Protective mechanisms of polyphenols against NSAIDs-induced intestinal cell death in Caco-2 cells

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Pharmacology
Inter-Department of Pharmacology
GRADUATE SCHOOL
Chulalongkorn University
Academic Year 2019

กลไกของสารโพลีฟีนอลในการปกป้องการตายของเซลล์ลำไส้ที่ถูกเหนี่ยวนำด้วยยาต้านอักเสบชนิดไม่ใช่สเตียรอยด์ในเซลล์คาโค-ทู

นายเชิดศักดิ์ บุญยง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชวิทยา สหสาขาวิชาเภสัชวิทยา
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

Thesis Title	Protective mechanisms of polyphenols against NSAIDs-induced intestinal cell death in Caco-2 cells
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เชิดศักดิ์ บุญยง : กลไกของสารโพลีฟีนอลในการปกป้องการตายของเซลล์ลำไส้ที่ถูกเหนี่ยวนำด้วยยาต้านอักเสบชนิดไม่ใช่สเตียรอยด์ในเซลล์คาโค-ทู. (Protective mechanisms of polyphenols against NSAIDs-induced intestinal cell death in Caco-2 cells) อ.ที่ปรึกษาหลัก : รศ. ภญ. ดร.สุรีย์ เจียรณ์มงคล, อ.ที่ปรึกษาร่วม : ผศ. ภญ. ดร.นนทิมา วรรธนะภูติ

ยาอินโดเมทาซิน (INDO) และ ไดโคลฟีแนค (DIC) ทำให้เกิดการตายของเซลล์ลำไส้ผ่านการสร้างอนุมูลอิสระ (ROS), เกิดความเครียดของเอนโดพลาสมิคเรติคูลัม (ER stress) และความผิดปกติของไมโทคอนเดรีย ในการศึกษานี้เพื่อพิสูจน์ฤทธิ์ปกป้องเซลล์ของสารธรรมชาติโพลีฟีนอลิกที่มีฤทธิ์ต้านอนุมูลอิสระต่อการป้องกันการตายของเซลล์คาโคทูจากการเหนี่ยวนำด้วยยา INDO และ DIC รวมถึงความเข้าใจของกลไกที่เกี่ยวข้อง ผลการศึกษาพบว่าเมื่อให้สาร caffeic acid (CAF), curcumin (CUR), epigallocatechin gallate (EGCG), gallic acid (GAL), hypophyllanthin (HYPO), naringenin (NAR), phyllanthin (PHY), piperine (PIP), quercetin (QUE), rutin (RUT) และ silymarin (SLY) สามารถลดการสร้างอนุมูลอิสระภายในเซลล์คาโคทูเมื่อถูกเหนี่ยวนำด้วยยา INDO และ DIC แต่อย่างไรก็ตามมีเพียงแค่สาร 3 ชนิด EGCG, QUE และ RUT (100 μM) สามารถป้องกันการตายของเซลล์จากการเหนี่ยวนำด้วยยา INDO และ DIC หลังจากให้เป็นเวลา 72 ชั่วโมง และในการศึกษานี้ฤทธิ์การปกป้องเซลล์ของสารทั้ง 3 ชนิดมีส่วนเกี่ยวข้องกับการยับยั้งการแสดงออกของ *CHOP*, ลดสัดส่วนของ *Bax/Bcl-2* และป้องกันการสูญเสียของ mitochondria membrane permeability (MMP) และการศึกษากลไกปกป้องเซลล์ของสาร EGCG ยังมีส่วนเกี่ยวข้องกับการยับยั้งการแสดงออกของ p-PERK/p-eIF-2α/ATF-4 และ p-IRE-1α/p-JNK1/2 นอกจากนี้สารโพลีฟีนอลอีก 2 ชนิดได้แก่ CAF และ NAR (100 μM) ป้องกันการตายของเซลล์คาโคทูจากการเหนี่ยวนำด้วยยา INDO แต่ไม่มีผลเมื่อให้ร่วมกับยา DIC ซึ่งกลไกในการปกป้องเซลล์ของสาร NAR เกี่ยวข้องกับการยับยั้งการเพิ่มสัดส่วน *Bax/Bcl-2* mRNA และป้องกันการสูญเสียของ MMP แม้ว่าสาร NAR ไม่สามารถกดการแสดงออกของ *CHOP* นอกจากนี้สารกลุ่มโพลีฟีนอลอีก 6 ชนิดประกอบด้วย CUR (50 μM), GAL (100 μM), HYPO (10 μM), PHY (10 μM), PIP (10 μM) และ SLY (100 μM) ไม่มีฤทธิ์ในการปกป้องการตายของเซลล์คาโคทูจากการเหนี่ยวด้วยยา INDO และ DIC ซึ่งสาร CUR, NAR, PHY, PIP และ SLY สามารถเพิ่มการตายของเซลล์จากการเหนี่ยวนำด้วยยา DIC และพบว่ากลไกการเพิ่มการตายของเซลล์เมื่อให้สาร PHY อาจเกี่ยวข้องกับการยับยั้งการแสดงออกของโปรตีนที่เกี่ยวข้องกับการอยู่รอดของเซลล์ p-Nrf-2/HO-1 เมื่อให้ร่วมกับยา DIC และส่งผลต่อการเพิ่มการกระตุ้นวิถีสัญญาณ PERK/CHOP และเพิ่มการตายของเซลล์แบบอะพอพโทซิสมากขึ้น ดังนั้นผลการวิจัยนี้แสดงให้เห็นว่าฤทธิ์ปกป้องการตายของเซลล์ด้วยสารโพลีฟีนอลจากการเหนี่ยวนำด้วยยา INDO หรือ DIC ในเซลล์คาโคทูที่ตายแบบอะพอพโทซิสอาจมีส่วนเกี่ยวข้องกับการยับยั้งการส่งสัญญาณของโปรตีนที่เกี่ยวข้องกับความเครียดของเอนโดพลาสมิคเรติคูลัม ทั้งนี้ฤทธิ์ดังกล่าวไม่มีส่วนเกี่ยวข้องกับกลไกการต้านอนุมูลอิสระ

สาขาวิชา	เภสัชวิทยา	ลายมือชื่อนิสิต .....
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5987839920 : MAJOR PHARMACOLOGY

KEYWORD: NSAIDs, Polyphenols, Oxidative stress, ER stress, Mitochondrial dysfunction, Intestinal cell death

Cherdsak Boonyong : Protective mechanisms of polyphenols against NSAIDs-induced intestinal cell death in Caco-2 cells. Advisor: Assoc. Prof. SUREE JIANMONGKOL, Ph.D. Co-advisor: Asst. Prof. NONTIMA VARDHANABHUTI, Ph.D.

Indomethacin (INDO) and diclofenac (DIC) cause intestinal cell death through reactive oxygen species (ROS), endoplasmic reticulum stress (ER stress) and mitochondrial dysfunction. This study investigated the cytoprotective effects of certain natural polyphenolic antioxidants against INDO- and DIC-mediated Caco-2 cell death as well as underlying mechanisms. In this study, caffeic acid (CAF), curcumin (CUR), epigallocatechin gallate (EGCG), gallic acid (GAL), hypophyllanthin (HYPO), naringenin (NAR), phyllanthin (PHY), piperine (PIP), quercetin (QUE), rutin (RUT) and silymarin (SLY) were able to decrease ROS production in Caco-2 cells treated with INDO and DIC. However, only EGCG, QUE and RUT (100 μM) could prevent INDO- and DIC-mediated cytotoxicity after 72-h treatment. Their cytoprotective action could be related to suppression of *CHOP* expression, *Bax/Bcl-2* ratios and loss of mitochondria membrane permeability (MMP). Further study on the cytoprotective effect of EGCG demonstrated that its underlying mechanism involved with suppressions of p-PERK/p-eIF-2α/ATF-4/CHOP and p-IRE-1α/p-JNK1/2 expressions. Another two polyphenols CAF and NAR (100 μM) protected Caco-2 toxicity from INDO treatment, but not DIC treatment. Moreover, the effect of NAR involved with suppression of *Bax/Bcl-2* mRNA ratios and loss in MMP. Apparently, NAR did not affect *CHOP* expression. A group of polyphenols including CUR (50 μM), GAL (100 μM), HYPO (10 μM), PHY (10 μM), PIP (10 μM) and SLY (100 μM) had no effects on INDO-/DIC-induced Caco-2 cytotoxicity. Instead, CUR, NAR, PHY, PIP and SLY enhanced cytotoxicity caused by DIC exposure. Further study on the potentiation effect of PHY suggested that this compound suppressed survival p-Nrf-2/HO-1 expression in DIC-treated cells, leading to increased activation of PERK/CHOP pathway and apoptosis. Taken together, the cytoprotective capabilities of polyphenols against INDO-/DIC-induced Caco-2 apoptosis might be involved with suppressions of the specific ER stress signaling pathways, but not the direct ROS scavenging mechanism.

Field of Study:	Pharmacology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my thesis advisor, Associate Professor Suree Jianmongkol, Ph.D, and my co-advisor, Assistant Professor Nontima Vardhanabhuti, Ph.D. for their invaluable advice, attention, motivation, encouragement and patience throughout this study.

I would like to thank Chulalongkorn University Drugs and Health Products Innovation Promotion Center and Pharmaceutical Research Instruments, Faculty of Pharmaceutical science for providing facilities.

This work was financially supported by the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship to CB and 90th Anniversary of Chulalongkorn University Fund (Ratchadaphisedksomphot Endowment Fund).

I am grateful to all staff members of the Department of Pharmacology, Pharmaceutical science for their help. The special thanks are also extended to all my friends for their suggestion, encouragement and assistance.

Finally, I wish to express my infinite thank to my family for their understanding and never ending support throughout my life. Thank you for always being beside me.

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LIST OF ABBREVIATIONS

ANOVA	= Analysis of variance
ATF-4	= Activating transcription factor-4
ATF-6	= Activating transcription factor-6
Bip	= Binding of immunoglobulin protein
BSA	= Bovine serum albumin
ºC	= degree Celsius
Caco-2	= Colorectal adenocarcinoma
CAF	= Caffeic acid
CDCFH-DA	= 2′,7′-Dichlorofluorescin diacetate
CHOP	= C/EBP homologous transcription factor
cm^2	= squared centimeter
CO_2	= Carbon dioxide
CUR	= Curcumin
DHE	= Dihydroethidium
DIC	= Diclofenac
DMEM	= Dulbecco's modified eagle medium
DMSO	= Dimethyl sulphoxide
EDTA	= Ethylenediaminetetraacetic acid
EGCG	= Epigallocatechin gallant
eIF-2α	= eukaryotic Initiation factor-2α
ER	= Endoplasmic reticulum
FBS	= Fetal bovine serum
G	= Golgi apparatus
GAL	= Gallic acid
GAPDH	= Glyceraldehyde 3-phosphate dehydrogenase

h	= hour
H_2O_2	= Hydrogen peroxide
HO-1	= Heme oxygenase-1
HPF	= Hydroxyphenyl fluorescein
HYPO	= Hypophyllanthin
I.V.	= Intravenous
INDO	= Indomethacin
IRE-1**α**	= Inositol-requiring enzymes-1**α**
JNK1/2	= c-Jun N-terminal kinases1/2
mg	= milligram
min	= minute
Mito	= Mitochondria
ml	= milliliter
mM	= millimolar
MMP	= Mitochondrial membrane potential
mPT pore	= mitochondrial permeability transition pore
MTT	= 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
Na_3VO_4	= Sodium orthovanadate
NaCl	= Sodium chloride
NaF	= Sodium fluoride
NAR	= Naringenin
NF-kB	= Nuclear factor-**K**B
nm	= nanometer
Nrf-2	= Nuclear factor erythroid 2-related factor 2
NSAIDs	= Nonsteroidal anti-inflammatory drugs
p	= phosphorylation

P.O. = per os (oral)

PBS = Phosphate buffered saline

PERK = Pancreatic-like ER kinase

PHY = Phyllanthin

PIP = Piperine

PMSF = Phenylmethylsulfonyl fluoride

PVDF = Polyvinylidine fluoride

qRT-PCR = quantitative reverse transcription polymerase chain reaction

QUE = Quercetin

ROS = Reactive oxygen species

ROT = Rotenone

RUT = Rutin

SDS = Sodium dodecyl sulfate

sec = second

SEM = Standard Error of Mean

SLY = Silymarin

TGN = Thapsigargin

TMRE = Tetramethylrhodamine

Tris-HCl = Tris hydrochloride

TUN = Tunicamycin

XBP-1s = spliced X-box-binding protein-1

µg = microgram

µl = microliter

µM = micromolar

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Nonsteroidal anti-inflammatory drugs (NSAIDs) are analgesic, anti-inflammatory and antipyretic agents commonly used for treatments of pain and arthritis. These drugs, particularly indomethacin (INDO) and diclofenac (DIC), have been known to cause enteropathy in mucosal small intestine (jejunum/ilium) and colon (Boelsterli et al., 2013; Chávez-Piña et al., 2018; Matsui et al., 2011; Utzeri and Usai, 2017). INDO and DIC have been reported to cause gastrointestinal damages, particularly enteropathy, both in experimental models and in clinical studies through induction of endoplasmic reticulum (ER) stress and apoptosis (Boonyong et al., 2020; Chávez-Piña et al., 2018). They trigger ER stress via inhibition of mitochondrial complex I and overproduction of reactive oxygen species (ROS), leading to disruption of protein folding in the ER (Chávez-Piña et al., 2018).

INDO and DIC have been linked to increased auto-phosphorylation of ER sensor proteins, particularly pancreatic-like ER kinase (PERK) and inositol-requiring enzymes-1α (IRE-1α) in various cell models such as primary pig gastric epithelial cells, Huh-7, H-4 and U-87 cells (Chang et al., 2020; Franceschelli et al., 2011; Mügge and Silva, 2015). Activation of PERK up-regulates the C/EBP homologous transcription factor expression (CHOP), an ER stress apoptotic mediator, resulting in an increase of Bax (pro-apoptotic) and decrease of Bcl-2 (anti-apoptotic) expression (Iurlaro and Muñoz-Pinedo, 2016; Li et al., 2015; Zhang et al., 2019). Consequently, mitochondrial permeability transition pore (mPT pore) opens, leading to the loss in mitochondrial membrane potential (MMP) and cell apoptosis (Iurlaro and Muñoz-Pinedo, 2016; Li et al., 2015; Zhang et al., 2019).

In clinical practice, antiulcer drugs such as misoprostol (prostaglandin analog) or omeprazole (a proton pump inhibitor) are used to attenuate NSAIDs-induced intestinal toxicity (Chan, 2006; Park et al., 2011). However, these drugs are ineffective in relieving NSAIDs-induced enteropathy (Blackler et al., 2012; Lim et al., 2012; Marlicz et al., 2014; Park et al., 2011; Wallace et al., 2011; Zhu et al., 2012). This might be due to these antiulcer drugs have not targeted at the etiology of intestinal damage. The mechanisms of NSAIDs-induce intestinal cell death have been linked to oxidative stress within the cells. Hence, "physico-chemical housekeeping" protective mechanisms including secretion of mucus and reduction of acid secretion may not be sufficient in preventing enteropathy from NSAIDs. Based on the mechanism of toxicity, it can be hypothesized that effective protection of NSAIDs-induced intestinal damage may be achieved through managing intracellular oxidative stress.

As known, polyphenols are found abundantly in several fruits and vegetables. These compounds present in human diet, supplementary foods and traditional medicines (Gharras, 2009; Martin and Bolling, 2015). A number of studies reported that polyphenols elicited cytoprotective activity toward oxidative stress-induced cell death through reduction of oxidative stress, ER stress and mitochondrial dysfunction (Carrasco-Pozo et al., 2016; Carrasco-Pozo et al., 2011; Chen et al., 2015; Jia et al., 2016; Karthikeyan et al., 2017; Krithika et al., 2015; Krithika and Verma, 2009; Krithika et al., 2011; Li and Kuemmerle, 2018; Seiquer et al., 2015). For example, the apple peel polyphenol-riched extract was able to protect INDO-induced intestinal damage via preventing mitochondrial complex I inhibition in intestinal Caco-2 cells (Carrasco-Pozo et al., 2011). Polyphenols-riched virgin argan oil was reported to protect tBOOH-induced Caco-2 cell death through ROS scavenger mechanism (Seiquer et al., 2015). Quercetin (QUE) was also reported to protect INDO-induced oxidative stress and inflammation in rat GI mucosa through increase of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Carrasco-Pozo et al., 2016). The

underlying mechanism of QUE protection involved with induction of Nrf-2 and inhibition of NF-kB nuclear translocations (Carrasco-Pozo et al., 2016). Epigallocatechin gallant (EGCG) was reported to prevent ER stress-mediated cytotoxicity from cisplatin in the mouse nephron (Chen et al., 2015) and thapsigargin in MRPE cells (Karthikeyan et al., 2017). Phyllanthin (PHY) and hypophyllanthin (HYPO) were demonstrated to protect carbon tetrachloride-induced liver damage in hepatocyte and mice models through antioxidant-related mechanisms (Jia et al., 2016; Krithika and Verma, 2009; Krithika et al., 2011). However, the capability of polyphenols on protection of INDO- and DIC-induced intestinal cell death has yet to be determined. It is likely that polyphenols may be cytoprotective agents against NSAIDs-induced enteropathy.

1.2 Hypothesis

Polyphenolic compounds could protect INDO- and DIC-induced intestinal cell death in Caco-2 cell lines. The cytoprotective mechanisms involved with antioxidant capacity, inhibition of ER stress and prevention of mitochondrial dysfunction.

1.3 Objective

The objectives of this study were

1. To assess the protective effects of polyphenols on INDO- and DIC-induced Caco-2 cell death.
2. To investigate the mechanisms of polyphenols in protecting NSAIDs-mediated cell death, and their association with reduction of oxidative stress, ER stress and mitochondrial dysfunction.

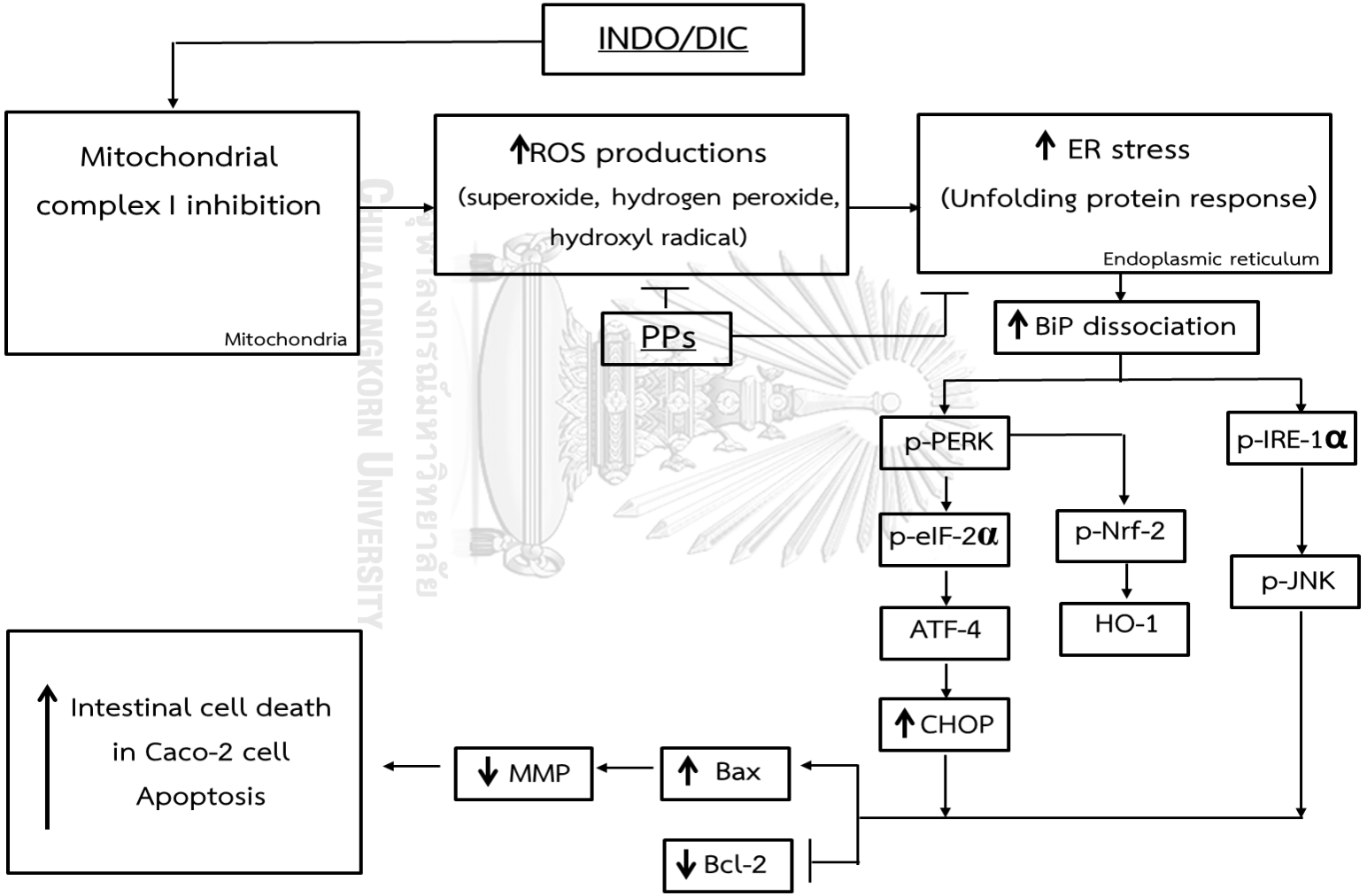
1.4 Scope of study

The present study demonstrated the mechanistic effects of INDO and DIC on induction of Caco-2 cell death, which may implicate enteropathy. This information may suggest the protective venues and molecular targets that are useful for the prevention of enteropathy. In addition, this research on the cytoprotective effect of natural polyphenolic compounds might add values to Thai herbal supplement and traditional medicine.

The study was divided into 2 parts as follows:

1. Determination of the protective effects of polyphenolic compounds on INDO- and DIC-induced intestinal Caco-2 cytotoxicity and their association with antioxidant capacity, inhibition of ER stress and prevention of mitochondrial dysfunction.
2. Investigation of underlying protective mechanism of polyphenolic compounds against ER stress-induced apoptosis by interference of ER stress signaling pathway.

1.5 Conceptual framework



CHAPTER II

LITERATURE REVIEW

2.1 NSAIDs-associated enteropathy

NSAIDs such as aspirin, indomethacin, diclofenac, ibuprofen or celecoxib are therapeutic agents for relieving inflammation, pain and fever as well as for the treatment of chronic inflammatory diseases including osteoarthritis and rheumatoid arthritis (Matsui et al., 2011). Despite its therapeutic efficacy, NSAIDs are associated with a high incidence of several adverse effects particularly gastric ulcer and enteropathy (Boelsterli et al., 2013; Chávez-Piña et al., 2018; Matsui et al., 2011; Utzeri and Usai, 2017). INDO and DIC are the most frequently reported NSAIDs to induce intestinal injury in the clinical trials (Boelsterli et al., 2013; Chávez-Piña et al., 2018; Matsumoto et al., 2008; Sigthorsson et al., 1998). For examples, INDO (100–150 mg/day) and DIC (75-150 mg/day) increased intestinal damage and intestinal permeability in Caucasian volunteers after treatment for 6 months (Sigthorsson et al., 1998). DIC damaged the small intestine in Japanese population after exposure for 2 year period (Matsumoto et al., 2008). In addition, INDO and DIC were reported to induce intestinal toxicity in experimental animal and cell culture models (Carrasco-Pozo et al., 2011; Satoh et al., 2014; Sigthorsson et al., 2002). For instant, INDO (3.5-15 mg/kg, p.o.) and DIC (10 mg/kg, p.o.) caused ileum mucosal damages after 24-h treatment in mice model (Satoh et al., 2014; Sigthorsson et al., 2002). In Caco-2 cells model, INDO (250 μM) was able to induce intestinal cell death after 1-h treatment (Carrasco-Pozo et al., 2011).

2.2 Mechanisms of INDO- and DIC-induced intestinal cell death

The molecular mechanisms of INDO- and DIC-induced enteropathy have been linked to production of ROS in various cell models such as Caco-2, RIE and IEC-6 cells (Carrasco-Pozo et al., 2011; Fornai et al., 2014; LoGuidice et al., 2010; Narabayashi et

al., 2015; Omatsu et al., 2009; Sandoval-Acuña et al., 2012; Somasundaram et al., 1997). INDO and DIC (250 μM) were capable of inhibiting mitochondrial complex I activity after treatment for 20 min in Caco-2 cells, leading to ROS-mediated cells death (Sandoval-Acuña et al., 2012). INDO (5 and 30 mg/kg, p.o.) caused small intestinal ulcer in rats as a result of inhibition of electron transport chain and induction of oxidative stress (Somasundaram et al., 1997). DIC (8 mg/kg, p.o.) could trigger jejunum and ilium damages via inhibition of mitochondrial respiratory chain and productions of oxidative stress in rats after exposure for 4 and 7 days (Fornai et al., 2014).

Cellular oxidative stress is associated with reducing the protein folding capacity of ER (i.e., ER stress). ER stress leads to accumulation and aggregation of unfolded protein response (UPR) as well as induction of apoptotic cell death (Iurlaro and Muñoz-Pinedo, 2016; Li et al., 2015; Sano and Reed, 2013). An increase of UPR within ER is linked to auto-phosphorylation of ER transmembrane receptors such as pancreatic-like ER kinase (PERK), activating transcription factor-6 (ATF-6) or inositol-requiring enzymes-1α (IRE-1α), as shown in Figure 1 (Engin, 2016; Iurlaro and Muñoz-Pinedo, 2016; Li et al., 2015; Sano and Reed, 2013). The activation of these three ER sensors commonly induces an up-regulation of the C/EBP homologous transcription factor (CHOP), leading to an increase of the Bax (pro-apoptotic) and a decrease of Bcl-2 (anti-apoptotic) expressions. Consequently, opening of the mitochondrial permeability transition pore (mPT pore) and loss of mitochondrial membrane potential (MMP) occur, leading to apoptosis (Engin, 2016; Li et al., 2015; Sano and Reed, 2013). In addition, the activation of IRE-1α-mediated JNK1/2 protein, another CHOP-independent signaling pathway, is linked directly to decreased Bcl-2 expression and subsequent apoptotic cells death (Iurlaro and Muñoz-Pinedo, 2016; Zhang et al., 2019). Moreover, two ER sensor proteins, PERK and IRE-1α signaling pathways, have been reported to in apoptosis induction at initiation phase during ER stress (Ishiwata-Kimata et al., 2018; Iurlaro and Muñoz-Pinedo, 2016; Merksamer and Papa, 2010; Zhang et al., 2019). In parallel to

PERK/CHOP activation, phosphorylation of PERK has been associated with up-regulation of detoxifying enzymes such as heme oxygenase (HO-1) expression through phosphorylation of transcription factor Nrf-2 (Figure 2) (Fujiki et al., 2019; Kim et al., 2007; Zhang et al., 2019; Zhu et al., 2019).

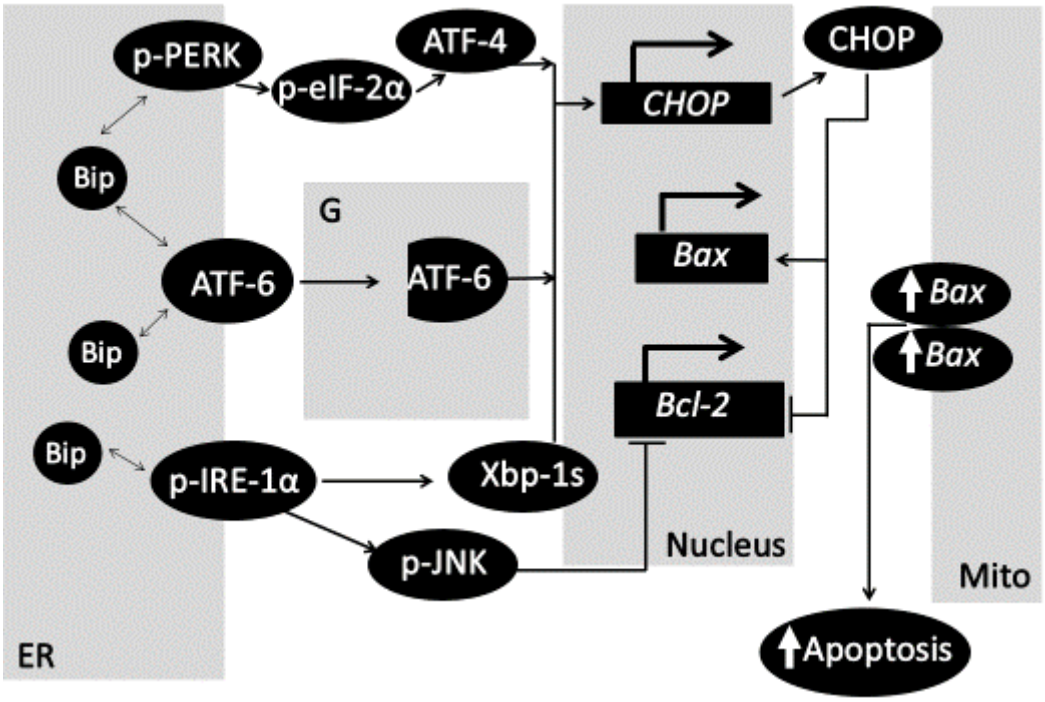


Figure 1. Activation of ER stress proteins-mediated cellular apoptosis (Engin, 2016; Sano and Reed, 2013). [ATF-4, activating transcription factor-4; ATF-6, activating transcription factor-6; Bip/GRP78, binding of immunoglobulin protein; CHOP, C/EBP homologous transcription factor; ER, endoplasmic reticulum; eIF-2α, eukaryotic Initiation factor-2α; G, Golgi apparatus; IRE-1α, inositol-requiring enzymes-1α; JNK1/2, c-Jun N-terminal kinases1/2; Mito, mitochondria; PERK, pancreatic-like ER kinase; p, phosphorylation; sXBP-1, spliced X-box-binding protein-1].

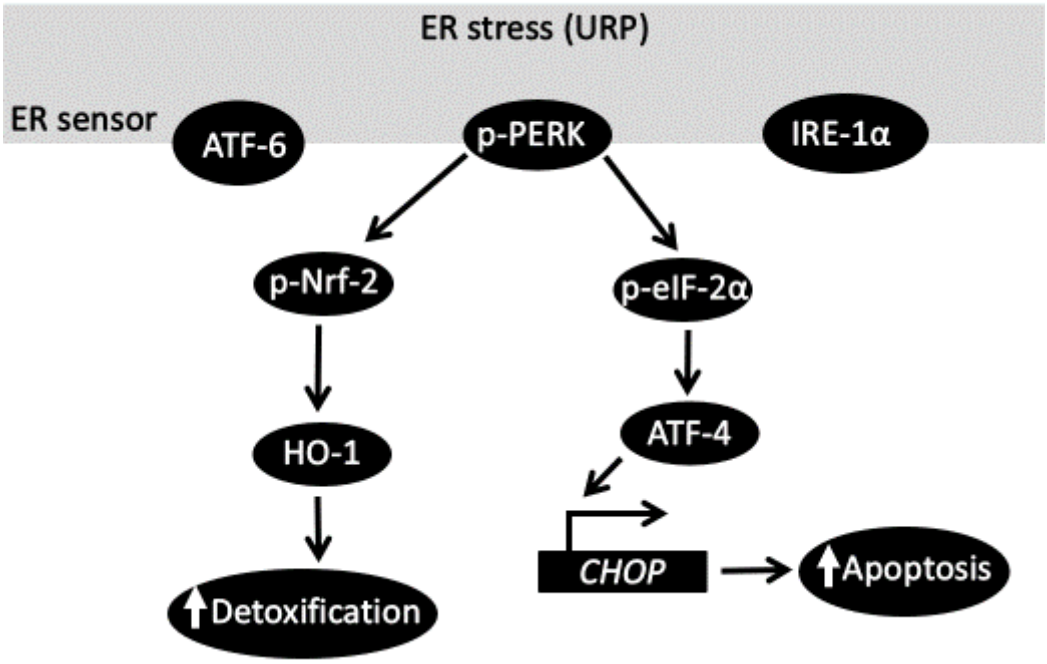


Figure 2. Activation of PERK-mediated HO-1 detoxification enzyme by phosphorylation of Nrf-2 protein along with CHOP-related ER pathway (Fujiki et al., 2019; Kim et al., 2007; Zhang et al., 2019; Zhu et al., 2019). [ATF-4, activating transcription factor-4; CHOP, C/EBP homologous transcription factor; ER, endoplasmic reticulum; eIF-2α, eukaryotic initiation factor-2α; HO-1, heme oxygenase-1; IRE-1α, inositol-requiring enzymes-1α; Nrf-2, nuclear factor erythroid 2-related factor 2; PERK, pancreatic-like ER kinase; p, phosphorylation; UPR, unfolded protein response].

Recent studies demonstrated that INDO and DIC were able to cause excess number of misfolded proteins in the ER, which might be associated with an induction of apoptotic cell death in Huh-7, H-4, U-87, primary pig gastric, rat intestinal, IEC-6 and animal models (Chang et al., 2020; Franceschelli et al., 2011; LoGuidice et al., 2010; Mügge and Silva, 2015; Narabayashi et al., 2015; Niu et al., 2014; Ohyama et al., 2012; Tsutsumi et al., 2004). For example, GI tract tissues of mouse given DIC (60-100 mg/kg, i.p.) for 6 h showed high expression levels of ER stress marker protein GRP78 (Bip) and CHOP (LoGuidice et al., 2010; Ohyama et al., 2012). DIC was also demonstrated to induce mitochondrial dysfunction-mediated cytotoxicity via up-regulation of CHOP expression in intestinal IEC-6 cells. In addition, INDO (200 μM) was reported to cause apoptosis in IEC-6 enterocyte via CHOP-related ER pathway after 24-h treatment (Narabayashi et al., 2015). INDO (250 μM) was reported to induce ROS production in H-4 and U-87 cells after 60-min treatment, leading to activation of PERK/CHOP pathway and subsequent apoptosis within 5 h (Chang et al., 2020). In guinea pig gastric mucosal cell model, INDO (1000 μM) mediated ER stress and consequent apoptosis after 18-h treatment by activating three ER sensors including PERK/CHOP, IRE-1α/JNK1/2, IRE-1α/XBP-1 and ATF-6 signaling pathways (Tsutsumi et al., 2004). Furthermore, the *CHOP*-knockout pig gastric cells decreased INDO-induced cytotoxicity under the same condition (Tsutsumi et al., 2004). INDO (500 nM) and DIC (300 μM) were demonstrated to induce ER stress-related apoptosis in Huh-7 cells after 16-h exposure via activation of PERK/CHOP pathway (Franceschelli et al., 2011). Furthermore, only IRE-1α/JNK1/2-related ER pathway was suggested to involve in INDO-treated cells, but not in DIC-treated cells (Franceschelli et al., 2011). Taken together, oxidative stress-mediated ER stress might be primary biochemical lesion of INDO- and DIC-induced intestinal cell death.

2.3 Pharmacological properties of polyphenols

Polyphenolic compounds such as caffeic acid (CAF), curcumin (CUR), epigallocatechin gallate (EGCG), gallic acid (GAL), hypophyllanthin (HYPO), naringenin (NAR), phyllanthin (PHY), piperine (PIP), quercetin (QUE), rutin (RUT) and silymarin (SLY), are commonly found in fruits, vegetables and herbal supplements (Table 1) (Gharras, 2009; Martin and Bolling, 2015). These compounds demonstrated cytoprotective effects against oxidative stress-mediated intestinal cell death possible through multi-mechanisms involving anti-oxidative stress, anti-ER stress and mitochondrial protection (Al-Rejaie et al., 2013; Brückner et al., 2012; Carrasco-Pozo et al., 2016; Carrasco-Pozo et al., 2011; Cheung et al., 2014; Chirdchupunseree and Pramyothin, 2010; Krithika et al., 2015; Krithika and Verma, 2009; Krithika et al., 2011; Li and Kuemmerle, 2018; Martin and Bolling, 2015; Natsume et al., 2009; Seiquer et al., 2015; Suganya et al., 2014).

Table 1. Polyphenol content in fruits, vegetables and herbal supplements

Polyphenols	Sources	Polyphenol content (mg/kg fresh weight)	References
CAF	Coffee Prune	0.03–11	(Fujioka and Shibamoto, 2006; Nakatani et al., 2000)
CUR	Curry Turmeric	2852-22130	(Suresh et al., 2007; Tayyem et al., 2006)
EGCG	Green tea	400-500	(Saklar et al., 2015)
GAL	Garlic	340	(Chen et al., 2013)
NAR	Grape Orange	50-300	(Gharras, 2009; Ho et al., 2000)
PIP	Black pepper	130	(Gorgani et al., 2017)
QUE	Onion	57	(Rodríguez Galdón et al., 2010)
RUT	Tomato	33	(Raffo et al., 2006)
SLY	Milk thistle	15	(Habán et al., 2009)

Polyphenols	Sources	Polyphenol content (mg/kg fresh weight)	References
HYPO	Phyllanthus plant	320	(Tripathi et al., 2006)
PHY		530	

For example, polyphenols riched-extraction of virgin argan oil was able to protect Caco-2 cytotoxicity from ROS-mediated cell death. Its protective mechanism was linked to its ability to scavenge ROS (Seiquer et al., 2015). Polyphenols extracted from apple plea (0.1 μg/mL) protected against INDO-produced superoxide radical production in Caco-2 cells by preventing mitochondrial dysfunction (Carrasco-Pozo et al., 2011). QUE (50 and 100 mg/kg, p.o.) attenuated ROS levels and inflammation in GI mucosa in rats treated with INDO (10 mg/kg, p.o.) (Carrasco-Pozo et al., 2016). This protective effect involved activation of Nrf-2 and suppression of NF-kB signaling pathway, resulting in increase of antioxidant enzyme activities such as superoxide dismutase and glutathione peroxidase (Carrasco-Pozo et al., 2016). HYPO and PHY were reported to major compounds in Phyllanthus plant (Jia et al., 2016; Kandhare et al., 2013; Krithika and Verma, 2009; Krithika et al., 2011). Furthermore, extract of *Phyllanthus amarus* plant (100 mg/kg, p.o.) protected carbon tetrachloride-induced liver damage in mice models through antioxidant-related mechanisms (Krithika and Verma, 2009). In addition, PHY (15 μg/ml) exerted hepatoprotective agent in carbon tetrachloride-mediated cell death in carp primary hepatocytes (Jia et al., 2016).

Three polyphenolic compounds, EGCG, QUE and RUT were suggested to protect ER stress-induced cytotoxicity and tissue damages from several ER stressors (Chen et al., 2015; Enogieru et al., 2019; Karthikeyan et al., 2017; Natsume et al., 2009; Suganya et al., 2014). For instance, EGCG (10 μM) prevented H_2O_2 and thapsigargin-induced apoptosis with suppression of PERK and IRE-1α-related ER signaling pathways

in MRPE cells (Karthikeyan et al., 2017). EGCG (100 mg/kg, i.p.) exerted to prevent nephron apoptosis from cisplatin-mediated ER stress after 48-h administration in mice model through inhibition of PERK/CHOP signaling pathway (Chen et al., 2015). In addition, RUT (25-100 μM) was suggested to prevent reduction of cells viability from MPP^+ in SH-SY5Y cells via inhibition of CHOP-related ER pathway (Enogieru et al., 2019). In intestinal LS180 and Caco-2 cell models, QUE (25-150 μM) was demonstrated to prevent ER stress-induced apoptosis in A23187- and thapsigargin-treated cells by inhibiting PERK/CHOP and IRE-1α/XBP-1 activations (Natsume et al., 2009). CAF (50 μM, p.o.) was reported to decrease hepatic steatosis from high fat diet-induced obese mice with inhibition of IRE-1α-related ER and induction of autophagy mechanisms (Kim et al., 2018). NAR (1 μM) also decreased ER stress in hepatitis-C infection- and tunicamycin-treated Huh-7.5.1 cells by inhibiting the IRE-1α signaling pathway (Jia et al., 2019). Thus, polyphenolic compounds might be cytoprotective agents against intestinal damage from NSAIDs treatment through their association with anti-oxidation, anti-ER stress and mitochondrial dysfunction.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Polyphenolic compounds

The polyphenolic compounds in this study were caffeic acid (CAF), curcumin (CUR), epigallocatechin gallate (EGCG), gallic acid (GAL), hypophyllanthin (HYPO), naringenin (NAR), phyllanthin (PHY), piperine (PIP), quercetin (QUE), rutin (RUT) and silymarin (SLY). They were commercial available as follow: CAF, HYPO, EGCG, NAR, PHY, PIP, QUE were from Sigma Chemical Co. (St Louis, MO, USA) and CUR, GAL, RUT and SLY were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

3.1.2 Chemicals and reagents

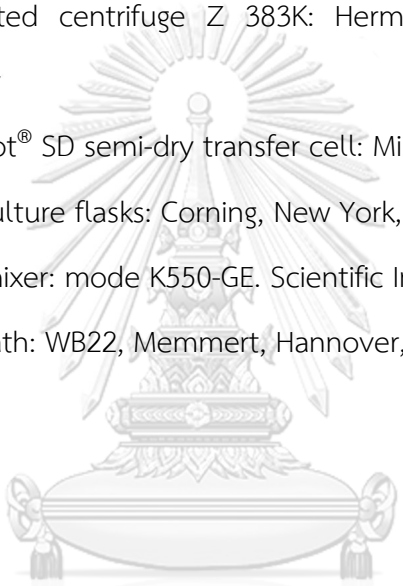
Two NSAIDs, diclofenac (DIC), indomethacin (INDO), and other compounds such as bovine serum albumin (BSA), bromophenol blue, dihydroethidium (DHE), dimethyl sulfoxide (DMSO), 2′, 7′-dichlorofluorescin diacetate (DCFH-DA), ethylenediaminetetraacetic acid (EDTA), glycerol, Hoechst 33342, hydrogen peroxide (H_2O_2), hydroxyphenyl fluorescein (HPF), ML385, β-mercaptoethanol, non-essential amino acid, paraformaldehyde, penicillin, phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktails, rotenone (ROT), sodium chloride (NaCl), sodium dodecyl sulfate (SDS), sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), streptomycin, tetramethylrhodamine (TMRE), thapsigargin (TGN), tris hydrochloride (Tris-HCl), tritron-X 100, trypsin, tunicamycin (TUN) and tween 20 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, methylthialazole tetrazolium (MTT) and TRIzol® reagent were purchased from Gibco Life Technologies (Grand Island, NY, USA). The ImProm-II™ reverse transcription system was purchased from Promega (Madison, WI, USA). The Immobilon-P PVDF membranes was purchased from Merck Millipore (Darmstadt,

Germany). Oligonucleotide primers were synthesized by Integrated DNA Technologies (The Gemini Singapore science Park II, Singapore). Pierce™ BCA protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The SsoFast™ EvaGreen® Supermix kit was purchased from Bio-Rad (Berkeley, CA, USA). The mouse monoclonal anti-ATF-4, anti-Bax, anti-Bcl-2, anti-CHOP, anti-eIF-2α, anti-HO-1, anti-IRE-1α, anti-Nrf-2 and anti-PERK were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The mouse monoclonal anti-GAPDH and secondary goat anti-mouse IgG (H&L) horseradish peroxidase (HRP) were purchased from Calbiochem (San Diego, CA, USA). The rabbit monoclonal anti-p-eIF-2α, anti-JNK1/2, anti-p-JNK1/2 anti-p-PERK and secondary goat anti-rabbit IgG (H&L) horseradish peroxidase (HRP) were purchased from Cell Signaling Technology (Beverly, MA, USA). The rabbit monoclonal anti-p-IRE-1α was purchased from Affinity Biosciences (Cincinnati, OH, USA). The rabbit monoclonal anti-p-Nrf-2 was purchased from Boster Bio (Pleasanton, CA, USA). SuperSignal® West Pico Chemiluminescent Substrate was purchased from Pierce Biotechnology (Rockford, IL, USA).

3.1.3 Experimental instruments

1. Autoclave: Hirayama, Saitama, Japan
2. CFX96 Touch™ Real-Time PCR Detection System: Bio-rad, Hercules, CA, USA
3. Fluorescence microscope: BX-FLA, Olympus, Tokyo, Japan
4. GE ImageQuant LAS 4000: GE Healthcare Life-Sciences Ltd., Branch, Taiwan
5. Hot air oven: MEMMERT, Buchenbach, Germany
6. Humidified carbon dioxide incubator: Forma Scientific, Marietta, OH, USA
7. Image J program: NIH, Bethesda, MD, USA
8. Inverted microscope: Axiovert 135, Zeiss, Konstanz, Germany

9. Microplate reader: Wallac 1420 VICTOR 3, PerkinElmer Inc., Hopkinton, MA, USA
10. Multiwell plates: Corning, New York, NY, USA
11. NanoDrop™ 2000/2000c Spectrophotometers: Thermo Scientific, Wilmington, UK
12. OmniPAGE mini vertical systems: Cleaver Scientific, Warwickshire, UK
13. pH meter: CG 842, Schott, Hofheim, Germany
14. Refrigerated centrifuge Z 383K: Hermle Labortechink, Burladingen, Germany
15. Trans-blot® SD semi-dry transfer cell: Missouri, TX, USA
16. Tissue culture flasks: Corning, New York, NY, USA
17. Vortex mixer: mode K550-GE. Scientific Industries, New York, NY, USA
18. Water bath: WB22, Memmert, Hannover, Germany



3.1.4 Cell culture and treatments

Human colon adenocarcinoma Caco-2 cells (ATCC® HTB-37™) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in DMEM supplemented with 2.5 mM L-glutamine, 100 U/L penicillin-streptomycin mixtures, 1% nonessential amino acid and 10% FBS at 37 ^{o}C in a controlled atmosphere at 5% CO_2 and 90% relative humidity. The cells were sub-cultured at 80% confluence, using 0.25% trypsin solution containing 1 mM EDTA.

For experiments, the cells (passage numbers 54 to 70) were cultured at the seeding density of 6.25 x 10^4 cells/cm^2 for 24 h. Then, the cells were treated with the test compounds at non-toxic concentration [CAF (100 µM), CUR (50 µM), EGCG (100 µM), GAL (100 µM), HYPO (10 µM), NAR (100 µM), PHY (10 µM), PIP (10 µM), QUE (100 µM), RUT (100 µM), SLY (100 µM) or ML385 (an Nrf-2 inhibitor; 10 µM)] for 24 h, followed by an ER stressor (INDO 1000 µM, DIC 1000 µM, TUN 20 µg/ml, or TGN 2 µM) for another 48 h and 72 h.

3.2 Methods

3.2.1 Cell viability

Cell viability was evaluated by MTT assay. After treatment period, the cells were washed three times with PBS and replaced with MTT solution (0.5 mg/ml) for 4 h. Then, the formazan crystals were dissolved with 100% (V/V) DMSO. The optical density was read by a microplate reader at an absorbance of 570 nm.

3.2.2 Determination of reactive oxygen species (ROS) productions

Intracellular ROS including superoxide anion, hydrogen peroxide, and hydroxyl radical were measured by DHE, DCFH-DA and HPF assay, respectively. Cells were treated with specific fluorescent probes (DHE; 10 μM, DCFH-DA; 100 μM or HPF; 10 μM) for 30 min, followed by addition of either INDO or DIC for 2 h. In this study, ROT (20 μM; superoxide anion generator) and hydrogen peroxide (1 mM; hydroxyl radical generator) were used as the positive control groups. At the end of treatment period, the cells were lysed with 1% Triton X-100. The fluorescent intensity was measured by a microplate reader at the excitation/emission wavelengths of 470/590 nm (for DHE), 485/535 nm (for DCF) and 510/595 nm (for HPF).

The effect of polyphenolic compounds on NSAIDs-mediated ROS production was also determined. The cells were treated with the test polyphenolic compounds for 2 h prior to addition of fluorescent probes and NSAIDs, as described above.

3.2.3 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The mRNA levels of *Bax*, *Bcl-2* and *CHOP* were quantified by qRT-PCR technique. In brief, Caco-2 cells were pre-treated with the test polyphenolic compounds for 24 h, followed by addition of either INDO or DIC for another 48 h. After the 48-h treatment period, the cells were washed three times with ice-cold PBS, followed by treatment with TRIzol® reagent for RNA extraction according to manufacturer's instructions. The amounts of RNA samples were quantified using a NanoDrop™ 2000/2000c spectrometer at the wavelengths 260 and 280 nm and reversely transcribed to cDNA according ImProm-II™ reverse transcription system. The cDNA was amplified by using SsoFast™ EvaGreen® Supermix kit on CFX96 touch™ real-time PCR detection system with specific primers listed in Table 2. The conditions of qPCR were as follows: an initial denaturing at 95 ^{o}C for 2 min, 40 cycles of denaturing at 95 ^{o}C for 5 sec, annealing and extension at 60 ^{o}C for 5 sec. The mRNA content was normalized relatively to *GAPDH* and calculated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 2. Primers were used for qRT-PCR analyses.

Gene	Oligonucleotides	Sequences (5'-3')	References
Bax	Forward Reverse	CCTTTTCTACTTTGCCAGCAAAC GAGGCCGTCCCAACCAC	(Wu et al., 2016)
Bcl-2	Forward Reverse	ATGTGTGTGGAGAGCGTCAACC GCATCCCAGCCTCCGTTATC	(Zhang et al., 2016)
CHOP	Forward Reverse	CTTGGCTGACTGAGGAGGAG TCACCATTCGGTCAATCAGA	(Caputo et al., 2012)
GAPDH	Forward Reverse	AGGTCGGAGTCAACGGATTTGGT ATGGCATGGACTGTGGTCATGAGT	(van der Linden et al., 2014)

3.2.4 Determination of mitochondrial membrane potential (MMP)

Mitochondrial function was evaluated by TMRE assay (Majewski et al., 2004). TMRE is a cationic lipophilic fluorochrome accumulating in the mitochondrial matrix when the mitochondrial membrane potential increases.

Caco-2 cells were pre-treated with the polyphenolic compounds for 24 h, followed by addition of either INDO or DIC for another 48 h or 72 h. At the end of treatment, the cells were washed three times with PBS and incubated with TMRE (1 μM) for 30 min. Then, the cells were washed three times with ice-cold PBS. The fluorescent intensity of TMRE in mitochondrial matrix was measured by a microplate reader at excitation wavelength 549 nm and emission wavelength 575 nm.

3.2.5 Apoptosis assay

Apoptotic cell death were determined by Hoechst 33342 staining method (Crowley et al., 2016). At the end of treatment period, cells were washed three times with PBS and stained with Hoechst 33342 (10 μM) for 30 min. Then, the cells were fixed with 4% paraformaldehyde for 15 min at 4 °C, followed by washing three times with ice-cold PBS. The cells with chromatin condensation and/or nuclei fragmentation were counted as apoptotic cells under a fluorescence microscope (20X, original magnification) at excitation wavelengths 350 nm and emission wavelengths 461 nm.

3.2.6 Western blot analysis

Expression of the apoptotic regulatory proteins (Bax and Bcl-2) and ER stress signaling proteins (PERK and IRE-1α pathway) were determined by western blot analysis.

At the end of treatment period, the cells were washed three times with ice-cold PBS and incubated with ice-cold lysis buffer containing: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Tritron-X 100, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktails for protein extraction. Cell lysate was then centrifuged at 18000 x g for 15 min at 4 °C and collected the supernatant for blotting analysis. Protein concentration was quantified using Pierce BCA protein assay kit according to the manufacturer's instructions.

Proteins (40 µg) was added into sample buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue) and denatured at 95 °C for 5 min. The proteins were loaded onto a 7-15% SDS-polyacrylamide gel electrophoresis, and separated at constant voltage of 100 V for 3 h. Then, the proteins were electrically transferred to PVDF membrane using constant voltage of 10 V for 1 h. After that, the membranes were blocked with 5% BSA in TBS-T for 30 min at room temperature and probed with primary antibodies for anti-ATF-4 (1:1000), anti-Bax (1:1000), anti-Bcl-2 (1:1000), anti-CHOP (1:1000), anti-eIF-2α (1:1000), anti-p-eIF-2α (1:1000), anti-HO-1 (1:1000), anti-IRE-1α (1:1000), anti-p-IRE-1α (1:1000), anti-JNK1/2 (1:1000), anti-p-JNK1/2 (1:1000), anti-Nrf-2 (1:1000), anti-p-Nrf-2 (1:1000), anti-PERK (1:1000), anti-p-PERK (1:1000) and GAPDH (1:2000) with TBS-T containing 3% BSA at 4 °C for overnight. After washing three times with TBS-T, the membranes were incubated at room temperature with secondary conjugated secondary antibody (1:2000) in TBS-T for 1 h and then developed by using the Super signal West Pico chemiluminescent substrates. The membranes were visualized by a GE ImageQuant LAS 4000. The

densitometry measurement of the bands was performed using Image J program. The expression of the apoptotic regulatory proteins and ER stress proteins were normalized to GAPDH as internal control.

3.2.7 Statistical analysis

All data were expressed as mean ± SEM and obtained from least three separate experiments. Statistical analyses were performed by either the Student's t test or one-way analysis of variance (ANOVA), followed by the Bonferroni post-hoc test. Differences were considered statistically significance when $P< 0.05$.

CHAPTER IV

RESULTS

4.1 Roles of polyphenolic compounds in NSAID-mediated cell death

4.1.1 Effects of INDO and DIC on ROS productions and the protective effect of polyphenolic compounds

As shown in Figure 3, INDO and DIC at 1000 μM significantly produced superoxide anion and hydrogen peroxide, but not hydroxyl anion in Caco-2 cells, after 2-h exposure as compared to the control group. Hence, the protective effect of eleven polyphenolic compounds (CAF, CUR, HYPO, EGCG, GAL, NAR, PHY, PIP, QUE, RUT and SLY) were further tested specifically to NSAIDs-induced production of superoxide anion and hydrogen peroxide. As shown in Figure 4A-D, the polyphenolic compounds including CAF, CUR, EGCG, GAL, NAR, QUE, RUT and SLY at concentrations up to 100 μM could scavenge the superoxide radical and hydrogen peroxide productions in Caco-2 cells after 2-h exposure to NSAIDs in concentration-dependent manner. However, three polyphenolic compounds, HYPO, PHY and PIP (10 μM) had no significant effect against NSAIDs-induced ROS productions. HYPO, PHY and PIP specifically reduced hydrogen peroxide level in the INDO- and DIC-treated cells, but not superoxide radical in NSAIDs-treated cells. In addition, the concentration 100 μM of HYPO, PHY and PIP caused cell detachment after co-incubation with either INDO or DIC. These results were summarized in Table 3.

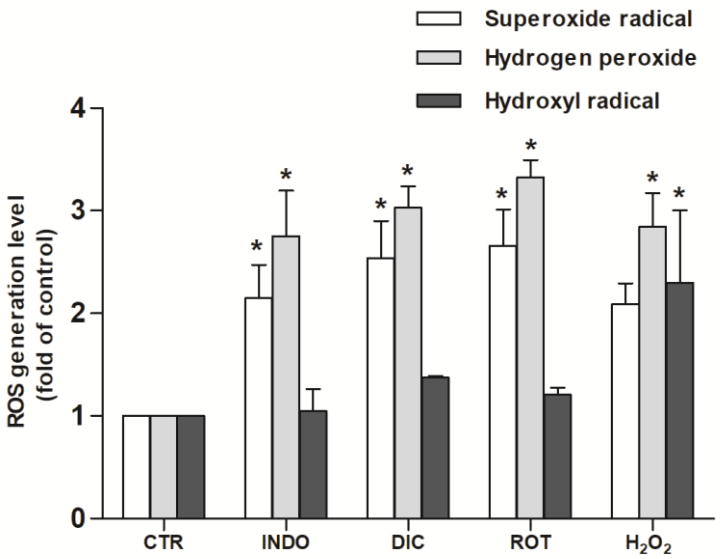
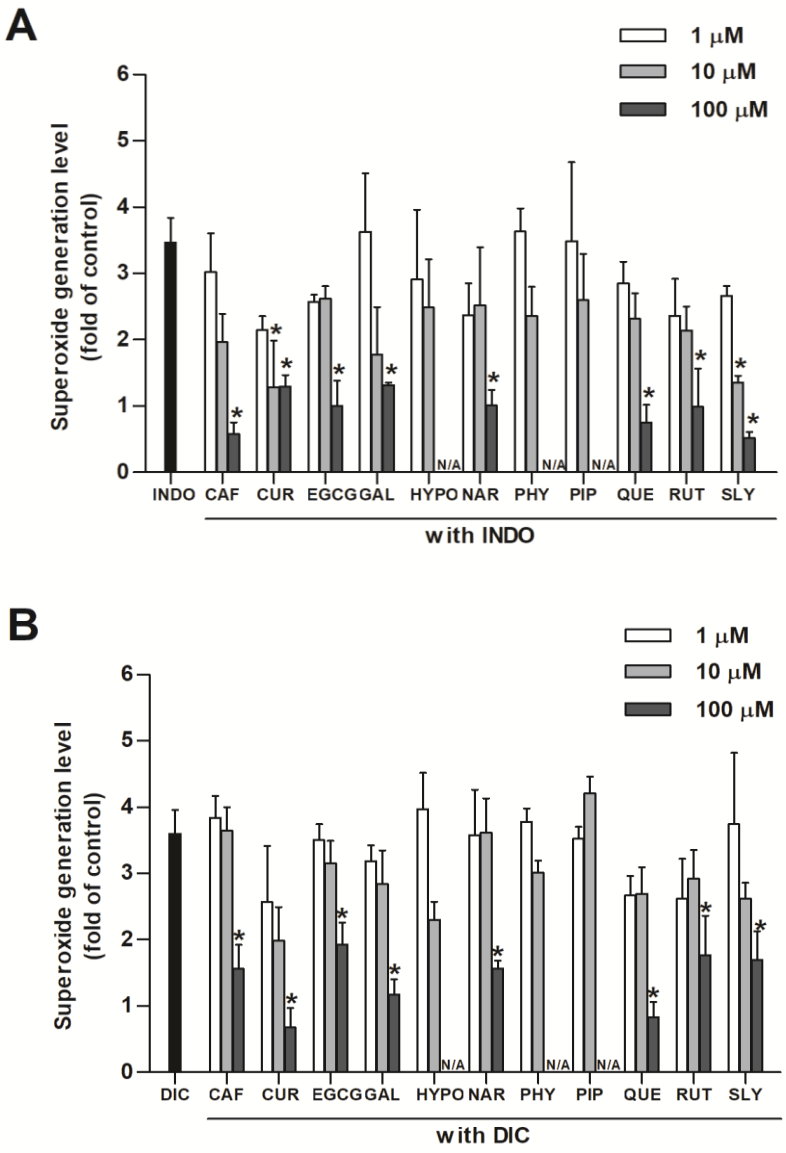


Figure 3. Effects of INDO and DIC (1000 μM) on ROS productions in Caco-2 cells after 2-h treatment. ROT (20 μM; a superoxide anion generator) and H_2O_2 (1000 μM; a hydroxyl radical generator) were used as positive control groups. Data are expressed as the percentage of the control. Each bar represents mean ± SEM (N=3). *P< 0.05 indicated statistically significant difference from the control (CTR).


A
1 μM
10 μM
100 μM
Superoxide generation level
(fold of control)
0
1
2
3
4
5
6
INDO
CAF
CUR
EGCG
GAL
HYPO
NAR
PHY
PIP
QUE
RUT
SLY
N/A
with INDO
B
1 μM
10 μM
100 μM
Superoxide generation level
(fold of control)
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RUT
SLY
N/A
with DIC

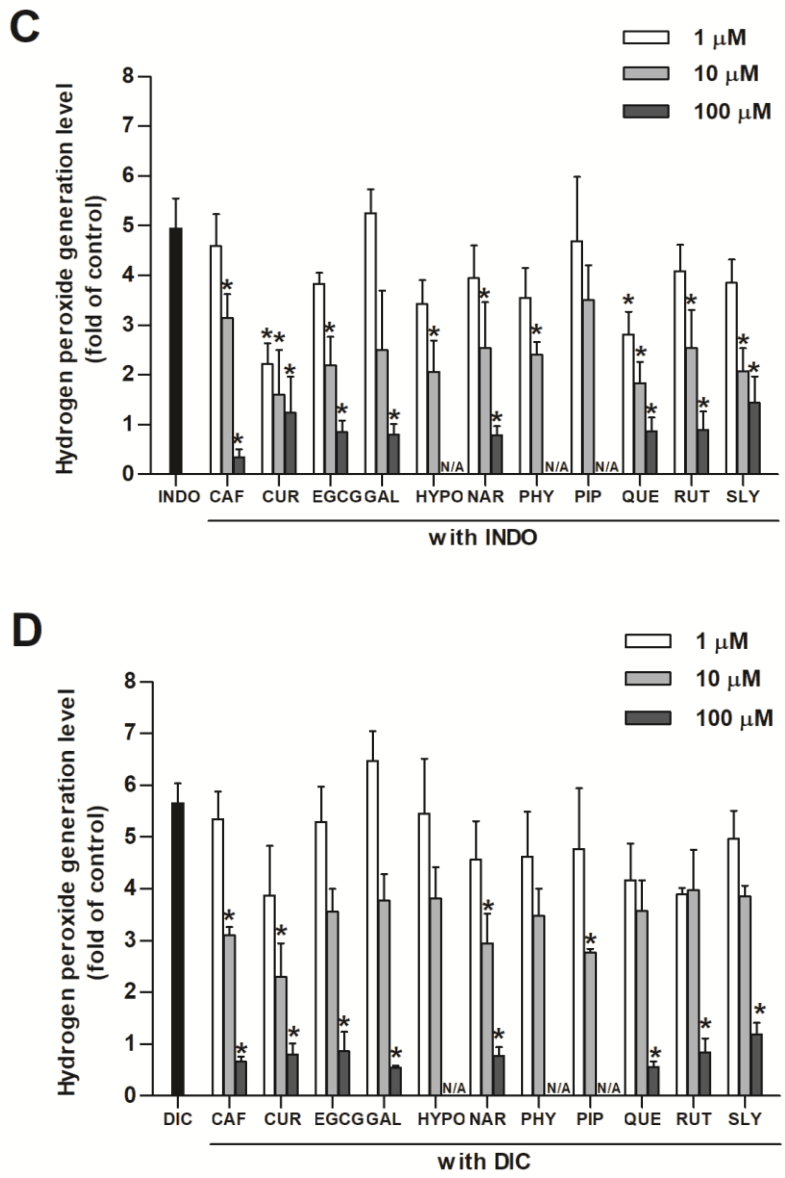


Figure 4. Effects of polyphenolic compounds against superoxide radical (**A, B**) and hydrogen peroxide (**C, D**) in Caco-2 cells treated with either 1000 μM INDO (**A, C**) or 1000 μM DIC (**B, D**) after 2-h treatment. Data are expressed as the percentage of the control. Each bar represents the mean ± SEM (N=3-4). **P*< 0.05 indicated statistically significant difference with NSAIDs alone group.

Table 3. Effects of polyphenolic compounds on INDO- and DIC-induced superoxide radical and hydrogen peroxide in Caco-2 cells after 2-h treatment

Polyphenolic compounds	INDO		DIC	
	Superoxide radical	Hydrogen peroxide	Superoxide radical	Hydrogen peroxide
CAF	/	/	/	/
CUR	/	/	/	/
EGCG	/	/	/	/
GAL	/	/	/	/
HYPO	-	/	-	-
NAR	/	/	/	/
PHY	-	/	-	-
PIP	-	-	-	/
QUE	/	/	/	/
RUT	/	/	/	/
SLY	/	/	/	/

/ Decreased; - Not decreased ROS production

4.1.2 Effects of INDO and DIC on cell death and the protective effect of polyphenolic compounds

As shown in Figure 5, INDO and DIC (1000 μM) significantly decreased cell viability in Caco-2 cells after 72-h treatment by approximately 50-55%. Five antioxidants, CAF, EGCG, NAR, QUE and RUT at the concentration of 100 μM were able to protect cytotoxicity in cells treated with INDO for 72 h. In addition, EGCG, QUE and RUT also prevented DIC-mediated cytotoxicity. On the contrary, CUR (50 μM), NAR (100 μM), PHY (10 μM), PIP (10 μM) and SLY (10 μM) significantly increased DIC-induced cytotoxicity. Two antioxidants, HYPO (10 μM) and GAL (10 μM) had no effects on NSAIDs-induced cytotoxicity. In this study, the concentration of each polyphenolic compounds was non-cytotoxic concentration after 96-h exposure. These results were summarized in Table 4.

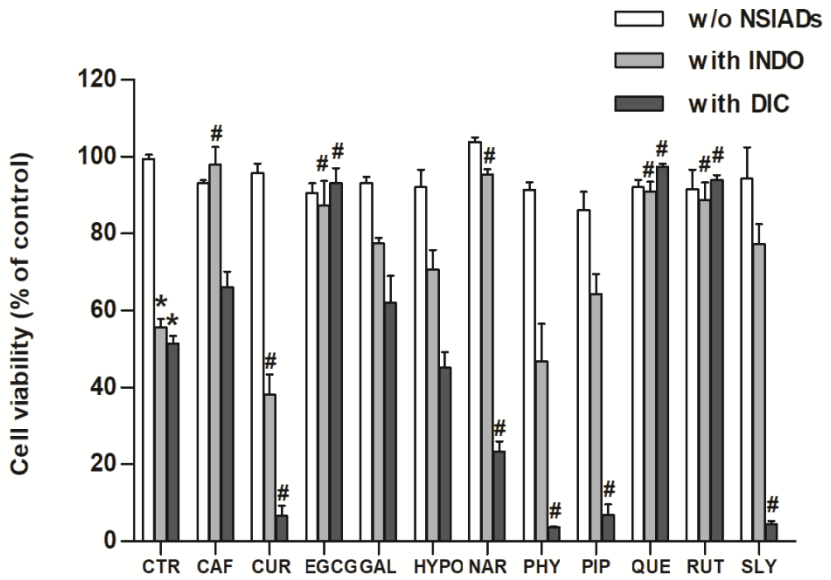


Figure 5. Effects of polyphenolic compounds on INDO- and DIC-induced cytotoxicity in Caco-2 cells after 72-h treatment. Data are expressed as the percentage of the control group. Each bar represents mean ± SEM (N=3). *P< 0.05 indicated statistically significant difference with the control; $^{\#}P$< 0.05 indicated statistically significant difference with the NSAIDs-treated group. [CAF (100 μM); CUR (50 μM); DIC (1000 μM); EGCG (100 μM); GAL (100 μM); HYPO (10 μM); INDO (1000 μM); NAR (100 μM); PHY (10 μM); PIP (10 μM); QUE (100 μM); RUT (100 μM); SLY (100 μM)].

Table 4. Effects of polyphenolic compounds on INDO- and DIC-induced cytotoxicity in Caco-2 cells after 72-h treatment

Polyphenolic compounds	INDO	DIC
CAF	/	-
CUR	+++	+++
EGCG	/	/
GAL	-	-
HYPO	-	-
NAR	/	+++
PHY	-	+++
PIP	-	+++
QUE	/	/
RUT	/	/
SLY	-	+++

/ Protected; - Not protected; +++ Enhanced cell death

Eight out of eleven polyphenolic compounds including CAF, EGCG, NAR, PHY, PIP, QUE, RUT and SLY were chosen for further investigation in their underlying protective mechanisms against NSAIDs-induced cell death.

4.1.3 Effects of polyphenolic compounds on INDO- and DIC-mediated up-regulation of *CHOP* expression

Increase of *CHOP* expression has been known as an indicator of ER stress in various cells (Li et al., 2015; Sano and Reed, 2013). As shown in Figure 6, the up-regulation of *CHOP* expression was observed in Caco-2 cells-treated with NSAIDs (1000 μM) for 48 h by approximately 2-4 fold. Only EGCG, QUE and RUT (100 μM) significantly suppressed the increased expression of *CHOP* mRNA in NSAIDs-treated Caco-2 cells. Five polyphenolic compounds, CAF (100 μM), NAR (100 μM), PHY (10 μM), PIP (10 μM) and SLY (100 μM) had no effects on the up-regulation of *CHOP* mRNA in NSAIDs-treated cells. These results were summarized in Table 5.

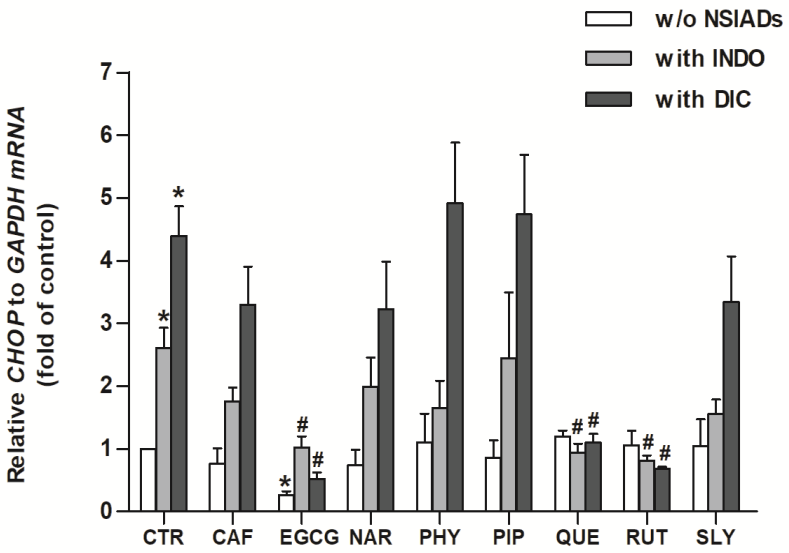


Figure 6. Effects of polyphenolic compounds on INDO- and DIC-induced the expression of *CHOP* mRNA in Caco-2 cells after 48-h treatment. Data are expressed as fold of control. Each bar represents mean ± SEM (N=3). *P< 0.05 indicated statistically significant difference with the control; $^{\#}P$< 0.05 indicated statistically significant difference with the NSAIDs-treated group (N=3). [CAF (100 μM); DIC (1000 μM); EGCG (100 μM); INDO (1000 μM); NAR (100 μM); PHY (10 μM); PIP (10 μM); QUE (100 μM); RUT (100 μM); SLY (100 μM)].

Table 5. Effects of polyphenolic compounds on INDO- and DIC-induced the expression of *CHOP* mRNA in Caco-2 cells after 48-h treatment

Polyphenolic compounds	INDO	DIC
CAF	-	-
EGCG	/	/
NAR	-	-
PHY	-	-
PIP	-	-
QUE	/	/
RUT	/	/
SLY	-	-

/ Suppressed; - Not suppressed

4.1.4 Effects of polyphenolic compounds on NSAID-mediated the up-regulation of *Bax* and down-regulation of *Bcl-2* mRNA

As shown in Figure 7A and B, INDO and DIC (1000 μM) were able to significantly up-regulate the pro-apoptotic *Bax* gene by approximately 3.4 fold, and down-regulate the anti-apoptotic *Bcl-2* by approximately 2 fold in Caco-2 cells after 48-h treatment. Three polyphenolic compounds, EGCG, QUE and RUT at concentration of 100 μM significantly suppressed the effects of INDO- and DIC-induced the increased *Bax*/anti-*Bcl-2* ratio, whereas PHY (10 μM), PIP (10 μM) and SLY (100 μM) had no effects as compared to the cells treated NSAIDs alone. The presence of NAR (100 μM) prevented on the effect of INDO-induced *Bax/Bcl-2* ratio, but not DIC. In addition, CAF (100 μM) suppressed the INDO-induced up-regulation of *Bax* mRNA, but this compound did not reverse the down-regulation of *Bcl-2* mRNA in INDO-treated cells. Moreover, CAF had no effect on DIC-mediated up-regulation of *Bax* and down-regulation of *Bcl-2* mRNA. These results were summarized in Table 6.

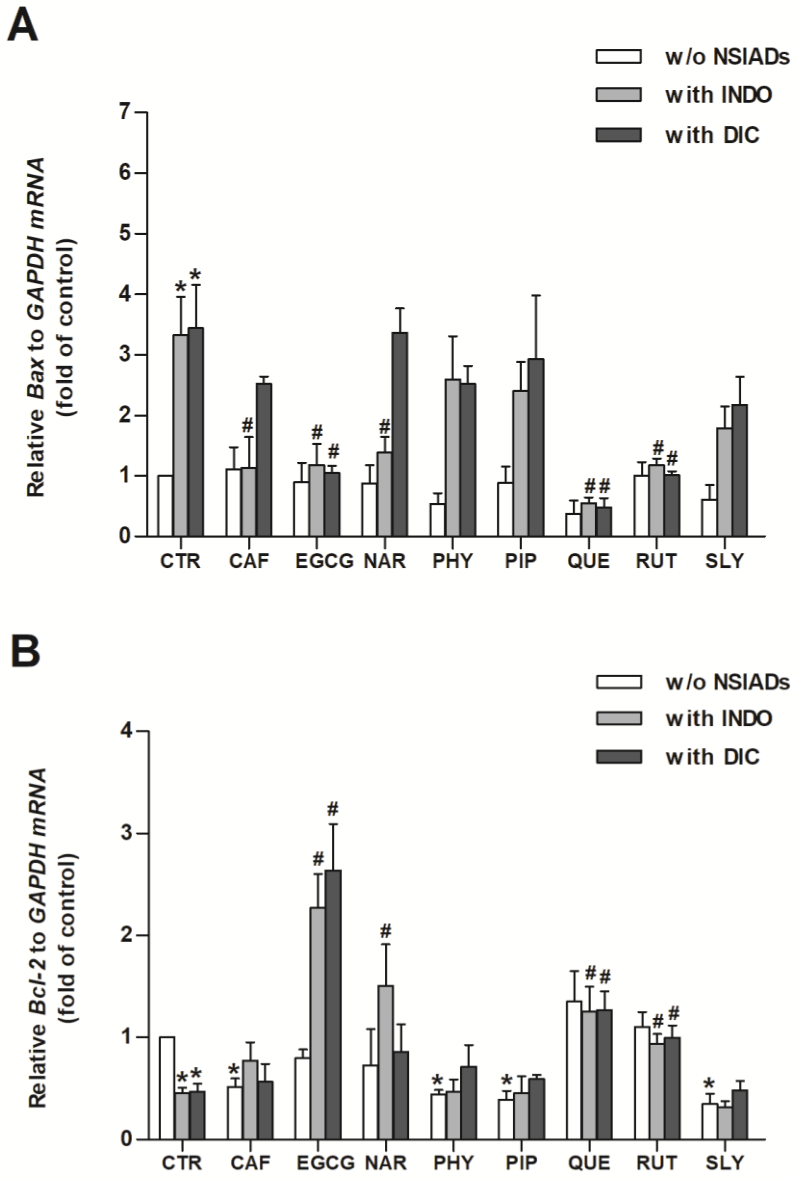


Figure 7. Effects of polyphenolic compounds on INDO- and DIC-induced up-regulation of *Bax* **(A)** and down-regulation of *Bcl-2* **(B)** expressions in Caco-2 cells after 48-h treatment. Data are expressed as the fold of the control. Each bar represents mean ± SEM (N=3-4). *$P< 0.05$ indicated statistically significant difference with the control; $^{\#}P< 0.05$ indicated statistically significant difference with the NSAIDs-treated group (N=3). [CAF (100 μM); DIC (1000 μM); EGCG (100 μM); INDO (1000 μM); NAR (100 μM); PHY (10 μM); PIP (10 μM); QUE (100 μM); RUT (100 μM); SLY (100 μM)].

Table 6. Effects of polyphenolic compounds on NSAID-mediated up-regulation of *Bax* and down-regulation of *Bcl-2* mRNA in Caco-2 cells after 48-h treatment

Polyphenolic compounds	INDO		DIC	
	Up-regulation of *Bax* mRNA	Down-regulation of *Bcl-2* mRNA	Up-regulation of *Bax* mRNA	Down-regulation of *Bcl-2* mRNA
CAF	/	-	-	-
EGCG	/	/	/	/
NAR	/	/	-	-
PHY	-	-	-	-
PIP	-	-	-	-
QUE	/	/	/	/
RUT	/	/	/	/
SLY	-	-	-	-

/ Suppressed; - Not suppressed

4.1.5 Effects of INDO and DIC on mitochondrial function and the protective effect of polyphenolic compounds

As shown in Figure 8, two NSAIDs, INDO and DIC (1000 μM) significantly altered the loss in MMP in Caco-2 cells after 72-h treatment by approximately 57-62%. Three polyphenolic compounds, EGCG, QUE and RUT at the concentration of 100 μM significantly prevented INDO- and DIC-induced loss in MMP after 72-h treatment. Furthermore, NAR (100 μM) was able to protect the loss in MMP in cells-treated with INDO. However, this compound significantly increased the loss in MMP in the cells treated with DIC. SLY (100 μM) significantly increased the effects of INDO- and DIC-altered the loss in MMP, as compared to Caco-2 cells treated NSAIDs alone after 72-h treatment. These results were summarized in Table 7.

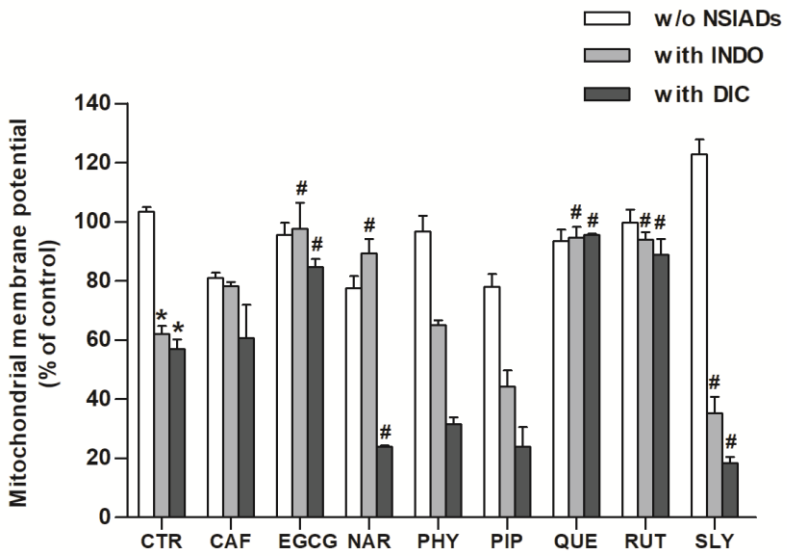


Figure 8. Effects of polyphenolic compounds on INDO- and DIC-induced mitochondrial dysfunction in Caco-2 cells after 72-h treatment. Data are expressed as the percentage of the control. Each bar represents mean ± SEM (N=3). *P< 0.05 indicated statistically significant difference with the control; $^{\#}P$< 0.05 indicated statistically significant difference with the NSAIDs-treated group. [CAF (100 μM); DIC (1000 μM); EGCG (100 μM); INDO (1000 μM); NAR (100 μM); PHY (10 μM); PIP (10 μM); QUE (100 μM); RUT (100 μM); SLY (100 μM)].

Table 7. Effects of polyphenolic compounds on NSAID-mediated loss in MMP in Caco-2 cells after 72-h treatment

Polyphenolic compounds	INDO	DIC
CAF	-	-
EGCG	/	/
NAR	/	+++
PHY	-	-
PIP	-	-
QUE	/	/
RUT	/	/
SLY	+++	+++

/ Protected; - Not protected; +++ Enhanced loss in MMP

4.2 The protective mechanism of polyphenolic compounds against ER stress-mediated apoptosis signaling pathway

EGCG, HYPO and PHY are three known antioxidants that were chosen for further investigation of the underlying protective mechanisms against ER stress-mediated apoptosis in Caco-2 cells exposed to ER stressors (INDO, DIC, TUN, TGN).

4.2.1 Effects of EGCG, HYPO and PHY on ER stressor-induced cytotoxicity

As shown in Figure 9, two NSAIDs (INDO and DIC; 1000 μM) and other ER stressors (TUN; 20 μg/ml and TGN; 2 μM) significantly reduced cells viability after 72-h exposure by approximately 50%, as measured MTT assay. At 100 μM, EGCG significantly protected on INDO-, DIC-, TUN- and TGN-induced cytotoxicity. HYPO (10 μM) had no effects against ER stressors-mediated cell death. In contrast, PHY (10 μM), as similar to ML385 (an Nrf-2 inhibitor; 10 μM), significantly increased cytotoxic effects of DIC, TUN and TGN, but not INDO by approximately 2-6 fold. These results were summarized in Table 8.

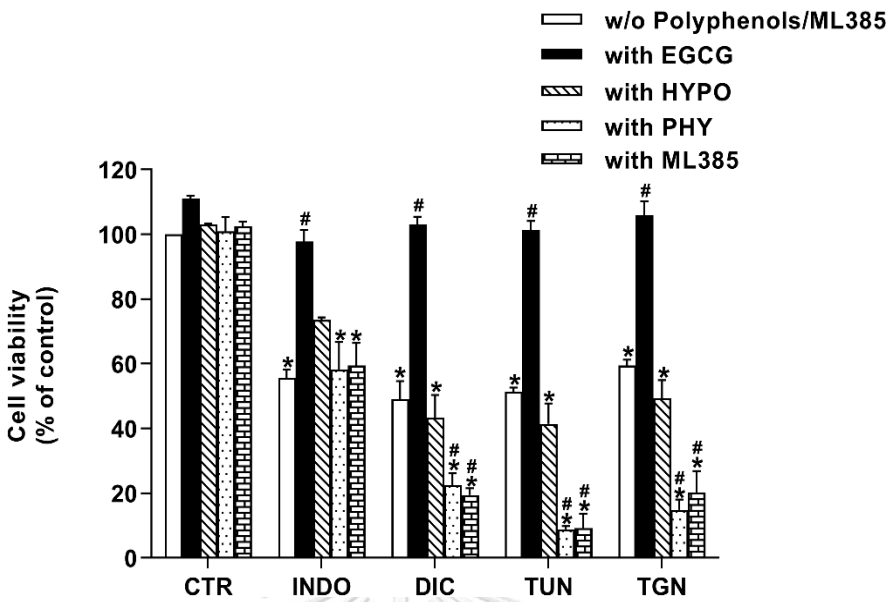


Figure 9. Effects of EGCG, HYPO, PHY and ML385 on ER stressor (INDO, DIC, TUN or TGN)-induced cytotoxicity in Caco-2 cells for 72 h. Data are expressed as the percentage of the control. Each bar represents mean ± SEM (N=3). *P< 0.05 indicated statistically significant difference with the control; #P< 0.05 indicated statistically significant difference with the stressors-treated alone group. [DIC (1000 μM); EGCG (100 μM); HYPO (10 μM); INDO (1000 μM); ML385 (10 μM); PHY (10 μM); TGN (2 μM); TUN (20 μg/ml)].

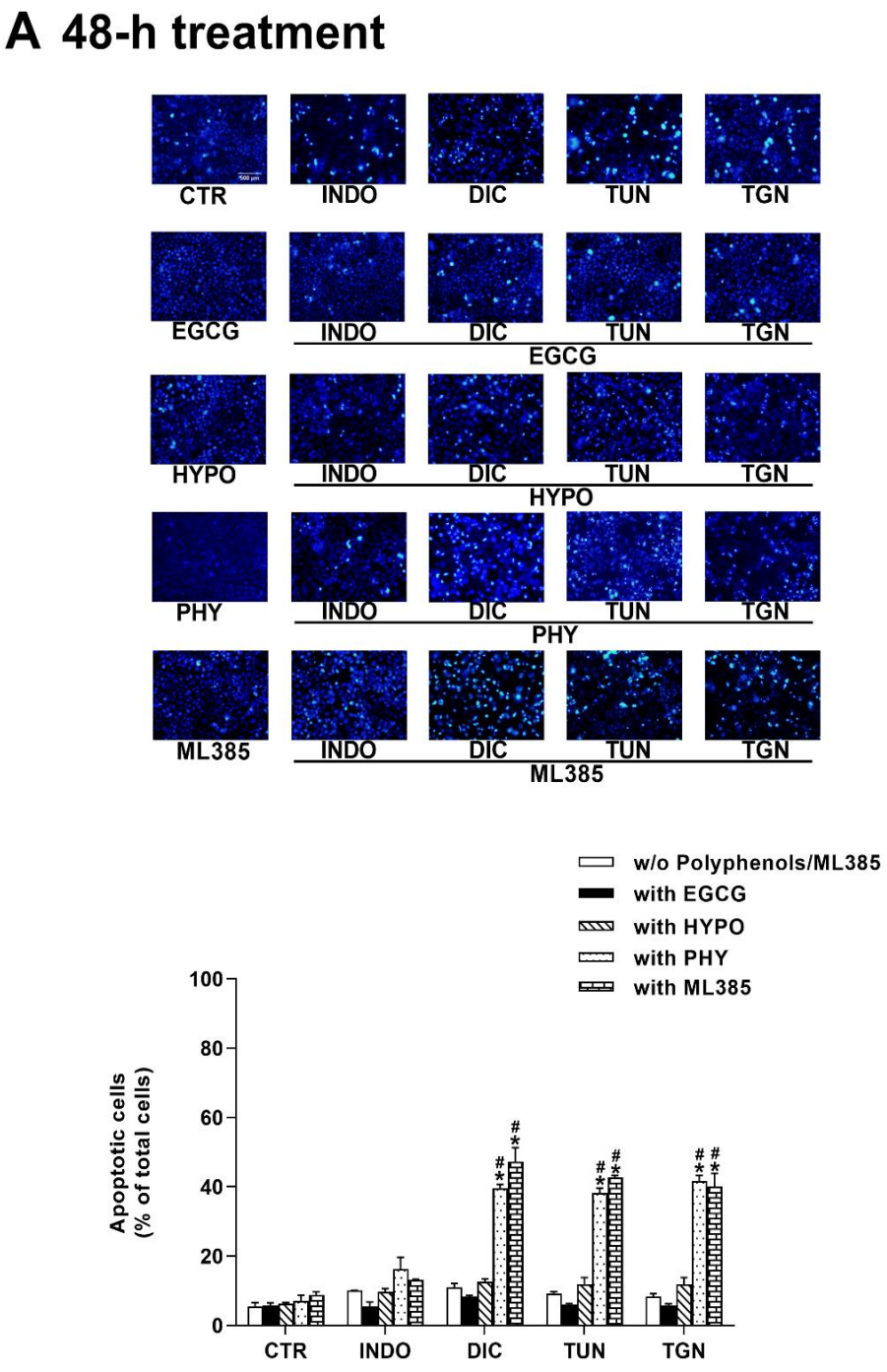
Table 8. Effects of EGCG, HYPO, PHY and ML385 on ER stressor (INDO, DIC, TUN or TGN)-induced cytotoxicity in Caco-2 cells after 72-h treatment

Test compounds	INDO	DIC	TUN	TGN
EGCG	/	/	/	/
HYPO	-	-	-	-
PHY	-	+++	+++	+++
ML385	-	+++	+++	+++

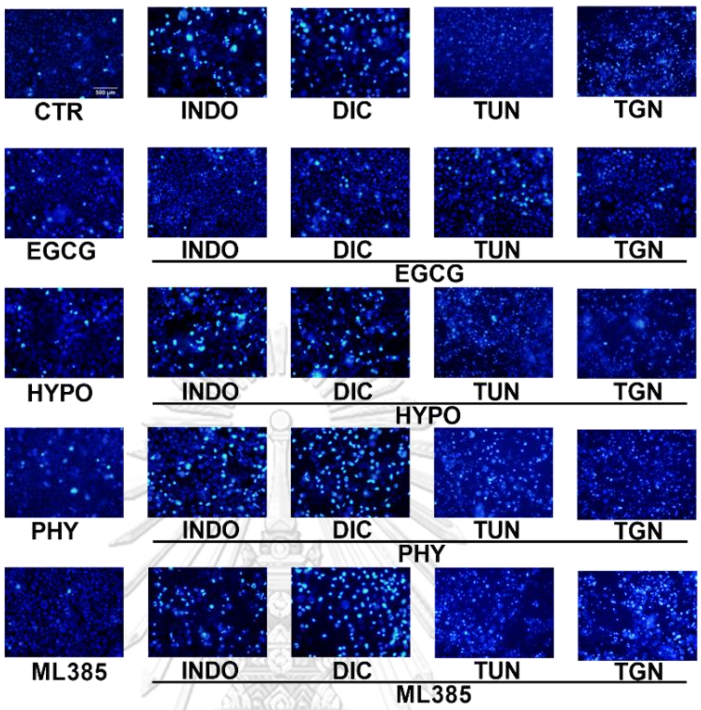
/ Protected; - Not protected; +++ Enhanced cell death

4.2.2 Effects of EGCG, HYPO and PHY on ER stressor-induced apoptosis

The cells with condensed and/or fragmented nuclei were determined by staining with Hoechst 33342 dyes, and counted as apoptotic cells. In this study, NSAIDs (INDO and DIC; 1000 μM) and other ER stressors (TUN; 20 μg/ml) and (TGN; 2 μM) had no effects on apoptotic cell death after 48-h exposure (Figure 10A). However, these compounds significantly increased the number of apoptotic cells after 72-h exposure by approximately 50%, as shown in Figure 10B. In this study, EGCG (100 μM) was able to protect INDO-, DIC-, TUN- and TGN-induced Caco-2 apoptosis cell after 72-h treatment (Figure 10B). Moreover, HYPO (10 μM) had no protective effect on NSAIDs- and ER stressors-mediated apoptotic cell death. PHY and ML385 (10 μM) significantly enhanced the effects of DIC, TUN and TGN on apoptosis induction after 48-h and 72-h exposures, but not INDO-mediated apoptosis (Figure 10A and B).


A 48-h treatment
CTR
INDO
DIC
TUN
TGN
EGCG
INDO
DIC
TUN
TGN
EGCG
HYPO
INDO
DIC
TUN
TGN
HYPO
PHY
INDO
DIC
TUN
TGN
PHY
ML385
INDO
DIC
TUN
TGN
ML385
w/o Polyphenols/ML385
with EGCG
with HYPO
with PHY
with ML385
Apoptotic cells
(% of total cells)
100
80
60
40
20
0
CTR
INDO
DIC
TUN
TGN

B 72-h treatment



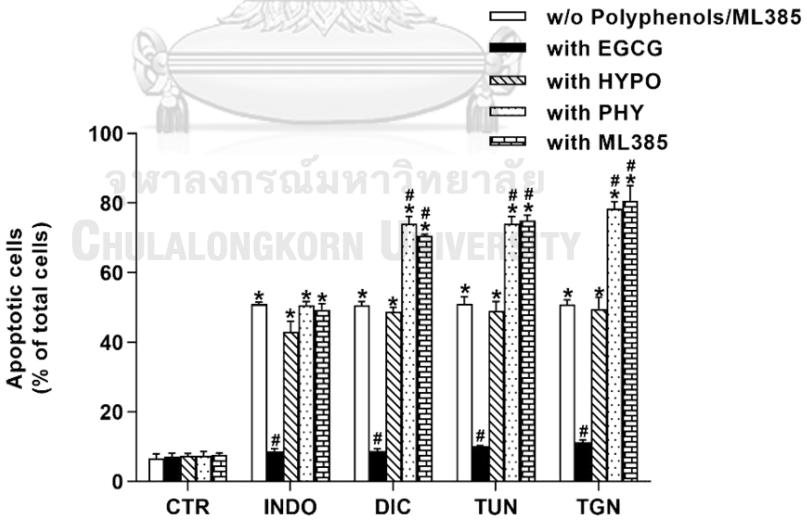
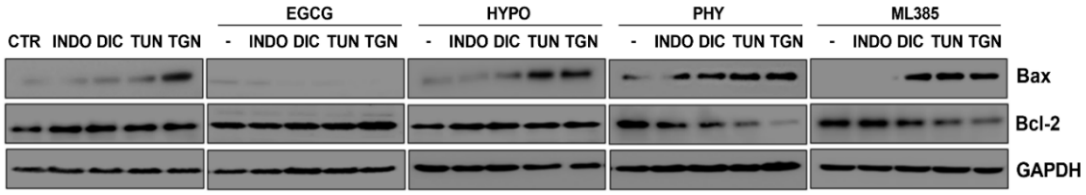
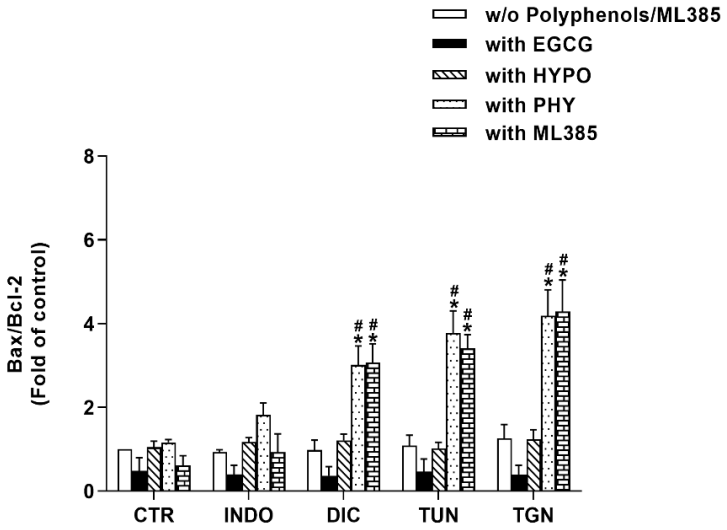


Figure 10. Effects of EGCG, HYPO, PHY and ML385 on ER stressor (INDO, DIC, TUN or TGN)-induced apoptotic cells in Caco-2 cells after 48-h **(A)** and 72-h **(B)** treatments. Cells were stained with Hoechst 33342 and visualized under a fluorescence microscope (20x magnification; scale bar = 500 μm). Data are represented as the percentage of the number of cells with condensed and/or fragmented nuclei after staining Hoechst 33342 dyes. Each bar represents mean ± SEM (N=3). *P< 0.05 indicated statistically significant difference with the control; $^{\#}P$< 0.05 indicated statistically significant difference with the stressors-treated alone group. [DIC (1000 μM); EGCG (100 μM); HYPO (10 μM); INDO (1000 μM); ML385 (10 μM); PHY (10 μM); TGN (2 μM); TUN (20 μg/ml)].

After 48-h incubation period, INDO and DIC (1000 μM), TUN (20 μg/ml) and TGN (2 μM) had no significant effect on the expression levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins in Caco-2 cells. However, these four ER stressors significantly increased Bax and decreased Bcl-2 proteins in Caco-2 cells when treatment period was extended to 72 h (Figure 11A and B). The ratio of Bax/Bcl-2 in INDO-, DIC-, TUN- and TGN-treated cells after 72-h treatment increased by approximately 3-4 fold (Figure 11B). The presence of EGCG (100 μM) significantly suppressed the increased Bax and decreased Bcl-2 expressions in INDO-, DIC-, TUN- and TGN-treated cells after 72-h exposure (Figure 11B). In addition, HYPO (10 μM) had no effect on the Bax/Bcl-2 ratio in the ER stressor-treated cells. PHY and ML385 (10 μM) significantly increased the Bax/Bcl-2 ratios in the cells treated with DIC, TUN or TGN at the higher levels than those in the cells-treated with DIC, TUN and TGN alone after 48-h and 72-h treatment periods, but not in the INDO-treated cells (Figure 11A and B). These results were summarized in Table 9.

A 48-h treatment





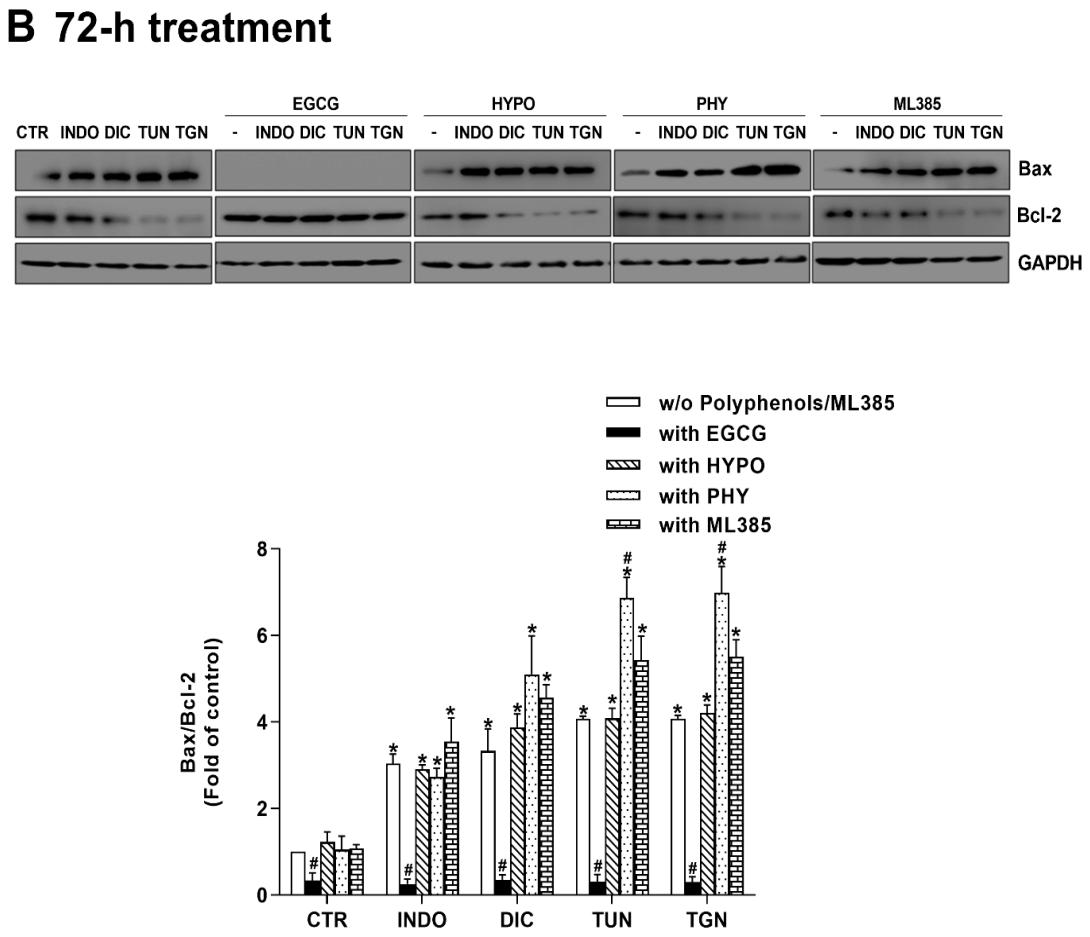


Figure 11. Effects of EGCG, HYPO, PHY and ML385 on ER stressor (INDO, DIC, TUN or TGN)-mediated Bax and Bcl-2 expressions in Caco-2 cells after 48-h **(A)** and 72-h **(B)** treatments. Immunoblots of Bax and Bcl-2 expression and their densitometrical analysis are expressed as the ratio of Bax/Bcl-2 in cells treated with test compounds for 48 h and 72 h. Each bar represents the mean ± SEM (N=3). *P< 0.05 indicated statistically significant tdifference with the control; #P< 0.05 indicated statistically significant difference with the stressors-treated alone group. [DIC (1000 μM); EGCG (100 μM); HYPO (10 μM); INDO (1000 μM); ML385 (10 μM); PHY (10 μM); TGN (2 μM); TUN (20 μg/ml)].

Table 9. Effects of EGCG, HYPO, PHY and ML385 on ER stressor (INDO, DIC, TUN or TGN)-induced apoptosis in Caco-2 cells after 48-h and 72-h treatments

Test Compounds	Alone				EGCG				HYPO				PHY				ML385			
	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN
Bax/Bcl-2 at 48 h														+++	+++	+++		+++	+++	+++
Bax/Bcl-2 at 72 h	+	+	+	+	/	/	/	/	+	+	+	+	+	++	+++	+++	+	++	++	++
Apoptosis at 48 h														+++	+++	+++		+++	+++	+++
Apoptosis at 72 h	+	+	+	+	/	/	/	/	+	+	+	+	+	+++	+++	+++	+	+++	+++	+++

+ Increased; / Protected; +++ Enhanced Bax/Bcl-2 expression and apoptosis

4.2.3 Effects of EGCG, HYPO and PHY on ER stressor-induced mitochondrial dysfunction

As shown in Figure 12A and B, INDO and DIC (1000 μM), TUN (20 μg/ml) and TGN (2 μM) had no effects on the loss in MMP in Caco-2 cells after 48-h treatment. However, the effect of these ER stressors on the loss in MMP increased significantly by approximately 60% after 72-h exposure. In this study, EGCG (100 μM), but not HYPO (10 μM), was able to protect the loss in MMP in the INDO-, DIC-, TUN- and TGN-treated cells (Figure 12B). On the other hand, PHY and ML385 (10 μM) significantly increased the effects of DIC-, TUN- and TGN, but not INDO, on mitochondrial dysfunction after 48-h and 72-h treatments (Figure 12A and B). These results were summarized in Table 10.

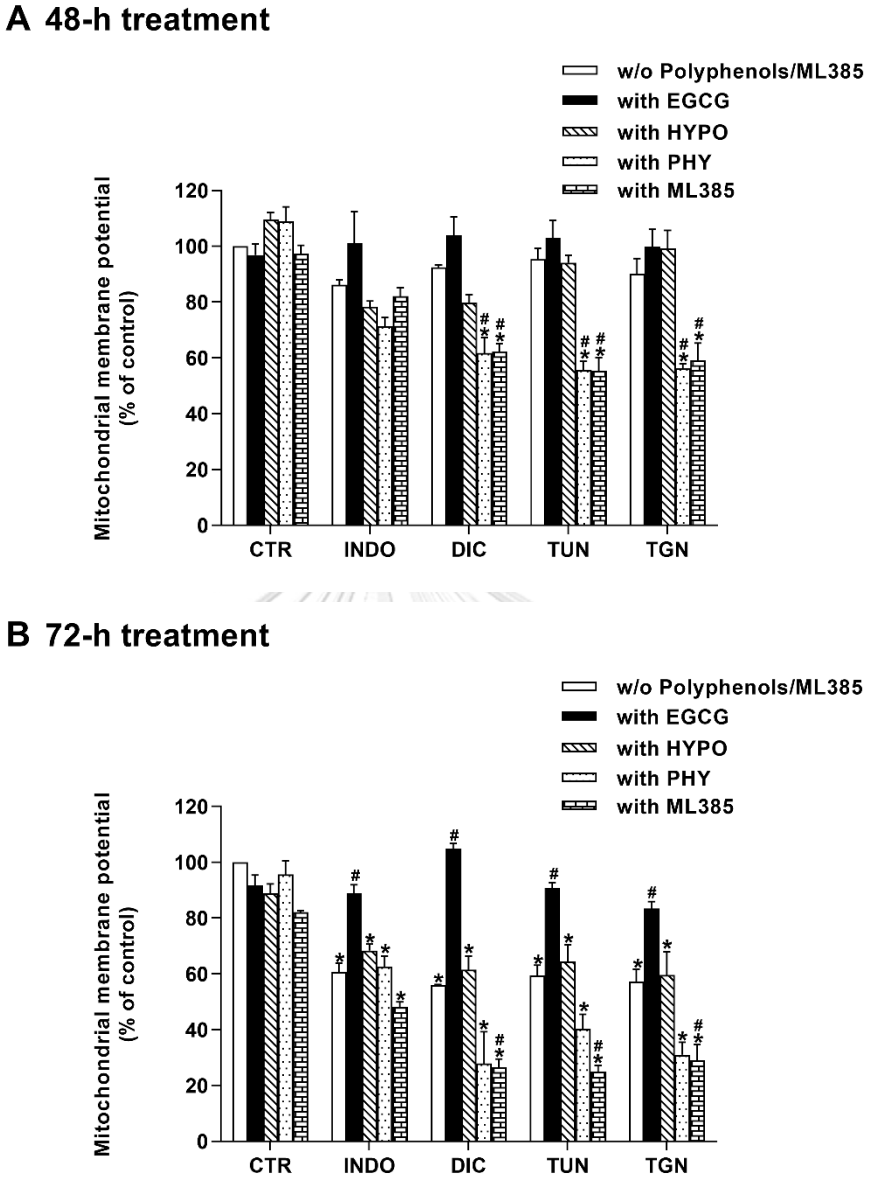


Figure 12. Effects of EGCG, HYPO, PHY and ML385 on ER stressor (INDO, DIC, TUN or TGN)-induced mitochondrial dysfunction in Caco-2 cells after 48-h **(A)** and 72-h **(B)** treatments. Data are expressed as the percentage of control. Each bar represents mean ± SEM (N=3). *P< 0.05 indicated statistically significant difference with the control; #P< 0.05 indicated statistically significant difference with the stressors-treated alone group. [DIC (1000 µM); EGCG (100 µM); HYPO (10 µM); INDO (1000 µM); ML385 (10 µM); PHY (10 µM); TGN (2 µM); TUN (20 µg/ml)].

Table 10. Effects of EGCG, HYPO, PHY and ML385 on ER stressor (INDO, DIC, TUN or TGN)-induced loss in MMP in Caco-2 cells after 48-h and 72-h treatments

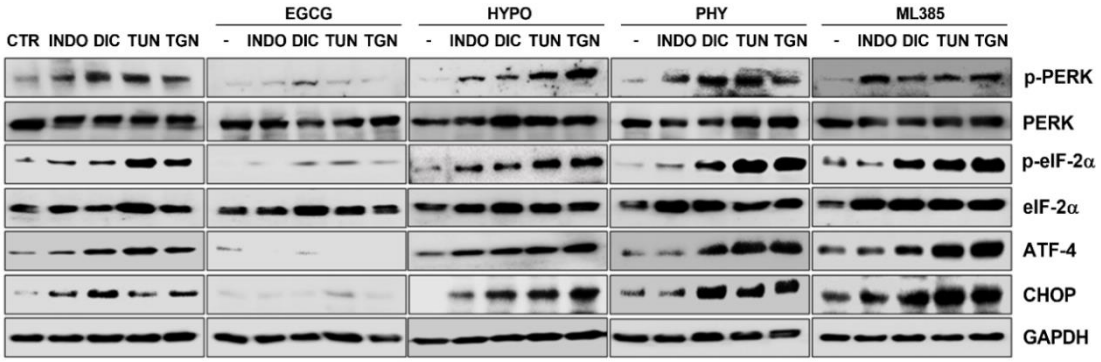
Test Compounds	Alone				EGCG				HYPO				PHY				ML385			
	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN
Loss in MMP at 48 h														+++	+++	+++		+++	+++	+++
Loss in MMP at 72 h	+	+	+	+	/	/	/	/	+	+	+	+	+	++	++	++	+	+++	+++	+++

+ Induced; / Protected; +++ Enhanced loss in MMP

4.2.4 Effects of EGCG, HYPO and PHY on ER stressor-mediated PERK-dependent CHOP pathway

Activation of PERK/eIF-2α/ATF-4/CHOP pathway is associated with increased Bax and decreased Bcl-2 proteins, resulting to apoptotic cell death during ER stress (Iurlaro and Muñoz-Pinedo, 2016; Li et al., 2015). In this study, INDO and DIC (1000 μM), similar to TUN (20 μg/ml) and TGN (2 μM), significantly increased the phosphorylated forms of PERK (p-PERK) and eIF2-α (p-eIF-2α) expressions as well as the extents of ATF-4 and CHOP expressions in Caco-2 cells after 48-h exposure (Figure 13A-E). Furthermore, the p-PERK/p-eIF-2α/ATF-4/CHOP expressions significantly decreased in cells treated with either INDO, DIC, TUN or TGN in presence of EGCG (100 μM), but not HYPO (100 μM) after 48-h treatment. In contrast, PHY and ML385 (10 μM) enhanced a significant increased p-eIF-2α/ATF-4/CHOP expressions in the cells treated with DIC, TUN or TGN, but not INDO (Figure 13A-E). In addition, these compounds did not change p-PERK expression in the cells treated with DIC, TUN or TGN under the same condition. These results were summarized in Table 11.

A


EGCG
HYPO
PHY
ML385
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PERK
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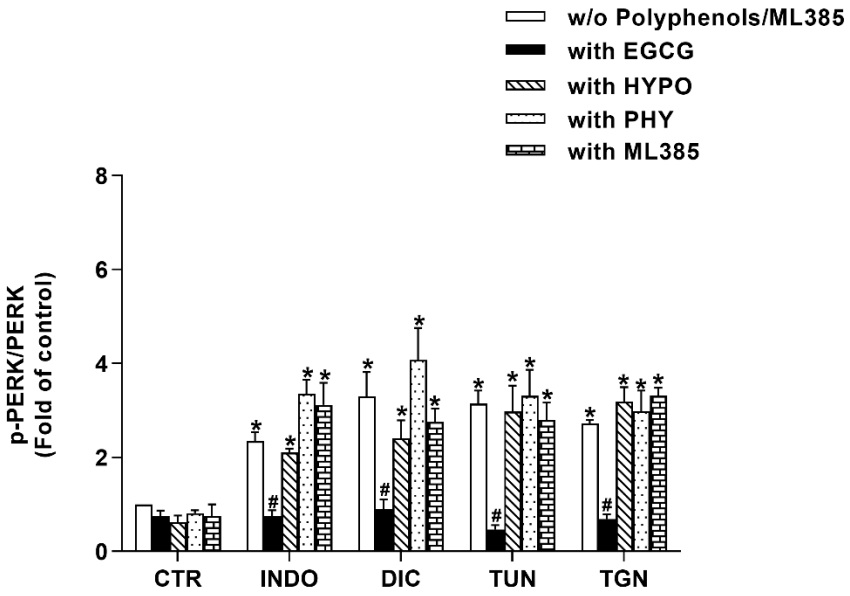
eIF-2α
ATF-4
CHOP
GAPDH

B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INDO
DIC
TUN
TGN

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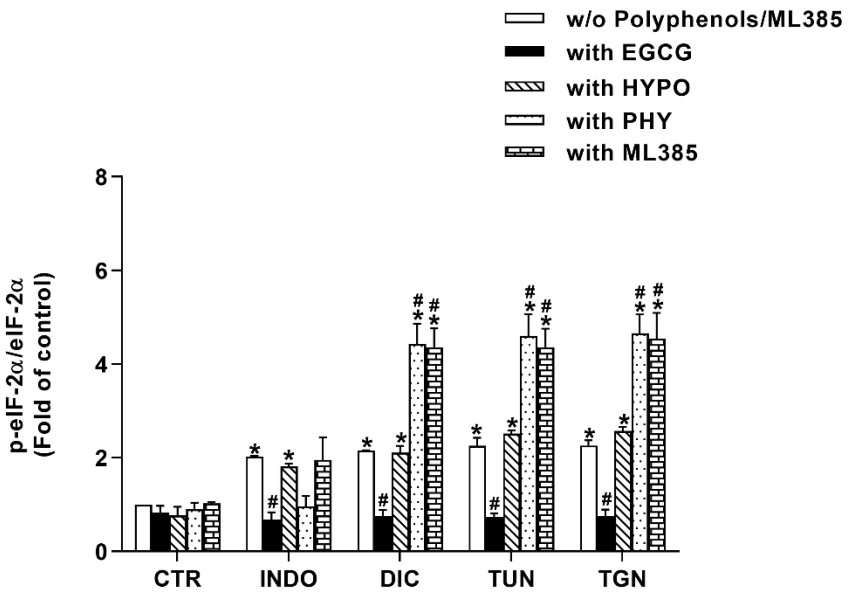
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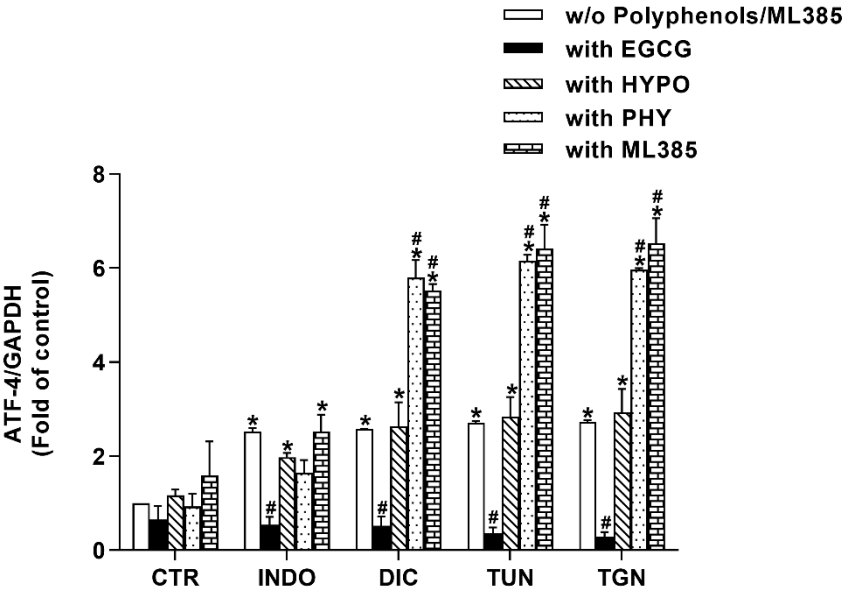
CTR
INDO
DIC
TUN
TGN

D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INDO
DIC
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TGN

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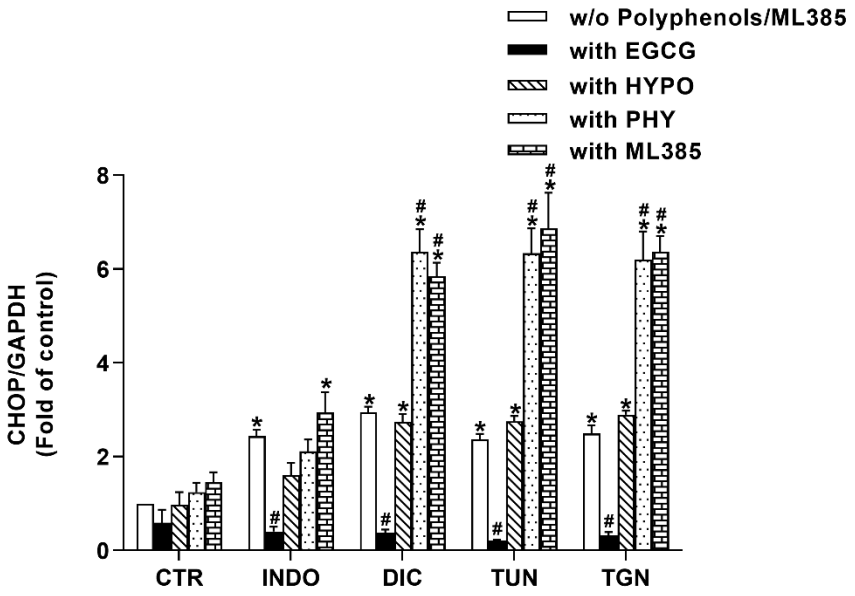


Figure 13. The expressions of PERK/eIF-2α/ATF-4/CHOP proteins in Caco-2 cells treated with ER stressor (INDO, DIC, TUN or TGN) in the absence and presence of EGCG, HYPO, PHY or ML385 after 48-h treatment. Immunoblots of PERK/eIF-2α/ATF-4/CHOP expressions as well as their phosphorylated forms **(A)**. Densitometrical analysis of immunoblots are expressed as the ratio of phosphorylated PERK to PERK **(B)**, phosphorylated eIF-2α to eIF-2α **(C)**, ATF-4 to GAPDH **(D)** and CHOP to GAPDH **(E)**. Each bar represents mean ± SEM (N=3). **P*< 0.05 indicated statistically significant difference with the control; *#P*< 0.05 indicated statistically significant difference with the stressors-treated alone group. [DIC (1000 μM); EGCG (100 μM); HYPO (10 μM); INDO (1000 μM); ML385 (10 μM); PHY (10 μM); TGN (2 μM); TUN (20 μg/ml)].

Table 11. Effects of EGCG, HYPO, PHY and ML385 on ER stressor (INDO, DIC, TUN or TGN)-induced PERK/eIF-2α/ATF-4/CHOP expression in Caco-2 cells after 48-h treatment

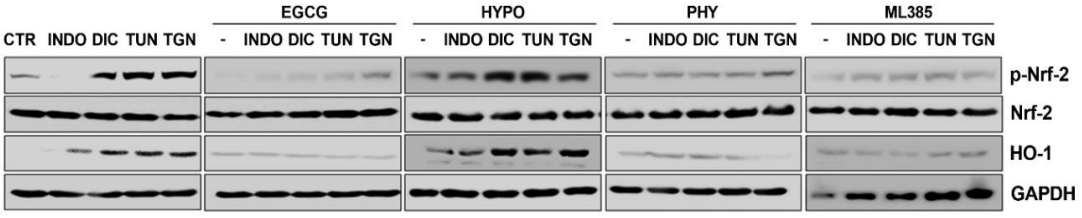
Test compounds	Alone				EGCG				HYPO				PHY				ML385			
	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN
p-PERK/ PERK	+	+	+	+	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+
p-eIF-2α/ eIF-2α	+	+	+	+	/	/	/	/	+	+	+	+	+	+++	+++	+++	+	+++	+++	+++
ATF-4	+	+	+	+	/	/	/	/	+	+	+	+	+	+++	+++	+++	+	+++	+++	+++
CHOP	+	+	+	+	/	/	/	/	+	+	+	+	+	+++	+++	+++	+	+++	+++	+++

+ Increased; / Suppressed; +++ Enhanced protein expression

4.2.5 Effects of EGCG, HYPO and PHY on ER stressor-mediated Nrf-2/HO-1 pathway

Activation of PERK-mediated CHOP pathway is linked to phosphorylation of transcription factor Nrf-2, resulting in an increase of detoxifying enzymes such as HO-1 expression (Fujiki et al., 2019; Kim et al., 2007; Zhang et al., 2019; Zhu et al., 2019). In this study, only DIC (1000 μM), TUN (20 μg/ml) and TGN (2 μM), but not INDO (1000 μM), significantly induced the phosphorylated form of Nrf-2 (p-Nrf-2) and HO-1 expressions in Caco-2 cells after 48-h treatment, as shown in Figure 14A-C. The presence of EGCG (100 μM), PHY (10 μM) or ML385 (10 μM), but not HYPO (10 μM), significantly suppressed the increased p-Nrf-2 and HO-1 expressions in the cells treated with DIC, TUN or TGN (Figure 14A-C). These results were summarized in Table 12.

A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HYPO
PHY
ML385
CTR INDO DIC TUN TGN
- INDO DIC TUN TGN
- INDO DIC TUN TGN
- INDO DIC TUN TGN
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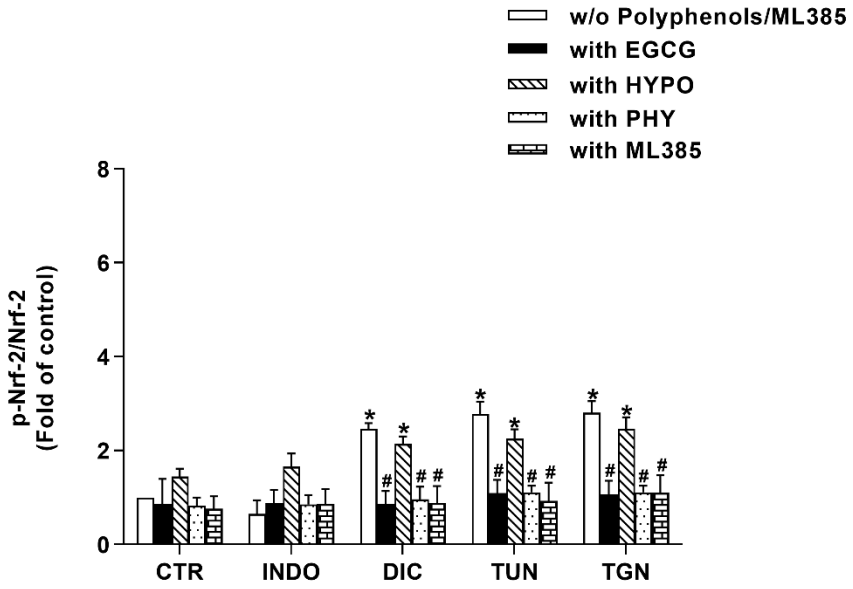
p-Nrf-2
Nrf-2
HO-1
GAPDH

B


w/o Polyphenols/ML385
with EGCG
with HYPO
with PHY
with ML385
p-Nrf-2/Nrf-2
(Fold of control)
0
2
4
6
8
CTR
INDO
DIC
TUN
TGN

C

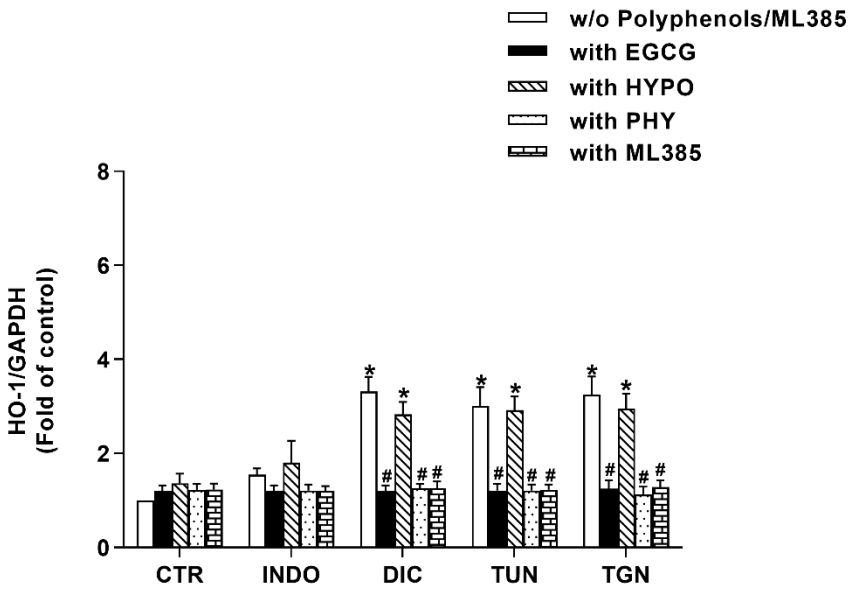


Figure 14. The expressions of Nrf-2 and HO-1 proteins in Caco-2 cells treated with either INDO, DIC, TUN or TGN in the absence and presence of EGCG, HYPO, PHY and ML385 for 48 h. Immunoblots of Nrf-2 and HO-1 expression as well as phosphorylated form of Nrf-2 (p-Nrf-2) **(A)**. Densitometrical analysis of immunoblots are expressed as the ratio of phosphorylated Nrf-2 to Nrf-2 **(B)** and HO-1 to GAPDH expression **(C)**. Each bar represents mean ± SEM (N=3). *P< 0.05 indicated statistically significant difference with the control; #P< 0.05 indicated statistically significant difference with the stressors-treated alone group. [DIC (1000 μM); EGCG (100 μM); HYPO (10 μM); INDO (1000 μM); ML385 (10 μM); PHY (10 μM); TGN (2 μM); TUN (20 μg/ml)].

Table 12. Effects of EGCG, HYPO, PHY and ML385 on ER stressor (INDO, DIC, TUN or TGN)-induced Nrf-2/HO-1 expression in Caco-2 cells after 48-h treatment

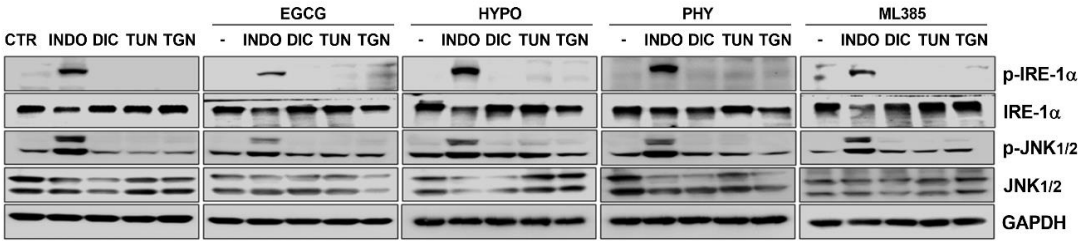
Test compounds	Alone				EGCG				HYPO				PHY				ML385			
	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN
p-Nrf-2/ Nrf-2		+	+	+		/	/	/		+	+	+		/	/	/		/	/	/
HO-1		+	+	+		/	/	/		+	+	+		/	/	/		/	/	/

+ Increased; / Suppressed protein expression

4.2.6 Effects of EGCG, HYPO and PHY on ER stressor-mediated IRE-1α/JNK1/2 pathway

The IRE-1α/JNK1/2 pathway is associated with direct decrease of anti-apoptotic Bcl-2 expression, resulting in ER stress-mediated apoptotic cells death (Iurlaro and Muñoz-Pinedo, 2016; Zhang et al., 2019). In this study, only INDO (1000 μM), but not DIC (1000 μM), TUN (20 μg/ml) and TGN (2 μM), significantly shifted the phosphorylated forms of IRE-1α (p-IRE-1α) and JNK1/2 (p-JNK1/2) expressions in Caco-2 cells after 48-h treatment (Figure 15A-C). In addition, the presence of EGCG (100 μM) suppressed the increases of p-IRE-1α and p-JNK1/2 expressions in the cells treated with INDO for 48 h. HYPO, PHY and ML385 (10 μM) had no effect on INDO-induced p-IRE-1α/p-JNK1/2 expression. These results were summarized in Table 13.

A


EGCG
HYPO
PHY
ML385
CTR INDO DIC TUN TGN
- INDO DIC TUN TGN
- INDO DIC TUN TGN
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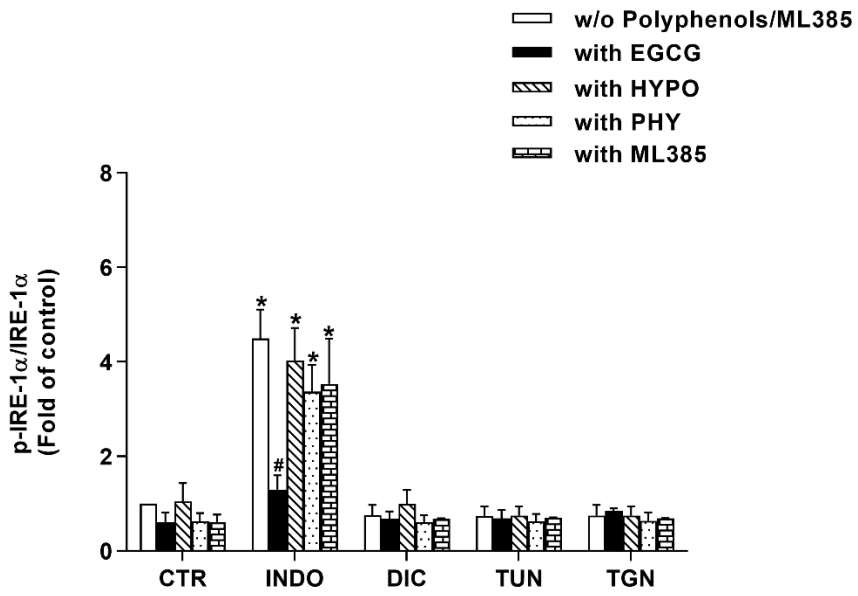
IRE-1α
p-JNK1/2
JNK1/2
GAPDH

B


w/o Polyphenols/ML385
with EGCG
with HYPO
with PHY
with ML385
p-IRE-1α/IRE-1α
(Fold of control)
8
6
4
2
0
CTR
INDO
DIC
TUN
TGN

C

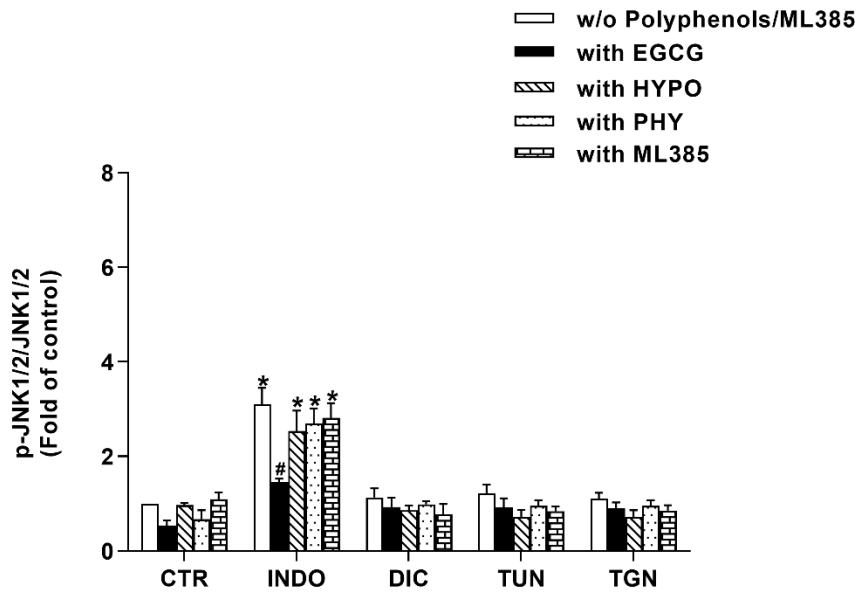


Figure 15. Expressions of IRE-1α and JNK1/2 proteins in Caco-2 cells treated with stressor (INDO, DIC, TUN or TGN) in the absence and presence of EGCG, HYPO, PHY or ML385 for 48 h. Immunoblots of IRE-1α and JNK1/2 expressions as well as theirs phosphorylated form **(A)**. Densitometrical analysis of immunoblots are expressed as the ratio of phosphorylated IRE-1α to IRE-1α **(B)** and phosphorylated JNK1/2 to JNK1/2 **(C)**. Each bar represents mean ± SEM (N=3). *$^{*}P$< 0.05 indicated statistically significant difference with the control; $^{\#}P$< 0.05 indicated statistically significant difference with the NSAIDs-treated group. [DIC (1000 μM); EGCG (100 μM); HYPO (10 μM); INDO (1000 μM); ML385 (10 μM); PHY (10 μM); TGN (2 μM); TUN (20 μg/ml)].

Table 13. Effects of EGCG, HYPO, PHY and ML385 on ER stressor (INDO, DIC, TUN or TGN)-induced IRE-1α and JNK1/2 proteins in Caco-2 cells after 48-h treatment

Test Compounds	Alone				EGCG				HYPO				PHY				ML385			
	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN	INDO	DIC	TUN	TGN
p-IRE-1α /IRE-1α	+				/				+				+				+			
p-JNK1/2/ JNK1/2	+				/				+				+				+			

+ Increased; / Suppressed protein expression

CHAPTER V

DISCUSSION

INDO and DIC have been reported to induce intestinal cell death in several experimental and clinical models such as primary rat intestinal cells, Caco-2, IEC-6 and rat models (Boelsterli et al., 2013; Carrasco-Pozo et al., 2011; Chávez-Piña et al., 2018; Narabayashi et al., 2015; Niu et al., 2014; Saitta et al., 2014; Sandoval-Acuña et al., 2012; Utzeri and Usai, 2017). Their mechanisms involve with oxidative stress, ER stress and mitochondrial dysfunction (Boelsterli et al., 2013; Carrasco-Pozo et al., 2011; Narabayashi et al., 2015; Sandoval-Acuña et al., 2012; Utzeri and Usai, 2017). Several studies suggested that INDO and DIC increased ROS production-mediated intestinal cell death in Caco-2 cells by inhibition of mitochondrial complex I (Carrasco-Pozo et al., 2011; Sandoval-Acuña et al., 2012). In this study, INDO and DIC at concentration of 1000 μM were able to produce superoxide anion, hydrogen peroxide, but not hydroxyl radical in Caco-2 cells. It was reported that two major free radicals, superoxide anion and hydrogen peroxide, but not hydroxyl radical were produced after early inhibition of mitochondrial respiratory chain (Turrens, 2003).

Increased ROS production in INDO- and DIC- treatment has been linked to ER stress-related apoptosis in primary rat intestine and ICE-6 cells (LoGuidice et al., 2010; Narabayashi et al., 2015). INDO and DIC were demonstrated to activate PERK/eIF-2α/ATF-4/CHOP and/or IRE-1α/JNK1/2 signaling pathways in primary pig gastric, Huh-7, H-4 and U-87 cells (Chang et al., 2020; Franceschelli et al., 2011; Mügge and Silva, 2015; Tsutsumi et al., 2004). Activation of PERK/eIF-2α/ATF-4/CHOP and IRE-1α/JNK1/2 signaling pathways lead to increased Bax/Bcl-2 expression ratios, disrupted mitochondrial function and eventually apoptotic cell death (Iurlaro and Muñoz-Pinedo, 2016; Li et al., 2015; Zhang et al., 2019). For example, INDO (1000 μM) was reported to induce ER stress-mediated apoptosis in guinea pig gastric mucosal cells through the

activation of PERK/CHOP, IRE-1α/JNK1/2, IRE-1α/XBP-1 and ATF-6 signaling pathways after 18-h treatment. Furthermore, deletion of *CHOP* gene by siRNA completely blocked INDO-mediated apoptosis (Tsutsumi et al., 2004). INDO (500 nM) and DIC (300 μM) shared commonly activation of PERK/CHOP signaling pathway after 8-h treatment, resulting in induction of apoptosis in Huh-7 cells within 16 h (Franceschelli et al., 2011). In addition, only INDO could induce ER stress-mediated apoptosis in Huh-7 cells via

IRE-1α/JNK1/2 signaling pathway under same condition, but not in DIC treated cells (Franceschelli et al., 2011). In this study, INDO and DIC (1000 μM) increased the p-PERK, p-eIF-2α, ATF-4 and CHOP expressions in Caco-2 cells after 48-h treatment, followed by up-regulation of *Bax* mRNA and down-regulation of *Bcl-2* mRNA expressions. Subsequently, the mitochondria-stressed Caco-2 cells underwent apoptotic death within 24 h. Only INDO, but not DIC at equimolar concentration, exerted the p-IRE-1α and p-JNK1/2 expressions in Caco-2 cells after 48-h treatment. INDO, DIC and two known ER stressors TUN (20 μg/ml) and TGN (2 μM) induced ER stress-mediated apoptosis in Caco-2 cells by increases of p-PERK/p-eIF-2α/ATF-4/CHOP proteins. Thus, these data suggested that INDO and DIC were able to induce ER stress-mediated apoptosis in Caco-2 cells via activation of PERK/eIF-2α/ATF-4/CHOP signaling pathway. Only INDO, but not DIC, could induce Caco-2 apoptosis through activation of IRE-1α/JNK1/2-related ER stress signaling pathway.

In addition to PERK/CHOP activation, the phosphorylation of PERK protein could stimulate the cellular adaptive Nrf-2 signaling pathway to resolve ER stress (Fujiki et al., 2019; Kim et al., 2007; Zhang et al., 2019). Phosphorylated Nrf-2 protein up-regulates HO-1 detoxifying enzyme (Fujiki et al., 2019; Kim et al., 2007; Zhu et al., 2019). It was previously reported that genetic deletion of *Nrf-2* in HepG2 cells over stimulated the p-eIF-2α/ATF-4/CHOP expression in TUN-treated cells whereas p-PERK protein did not change under same condition (Zhu et al., 2019). In this study, DIC, similar to ER

stressors TUN and TGN, induced p-Nrf-2/HO-1 expression in Caco-2 cells along with the increase of PERK/eIF-2α/ATF-4/CHOP expression. The presence of Nrf-2 inhibitor ML385 was able to increase extents of p-eIF-2α/ATF-4/CHOP expressions in DIC-, TUN- and TGN-treated Caco-2 cells, while p-PERK expression did not change in any ER stress-treated cells. Consequently, apoptosis was observed in DIC-, TUN- and TGN-treated cells in the presence of ML385. These data indicated that DIC, but not INDO, induced survival Nrf-2/HO-1 expression in Caco-2 cells by activating PERK signaling pathway. INDO was demonstrated to induce ER stress-mediated cytotoxicity through activation of IRE-1α/JNK1/2 pathway in this study. It has been reported that activation of JNK1/2 pathway in diabetes-induced renal damage was able to suppress the Nrf-2-mediated HO-1 expression in mice model (Zhang et al., 2018). Thus, it is possible that the absence of Nrf-2/HO-1 expression in INDO-treated Caco-2 cells related to activation of IRE-1α/JNK1/2 pathway.

Polyphenolic compounds have been reported their high antioxidant and cytoprotective activities in various cell models such as primary hepatocyte, PC-12, Caco-2 and rat models (Al-Rejaie et al., 2013; Blackler et al., 2012; Carrasco-Pozo et al., 2011; Cheung et al., 2014; Chirdchupunseree and Pramyothin, 2010; Krithika et al., 2015; Krithika and Verma, 2009; Krithika et al., 2011; Li and Kuemmerle, 2018; Martin and Bolling, 2015; Natsume et al., 2009; Seiquer et al., 2015; Suganya et al., 2014). For example, polyphenols riched-extraction of virgin argan oil protected t-BOOH-induced Caco-2 cell death with direct ROS scavengers (Seiquer et al., 2015). The polyphenols in apple plea (0.1 μg/ml) was reported to reduce INDO-mediated production of superoxide radical in Caco-2 cells through prevention of mitochondrial dysfunction (Carrasco-Pozo et al., 2011). QUE (50 and 100 mg/kg, p.o.) prevented INDO-mediated ROS levels and inflammation in rat GI mucosa (Carrasco-Pozo et al., 2016). This compound was able to inhibit NF-kB and activate Nrf-2 signaling pathways, leading to

increased antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Carrasco-Pozo et al., 2016). In this study, all eleven natural polyphenolic compounds including CAF, CUR, EGCG, GAL, HYPO, NAR, PHY, PIP, QUE, RUT and SLY were able to directly scavenge ROS after INDO or DIC exposure in Caco-2 cells for 2 h. Interestingly, only EGCG, QUE and RUT (100 μM) were able to prevent cytotoxicity from INDO or DIC (1000 μM) after 72-h exposure. CAF (100 μM) and NAR (100 μM) were able to protect only INDO-induced cytotoxicity, but not DIC. Six antioxidant polyphenols including CUR (50 μM), GAL (100 μM), HYPO (10 μM), PHY (10 μM), PIP (10 μM) and SLY (100 μM) had no effects against Caco-2 cell death from INDO or DIC treatment. Certain antioxidant polyphenols including CUR, NAR, PHY, PIP and SLY at their noncytotoxic concentration increased Caco-2 cytotoxicity caused by DIC treatment. Taken together, these data indicated that the direct ROS scavenging capacity of natural polyphenolic compounds might not be effective in prevention of INDO- and DIC-induced Caco-2 cytotoxicity.

Eight polyphenolic compounds, CAF, EGCG, NAR, PHY, PIP, QUE, RUT and SLY were selected for further investigation in mechanisms of anti-ER stress and anti-mitochondrial dysfunction. The results showed that EGCG, QUE and RUT suppressed INDO- and DIC-induced *CHOP* expression, thereby they could suppress alteration of *Bax* and *Bcl-2* expression. The increases of loss in MMP and apoptotic cell death were not seen in INDO- and DIC-treated cells in presence with either EGCG, QUE or RUT within 24 h. Interestingly, the cytoprotective effect of EGCG was mediated through suppression of PERK/eIF-2α/ATF-4/CHOP and the IRE-1α/JNK1/2 signaling pathways. In addition, EGCG was able to suppress extents of p-PERK/p-eIF-2α/ATF-4/CHOP proteins and prevent apoptosis in two ER stressors TUN- and TGN-treated Caco-2 cells. Several studies were reported that EGCG, QUE and RUT prevented cytotoxicity and tissue damages from several ER stressors such as cisplatin, MPP^+, TUN, A23187, H_2O_2 and TGN (Chen et al., 2015; Enogieru et al., 2019; Karthikeyan et al., 2017; Natsume et

al., 2009; Suganya et al., 2014). For instance, EGCG (10 μM) abolished ER stress-induced apoptosis after H_2O_2 or TGN treatment in primary retinal pigment epithelial cells via inhibition of PERK and IRE-1α-related ER pathways (Karthikeyan et al., 2017). In addition, RUT protected ER stress-induced apoptosis from MPP+ in neuronal SH-SY5Y cells through suppression of CHOP pathway (Enogieru et al., 2019). QUE was reported to protect ER stress-mediated cytotoxicity from two ER stressors A23187 and TGN in intestinal LS180 and Caco-2 cells through inhibition of PERK/CHOP and IRE-1α/XBP-1 signaling pathways (Natsume et al., 2009).

In this study, NAR and CAF did not suppress the up-regulation of *CHOP* mRNA in INDO- and DIC-treated Caco-2 cells after 48-h exposure. However, the presence of NAR or CAF protected Caco-2 cell death in INDO-treated cells after 72-h treatment, but not in the DIC treated cells. NAR, but not CAF, also suppressed the alteration of *Bax/Bcl-2* expression ratios in INDO-treated cells after 48-h treatment, leading to absence of loss in MMP in the INDO-treated cells within 24 h. As known, only INDO, but not DIC, was able to induce cytotoxicity in Caco-2 cells via CHOP-independent mechanism, IRE-1α/JNK1/2 pathways. NAR and CAF were reported to abolish ER stress in hepatocyte (Huh-7.5.1 and AML 12 cells) and animal models through suppression of IRE-1α-related ER signaling pathway (Jia et al., 2019; Kim et al., 2018). For example, CAF (50 μM, p.o.) reduced hepatic steatosis from ER stress in high fat diet-induced obese mice through induction of autophagy mechanisms (Kim et al., 2018). NAR (1 μM) was able to ameliorate ER stress from hepatitis-C infection and TUN treatment in Huh-7.5.1 cells through suppression of IRE-1α-related ER pathway (Jia et al., 2019). Therefore, NAR and CAF might protect INDO-induced cytotoxicity by suppression of IRE-1α-related ER pathway.

Three polyphenolic compounds, PHY, PIP and SLY did not protect INDO-mediated Caco-2 cytotoxicity after 72-h exposure. On the other hand, these three

polyphenols enhanced the effect of DIC in causing cell death. The results showed that PIP and SLY had no effect on the increase of *CHOP* expression and alteration of *Bax/Bcl-2* mRNA expression ratios in DIC-treated cells after 48-h treatment. SLY, but not PIP, also directly increased the loss in MMP in DIC-treated cells within 24 h. Thus, it is possible that the enhancement effect of SLY and PIP in DIC-mediated cytotoxicity did not involve with ER stress signaling pathways. Interestingly, the structure-related lignan PHY and HYPO displayed different effects on ER stressor-mediated Caco-2 cytotoxicity. HYPO had no effect against Caco-2 apoptosis caused by any ER stressors. On the contrary, PHY exhibited the similar effect to Nrf-2 inhibitor ML385 in suppressing the survival p-Nrf-2/HO-1 expression in DIC-, TUN- and TGN-treated cells after 48-h exposure. Under same condition, PHY increased expressions of p-eIF-2α/ATF-4/CHOP proteins in the DIC-, TUN- and TGN-treated Caco-2 cells without any interference on p-PERK expression. Subsequently, the Bax/Bcl-2 expression ratios, loss in MMP and apoptotic cell death increased in DIC-, TUN- and TGN-treated cells in presence of PHY within 48-h and 72-h treatment. It was reported that the chemical structure related to PHY, lignan honokiol found in Magnolia plants, suppressed the survival mechanisms including NF-kB and Nrf-2 pathways, leading to ROS-mediated apoptosis in Raji cells (Gao et al., 2016). Hence, it is possible that PHY enhanced effect of DIC-induced cytotoxicity in Caco-2 cells by inhibiting survival Nrf-2/HO-1 signaling pathway, resulting in over activation of PERK/eIF-2α/ATF-4/CHOP-mediated apoptosis. It is worth noting that PHY did not interfere with expressions of the survival p-Nrf-2/HO-1 and p-PERK in INDO-treated Caco-2 cells.

In conclusion, EGCG, QUE and RUT were able to protect ER stress-induced mitochondrial dysfunction and apoptosis from INDO and DIC exposure in Caco-2 cells via suppression of CHOP-related ER pathway. In addition, the protective mechanism of EGCG in INDO- and DIC-treated cells may be associated with inhibition of PERK/CHOP and/or IRE-1α/JNK1/2-realted ER signaling pathways. CAF and NAR could protect ER

stress-mediated apoptosis from INDO treatment, but not DIC by inhibition of CHOP-independent ER pathway. On the contrary, the lignan PHY could enhance DIC-induced ER stress-mediated apoptotic cell death by suppression of survival Nrf-2/HO-1 pathway, thereby increased activation of PERK/CHOP pathway. Taken together, the potential protective mechanism of polyphenolic compounds against NSAIDs-induced Caco-2 cells damage might be involved with their interaction of ER stress signaling pathways, as shown in Figure 16. Moreover, it could not be assumed that any polyphenolic compound was able to prevent NSAIDs-mediated enteropathy via intrinsic ROS scavenging activity.

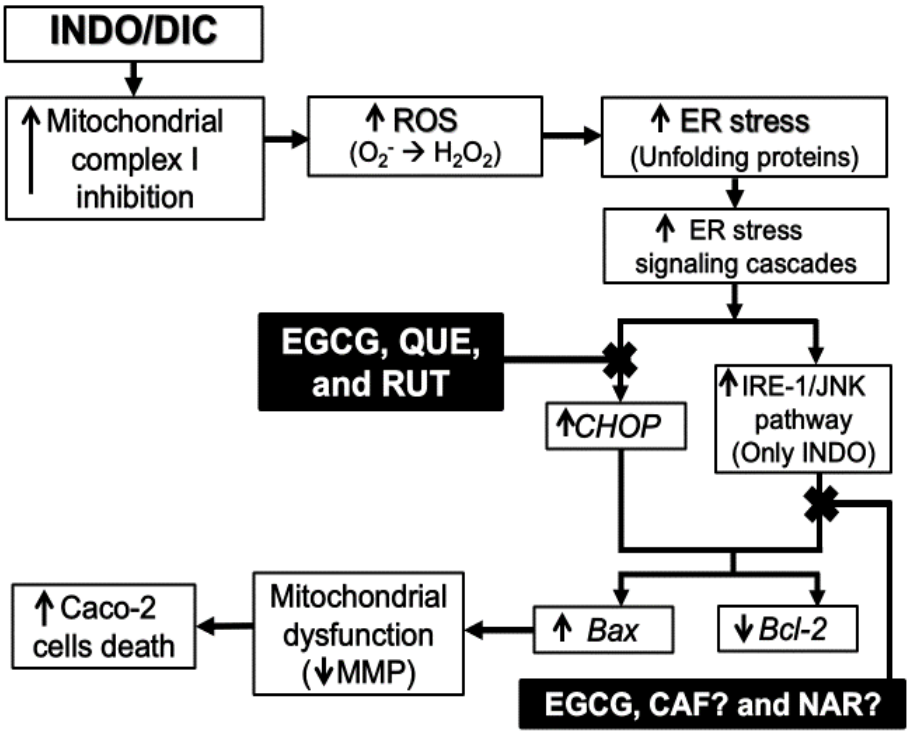


Figure 16. The protective effects of certain polyphenolic compounds on INDO-/DIC-mediated Caco-2 cytotoxicity.

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