulcer healing (157,185,186). It has been found in acetic acid induced chronic GU that COX-2 and iNOS have multifaceted role in inflammatory and ulcer healing reactions of which on the one hand, initiating inflammatory response in the early phase of inflammation and on the other hand, accelerating the ulcer healing in the late phase of inflammation in enhancement of epithelial cell proliferation, angiogenesis and the expression of growth factors involving in promotion of ulcer healing (74,187–189). Moreover, in circumstances in which the production of one of these enzymes is suppressed, there are compensatory increases in the production of the other. Due to the complex interrelationship of these enzymes including multi-target antioxidant and anti-inflammatory action of curcumin, the exact mechanisms of antiulcer action of a chitosan-curcumin mixture in treatment of acetic acid-induced chronic GU seems to be difficult to explain. Therefore, the more simple model of indomethacin-induced acute GU was used for evaluation the potential underlying antiulcer mechanisms of action of a chitosan-curcumin mixture.

The results obtained from the present study showed that pretreatment of 0.1 M acetic acid did not have any significant effect on the ulcer severity induced by indomethacin. In addition, pretreatment of a chitosan-curcumin mixture (containing 20

mg of curcumin in 10 ml of 150 or 300 mg of chitosan dissolved in 0.1 M acetic acid) was found to have a superior ulcer prophylactic efficacy than curcumin, chitosan or proton pump inhibitor: lanzoprazole (a standard antiulcer agent). A chitosan-curcumin mixture containing 300 mg of chitosan provided a comparable potent gastroprotective efficacy to a mixture containing 150 mg of chitosan, however, its high viscosity results in difficulty in administering through an oral gavage needle that may cause confounded results and adverse effects in experimental animal. Similarly to its ulcer preventive efficacy, a chitosan-curcumin mixture was found to provide the greatest ulcer healing efficacy in treatment indomethacin-induced acute GU comparing with those of curcumin, chitosan and a positive standard lansoprazole. Interestingly, the obtained results indicated that the repair of mucosal epithelium cells and muscularis mucosae of chronic GU induced by a topical application of acetic acid was prominently shown in animal group treated with once daily administration of a chitosan-curcumin mixture compared to a twice daily administration of curcumin, chitosan or a positive standard lansoprazole. The beneficial results of curcumin obtained from the present study were related to those of previous studies against various ulcerogenic agents-induced acute gastritis and GU (28,140,141) and also confirmed the efficacy of curcumin in acceleration the healing of acute GU induced by NSAIDs with the therapeutic dosage level of 20 mg/kg. Chitosan at a high dose (150 mg/kg) was also found to exert a higher preventive and curative efficacy than curcumin against indomethacin-induced acute GU. Since it was found that a chitosan-curcumin mixture (containing 20 mg of curcumin and 150 mg of chitosan) exerted a superior prophylaxis and treatment efficacy than pure curcumin and chitosan even though a once daily administration regimen suggested that there may be synergic antiulcer mechanisms of

action of curcumin and chitosan in a chitosan-curcumin mixture. The mucoadhesive property of chitosan occurring due to the electrostatic interactions of the strong positive charge of chitosan with the negatively charged mucus present in the mucosal layer of the GI tract also results to the releasing of the drug over time with the potential to reduce dosing interval. In addition, chitosan is a solution type that can acts as a drug penetration enhancer by opening the tight junctions of the epithelium and facilitates both transcellular and paracellar transport of drug across the epithelium (190), thereby enhancing an optimal curcumin bioavailability.

The pathological effects of indomethacin on the gastric mucosal damage obtained from the present study were consistent with previous studies that indomethacin initiated gastric mucosal damage through increasing the level of mucosal MDA (a biomarker for lipid peroxidation), MPO (a biomarker for neutrophil infiltration as it present in phagocytic cells), pro-inflammatory cytokine: TNF-α, pro-inflammatory mediator: PGE_2 and the expression of pro-inflammatory enzymes: COX-2 and iNOS. On the other hand, it decreased the expression of cytoprotective COX-1 and cytoprotective NOS: eNOS and nNOS. It has been reported that two hours after indomethacin administration, there will be an acute increase of toxic oxygen radicals (O_2^- and H_2O_2) level in the gastric ulcerated mucosa (177). In addition, MPO in infiltrative phagocytic cells will catalyze the most potent toxic oxygen radical: hypochlorous acid (HOCl) produced from H_2O_2 and halide ion. It has been reported that an increase of mucosal MPO activity after an indomethacin administration is a time-dependent with the peak value at 16-24 h and retains up to 72 h (71). A significant increased level of MDA was found to be remained at day 3 after an indomethacin administration in control rats. The excessive release of MPO and excessive production

of reactive oxygen radicals bring about gastric mucosal oxidative damage or lipid peroxidation marked by an increase of MDA level (177). As indomethacin is known as a nonselective COX-1 and COX-2 inhibitor and PGE_2 is generated via COX-1 and COX-2; an increased level of COX-2 expression and PGE_2 found in the ulcerated mucosa in water control group after an administration of indomethacin for 3 days may be resulted from the overproduction of iNOS (191) and TNF-α (192) during the inflammation stage as both of these activating pro-inflammatory cytokines have been found to modulate the expression of COX-2-dependent production of inflammatory PGE_2. In the present study, a significant increased level of PGE_2 was found to be remained at day 3 after an indomethacin administration in control rats. The mean basal level of PGE_2 in normal gastric mucosa is about 288 pg/ g tissue (193). In contrast, it has been reported that an amount of TNF-α level is increased after an indomethacin administration with the maximal level at 16 h after induction and then decreased in a time dependent manner. Nevertheless, a significant increased level of TNF-α was found to be remained at day 3 after an indomethacin administration in control rats. The mean basal level of TNF-α in normal gastric mucosa is about 250 pg/ g tissue (194). Consequently, agent with potent antisecretory, gastric mucus producing, antioxidant, anti-inflammatory and ulcer healing properties will have a high efficacy for indomethacin-induced gastric lesions prophylaxis and treatment.

The DPPH free radical method is a simple, easy and reasonable *in vitro* study for preliminary screening for antioxidant efficacy of test samples in scavenging an organic free DPPH and suppressing lipid peroxidation. Concurrently, an *in vitro* evaluation on NO scavenging efficacy of the test compounds has been included in a preliminary evaluation of antioxidant activity of test samples as the NO derived from

iNOS in activated macrophage during inflammation when reacting with superoxide radical, forms a highly reactive nitrogen radical: peroxynitrite anion ($ONOO^-$), which in turn, can lead to gastric cell damage (195). The NO generated from sodium nitroprusside reacts with oxygen to produce stable products nitrite, the quantities of which can be determined using Griess reagent (196). LPS is the surface molecule derived from the outer membrane of gram-negative bacteria. The activation of macrophage cells with LPS will lead to an excessive production of NO from iNOS gene for prolonged periods, leading to the initiation of inflammatory response. It was found from the present study that curcumin and a chitosan-curcumin mixture exhibited both potent antioxidant efficacy in scavenging an organic free DPPH radical and nitrite radicals and potent anti-inflammatory efficacy in inhibition of NO production in activated macrophage RAW 246.7 cells. In contrast to curcumin, chitosan was not found to exert any effective antioxidant activity in scavenging both types of free radicals or any extracellular anti-inflammatory in inhibition of NO production in activated macrophage. The results obtained from an *in vivo* investigation on endogenous antioxidant efficacy in suppression the release of MDA (a biomarker of oxidative stress and lipid peroxidation) and anti-inflammatory efficacy in suppression the release of MPO (a biomarker of neutrophils infiltration), pro-inflammatory cytokines: PGE_2 and TNF-α in indomethacin-induced GU were also consistent with the *in vitro* antioxidant and anti-inflammatory results of curcumin and confirmed its superior anti-oxidant and anti-inflammatory efficacy than those of chitosan and lansoprazole. Based on several *in vitro*, cell culture, experimental animals and few pre-clinical trials; curcumin has been claimed as a potential therapeutic candidate against GU or gastric pathogenesis through its multi-target antioxidant and anti-inflammatory

properties. Curcumin was found to inhibit lipid peroxidation; scavenge a variety of ROS (including superoxide anion radicals, hydroxyl radicals) and nitrogen dioxide radicals (including peroxynitrite); and activate endogenous antioxidant defense enzymes (15). It was also reported to prevent oxidative damage during indomethacin-induced gastric lesion by blocking an inactivation of gastric peroxidase; direct scavenging of H_2O_2, and $OH^{\bullet}$; and enhancing the activity of natural antioxidant enzyme such as SOD, catalase and GPx (15). In addition, curcumin exhibited anti-inflammatory activity through suppressing the synthesis of pro-inflammatory PGs and leukotrienes (17), pro-inflammatory cytokines from the activated macrophage such as TNF-α, interleukins (IL-1, -2, -6, -8 and IL-12) and NO derived from iNOS (18,19,21). On the other hand, curcumin has also been shown to inhibit the cellular uptake of AA and all branches of the AA cascade by inhibition of both COX and LOX enzyme activities (51,197) so that it is claimed to be used as alternative anti-inflammatory agent against bone and joint disease such rheumatoid arthritis. Only a few experimental studies have until now been conducted to determine the mechanism of curcumin in modulating the expression of NOS and COX in ulcerated gastric mucosa. Recently, curcumin has been shown to exert gastroprotective in decreasing of gastric acid secretion and increasing of gastric microcirculation through endogenous PGs, NO and vasoactive neuropeptides against experimental stress-induced gastric lesions (140). According to the results obtained from the RT-PCR and qRT-PCR analysis, it was revealed that curcumin in the optimal therapeutic dose (less than 40 mg/kg) up-regulated the expression of constitutive COX-1 and constitutive nNOS and eNOS but down-regulated the expression of pro-inflammatory COX-2 and iNOS expression in ulcerated gastric mucosa induced by indomethacin which were correlated with its effect in reducing the

level of pro-inflammatory PGE_2 in the ulcerated gastric mucosa. These obtained results were consistent with previous studies of curcumin on its prophylaxis efficacy against indomethacin-induced gastric ulceration through antioxidant and anti-inflammatory in inhibition of iNOS production (141). The obtained result also indicated that chitosan has endogenous antioxidant and anti-inflammatory efficacy with the similar modulation profile to those of curcumin. Previous studies had shown that chitosan at a concentration of 0.02% (w/v) (0.02 g) was found to reduce the production of MDA and enhance the activity of antioxidant enzymes including anti-peroxidative enzyme (SOD and CAT) and glutathione-dependent antioxidant enzyme (GPx and GST) (106). It was also found to exert anti-inflammatory properties by inhibiting the production of PGE_2 and pro-inflammatory cytokines (such as TNF-α and IL-1β) (198). Lansoprazole has been reported to exhibit a powerful hypochlorous acid and hydroxyl radical scavenging activity (80,199) but its exact action on the expression of endogenous PGs and NO is still unclear. However, lansoprazole has been found to inhibit iNOS expression through up-regulation of heme oxygenase-1/carbon monoxide production in the mucosa (188,200). The realtime RT-PCR analysis results obtained in the present study revealed that lansoprazole exhibited the lowest efficacy in down-regulation of COX-2 or iNOS gene expression and in up-regulation of COX-1 and nNOS gene expression. Nevertheless, lansoprazole exerted a higher potency in up-regulation of eNOS expression which plays an important anti-inflammatory role by inhibiting leukocyte infiltration) than chitosan. Thereby, lansoprazole exhibited a lower antioxidant and anti-inflammatory potency than curcumin but a higher antioxidant and anti-inflammatory potency than chitosan in suppressing the production of MDA, MPO and TNF-α. Likewise, a chitosan-curcumin mixture which exerted the greatest efficacy in up-

regulation of constitutive COX-1 and constitutive nNOS and eNOS gene expression and in down-regulation of pro-inflammatory COX-2 and iNOS gene expression had the greatest antioxidant and anti-inflammatory potency.

As it has been reported that infiltrated polymorphonuclear leukocytes plays important role in the suppression of acid secretion (201), the pylorus ligation method was used for investigating antisecretory activity of test compounds on the release of total gastric acid output and the activity of pepsin (active in acidic pH) including gastric mucus stimulating activity through the direct activity of neurotransmitter acetylcholine on the parietal cells (202). The results observed in the present study indicated that intraduodenal administration of curcumin, chitosan and lansoprazole possessed inhibitory capacity against gastric acid secretion and pepsin activity but exerted a stimulating activity on gastric mucus production with lansoprazole exhibited the most potent acid inhibitory activity leading to the greatest efficacy in suppression of pepsin activity followed by curcumin and chitosan, respectively; whereas chitosan exhibited the most potent gastric mucus producing activity followed by curcumin and lansoprazole, respectively. Lansoprazole: is a proton pump inhibitor which irreversible blocking H^+-K^+-ATPase (proton pump) in the secretory membrane of parietal cell which is the final step of acid secretion by parietal cells. Therefore, it exerted the greatest efficacy in suppression of gastric total acid output and pepsin activity. It was found from the study that lansoprazole seemed to have no significant effect on stimulating gastric mucus producing though it was found to possess a significant up-regulation on the expression of nNOS which plays important role in regulating acid and mucus secretion. The obtained result seemed to be consistent with previous studies that neither omeprazole nor lansoprazole were found to increase gastric mucus secretion in

experimental studies and by observation in patients receiving omeprazole or lansoprazole (203). The obtained results also supported the previous study that curcumin effectively inhibited gastric acid secretion in pylorus-ligated rats (28). Curcumin at a dose less than 50 mg/kg was also reported to increase gastric mucus production as well (204). The finding of an increase in gastric ulceration index in a preventive study against indomethacin-induced gastric ulceration including a decrease in acid inhibitory and gastric mucus producing activity at a high dose of 40 mg/kg of curcumin confirmed the previous study that the potency of curcumin in inhibition of acid secretion and in stimulation of gastric mucus production reached almost the maximum at the dose of 20 mg/kg and was rather weakened at doses over 40 mg/kg (144). As it has been reported that endogenous NO has an inhibitory action on gastric acid secretion through suppression of histamine release from enterochromaffin-like (ECL) cells (205), curcumin at an optimal therapeutic dose (less than 40 mg/kg) exerted antisecretory and gastric mucus producing activity through an up-regulation of nNOS which plays important role in regulating acid and mucus secretion. On the contrary, the effect of curcumin might be predominantly mediated by an inhibitory activity on COX expression (through inhibition the cellular uptake of AA and all branches of the AA cascade (200) including iNOS expression at a higher dose, resulting in a reduction of its antisecretory and gastric mucus producing activity, which in turn, lead to an attenuation of its prophylactic efficacy against indomethacin-induced GU as found in the present study.

Chitosan has been known to form the viscous gel in acidic condition covering the gastric mucosa from irritating agents such as acid and pepsin. Its amino group may also neutralize acid in gastric content that resulting in a reduction of acid back-diffusion

into the gastric mucosa (104). These beneficial properties of chitosan accompany with its greater efficacy in up-regulation of nNOS expression than that of curcumin, chitosan was found to exhibit a superior gastric mucus producing potency than curcumin. Thereby, the greatest potency of acid inhibitory and gastric mucus producing activities of a chitosan-curcumin mixture might be accounted from the synergistic effective antisecretory activity of curcumin and effective gastric mucus producing activity of chitosan.

According to the higher prophylactic efficacy of chitosan against indomethacin-induced GU than those of curcumin and lansoprazole though it exerted the lowest antisecretory and antioxidant efficacy suggested that gastric mucus plays an important role in prevention the development of ulcer formation induced by NSAIDs. In contrast, despite showing the lowest potency on modulating the expression of NOS and COX mRNA including the production of gastric mucus, lansoprazole provided the excellent ulcer healing. This suggests that gastric acid accompany with the accumulation of free radicals and reactive species (ROS & RNS) derived from infiltrated neutrophils at the ulcerated gastric play an important role in aggravating the severity of the produced ulcer. In addition, gastric microcirculation produces a beneficial effect on gastric ulcer healing. Consequently, a chitosan-curcumin mixture which possessed the greatest antioxidant, anti-inflammatory, gastric mucus producing activities had proven the most effective than pure curcumin, chitosan and proton pump inhibitor: lanzoprazole (a standard antiulcer agent) in the prophylaxis and treatment of NSAIDs-induced acute GU and in the treatment of acetic acid-induced chronic GU

Topical formulation use in oral ulcer can be formulated as solution, suspension or emulsion depending on the properties of active ingredients like solubility and

stability. The important characteristic for the material to be selected for use in mouthwash formulation is that the component is pharmaceutically acceptable for mucosal application, nonirritant and nonsensitizing to oral mucosa and notified under the Generally Regraded as Safe category. Base on the preliminary anti-microbial investigation, the required concentration of curcumin in the formulation should be 0.1% (123). Considering its taste, safety profile, solubility and an optimal pH range to use in oral cavity, a nonirritant (alcohol free) mouthwash of curcumin (0.1%) was formulated using a co-solvent system composed of chitosan (0.5%) and surfactant: polyethylene glycol 400 by the method of Mustafa et al. (2019) (123). The percentage solubility of curcumin in PEG 400 is shown higher than other surfactants such as tween 80 and span 80. A developed nonirritant (alcohol-free) 0.1% chitosan-curcumin mouthwash was a clear solution with an optimal pH range to use in the human oral cavity (pH~ 5.5) and was determined for its therapeutic potential in management of oral inflammatory ulcers.

The buccal mucosal ulcer induce by a topical application of acetic acid is commonly use in animal studies especially in hamster model for evaluation the efficacy of the potential compounds in accelerating oral inflammatory ulcer due to its similar pathophysiologic ulcer phase as that of radiation or chemotherapy induced oral ulcer (87). A single large buccal mucosal ulcer was produced and the ulcer remained at 12 days post-treatment. Histologically, the produced buccal mucosal ulcer resembled acetic acid induced GU, in terms of both pathology (a defection of mucosal layer) and healing (a formation of granulation tissue). The findings suggested that a topical 0.1% curcumin in 0.5% of chitosan solution exerted a superior wound healing efficacy than a topical 0.5% chitosan without curcumin (vehicle control) and 0.15% benzydamine (Difflam®) solution. However, an oral administration of a chitosan-curcumin mixture

(containing curcumin 20 mg) could significantly decrease the ulcer severity and there was also no significant difference of the mean histological healing score between the two groups of chitosan-curcumin preparations. Histopathological analysis showed that the inflammatory changes were decreased and the healing of oral ulcer was accelerated with new capillary proliferation that reached the surface including a complete of epithelization, wound contraction and tissue remodeling. On the contrary, the positive group treated with Difflam® solution (a standard mouthwash with analgesic, anesthetic, anti-inflammatory and antimicrobial properties) showed the beginning of epithelization but still had no wound contraction or tissue remodeling. This may be resulted from the alcohol containing in the formulation which can cause an irritation to the produced ulcer and delay the wound healing. The healing and prevention of ulcer progression may be attributed mainly to antioxidant and anti-inflammatory properties of curcumin as described before accompany with its wound healing property in enhancement of fibroblast proliferation and migration, neovascularization, epithelial regeneration, collagen synthesis and remodeling phase (improving of wound contraction) through an up-regulation of TGF-1β expression (95). The addition of chitosan not only maintained the curcumin concentration in oral cavity to achieve an optimal substantivity in oral cavity (123) but also enhanced oral ulcer healing capacity of curcumin as chitosan has been found to promote tissue growth matrix and fibroblasts activity (114,206). Furthermore, this nonirritant 0.1% chitosan-curcuminoid moutwash had been found to provide the distinctive advantage comparable anti-candida efficacy to a standard 0.2% chlorhexidine mouthwash against *C. albican* growth, in biofilms method (112) and the potential therapeutic efficacy and safety in management of denture stomatitis (207).

Scratch assay is a useful method for gaining an insight into the potential of a sample to repair injured dermis. This assay is commonly used to study cell migration by creation of an artificial gap on a confluent cell monolayer with pipette tip (208). Fibroblast proliferation and migration are important steps in wound healing for tissue regeneration. Epithelial cell migration also plays important role in the intracellular adhesion and regeneration of mucosal cell after injury. Therefore, HGF human gingival fibroblast cells and AGS human gastric epithelial cell were represented for evaluation of fibroblast and epithelial cell proliferation and migration. The results obtained from the present study showed that the group treated with chitosan-curcumin mixture, curcumin and chitosan provided a comparable capacity in enhancement of wound healing via fibroblast migration and epithelial cell migration. Curcumin seemed to possess the most powerful modulating effects on wound healing with the shortest time needed for wound healing. Nevertheless, it was found that both HGF human gingival fibroblast and human gastric epithelial cell proliferation and migration decreased at a higher dose of curcumin, chitosan and a chitosan-curcumin mixture. NO generated from iNOS has been found to exert a dual nature not only participates in initiating ulcer formation but also plays a beneficial role in eliminating iNOS-positive inflammatory cells in the regenerating mucosa through apoptotic mechanism, leading to an acceleration of ulcer healing (200). Additionally, COX-2 has been found to compensate the temporary loss of COX-1 during the healing period for maintenance of a pH gradient and gastric mucosal integrity since COX-2 plays an essential role in the ulcer healing process to increase epithelial cell proliferation, migration and re-epithelization including the expression of growth factors especially VEGF which enhances angiogenesis (56,57). Accordingly, the wound/ulcer healing efficacy of curcumin,

chitosan and a chitosan-curcumin that possesses a potent down-regulation of iNOS and COX-2 expression will decrease when their dose is increased. These also suggest that a chitosan-curcumin mixture can exert opposite effects between prevention and exacerbation of GU or ulcer healing and ulcer relapse depending on the dose-effect relationship.

CHAPTER 6

CONCLUSION

A simple preparation method of chitosan-curcumin combination was developed and investigated the pharmacological interaction mechanism between curcumin and chitosan in management of NSAIDs: indomethacin-induced acute GU and acetic acid-induced chronic GU and oral ulcer *in vitro* and *in vivo* models.

An oral mixture with a combination of chitosan (150 mg) and curcumin (20 mg) using 0.1 M acetic acid as a solvent was a uniform yellowed-color mixture with pH about 3 which is an optimal gastric pH range (pH 1.5-3.5). Despite of having lower antisecretory efficacy than a proton pump inhibitor: lanzoprazole (a standard antiulcer agent), the pharmacological findings indicated that

1. An oral chitosan-curcumin mixture with low dose (20 mg) of curcumin provided the superior prophylactic and treatment efficacy against indomethacin-induced acute GU than lansoprazole, pure chitosan and pure curcumin.
2. A once-daily dosing of an oral chitosan-curcumin mixture showed a comparable ulcer healing efficacy to a twice-daily dosing of curcumin, chitosan or lanzoprazole in management of chronic GU induced by topical application of acetic acid which suggested synergistic antiulcer mechanisms of action of curcumin and chitosan in a chitosan-curcumin mixture including the beneficial effect of chitosan in enhancing the substantivity of curcumin in the gastric mucosa and the bioavailability of

curcumin through the mucoadhesive and drug penetration enhancing properties of chitosan.

3. Low daily dosing and low dosing frequency of an oral chitosan-curcumin mixture may increase patient compliance or medication adherence and decrease adverse drug reaction of curcumin.
4. The antiulcer mechanisms of action of a chitosan-circumin mixture might be exerted mainly through its potent antioxidant, anti-inflammtory (through a down-regulation of pro-inflammatory COX-2 and iNOS expression and the release of pro-inflammatory mediators: PGE_2 and TNF-α including an up-regulation of eNOS expression), antisecretory and gastric mucus producing (through an up-regulation of COX-1 and nNOS expression) activity and ulcer healing activity.

A topical nonirritant (alcohol-free) 0.1% chitosan-curcumin mouthwash formulated using a co-solvent system composed of chitosan (0.5%) and PEG 400 was a clear solution with an optimal pH range to use in the human oral cavity (pH~ 5.5). The findings obtained from the study revealed that

1. A topical nonirritant (alcohol-free) 0.1% of curcumin in 0.5% of chitosan provided a superior ulcer healing efficacy than a standard anti-inflammatory mouthwash (0.15% benzydamine) that might be attributed mainly to the antioxidant, anti-inflammatory and ulcer healing properties of curcumin.

2. The addition of chitosan not only maintained the curcumin concentration in oral cavity to achieve an optimal substantivity in oral cavity but also enhanced oral ulcer healing capacity of curcumin.

The potential pharmacological results obtained from the animal studies will be useful for further clinical study of chitosan-curcumin preparation as a potential alternative therapeutic agent for NSAIDs-induced GU, chemotherapy and/or radiotherapy-induced oral ulcer and chemotherapy induced gastrointestinal mucositis.

It is important to recognize that both curcumin and chitosan possess a potent down-regulation of iNOS and COX expression, therefore, a chitosan-curcumin preparation can exert the opposing effects of prevention and exacerbation of ulcer or ulcer healing and ulcer relapse depending on the dose-effect relationship.

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List of Publication and Proceeding

1. **Kuadkaew S**, Mahattanadul S, Ungphaiboon S, Puttaruk P. Effectiveness of a chitosan-curcumin mixture in treatment of acetic acid-induced chronic gastric ulcer in rats. International Conference on Traditional Medicine and Ethnomedical Research, Tokyo, Japan, Oct 24-25, 2019. (Publication for abstract)

2. Mahattanadul S, Mustafa MW, **Kuadkaew S**, Pattharachayakul S, Ungphaiboon S, Sawanyawisuth K. Oral ulcer healing and anti-candida efficacy of an alcohol-free chitosan-curcumin mouthwash. *European Review for Medical and Pharmacological Sciences.* 2018: 22:7020-7023.

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preparation on a modified acetic acid-induced buccal mucosal ulcer in hamster model. Proceeding of The 4th Current Drug Development International Conference (CDD2016), Phuket Graceland Resort & Spa, Patong Beach, Phuket, Thailand, June 1-3, 2016.

4. **Kuadkaew S**, Mahattanadul S, Ungphaiboon S, Kaewsuwan S, Phdoonsombut N. Puttarak P. Effectiveness of chitosan-curcumin mixture on indomethacin-induced gastric ulcers in rats. (In preparation for submission to journal of pharmacological research)