ฤทธิ์ของสารไฮดรอกซีแซนโทนต่อการป้องกันการรั่วไหลของเยื่อบุลำไส้ที่มีผลจากสารสื่ออักเสบที่เกี่ยวข้องกับสารพิษของแบคทีเรีย

นางสาววรรณพร ฉายาลักษณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาสรีรวิทยาการสัตว์ ภาควิชาสรีรวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

The protective effect of hydroxyxanthone on the leakiness of intestinal epithelia by the proinflammatory cytokine related to bacterial endotoxin

Miss Wannaporn Chayalak

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Animal Physiology
Department of Veterinary Physiology
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2017

Thesis Title	The protective effect of hydroxyxanthone on the leakiness of intestinal epithelia by the proinflammatory cytokine related to bacterial endotoxin
By	Miss Wannaporn Chayalak
Field of Study	Animal Physiology
Thesis Advisor	Associate Professor Sutthasinee Poonyachoti, D.V.M.,Ph.D.
Thesis Co-Advisor	Associate Professor Chatsri Deachapunya, Ph.D.

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

.....Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, D.V.M.,Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Sumpun Thammacharoen, D.V.M.,Ph.D.)

.....Thesis Advisor

(Associate Professor Sutthasinee Poonyachoti, D.V.M.,Ph.D.)

.....Thesis Co-Advisor

(Associate Professor Chatsri Deachapunya, Ph.D.)

.....Examiner

(Associate Professor Kris Angkanaporn, D.V.M.,Ph.D.)

.....Examiner

(Associate Professor Sirichai Adisakwattana, Ph.D.)

.....External Examiner

(Associate Professor Sunit Suksamram, Ph.D.)

วรรณพร ฉายาลักษณ์ : ฤทธิ์ของสารไฮดรอกซีแซนโทนต่อการป้องกันการรั่วไหลของเยื่อบุลำไส้ที่มีผลจากสารสื่ออักเสบที่เกี่ยวข้องกับสารพิษของแบคทีเรีย (The protective effect of hydroxyxanthone on the leakiness of intestinal epithelia by the proinflammatory cytokine related to bacterial endotoxin) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ส.พญ. ดร. สุทธาสินี ปุญญโชติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. ฉัตรศรี เดชะปัญญา, 88 หน้า.

การรั่วไหลของเยื่อบุลำไส้มีคุณลักษณะที่สำคัญคือมีการเพิ่มการแพร่ของสารโมเลกุลใหญ่ผ่านช่องด้านข้างของเซลล์เยื่อบุ เป็นสาเหตุสำคัญของการเสียชีวิตจากโรคติดเชื้อในกระแสโลหิต โดยพบว่ามีสารสื่ออักเสบคือ อินเตอร์ลิวคิน-1เบต้า เป็นตัวกลางที่สำคัญในการกระตุ้นผ่านวิถีการทำงานของไมโอซินไลท์เชนไคเนส ดังนั้นถ้าหากสามารถการยับยั้งการทำงานของกลไกนี้ที่ทำให้เกิดการเหนี่ยวนำการเกิดฟอสโฟรีเลตของไมโอซินไลท์เชน (พี-เอ็มแอลซี) ได้ก็น่าจะประสบความสำเร็จในการรักษาโรคได้ พบว่า แซนโทน ซึ่งเป็นสารที่พบมากในเปลือกของมังคุดมีฤทธิ์ที่ชะงัดในการต้านการอักเสบ โดยในปัจจุบันได้มีการสังเคราะห์สาร ไฮดรอกซีแซนโทน ซึ่งเป็นสารกลุ่มแซนโทนสำคัญที่พบอยู่ในมังคุดได้ในหลายรูปแบบ

การศึกษานี้มีวัตถุประสงค์เพื่อกลั่นกรองและตรวจสอบรูปแบบของสารไฮดรอกซีแซนโทน ที่มีความสามารถออกฤทธิ์ป้องกันการเพิ่มการแพร่ผ่านของสารผ่านช่องด้านข้างของเซลล์เยื่อบุในขณะที่ได้รับอินเตอร์ลิวคิน-1เบต้า โดยการทำงานผ่านการยับยั้งการแสดงออกของพี-เอ็มแอลซีในเซลล์เพาะเลี้ยงของลำไส้ชนิดคาโคทูซึ่งเป็นแบบจำลองนอกตัวสัตว์ เซลล์เพาะเลี้ยงของลำไส้ชนิดคาโคทูถูกเพาะเลี้ยงในอาหารเลี้ยงเนื้อเยื่อ ซึ่งประกอบด้วยซีรั่มจากตัวอ่อนลูกวัว 10 เปอร์เซ็นต์ เป็นเวลา 7 หรือ 21 วัน เพื่อเจริญต่อเป็นเซลล์ลำไส้ใหญ่หรือมีการพัฒนาเปลี่ยนเป็นเซลล์ลำไส้เล็ก ตามลำดับ เพื่อที่จะหารูปแบบของไฮดรอกซีแซนโทนที่ไม่เป็นพิษที่ 24 และ 48 ชั่วโมงจึงมีการทดสอบความเป็นพิษโดยวิธีเอ็มทีทีก่อนในเบื้องต้น หลังจากนั้นจึงทำการทดลองการแพร่ผ่านของสารผ่านด้านข้างของเซลล์ โดยวัดค่าความต้านทานไฟฟ้าระหว่างเซลล์เยื่อบุโดยเครื่องโวลล์โอห์มมิเตอร์และวัดการแพร่ผ่านของสารเรืองแสงเอฟดีโฟว์ และสุดท้ายจึงทดลองการตอบสนองของยาต่อแสดงออกของโปรตีนพี-เอ็มแอลซีด้วยวิธีการวิเคราะห์แบบเวสเทิร์นบลอท

ผลการทดลองจากเอ็มทีทีพบว่า 1-โมโนไฮดรอกซีแซนโทน 1-3-ไดไฮดรอกซีแซนโทน 1,3,6-ไตรไฮดรอกซีแซนโทน และ 1,3,6,8-เตเตระไฮดรอกซีแซนโทน ที่ความเข้มข้น 10-100 ไมโครโมลาร์จะไม่มีความเป็นพิษต่อเซลล์เยื่อบุลำไส้ที่เลี้ยงเป็นเวลา 7 วัน ยกเว้น 1,3,6,8-เตตระไฮดรอกซีแซนโทนความเข้มข้น 100 ไมโครโมลาร์ ที่ 48 ชั่วโมงเท่านั้นที่จะลดการมีชีวิตของเซลล์ ในสภาวะปกติ 1-โมโนไฮดรอกซีแซนโทน หรือ 1,3-ไดไฮดรอกซีแซนโทน จะกระตุ้นการเพิ่มค่าความต้านทานทางไฟฟ้าของเซลล์เยื่อบุที่มีอายุ 7 วัน ที่ 12 ชั่วโมง แต่ 1,3-ไดไฮดรอกซีแซนโทน หรือ 1,3,6-ไตรไฮดรอกซีแซนโทน ที่ความเข้มข้น 100 ไมโครโมลาร์จะลดค่าความต้านทานทางไฟฟ้าของเซลล์เยื่อบุที่มีอายุ 21 วัน ที่ 12 ถึง 48 ชั่วโมง นอกจากนั้นยังพบว่าอินเตอร์ลิวคิน-1เบต้าสามารถเพิ่มการแพร่ผ่านของสารผ่านด้านข้างของเซลล์เยื่อบุที่มีอายุ 7 วัน และเมื่อได้รับสาร 1-โมโนไฮดรอกซีแซนโทน หรือ 1,3-ไดไฮดรอกซีแซนโทน ความเข้นข้น 10 ไมโครโมลาร์ เป็นเวลา 12 และ 24 ชั่วโมง จะสามารถฟื้นฟูผลจากอินเตอร์ลิวคิน-1เบต้าได้ ส่วนในการศึกษาเซลล์ที่มีอายุ 21 วัน กลับไม่พบการตอบสนองเมื่อได้รับอินเตอร์ลิวคิน-1เบต้า อย่างไรก็ตามยังคงพบว่า 1-โมโนไฮดรอกซีแซนโทนสามารถกระตุ้นให้เกิดการเพิ่มค่าความต้านทางไฟฟ้าของเซลล์เยื่อบุที่ 12 และ 24 ชั่วโมงได้ ในทิศทางสอดคล้องกัน ผลการวิเคราะห์แบบเวสเทิร์นบลอทพบการแสดงออกของพี-เอ็มแอลซีเพิ่มขึ้นเมื่อมีการกระตุ้นด้วยอินเตอร์ลิวคิน-1เบต้า และถ้าได้รับไฮดรอกซีแซนโทนในเซลล์ที่ถูกกระตุ้นด้วยอินเตอร์ลิวคิน-1เบต้าจะสามารถลดการแสดงออกของโปรตีนพี-เอ็มแอลซีได้ที่ 24 ชั่วโมง จากผลการทดลองทั้งหมดบ่งชี้ว่า 1-โมโนไฮดรอกซีแซนโทน และ 1,3-ไดไฮดรอกซีแซนโทน ที่ความเข้มข้น 10 ไมโครโมลาร์ สามารถป้องกันการแพร่ผ่านของสารผ่านด้านข้างของเซลล์เยื่อบุโดยการยับยั้งการแสดงออกของโปรตีนพี-เอ็มแอลซีในเซลล์เยื่อบุลำไส้ใหญ่ จึงทำให้สรุปได้ว่าไฮดรอกซีแซนโทนในรูปแบบของ 1-โมโนไฮดรอกซีแซนโทน และ 1,3-ไดไฮดรอกซีแซนโทน ที่ความเข้มข้น 10 ไมโครโมลาร์ จะสามารถการนำไปใช้ และอาจถูกคัดเลือกเพื่อเป็นยาที่ใช้รักษาการรั่วไหลของเยื่อบุลำไส้ได้ในอนาคต

ภาควิชา สรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สรีรวิทยาการสัตว์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5875320331 : MAJOR ANIMAL PHYSIOLOGY
KEYWORDS: CACO-2 CELL, HYDROXYXANTHONE, PREVENTION, IL-1β, TIGHT JUNCTION DISRUPTION

WANNAPORN CHAYALAK: The protective effect of hydroxyxanthone on the leakiness of intestinal epithelia by the proinflammatory cytokine related to bacterial endotoxin. ADVISOR: ASSOC. PROF. SUTTHASINEE POONYACHOTI, D.V.M.,Ph.D., CO-ADVISOR: ASSOC. PROF. CHATSRI DEACHAPUNYA, Ph.D., 88 pp.

Leaky gut characterized as decreased transepithelial electrical resistance and increased paracellular permeability to macromolecules is associated with several fatal diseases including sepsis. Pro-inflammatory cytokine IL-1β is a critical mediator of underlying mechanism through activation of myosin light chain kinase pathway. Blocking the inducible phosphorylated myosin light chain (p-MLC) indicates the successful treatment. Xanthones predominantly found in mangosteen (*Garcinia mangostana*) has been indicated as a potent anti-inflammatory action. Hydroxyxanthones (HDX), the major natural xanthones, have been recently synthesized in various forms.

This study aimed to examine and screen the action of various forms of synthetic HDXs, in preventing the increased paracellular permeability during challenging with IL-1β by inhibiting the p-MLC expression in an *in vitro* model of intestinal Caco-2 cell culture. Caco-2 cells were cultivated in standard media with 10% fetal bovine serum for 7 or 21 days representing colonic-like or differentiated jejunal-like intestinal epithelial cells, respectively. Screening of non-cytotoxic forms of HDXs over 24 and 48 h were determined primarily by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The transepithelial resistance (TER) measured by volt-ohm meter and paracellular permeability of fluorescein isothiocyanate-dextran (FD-4; MW=4,400 Da) were accomplished for evaluating of paracellular permeability. Semi-quantitative Western blot analysis was performed to monitoring of p-MLC protein expression in response to drug treatments.

In the MTT assay, all HDXs 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX or 1,3,6,8-tetraHDX at 10 or 100 μM had no cytotoxicity. In an exception, 1,3,6,8-tetraHDX 100 μM decreased the viability of 7-day cell at 48 h. In normal condition, 1-monoHDX or 1,3-diHDX (10 μM) treatment promoted the increased TER in 7-day cells at 12 h, but 1,3-diHDX and 1,3,6-triHDX decreased TER in 21-day cells during 12-48 h. In 7-day cells challenged with IL-1β to decrease TER and increase paracellular permeability of FD-4, 1-monoHDX or 1,3-diHDX at 10 μM restored all the effect of IL-1β on the paracellular permeability at 12 and 24 h (n=5 experiments; $p<0.05$). In 21-day cells which were not responding to IL-1β, however 1-monoHDX also enhanced the TER from the initial values ($p<0.05$) at 12 and 24 h. In an accordance, Western blot analysis showed that p-MLC protein expression was increased in IL-1β treatments at 24 h. HDXs decreased the p-MLC stimulated by IL-1β at 24 h. These results suggest that HDXs can protect the increased paracellular permeability induced by IL-1β by inhibiting the increased p-MLC expression in colonic-like cells. In conclusion, 1-monoHDX and 1,3-diHDX at a concentration of 10 μM may be beneficial and be a candidate for treatment of the leaky gut in the future.

Department:	Veterinary Physiology	Student's Signature	.....
Field of Study:	Animal Physiology	Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

This study was achieved at Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand and Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand. The study was afforded by graduate school thesis grant 2017, Chulalongkorn University, and in part by Srinakharinwirot University research grants (034/2560).

I would like to express my sincere thanks to my thesis advisor, Associate Prof. Dr. Sutthasinee Poonyachoti for continuous support me, for her patience, carefulness, encouragement, precious knowledge and methodologies in life. She helped me all the time of research and writing this thesis. I could not imagine how I can finish the thesis without her. I am truly grateful to my co-advisor, Associate Prof. Dr. Chatsri Deachapunya who has always given counsel and support me since the beginning and the end of my study. Importantly, I really thank you for giving me the opportunity to be her advisee and a lot of experience in my Master's degree study.

I would like to thank Prof. Narattaphol Charoenphandhu, Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand for his kindness to provide Caco-2 cell in my research. In addition, I wish to thank Associate Prof. Dr. Sunit Suksamran, Department of Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand for her support to offer hydroxyxanthones in this thesis. I would especially like to thank lab members in Chulalongkorn and Srinakharinwirot University: Muttarin Lothong, Dran Rukarcheep and Norathee Buathong who support my work, knowledge, friendship and share the precious memories together.

Finally, special thanks to my family for always support and encourage me all the time. Thank you for their patience and motivation to let me through the problem and pressure. They are the most important persons in my life and my accomplishment.

CONTENTS

Page

Page

Page

TABLE OF CONTENT

TABLE OF FIGURES

LIST OF ABBREVIATION

°C	Degree Celsius
*	Significant difference
%	Percent
β-actin	Beta-actin
µg	Microgram
µl	Microliter
µM	Micromolar
Ω	Ohm
ADJ	Apical junctional complex
AJ	Adherens junction
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein-1
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C	Carbon
Ca	Calcium
$CaCl_2$	Calcium chloride

Caco-2	Human colorectal adenocarcinoma cell line
CAMP	Cyclic adenosine monophosphate
CD14	Cluster of differentiation 14
CFTR	Cystic fibrosis transmembrane conductance regulator
Cl	Chloride
cm^2	Square centimeter
^{13}C NMR	Carbon nuclear magnetic resonance spectroscopy
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
d	day
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal regulated kinase

FBS	Fetal bovine serum
Fig	Figure
FITC or FD	Fluorescein isothiocyanate dextran
g	gravity
^{1}H	Proton
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
h	Hour
HDX	Hydroxyxanthone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HT-29	Human colorectal adenocarcinoma cell line
IBD	Inflammatory bowel disease
IL-1β	Interleukin-1beta
IL-1RI	Interleukin-1 receptor type I
IL-6	Interleukin-6
IL-12	Interleukin-12
IFNγ	Interferon gamma
IP_3	Inositol triphosphate
IR	Infrared spectroscopy

IRAKs	Interleukin-1 receptor-associated kinases
IRF5	Interferon regulatory factor 5
JAMs	Junctional adhesion molecules
JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
kDa	Kilodalton
LBP	Lipid binding protein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MD-2	Lymphocyte antigen 96
Mg	Magnesium
mg	Milligram
min	Minute
MKK	Mitogen-activated protein kinase kinase
ml	Milliliter
MLC	Myosin light chain
MLCK	Myosin light chain kinase
mM	Millimolar
mm	Millimeter
mm^2	Square millimeter

mRNA	Messenger ribonucleic acid
MS	Mass spectroscopy
MTT	MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response 88
n	Number
NaCl	Sodium chloride
Na deoxycholate	Sodium deoxychelate
$NaHCO_3$	Sodium hydrogen carbonate
NaF	Sodium fluoride
ng	Nanogram
NF-κB	Nuclear factor-kappa B
nm	Nanometer
NP40	Nonyl phenoxypolyethoxylethanol 40
IkBα	NF-Kappa-B Inhibitor Alpha
OD	Optical density
OH	Hydroxyl
OH^-	Hydroxyl radical
Papp	Apparent permeability coefficient
PAGE	Polyacrylamide gel

PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PLC	Phospholipase C
p-MLC	Phosphorylated myosin light chain
PMSF	Phenylmethylsulfonyl fluoride
PKA	Protein kinase A
PKC	Protein kinase C
PRR	Patterns-recognition receptor
RIP1	Receptor interacting protein 1
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
S	Serine
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
T84	Human colonic adenocarcinoma cell line
TRAF6	TNF receptor-associated factor 6
TER	Transepithelial electrical resistance
TJ	Tight junction
TLR	Toll-like receptor

TNF-α	Tumor necrosis factor-alpha
TNFR1	TNF receptor type 1
TNFR2	TNF receptor type 2
TRIF	TIR-domain-containing adapter-inducing interferon-beta
UV	Ultraviolet visible spectroscopy
ZO	Zoluna occludens

CHAPTER I
INTRODUCTION

1.1 Background and rationale

Infectious disease in gastrointestinal system causes diarrhea in human and animal resulting in death, and loss of money and productions (Haycocks et al., 2015). The anti-microbial overusing in animals causes antimicrobial resistance, especially gram negative bacteria such as *Escherichia coli* (*E. coli*) (Hammerum and Heuer, 2009). Furthermore, the pathogens can induce subclinical signs by disrupting the intestinal barrier, which may associate with a decreased growth performance in farm animals (Awad et al., 2015). Importantly, the intestinal epithelial barrier primarily mediated by tight junction (TJ), which functions for defensing of pathogen and their toxin, may be loss of integrity, and known as "leaky gut". Leaky epithelia lead to an increase in permeability, and allows the luminal antigens invading the mucosa to enter the body. The local infection has not only altered the homeostasis of gut mucosa, but also intensified systemic infection, chronic inflammation and malabsorption (Awad et al., 2015). The prevention of TJ barrier dysfunction or leaky epithelia, which usually associate with the subclinical infected diseases in animals, may be a powerful approach to protect the body from the penetration of luminal pathogens.

Lipopolysaccharide (LPS) is the main endotoxin liberated from the cell wall of gram-negative bacteria during shedding (Demonty and De Graeve, 1982). In general, LPS exist in the intestinal lumen, but could not pass the healthy epithelial barrier. However, the systemic infection, which present of circulatory LPS in a high level (Demonty and De Graeve, 1982) , causes the impairment of TJ leading to increases in the paracellular permeability. The intestinal mucosa turns leaky and allows the luminal LPS and other antigens penetrating the mucosa (Guo et al., 2013). The

important mechanism of LPS to increase TJ permeability is mediated by toll-like receptor-4 (TLR4) signaling pathway. The TLR4 signaling system stimulated by binding of LPS to TLR4 has potential to activate nuclear factor-kappa B (NF-κB) in MyD88 dependent pathway, and subsequently induce the production and secretion of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1beta (IL-1β) (Guo et al., 2015). TNF-α and IL-1β play a role to induce the inflammatory response by many signaling systems via their receptors. Both of cytokines induced by LPS effect may activate immune cells and the secretion of epithelial cells. The disruption of the TJ barrier as reflected by the decreased transepithelial electrical resistance (TER) and the increased paracellular permeability has been indicated in LPS-, TNF-α or IL-1β challenged monolayers.

The paracellular transport and permeability, is generally determined by an intercellular seal, termed apical junctional complex (AJC) located at the apical part of intestinal epithelium. The junctional complex is formed by two main protein complexes, the TJ-associated proteins and adherens junction (AJ). The TJ associated proteins comprise many transmembrane proteins, including the claudin family and occludins in which their interaction regulates paracellular flux of macromolecules and ion in a specific manner. The AJ does not directly determine the paracellular flux, but mainly provide the epithelial barrier. It physically links to the perijunctional actin-myosin ring. However, the perijunctional actin-myosin ring also attaches with TJ protein complex. Thus, the perijunctional actomyosin seems to be a linker that has direct and indirect protein-protein interaction with the AJ and the transmembrane protein of TJ, which determine both epithelial barrier and paracellular flux junction (Cunningham and Turner, 2012). It has been thought that the activation of myosin light chain causes the contraction of actin-myosin, and subsequently changes the TJ protein localization and decreases the TJ barrier (Ma et al., 2005). Myosin light chain kinase (MLCK) is a

Ca^{2+}/calmodulin-dependent serine/threonine protein kinase that phosphorylates myosin light chain (MLC) to become the phosphorylated form of myosin light chain (p-MLC). The p-MLC which is an active form that promotes the contraction of perijunctional actin-myosin filament resulting in increased permeability and fluid flux across the paracellular pathway. It is induced by many factors, especially the pro-inflammatory cytokines, including TNF-α and IL-1β, during the bacterial infection (Cunningham and Turner, 2012; Xu et al., 2015). The loosening of TJ that was seen in the early period of LPS treatment, was suggested to be contributed by myosin light chain kinase (MLCK) activity (Al-Sadi et al., 2011). As the p-MLC has been indicated as an important regulator for TJ permeability and barrier (Cunningham and Turner, 2012; Ulluwishewa et al., 2011) therefore, we propose that inhibition of MLCK may protect the TJ from an increased permeability and loss of fluid. Apart from MLCK, tyrosine kinases that are associated with TJs, AJs and MLCK (Anderson and Van Itallie, 2009; Ulluwishewa et al., 2011) have been shown to regulate the integrity of these junction complexes. Inhibition of tyrosine kinase will prevent the phosphorylation of MLC resulting in decreasing of the opening of TJ and paracellular permeability (Samak et al., 2014; Ulluwishewa et al., 2011).

Isoflavone is polyphenolic compounds which are composed of 12 isoforms which are 3 aglycones and 9 glucosides in soybeans (Middleton et al., 2000; Song et al., 1998). Genistein is isoflavone which has many biological activities, based on estrogenic action on blocking the steroidogenesis and protein tyrosine kinase inhibitor (Bektic et al., 2005). Moreover, use of flavanols and isoflavones has been suggested as a potential drug to improve the TJ permeability induced by bacterial challenge in an ex *vivo* or *in vitro* study of many mucosal tissues or epithelial cell culture, respectively (Amasheh et al., 2008; Kiatprasert et al., 2015; Noda et al., 2012). The mechanistic actions responsible for improving the epithelial barrier and integrity by inducing the

expression of TJ protein, adherens molecules or scaffold proteins as well as inhibiting tyrosine kinase and protein kinase A-dependent pathways (Kiatprasert et al., 2015; Noda et al., 2012; Suzuki and Hara, 2009). It is possible that the drug or substance, which has the similar structure to genistein, may have the effect to protect TJ from bacterial endotoxin consequence.

Hydroxyxanthone (HDX) is the major phenolic compounds, alike genisteine, found in many plants. It reveals a wide range of effects, including anti-oxidant, anti-inflammatory (Visioli et al., 2011). Natural HDXs are usually found in Mangosteen *(Garcinia mangostana)* and composed of many fractions. The crude extraction of natural HDXs is mangostin which is used for diarrheal treatment in traditional medicine (Pedraza-Chaverri et al., 2008). Up to date, the synthetic HDXs which have the different numbers and position of hydroxyl groups on a skeleton of xanthones, such as 1,3-diHDX, 1,3,6-triHDX, 1,3,8-triHDX or 1,3,6,8-tetraHDX have been produced and tested for their biological activities (Pedraza-Chaverri et al., 2008). The different forms of xanthones have been identified and shown to have a variety of biological actions. In the human intestinal epithelial cell line, T84 cells, only 1,3,6-triHDX has the most efficiency for the inhibition of cAMP-activated Cl^- secretion (Luerang et al., 2012). Interestingly, the chemical structure of HDX is similar to isoflavone genistein which is the potent tyrosine kinase inhibitor. Thus, HDX may have an ability to inhibit protein tyrosine kinase-induced MLCK pathway during exposure to LPS. This inhibitory effect of HDXs may be beneficial to prevent the leakage epithelia in subclinical infected diseases. The present study will therefore be conducted to investigate the effect and underlying mechanisms, i.e. inhibition of MLCK, of the varieties of HDXs on IL-1β-activated paracellular leakiness. The results of the present study may provide the potential benefit of this class of compounds to protect leaky gut from IL-1β, and

further be used as the potential candidate drug for improving the quality of gastrointestinal properties and function in animals.

1.2 Research Objectives

1. To examine the effects of synthetic HDXs on TJ permeability in intestinal epithelium in normal condition.

2. To examine the effects of synthetic HDXs on TJ permeability in intestinal epithelium in IL-1β-induced condition.

3. To examine the intracellular mechanism of HDXs associated with TJ permeability in intestinal epithelium treated with IL-1β.

1.3 Research Hypothesis

1. HDXs may increase transepithelial electrical resistance (TER) and reduce paracellular macromolecule permeability in intestinal epithelium in normal condition.

2. HDXs can inhibit a decreased transepithelial electrical resistance (TER) and increased paracellular macromolecule permeability in intestinal epithelium treated with IL-1β.

3. HDXs can reduce phosphorylation form of TJ regulatory protein MLC in intestinal epithelium treated with IL-1β.

1.4 Expected Benefits and Application

1. This study provides an understanding of the effects of HDXs on intestinal barrier in normal and IL-1β induced condition.

2. This study aims to improve intestinal barrier in normal condition by HDXs treatment.

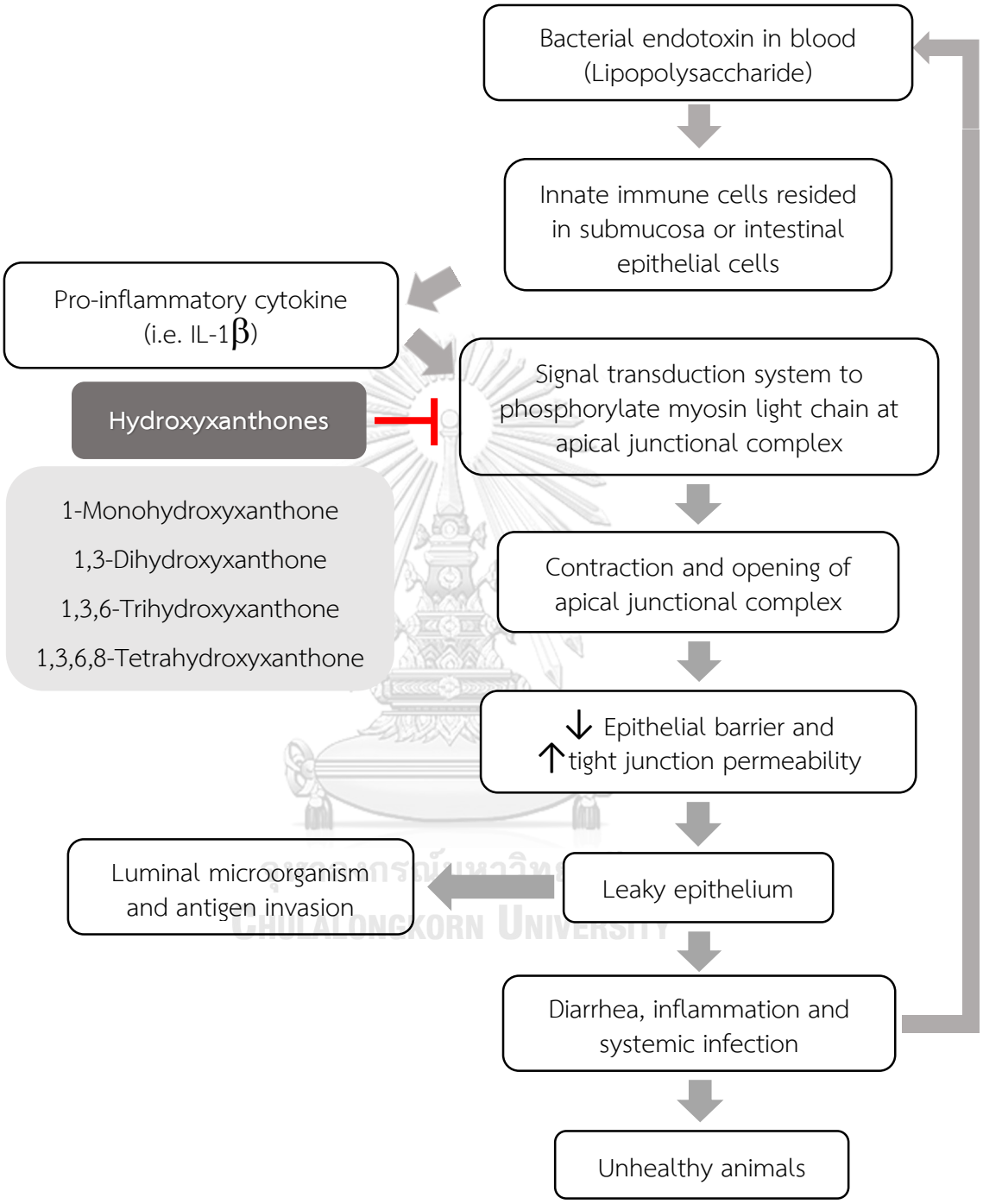
3. This study aims to protect the intestinal barrier from IL-1β by HDXs treatment.

4. This study will lead to the knowledge of candidate drug for the treatment gastrointestinal disorder.

1.5 Places of study

Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand and Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand.

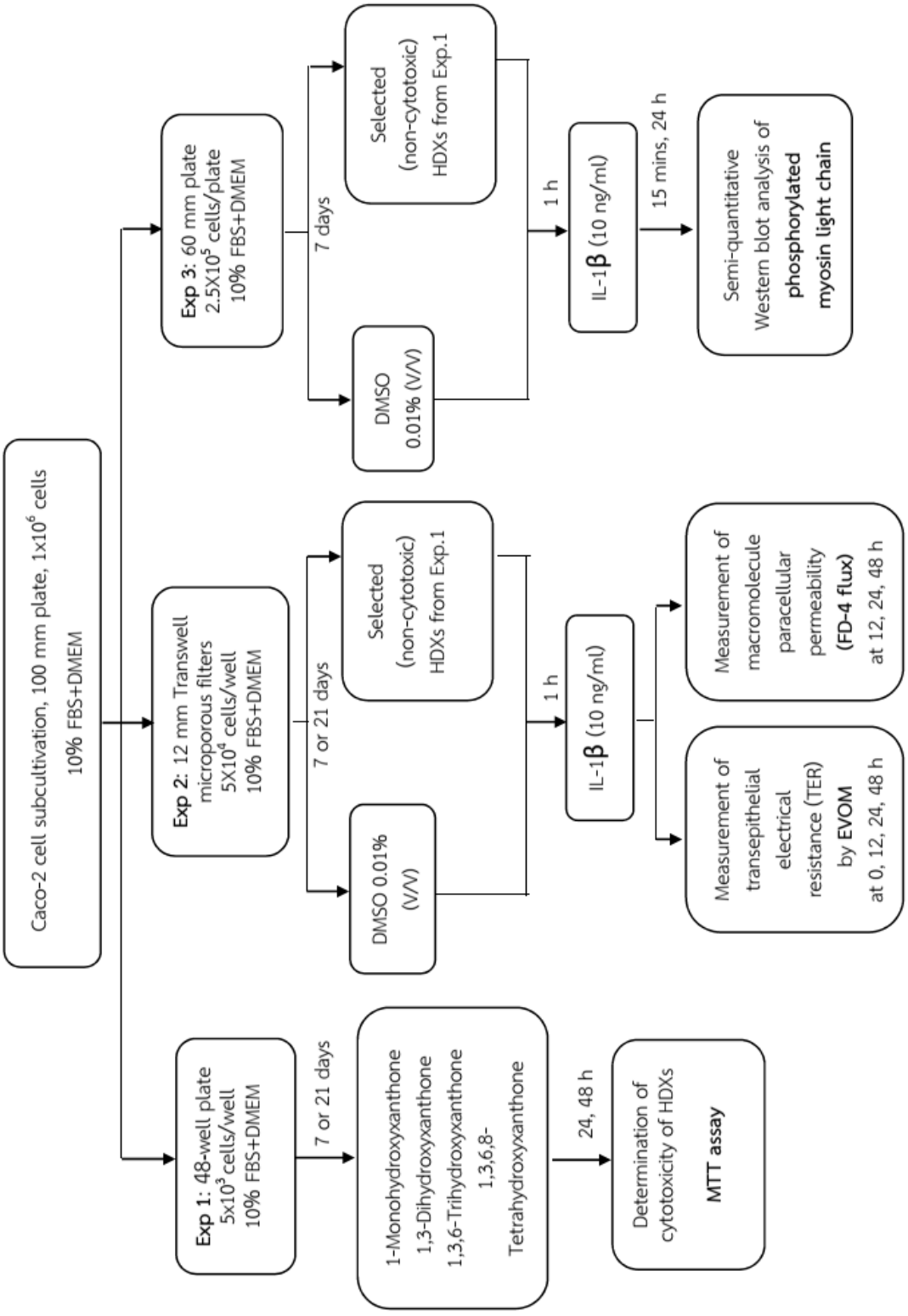
1.6 Conceptual Framework



1.7 Keywords

CaCo-2 cell, Hydroxyxanthone, Prevention, IL-1β, Tight junction disruption

1.8 Experimental design



CHAPTER II
LITERATURE REVIEW

2.1 Gastrointestinal anatomy and function

The structure of gastrointestinal tract consists of many layers from inside to outside; mucosa, submucosa, lamina propria, muscularis mucosa, and serosa, respectively. Along the gastrointestinal epithelial lining, there are many specialized cells, such as goblet cells, which produce mucus to protect the pathogen in the lumen. The villi in mucosal layer are found predominantly in the duodenum but less in the ileum. Villi function to absorb nutrient to a blood vessel or lymphatic system in lamina propria and secrete digestive enzymes to digest the food. The Brunner's glands in the submucosa also produce the mucus to neutralize the acid. Lamina propria has many capillaries, which transport nutrients to the liver. Moreover, lamina propria have the structure called lacteal, which is important for transportation of nutrients that cannot pass through capillaries, i.e. fatty acid and proteins (Kiela and Ghishan, 2016). Moreover, the gastrointestinal tract is regulated by cytokine and immune system such as macrophage or B-cell (Keita and Soderholm, 2010). A disorder of gastrointestinal function has been known to associate with malnutrition. Malnutrition, gastrointestinal bacteria and septicemia are the critical public health problem in human and animals because they will be unhealthy and elevates the infection and death (Pelletier and Frongillo, 2003).

2.2 Epithelial barrier and function

Mucosa or epithelia contact with contents in lumen and environment in the intestine. Since it comprises the largest surface areas, the potent defense mechanism

to protect the host from invading pathogens must be required. The intestinal barriers are characterized into two types, which are physical and chemical barriers to prevent the invading of pathogen and toxin (Sperandio et al., 2015). Physical barriers, such as intestinal epithelium, mucin and TJ, are important since they serve as first-line barriers. The single layer of intestinal epithelium separates luminal from basolateral sides. This epithelial layer mainly functions to regulate or transport of nutrients, ions and fluid flow (Madara et al., 1989). The intestinal epithelium has two permeability routes, which are transcellular and paracellular routes (Tsukita et al., 2001).

Transcellular permeability regulates the solute transport across the epithelial cells associated with selective transporters (Groschwitz and Hogan, 2009). Paracellular permeability regulates the transportation between spaces of epithelial cells. The paracellular transport pathway serves as a main route of passive flow of water and solutes as well as other macromolecules. Paracellular transport of macromolecules is physically regulated by the structural proteins sealing between cells, called TJs (Naslund and Hellstrom, 2007). Therefore, tight junction barrier plays roles in the physical barrier mechanism when the pathogens invading to the epithelia. Even though, the mucous layer containing mucin can block or neutralize the pathogen-epithelial binding prior to attacking, mucin layer or biofilm may be a reservoir for pathogens (Wingender and Flemming, 2011). Thus, TJ is the last, but most importantly as a physical barrier to prevent pathogen invading to the basolateral side. The disruption of TJ, called “leaky epithelia” causes the increased intestinal permeability, and promotes permeation to a pathogen and toxin that the predominant result of intestinal inflammation (Ma et al., 2004). The chemical substances released from innate immune cells or intestinal epithelium during the early response, i.e. pro-inflammatory cytokines or chemokines is the other potent chemical barriers. However,

it can regulate or modify the epithelial barrier structure and functions, especially TJ proteins that control paracellular permeability (Castoldi et al., 2015).

2.3 Tight junctions and adherens junctions, as an epithelial barrier (Fig. 1)

Tight junctions (TJs) are the intestinal barrier that controls permeability of ions, water and macromolecules. TJs are composed of multiple complex transmembrane proteins that locate between apical and basolateral side (Turner, 2006). Transmembrane proteins of TJs, occludin, claudins and junctional adhesion molecule (JAM) (Ebnet et al., 2004), have been identified. Occludin is the first TJ protein, which contains two extracellular loops, intracellular turn, N- and C-terminal cytoplasmic domain (Furuse et al., 1993). The extracellular loop composed of many glycine and tyrosine and few amino acid charges. Besides, the C-terminal part has numerous of tyrosine and serine which can be phosphorylated by protein kinase (Schneeberger and Lynch, 2004). Moreover, the C-terminal domain also link with ZO-1 which bind to the actin filament (Furuse et al., 1994). Actin cytoskeleton is also located at the adherens junction (AJ). AJ contains different transmembrane proteins (E-cadheren) and scaffold proteins (β and α-catenin).

TJ disruption leads to increase the paracellular permeability and allow the antigens macromolecules interact with innate immune cells underneath the intestinal epithelium. The evidence causes to induce inflammation and mucosal immunity responses resulting in intestinal and systemic disease (Turner, 2006). However, epithelia is classified as tight and leaky epithelia depending on the water and solute permeability. The example of tight epithelia is the distal convoluted tubule, collecting duct of the kidney and bile duct of the liver. In contrast, the leaky epithelia does not

have much TJs or less transmembrane protein of TJ, such as proximal tubule of the kidney, the proximal intestinal epithelium (Diamond, 1974).

At the tight junction, the occludin has been indicated as a first macromolecule barrier. Alteration of occludin expression and function, i.e. in the inflammatory disease Chrohn's disease, ulcerative colitis, collagenous colitis and pleural inflammation, revealed the decreased barrier to macromolecules. The lower occludin expression has been indicated in intestinal inflammatory diseases depending on cytokine such as IFNγ, TNF-α or infection of the pathogen (Krug et al., 2014). However, the occludin-deficient in knockout mice did not reveal the abnormality of TJ structure and barrier function in intestine (Saitou et al., 1998).

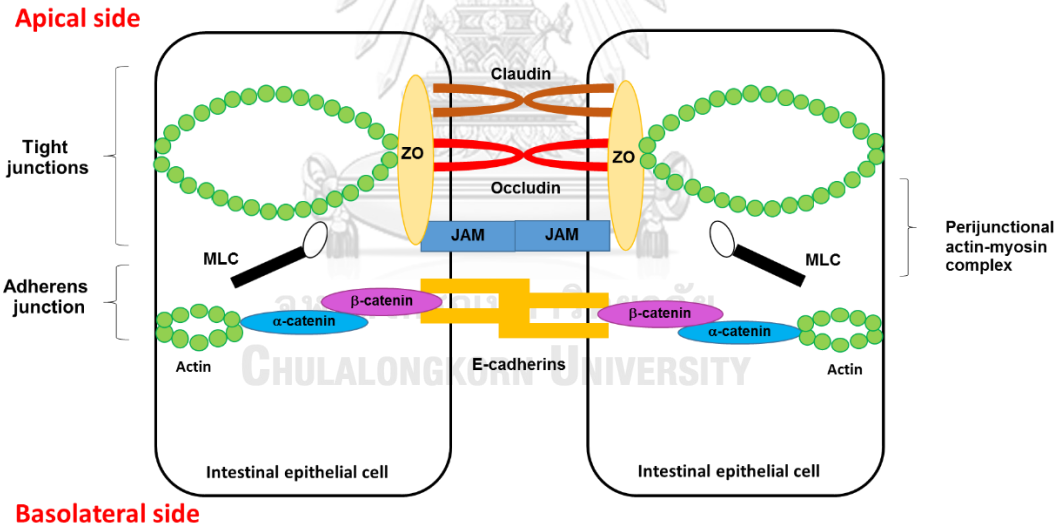


Figure 1 The intracellular structure of tight junctions of intestinal epithelial cells (Chiba et al., 2008).

Claudins are the second TJ protein. They are the transmembrane proteins that have a short cytoplasmic N-terminus, C-terminal cytoplasmic domain and two

extracellular loops (Tsukita et al., 2001). Twenty-four members of claudins family have been suggested and detected in human and mice (Van Itallie and Anderson, 2006). Claudins bind to the core proteins, PDZ domain ZO-1-3 (Itoh et al., 1999).

The third TJ protein is the junctional adhesion molecules (JAMs) which are glycosylated transmembrane proteins that have two extracellular domains, one transmembrane domain and a C-terminal cytoplasmic region (Ebnet et al., 2004). JAMs are located in the epithelial, endothelial and blood cells. JAMs regulate only the transport of nutrients and solutes across the epithelial cells (Monteiro and Parkos, 2012). Even though they form the cell-cell adhesion, JAM-A do not function directly as a barrier, they rather mediate a divergent epithelial function, such as proliferation and migration (Monteiro and Parkos, 2012). Many proteins presented and function as a cell-cell adhesion; however, the regulation of the epithelial barrier mediated by apical junctional complex, the collection of TJ and AJC-associated proteins has been predominantly suggested in all epithelia (Anderson and Van Itallie, 2009).

As aforementioned, the AJC including TJs and AJs locate nearly at the luminal side. At the AJC, the AJ does not establish paracellular transport, but function for structural stability and integrity. In contrast, the TJ proteins, claudin and occludins contribute for both paracellular flux and integrity (Anderson and Van Itallie, 2009). It is noted that both AJ and TJ protein complex link to the structural protein, called perijunctional actomyosin ring, which forms the belt around each epithelial cells (Cunningham and Turner, 2012). The binding between the AJC and actin cytoskeleton controls the AJC function on epithelial barrier properties (Cunningham and Turner, 2012) Previous studies using T84, Caco-2 or HT-29 intestinal epithelial cell line revealed that the impairment of AJC leads to the increased permeability in inflammatory bowel disease. The evidence has been indicated to associate with the septicemia, when the

pro-inflammatory cytokines, especially TNF-α and IFNγ presence at high levels in blood circulation. It implies that the pro-inflammatory cytokines may play roles to regulate AJC functions. The actin-myosin perijunctional complex activity causing the contraction of perijunctional actin and myosin belt will be occurring when the phosphorylation of myosin light chain (MLC) is induced. The mechanism is mediated by the serine-threonine protein kinases to phosphorylate MLC which have two types: myosin light chain kinase (MLCK) and Rho-associated protein kinase (ROCK) (Cunningham and Turner, 2012). Interestingly, the inhibition of MLCK and RhoA can decrease an impairment of epithelial barrier induced by TNF-α and IFNγ (Bruewer et al., 2006). Moreover, the inhibition of MLCK may reverse an increased permeability or loss of fluid from the opened TJ (Cunningham and Turner, 2012; Krug et al., 2014). Unfortunately, many intracellular signaling molecules, including tyrosine kinases can be induced by many factors, including post-prandial stage and infection. The regulation of tyrosine kinase-MLCK activity on the function of TJs and AJs is of interest (Anderson and Van Itallie, 2009; Ulluwishewa et al., 2011).

2.4 Myosin light chain kinase function on the epithelial barrier (Fig. 2)

The MLC phosphorylation (p-MLC) is a biomarker of actin-myosin contraction (Shen et al., 2011; Turner et al., 1997). The MLCK-dependent MLC phosphorylation is related to the increased TJ permeability. p-MLC has been shown to up-regulate in both physiological and pathophysiological conditions. However, under the normal physiological condition, the increased paracellular fluxes respond to the post-prandial that are associated with the p-MLC. In many infectious diseases, the enteropathogenic bacteria, *E. coli* in particular has direct or indirect effects to activate the phosphorylation of MLC through, pro-inflammatory cytokines, including TNF-α and IL-

1β (Cunningham and Turner, 2012). Therefore, MLCK seems to be the target of pro-inflammatory cytokines and contributes to leaky epithelia.

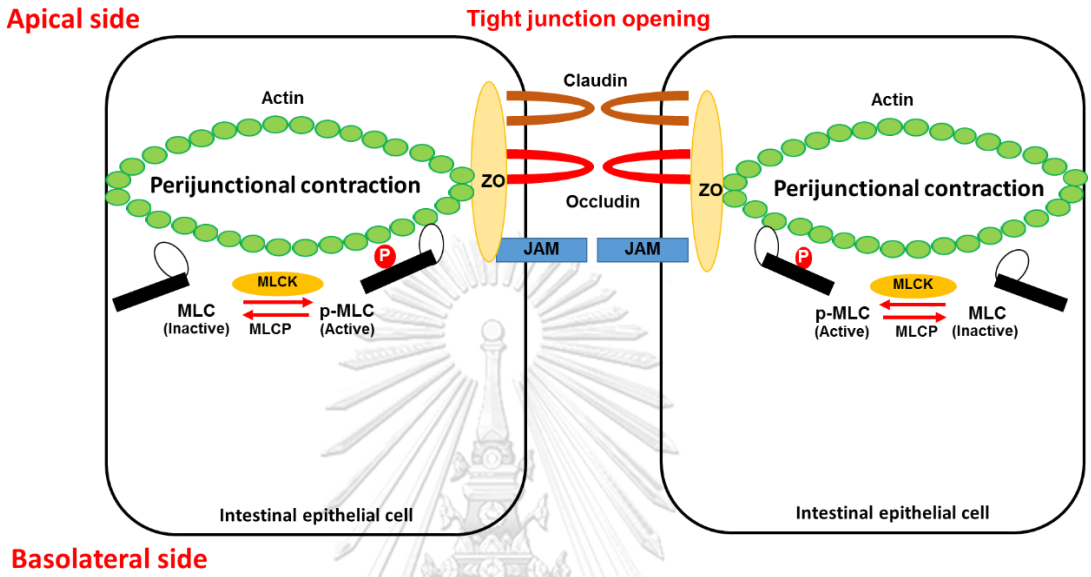


Figure 2 Myosin light chain kinase (MLCK) phosphorylated Myosin light chain (p-MLC) and its function to opening the tight junction (Cunningham and Turner, 2012).

Pro-inflammatory cytokines induce MLCK by two mechanisms, which are the increased MLCK transcription and the increased activation of MLCK (Wang et al., 2005). The study revealed that mice treated with an inhibitor of MLCK leading to lower p-MLC, and prevented the loss of barrier and fluid secretion, suggesting that MLCK is an important effector to control barrier dysfunction (Clayburgh et al., 2005). Two types of MLCK; short and long form has been classified. MLCK is encoded by two separate genes. Short form MLCK (M.W.=130-160 kDa) express in smooth muscle, but not in intestinal epithelia. The long form MLCK (M.W.=210 kDa), which is MLCK1 or MLCK2, is located in villous epithelium and crypt cells, respectively. In addition, MLCK1 is

associated with the perijunctional actomyosin ring in normal integrity epithelia, whereas lacking of MLCK1 increase barrier function (Blair et al., 2006; Clayburgh et al., 2004). The study indicated that NF-κB and AP-1 enhances the up-regulation of IL-1β-induced MLCK (Al-Sadi et al., 2008; Blair et al., 2006). The increased MLC activity to phosphorylate Ser19 of MLC was also demonstrated. In the inflammatory bowel disease, the elevation of TNF-α and IL-1β associated with the increased MLC transcription and loss of barrier was indicated as the underlying mechanism (Blair et al., 2006; Bruewer et al., 2006).

Since MLCK protein and MLC phosphorylation are the major target of pro-inflammatory cytokines to regulate TJ permeability (Bruewer et al., 2003; Taylor et al., 1998; Wang et al., 2005), the inhibition of the signaling system of protein kinase to inhibit the phosphorylation of MLCK may provide the promotion of TJ barrier and treat the intestinal disease (Cunningham and Turner, 2012).

2.5 Pathogenesis of pathogen on the intestinal permeability (Fig. 3)

The pathogens can infect the gastrointestinal tract through fecal-oral route. The pathogens must increase the numbers or damage the host barriers of upper gastrointestinal tract and invade to the intestine. The remaining pathogens or toxin production penetrates into the mucosal epithelium and invade into the lymphatic or bloodstream (Blacklow and Greenberg, 1991; Field et al., 1989). The intestinal mucosal barrier has a critical role in preventing the pathogens and providing an absorption of the nutrients. The intestinal barrier needs to sense and respond to pro-inflammatory bacterial products. Many intestinal diseases associate with the intestinal mucosal barrier dysfunction, including inflammatory bowel disease (Crohn's disease) (Claud, 2009). In general, the host can recognize the pathogens the specific molecular pattern,

called pathogen-associated molecular patterns (PAMPs). The mammalian, whose cells express many patterns-recognition receptor (PRR) types, including toll-like receptors (TLRs) can detect PAMPs, and it transduces signaling pathway to stimulate the immune system (Akira et al., 2006).

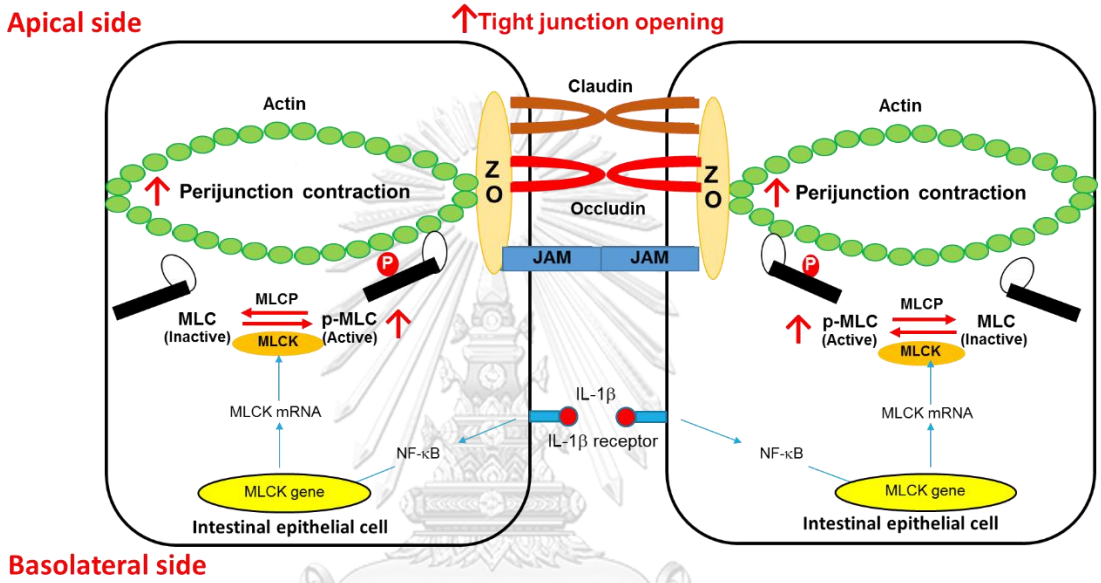


Figure 3 Cellular mechanism of lipopolysaccharide and its pro-inflammatory mediator cytokines in the opening of tight junction mediated by the induction of Myosin light chain kinase (MLCK) expression pathway (Al-Sadi et al., 2008; Ma et al., 2005)

In the gastrointestinal system, lipopolysaccharides (LPS) are the endotoxin, which is the outer membrane component of enteric bacteria, especially gram-negative (Akira et al., 2006). Lipid binding protein (LBP), which is the soluble protein of LPS, will bind to the surface protein CD14 at the outer membrane of host cells (Wright et al., 1989). CD14 induces LPS binding with toll-like receptor complex TLR4/MD-2 receptor

complex to activate signal transduction (Wright et al., 1990). The TLR4-signaling has two pathways, which are dependent or independent MyD88 leading to induce pro-inflammatory cytokines and anti-viral cytokines interferon. These two groups of cytokines cause inflammation or neutralize viruses, respectively (Takeda and Akira, 2004). The MyD88-dependent pathway activates many transcription factors, including interleukin-1 receptor-associated kinases (IRAKs), TNF receptor-associated factor 6 (TRAF6) and nuclear factor-kappa B (NF-κB), Activator protein1 (AP-1), interferon regulatory factor 5 (IRF5), which subsequently induce transcription of pro-inflammatory cytokine genes, i.e. tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-12 (IL-12). On the other hand, the MyD88-independent pathway activates TRIF signals to induce transcription factor TRAF3 and RIP1 to stimulate IRF3, NF-kB and AP-1 leading to interferon type I expression (Lu et al., 2008). Previous studies revealed LPS at physically and clinical concentrations (0-10 ng/ml) induce an increase of TJ permeability without cell death (Guo et al., 2015). However, the induction of TNF-α and IL-1β synthesis and secretion by the LPS has been indicated and associated with the pathogenesis of diarrhea and septicemia (Van Amersfoort et al., 2003).

Both pro-inflammatory cytokines have function of the intestinal immune system, infection, inflammation, cell differentiated, tissue remodeling and apoptosis. IL-1 family consists of 3 members, including IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra) (Dinarello, 1996). However, IL-1β presents in the intestinal fluid and blood circulation in the inflammation period but not in the normal condition. Additionally, the increased IL-1β level (1–10ng/ml) correlated with the drop of epithelial resistance which related to an increased the paracellular permeability in a time-dependent (0–72 h) in Caco-2 (Al-Sadi and Ma, 2007). The mechanism of IL-1β in regulating the of

MLCK activity correlates with an increased MLCK mRNA and protein levels (Al-Sadi et al., 2008). It was shown that NF-κB activation was an important signal for IL-1RI. Release of NF-κB from IkBα is induced by IL-1R/TLR binding to MyD88 signaling pathway. Activated NF-κB can induce MKK enzyme resulting to activate ERK, JNK, and p38 MAP kinase pathways to induce the target gene transcription regulation (Fortin et al., 2009).

Thus, both of TNF-α and IL-1β are mediators of LPS to associate with tyrosine kinase pathway and myosin light chain kinase (MLCK) to induce the perijunctional actin-myosin complex contraction and relevant to elevate paracellular permeability. The tyrosine kinase inhibitor inhibits the phosphorylation of MLC to p-MLC by MLCK leading to prevent the opening of TJ and decrease paracellular permeability was suggested as the powerful target (Samak et al., 2014; Ulluwishewa et al., 2011).

2.6 Bioactive compounds and hydroxyxanthones: structure and functions in intestinal tissues (Fig. 4, 5)

Bioactive compound is the compound existing in food or plants that have the effect to support the good health (Kris-Etherton et al., 2002; Ramos et al., 2011). The phytochemical is bioactive compound from plants that have many classification, but the biggest groups is polyphenolic compounds (Tsao, 2010). Polyphenolic compounds, isoflavones have been used to protect the membrane integrity such as genistein. It has been proposed as the potent protein tyrosine kinase inhibitor (Bektic et al., 2005). Moreover, using of flavanols has been indicated to be a potent drug to improve the TJ permeability induced by bacterial challenged in the *ex vivo* or *in vitro* study of many mucosal tissues and epithelial cell culture, respectively (Amasheh et al., 2008; Kiatprasert et al., 2015; Noda et al., 2012). The mechanism helps improving the

epithelial barrier integrity, increasing in the expression of TJ protein, adherens molecules or scaffold proteins, protein kinase A and tyrosine kinase inhibition (Kiatprasert et al., 2015; Noda et al., 2012; Suzuki and Hara, 2009). The signal transduction as a protein tyrosine kinase inhibitor related with phosphorylation of TJ proteins (Fujibe et al., 2004; Sakakibara et al., 1997). Genistein prevents TJ barrier by inhibition of protein tyrosine kinase. Genistein also protects the increased TJ permeability induced by oxidative stress. Its mechanism is related to the prevention of the disassembly of junctional complex (i.e. occludin, ZO-1 and AJ protein, including E-cadherin). Moreover, the potent polyphenolic compound genistein can protect the disruption of the TJ barrier induced by inflammatory cytokine TNF-α in HT-29/B6 cells and enteric bacteria, *Salmonella typhimurium* and *Escherichia coli* in Caco-2 cells (Schmitz et al., 1999; Wells et al., 1999).

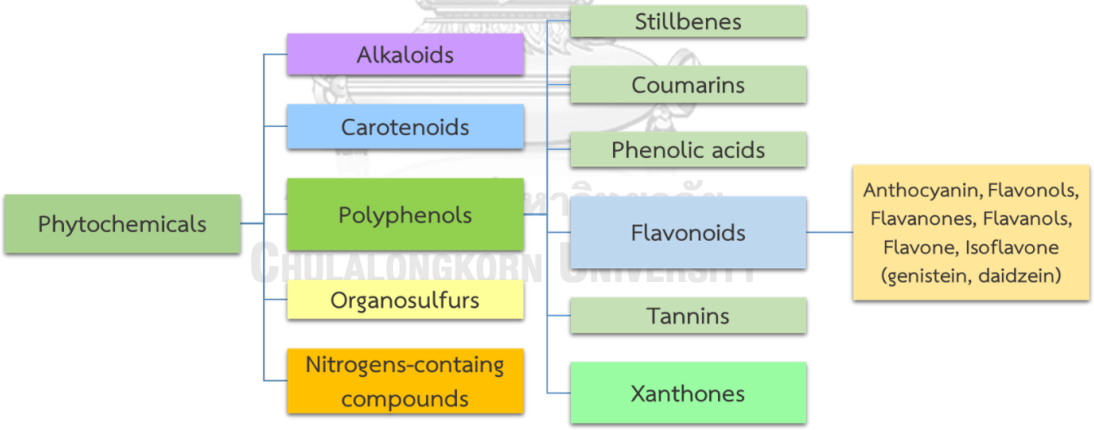


Figure 4 Classification of phytochemical compounds (Kris-Etherton et al., 2002; Ramos et al., 2011)

Recently, xanthones are polyphenolic compounds abundantly found in many plants, especially mangosteen (*Garcinia mangostana*). Suksamrarn and co-workers (2002) firstly isolate xanthones using methanol extract, and identified many types of xanthones (Suksamrarn et al., 2002). Subsequently, several biological activities including anti-oxidant, pro-apoptotic, anti-microbial and anti-inflammatory have been revealed (Suksamrarn et al., 2002; Visioli et al., 2011). In addition, the secondary metabolites which have at least 68 different xanthones in mangosteen, and most of xanthones found are mangostin was indicated (Gutierrez-Orozco and Failla, 2013). The natural xanthones are classified into 6 main groups, simple xanthones, prenylated xanthones, xanthone glycosides, bisxanthones, xanthonolignoids and miscellaneous xanthones. Natural xanthones are usually isolated by column chromatography on silica gel by different solvent such as ethanol, methanol or water to be mobile phase. The structure of xanthones is investigated by many methods. Ultraviolet visible spectroscopy (UV) is helpful for detecting the location of hydroxyl groups. The carbonyl groups can be examined by infrared spectroscopy (IR). Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) is used for identifying the number and position of protons (^{1}H) and carbon nuclear magnetic resonance spectroscopy (^{13}C NMR) is practiced for C atoms. The structure of xanthone glycoside can be detected by mass spectroscopy (MS) (Negi et al., 2013). The specific structure of xanthones are comprised of a core tricyclic aromatic system (C6–C3–C6) which connected with addition side chains (Suksamrarn et al., 2002). While isoprene, hydroxyl and methoxyl groups were located at different locations on the A and B rings with modification of chemical structures and positions of xanthone nucleus substituents different derivatives of xanthones have been identified and shown to have a variety of biological activities. HDXs have been used as traditional medicine for diarrhea in a long time (Pedraza-Chaverri et al., 2008). HDXs are phenolic compound that has activities about

anti-inflammation, which are caused by pro-inflammatory cytokines, such as TNF-α or inflammatory mediator cyclooxygenase-2 (COX-2) (Chen and Chen, 2007). Therefore, it is suspected that HDX may have similar effects as genistein on the inhibition of tyrosine kinase induced by pro-inflammatory cytokine during the LPS contamination. From the previous studies, the structure of HDXs has different effects depending on the number of hydroxyl groups in the structure. The greater numbers of phenolic hydroxyl groups demonstrated to be more effective than the small numbers of phenolic hydroxyl groups (Liu et al., 2006). In the human intestinal epithelium, HDX has anti-secretory effect by inhibiting cAMP-activated Cl^- secretion (Luerang et al., 2012). The synthetic HDXs simulates the effect of mangostin the crude substances isolated from mangosteen. However, the synthetic HDXs has the various numbers and position of hydroxyl groups of HDXs. 1,3-diHDX, 1,3,6-triHDX, 1,3,8-triHDX or 1,3,6,8-tetraHDX has been synthesized and tested for their biological activities (Pedraza-Chaverri et al., 2008). Previous study in the intestinal epithelial cells, T84 cells, only 1,3,6-triHDX (THX-001) was the most efficient for the inhibition of cAMP-activated Cl^- secretion (Luerang et al., 2012). It is of interest to examine a variety of HDX structure, particularly the structure very closely related to isoflavones compound genistein or daidzein which is the potent tyrosine kinase inhibitor. It is possible that HDX may have an ability to inhibit protein tyrosine kinase-induced MLCK pathway during activation of LPS consequence. The inhibitory effects of HDXs may be beneficial to prevent the intestinal barrier from the leakage of epithelia in subclinical infected disease.

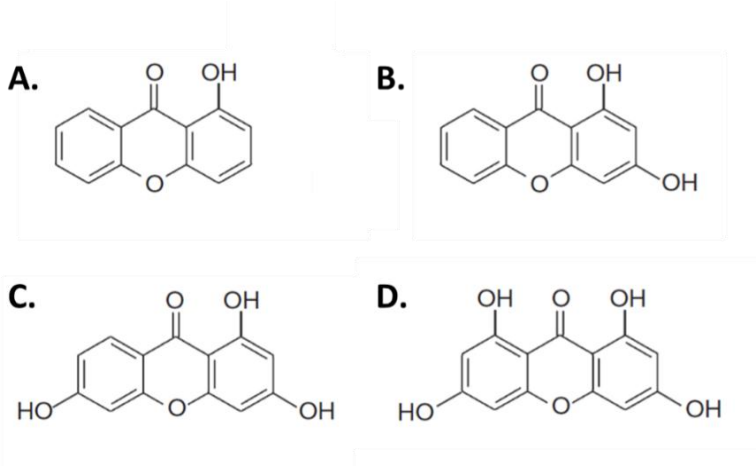


Figure 5 Chemical structures of hydroxyxanthones (A) 1-hydroxyxanthone, (B) 1,3-hydroxyxanthone (DiHDX), (C) 1,3,6-trihydroxyxanthone (TriHDX) and (D) 1,3,6,8-tetrahydroxyxanthone (TetraHDX) (Suksamrarn et al., 2002)

2.7 Caco-2 cells as a model of leaky epithelia

Caco-2 is a monolayer of human colorectal adenocarcinoma cell, and classified as adherent epithelial cell. In additional, the specific condition affects to differentiated and polarized of this cell line (Hidalgo et al., 1989). Caco-2 cells have the specific morphology to differentiate from colonic-like cells to jejunal-like cells at day 14-21 (Engle et al., 1998). The unique functions of the Caco-2 cell are expression of many enzymes, microvilli and transporter proteins, such as peptidases, esterases and P-glycoprotein. Caco-2 cell lines are frequently used for a model of monolayer cells on insert filter. The Caco-2 cell provides the barrier for transportation of ions and macromolecules as usual enterocyte (Artursson, 1990). The Caco-2 monolayer commonly used for *in vitro* model of intestinal mucosa in human to study about drug absorption (Artursson and Karlsson, 1991). Moreover, Caco-2 can be used elsewhere for transepithelial resistant measurement and paracellular permeability test which

assessed apical to basolateral transport by fluorescent probe, such as a fluorescein isothiocyanate dextran probe (FD-4) (Brufau et al., 2016).

To determine the leaky epithelia using the Caco-2 model, the decrease of transepithelial electrical resistance (TER), which is used as an important parameter in endothelial and epithelial cells, was monitored. The TER is normally used for evaluating the transport of substances and performed in ohms resistance measurement. Therefore, the decreased TER does not indicate the disruption of intestinal barrier, allowing macromolecule passing through the paracellular route, but also an increased transcellular ion transport (Ronaghan et al., 2016). Normally, TER of colonic-like and jejunal-like Caco-2 cells are between 150-400 $\Omega.cm^2$ and 600-800 $\Omega.cm^2$ respectively (Artursson et al., 2001). The variations of TER can be happened from many factors, i.e. number of cells, passages, type of medium and temperature that regulate the expression of transporter or TJs (Srinivasan et al., 2015).

Indeed, the leaky epithelia which is defined by the permeability of epithelia macromolecules, the macromolecules conjugated with the reported fluorescence system, fluorescein isothiocyanate-dextran (FITC-dextran, MW = 4kDa; FD-4) has been extremely recommended. FD-4 permeability is used in monolayer cell and permeability such as renal elimination, blood flow, vascular drainage, membrane damage and cell junction permeability (Wang et al., 2015). In the Caco-2 model, an increased FD-4 flux associated with a decreased TER on intestinal barrier is a standard protocol to declare the impairment barrier or TJ damage (Matter and Balda, 2003).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

Transwell microporous filters (12 mm polycarbonate membrane, 0.4 μm pore size), 48, 96-well plate, 60 and 100 mm cell culture dish were obtained from Costar® (Corning, MA, USA). IL-1β was purchased from WardMedic (GenScript, NJ, USA). Primary antibody rabbit-anti-Myosin light chain (phospho S20) antibody was obtained from Biomed (Abcam, CA, USA). Specific anti-β-actin primary antibody, secondary antibody, donkey-anti-rabbit conjugated horseradish peroxidase and chemilumi-nesence ECL were purchased from Santa Cruz Biotechnology, CA, USA. All cell culture medium and supplements, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.05% trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA), kanamycin, penicillin-streptomycin and fungizone were purchased from Gibco (Life Technologies, CA, USA). MTT and para-cellular marker FD-4 (FITC-Dextran; MW=4 kDa) were purchased from Sigma (Sigma-Aldrich, MO, USA). Bicinchoninic acid assay (BCA assay), Laemmli loading buffer and mercaptoethanol were purchased from Biorad (Thermofisher, CA, USA). Transfer polyvinylidene difluoride membranes, Immobilon™ was purchased from Millipore, CA, USA. Amersham™ hyperfilm ECL was obtained from GE Healthcare Life Sciences, WA, USA.

3.2 Culture of Caco-2 cells

Caco-2 cell line (passage no. 26) from the American Type Culture Collection (Manassas, VA, USA) was kindly provided by Professor Narattaphol Charoenphandhu, Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand.

Caco-2 cells were maintained in standard medium containing 10% FBS, 1% non-essential amino acid and 1% penicillin-streptomycin and fungizone. For culturing Caco2-cells, $1X10^6$ cells were plated on 100 mm^2 cell culture dish and incubated in 5% CO_2 at 37°C. Culture medium was changed every 1 to 2 days. The cells were subcultured by 0.25% EDTA trypsin in Ca^{2+}-free and Mg^{2+}-free PBS.

3.3 Chemical treatment

Caco-2 cells were maintained for 7 or 21 days before treatments for a permeability test. At 7 and 21 days, cells were incubated with varies forms of HDXs, 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX and 1,3,6,8-tetraHDX at concentration of 10 and 100 µM, vehicle DMSO and control. These compounds were diluted with an equal volume of DMSO and then in a medium with a final concentration of DMSO of 0.01% (V/V). The cells were treated by DMSO and HDXs for 1 h following by IL-1β 10 ng/ml prior to paracellular permeability measurement and western blot analysis. In this study, duplicate cell monolayers were performed in each passage, and the experiment was conducted at least 5 times.

3.4 Experimental design

Experiment 1: Cytotoxicity effects of HDXs on Caco-2 cell

Cytotoxicity of HDXs were determined first using the MTT assay for finding the appropriate concentration and incubation period of HDXs that do not toxic to Caco-2 cells. The cells were cultured in 48-well plate with $5X10^3$ cells/well for 7 or 21 days. Subsequently, the 1, 10, 100 or 1000 µM of 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX or 1,3,6,8-tetraHDX were added in cell culture medium and incubated for 24 or 48 h.

DMSO at the same dissolved volume of HDXs were also performed in every trial as a control. Following treatment, the MTT 5 mg/ml which was diluted in medium, was added at 125 µl/well and incubated in 5% CO_2 at 37°C for 3 h. After removing the exceed MTT supernatant, cells were washed with PBS 200 µl/ well, and the intracellular formazan crystal was dissolved with 100 µl/ well of DMSO in 5% CO_2 at 37°C for 45 mins. The formasan solution was transferred to 96-well plate for optical density (OD) measurement. The concentration of formazan crystal was detected by spectrophotometer at the specific wavelength 570 mm and non-specific wavelength 620 nm using the Microplate reader (Epoch, BioTek, VM, USA). The OD unit of HDXs at 570 nm minus with 620 nm was calculated for cytotoxicity analysis compared with vehicle DMSO.

Experiment 2: The protective effect of HDXs on Caco-2 cell permeability induced by IL-1β

2.1 The protective effect of HDXs on the transepithelial electrical resistance (TER) of Caco-2 induced by IL-1β

Caco-2 cells ($5X10^4$ cells/well) were plated in Transwell microporous filters and maintained for 7 or 21 days. After that the 7-day or 21-day cultured cells were treated with 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX at 10 and 100 µM or DMSO. After 1 h of HDXs treatment, the cells were challenged with either IL-1β 10 ng/ml or IL-1β+HDXs. TER was measured at 0, 12, 24 and 48 h with an epithelial volt-ohmmeter (World precision, FL, USA). The measurements were duplicated in the same filter. The TER range of 400-1000 Ωcm^2 was accepted for permeability test. The percentage of TER change from the initial TER (before drug added) was calculated and reported using the equation (1):

$$\% \text{ changes of TER} = \frac{(\text{TER after treatment} - \text{TER before treatment})}{\text{TER before treatment}} \times 100 \quad \text{.....(1)}$$

2.2 The protective effect of HDXs on the macromolecule permeability of Caco-2 cell induced by IL-1β

Paracellular permeability measurement was accessed by para-cellular marker FD-4 on the each of TER measurements. Before loading the FD-4, HBSS buffer solution containing 25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, 15 mM glucose, pH 7.4 at the volume of 1.0 ml was added into the basolateral side. FD-4 at 1 mg/ml, 1 ml was added into the apical side. After incubation for 12, 24 and 48 h at 37°C in the dark chamber, 100 µl of apical and basolateral samples were taken and transferred to 96-well microplate for determining fluorescence intensity using fluorescent microplate reader (Synergy™, BioTek, VM, USA). The excitation wavelength of 485 nm and emission wavelength of 530 nm was applied. The amount of FD-4 into the basolateral side was calculated from a standard curve. The apical-to-basolateral transport of FD-4 at 12, 24 and 48 h after IL-1β treatment was reported as the apparent permeability coefficient (P_{app}; $h^{-1}.cm^{-2}$). P_{app} was the number reflecting the transportation of FD-4 from the apical side of basolateral side, which was calculated according to the equation (2):

$$P_{app}\ (h^{-1}.\ cm^{-2}) = \frac{\text{FD-4 at basolateral side (ng/ml) / FD-4 at apical side (ng/ml)}}{\text{Time of incubation (h)} \times 1/\ \text{Filter area } (mm^2)} \quad \text{.....(2)}$$

Experiment 3: The protective of HDXs on p-MLC expression of Caco-2 cell induced by IL-1β.

The p-MLC expression was detected by semi-quantitative Western blot analysis**.** Total protein was performed at 15 mins and 24 h that affected to MLCK expression from a preliminary study, decreasing TER and increasing permeability. Cell lysate was added in 60 mm plate at 7 days and treated by IL-1β, HDXs at the same period with permeability test. The cell lysate was lysed in ice-cold lysis buffer containing 46.5 mM Tris, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1% NP40, 1 mM NaF, 1 mM PMSF, 6.02 mM Na deoxycholate and 0.01 mg/ml protease inhibitors. After incubation on ice for 30 mins, the cell lysate was centrifuged at 12,000 g for 15 min at 4°C. The detergent-soluble fraction was transferred to a microcentrifuge tube. The protein samples were stored at -20°C until used. To determine the protein quantity and quality, all protein samples were measured using a BCA assay according to the manufacturer's instructions using bovine serum albumin as standard. The BCA reagents were mixed and incubated at room temperature without light 30 mins. All standards, protein sample 5 µl and BCA reagents 100 µl were added in 96-well plate incubating at 37°C in the dark chamber. The OD unit was detected by Microplate reader at the wavelength 570 nm and 620 nm to obtain protein concentration from a standard curve.

Laemmli loading buffer and mercaptoethanol mixture were added with 30 µg of protein samples and boiled at 95°C for 2 mins. For separation of the interesting protein, equal amounts of proteins (30 µg) from each sample was loaded into 10% dodecyl sulfate (SDS)-polyacrylamide gel (PAGE) and separated by electrophoresis at 200 V 45 mins. The separated protein was transferred to Immobilon™ transfer polyvinylidene difluoride membranes. The blot was blocked with 3% BSA in Tris-

Tween for 1 h, and the primary antibody, rabbit- anti-myosin light chain (Phospho S20) antibody at the dilution of 1:2000 was incubated sequentially overnight at 4°C. Subsequently, an appropriated horseradish peroxidase-conjugated secondary antibody, donkey-anti-rabbit at a dilution of 1:2000 was incubated for 1 h and developed using the chemiluminesence ECL plus.

In this experiment, detection of structural protein β-actin using specific anti-β-actin primary antibody was performed as an internal standard for protein loading. The luminescence products were exposed for 8 mins on Amersham™ hyperfilm ECL in a dark room. The intensity of protein bands on ECL film was scanned in the ratio 1:1 by the scanner (Epson, Nagano, Japan) and quantified using densitometry analysis by Scion Image Software 4.0.3.2 (NIH, Bethesda, Maryland). The band ratio of p-MLC/β-actin was expressed for each experiment, and molecular weight of p-MLC compared with protein standard marker at 37 kDa.

3.5 Statistical analysis

Data were reported as means±SEM from at least 5 experiments using the different passages of Caco-2 cells. Statistical analysis was performed by two-way analysis of variance for the influence of two different factors, duration and a variety of drug treatment. When p value was < 0.05 on ANOVA, the difference between groups was assessed by Bonfferoni post hoc-test. The GraphPad Prism program (Prism 5.0, GraphPad Software, Inc., San Diego, CA, USA) was used to indicate statistical differences.

CHAPTER IV
RESULTS

4.1 Standardization and optimization of methods for the cultivation of Caco-2 cells to colonic-like cells and jejunal-like cells

In the present study, we studied the preventive effects of HDXs on the leaky epithelia of small intestine and large intestine using the model of Caco-2 cells. To generate two subpopulations of intestinal epithelia, Caco-2 cells were plated and cultured in standard medium for 7 and 21 days according to the protocol of Natoli and colleagues (Natoli et al., 2012). Under the phase contrast microscopy, the Caco-2 cells grown for 7 days (Fig. 6A; 7-day cells) had the morphology different from Caco-2 cells grown for 21 days (Fig. 6B; 21-day cells). Briefly, 7-day cells which have been previously indicated as the colonic-like cells had the cell number lesser than the 21-day cells which were differentiated to jejunal-like cells. The 7-day cells organized in a square shape were larger than 21-day cells. The borders of 7-day colonic-like cells were clearly identified; however, their nucleus was hardly identified. In contrast, the cell borders of 21-day jejunal like-cells could not be located, but the nucleus of cells was disclosed (Fig. 6B). These two different subpopulations seemed to be generated by time-courses in the present study. The specific characteristic of transepithelial electrical resistance (TER) and paracellular permeability of macromolecules in the colonic and jejunal-like cells was to be confirmed.

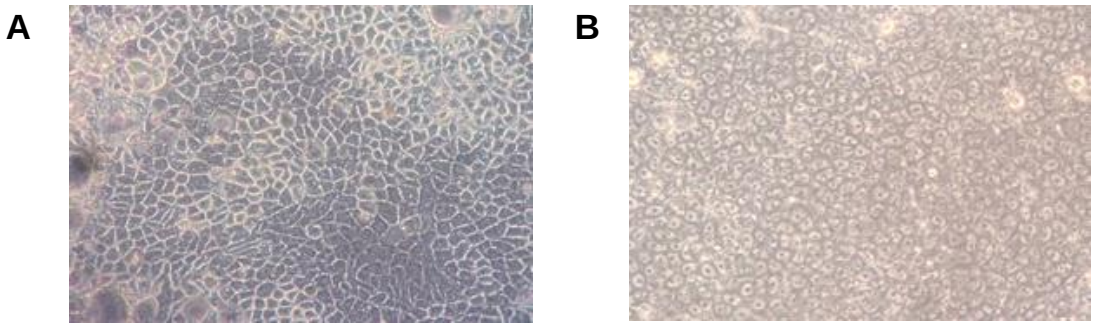


Figure 6 Morphological characteristics of Caco-2 cells at two different time courses. (A) 7 days and (B) 21 days in culture. Caco-2 cells were cultured in the standard media for 7 days or 21 days. Phase contrast micrographs of representative Caco-2 cells (n > 5 passages) at the magnification 100X are shown.

4.2 Determination of cytotoxicity of HDXs on the Caco-2 cells

The cytotoxicity test of HDXs at two different concentrations, 10 or 100 μM, on the viability of Caco-2 cells during 24-48 h of treatment was determined. The results showed that all treatments with HDXs, with an exception of 1,3,6,8-tetraHDX 100 μM, had no cytotoxic to 7-day colonic-like cells compared to the corresponding DMSO control (Fig. 7A). Treatment with 1,3,6,8-tetraHDX 100 μM for 24 h appeared to decrease the viability of 7-day cells by 30% (Table 1), but the extended treatment for 48 h significantly revealed the cell toxicity ($p<0.05$). In contrast, all treatments of the 21-day cells with HDXs had no cytotoxicity effect (Fig. 7B). Accordingly, 10 and 100 μM of 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX were suitable for further study of its preventive action on IL-1β mediated the leakiness of tight junctions.

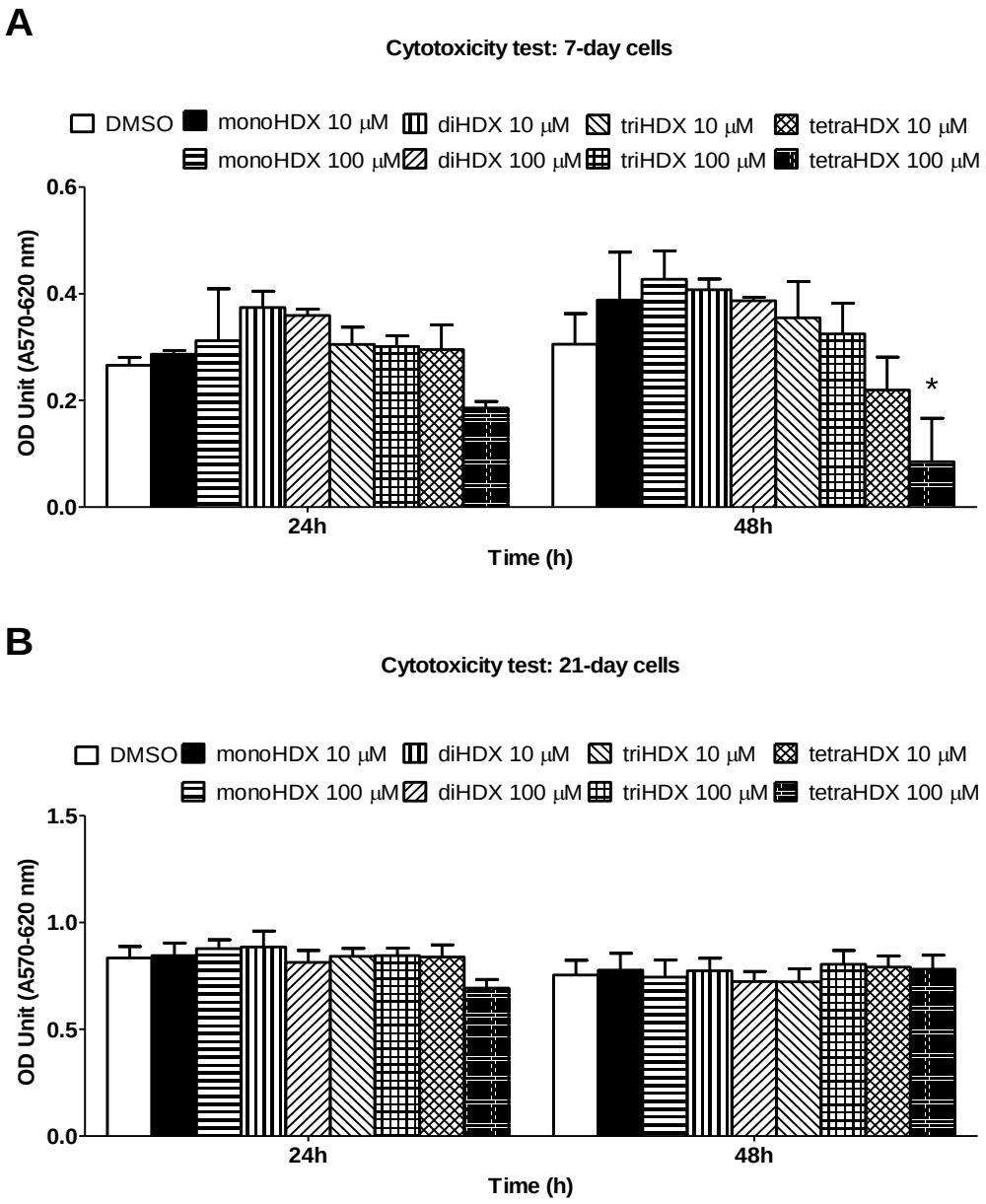


Figure 7 The cytotoxicity test of HDXs on the viability of (A) 7-day and (B) 21-day Caco-2 cells at 24 and 48 h. 7-day or 21-day cells cultured in the standard media were incubated with 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX or 1,3,6,8 tetraHDX (10-100 μM) for 24 and 48 h followed by MTT assay. Each value represents mean ± SEM (n=6 experiments) of $OD_{(570-620)}$ unit of samples. * is a significant difference from vehicle control (DMSO) at a value of $p<0.05$ by two-way ANOVA and Bonferroni post-hoc test.

Table 1 Percent changes of cell viability from control (DMSO) of 7-day and 21-day Caco-2 cells treated with HDXs for 24 and 48 h. Cells cultured in the standard media for 7 or 21 days cells were treated with DMSO, 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX or 1,3,6,8 tetraHDX for 24 and 48 h followed by MTT assay.

Treatment	7-day cells		21-day cells	
	24 h	48 h	24 h	48 h
monoHDX 10 μM	7.75±2.55	34.36±22.19	1.44±7.07	2.98±10.44
monoHDX 100 μM	17.35±36.56	51.57±19.11	5.34±10.49	-1.26±10.49
diHDX 10 μM	40.69±11.57	43.89±16.84	6.18±9.06	2.62±7.68
diHDX 100 μM	35.13±4.46	35.85±6.70	-2.46±6.67	-3.99±6.14
triHDX 10 μM	14.74±12.09	29.94±28.94	1.05±4.38	-4.23±8.12
triHDX 100 μM	13.21±7.65	18.75±24.95	1.37±4.21	6.67±8.49
tetraHDX 10 μM	11.10±17.39	-21.21±26.99	0.54±6.84	4.95±6.97
tetraHDX 100 μM	-30.11±4.57	-72.14±26.73*	-16.93±4.85	3.61±8.74

Each value represents (mean ± SEM of the $OD_{(570-620)}$ unit from the MTT assay

* is a significant difference from vehicle control (DMSO) at a value of $p<0.05$ at the same time point by two-way ANOVA and Bonferroni post-hoc test. The experiments were performed duplicate from n=6 experiments.

4.3 Effects of HDXs on the transepithelial electrical resistance (TER) of Caco-2 cells

The effects of HDXs on 7-day or 21-day Caco-2 cells at 12, 24 and 48 h on TER were shown in Fig. 8A and B, respectively. Percent changes of TER from the initial following treatment with 1-monoHDX, 1,3-diHDX or 1,3,6-triHDX were compared with control (DMSO) at each time point.

In the 7-day culture colonic-like cells, 1-monoHDX or 1,3-diHDX at 10 μM significantly increased TER greater than 300% after incubation for 12 h ($p<0.01$; Fig. 8A). However, longer treatment period of both HDXs for 24-48 h did not significantly alter TER as compared to DMSO control at the same time point ($p>0.05$; Fig. 8A). Likewise, treatment of 21-day cells with 1-monoHDX at 100 μM up to 48 h did not affect the TER. In contrast, 1,3-diHDX at 100 μM significantly decreased the TER of 21-day cells at 24 and 48 h after treatment, and 1,3,6-triHDX at 100 μM produced a marked decrease in TER which was observed as early as 12 h and continued up to 48 h (Fig. 8B). A decreased TER in response to specific HDXs treatment could be due to an impairment of the TJ barrier or a stimulation of transcellular ion transport. Thus, 1, 3-diHDX and 1, 3, 6-triHDX at 10 μM should be considered for testing in the next experiment.

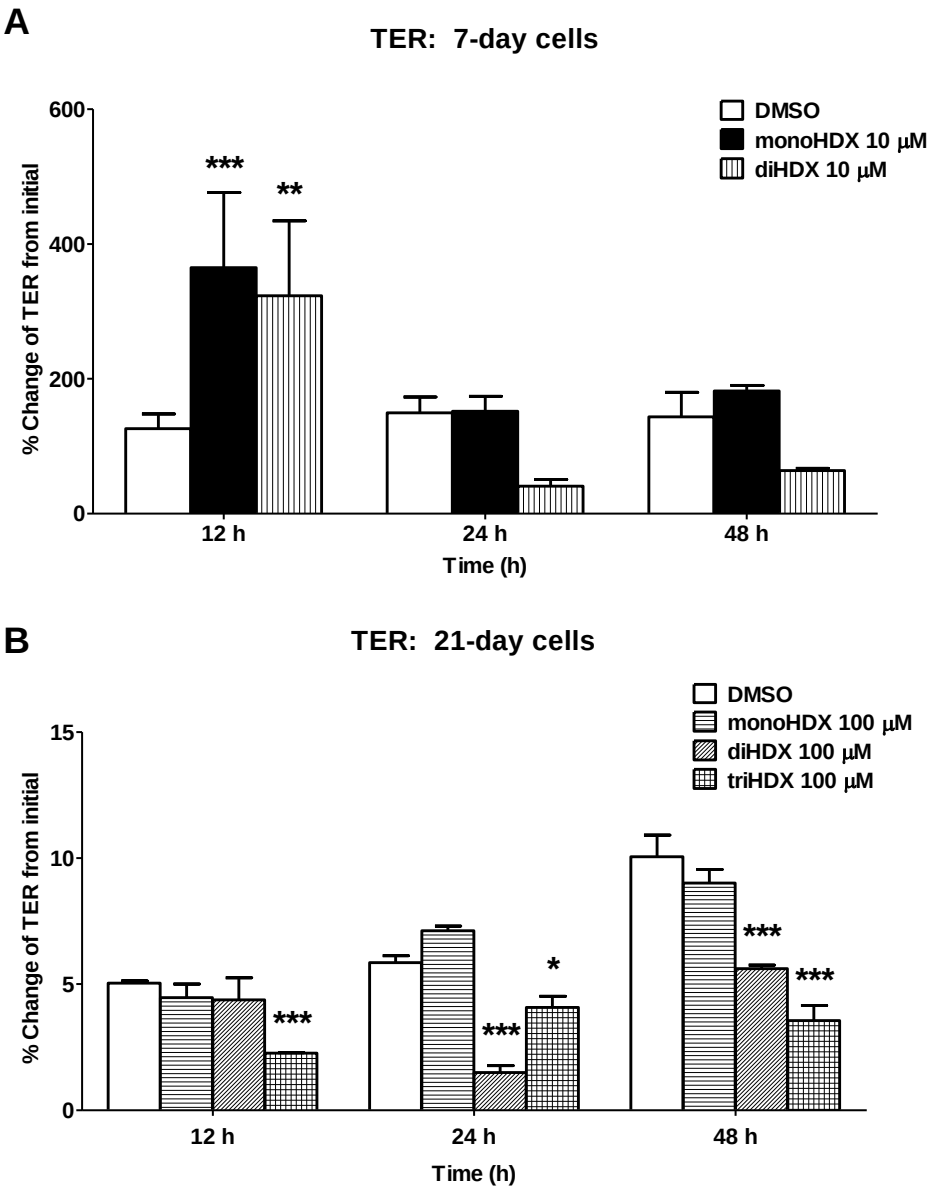


Figure 8 Effects of HDXs treatment on the transepithelial electrical resistance (TER) of (A) 7-day and (B) 21-day Caco-2 cells at 12-48 h. Caco-2 cells cultured in transwell microporous with the standard media were incubated with DMSO, 1-monoHDX, 1,3-diHDX or 1,3,6-triHDX at indicated concentrations for 12-48 h. Bar graph represents mean ± SEM of percent changes of TER from initial (n=10 experiments). *, ** or *** is a significant difference from vehicle control (DMSO) at the respective value of *p<0.05, 0.01 or 0.001* by two-way ANOVA and Bonferroni post-hoc test.

4.4 The preventive effect of HDXs on the decreased transepithelial electrical resistance induced by IL-1β

The effects of HDXs on the TER in the presence of IL-1β on 7-day and 21-day Caco-2 cells at 12, 24 or 48 h of incubation was revealed in Fig. 9. Exposure to IL-1β (10 ng/ml) alone was found only to decrease TER of 7-day cells after treatment for 24 h ($p<0.05$; Fig. 9A). When IL-1β was co-incubated with either 1-monoHDX or 1, 3-diHDX at 10 μM, the TER of 7-day cells was markedly increased by twofold after 12 h treatment period. In addition, these HDXs was shown to inhibit the IL-1β effect at 24 h ($p<0.01$; Fig. 9A).

For the cells cultured for 21 days, IL-1β alone had no significant effect on the percent change of TER at any time point as compared to vehicle DMSO control. Treatment with 1-monoHDX, but not 1,3-diHDX or 1,3,8-triHDX, in the presence of IL-1β significantly increased the TER ($p<0.05$; Fig. 9B). However, exposure of IL-1β alone or in the presence of HDXs had no effect on TER changes at 48 h treatment.

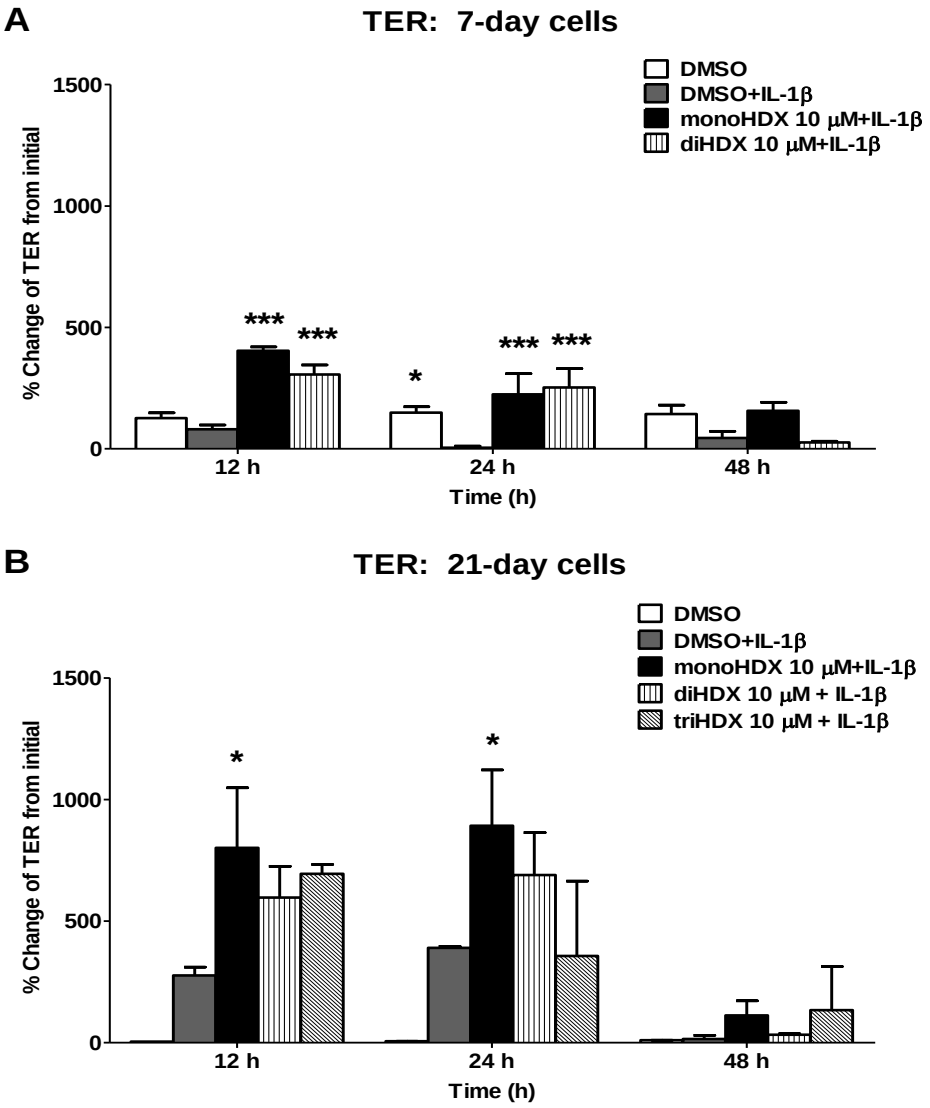


Figure 9 Effects of HDXs treatment on the transepithelial electrical resistance (TER) in response to IL-1β of (A) 7-day and (B) 21-day Caco-2 cells at 12-48 h. Caco-2 cells cultured in transwell microporous with the standard media were incubated with 10 μM of 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX or DMSO for 12-48 h in the presence of IL-1β 10 ng/ml. Bar graph represents mean ± SEM of percent changes of TER from initial (n=5 experiments). *, ** or *** is a significant difference from DMSO+IL-1β group at a value of *p<0.05, 0.01 or 0.001*, respectively by two-way ANOVA and Bonferroni post-hoc test.

4.5 Effects of HDXs on the intestinal permeability to macromolecules of Caco-2 cells

The effects of HDXs treatment for 12-48 h on the intestinal permeability were performed using FD-4 macromolecules in both 7- and 21-day Caco-2 cell culture. The results showed that the permeability of FD-4 was increased depending on time ($p<0.0001$). However, none of HDXs treatment did not significantly alter the permeability to FD-4 in Caco-2 cells at any time point ($p>0.05$; Fig. 10A, B).

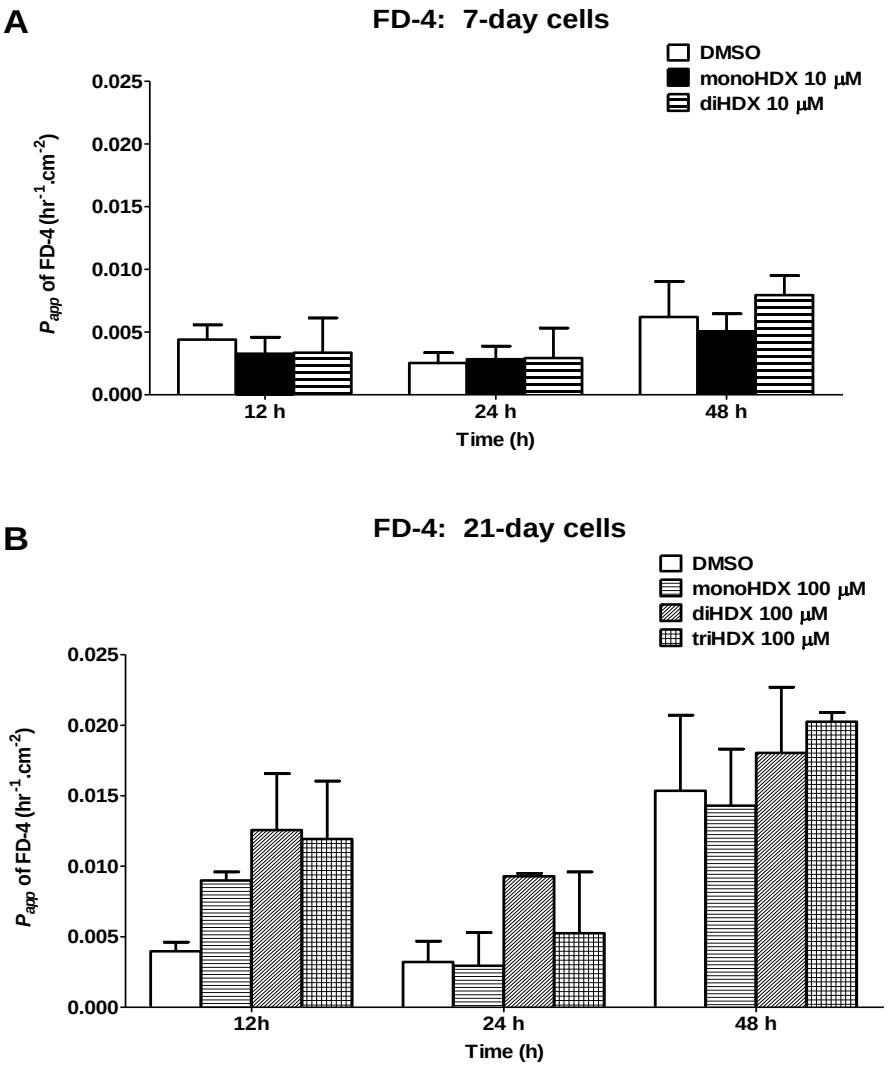


Figure 10 Effects of HDXs treatment on the intestinal permeability to macromolecules of (A) 7-day and (B) 21-day Caco-2 cells at 12-48 h. Caco-2 cells cultured in transwell microporous with the standard media were incubated with HDXs; 1-monoHDX, 1,3-diHDX or 1,3,6-triHDX at indicated concentrations for 12-48 h. Bar graph represents mean ± SEM (n=5 experiments) of apical to the basolateral flux of FD-4 (P_{app}, $h^{-1}.cm^{-2}$) at 12, 24 or 48 h of cell monolayer by fluorometry. The results were analyzed by two-way ANOVA and Bonferroni post-hoc test compared with vehicle control (DMSO).

4.6 The preventive effect of HDXs on the increased intestinal permeability to macromolecules of Caco-2 cells induced by IL-1β

According to the previous results, IL-1β (10 ng/ml) at specific treatment period decreased the TER of Caco-2, the permeability test to macromolecules were also performed laterally in the same monolayer cultured for 7 or 21 days to assess the leakiness of TJs barriers. As shown in figure 11A, the 7-day culture cells exposed to IL-1β alone produced about 2.5-3 folds increases in permeability to FD-4 which was observed in 12-24 h ($p<0.001$). When IL-1β was co-incubated with either 1-monoHDX or 1,3-diHDX at 10 μM, the paracellular permeability of 7-days cells was significantly decreased after 12 and 24 h. However, no change in the permeability test was evident with any treatments at 48 h of 7-day cells and all periods of 21-day cells. (Fig. 11A, B).

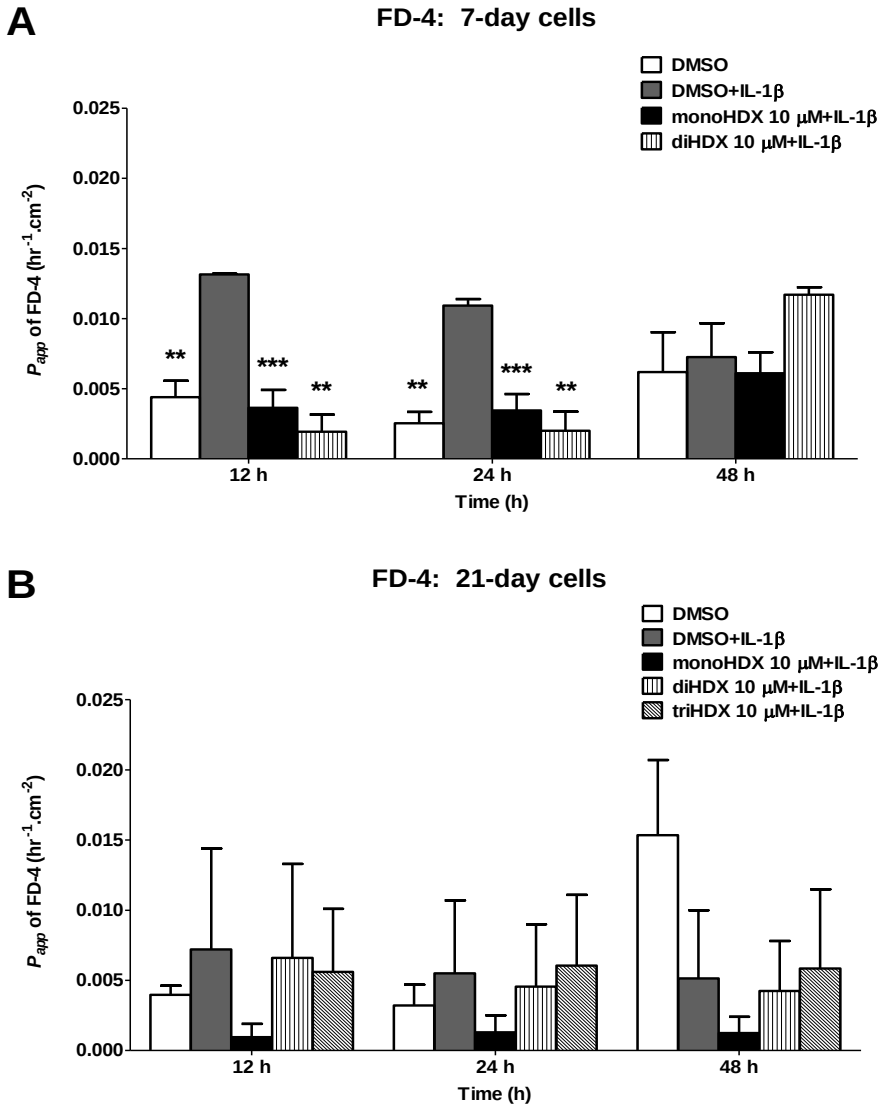


Figure 11 Effects of HDXs treatment on the IL-1β-induced the intestinal permeability to FD-4 of (A) 7-day and (B) 21-day Caco-2 cells at 12-48 h. Caco-2 cells cultured in transwell microporous with the standard media were incubated with 10 μM of 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX or DMSO in the presence of IL-1β (10 ng/ml) for 12-48 h. Bar graph represents mean ± SEM (n=5 experiments) of apical to the basolateral flux of FD-4 (P_{app}, $h^{-1}.cm^{-2}$) at 12, 24 or 48 h by fluorometry. *, ** or *** is a significant difference from DMSO+IL-1β group at a value of *p<0.05, 0.01 or 0.001*, respectively, by two-way ANOVA and Bonferroni post-hoc test.

4.7 Effects of HDXs on the expression of phosphorylated myosin light chain (p-MLC) in 7-day Caco-2-cells

Activation of MLCK associated with increased expression of p-MLC has been demonstrated to be a target of IL-1β. The inhibition of p-MLC expression level is a critical consideration for the treatment of leaky epithelia. In order to determine the mechanism of HDXs in the prevention of IL-1β induced increase in the permeability to macromolecules, the expression of p-MLC protein was determined by semi-quantitative Western blot analysis. The effect of 1-monoHDX or 1,3-diHDX 10 μM was first determined at 15 mins and 24 h in the 7-day cells. Western blot analyses using the rabbit- anti-myosin light chain (phospho S20) antibody detected a protein band with a molecular weight of 37 kDa. Treatment with 1-monoHDX or 1, 3-diHDX at 10 μM in a fresh media for 15 mins or 24 h did not affect the expression of p-MLC when compared with the control DMSO treated cells (Fig. 12).

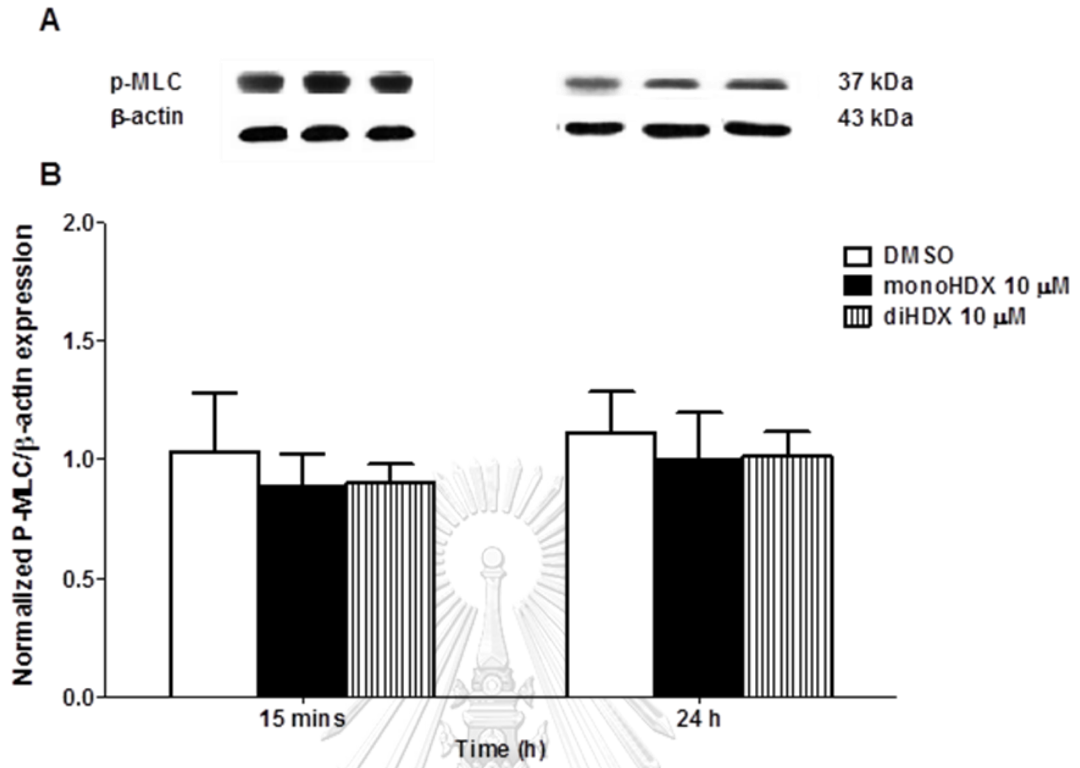


Figure 12 Effects of HDXs on the expression of phosphorylated myosin light chain (p-MLC) in 7-day Caco-2 cells. Total protein was extracted from cells cultured in the standard media in the presence of 1-monoHDX, 1,3-diHDX or DMSO for 15 mins or 24 h. (A) Representative protein bands of p-MLC compared to housekeeping β-actin was performed and revealed by semi-quantitative Western blot analysis. (B) Bar graph represents mean ± SEM (n=4 experiments) of the densitometry analysis of the p-MLC normalized to β-actin protein. The data showed no difference from DMSO as analyzed by two-way ANOVA followed by Bonferroni post-hoc test.

4.8 Effects of HDXs on the IL-1β-induced increase in phosphorylated myosin light chain (p-MLC) expression in 7-day Caco-2-cells

According to the previous results, 1-monoHDX or 1,3-diHDX treatments without IL-1β activation did not change paracellular permeability and p-MLC protein expression. In order to confirm the mechanism of HDXs in impairment barrier induced by IL-1β, the p-MLC protein expression was evaluated by semi-quantitative Western blot analysis. The effect of 1-monoHDX or 1,3-diHDX 10 μM on the IL-1β induced condition was determined at 15 mins and 24 h in 7-day cells. IL-1β alone induced p-MLC protein expression by two times compared with DMSO at 24 h of 7-day cells. When co-incubation between IL-1β and 1-monoHDX or 1,3-diHDX at 10 μM was done, p-MLC protein expression was significantly decreased after 24 h. However, 1-monoHDX or 1,3-diHDX did not change p-MLC protein expression in IL-1β treatment at 15 mins (Fig. 13).

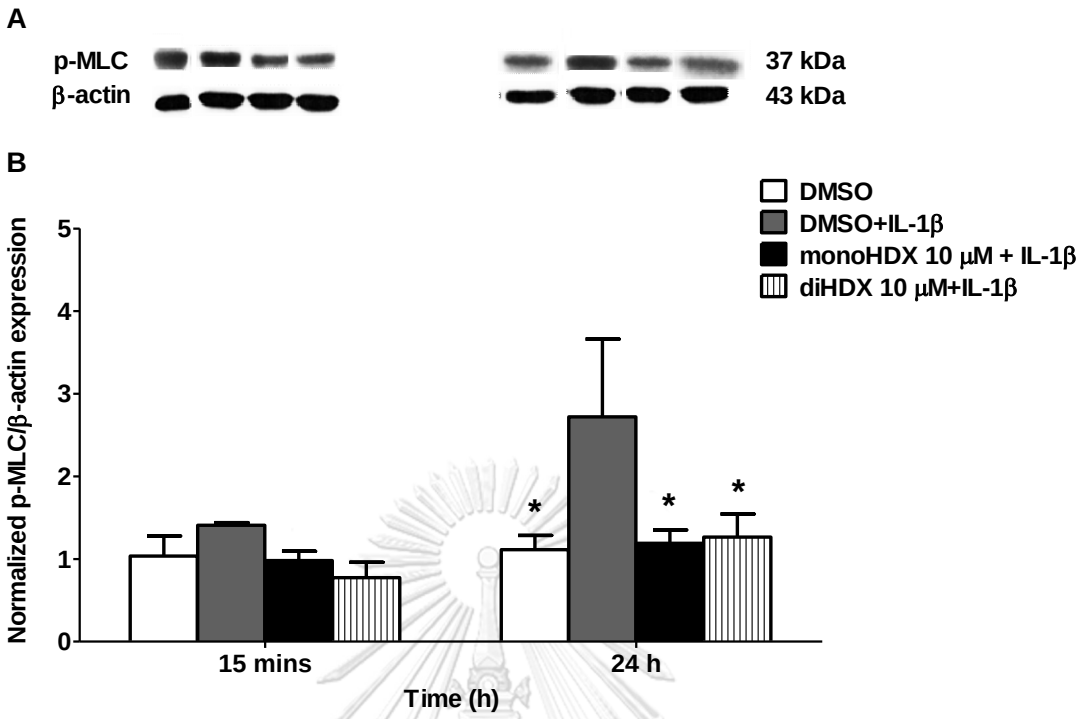


Figure 13 Effects of HDXs on IL-1β induced the expression of p-MLC in 7-day Caco-2 cells. Total protein was extracted from cells cultured in the standard media with 10 μM of 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX or DMSO in the presence of IL-1β (10 ng/ml) for 15 mins or 24 h. (A) Representative protein bands of p-MLC compared to β-actin was performed and revealed by semi-quantitative Western blot analysis. (B) Bar graph represents mean ± SEM (n=4 experiments) of the densitometry analysis of the p-MLC normalized to β-actin protein. * is a significant difference from DMSO+IL-1β group at a value of *p<0.05* by two-way ANOVA and Bonferroni post-hoc test.

CHAPTER V
DISCUSSION

Intestinal epithelial barrier, especially TJ, has the function to protect pathogen and toxin invading to the body and the circulatory system (Awad et al., 2015). The disruption of the TJ barrier without cell damages is predominantly induced by pro-inflammatory cytokines, IL-β and TNF-α. These cytokines have been indicated as a significant underlying mechanism of chronic inflammation, malabsorption and the fatal diseases, i.e. septicemia and septic shock (Awad et al., 2015). The characteristics of TJ barrier disruption could be detected by a decrease in TER associated with an increase in epithelial permeability to macromolecules. One of the underlying mechanisms has been suggested to be stimulated by p-MLC in MLCK pathway (Al-Sadi et al., 2011). Recently, the crude extraction from mangosteen tropical fruit skin and its synthetic ingredients, particularly HDXs, have been used for treatment of diarrhea in tradition medicine (Pedraza-Chaverri et al., 2008). The different forms of synthetic HDXs has been shown to inhibit cAMP-activated Cl^- secretion inducing by cholera toxin (Luerang et al., 2012). In the present study, the possibility of the various forms of the synthetic HDXs on the inhibition of the disruption of TJ barrier induced by IL-1β was evaluated. Since the principal target of IL-1β was the activation of MLCK pathway, the action of the potent form of HDXs on reversibility of the IL-1β effect was emphasized.

5.1 Standardization and optimization of methods for the cultivation of Caco-2 cells to colonic-like cells and jejunal-like cells

Since our study aimed to examine the action of HDXs on the TJs barrier function of overall intestinal epithelia, Caco-2 cell model was used in the present study as the morphology of cells can be differentiated from colonic-like cells to

jejunal-like cells under specific condition (Engle et al., 1998). In the first part of experiment, the cultivation of colonic and jejunal like cells were established and standardized. Time-course cultivation of Caco-2 cell for 7 and 21 days were performed following the protocol of Natoli and coworkers (Natoli et al., 2012). In consistent with the previous study, the 7-day and 21-day cells cultured in the standard medium with 10%FBS in the present study had the same characteristics as shown in the results of Natoli and co-workers (Natoli et al., 2012). In general, at day 12-14 the cells start to differentiate and express microvilli in some area, and the cells are completed differentiated and form constant barrier between apical and basolateral side at day 18-21 (Engle et al., 1998). Therefore, cultivation of Caco-2 cells for 21 days is used to generate the jejunal-like cells in this study. In addition to classify two intestinal cell populations by the cell morphology, the cell function like TER of each population was further validated. The 7-day cells (TER=300-400 Ω.cm^2) had not only the smaller numbers of cells, but also had the lower TER than those of 21-day cells (TER=700-800 Ω.cm^2). The higher TER in 21-day cells was in agreement with well development of TJ protein in the 21-day differentiated Caco-2 cells (Natoli et al., 2012).

In addition, the permeability of macromolecules FD-4 was evaluated along with TER measurement

5.2 Determination of cytotoxicity of HDXs on the Caco-2 cells

The cytotoxicity of 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX and 1,3,6,8-tetraHDX on Caco-2 cells as assessed by the viability test MTT assay showed that all of these compounds, except 1,3,6,8-tetraHDX, at 10 and 100 μM were not toxic to both Caco-2 cell populations when treated for 24 and 48 h. However, treatment with 1,3,6,8-

tetraHDX at 100 μM was toxic to 7-day cells but not 21-day cells (Fig. 7, Table 1). The different results between 7- and 21-day culture cells could be explained by differences in cell number, intestinal barrier, intestinal surfactants, and protease enzymes (Engle et al., 1998). The finding that the intestinal barrier at 21 days are stronger than 7 days (Engle et al., 1998) may be the reason why the cells at 21 days can tolerate to high dose of HDXs than 7 days.

It is noted that only tetraHDXs which contains more hydroxyl group than the others HDXs used in the present study revealed cytotoxicity effect. It is possible that different position and number of hydroxyl groups of HDXs may be associated with the cell viability. It has been previously indicated that the number of hydroxyl groups of xanthones is correlated with the inhibitory effect of xanthones on the growth of many cell lines including human colon cancer DLD-1 (Matsumoto et al., 2005). Since the biological activities of xanthones are based on the various types and position of substituents on the core ring, the hydroxyl group at the C8 position are postulated to relate with anti-tumor activity. In addition, the hydroxyl group at the C6 position has been indicated to be crucial role for inhibiting growth of endometrial cancer cell lines HL60 cells (Bak, 2011). In the present study, the HDXs carrying hydroxyl group at the C6 position, 1,3,6-triHDX, revealed a substantial decrease, but not significant differences, in viability of 7-day cells at 48 h. The mechanism of anti-proliferation of 1,3,6-triHDX could not be reveal in this study because Caco-2 cells may reach confluence in 96-well plate prior to observation at 24 h. On the other hand, the anti-tumor activity of the C8 position carrying by 1,3,6,8-tetraHDX, causing the mitochondrial dysfunction and leading to apoptosis can be detected in the cell confluent (Matsumoto et al., 2005). Likely, 1,3,6,8-tetraHDX at high dose may induce cell toxicity in the similar manner, since it carries two hydroxyl groups at the critical

position, C6 and C8 in its structure. Although Caco-2 cells represents a good model for intestinal epithelial model, however it is a carcinoma cell which may be a target of the anti-tumor compound including the HDXs. As aforementioned ahead, the non-cytotoxic forms of HDXs, 1-monoHDX, 1,3-diHDX, 1,3,6-triHDX at the 10 or 100 μM were chosen for further experiments.

5.3 Effects of HDXs on the transepithelial electrical resistance (TER) related to the intestinal permeability to macromolecules in Caco-2 cells

In the current study, HDXs 10 and 100 μM were chosen for studying their effect on TJ barrier function and permeability by measurement of TER and paracellular permeability to FD4. TER measurement by volt-ohmmeter before and after treatment with HDXs for 12, 24 and 48 h were reported as percent changes from initial before drug application in order to normalize variations among the cell passages. In the absence of IL-1β representing the normal condition, 1-monoHDX or 1, 3-diHDX at 10 μM markedly increased the TER within 12 h by approximately two folds in 7-day cells, indicating its promoting effect on the epithelial barrier function in the colonic-like cells. However, there was no changes in the FD-4 flux in the corresponding 7-day cells compared to DMSO (Fig. 10A). This finding was not surprising because the paracellular transport of FD4 under untreated condition is minimal such that the tighter monolayer has no further decrease in the permeability to FD4. Indeed, TJ barrier of normal epithelia do not even allow the macromolecule passing, excepting in the leaky epithelia where the macromolecule can pass to the basolateral side (Ma et al., 2004).

The underlying mechanism of HDXs on promoting the epithelial barrier function has not yet explained. Other members of phenolic compounds, such as flavonoids or catechins, has been demonstrated to promote the TJs barrier by

increasing the expression of TJ barrier proteins, i.e. claudin-4, occludin and ZO-1 in Caco-2 cells (Noda et al., 2012; Park et al., 2015). The signaling pathway of catechins on enhancement of mRNA and protein expression of TJ proteins in Caco-2 cells is mediated by AMPK pathway (Park et al., 2015). The increased protein expression by those phenolic compounds is revealed when incubated over 24 h. Since HDXS were found to increase the TER within 12 h and subsequently returned to baseline value, the mechanism is unlikely genomic effects on TJs-related protein expression. Since, HDXs have the structure similar to genistein which has been shown to inhibit tyrosine kinase, reduce inflammation as well as promote colonic TJ barrier function by increasing TER and decreasing permeability associated with TLR4/NF-**κ**B on Caco-2 cells (Bektic et al., 2005; Zhang et al., 2017). It is intriguing whether HDXs have the similar inhibitory action on the TLR4/NF-**κ**B on Caco-2 cells, such as the inhibition of phosphorylation of myosin at actomyosin complex via MLCK pathway.

In contrast, 1, 3-diHDX and 1, 3, 6-triHDX, but not 1-monoHDX, at 100 μM decreased TER in 21-day cells at 12-48 h (Fig. 8B). None of HDXs decreased the TER in 7-day cells which is likely due to lower concentration of HDXs used (10 μM). In general, the decreased TER is partly related to TJ barrier disruption allowing macromolecule, pathogen or endotoxin in lumen penetrating into the circulatory system (Ronaghan et al., 2016). In theory, changes in TER was not associated only with paracellular transport, the decreased TER respond to HDX may be caused by the transcellular transport in the tight epithelia 21-day cells (Collington et al., 1998). However, the transcellular transport reflecting by TER should be very rapid. However, In the study of the permeability of FD-4 macromolecules, the corresponding HDXs-treated 21-day cells had high numbers of FD-4 flux. Although the changes of TER by HDXs treatment was not statistically significant from control (Fig. 10B). Therefore, in order to avoid the

cytotoxic effects and stimulation of ion transport, both transcellular and paracellular routes, 1-monoHDX and 1, 3-diHDX 10 μM were chosen for TER and permeability test on 7 days-old Caco-2 cells (Fig 8A).

5.4 The preventive effect of HDXs on the decreased TER related to the intestinal permeability to macromolecules induced by IL-1β in Caco-2 cells

Previous study in Caco-2 cells has shown that IL-1β disrupts barrier function by decreasing TER and increasing paracellular permeability to macromolecules in both colonic- and jejunal-like cells (Al-Sadi and Ma, 2007). In consistent with this study, our results showed IL-1β disruption of TJ barrier function in the Caco-2 cells cultured for 7 days as evidenced by decreased TER and increased FD-4 flux after exposure to IL-1β for 12-24 h (Fig. 9A and Fig. 11A). However, no change in the TER and FD-4 flux was found in the 21-day cells after exposure to IL-1β for 12-48 h. This unresponsive effect of IL-1β in 21-days cells on TER and FD-4 flux may be likely explained by higher TER of these cells than 7-day culture cells in the present study or Caco-2 cells used by the previous study (Natoli et al., 2012). Other explanation may be due to differences in cell population, growth rate and any others as discussed earlier. The high resistance Caco-2 cells may be tolerant to or not affected by IL-1β at a concentration used in the present study.

Several mechanisms of IL-1β induced TJ barrier disruption have been suggested. IL-1β has been reported to decrease occludin mRNA and protein expression via NF-κB (Al-Sadi and Ma, 2007). Moreover, IL-1β mediated by NF-κB has been demonstrated to induce MLCK pathway by increased expression of MLC*K* mRNA and protein, resulting increased p-MLC expression. The increased p-MLC in turn

stimulates perijunctional contraction, leading to TJ opening, decreased TER and increased epithelial permeability to macromolecules (Al-Sadi et al., 2008).

In the present result, 1-monoHDX and 1,3-diHDX significantly protected the decreased TER induced by IL-1β at 12 and 24 h of 7-day cell associated with the restoration of paracellular resisting to FD-4 macromolecules (Fig. 9A and Fig. 11A). This is the first report of the preventive action of HDXs on the leakiness of intestinal epithelia induced by pro-inflammatory cytokines. The underlying mechanism of these actions of HDXs in the intestinal mucosa will be discussed in the next section.

For the 21-day jejunal like cells, although the TER did not respond to either HDXs or IL-1β treatment, it appeared that cotreatment with 1-monoHDX (10 μM) and IL-1β for 12-24 h showed an enhancing effect on membrane integrity with no change in paracellular permeability to FD-4. This finding also indicates the significant role of 1-monoHDX on strengthening the intestinal barrier function.

5.5 Effects of HDXs on the increased myosin light chain phosphorylation (p-MLC) expression induced by IL-1β in Caco-2 cells

To date the emergence of MLCK and p-MLC as a target of IL-1β and a critical regulator of epithelial paracellular permeability has been suggested (Cunningham and Turner, 2012). Particularly, IL-1β induced increase in TJ permeability in Caco-2 cells is mediated by NF-κB, leading to increased MLCK mRNA and protein expression. The increased MLCK activity will phosphorylate MLC to produce more p-MLC which induces contraction of perijunctional actomyosin causing the opening of TJ and thus increased paracellular permeability (Al-Sadi et al., 2008). This signaling mechanism was

taken into our consideration of the potential effect of HDXs on prevention of IL-1β-induced barrier disruption.

Our finding identified a specific p-MLC protein band of 37 kDa from whole Caco-2 cell lysate as has been previously shown in vitro and in vivo (Cunningham and Turner, 2012). Under normal condition, the ratio of p-MLC and β-actin was closed to 1 which was not affected by 1-monoHDX or 1, 3-diHDX treatment for 15 min and 24 h (Fig 12). The non-inducible effects of 1-monoHDX or 1, 3-diHDX on the p-MLC expression was in accordance to its effects on the FD-4 permeability. Indeed, the phosphorylation of MLC in the intestinal epithelia is very dynamic and has been regulated by many factors in order to allow the absorption and secretion of solutes and fluid through transcellular and paracellular route. Some stimuli such as glucose, histamine, or adenosine have been indicated to increase p-MLC concentration level (Turner et al., 1997). In addition, the activity of TJs barrier protein function including the MLCK pathway and their effectors MLC is required to maintain the homeostasis in the environment surrounding including cell culture medium and many chemical factors. Therefore, the presence of p-MLC expression at 15 min after the adding of fresh media containing glucose and growth factor were found in all groups.

As aforementioned earlier, the major pathway of IL-1β-induced decrease in TER and increase in permeability is mediated by NF-κB to stimulate MLCK gene and protein expression (Al-Sadi et al., 2008). The changes in the MLCK protein expression may take a longer period over 24 h. However, the end point of this pathway is an accumulation of p-MLC which is the active form and able to interact with actin at the TJs complex. In order to coordinate their action with the regulation of TJs disruption, the increase of p-MLC protein expression in response to IL-1β was subject to be monitored during 15 min and 24 h in the present study.

In our current results, 7-day cells exposed to IL-1β for 15 min failed to increase the expression of p-MLC (Fig. 13), consistent with other study (Cunningham and Turner, 2012). Nevertheless, the p-MLC was significantly expressed after exposure to IL-1β for 24 h, suggesting that it requires longer time or 24 h of IL-1β treatment for up-regulation of MLCK. And here in 7 day-cells, 1-monoHDX or 1, 3-diHDX was also found to inhibit the increased p-MLC expression induced by IL-1β at 24 h which was correlated with TER and permeability results (Fig 9A, 11A and 13). The evidence suggesting the suppression of p-MLC expression target by HDXs can restore the disruption of TJs induced by pro-inflammatory cytokines IL-1β. Other study using at least two xanthones, 1,3,6-trihydroxy-7-methoxy-2,8-bis(3-methyl-2-butenyl)-9H-xanthen-9-one (mangostin) and 1,3,6,7-tetrahydroxy-2,8-bis(3-methyl-2-butenyl)-9H-xanthen-9-one (γ-mangostin) has been reported to inhibit MLCK activity in avian Ca^{2+}calmodulin-dependent-light chain kinase assay (Jinsart et al., 1992). The suggested mechanism of mangostin on MLCK-pathway was seem to be an acute effect which was not relevant to our study. However, the study of anti-inflammatory effects of phenolic compound mangiferin, has been reported to decrease NF-κB activity induced by LPS and TNF-α in the model of chronic inflammation using U-937 and HepG2 cells (Sahoo et al., 2015). Inhibition of NF-κB which is signal for genomic action of pro-inflammatory cytokines to many target genes, including MLCK may be associated with the inhibitory effects of 1-monoHDX and 1, 3-diHDX on p-MLC expression at 24 h treatment. The mechanism is depicted in figure 14.

In addition to the MLCK pathway, IL-1β has been indicated to decrease occludin, increase claudin-1 protein expression in jejunal-like Caco-2 cells (Al-Sadi and Ma, 2007). Occludin and claudin-1 as the TJ barrier builder protein are significance in the tightening of epithelial barrier (Khan and Asif, 2015). The decreased occludin and

claudin-1 protein affected by IL-1β relevant to the decreased integrity of TJ barrier was observed. Therefore, the inducing effects of HDXs on the TJ barrier proteins to promote the strength of epithelial barrier should be in the further study.

The inhibitory effects of HDXs on the activation of MLCK to acutely increase the p-MLC concentration is less important than the suppressive effects of HDXs on the long-term activity of IL-1β found in the present study. This possibility is great relevance to develop HDXs as therapeutic agent for treatment of chronic intestinal inflammation, irritable bowel diseases or gut-derived sepsis, in particular.

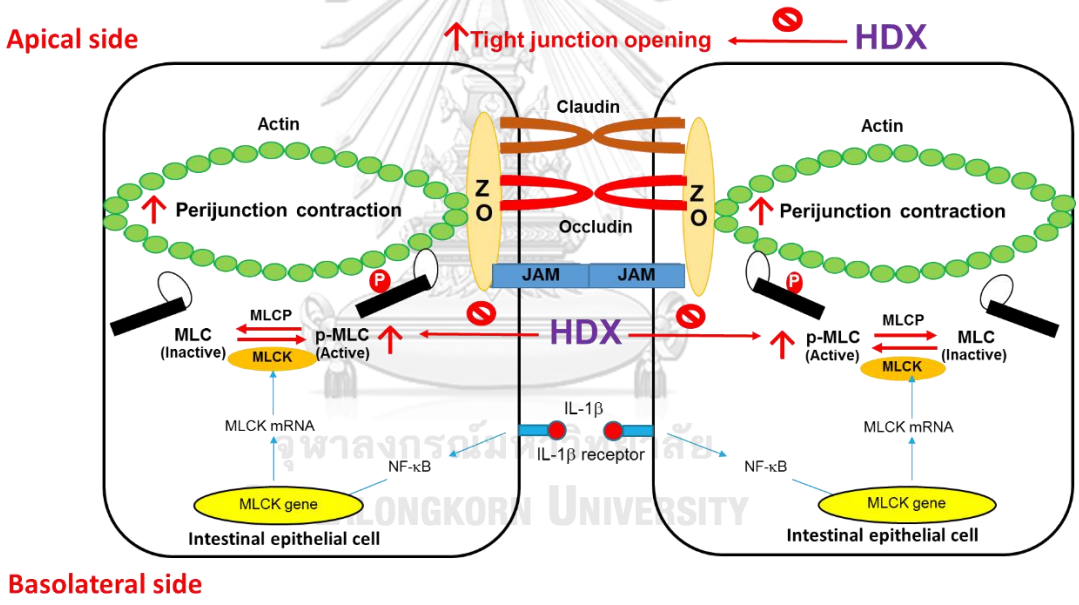


Figure 14 Model of the underlying mechanism of HDXs on the prevention of IL-1β-induced paracellular permeability. The predominant MLCK pathway induced by IL-1β is to produce an increase in p-MLC which is inhibited by 1-monoHDX or 1, 3-diHDX. The prevention from increasing p-MLC level restores perijunctional contraction, TJs opening, and paracellular permeability during bacterial infection.

CHAPTER VI
CONCLUSION

The present study demonstrated the effect of HDXs on TJ barrier of intestinal epithelia in the normal or challenging with pro-inflammatory cytokine IL-1β. The cytotoxicity test was provided for the suitable concentration of HDXs. The analysis of HDX action and mechanism on the TJ barrier disruption were followed by the permeability test and Western blot analysis. The result showed that HDXs decreased the p-MLC induced by IL-1β helping to restore the pathogenesis of IL-1β on the TJ disruption to normal.

The conclusions could be drawn as follows:

1. HDXs excepting 1, 3, 6, 8-tetraHDX did not have cytotoxic effect on intestinal epithelial cells.
2. HDXs seem to promote short-term TJ barrier function of colonic epithelia in the normal condition
3. HDXs also protected the TJ barrier disruption induced by IL-1β.
4. The preventive effect of HDXs to decrease the p-MLC expression may be the pathway that mediate the restoration of the leakiness of intestinal epithelia induced by IL-1β.
5. The potent HDXs suggested in prevention of the leakiness of TJ disruption induced by IL-1β is 1-monoHDX or 1, 3-diHDX.

The present study showed the important effects of HDXs on the protection of intestinal epithelial barrier from bacterial endotoxin consequence. The mechanism of action of HDXs should be investigated in the further study for candidate medicine to treat gastrointestinal disorder.

REFERENCES

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783-801.

Al-Sadi, R., Ye, D., Dokladny, K., Ma, T.Y., 2008. Mechanism of IL-1beta-induced increase in intestinal epithelial tight junction permeability. J Immunol 180, 5653-5661.

Al-Sadi, R., Ye, D., Said, H.M., Ma, T.Y., 2011. Cellular and molecular mechanism of interleukin-1beta modulation of Caco-2 intestinal epithelial tight junction barrier. J Cell Mol Med 15, 970-982.

Al-Sadi, R.M., Ma, T.Y., 2007. IL-1beta causes an increase in intestinal epithelial tight junction permeability. J Immunol 178, 4641-4649.

Amasheh, M., Schlichter, S., Amasheh, S., Mankertz, J., Zeitz, M., Fromm, M., Schulzke, J.D., 2008. Quercetin enhances epithelial barrier function and increases claudin-4 expression in Caco-2 cells. J Nutr 138, 1067-1073.

Anderson, J.M., Van Itallie, C.M., 2009. Physiology and function of the tight junction. Cold Spring Harb Perspect Biol 1, a002584.

Artursson, P., 1990. Epithelial transport of drugs in cell culture. I: A model for studying the passive diffusion of drugs over intestinal absorbtive (Caco-2) cells. J Pharm Sci 79, 476-482.

Artursson, P., Karlsson, J., 1991. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem Biophys Res Commun 175, 880-885.

Artursson, P., Palm, K., Luthman, K., 2001. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev 46, 27-43.

Awad, W.A., Molnar, A., Aschenbach, J.R., Ghareeb, K., Khayal, B., Hess, C., Liebhart, D., Dublecz, K., Hess, M., 2015. Campylobacter infection in chickens modulates the intestinal epithelial barrier function. Innate Immun 21, 151-160.

Bak, J.Y. 2011. Chemical synthesis of 1, 6-dioxygenated xanthones and their cytotoxic activities, UTAR.

Bektic, J., Guggenberger, R., Eder, I.E., Pelzer, A.E., Berger, A.P., Bartsch, G., Klocker, H., 2005. Molecular effects of the isoflavonoid genistein in prostate cancer. Clin Prostate Cancer 4, 124-129.

Blacklow, N.R., Greenberg, H.B., 1991. Viral gastroenteritis. N Engl J Med 325, 252-264.

Blair, S.A., Kane, S.V., Clayburgh, D.R., Turner, J.R., 2006. Epithelial myosin light chain kinase expression and activity are upregulated in inflammatory bowel disease. Lab Invest 86, 191-201.

Bruewer, M., Luegering, A., Kucharzik, T., Parkos, C.A., Madara, J.L., Hopkins, A.M., Nusrat, A., 2003. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. J Immunol 171, 6164-6172.

Bruewer, M., Samarin, S., Nusrat, A., 2006. Inflammatory bowel disease and the apical junctional complex. Ann N Y Acad Sci 1072, 242-252.

Brufau, M.T., Campo-Sabariz, J., Bou, R., Carne, S., Brufau, J., Vila, B., Marques, A.M., Guardiola, F., Ferrer, R., Martin-Venegas, R., 2016. Salmosan, a beta-Galactomannan-Rich Product, Protects Epithelial Barrier Function in Caco-2 Cells Infected by Salmonella enterica Serovar Enteritidis. J Nutr 146, 1492-1498.

Castoldi, A., Favero de Aguiar, C., Manoel Moraes-Vieira, P., Olsen Saraiva Camara, N., 2015. They must hold tight: junction proteins, microbiota and immunity in intestinal mucosa. Curr Protein Pept Sci 16, 655-671.

Chen, Y.C., Chen, K.T., 2007. Novel selective inhibitors of hydroxyxanthone derivatives for human cyclooxygenase-2. Acta Pharmacol Sin 28, 2027-2032.

Chiba, H., Osanai, M., Murata, M., Kojima, T., Sawada, N., 2008. Transmembrane proteins of tight junctions. Biochimica et Biophysica Acta 1778, 588-600.

Claud, E.C., 2009. Neonatal necrotizing enterocolitis-inflammation and intestinal immaturity. Antiinflamm Antiallergy Agents Med Chem 8, 248-259.

Clayburgh, D.R., Barrett, T.A., Tang, Y., Meddings, J.B., Van Eldik, L.J., Watterson, D.M., Clarke, L.L., Mrsny, R.J., Turner, J.R., 2005. Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo. J Clin Invest 115, 2702-2715.

Clayburgh, D.R., Rosen, S., Witkowski, E.D., Wang, F., Blair, S., Dudek, S., Garcia, J.G., Alverdy, J.C., Turner, J.R., 2004. A differentiation-dependent splice variant of myosin light chain kinase, MLCK1, regulates epithelial tight junction permeability. J Biol Chem 279, 55506-55513.

Collington, G.K., Booth, I.W., Knutton, S., 1998. Rapid modulation of electrolyte transport in Caco-2 cell monolayers by enteropathogenic *Escherichia coli* (EPEC) infection. Gut 42, 200-207.

Cunningham, K.E., Turner, J.R., 2012. Myosin light chain kinase: pulling the strings of epithelial tight junction function. Ann N Y Acad Sci 1258, 34-42.

Demonty, J., De Graeve, J., 1982. Release of endotoxic lipopolysaccharide by sensitive strains ofEscherichia coli submitted to the bactericidal action of human serum. Med Microbiol Immunol 170, 265-277.

Diamond, J., 1974. Tight and leaky junctions of epithelia: a perspective on kisses in the dark. Fed Proc 33, 2220-2224.

Dinarello, C.A., 1996. Biologic basis for interleukin-1 in disease. Blood 87, 2095-2147.

Ebnet, K., Suzuki, A., Ohno, S., Vestweber, D., 2004. Junctional adhesion molecules (JAMs): more molecules with dual functions? J Cell Sci 117, 19-29.

Engle, M.J., Goetz, G.S., Alpers, D.H., 1998. Caco-2 cells express a combination of colonocyte and enterocyte phenotypes. J Cell Physiol 174, 362-369.

Field, M., Rao, M.C., Chang, E.B., 1989. Intestinal electrolyte transport and diarrheal disease. N Engl J Med 321, 879-883.

Fortin, C.F., Ear, T., McDonald, P.P., 2009. Autocrine role of endogenous interleukin-18 on inflammatory cytokine generation by human neutrophils. FASEB J 23, 194-203.

Fujibe, M., Chiba, H., Kojima, T., Soma, T., Wada, T., Yamashita, T., Sawada, N., 2004. Thr203 of claudin-1, a putative phosphorylation site for MAP kinase, is required to promote the barrier function of tight junctions. Exp Cell Res 295, 36-47.

Furuse, M., Itoh, M., Hirase, T., Nagafuchi, A., Yonemura, S., Tsukita, S., Tsukita, S., 1994. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. J Cell Biol 127, 1617-1626.

Groschwitz, K.R., Hogan, S.P., 2009. Intestinal barrier function: molecular regulation and disease pathogenesis. J Allergy Clin Immunol 124, 3-20.

Guo, S., Al-Sadi, R., Said, H.M., Ma, T.Y., 2013. Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. Am J Pathol 182, 375-387.

Guo, S., Nighot, M., Al-Sadi, R., Alhmoud, T., Nighot, P., Ma, T.Y., 2015. Lipopolysaccharide Regulation of Intestinal Tight Junction Permeability Is Mediated by TLR4 Signal Transduction Pathway Activation of FAK and MyD88. J Immunol 195, 4999-5010.

Gutierrez-Orozco, F., Failla, M.L., 2013. Biological activities and bioavailability of mangosteen xanthones: a critical review of the current evidence. Nutrients 5, 3163-3183.

Hammerum, A.M., Heuer, O.E., 2009. Human health hazards from antimicrobial-resistant Escherichia coli of animal origin. Clin Infect Dis 48, 916-921.

Haycocks, J.R., Sharma, P., Stringer, A.M., Wade, J.T., Grainger, D.C., 2015. The molecular basis for control of ETEC enterotoxin expression in response to environment and host. PLoS Pathog 11, e1004605.

Hidalgo, I.J., Raub, T.J., Borchardt, R.T., 1989. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96, 736-749.

Itoh, M., Furuse, M., Morita, K., Kubota, K., Saitou, M., Tsukita, S., 1999. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. J Cell Biol 147, 1351-1363.

Jinsart, W., Ternai, B., Buddhasukh, D., Polya, G.M., 1992. Inhibition of wheat embryo calcium-dependent protein kinase and other kinases by mangostin and γ-mangostin. Phytochemistry 31, 3711-3713.

Keita, A.V., Soderholm, J.D., 2010. The intestinal barrier and its regulation by neuroimmune factors. Neurogastroenterol Motil 22, 718-733.

Khan, N., Asif, A.R., 2015. Transcriptional regulators of claudins in epithelial tight junctions. Mediators Inflamm 2015, 219843.

Kiatprasert, P., Deachapunya, C., Benjanirat, C., Poonyachoti, S., 2015. Soy isoflavones improves endometrial barrier through tight junction gene expression. Reproduction 149, 269-280.

Kiela, P.R., Ghishan, F.K., 2016. Physiology of Intestinal Absorption and Secretion. Best Pract Res Clin Gastroenterol 30, 145-159.

Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E., Etherton, T.D., 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am J Med 113 Suppl 9B, 71S-88S.

Krug, S.M., Schulzke, J.D., Fromm, M., 2014. Tight junction, selective permeability, and related diseases. Semin Cell Dev Biol 36, 166-176.

Liu, Y., Zou, L., Ma, L., Chen, W.H., Wang, B., Xu, Z.L., 2006. Synthesis and pharmacological activities of xanthone derivatives as alpha-glucosidase inhibitors. Bioorg Med Chem 14, 5683-5690.

Lu, Y.C., Yeh, W.C., Ohashi, P.S., 2008. LPS/TLR4 signal transduction pathway. Cytokine 42, 145-151.

Luerang, W., Khammee, T., Kumpum, W., Suksamrarn, S., Chatsudthipong, V., Muanprasat, C., 2012. Hydroxyxanthone as an inhibitor of cAMP-activated apical chloride channel in human intestinal epithelial cell. Life Sci 90, 988-994.

Ma, T.Y., Boivin, M.A., Ye, D., Pedram, A., Said, H.M., 2005. Mechanism of TNF-**α** modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. Am J Physiol Gastrointest Liver Physiol 288, G422-G430.

Ma, T.Y., Iwamoto, G.K., Hoa, N.T., Akotia, V., Pedram, A., Boivin, M.A., Said, H.M., 2004. TNF-**α**-induced increase in intestinal epithelial tight junction permeability requires NF-**K**B activation. Am J Physiol Gastrointest Liver Physiol 286, G367-G376.

Madara, J.L., Nash, S., Moore, R., Atisook, K., 1989. Structure and function of the intestinal epithelial barrier in health and disease. Monogr Pathol, 306-324.

Matsumoto, K., Akao, Y., Ohguchi, K., Ito, T., Tanaka, T., Iinuma, M., Nozawa, Y., 2005. Xanthones induce cell-cycle arrest and apoptosis in human colon cancer DLD-1 cells. Bioorg Med Chem 13, 6064-6069.

Matter, K., Balda, M.S., 2003. Functional analysis of tight junctions. Methods 30, 228-234.

Middleton, E., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 52, 673-751.

Monteiro, A.C., Parkos, C.A., 2012. Intracellular mediators of JAM-A–dependent epithelial barrier function. Ann N Y Acad Sci 1257, 115-124.

Naslund, E., Hellstrom, P.M., 2007. Appetite signaling: from gut peptides and enteric nerves to brain. Physiol Behav 92, 256-262.

Natoli, M., Leoni, B.D., D'Agnano, I., Zucco, F., Felsani, A., 2012. Good Caco-2 cell culture practices. Toxicol In Vitro 26, 1243-1246.

Negi, J., Bisht, V., Singh, P., Rawat, M., Joshi, G., 2013. Naturally occurring xanthones: Chemistry and biology. J Applied Chem 2013.

Noda, S., Tanabe, S., Suzuki, T., 2012. Differential effects of flavonoids on barrier integrity in human intestinal Caco-2 cells. J Agric Food Chem 60, 4628-4633.

Park, H.-Y., Kunitake, Y., Hirasaki, N., Tanaka, M., Matsui, T., 2015a. Theaflavins enhance intestinal barrier of Caco-2 Cell monolayers through the expression of AMP-activated protein kinase-mediated Occludin, Claudin-1, and ZO-1. Biosci Biotechnol Biochem 79, 130-137.

Pedraza-Chaverri, J., Cardenas-Rodriguez, N., Orozco-Ibarra, M., Perez-Rojas, J.M., 2008. Medicinal properties of mangosteen (Garcinia mangostana). Food Chem Toxicol 46, 3227-3239.

Pelletier, D.L., Frongillo, E.A., 2003. Changes in child survival are strongly associated with changes in malnutrition in developing countries. J Nutr 133, 107-119.

Ramos, A., F. Lima, C.v.o., Pereira-Wilson, C., 2011. DNA Damage Protection and Induction of Repair by Dietary Phytochemicals and Cancer Prevention: What Do We Know?, INTECH Open Access Publisher.

Ronaghan, N.J., Shang, J., Iablokov, V., Zaheer, R., Colarusso, P., Dion, S., Desilets, A., Leduc, R., Turner, J.R., MacNaughton, W.K., 2016. The serine protease-mediated increase in intestinal epithelial barrier function is dependent on occludin and requires an intact tight junction. Am J Physiol Gastrointest Liver Physiol 311, G466-479.

Sahoo, B.K., Zaidi, A.H., Gupta, P., Mokhamatam, R.B., Raviprakash, N., Mahali, S.K., Manna, S.K., 2015. A natural xanthone increases catalase activity but decreases NF-kappa B and lipid peroxidation in U-937 and HepG2 cell lines. Eur J Pharmacol 764, 520-528.

Saitou, M., Fujimoto, K., Doi, Y., Itoh, M., Fujimoto, T., Furuse, M., Takano, H., Noda, T., Tsukita, S., 1998. Occludin-deficient Embryonic Stem Cells Can Differentiate into Polarized Epithelial Cells Bearing Tight Junctions. J Cell Biol 141, 397-408.

Sakakibara, A., Furuse, M., Saitou, M., Ando-Akatsuka, Y., Tsukita, S., 1997. Possible involvement of phosphorylation of occludin in tight junction formation. J Cell Biol 137, 1393-1401.

Samak, G., Gangwar, R., Crosby, L.M., Desai, L.P., Wilhelm, K., Waters, C.M., Rao, R., 2014. Cyclic stretch disrupts apical junctional complexes in Caco-2 cell monolayers by a JNK-2-, c-Src-, and MLCK-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 306, G947-958.

Schmitz, H., Fromm, M., Bentzel, C.J., Scholz, P., Detjen, K., Mankertz, J., Bode, H., Epple, H.J., Riecken, E.O., Schulzke, J.D., 1999. Tumor necrosis factor-alpha (TNFalpha) regulates the epithelial barrier in the human intestinal cell line HT-29/B6. J Cell Sci 112 (Pt 1), 137-146.

Schneeberger, E.E., Lynch, R.D., 2004. The tight junction: a multifunctional complex. Am J Physiol Cell Physiol 286, C1213-C1228.

Shen, L., Weber, C.R., Raleigh, D.R., Yu, D., Turner, J.R., 2011. Tight Junction Pore and Leak Pathways: A Dynamic Duo. Ann Rev Physiol 73, 283-309.

Song, T., Barua, K., Buseman, G., Murphy, P.A., 1998. Soy isoflavone analysis: quality control and a new internal standard. Am J Clin Nutr 68, 1474S-1479S.

Sperandio, B., Fischer, N., Sansonetti, P.J., 2015. Mucosal physical and chemical innate barriers: Lessons from microbial evasion strategies. Semin Immunol 27, 111-118.

Srinivasan, B., Kolli, A.R., Esch, M.B., Abaci, H.E., Shuler, M.L., Hickman, J.J., 2015. TEER measurement techniques for in vitro barrier model systems. J Lab Autom 20, 107-126.

Suksamrarn, S., Suwannapoch, N., Ratananukul, P., Aroonlerk, N., Suksamrarn, A., 2002. Xanthones from the Green Fruit Hulls of Garcinia mangostana. J Nat Prod 65, 761-763.

Suzuki, T., Hara, H., 2009. Quercetin enhances intestinal barrier function through the assembly of zonnula occludens-2, occludin, and claudin-1 and the expression of claudin-4 in Caco-2 cells. J Nutr 139, 965-974.

Takeda, K., Akira, S., 2004. TLR signaling pathways. Semin Immunol 16, 3-9.

Taylor, C.T., Dzus, A.L., Colgan, S.P., 1998. Autocrine regulation of epithelial permeability by hypoxia: role for polarized release of tumor necrosis factor alpha. Gastroenterology 114, 657-668.

Tsao, R., 2010. Chemistry and biochemistry of dietary polyphenols. Nutrients 2, 1231-1246.

Tsukita, S., Furuse, M., Itoh, M., 2001. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2, 285-293.

Turner, J.R., 2006. Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. Am J Pathol 169, 1901-1909.

Turner, J.R., Rill, B.K., Carlson, S.L., Carnes, D., Kerner, R., Mrsny, R.J., Madara, J.L., 1997. Physiological regulation of epithelial tight junctions is associated with myosin light-chain phosphorylation. Am J Physiol 273, C1378-1385.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., Roy, N.C., 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. J Nutr 141, 769-776.

Van Amersfoort, E.S., Van Berkel, T.J.C., Kuiper, J., 2003. Receptors, Mediators, and Mechanisms Involved in Bacterial Sepsis and Septic Shock. Clin Microbiol Rev 16, 379-414.

Van Itallie, C.M., Anderson, J.M., 2006. Claudins and epithelial paracellular transport. Annu. Rev. Physiol. 68, 403-429.

Visioli, F., De La Lastra, C.A., Andres-Lacueva, C., Aviram, M., Calhau, C., Cassano, A., D'Archivio, M., Faria, A., Fave, G., Fogliano, V., Llorach, R., Vitaglione, P., Zoratti, M., Edeas, M., 2011. Polyphenols and human health: a prospectus. Crit Rev Food Sci Nutr 51, 524-546.

Wang, F., Graham, W.V., Wang, Y., Witkowski, E.D., Schwarz, B.T., Turner, J.R., 2005. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. Am J Pathol 166, 409-419.

Wang, L., Llorente, C., Hartmann, P., Yang, A.M., Chen, P., Schnabl, B., 2015. Methods to determine intestinal permeability and bacterial translocation during liver disease. J Immunol Methods 421, 44-53.

Wells, C.L., Jechorek, R.P., Kinneberg, K.M., Debol, S.M., Erlandsen, S.L., 1999. The isoflavone genistein inhibits internalization of enteric bacteria by cultured Caco-2 and HT-29 enterocytes. J Nutr 129, 634-640.

Wingender, J., Flemming, H.-C., 2011. Biofilms in drinking water and their role as reservoir for pathogens. Int J Hyg Environ Health 214, 417-423.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., Mathison, J.C., 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249, 1431-1433.

Wright, S.D., Tobias, P.S., Ulevitch, R.J., Ramos, R.A., 1989. Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. J Exp Med 170, 1231-1241.

Xu, C., Wu, X., Hack, B.K., Bao, L., Cunningham, P.N., 2015. TNF causes changes in glomerular endothelial permeability and morphology through a Rho and myosin light chain kinase-dependent mechanism. Physiol Rep 3, e12636.

Zhang, R., Xu, J., Zhao, J., Chen, Y., 2017. Genistein improves inflammatory response and colonic function through NF-kappaB signal in DSS-induced colonic injury. Oncotarget 8, 61385-61392.

APPENDIX

VITA

Wannaporn Chayalak was born on August 3rd 1988 in Bangkok, Thailand. She graduated with Degree of Bachelor of Veterinary Science, Chulalongkorn University, Bangkok, Thailand in 2014. During Bachelor's degree, she provided the senior project in the topic "The ability of porcine endometrial cells in releasing of interleukin-6 and -8 respond to various microbial components in the absence of immune cells" with her thesis advisor, Associated Prof. Dr. Sutthasinee Poonyachoti, who supported and gave the counsel for her all the time. She has been worked as veterinarian in animal hospital since 2014. Wannaporn is recently pursuing a Master's degree in the field of animal gastrointestinal system at Department of Veterinary Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. She is interested in molecular science of gastrointestinal system with aim of improving animal gut health during barrier impairment. Moreover, she also attended the poster presentation in the topic "Protective effect of hydroxyxanthone on the leakiness of intestinal epithelia induced by TNF-α" of Chalalongkorn university veterinary conference (CUVC) 2018 which was accepted to published in Thai Journal Veterinary Medicine Volume 48. In addition, she planned to further study a Doctor's degree in the field of molecular science.