การเสริมฤทธิ์กันของเซฟราแรนทีนและเซเลค็อกซิบในเซลล์มะเร็งลำไส้ใหญ่และทวารหนักของมนุษย์

นางสาวปารวี เลิศวนางกูร

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SYNERGISTIC EFFECTS OF CEPHARANTHINE AND CELECOXIB IN HUMAN COLORECTAL CANCER CELLS

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Thesis Title	SYNERGISTIC EFFECTS OF CEPHARANTHINE AND CELECOXIB IN HUMAN COLORECTAL CANCER CELLS
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เซเลค็อกซิบที่ยับยั้งเอนไซม์ไซโคลออกซิจิเนส 2 (cyclooxygenase-2, COX-2) อย่างจำเพาะเจาะจง เป็นยาที่แนะนำให้ใช้ในทางคลินิกเพื่อป้องกันการดำเนินโรคโพลิปลำไส้ใหญ่ไปเป็นมะเร็งลำไส้ใหญ่และทวารหนัก แต่ต้องใช้ในขนาดที่สูงเป็นระยะเวลานานทำให้ต้องคำนึงถึงปัญหาความเป็นพิษต่อระบบหลอดเลือดและหัวใจของยา การใช้ยานี้ร่วมกับยาอื่นที่มีฤทธิ์ต้านมะเร็งอาจเป็นกลยุทธ์ในการทำให้ใช้ยานี้อย่างมีประสิทธิภาพมากขึ้น งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านมะเร็งของการใช้ยาเซเลค็อกซิบร่วมกับเซฟราแรนทีนในเซลล์มะเร็งลำไส้ใหญ่และทวารหนักของมนุษย์ (เซลล์ HT-29) ทำการศึกษาฤทธิ์ของยาต่อการอยู่รอดของเซลล์มะเร็งโดยการย้อมเซลล์ด้วยสี resazurin พบว่า เซเลค็อกซิบและเซฟราแรนทีนลดจำนวนเซลล์มีชีวิตของเซลล์ HT-29 โดยมีค่า IC_{50} เป็น > 50 ไมโครโมลาร์ (μM) และ 5.22 ± 0.28 μM ตามลำดับ เมื่อเซลล์ได้รับสารทดสอบนาน 48 ชั่วโมง ทำการทดสอบการอยู่รอดของเซลล์ที่ได้รับเซเลค็อกซิบที่ความเข้มข้น 5, 10, 20, และ 40 μM ร่วมกับเซฟราแรนทีนที่ความเข้มข้น 1.25 และ 2.5 μM พบว่า การใช้ยาร่วมกันออกฤทธิ์เสริมกันทุกความเข้มข้นที่ใช้ร่วมกัน โดยมีค่าการเสริมฤทธิ์กันที่เรียกว่า combination index (CI) น้อยกว่า 1 ทำการเลือกเซเลค็อกซิบที่ 20, และ 40 μM และเซฟราแรนทีนที่ 1.25 และ 2.5 μM เพื่อทำการศึกษาต่อ เมื่อเทียบกับยาแต่ละตัวพบว่า การใช้ยาร่วมกันไม่มีผลเสริมฤทธิ์กันต่อการแสดงออกของ COX-2 และเอนไซม์ NADPH oxidases (NOX1 และ NOX2) ที่ตรวจวัดด้วยวิธี real time RT-PCR ไม่มีผลต่อการสร้างโพรสตาแกลนดิน E2 (PGE_2) ที่ตรวจวัดด้วยวิธี ELISA และการสร้างอนุมูลอิสระที่ตรวจวัดด้วยวิธี DCFH-DA การใช้ยาร่วมกันทำให้เกิดการสะสมเซลล์ HT-29 ในระยะ G1 ของวัฏจักรเซลล์เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับผลรวมของยาแต่ละตัว ตรวจสอบเซลล์ในวัฏจักรโดยการย้อมด้วยสี propidium iodide (PI) และใช้เครื่อง flow cytometer การยับยั้งวัฏจักเซลล์เพิ่มมากขึ้นอาจเกิดจากการเพิ่มการแสดงออกของ p21 ที่ยับยั้งเอนไซม์ cyclin dependent kinases (CDKs) และลดการแสดงออกของ cyclin A เมื่อเทียบกับยาแต่ละตัว ซึ่งให้ผลอย่างมีนัยสำคัญทางสถิติเมื่อใช้เซเลค็อกซิบที่ 40 μM ร่วมกับเซฟราแรนทีนที่ 2.5 μM นอกจากนี้การใช้ยาร่วมกันเพิ่มการตายของเซลล์ HT-29 แบบอะพอพโตซิสเมื่อเทียบกับยาแต่ละตัว จากการตรวจวัดด้วยการย้อม annexin V-FITC/ PI และใช้ flow cytometer ซึ่งการทำให้เซลล์ตายแบบอะพอพโตซิสสอดคล้องกับการเพิ่มการแสดงออกของ pro-apoptotic BAX และการลดการแสดงออกของ anti-apoptotic Bcl-XL ในเซลล์ที่ได้ยาสองตัวร่วมกัน การศึกษาครั้งนี้แสดงให้เห็นถึงการออกฤทธิ์ต้านมะเร็งแบบเสริมฤทธิ์กันของเซเลค็อกซิบกับเซฟราแรนทีนที่ความเข้มข้นต่ำกว่าค่า IC_{50} ของยาทั้งสองตัว ผลการศึกษานี้อาจเป็นประโยชน์ในการลดขนาดยาและลดความเป็นพิษของเซเลค็อกซิบเพื่อป้องกันการเป็นมะเร็งลำไส้ใหญ่ การศึกษาวิจัยเพิ่มเติมจะช่วยยืนยันผลการศึกษานี้

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PARAWEE LERDWANANGKUN: SYNERGISTIC EFFECTS OF CEPHARANTHINE AND CELECOXIB IN HUMAN COLORECTAL CANCER CELLS. ADVISOR: ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., CO-ADVISOR: ASST. PROF. PIYANUCH WONGANAN, Ph.D., 91 pp.

Celecoxib, a specific cyclooxygenase 2 (COX-2) inhibitor, is clinically suggested as adjunctive therapy for preventing the progression of colon polyps to colorectal cancer. However, this drug is used at high dose in a long period of time for this indication. This leads to the cardiovascular toxicity concern. Combination of celecoxib with other anticancer agents is a strategy for improving the efficacy of celecoxib. This study aimed to investigate anticancer effect of celecoxib – cepharanthine combination on human colorectal cancer HT-29 cells. Effects of the drugs on cell viability were determined by resazurin assay. Celecoxib and cepharanthine decreased viability of HT-29 cells with IC_{50} > 50 μM and at 5.22 ± 0.28 μM, respectively after 48 h of exposure. Celecoxib at 5, 10, 20, and 40 μM were combined with cepharanthine at 1.25, 2.5 and 5 μM for HT-29 viability test. All combinations had synergistic effects on the cell viability with combination indices less than one (CI < 1). The combinations of celecoxib at 20 and 40 μM and cepharanthine at 1.25 and 2.5 μM were investigated further. When compared to each drug, these combinations did not have additive/ synergistic effects on COX-2 and NADPH oxidases (NOX1 and NOX2) expression determined by real time RT- PCR, PGE_2 production determined by ELISA, and ROS generation determined by DCFH-DA assay. The combinations significantly increased HT-29 cell accumulation at the G1 phase of the cell cycle when compared to the effects of each drug. The cell cycle was determined by propidium iodide (PI) staining with fluorescence flow cytometer. The effects on cell cycle arrest of these combinations may come from the increase in cyclin-dependent kinase (CDK) inhibitor p21 and the decreased in cyclin A expression. The combination of 40 μM celecoxib – 2.5 μM cepharanthine significantly increased p21 but decreased cyclin A expression when compared to each drug. The combinations significantly increased HT-29 apoptosis determined by annexin V/FITC and PI staining with flow cytometer when compared to each drug. Similarly, their apoptotic induction effects correlated with the increase in pro-apoptotic BAX and the decrease in anti-apoptotic Bcl-XL expression induced by these combinations. The results from this study reveal the synergistic anticancer effect of celecoxib – cepharanthine combinations at sub-IC_{50} concentrations. This finding may be useful for reducing the dose and toxicities of celecoxib in colorectal cancer prevention. Further investigations are needed to confirm these results.

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Abbreviations

ANOVA	=	One-way Analysis of variance
APAF-1	=	Apoptotic protease-activating factor 1
ATCC	=	American Type Culture Collection
ATM	=	Ataxia telangiectasia mutated
BAK	=	Bcl-2 homologous antagonist killer
BAX	=	Bcl-2- associated X protein
Bcl-2	=	B-cell lymphoma 2
Bcl-XL	=	B-cell lymphoma-extra large
Bid	=	BH3-interacting death domain agonist
BOP	=	N-nitroso-bis (2-oxopropyl) amine
cAMP	=	Cyclic adenosine monophosphate
Caspase	=	Cysteine-aspartic protease
cdc25C	=	cell division cycle 25 C protein
CDK	=	Cyclin dependent kinase
cDNA	=	Complementary DNA
Chk1	=	Check point kinase 1
Chk2	=	Check point kinase 2
CI	=	Combination index
CIP/KIP	=	CDK interacting protein/kinase inhibitory protein
COX-1	=	Cyclooxygenase 1
COX-2	=	Cyclooxygenase 2
COX-3	=	Cyclooxygenase 3
CRC	=	Colorectal cancer
DCC	=	Deleted in colorectal carcinoma
DCF	=	2'-7'-Dichlorodihydrofluorescein
DCFH-DA	=	2'-7'-Dichlorodihydrofluorescein diacetate
DEPC	=	Diethyl pyrocarbonate
DISC	=	Death-inducing signaling complex
DMEM	=	Dulbecco's modified Eagle medium

DMH	=	1, 2-dimethylhydrazine
DMSO	=	Dimethyl sulfoxide
Duox1	=	double oxidase 1
EGCG	=	Epigallocatechin gallet
EGFR	=	Epidermal growth factor receptor
EP1	=	Prostaglandin E2 receptor 1
EP2	=	Prostaglandin E2 receptor 2
EP3	=	Prostaglandin E2 receptor 3
EP4	=	Prostaglandin E2 receptor 4
ERK	=	Extracellular signal-regulated kinases
FADD	=	Fas-associated death domain protein
FAP	=	Familial adenomatous polyposis
FBS	=	Fetal bovine serum
G1 phase	=	Gap1 phase
G2 phase	=	Gap2 phase
GADD153	=	Growth arrest and DNA damage-inducible gene 153
HBSS	=	Hank's Balanced salts
IC_{50}	=	Inhibition of concentration
INK4	=	inhibitor of CDK4
iNOS	=	Inducible nitric oxide synthase
ITP	=	Idiopathic thrombocytopenic purpura
JNK	=	c-Jun-N-terminal kinases
K-ras	=	Kristen rat sarcoma viral oncogene homolog
LPS	=	Lipopolysaccharide
M phase	=	Mitotic phase
MAPK	=	Mitogen activated protein kinases
Mcl-1	=	Myeloid cell leukemia 1
ml	=	Milliliter
mM	=	Millimolar
MOMP	=	Mitochondria outer membrane permeability
MPF	=	Maturation promoting factor

NAC	=	N-acetyl cysteine
NF-κB	=	nuclear factor kappa-light-chain-enhancer of activated B cells
NOX1	=	Nicotinamide adenine dinucleotide phosphate oxidase 1
NOX2	=	Nicotinamide adenine dinucleotide phosphate oxidase 2
NSAIDs	=	Nonsteroidal anti-inflammation drugs
P-gp	=	Permeability glycoprotein
P53	=	Tumor suppressor P53
PARP	=	Poly ADP ribose polymerase
PBS	=	Phosphate buffered saline
PGD_2	=	Prostaglandin D_2
PGE_2	=	Prostaglandin E_2
PGE_2-AP	=	Alkaline Phosphatase conjugated PGE_2
$PGF_{2\alpha}$	=	Prostaglandin $F_{2\alpha}$
PGH_2	=	Prostaglandin H_2
PGI_2	=	Prostaglandin I_2
pH	=	Potential of hydrogen
PI	=	Propidium iodide
PI3K/AKT	=	Phosphatidylinositol 3- kinase/protein kinase B
PKC	=	Protein kinase C
pRB	=	Retinoblastoma protein family
PTEN	=	Phosphatase and tensin homolog
ROS	=	Reactive oxygen species
RPM	=	Revolutions per minute
S phase	=	Synthesis phase
SMAC	=	Second mitochondria-derived activator of caspase
SPSS software	=	Statistical package for the social sciences software
Src	=	Sarcoma kinase
STAT3	=	Signal transducer and activator of transcription 3

TNF-α	=	Tumor necrosis factor alpha
TPA	=	12-O-Tetradecanoylphorbol-13-acetate
TRAIL	=	TNF-related apoptosis-inducing ligand
TXA_2	=	Thromboxane A_2
VEGF	=	Vascular endothelial growth factor
μl	=	Microliter
μM	=	Micromolar

CHAPTER I
Introduction

1.1 Background and rationale

Colorectal cancer (CRC) is one of the leading causes of cancer death in both males and females worldwide. It is a cancer starting in lining of colon or rectum. Stage of this cancer has high impact on prognosis, treatment and survival rate of patients. Optimal treatment for colorectal cancer depends on tumor location and stage of disease at diagnosis. Standard treatments of colorectal cancer are surgery, radiotherapy, and pharmacotherapy. Pharmacological drugs for colorectal cancer treatment are chemotherapeutic drugs and targeted anticancer drugs. These drugs are commonly used after surgery. 5-Fluorouracil (5-FU) in combination with other chemotherapeutic agents including oxaliplatin and irinotecan, is the first line chemotherapy for this cancer. However, nonselective toxicity to normal cells and drug resistance of cancer cell are critical challenges of chemotherapy. Several strategies have been continuously explored in order to improve the use of anticancer agents against CRC (1).

Epidemiologic studies have reported non-steroidal anti-inflammatory drugs (NSAIDs) as well as aspirin can decrease CRC mortality. These drugs inhibit activities of cyclooxygenases (COXs), both COX-1 and COX-2 isoforms. This led to an understanding of the roles of COXs and their products prostaglandins (PGs) in the progression of CRC. COX-2 is overexpressed in many types of cancer as well as CRC (2-5). PGE_2 is a major product of COX-2 in cancer cells. It plays a role in tumorigenesis by inhibiting apoptosis, increasing cell proliferation, stimulating angiogenesis, and inducing cancer cell invasion. In addition, COX-2 also produces reactive oxygen species (ROS). The major source of ROS is NADPH oxidase family (NOX). Many studies reported that NOX1 is a member of NADPH oxidase family which over-expressing in HT-29 colorectal cancer cell line, which was used in this study. Dual-role of ROS depends on cells and their levels to stimulate or inhibit cell growth. Celecoxib, a selective COX-2 inhibitor, has shown promising pharmacological effects on CRC prevention. Several studies demonstrated that celecoxib suppressed colon polyps both in vivo and in clinic. This drug has been

approved by the US Food and Drug Administration to treat patients with familial adenomatous polyposis. Celecoxib demonstrated antitumor activity in various cancer cells such as lymphomas (6), chronic myeloid leukemia (7), pancreatic cancer (8), and colorectal cancer (9). It arrested cancer cell cycle, induced apoptosis, inhibited cell invasion, and suppressed angiogenesis. Treatment of celecoxib at high dose and for long period of time is recommended for colorectal cancer prevention. However, this leads to the increase risk of cardiovascular side effects (10). Combination of celecoxib with other anticancer agents is suggested to be a strategy for improving the effectiveness of celecoxib in CRC. Many studies demonstrated that celecoxib enhanced antitumor activities of some chemotherapeutic agents or phytochemicals in various cancer cells (11-14).

Cepharanthine (CEP) is a biscoclaurine alkaloid isolated from the root of *Stephania cepharantha* Hayata. It is approved for treatment of alopecia areata, leukopenia and idiopathic thrombocytopenic purpura (ITP) by Japanese Ministry of Health. It demonstrated many pharmacological activities including anti-malaria, anti-HIV-1, anti-allergic, anti-inflammatory, and anti-tumor activities. It had antitumor activity against many types of cancer cells by arresting cell cycle (15, 16), inducing apoptosis (16-19), and decreasing multidrug resistance (20-22). It inhibited COX-2 expression (23) and induced ROS generation (23-26). It enhanced anti-tumor activities of chemotherapeutic agents against various cancer cells (27-32). Therefore, the rationale for using celecoxib in combination with cepharanthine may be a new therapeutic strategy for colorectal cancer treatment in order to reduce toxicity and increase anti-tumor activity of celecoxib.

1.2 Objective

To determine the combination effect of celecoxib and cepharanthine in human colorectal cancer cells

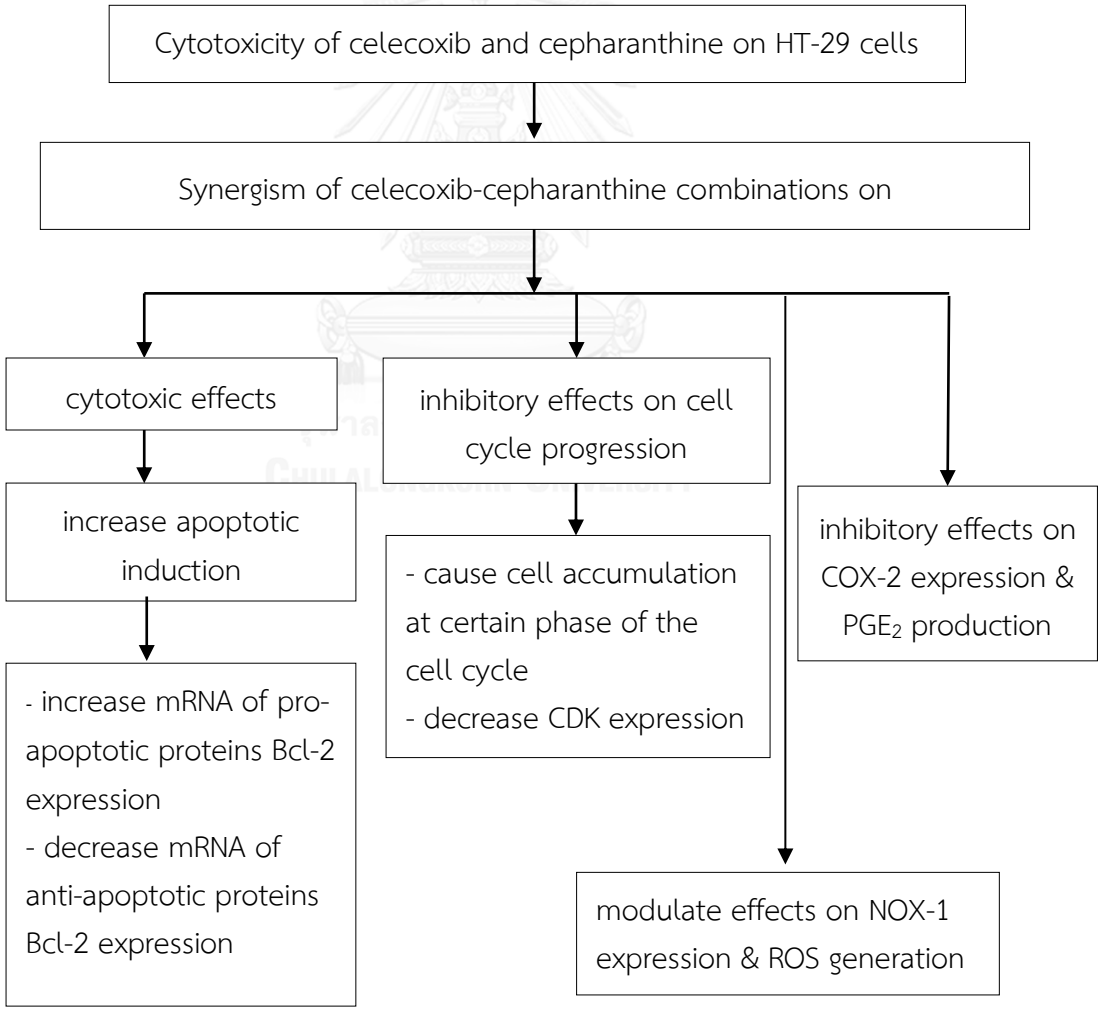
1.3 Hypothesis

Celecoxib in combination with cepharanthine synergistically inhibits colorectal cancer cell growth

1.4 Contribution of the study significance

This study may generate a new strategy for using celecoxib to prevent or to treat colorectal cancer in order to reduce toxicities and increase anticancer efficacy of celecoxib.

1.5 Conceptual framework



CHAPTER II

Literature reviews

2.1. Colorectal cancer

Colorectal cancer is the third most common cancer in both men and women. In 2017, the American Cancer Society estimated that the new cases of colon cancer were approximately 95,520 cases and rectal were approximately 39,910 cases. Estimated patients will die from colorectal cancer 27,150 cases in men and 23,110 cases in women (33). In 2014, the National Cancer Institute of Thailand reported that the new colorectal cancer patients were approximately 275 cases in male and 208 cases in female (34). The incidence of colorectal cancer is associated with the development of social economy (35). Lifestyle-related factors have been implied to the risk factors of colorectal cancer. These factors include obese, older, physical inactivity, food, smoking, alcohol drinking, race, ethnic background, personal or family history of inflammatory bowel disease, polyp and colorectal cancer, and genetic disorders (36). The types of colorectal cancer include adenocarcinomas, carcinoid tumors, gastrointestinal stromal tumors, lymphomas, and sarcomas. The colorectal cancer development usually begins at the inner lining of colon or rectum by forming polyps. Changes from polyps to colorectal cancer depend on the types of polyps which include hyperplastic polyps, inflammatory polyps, and adenomatous polyps (adenomas). Only the adenomatous polyps can be changed to cancer.

2.1.1 Stages of colorectal cancer

The stages of cancer are one of the most important factors for deciding how to treat cancer patients and for determining the success of the treatment. The stages of colorectal cancer are often classified using TNM system according to the American Joint Committee on Cancer (AJCC); where T as the extent of primary tumor growth in the wall of intestine and nearby areas, N as the spread of cancer to regional or nearby lymph nodes and M as the metastasis of cancer to other organs (1). Numbers after T, N, and M describe these factors in more details. The combinations of TNM information

are used to determine the overall stages of cancer; ranging from the earliest or the least severe stage (Stage 0) to the most severe or advanced stage (Stage IV).

Table 1 The stages of colorectal cancer (1)

Stage	Stage grouping	Stage description
0	Tis, N0, M0	The cancer is in its earliest stage. This stage is also known as carcinoma in situ or intramucosal carcinoma (Tis). It has not grown beyond the inner layer (mucosa) of the colon or rectum.
I	T1 or T2, N0, M0	The cancer has grown through the muscularis mucosa into the submucosa (T1), and it may also have grown into the muscularis propria (T2). It has not spread to nearby lymph nodes (N0). It has not spread to distant sites (M0).
IIA	T3, N0, M0	The cancer has grown into the outermost layers of the colon or rectum but has not gone through them (T3). It has not reached nearby organs. It has not yet spread to nearby lymph nodes (N0) or to distant sites (M0).
IIB	T4a, N0, M0	The cancer has grown through the wall of the colon or rectum but has not grown into other nearby tissues or organs (T4a). It has not yet spread to nearby lymph nodes (N0) or to distant sites (M0).
IIC	T4b, N0, M0	The cancer has grown through the wall of the colon or rectum and is attached to or has grown into other nearby tissues or organs (T4b). It has not yet spread to nearby lymph nodes (N0) or to distant sites (M0).
IIIA	T1 or T2, N1, M0	The cancer has grown through the mucosa into the submucosa (T1), and it may also have grown into the muscularis propria (T2). It has spread to 1 to 3 nearby lymph nodes (N1a/N1b) or into areas of fat near the lymph nodes but not the nodes themselves (N1c). It has not spread to distant sites (M0).
	OR	
	T1, N2a, M0	The cancer has grown through the mucosa into the submucosa (T1). It has spread to 4 to 6 nearby lymph nodes (N2a). It has not spread to distant sites (M0).
	T3 or T4a, N1, M0	The cancer has grown into the outermost layers of the colon or rectum (T3) or through the visceral peritoneum (T4a) but has not reached nearby organs. It has spread to 1 to 3 nearby lymph nodes (N1a or N1b) or into areas of fat near the lymph nodes but not the nodes themselves (N1c). It has not spread to distant sites (M0).
	OR	
IIIB	T2 or T3, N2a, M0	The cancer has grown into the muscularis propria (T2) or into the outermost layers of the colon or rectum (T3). It has spread to 4 to 6 nearby lymph nodes (N2a). It has not spread to distant sites (M0).
	OR	
	T1 or T2 N2b, M0	The cancer has grown through the mucosa into the submucosa (T1), and it may also have grown into the muscularis propria (T2). It has spread to 7 or more nearby lymph nodes (N2b). It has not spread to distant sites (M0).
IIIC	T4a, N2a, M0	The cancer has grown through the wall of the colon or rectum (including the visceral peritoneum) but has not reached nearby organs (T4a). It has spread to 4 to 6 nearby lymph nodes (N2a). It has not spread to distant sites (M0).
	OR	
	T3 or T4a, N2b, M0	The cancer has grown into the outermost layers of the colon or rectum (T3) or through the visceral peritoneum (T4a) but has not reached nearby organs. It has spread to 7 or more nearby lymph nodes (N2b). It has not spread to distant sites (M0).
	OR	
	T4b, N1 or N2, M0	The cancer has grown through the wall of the colon or rectum and is attached to or has grown into other nearby tissues or organs (T4b). It has spread to at least one nearby lymph node or into areas of fat near the lymph nodes (N1 or N2). It has not spread to distant sites (M0).
IVA	Any T, Any N, M1a	The cancer may or may not have grown through the wall of the colon or rectum (Any T). It might or might not have spread to nearby lymph nodes. (Any N). It has spread to 1 distant organ (such as the liver or lung) or distant set of lymph nodes (M1a).
IVB	Any T, Any N, M1b	The cancer might or might not have grown through the wall of the colon or rectum. It might or might not have spread to nearby lymph nodes. It has spread to more than 1 distant organ (such as the liver or lung) or distant set of lymph nodes, or it has spread to distant parts of the peritoneum (the lining of the abdominal cavity) (M1b).

The treatment of colorectal cancer depends on location, characteristic and stages of cancer (1).

2.1.2 Colorectal cancer treatment

The standard strategies of colorectal cancer treatments include surgery, radiotherapy, targeted therapy and chemotherapy.

Surgery is a major treatment of colorectal cancers by removing and eradicating tumor out of the body.

Radiotherapy is often used before or after surgery. There are 2 types of radiotherapy, including external or internal radiation therapy. The problem of radiotherapy is many side effects and some side effects are permanent after complete treatment.

Targeted therapy is pharmacological treatment using drugs which act specifically on cancer cells more than normal cells. The molecular targets of the drugs are associated with tumor progression, such as vascular endothelial growth factor (VEGF) targeted by bevacizumab, epidermal growth factor receptor (EGFR) targeted by cetuximab, panitumumab, and kinase inhibitor regorafenib. However, the problems of target therapy are the limitation of drug use only on patient with high target expression and the cost of treatment is very expensive.

Chemotherapy is the treatment with cytotoxic drugs or chemotherapeutic agents which inhibit cell proliferation or kill the cells. Chemotherapeutic agents act mainly on dividing cells. Anti-cancer drugs often used in colorectal cancer are 5-fluorouracil (5-FU), capecitabine, oxaliplatin and trifluridine and tipiracil. The problems of chemotherapy are low therapeutic indices, drug resistance, and high side effects. Side effects of chemotherapeutic agents depend on types and doses of these drugs. Most common side effects are bone marrow suppression, infection, hair loss, mouth sores, nausea, vomiting, diarrhea, fatigue, loss of appetites, and bleeding. There are also specific side effects of these drugs. 5-FU and capecitabine cause hand-foot syndrome. Oxaliplatin causes neuropathy and sensitivity reaction. Irinotecan causes severe diarrhea. So, several strategies have been explored in order to improve the use of anticancer agents against CRC (1, 36).

2.1.3 Molecular biology of colorectal cancer

Colorectal carcinogenesis associates with mutations of both tumor suppressor genes and oncogenes. The mutations of tumor suppressor genes include adenomatous polyposis coli (*APC)*, *p53*, and deleted in colorectal carcinoma (*DCC)*. Mutation of oncogene includes K-*Ras* is also found approximately 12% in early adenoma stage and it increases to 50% in advance adenoma stage and colorectal cancer carcinoma stage. Colorectal cancer is linked to genetic mutation a contribution from inflammation to cancer development (3). Laurent et al. reported that the *K-ras* mutation was associated with the expression of nicotinamide adenine dinucleotide phosphate oxidase 1 (NOX1) which is an enzyme producing ROS production (37). Cyclooxygenase-2 (COX-2) is an enzyme which involves in inflammation and colorectal cancer. COX-2 was found to express in adenomas approximately 50% (4) and in adenocarcinomas about 86% when compared to paired normal colorectal mucosa (5). COX-2 was over-expressed in human colorectal cancer approximately 80-86% when compared to normal colorectal mucosa (38, 39). The colorectal cancer HT-29 cells which were used in this study expressed high level of COX-2 and NOX1 (40, 41).

2.1.4 Role of COX-2 and prostaglandin in cancer

Cyclooxygenase (COX) or prostaglandin H synthase family consists of 3 isoforms, including COX-1, COX-2 and COX-3. COX-1, a constitutively expressed enzyme in several normal tissues, produces several prostaglandins (PGs) at physiological levels for homeostasis of tissues and organs of the body such as protection gastric mucosa by PGE_2 and induction platelet aggregation by thromboxane (TXA). COX-2 is an inducible enzyme, which is stimulated by cytokines and growth factors. It is induced during inflammation process and in cancer cells. It generates much higher number of PGs than COX-1. COX-3 is the splice variant of COX-1 with unknown function. COX enzymes catalyze arachidonic acid which derived from membrane phospholipid to PGH_2. PGH_2 is converted by prostaglandin synthases to PGs, including PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 (42). These PGs bind to their cell surface receptors, generate signal transduction and induce cellular responses.

COX-2 expression and PGE_2 production involve in tumorigenesis of some types of cancer such as lung cancer, ovarian cancer, colorectal cancer. It has been reported that size of adenomas related to level of PGE_2 (43). PGE_2 was reported to involve in the progression of adenomatous polyp to carcinomas (44). Normally, PGE2 functions after binding to its receptors, EP1, EP2, EP3 and EP4 which are G-protein-coupling receptors. These EP receptors are associated with different second messengers and signaling pathways. EP1 activates phospholipase C which generates inositol triphosphate, leading to mobilization of the intracellular calcium. EP2 and EP4, coupled to G_s protein, activate adenylate cyclase leading to increase of intracellular cAMP. EP3, coupled to G_i protein, which inhibits adenylate cyclase leading to reduction of intracellular cAMP (45). PGE_2 involved in colon-rectum tumorigenesis via stimulation of the EP1, EP2 and EP4 receptor downstream signaling pathways which cause apoptosis inhibition, cell proliferation, cell invasion and angiogenesis (45). The main signaling pathways were RAS-MAPK pathway and phosphatidylinositol 3 kinase/protein kinase B pathway (46-52). PGE_2 bound to EP1, activating ERK signaling pathway and inducing VEGF mRNA expression (53). PGE_2 bound to EP2 and amplified of inflammatory response by increasing NF-κB activation which also associated to colorectal cancer progression (54). PGE_2 bound to EP4 and activated PI3K/AKT signaling pathway to stimulate colorectal cancer cell growth and invasion (49).

It has been reported that COX-2 could induce ROS production during prostaglandin synthesis process in cancer cells (42, 55). COX-2 over-expression in osteosarcoma cells involved in tumorigenesis by increasing ROS level (56). Not only COX-2 but also other enzymes are responsible to ROS production. These include xanthine oxidase, nitric oxide synthase, cytochrome P450 enzyme, lipoxygenase and NADPH oxidases (NOXs) (57).

2.1.5 Roles of NOXs on ROS production in cancer

Nicotinamide adenine dinucleotide phosphate or NADPH oxidase (NOX) family consists of 7 isoforms, NOX1-5, double oxidase 1 (Duox1), and Duox2. NOXs are transmembrane enzymes that transfer electron from NADPH to reduce oxygen to superoxide. Superoxide ($O_2^{\bullet-}$) has very short life. It is rapidly converted to hydrogen

peroxide (H_2O_2) via superoxide dismutase (rate= 2×10^9 $M^{-1}s^{-1}$) or spontaneously (rate = 8×10^4 $M^{-1}s^{-1}$) (58). Hydrogen peroxide is more stable than superoxide. It can diffuse across membrane and reacts with oxidation-sensitive cysteine residues in proteins involve in cellular signaling. Hydrogen peroxide is converted to water by antioxidant enzymes, including catalase, glutathione peroxidase, peroxiredoxins (59). ROS mediates various biological responses, including proliferation, apoptosis, differentiation, migration and inflammation.

The NOX family members are widely distributed and their tissue distribution varies greatly. The role of NOXs in cancer biology has been investigated by using human cancer cell lines. NOX1 is the main NOX expressing in colorectal cancer cell lines such as caco-2, DLD-1 and HT-29. The HT-29 cells are reported to express high level of NOX1 and intermediate level of NOX2 expression was detected in this cell line (40). Activation of NOX1 in colon epithelial cells led to the production of ROS which causes genetic instability (60). NOX1-derived ROS caused DNA damage and stimulated cell proliferation and migration, resulting in colon cancer progression (40, 61-65). There was a study demonstrating that reduction of NOX1 induced NOX-2 expression (64).

ROS has dual roles on tumorigenesis. It can induce cancer development and suppress cancer cell growth, depending on its intracellular level. For induction of tumorigenesis, ROS stimulated cancer cell proliferation through various signaling pathways. It induced breast cancer cell proliferation by reducing PTEN activity which leads to PI3K pathway activation (66). It involved in hepatoma cells growth through PI3K/PKB and JNK signaling pathways (67). Inhibition of ROS by NAC, a ROS inhibitor, caused the decrease of glioma cell proliferation via inhibition of PKC, AKT, ERK1/2, NF-κB activities and up-regulation of p21 expression which induces cell cycle arrest at G1 (68). ROS at high level could inhibit cancer cell proliferation and induce cell death. It caused cell cycle arrest at G2/M in hepatoma cancer cells by reducing cdc25C phosphorylation, and increasing ATM, Chk1, and Chk2 phosphorylation (69).

Both pro-oxidants and anti-oxidants have been reported to play critical roles in cancer treatment. Pro-oxidant agents induce the ROS generation and/or decrease

anti-oxidant enzymes such as artemisinin and its derivative. Artesunate, a semisynthetic derivative of artemisinin induced ROS generation, activating lung cancer cells apoptosis (70). Anti-oxidant with anticancer activities are agents that scavenge ROS, increase anti-oxidant enzyme activities, or inhibit NOXs activities (71, 72) such as resveratrol (73), curcumin (11) and epigallocatechin gallate (74). It was reported that resveratrol induced ROS generation which associated with caspase-8 and caspase-3 activation and induction of colorectal cancer cells apoptosis (75).

2.1.6 The relation of NOX and COX-2

It has been demonstrated that the activation of PKC induced NOX-derived ROS generation (76, 77) and COX-2 expression (78, 79). NOX-derived ROS controlled NF-**κ**B activity which associated with inflammation and tumorigenesis in colon cancer cells (62, 80). COX-2 is one of these genes. Several studies demonstrated that NOX – derived ROS induced COX-2 expression (81-87).

2.1.7 The cell cycle and cancer

Uncontrolled of cell proliferation is one characteristic of cancer cells. The cell cycle is a process of cell duplication. This process can be divided into 4 phases, including G1, S, G2 and M phases. G1 is the preparation phase for DNA synthesis. S is replication or DNA synthesis phase. G2 is the preparation phase for checking the completeness of DNA synthesis and cell division. Mitosis or M is the cell division phase before cytokinesis to two daughter cells.

Progression of the cell cycle is controlled by a group of serine threonine kinases called cyclin dependent kinases (CDKs). These enzymes phosphorylate various substrates involving in the cell cycle progression. CDKs are regulated by cyclins, CDK inhibitors, and kinases/ phosphatases. CDKs are active in the forms of CDK-cyclin complexes. Cyclins are differently expressed in each phase. Heterodimer of the CDKs-cyclins complexes could change their conformation, leading to CDKs activation. The phosphorylation of CDKs is activated by CAK (CDK-activating kinase).

The transition in each phase is regulated through activation or de-activation of

CDKs. It should also be noted that CDKs are further regulated by cyclins, which are degraded or synthesized in the cell cycle process. Each phase of cell cycle is regulated by different CDKs; CDK2, CDK4, CDK6 in the G1 phase, CDK2 in the S phase, and CDK1 in the G2/M phase. Cyclin D family (cyclin D1-3) are the first cyclin, which response to extracellular signal such as growth factors. They activate CDK4, CDK6 and retinoblastoma protein family (pRB), causing activation of E2F. In addition, cyclin E family (cyclin E1-2) expressed in G1/S transition phase, activate CDK-2, leading to transition into S phase. However, cyclin A and cyclin E are expressed in S phase where cyclin E are degraded and replaced by cyclin A to form cyclin A/CDK2 complex, resulting in transition from S to G2 phase. Cyclin A is also expressed in G2 phase that activate CDK1, causing transition to M phase. The M phase is regulated by cyclin B/CDK1 complex. Cyclin B interacts with cell division cycle protein2 (cdc2) to form maturation promoting factor (MPF) during anaphase of mitosis. When the cyclin B is degraded, cell will move out of the mitosis and turn to G1 phase.

Cell division is up-regulated by over-activation of CDKs. The CDKs activity is inhibited by cyclin dependent kinase inhibitor (CKIs), including CDK interacting protein/kinase inhibitory protein (CIP/KIP) family and inhibitor of CDK4 (INK4) family. INK4 family, including p15, p16, p18 and p19 bind to CDK4 and CDK6, that inhibiting these proteins to form complex with cyclin D. The CIP/KIP family, including p21, p27, and p57, bind to cyclin/CDK complex and inhibit their activity, leading to cell cycle arrest. The p21 is a CKI protein that binds to the complex of cyclin A/CDK2, cyclin E/CDK2, cyclin D1/CDK4, or cyclin D2/CDK4, inhibiting phosphorylation of pRB protein. Induction of p21 inhibits cyclin E/CDK2 complex leading to cell cycle arrest at G1/S transition. p21 can inhibit CDK1, resulting in cell cycle arrest at G2/M transition. Moreover, p21 also inhibit the complex of cyclin A/CDK1 or 2, leading to inhibition of cell cycle to S phase. On the other hand, p21 is associated with apoptosis by interaction of NF-κB and STAT, leading to suppression of anti-apoptotic protein, including Bcl-2 and Bcl-XL and consequently induction of cell apoptosis (88). However, the cell cycle will stop when the cell damage in order to repair the damage. In case that the damage is un-repaired, the cell will induce cell death (89, 90).

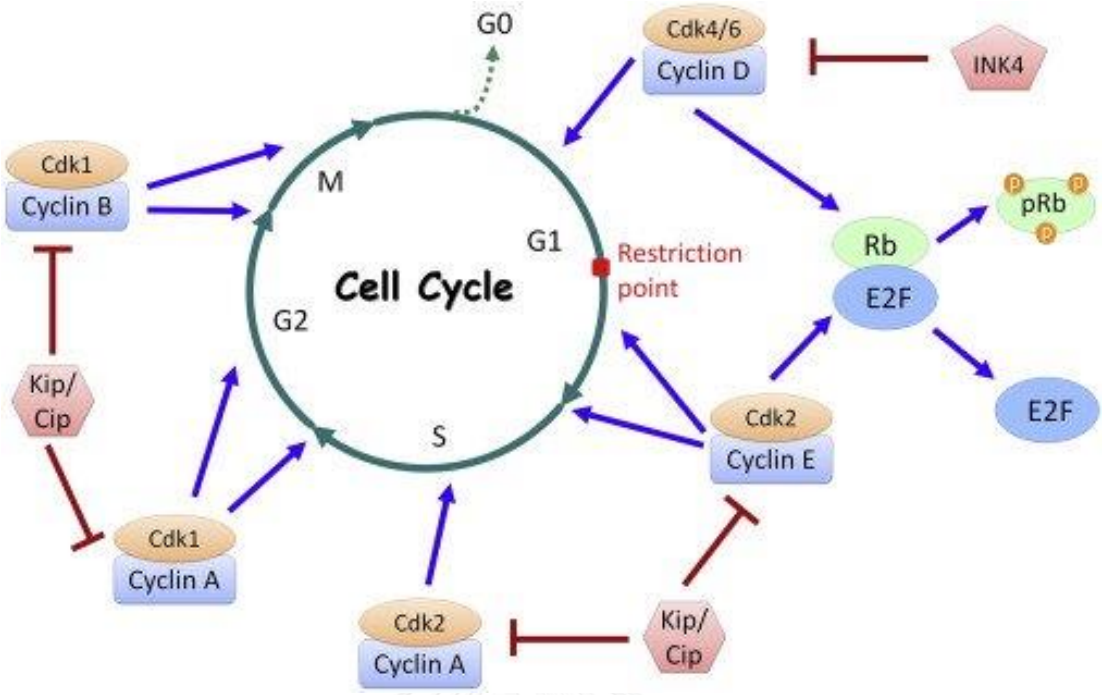


Figure 1 The cell cycle progression and their regulators (91)

2.1.8 Apoptosis

The apoptosis is one homeostasis mechanism to regulate cell population. Apoptosis has two major pathways; extrinsic death receptor pathway and intrinsic mitochondria pathway.

Firstly, the extrinsic death receptor pathway is initiated by binding of tumor necrosis factor receptor superfamily, including CD95 (Fas/APO-1) to the TNF-related apoptosis-inducing ligand (TRAIL), recruiting adaptor protein such as Fas-associated death domain protein (FADD) to activate receptors to form death-inducing signaling complex (DISC). These events recruits and activates initiator caspases such as caspase 8 and caspase 10, which will further activate effector caspases such as caspase 3 and caspase 7, leading to apoptosis.

Secondly, the intrinsic mitochondria pathway generally responses to the cellular stress, and anticancer drugs, resulting in increased mitochondria outer membrane permeability (MOMP). MOMP is associated with the opening of permeability transition pore complex. These events lead to release of cytochrome c into cytosol

which bind with apoptotic protease-activating factor 1 (APAF-1), forming apoptosome. Procaspase 9 is then cleaved by protease to its active form and activate executioner caspase 3 and caspase 7, leading to apoptosis.

The extrinsic pathway can activate intrinsic pathway through BH-3 only protein, BH3-interacting death domain agonist (Bid) by cleavage of the caspase 8. Bid interacts with mitochondria. MOMP is controlled by Bcl-2 family proteins. BH-3 only protein controls activation of pro-apoptotic Bcl-2 proteins, including BAX and BAK by inhibiting anti-apoptotic Bcl-2 proteins. The activation of BH-3 only caused oligomerization of BAX and BAK. They form pores, leading to release of cytochrome c and cytotoxic proteins such as second mitochondria-derived activator of caspase (SMAC). The anti-apoptotic Bcl-2 proteins, including Bcl-2, Bcl-XL and Mcl-1, bind to pro-apoptotic Bcl-2 protein which inhibits pore formation, preventing apoptosis (71, 89, 90, 92).

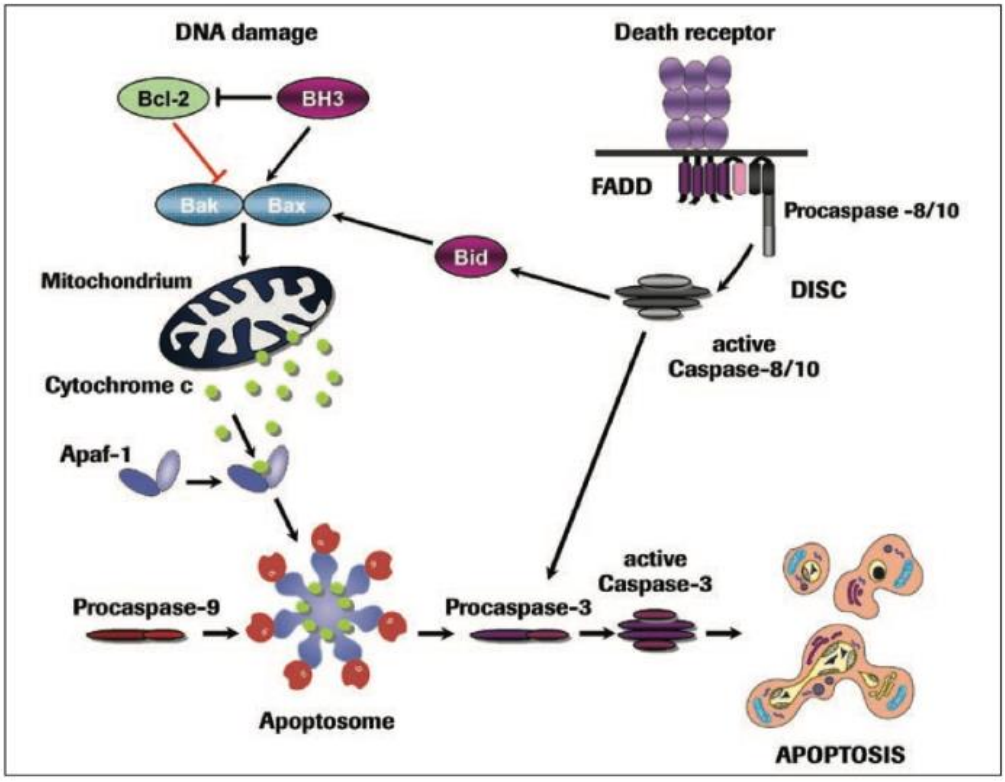


Figure 2 The cell apoptosis pathway (90)

2.1.9 NSAIDS on cancer

Previous studies have demonstrated that the COX-2 expression in adenomas is approximately 50% and it is increased into 85% in adenocarcinomas Therefore, COX-2 is considered one of the molecular targets for colorectal cancer prevention and treatment. Aspirin and other non-steroidal anti-inflammation drugs (NSAIDs) were shown to reduce the colorectal cancer progression (93). NSAIDs exert both anti-inflammatory and anti-tumor activities via inhibition of COX activity. NSAIDs are commonly used worldwide and their side effects include nausea, dyspepsia, gastritis, abdominal pain, peptic ulcer and gastrointestinal bleeding. These side effects are associated with the inhibition of COX-1. Therefore, in order to reduce these side effects, specific COX-2 inhibitors are recommended for colorectal cancer prevention and treatment (5).

2.2 Celecoxib

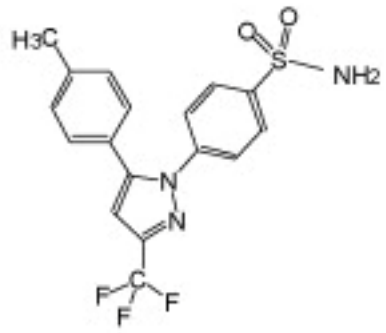


Figure 3 The chemical structure of celecoxib

Celecoxib (1,5- diaryl pyrazole- based compound) is a specific COX-2 inhibitor which binds to the catalytic site of COX-2. It has been approved to treat osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, acute pain, primary dysmenorrhea and familial adenomatous polyposis. The US-FDA approved this drug for reduction of polyps in familial adenomatous polyposis patient and colorectal cancer risk. Celecoxib is a highly selective COX-2 inhibitor. Thus, the gastrointestinal toxicity is reduced by about 50%. Celecoxib not only inhibit PGE_2 production but also inhibit PGI_2 production. The inhibition of PGI_2 could promote platelet aggregation. The PGI_2 antagonizes

thromboxane A2 produced from platelet and the inhibition may shift the homeostatic balance to increase thromboxane A2 effect, leading to increased risk of cardiovascular disease such as coronary thrombosis and stroke. The dose of celecoxib approved for FAP treatment is at 800 mg/day and treatment of the drug at this high dose for 12-18 months has increased the risks of cardiovascular disease from the PGI_2 inhibition. Solomon et al. demonstrated that doses of celecoxib were related with cardiovascular disease risk (94). Hence, it is recommended that using celecoxib at 800 mg/day for 1 year may be sufficient to prevent polyp recurrent before these side effects appear. However, the meta-analysis of independent 72 studies indicated that there was no association between celecoxib and cardiovascular disease risk (95).

2.2.1 Anti-tumor activity

The anti-tumor activity of celecoxib has been mediated through inhibition of cell migration, suppression of angiogenesis, induction of cell cycle arrest and apoptosis. In addition, Dai et al reported that celecoxib inhibited COX-2 expression and decreased PGE_2 product in breast cancer (96). The PGE_2 production was also decreased by COX-2 down-regulation through the reduction the nuclear localization of NF-**κ**B *in vivo* model (97). Moreover, Grosch et al. demonstrated that celecoxib arrested cell cycle at G1 phase in colorectal cancer cells through up-regulation of p21 and p27 and down-regulation of cyclin A, cyclin B1 and CDK-1. This agent also suppressed tumor cell growth *in vivo* model (98). Similarly, Peng et al. showed that celecoxib induced p21 over-expression and inhibited CDK-2 and CDK4 expression in colorectal cancer cells (9). Celecoxib also induced cell cycle arrest at G1/S phase by up-regulating p16 and p27 expression and down-regulating cyclin D1, cyclin E and pRB expression in chronic myeloid leukemia cells (7). Previous studies reported that celecoxib induced cell apoptosis via extrinsic apoptosis pathway by activating death receptor (TRAIL receptor system). It also triggered intrinsic apoptosis pathway via down-regulation of the anti-apoptotic protein Mcl-1, follow by the translocation of BAX from mitochondria to cytosol, causing cytochrome C release (99). Furthermore, Jendrossek et al. found that it induced the cell apoptosis in lymphoma through release of cytochrome C, activation of caspase 9, 8, 3 and cleavage of PARP (100). The growth Inhibitory effect of celecoxib

was found to be independent of p53 in prostate cancer (101). It also induced apoptosis in colon cancer cells by inhibiting the 3-phosphoinositide-dependent kinase 1 activity (102). Additionally, celecoxib was able to reduce ROS generation in breast cancer cells (103). However, it induced intracellular ROS generation, causing reduced mitochondria membrane potential *in vivo* model (104). Bastos-Pereira et al. showed that celecoxib reduced cell growth in carcinosarcoma-inoculated in an animal model by increasing antioxidant enzymes such as superoxide dismutase and catalase, inhibiting NADH oxidase and succinate oxidase and suppressing anti-apoptotic protein Bcl-XL expression (105). It also decreased the expression of genes involved in lipid and glutathione metabolism and proliferation, reducing cell proliferation in the colorectal cancer patients (106). Moreover, previous studies have reported that celecoxib potentiated the anti-tumor of chemotherapy and radiation in various cancer cells. Sanchez et al. found the synergistic anti-tumor effect of celecoxib-capecitabine combination in BOP-induced pancreatic cancer animal model by increasing antioxidant enzyme, including superoxide dismutase, catalase and glutathione peroxidase (107). Similarly, celecoxib enhanced anti-tumor activity of cetuximab in oral squamous cell carcinoma via inhibition of PGE_2 production, VEGF expression and EGFR PI3K and AKT phosphorylation. Their combination also suppressed tumor cell growth in animal model (108). Zhang et al. demonstrated that celecoxib augmented anti-tumor activity of 5-FU by inhibiting COX-2 protein expression and inducing apoptosis via the activation of cytochrome C *in vivo* (13). It also enhanced the radiosensitivity of prostate cancer and nasopharyngeal carcinoma (109, 110). Moreover, Lev-ari et al. revealed that the combination of celecoxib with curcumin synergistically suppressed colorectal cancer cells growth by inhibiting COX-2 expression and its products (11). The synergistically of celecoxib with EGCG was also illustrated in lung cancer cells by inducing GADD153 expression, leading to apoptosis (12). Thus, celecoxib should be used in combination with other agents in order to increase its efficacy and reduce its side effects.

2.3 Cepharanthine

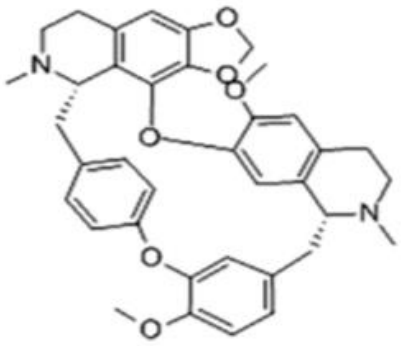


Figure 4 The chemical structure of cepharanthine

Cepharanthine is a biscoclaurine (bisbenzylisoquinoline) alkaloid, isolated from the root of *Stephania cepharantha* Hayata. It has been used in Japan more than 40 years for the treatment of various chronic and acute diseases and Japanese Ministry of Health also approved the cepharanthine for treatment in alopecia areata, radiation-induced leukopenia and idiopathic thrombocytopenic purpura (ITP). It has many pharmacological effects including anti-malaria, anti-virus HIV-1, anti-allergic effect, anti-platelet aggregation, anti-inflammation, anti-oxidant and anti-tumor activity (16). Although cepharanthine is widely used, its serious side effects of cepharanthine have never been reported. Cepharanthine was not toxic to lymphocyte isolated from normal volunteer (25). It also did not have bone marrow toxicity (111). Cepharanthine was found to alleviate side effects of radiotherapy in patients with head and neck cancer (112). Tanimura et al. reported that 2 patients received the cepharanthine at the high dose of for 23 and 35 days did not have any side effects (113). Sato et al. also demonstrated that 13 patients receiving orally cepharanthine 100 mg/day for 3 years did not have any side effects. The mild side effects of cepharanthine have been reported such as headache, stomach dis-comfortable and dizziness (114).

2.3.1 Anti-inflammation

Cepharanthine was shown to reduce inflammation through suppression of pro-inflammatory cytokine production such as TNF-α, IL-1β, IL-6. Inhibition of the NF- κB activity by preventing phosphorylation of ERK, JNK, P38 and inducing IκBα degradation

in LPS-stimulated RAW264.7 cells *in vitro* model and LPS-induced acute lung injury *in vivo* model (115). Moreover, cepharanthine also inhibited production of NO, PGE_2 and translocation of NF-κB from cytosol to nucleus in RAW264.7 cells (116). This agent also inhibited secretion of cytokines including TNF-α, IL-6 and NO-associated protein and phosphorylation of IκB, leading to suppression of the NF-κB activation in LPS-stimulated systemic inflammation model (117). Additionally, cepharanthine has been demonstrated to reduce ROS such as superoxide anion in neutrophils by inhibiting PKC and NADPH oxidase activity (118).

2.3.2 Anti-tumor activity

Cepharanthine has displayed the anti-tumor activity in various cancer cells through induction of cell cycle arrest, generation of ROS and induction of cell apoptosis. Cepharanthine also inhibited COX-2 expression but did not inhibit the activity of COX-2 in colorectal cancer HT-29 cells (23). Effect of cepharanthine on induction of cell cycle arrest was mediated through the up-regulation of cyclin dependent kinase inhibitor such as p21 and down-regulation of cyclins. Harada et al. demonstrated that cepharanthine inhibited cell proliferation of adenosquamous cell carcinomas (TYS) by up-regulating p21, leading to cell cycle arrest at G1 phase. It also induced cell apoptosis through caspase-3 activation (18). Moreover, it blocked the cell cycle progression at G1 phase in myeloma cells through up-regulation of cyclin dependent kinase inhibitors such as p15 and p21 and down-regulation of cyclin D1 and CDK-6. It also induced cell apoptosis via ROS generation and caspase-3 activation (25). Hua et al. showed that cepharanthine induced non-small cell lung cancer cells to undergo apoptosis through generation of ROS, reduction of mitochondria membraned potential, up-regulation of BAX, down-regulation of Bcl-2, activation of caspase and cleavage of PARP (24). Cepharanthine has demonstrated the anti-tumor activities by suppressing MAPK, STAT3 and NF-κB pathways. It suppressed cell growth in osteosarcoma cells (SaOS2) by inducing cell cycle arrest at G1 phase. Inhibition of STAT3 expression led to decreased expression of target genes such as anti-apoptotic gene Bcl-XL and cell cycle regulators such as c-myc and cyclin D1 (17). This compound induced cell apoptosis in cholangiocarcinoma cells through activation of caspase 9 and

caspase 3 and suppression of NF-κB nuclear translocation (119). Moreover, cepharanthine induced apoptosis in leukemia cells by inducing caspase activation and DNA fragmentation (19). Similarly, Biswas et al. have reported that cepharanthine induced cell apoptosis in hepatocellular carcinoma cells via generation of ROS, activation of MAPK, p38, JNK1/2 and down-regulation of PKB/AKT. It also induced release of cytochrome C followed by caspase-3 activation and PARP cleavage (26). In addition, cepharanthine has exerted the multidrug resistance reversal effect by inhibiting the activity of P-gp transporter. It could sensitize many resistant cancer cells to chemotherapeutic drugs especially, vincristine (21), doxorubicin (22) and paclitaxel (20). Furthermore, cepharanthine has shown the chemo-potentiation effect in various cancer cells, including leukemia cells (27-29), colon cancer cells (30), uterine cervical cancer cells (30) and oral squamous cell carcinoma (31). Kato et al. found that cepharanthine enhanced the anti-tumor activity of vincristine in leukemia cells about 1.5-7 fold (27). This compound also enhanced the anti-tumor activity of methylglyoxal bis cyclopentyl amidinohydrazone (polyamine biosynthesis inhibitor) on leukemia cells by inhibiting macromolecule synthesis (28). Ikeda et al. reported that cepharanthine potentiated the antitumor of vincristine (4.4 fold) and doxorubicin (5.4 fold) in leukemia cells by inducing cell apoptosis (29). Ono et al. revealed that cepharanthine enhanced the anti-tumor activity of vinca alkaloids in both colon cancer cells and uterine cervical cancer cells (30). Similarly, it enhanced the anti-tumor activity of fluoropyrimidine in oral squamous cell carcinoma (31). Cepharanthine potentiated the radiotherapy in cervical adenocarcinoma (HeLa) by inhibiting expression of COX-2 and STAT3. This in turn led to down-regulation of STAT3 target genes such as anti-apoptosis Bcl-2 and cell cycle regulator c-myc (32). It also enhanced radiosensitivity of oral squamous cell adenocarcinoma about 1.47-1.55 times by inducing apoptosis and inhibiting DNA double strand break repair (120).

CHAPTER III
Materials and Methods

3.1 Reagents and Materials

3.1.1 Cells

HT-29 human colorectal cancer cell line (HTB-38™) were obtained from American Type Culture Collection-ATCC (Rockville, MD)

3.1.2 Test compounds

- Celecoxib and cepharanthine were test compounds used in this study. Celecoxib was purchased from Sigma-Aldrich (USA), and cepharanthine was purchased from Abcam Biochemicals (UK).

3.1.3 Chemicals

The following reagents and reagent kits were used in this study;

- Dulbecco's modified Eagle medium (DMEM) (Gibco, USA)
- Penicillin- Streptomycin (Gibco, USA)
- Fetal bovine serum (Gibco, USA)
- 0.25% trypsin-EDTA (Gibco, USA)
- 0.4% trypan blue dye (Sigma, USA)
- Dimethyl sulfoxide [cell culture grade] (Sigma, USA)
- Resazurin sodium salt (Sigma, USA)
- TRIzol reagent (Invitrogen, USA)
- 2-propanol (Merck, Germany)
- Ethanol (Merck, Germany)
- Chloroform (Lab-scan, Thailand)
- Nuclease-free water (Qiagen, Germany)
- Diethyl pyrocarbonate (DEPC) (Ambion, USA)
- Improm-II™ Reverse Transcription system (Promega, USA)

- Express SYBR green qPCR supermix universal (Invitrogen, USA)
- QPCR Green Master Mix HROX (Biotechrabbit, Germany)
- 2'-7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma, USA)
- Hank's Balanced salts (HBSS) (Sigma, USA)
- Triton® X-100 (Sigma, USA)
- Propidium iodide (Santa Cruz Biotecnology, USA)
- HEPES (Sigma, USA)
- FIT-C Annexin V (Invitrogen, USA)
- RNase (Thermo scientific, USA)
- PGE_2 ELISA kit (Invitrogen, USA)

3.1.4 Instruments and equipments

The following instruments and equipments were used;

- 25 cm^2 flask vent cap (Corning Inc., USA)
- 96 well plate flat bottom polystyrene plate (Corning Inc., USA)
- 6 well plate (Corning Inc., USA)
- 96 well black flat bottom polystyrene plate (Corning Inc., USA)
- 15 ml plastic conical centrifuge tube (Corning Inc., USA)
- 50 ml plastic conical centrifuge tube (Corning Inc., USA)
- 5 ml round bottom polystyrene tube (Falcon, USA)
- Pipette tips (Axygen, USA)
- 1.5 ml microcentrifuge tube (Axygen, USA)
- 0.2 ml PCR tubes with flat cap (Axygen, USA)
- 0.1 ml low profile polypropylene thin wall PCR tube strips (Axygen, USA)
- Pipette (Brandtech Scientific Inc., USA)
- Pipette controller (Brandtech Scientific Inc., USA)
- Multichannel Pipettors (Thermo Scientific, USA)
- Analytical balance (Sartorious, Germany)
- Vortex (Scientific Industries, USA)
- Incubator (Thermo scientific, USA)

- Microliter centrifuge (Hettich, USA)
- pH meter (Mettler toledo, Switzerland)
- Microscope (Nikon, Japan)
- Microplate reader (Thermo Fisher Scientific, USA)
- Fluorescence microplate reader (Thermo Scientific, USA)
- FACScalibur flow cytometer (BD Biosciences, USA)
- Oven (WTB Binder, Germany)
- Autoclave (Sanyo, Japan)
- Laminar flow hood (Issco, USA)
- Nanodrop UV-Vis spectrophotometer (Thermo Scientific, USA)
- Mastercycler personal PCR thermal cycler (Eppendorf, Germany)
- StepOnePlus real time PCR (Applied Biosystems, USA)
- Refrigerated incubator shaker (New Brunswick, Germany)

3.2 Methods

3.2.1 Cell culture

HT-29 human colorectal cancer cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin & streptomycin at 37 °C in a humidified 5% CO_2. Cells were sub-cultured when they reached 80% confluence. Cells in the exponential growth phase with over 95% viability were used for all experiments in this study.

3.2.2 Preparation of celecoxib and cepharanthine solutions

The stock solutions of celecoxib at 25 mM and cepharanthine 10 mM were prepared by dissolving in dimethyl sulfoxide (DMSO). For treating cells, these stock solutions were diluted to required concentrations with the completed DMEM and the final concentration of DMSO was constantly kept at 0.2%.

3.2.3 Determination of the effects of celecoxib and cepharanthine on HT-29 cell viability by resazurin assay

Resazurin assay was used to determine the metabolic activity of viable cells which reduced the blue color of resazurin to the pink color of resorufin. HT-29 cells were seeded in a 96 well plate at the density of 5×10^3 cells/well. Twenty-four hours later, the cells were treated with celecoxib at 2.5, 5, 10, 20 and 50 μM and cepharanthine at 1.25, 2.5, 5, 10 and 20 μM for 48 h. 0.2% DMSO in DMEM was used as the vehicle control. Five hours before the end of the treatment, 15 μl of 0.05 mg/ml resazurin solution was added into each well. The absorbance of each well was measured by a microplate reader at 570 and 600 nm. The effects of celecoxib and cepharanthine on HT-29 cells were presented as the percentage of cell viability compared with the vehicle control, according to the following formula

$$\% \text{ cell viability} = \frac{\Delta \text{ OD (sample)}}{\Delta \text{ OD (0.2\%DMSO)}} \times 100\%$$

Where Δ OD = OD 570 nm - OD 600 nm

The IC_{50} values of celecoxib and cepharanthine were calculated by GraphPad Prism 7 software

3.2.4 Determination of celecoxib-cepharanthine combination effect on HT-29 cell viability analysis of drug interaction by combination index (CI)

Combination index (CI) is the method widely used to determine the effect of drug combination. HT-29 cells were seeded in a 96 well plate at a density of 5×10^3 cells/well. Twenty-four hours later, the cells were treated with celecoxib at 5, 10, 20, 40 μM, cepharanthine at 1.25 and 2.5 μM and 8 combinations of both drugs for 48 h. 0.2% DMSO in DMEM was used as the vehicle control. Viability of the treated cells was determined by resazurin assay. The percentage of cell viability of the combinations and each drug was compared with vehicle control. The IC_{30} value of each drug was calculated by GraphPad Prism 7 software. The combination index (CI) of the drugs was determined according to Chou-Talalay method (121) using the following formula

$$\text{Combination Index (CI)} = \frac{(\text{D combination})1}{(\text{D alone})1} + \frac{(\text{D combination})2}{(\text{D alone})2}$$

$D_{combination\ 1}$: The concentration of first drug (e.g. celecoxib) that gives 30% of cell inhibition

$D_{combination\ 2}$: The concentration of second drug (e.g. cepharanthine) that give 30% of cell inhibition

$D_{alone\ 1}$: IC_{30} of the first drug

$D_{alone\ 2}$: IC_{30} of the second drug

CI values were interpreted as follows; CI = 1 as additive effect CI < 1 indicate as synergism and CI >1 as antagonism. Interpretation in more detail were, CI< 0.1 as very strong synergism, CI=0.1-0.3 as strong synergism, CI=0.3-0.7 as synergism, CI=0.70-0.85 as moderate synergism, CI=0.85-0.90 as slight synergism, CI=0.90-1.10 as nearly additive, CI=1.10-1.20 as slight antagonism, CI=1.20-1.45 as moderate antagonism, and CI=1.45-3.3 as antagonism.

3.2.5 Determination of mRNA expression of the interested genes by quantitative real time RT-PCR

The effect of celecoxib and cepharanthine combination on mRNA expression of COX-2, NADPH oxidase (NOX1, NOX2), cell cycle regulators (cyclins: cyclin D, cyclin E, cyclin A; cyclin dependent kinase inhibitor: p21) and bcl-2 family (pro-apoptotic genes: BAX, BAK; anti-apoptotic genes: Bcl-2, Bcl-XL and Mcl-1) were determined by quantitative real time RT-PCR. HT-29 cells were seeded in a 6 well plate at the density of 5×10^5 cells/well. Twenty-four hours later, the cells were treated with celecoxib at 20 and 40 μM, cepharanthine at 1.25 and 2.5 μM, and combinations of both drugs for 24 h. 0.2 % DMSO was used as the vehicle control. Total RNA from the treated cells were isolated by TRIzol reagent. The concentration and the purity of the RNA were determined by measuring at the absorbance 260 and 280 nm using Nanodrop spectrophotometer. These RNA samples were transcribed to cDNA samples by Improm-II™ Reverse Transcription system according to manufacturer's instruction. The

cDNA samples were used as the templates to determine the expression of genes of interest by quantitative real time RT-PCR using SYBR green qPCR supermix universal with specific primers in **Table 2** and performing in a StepOne^{TM} Plus real-time PCR system. Each reaction mixture contained cDNA sample, forward primer, reverse primer, ROX reference dye and SYBR green. The real-time PCR reaction conditions consisted of pre-incubation at 50°C for 2 minutes, 95°C for 10 minutes, then cycling for 40 cycles 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Expression of the studied genes was determined by $2^{-\Delta\Delta CT}$ method of each gene compared with the reference GAPDH gene.

Table 2 The sequencing primers used for quantitative real time RT- PCR

Specific primers	Sequences	
GAPDH	Forward	5'- ATG GCA TGG ACT GTG GTC ATG AGT -3'
	Reverse	5'- AAG GTC GGA GTC AAC GGA TTT GGT -3'
P21	Forward	5'- CCT GTC ACT GTC TTG TAC CCT -3'
	Reverse	5'- GCG TTT GGA GTG GTA GAA ATC T -3'
Cyclin D	Forward	5'- TTG TTG AAG TTG CAA AGT CCT GG -3'
	Reverse	5'- ATG GTT TCC ACT TCG CAG CA -3'
Cyclin E	Forward	5'- TCC TGG ATG TTG ACT GCC TT -3'
	Reverse	5'- CAC CAC TGA TAC CCT GAA ACC T -3'
Cyclin A	Forward	5'- CTG CTG CTA TGC TGT TAG CC -3'
	Reverse	5'- TGT TGG AGC AGC TAA GTC AAA A -3'
COX-2	Forward	5'- CCC TGA GCA TCT ACG GTT TG -3'
	Reverse	5'- TCG CAT ACT CTG TTG TGT TCC -3'
NOX-1	Forward	5'- GGT TTA CCG CTC CCA GCA GAA -3'
	Reverse	5'- GGA TGC CAT TCC AGG AGA GAG -3'
NOX-2	Forward	5'- CCT AAG ATA GCG GTT GAT GG -3'
	Reverse	5'- GAC TTG AGA ATG GAT GCG AA -3'
Bcl-2	Forward	5'- TCA TGT GTG TGG AGA GCG TCA A -3'
	Reverse	5'- CTA CTG CTT TAG TGA ACC TTT TGC -3'
Bcl-XL	Forward	5'- TTG GAC AAT GGA CTG GTT GA -3'
	Reverse	5'- GTA GAG TGG ATG GTC AGT G -3'
Mcl-1	Forward	5'- GCT GGA GTA GGA GCT GGT T -3'
	Reverse	5'- CCT CTT GCC ACT TGC TTT TC -3'
BAX	Forward	5'- GAC GAA CTG GAC AGT AAC ATG -3'
	Reverse	5'- AGG AAG TCC AAT GTC CAG CC -3'
BAK	Forward	5'- ATG GTC ACC TTA CCT CTG CAA -3'
	Reverse	5'- TCA TAG CGT CGG TTG ATG TCG -3'

3.2.6 Measurement of ROS generation by DCFH-DA assay

The intracellular ROS was measured by 2'-7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). Once enters the cells, DCFH-DA is changed to DCFH by intracellular esterase. DCFH is then oxidized by ROS to DCF fluorescent product. HT-29 cells were seeded in a 96 well plate at the density 1×10^4 cells/well. Twenty-four hours later, the cells were washed with PBS, treated with 100 μl DCFH-DA in Hank's Balanced salts solution (50 μM) for 30 min. DCFH-DA was removed and the cells were treated with celecoxib at 20 and 40 μM, cepharanthine 1.25 and 2.5 μM; 4 combinations of both drugs, 0.2 % DMSO as vehicle control and 0.3% H_2O_2 as positive control for 1 h. The cells were washed with cold PBS and lysed with 200 μl 1% triton-X in 0.3 M NaOH. The plate was shaken for 10 min. The cell lysis was transferred to a 96 well black flat bottom polystyrene plate. The fluorescent intensity was measured by a fluorescent microplate reader at 485 and 570 nm. The percentage of DCF fluorescence of each condition was calculated compared to the vehicle control by the following formula;

$$\% \text{ DCF fluorescence} = \frac{\textbf{Fluorescent intsensity(treated)}}{\textbf{Fluorescent intensity(0.2\%DMSO)}} \times 100\%$$

3.2.7 Measurement of prostaglandin-E2 (PGE_2) level by competitive ELISA kit

PGE_2 level in culture medium was determined by competitive enzyme-linked immunosorbent assay (ELISA). HT-29 cells were seeded in a 6 well plate at density 5×10^5 cells/well. Twenty-four hours later, the cells were treated with celecoxib at 20 and 40 μM, cepharanthine at 1.25 and 2.5 μM; 4 combinations of both drugs and 0.2 % DMSO as vehicle control for 24 h. The supernatant of the treated cells was collected and stored at 20°C. PGE_2 level was measured according to the manufacturer's instruction. Briefly, 100 μM of standard solution and the sample solutions were added into each well. Later, 50 μM of the PGE_2-AP conjugated and PGE_2 antibody were added in each well. The plate was covered and shaken at 500 rpm for 2 h. The wells were

washed with 400 μl wash buffer 5 times. Two hundred μl of substrate solution was added and incubated at the room temperature for 45 min. Fifty microliters of stop solution were added in each well. The absorbance was measured by a microplate reader at 405 and 570 nm. The PGE_2 levels in the samples were determined from the PGE_2 standard curve.

3.2.8 Cell cycle analysis by flow cytometer

The DNA contents of cells in each phase of the cell cycle were measured by propidium iodide staining using flow cytometer. HT-29 cells were seeded in a 6 well plate at density 2.5×10^5 cells/well. Twenty-four hours later, the cells were treated with celecoxib at 20 and 40 μM; cepharanthine at 1.25 and 2.5 μM; celecoxib 2 concentration: 20, 40 μM, 4 combinations of both drugs and 0.2 % DMSO (vehicle control) for 48 h. The cells were washed with PBS, trypsinized by 500 μl of 0.25% trypsin-EDTA and added 1 ml DMEM. The cells were transferred to 5 mL round bottom polystyrene tube and centrifuged at 1500 RPM at 25°C for 5 min. The cells were washed twice with 1 ml cold PBS and fixed with 70% ethanol at -20 °C for 20 min. The cells were then washed twice with cold PBS, re-suspended in 500 μl assay buffer, treated 5 μl RNase (4 mg/ml) at room temperature for 30 min, stained with 5 μl propidium iodide for 15 min and analyzed 10,000 cells/sample by FACScalibur flow cytometer. The percentage of cells in each phase of the cell cycle was analyzed by FACDIVA version 6.1.3 software. The phases of the cell cycle were interpreted from DNA contents of cells as follow; 2N DNA in G1 phase, more than 2N but less than 4N in S phase, and 4N in G2/M phase.

3.2.9 Cell apoptosis analysis by flow cytometer

The cell apoptosis was measured by annexin V-FIT C/propidium iodide staining using flow cytometer. In viable cells, phosphatidylserine (PS) locates in the inner plasma membrane. In early apoptotic cells, PS is flipped to the outer plasma membrane, and can be detected by FITC-labelled annexin V which specifically binds to PS in the presence of Ca^{2+}. Propidium iodide is an impermeable-dye. It can enter

into a cell when the plasma membrane lose integrity, and bind to DNA in late apoptosis cells and necrotic cells. HT-29 cells were seeded in a 6 well plate at density 2.5×10^5 cells/well. Twenty-four hours later, the cells were treated with celecoxib at 20 and 40 μM, cepharanthine at 1.25 and 2.5 μM, 4 combinations of both drugs and 0.2 % DMSO (vehicle control) for 24 h. The cells were collected by trypsinization. The cell pellets were washed twice with 1 ml cold PBS, re-suspended in 100 μl assay buffer, stained with 1 μl FIT-C Annexin V for 15 min and 1 μl propidium iodide for 5 min on ice, respectively, and analyzed 10,000 cells/sample by FACScalibur flow cytometer. The patterns of cell death were analyzed by FACDIVA version 6.1.3 software. Cells are interpreted as viable cells if no staining, as early apoptotic cells if stained with annexin V-FITC, as necrotic cells if stained with PI, and as late apoptotic cells if stained with annexin V-FITC and PI.

3.2.10 Data analysis

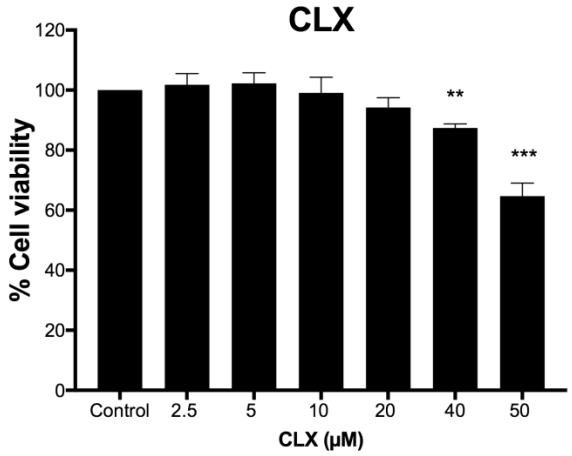
The data are presented as mean ± standard error of mean (SEM) of three independent experiments (n=3). Statistical analysis was analyzed by SPSS statistics version 22 software. The comparison between the celecoxib-cepharanthine combination and celecoxib alone or cepharanthine alone was performed by One-way analysis of variance (ANOVA) with LSD post hoc test. Statistically significant difference was considered at $P \leq 0.05$.

CHAPTER IV
Results

4.1 Effects of celecoxib and cepharanthine on HT-29 cell viability

The effects of celecoxib and cepharanthine on the viability of colorectal HT-29 cells were determined by resazurin assay. The cells were treated with celecoxib at 2.5, 5, 10, 20, 40, and 50 μM for 48 h. The result in **Fig. 5A** demonstrated that celecoxib at 40 and 50 μM significantly decreased the viability of HT-29 cells with IC_{50} more than 50 μM. HT-29 cells were treated with cepharanthine at 1.25, 2.5, 5, 10, and 20 μM for 48 h. As shown in **Fig 5B**, cepharanthine decreased the viability of HT-29 in concentration dependent manner with the IC_{50} 5.22 ± 0.28 μM. Celecoxib and cepharanthine at lower than their IC_{50} values were used for investigating their combination effect on HT-29 cells.

A)



B)

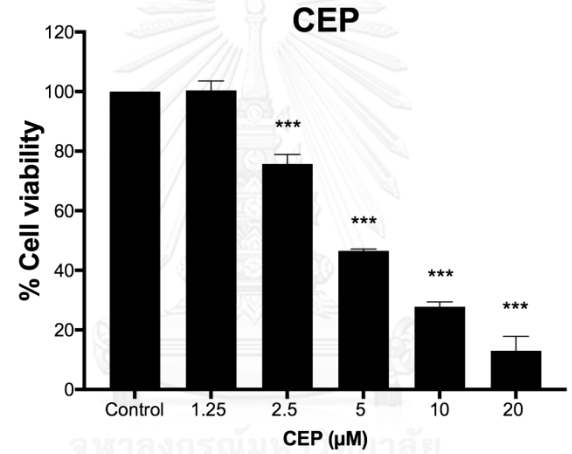


Figure 5 Effects of celecoxib and cepharanthine on HT-29 cell viability **A)** The cells were treated with celecoxib at 2.5, 5, 10, 20, 40, and 50 μM for 48 h. **B)** The cells were treated with cepharanthine at 1.25, 2.5, 5, 10, and 20 μM for 48 h. The viability of the treated cells was determined by resazurin assay. The data are presented as mean ± SEM from three independent experiments (n=3) ** $P \leq 0.01$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control.

4.2 Effect of celecoxib - cepharanthine combination on HT-29 cell viability

Celecoxib at 5, 10, 20, and 40 μM and cepharanthine at 1.25 and 2.5 μM, which were lower than their IC_{50} values, were used to evaluate their combination effect on the viability of HT-29 cells. The cells were treated with four concentrations of celecoxib, two concentrations of cepharanthine, and 8 combinations of celecoxib and cepharanthine for 48 h. The viability of the treated cells was determined by resazurin assay. The result in **Fig. 6A** demonstrated the combination effects of cepharanthine 1.25 μM and four concentrations of celecoxib. When each drug was compared with the vehicle control, only celecoxib at 40 μM significantly decreased the viability of HT-29 cells. Cepharanthine at 1.25 μM did not have effect on the cell viability. When the celecoxib - cepharanthine combinations were compared with each drug, all combinations significantly decreased the viability of HT-29 cells. The result in **Fig. 6B** presented the combination effects of cepharanthine at 2.5 μM and four concentrations of celecoxib. When each drug was compared with the vehicle control, celecoxib at 40 μM and cepharanthine at 2.5 μM significantly decreased the viability of HT-29 cells. When the combinations were compared with each drug, the combination of cepharanthine at 2.5 μM with celecoxib at 20 and 40 μM significantly decreased HT-29 cell viability. The viability of HT-29 cells treated with cepharanthine at 2.5 μM in combination with celecoxib at 5 and 10 μM did not different from cepharanthine alone.

The effects of celecoxib – cepharanthine combination on the viability of HT-29 was also interpreted as the combination index (CI) values according to Chou-Talalay method (121). The IC_{30} values of celecoxib (48.18 ± 1.53 μM) and cepharanthine (3.08 ± 0.22 μM) were used to calculate the CI value of each combination. The result in **Table 3** and **4** showed that, the CI values of the combinations were less than 1 (CI<1). Thus, the combinations had nearly additive effect (CI = 0.964) or moderate synergistic effects to synergistic effects (CI range: 0.424-0.777) on the viability of HT-29 cells.

Combinations of celecoxib at 20 and 40 μM with cepharanthine at 1.25 and 2.5 μM, which had CI range 0.424 – 0.694, were chosen for investigating their combination effects at molecular levels on HT-29 cell viability.

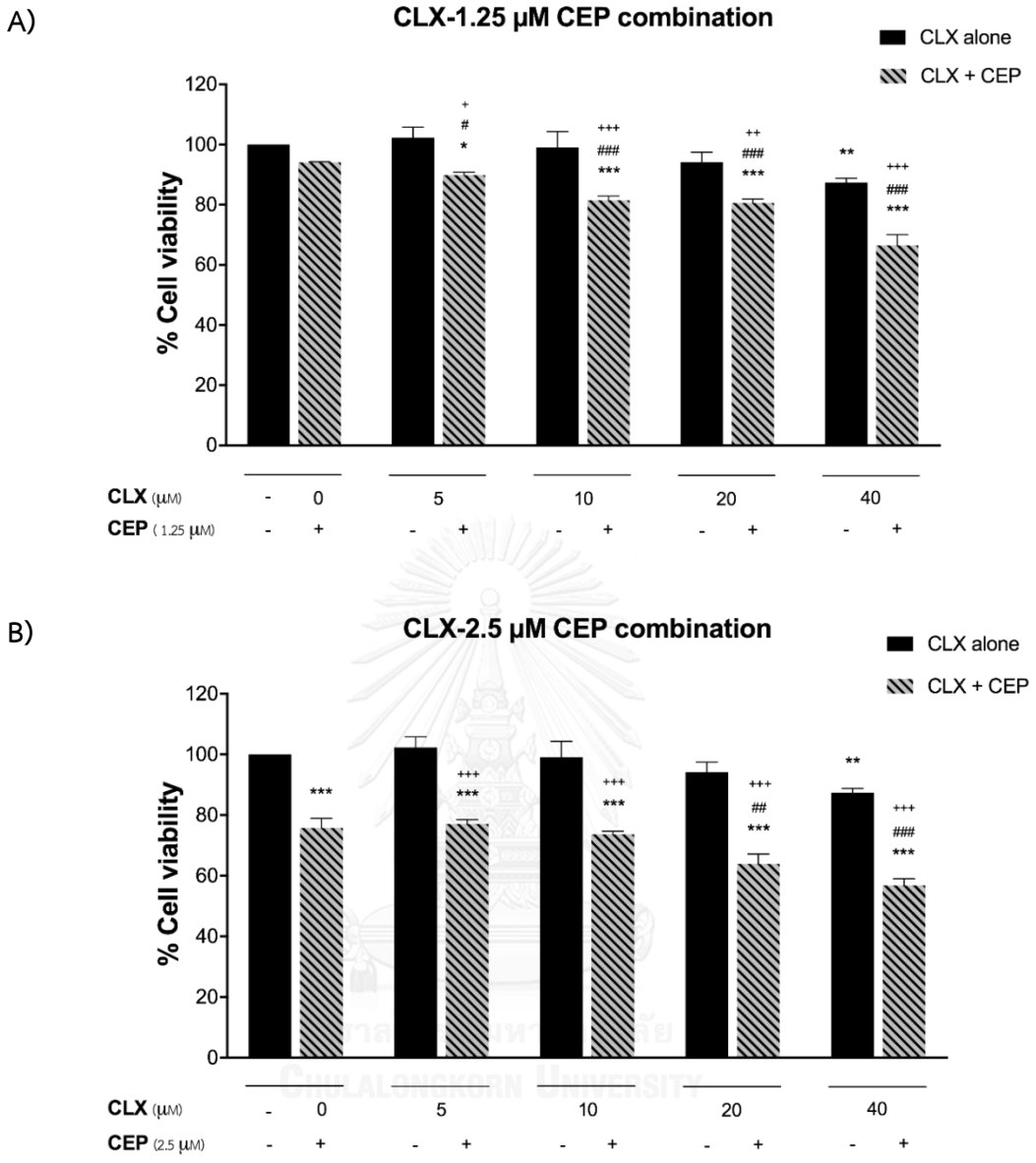


Figure 6 Effects of celecoxib - cepharanthine combinations on HT-29 cell viability. The cells were treated celecoxib at 5-40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 48 h. The viability of the treated cells was determined by resazurin assay. The data are presented as mean ± SEM from three independent experiments (n=3). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ compared with cepharanthine alone; $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ compared with celecoxib alone.

Table 3 Combination index values of celecoxib-1.25 μM cepharanthine combination

celecoxib (μM)	CI	CI effect
5	0.964	nearly additive effect
10	0.720	moderate synergistic effect
20	0.694	synergistic effect
40	0.610	synergistic effect

Table 4 Combination index values of celecoxib-2.5 μM cepharanthine combination

celecoxib (μM)	CI	CI effect
5	0.777	moderate synergistic effect
10	0.534	synergistic effect
20	0.502	synergistic effect
40	0.424	synergistic effect

4.3 Effects of the celecoxib - cepharanthine combinations on COX-2 mRNA expression and PGE_2 production

HT-29 cells are human colorectal cancer cells with COX-2 expression. COX-2 enzyme and its product PGE_2 are known to be involved in cancer progression by inhibiting cell apoptosis and stimulating cell proliferation, invasion and angiogenesis (89). Celecoxib and cepharanthine have been shown to inhibit COX-2 expression (122). Therefore, the effects of the celecoxib – cepharanthine combinations on the expression of COX-2 and the production of PGE_2 in HT-29 cells were determined compared with the effect of each drug alone.

The results in **Fig. 7A** and **7B** demonstrated the effects of the celecoxib – cepharanthine combinations on COX-2 mRNA expression in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM, cepharanthine at 1.25 and 2.5 μM, and the celecoxib – cepharanthine combinations for 24 h. The mRNA expression of COX-2 of the treated cells was determined by real time PCR. Celecoxib at 40 μM significantly decreased the COX-2 expression to 0.567 ± 0.09 fold when compared to the vehicle control. Cepharanthine at both concentrations did not significantly have effects on the COX-2 expression. The effects of combination treatment on the expression of COX-2 were not significantly different from the treatment of each drug alone.

The results in **Fig. 8** presented the effects of the combinations on PGE_2 production in HT-29 cells compared with the effect of each drug. The cells were treated with celecoxib at 20 and 40 μM, cepharanthine at 1.25 and 2.5 μM, and the celecoxib – cepharanthine combinations for 24 h. Amount of PGE_2 in the supernatants of the treated cells were determined by ELISA. Celecoxib at 20 and 40 μM significantly decreased the PGE_2 level to 73.151 ± 6.89 and 67.280 ± 10.47 pg/ml, respectively, when compared with the vehicle control (113.417 ± 17.24 pg/ml). Cepharanthine at 1.25 μM did not have effect on PGE_2 production when compared with the vehicle control. As shown in **Fig. 8A** demonstrated the combination effects of cepharanthine at 1.25 μM with celecoxib. The combinations of cepharanthine at 1.25 μM with celecoxib at 20 μM did not different from each drug alone, whereas the combinations of cepharanthine at 1.25 μM with celecoxib at 40 μM significantly decrease PGE_2 when

compared with cepharanthine alone. As shown in **Fig. 8B** presented the combination effects of cepharanthine at 2.5 μM with celecoxib. Cepharanthine at 2.5 μM significantly decreased PGE_2 production when compared with the effect of the vehicle control. However, the combinations of cepharanthine at 2.5 μM and celecoxib at 20 and 40 μM did not different from each drug alone.

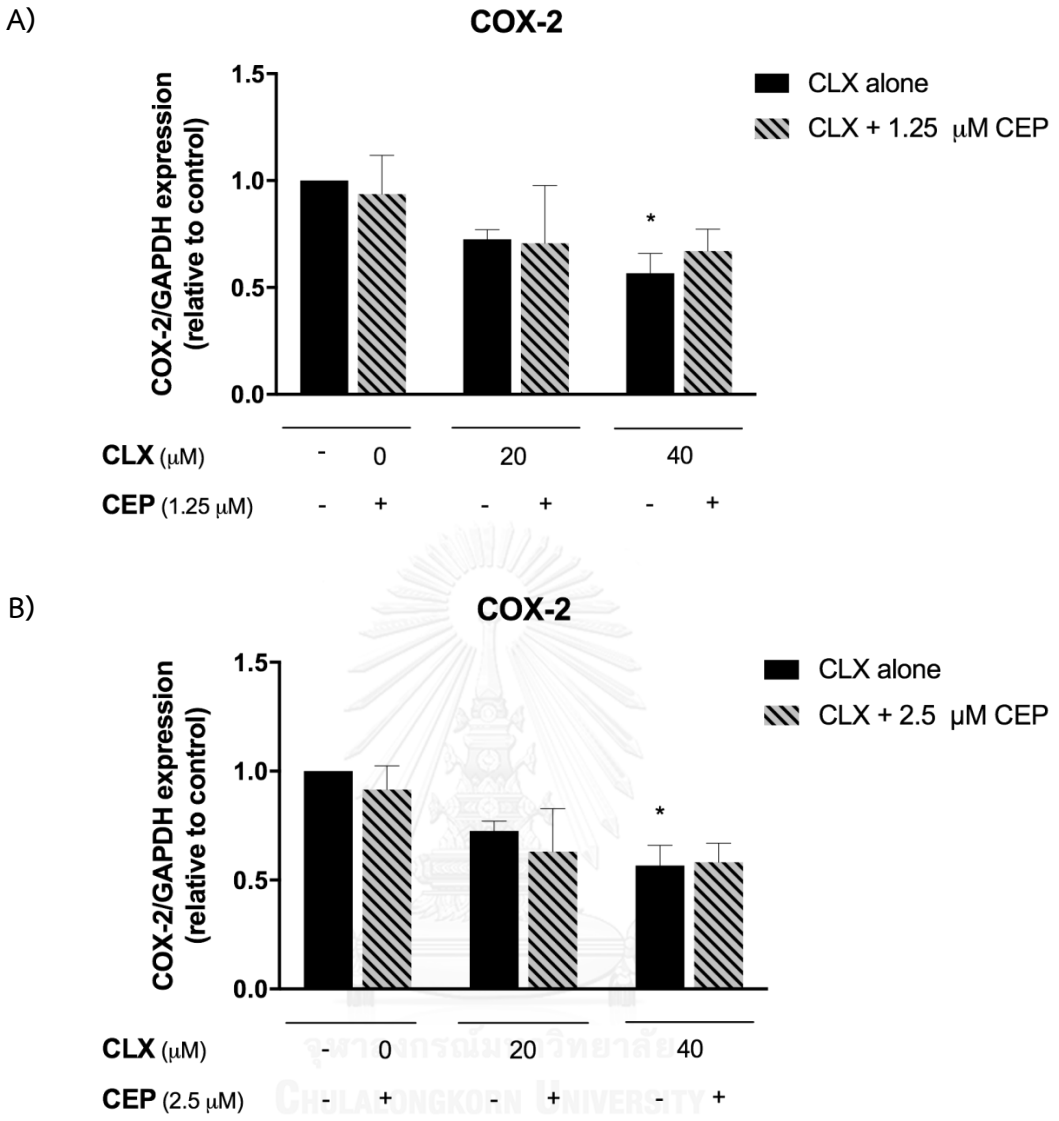


Figure 7 Effects of the celecoxib - cepharanthine combinations on COX-2 mRNA expression in HT-29 cells. HT-29 cells were treated celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of COX-2 in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3). * $P \leq 0.05$ compared with 0.2% DMSO vehicle control.

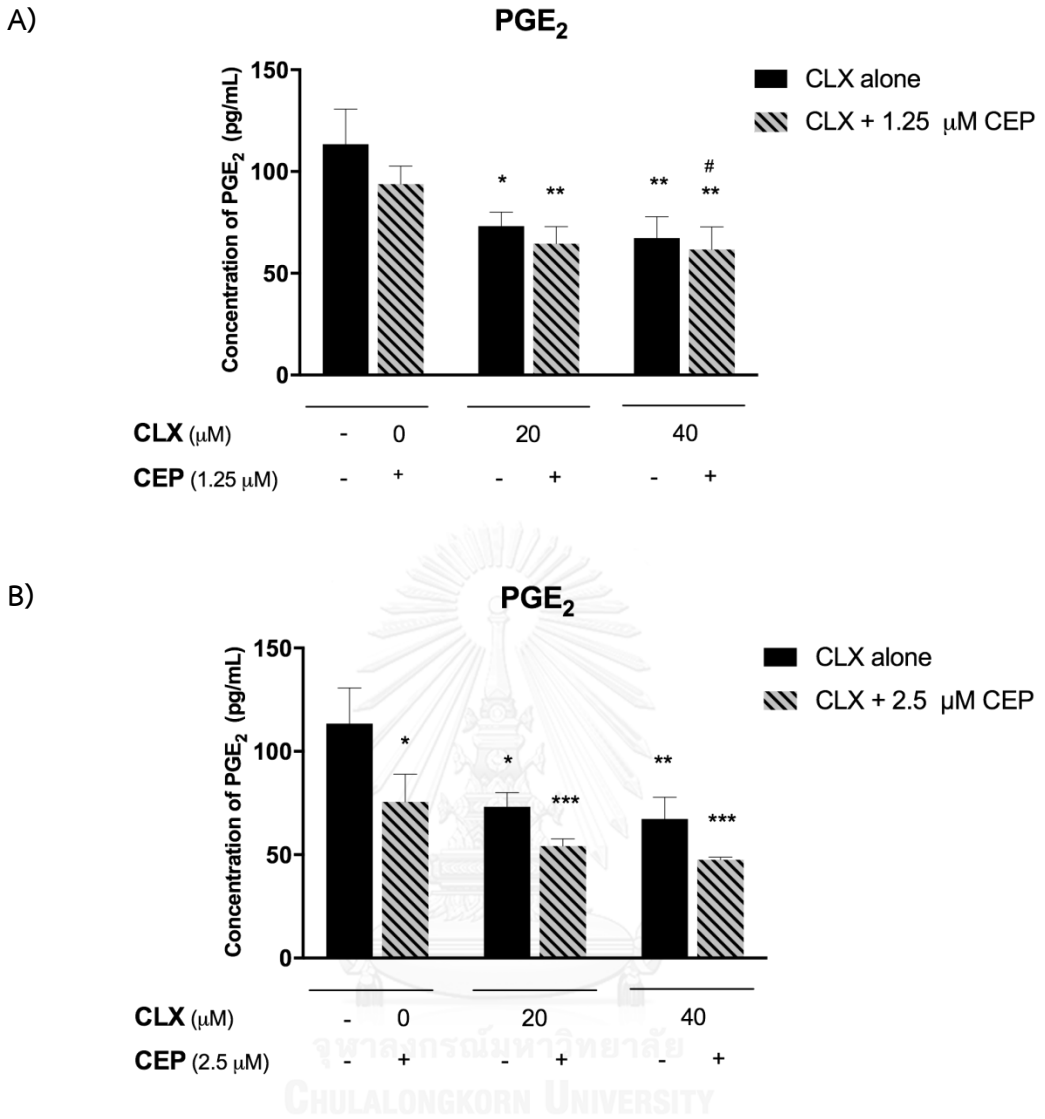


Figure 8 Effects of celecoxib - cepharanthine combinations on PGE_2 production in HT-29 cells. The quantification of PGE_2 from standard curve (39.1-2500 pg/ml). The cells were treated 20 and 40 μM of celecoxib in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine. The supernatant of treated cells was collected. The PGE_2 level was determined by PGE_2 competitive ELISA kit using microplate reader and the all data were expressed in pg/ml of PGE_2. The data are presented as mean ± SEM from three independent experiments (n=3). * $P \leq 0.01$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control; # $P \leq 0.001$ compared with cepharanthine alone.

4.4 Effects of the celecoxib - cepharanthine combinations on the mRNA expression of NOX1 and NOX2 and the production of reactive oxygen species (ROS)

NADPH oxidases (NOXs) are major enzymes generating superoxide anion ($O_2^{-\bullet}$) which rapidly changes to hydrogen peroxide (H_2O_2). HT-29 cells express high level of NOX1 and intermediate level of NOX2 (40). It has been reported that over-expression of NOX1 and ROS involved in cell proliferation (65), and migration (63) of colon cancer. The effects of the combinations on NOX expression and ROS production in HT-29 cells were evaluated. The cells were treated with celecoxib at 20 and 40 μM, cepharanthine at 1.25 and 2.5 μM, and the celecoxib - cepharanthine combinations for 24 h. The expression of NOX1 and NOX2 was determined by real time PCR using GAPDH gene expression for normalization. **Fig. 9A** and **9B** demonstrated the effects of the combinations on the mRNA expression of NOX1. As shown in **Fig. 9**, celecoxib at 20 and 40 μM dramatically decreased NOX1 mRNA expression, in a concentration – dependent manner. They decreased NOX1 expression to 0.427 $\pm$ 0.16 and 0.228 $\pm$ 0.02 fold, respectively when compared to the vehicle control. Cepharanthine did not have any effect on the expression of NOX1. When compared to each drug, the combinations trended to decrease the NOX1 expression. The reduction was significant when compared to cepharanthine alone, but it was not significant when compared to celecoxib alone. **Fig. 10** presented the effects of the combinations on NOX2 expression. Both drugs and their combinations did not have any effect on the NOX2 expression.

The effects of the combinations on ROS in HT-29 cells were also investigated by DCFH-DA assay. The results in **Fig. 11A** and **11B** demonstrated that both drugs did not have effect on ROS production when compared to the vehicle control. The effects of the combinations did not significantly different from the effect of each drug alone, except the combination of celecoxib at 40 μM with cepharanthine at 2.5 μM significantly decreased ROS production when compared with cepharanthine alone.

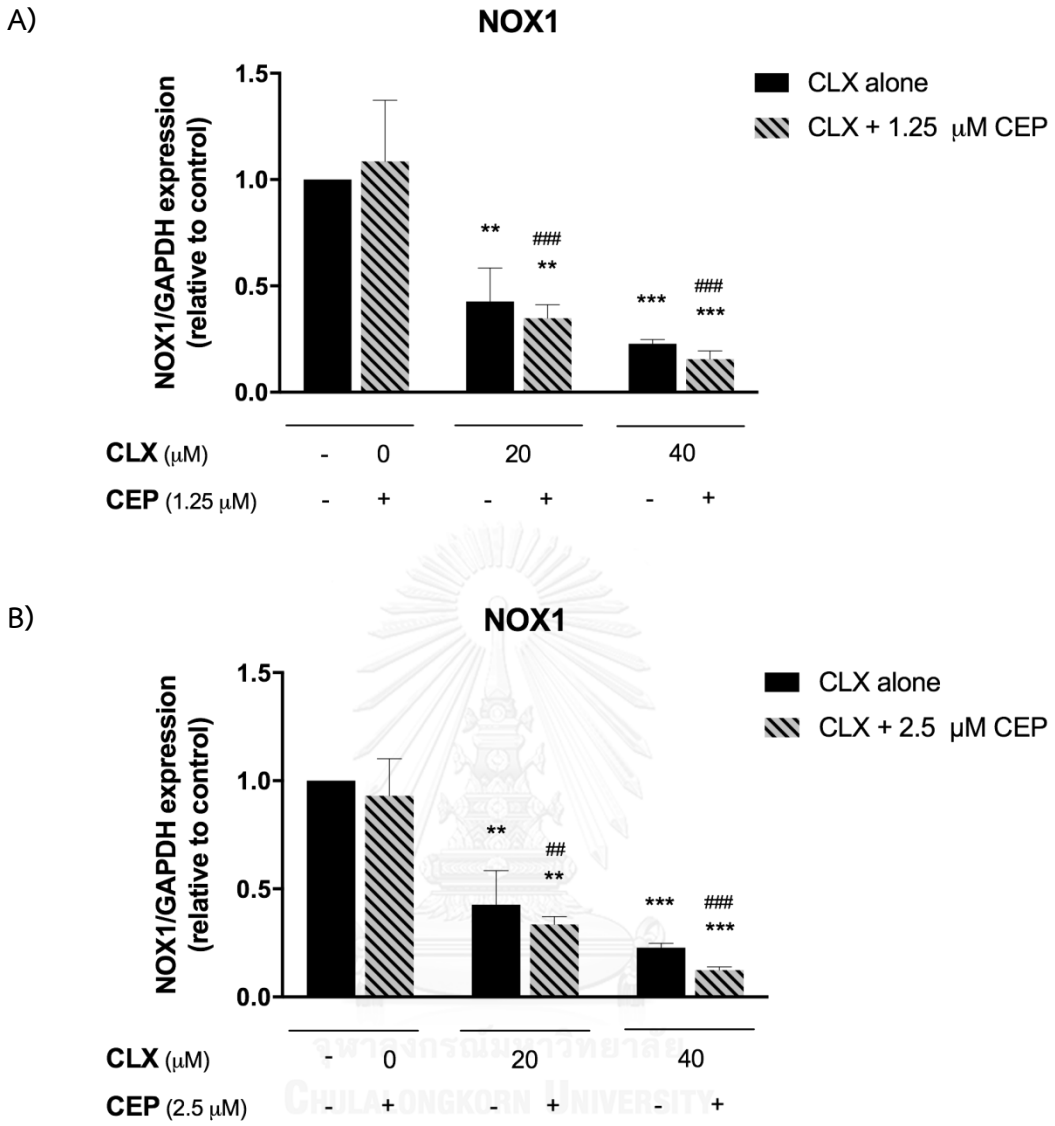


Figure 9 Effects of the celecoxib - cepharanthine combinations on NOX1 mRNA expression in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of NOX1 in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3). ** $P \leq 0.01$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control, ## $P \leq 0.01$, ### $P \leq 0.001$ compared with cepharanthine.

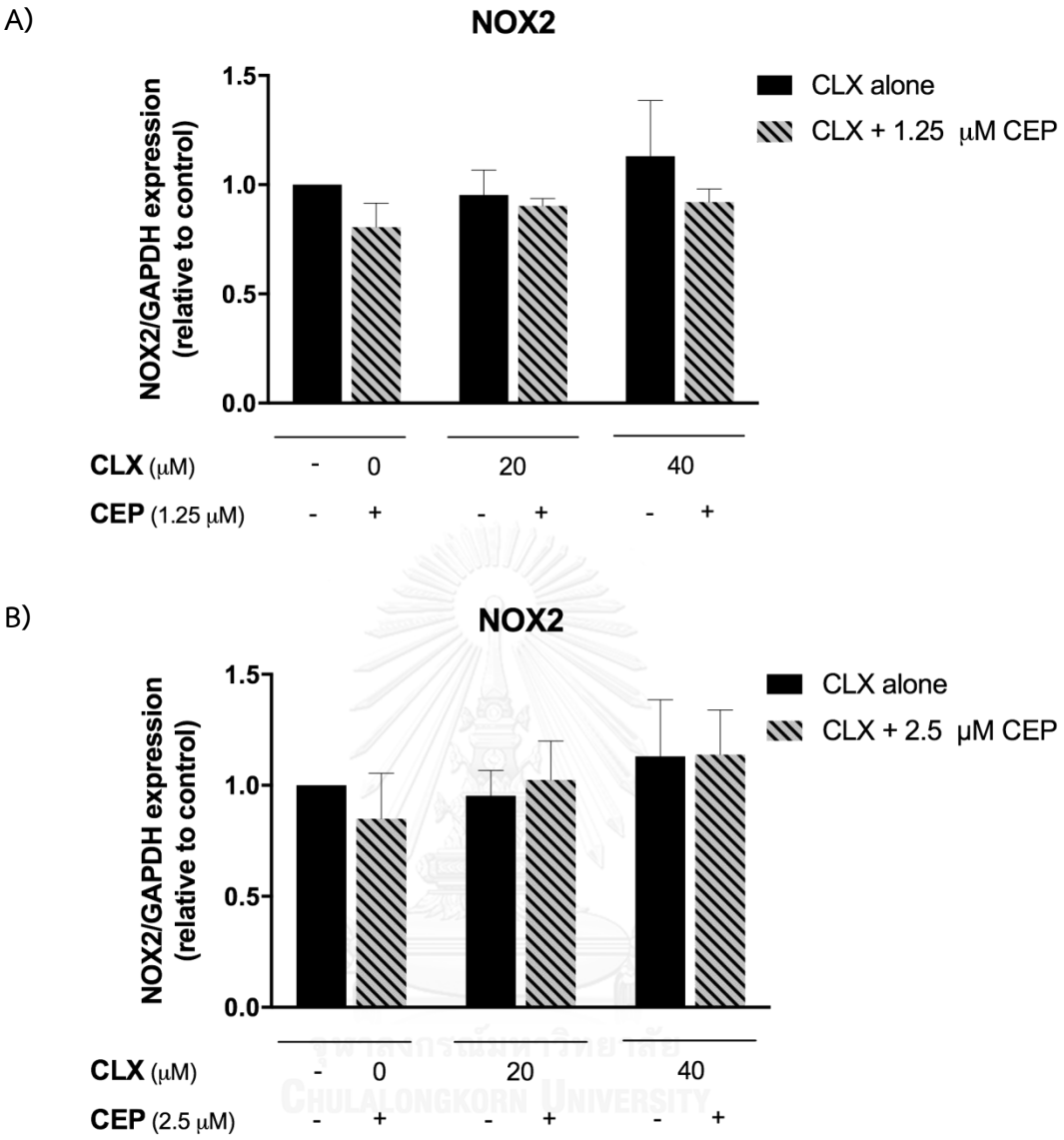


Figure 10 Effects of the celecoxib - cepharanthine combinations on NOX2 mRNA expression in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of NOX2 in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3).

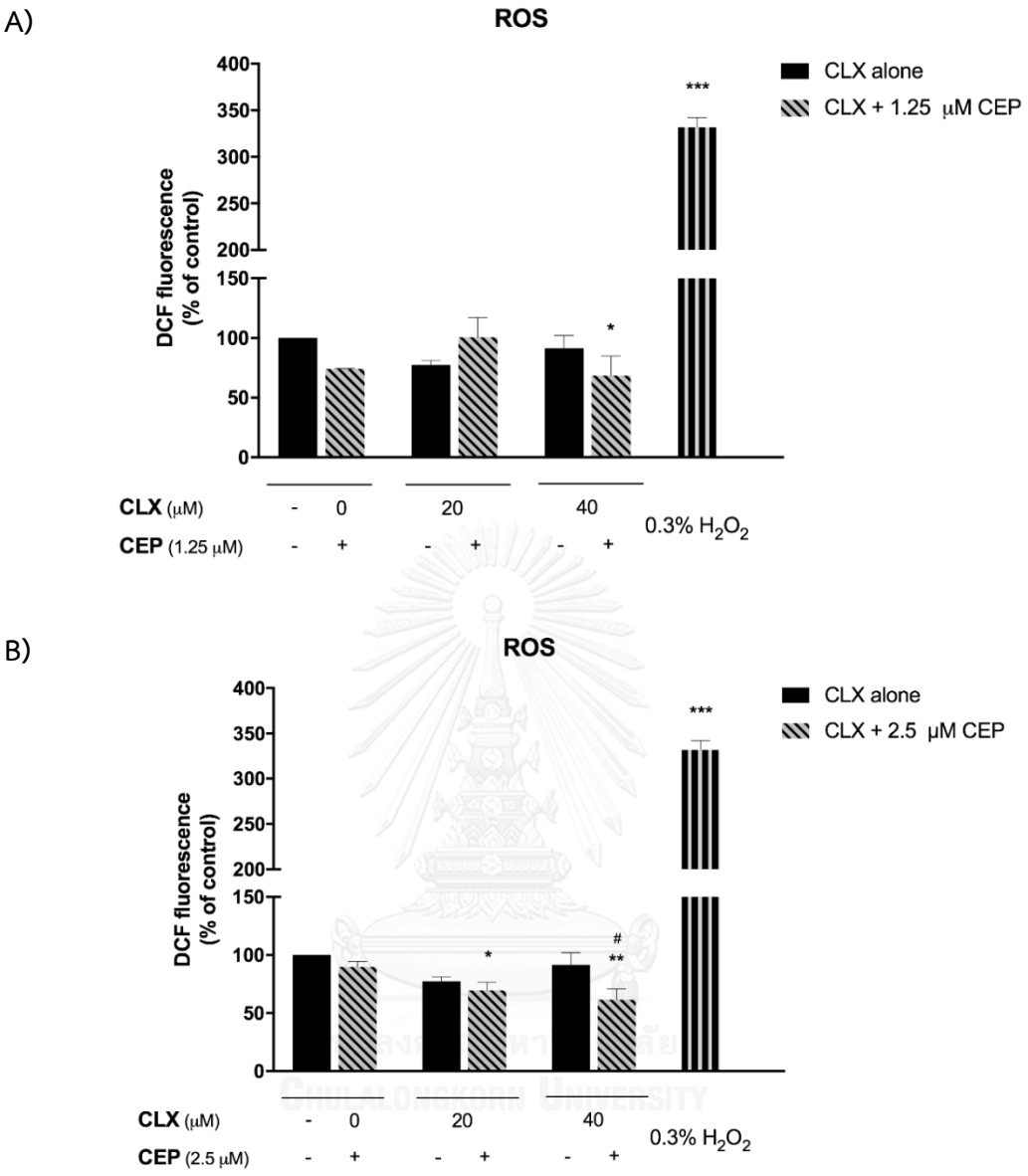


Figure 11 Effects of the celecoxib - cepharanthine combinations on ROS generation in HT-29 cells. The cells were pretreated with 50 μM DCFH-DA for 30 min, then treated with celecoxib at 20 and 40 μM in the presence and absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 1 h. The fluorescence of DCF product was determined by a fluorescence microplate reader. 0.3% H_2O_2 was used as the positive control. The data are presented as mean ± SEM from three independent experiments(n=3). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control; # $P \leq 0.05$ compared with cepharanthine.

4.5 Effects of the celecoxib - cepharanthine combinations on the cell cycle of HT-29 cells and mRNA expression of cell cycle regulators

To investigate the effects of celecoxib - cepharanthine combinations on the cell cycle of HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of cepharanthine at 1.25 and 2.5 μM for 48 h. Patterns of the cell cycle of the treated cells were evaluated by fixing and propidium iodide staining before analyzing by flow cytometer. The results in **Fig. 12A** and **12B** showed that celecoxib at 40 μM and cepharanthine at 2.5 μM significantly caused cell accumulation in G1 phase when compared to the effect of the vehicle control (73.93% ± 1.07%, 73.83% ± 0.82%, compared to 65.87% ± 1.05%, respectively). All of the combinations significantly increased cells in G1 phase when compared to the effect of each drug. The combinations of cepharanthine at 1.25 μM and celecoxib at 20 and 40 μM increased cells in G1 phase to 73.40 ± 0.8% and 82.90 ± 0.3%, respectively. The combinations of cepharanthine at 2.5 μM and celecoxib at 20 and 40 μM increased cells in G1 phase to 80.20 ± 1.2% and 86.80 ± 0.9%, respectively. The increase of cells in G1 phase of the combinations was correlated with the decrease of cells in S and G2/M phases.

The effects of the combinations on the cell cycle of HT-29 cells were investigated at the molecular level by determining mRNA expression of cell cycle regulators including stimulatory regulators cyclin D, cyclin E, and cyclin A as well as an inhibitory regulator p21. The cells were treated with celecoxib at 20 and 40 μM in the presence or the absence of cepharanthine at 1.25 and 2.5 μM for 24 h. The mRNA expression of cyclin D, cyclin E, cyclin A, and p21 was determined by real time PCR using specific primers.

As shown in **Fig. 13A** and **13B** demonstrated that the combinations and each drug did not have effect on the expression of cyclin D which functions by activating CDK4/6 activity in early G1 phase for the cell cycle progression.

As shown in **Fig. 14A** and **14B** demonstrated the effects of the combinations and each drug on the expression of cyclin E which activates CDK2 in the late G1 phase plus the early S phase. Celecoxib at 20 and 40 μM and the combination of celecoxib

at 40 μM with cepharanthine at 2.5 μM significantly decreased the expression of cyclin E to 0.576 ± 0.09, 0.579 ± 0.18, and 0.280 ± 0.12 fold of the vehicle control, respectively. The effects of combination treatments on cyclin E expression were not different from the treatment with each drug alone.

As shown in **Fig. 15A** and **15B** demonstrated the effects of the combinations and each drug on the expression of cyclin A which activates CDK2 in S phase plus CDK1 in S/G2 phase. Each drug significantly decreased the expression of cyclin A when compared to the vehicle control. Celecoxib at 20 and 40 μM down-regulated cyclin A to 0.730 ± 0.11, and 0.419 ± 0.04 fold, whereas cepharanthine at 1.25 and 2.5 μM down-regulated cyclin A to 0.611 ± 0.04, and 0.553 ± 0.14 fold. The combinations of at 40 μM celecoxib with cepharanthine at 1.25 and 2.5 μM down-regulated cyclin A to 0.266 ± 0.02 and 0.195 ± 0.02 fold of the vehicle control, respectively. These combinations significantly decreased the cyclin A expression when compared to the effect of celecoxib or cepharanthine alone.

As shown in **Fig. 16A** and **16B** demonstrated the effects of the combinations and each drug on the expression of p21 which binds and inhibits multiple cyclin-CDK activities. When compared to the effect of the vehicle control, only celecoxib at 40 μM significantly increased p21 expression to 4.026 ± 1.26 fold. When compared the effects of the combinations to the effect of each drug, all combinations profoundly increased the expression of p21. In **Fig. 16A**, the combinations of cepharanthine at 1.25 μM with celecoxib at 20 and 40 μM up-regulated p21 to 3.954 ± 0.99 and 9.079 ± 1.42 folds of the vehicle control, respectively. In **Fig. 16B**, the combinations of cepharanthine at 2.5 μM with celecoxib at 20 and 40 μM up-regulated p21 to 4.937 ± 1.52, and 8.337 ± 1.36 fold of the vehicle control, respectively.

So, the decrease of cyclin A and the increase of p21 by the combinations may involve in HT-29 cell cycle arrest leading to cell accumulation in G1 phase.

Table 5 Effects of the celecoxib - cepharanthine combinations on HT-29 cell cycle for 48 h.

Treatment	The percentage of cell (%)		
	G1	S	G2/M
Untreated	67.93±1.1	7.77±0.5	21.93±1.8
0.2% DMSO	65.87±1.1	8.57±1.1	22.20±1.4
20 μM celecoxib	66.77±1.6	6.77±0.2 *	24.60±1.4
40 μM celecoxib	73.93±1.1 ***	5.07±1.1 ***	19.00±0.6
1.25 μM cepharanthine	62.27±3.3	7.17±0.5	26.80±2.8 *
2.5 μM cepharanthine	73.83±0.8 ***	4.27±0.2 ***	18.83±0.3
20 μM celecoxib-1.25 μM cepharanthine combination	$^{***, +++, ###}$ 73.40±0.8	$^{***, ++, ##}$ 4.20±0.4	$^{+++, ##}$ 20.23±1.7
40 μM celecoxib-1.25 μM cepharanthine combination	$^{***, +++, ###}$ 82.90±0.3	$^{***, ###}$ 3.40±0.2	$^{***, +++, ###}$ 11.33±0.7
20 μM celecoxib-2.5 μM cepharanthine combination	$^{***, +++, ##}$ 80.20±1.2	$^{***, ++}$ 3.80±0.4	$^{***, +++, ###}$ 13.30±0.4
40 μM celecoxib-2.5 μM cepharanthine combination	$^{***, +++, ###}$ 86.80±0.9	$^{***, ++, #}$ 2.23±0.3	$^{***, +++, ###}$ 8.07±0.2

The data are presented as mean ± SEM from three independent experiments (n=3). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control; $^{++}$ $P \leq 0.01$, $^{+++}$ $P \leq 0.001$ compared with celecoxib alone; $^{\#}$ $P \leq 0.05$, $^{\#\#}$ $P \leq 0.01$, $^{\#\#\#}$ $P \leq 0.001$ compared with cepharanthine alone.

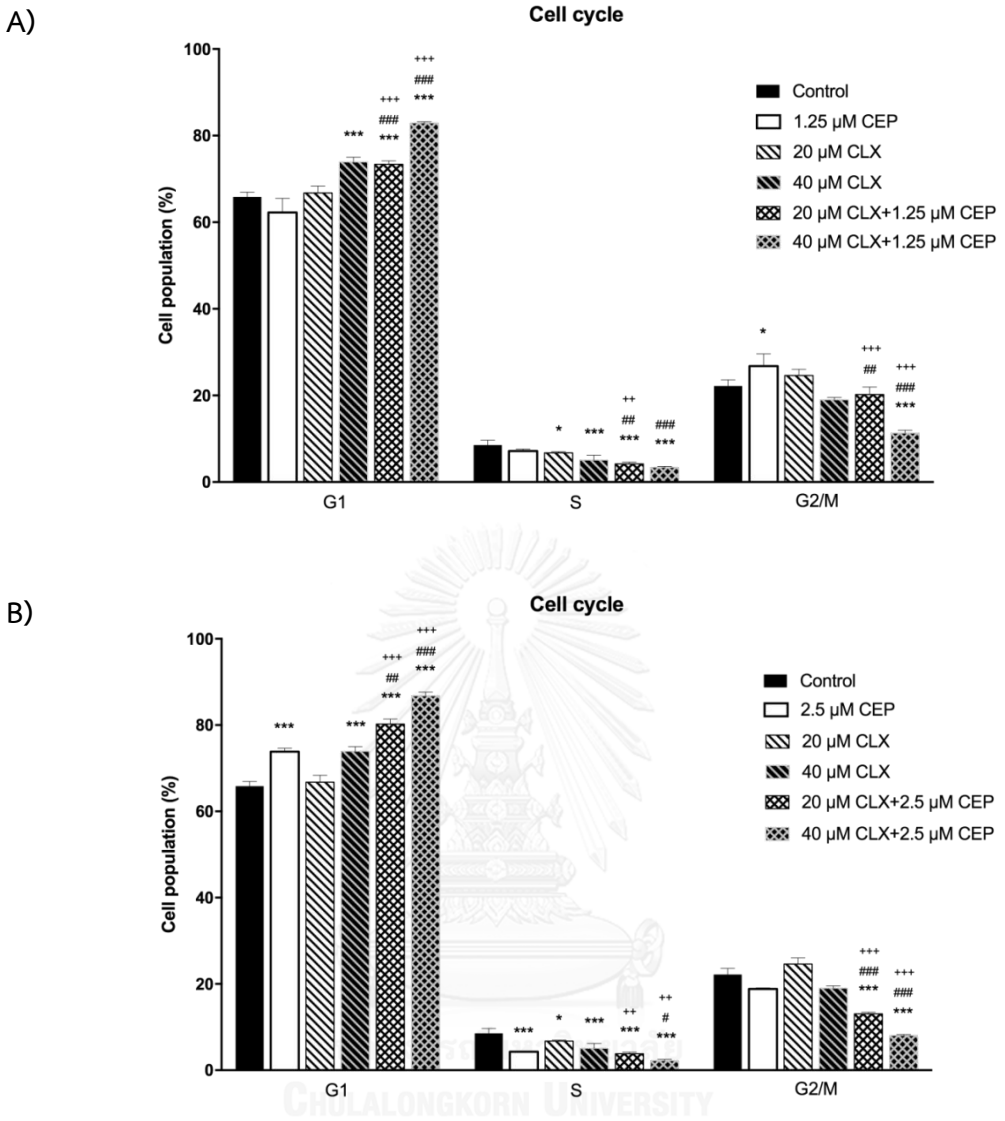


Figure 12 Effects of the celecoxib - cepharanthine combinations on the cell cycle of HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 48 h. The treated cells were fixed and stained with PI and analyzed by a flow cytometer. The percentage of the treated cells in each phase of the cell cycle was analyzed and compared. The data are presented as mean ± SEM from three independent experiments (n=3). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control; $^{++}$ $P \leq 0.01$, $^{+++}$ $P \leq 0.001$ compared with celecoxib alone; $^{\#}$ $P \leq 0.05$, $^{\#\#}$ $P \leq 0.01$, $^{\#\#\#}$ $P \leq 0.001$ compared with cepharanthine alone.

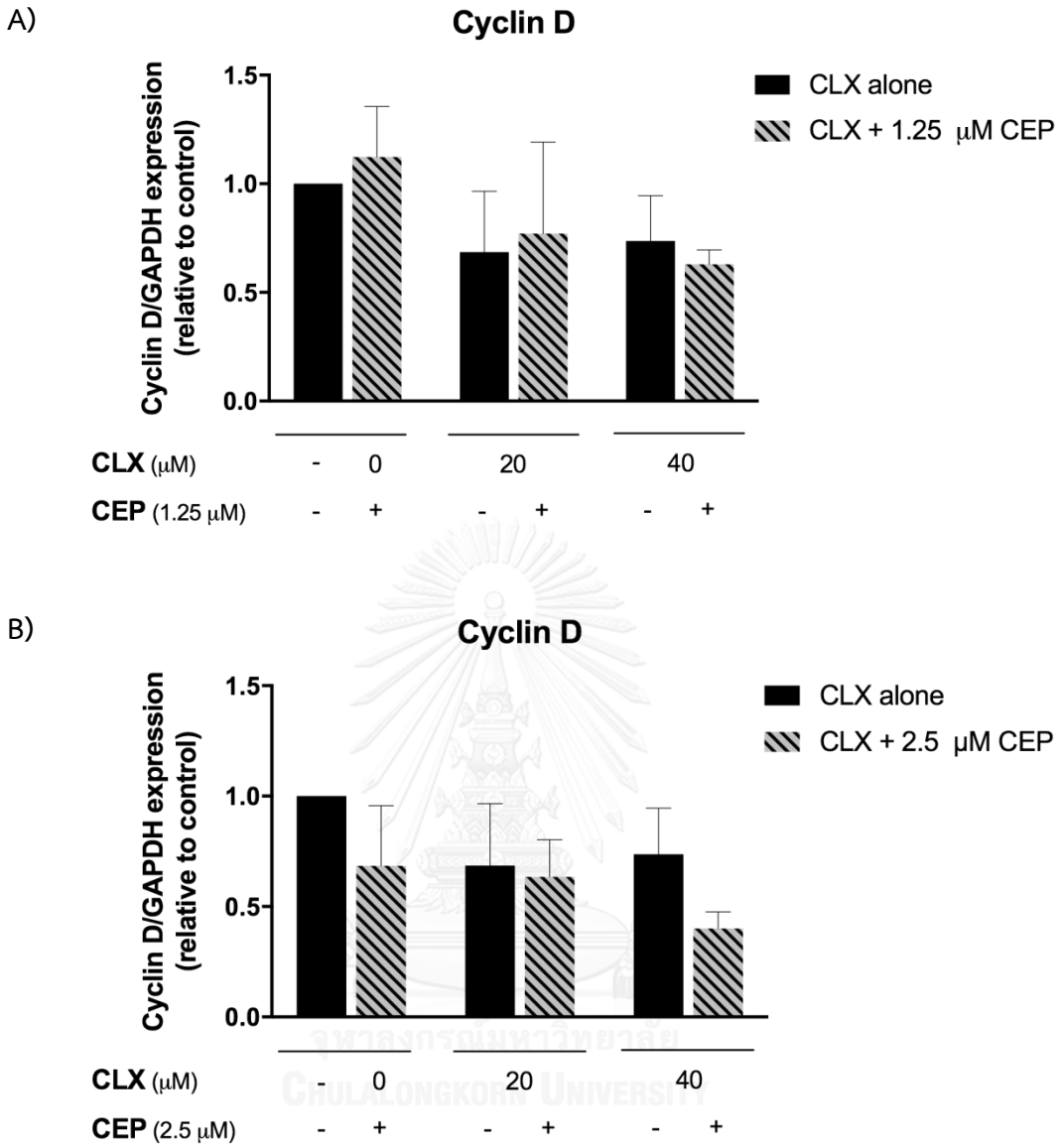


Figure 13 Effects of the celecoxib - cepharanthine combinations on cyclin D mRNA expression in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of cyclin D in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3).

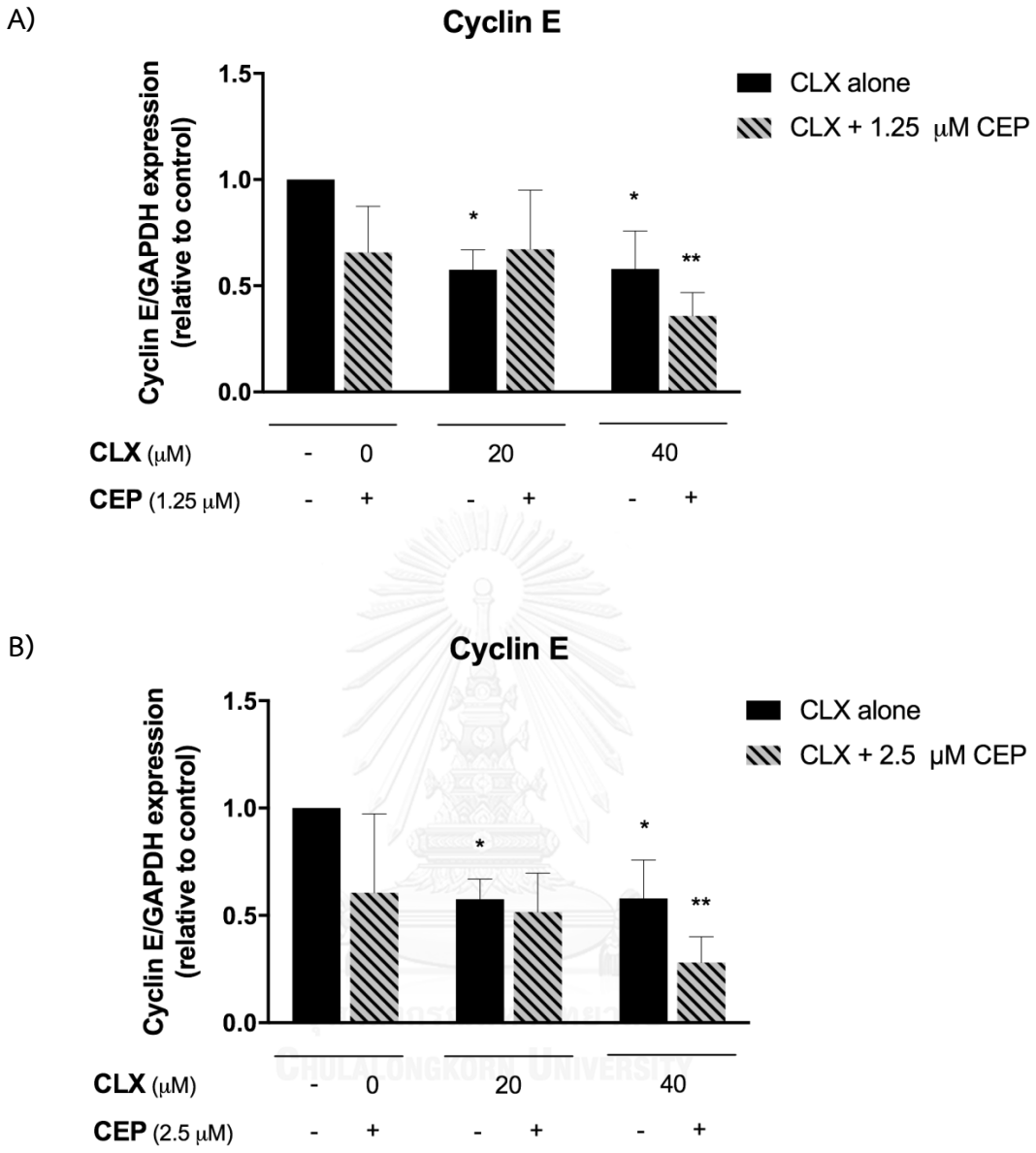


Figure 14 Effects of the celecoxib - cepharanthine combinations on cyclin E mRNA expression in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of cyclin E in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3). * $P \leq 0.05$, ** $P \leq 0.01$ compared with 0.2% DMSO vehicle control.

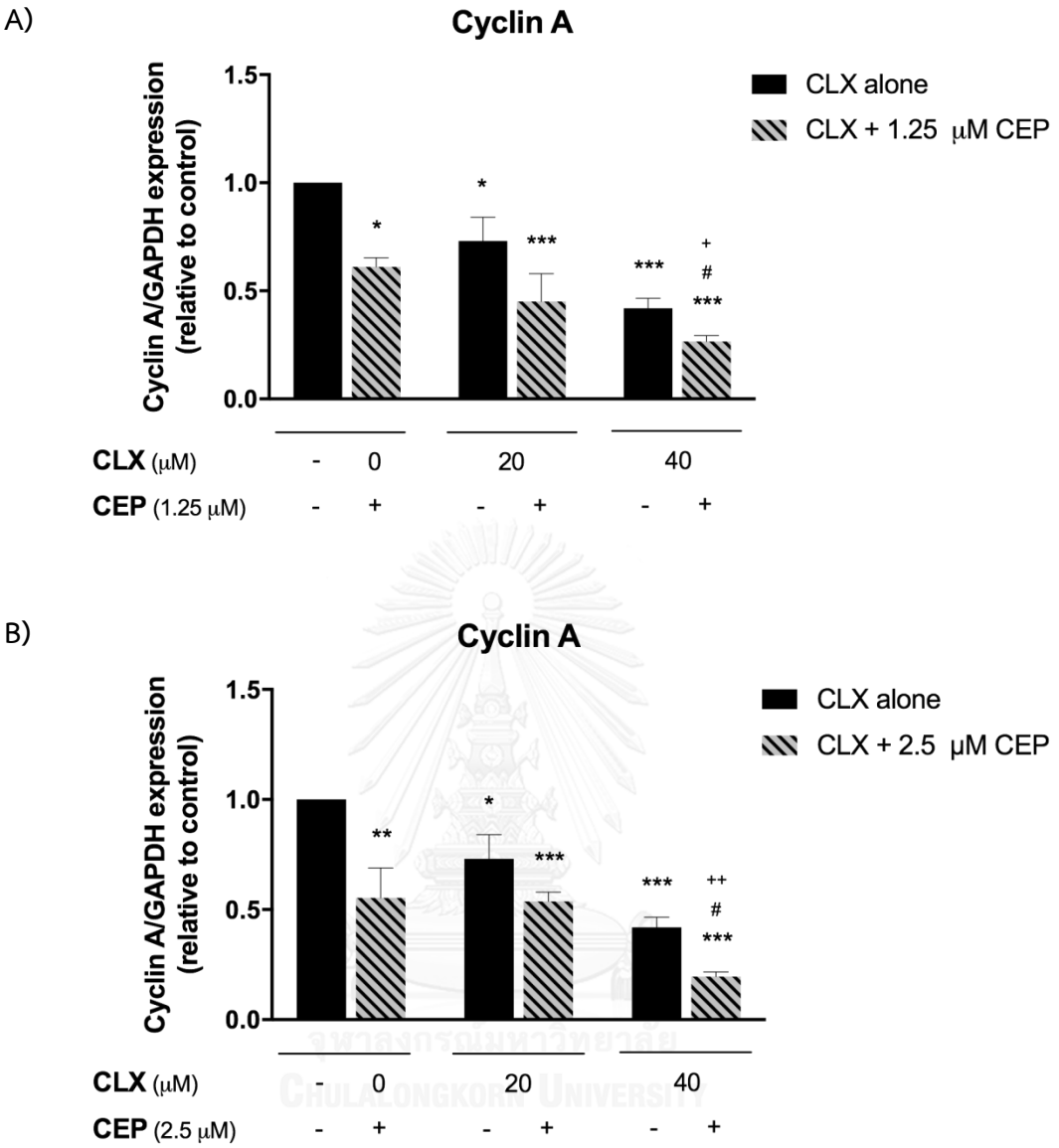


Figure 15 Effects of the celecoxib - cepharanthine combinations on cyclin A mRNA expression in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of cyclin A in the treated cells was determined by real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control; $^{+}$ $P \leq 0.05$, $^{++}$ $P \leq 0.01$ compared with celecoxib alone; $^{\#}$ $P \leq 0.05$ compared with cepharanthine alone.

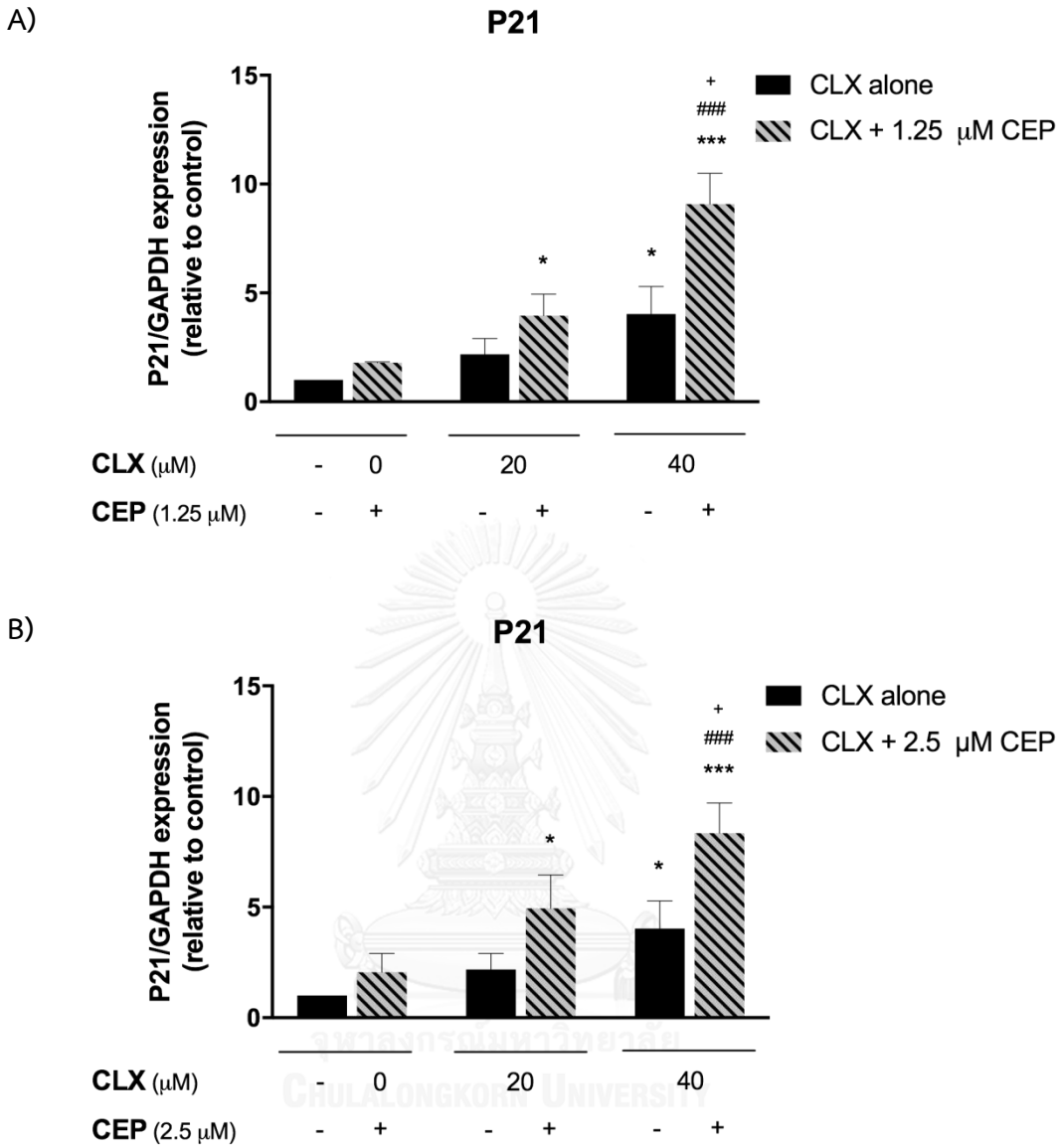


Figure 16 Effects of the celecoxib - cepharanthine combinations on P21 mRNA expression in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of p21 in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3). * $P \leq 0.05$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control; $^{+}$ $P \leq 0.05$ compared with celecoxib alone; $^{\#\#\#}$ $P \leq 0.001$ compared with cepharanthine alone.

4.6 Effects of the celecoxib - cepharanthine combinations on apoptosis and on the mRNA expression of Bcl-2 family proteins

Effects of the combinations on HT-29 apoptotic cell death were also evaluated in this study. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of cepharanthine at 1.25 and 2.5 μM for 24 h. Patterns of cell death of the treated cells were determined by staining with annexin V – FITC/ PI and determining by flow cytometer. The results in **Fig. 17A** and **17B** and **Table 6** demonstrated the effects of the combinations and each drug on HT-29 cell death. When compared to the vehicle control, only cepharanthine at 2.5 μM significantly decreased cell viability. All the combinations significantly decreased HT-29 cell viability and significantly increased late apoptotic cells when compared with celecoxib or cepharanthine alone. As shown in **Fig. 17A**, the combinations of cepharanthine at 1.25 μM with celecoxib at 20 and 40 μM decreased HT-29 cell viability to 84.90 ± 0.8% and 80.02 ± 1.5%, respectively. They increased late apoptotic cells to 10.37 ± 0.8% and 14.53 ± 1.0%, respectively. As shown in **Fig. 17B**, the combinations of cepharanthine at 2.5 μM with celecoxib at 20 and 40 μM decreased HT-29 cell viability to 71.30 ± 2.0 % and 64.20 ± 0.5 %, respectively. They increased late apoptotic cells to 20.57 ± 1.6% and 25.97 ± 0.9%, respectively.

The molecular effects of the combinations on the mRNA expression of proteins in the Bcl-2 family were also investigated. HT-29 cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of cepharanthine at 1.25 and 2.5 μM for 24 h. The mRNA expression of both pro-apoptotic (BAX and BAK) and anti-apoptotic (Bcl-2, Bcl-XL, and Mcl-1) proteins were evaluated by quantitative real time RT-PCR.

Fig. 18A and **18B** demonstrated the effects of the combination and each drug on the mRNA expression of pro-apoptotic BAX. When compared each drug to the vehicle control, both celecoxib at 20 and 40 μM and cepharanthine at 1.25 and 2.5 μM did not have effect on BAX expression. However, all the combination significantly increased BAX expression when compared to the vehicle control. The combinations of cepharanthine at 1.25 μM with celecoxib at 20 and 40 μM up-regulated BAX mRNA level to 2.156 ± 0.14 and 1.883 ± 0.36 fold, respectively. Similarly, the combinations

of cepharanthine at 2.5 μM with celecoxib at 20 and 40 μM up-regulated BAX mRNA level to 2.127 ± 0.16 and 2.303 ± 0.30 fold, respectively. The combination of celecoxib at 20 μM with cepharanthine at 2.5 μM significantly increased BAX expression when compared to cepharanthine alone. Moreover, the combinations of celecoxib at 40 μM with cepharanthine at 2.5 μM significantly increased the expression of BAX when compared to each drug alone.

Fig. 19A and **19B** demonstrated the effects of the combination and each drug on the mRNA expression of pro-apoptotic BAK. The effect of each drug on BAK expression was not different from the vehicle control. Only the combination of celecoxib at 20 μM with cepharanthine at 2.5 μM up-regulated BAK mRNA level to 1.498 ± 0.27 fold. This combination significantly increased BAK expression when compared to each drug alone.

Fig. 20A and **20B** demonstrated the effects of the combination and each drug on the mRNA expression of anti-apoptotic Bcl-2. When compare to the vehicle control, only 2.5 μM cepharanthine significantly decreased Bcl-2 expression, as shown in **Fig. 20B**. It down-regulated Bcl-2 to 0.352 ± 0.19 fold of the vehicle control. Celecoxib at 20 and 40 μM and cepharanthine at 1.25 μM did not have any effect on the expression. Similarly, the effects of all the combinations did not significantly different from the effect of each drug alone.

Fig. 21A and **21B** demonstrated the effects of the combination and each drug on the mRNA expression of anti-apoptotic Bcl-XL. When compared to the vehicle control, celecoxib at 20 and 40 μM and cepharanthine at 1.25 and 2.5 μM did not have effect on Bcl-XL expression. Interestingly, when compared the combinations to each drug, the combinations of celecoxib at 40 μM with both cepharanthine at 1.25 and 2.5 μM and celecoxib at 20 μM with cepharanthine at 2.5 μM was down-regulated Bcl-XL mRNA level to 0.448 ± 0.22, 0.562 ± 0.03, and 0.369 ± 0.14 fold, respectively. These combinations significantly decreased Bcl-XL expression when compared to celecoxib alone. While all the combinations did not significantly different from cepharanthine alone.

Fig. 22A and **22B** demonstrated the effects of the combination and each drug on the mRNA expression of anti-apoptotic Mcl-1. When compared to the vehicle control, only celecoxib at 40 μM significantly decreased the expression of Mcl-1. Mcl-1 mRNA level was down-regulated to 0.524 ± 0.12 fold. Celecoxib at 20 μM and cepharanthine at 1.25 and 2.5 μM did not have effect on the expression of Mcl-1. When compared the combinations to each drug, the combination of celecoxib at 40 μM with cepharanthine at 1.25 μM significantly increased Mcl-1 expression (0.917 ± 0.11 fold) when compared to the effect of celecoxib 40 μM alone (0.524 ± 0.12 fold).

Table 6 Effects of the celecoxib - cepharanthine combinations on HT-29 cell apoptosis for 24 h.

Treatment	The percentage of cells (%)			
	viable cells	early apoptotic cells	late apoptotic cells	necrotic cells
Untreated	87.90±0.6	3.83±0.6	6.63±1.0	1.63±0.3
0.2% DMSO	87.40±1.1	3.53±1.0	7.43±1.1	1.63±0.6
20 μM celecoxib	90.70±0.6	2.43±0.3	5.57±0.6	1.33±0.6
40 μM celecoxib	90.00±0.7	1.73±0.4	7.03±0.9	1.30±0.3
1.25 μM cepharanthine	89.80±0.3	2.73±0.7	6.50±0.7	0.97±0.2
2.5 μM cepharanthine	82.90±1.5**	2.87±0.8	10.70±0.5*	3.57±1.1
20 μM celecoxib-1.25 μM cepharanthine combination	84.90±0.8 ++, ##	2.30±0.6	10.37±0.8 *, ++, ##	2.57±0.6
40 μM celecoxib-1.25 μM cepharanthine combination	80.20±1.5 ***, +++, ###	2.53±1.3	14.53±1.0 ***, +++, ###	2.73±0.8
20 μM celecoxib-2.5 μM cepharanthine combination	71.30±2.0 ***, +++, ###	2.57±0.8	20.57±1.6 ***, +++, ###	5.50±1.3 ***, ++
40 μM celecoxib-2.5 μM cepharanthine combination	64.20±0.5 ***, +++, ###	2.27±0.9	25.97±0.9 ***, +++, ###	7.57±1.2 ***, ++, ##

The data are presented as mean ± SEM from three independent experiments (n=3). ** $P \leq 0.01$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control; ++ $P \leq 0.01$, +++ $P \leq 0.001$ compared with celecoxib alone; ## $P \leq 0.01$, ### $P \leq 0.001$ compared with cepharanthine alone.

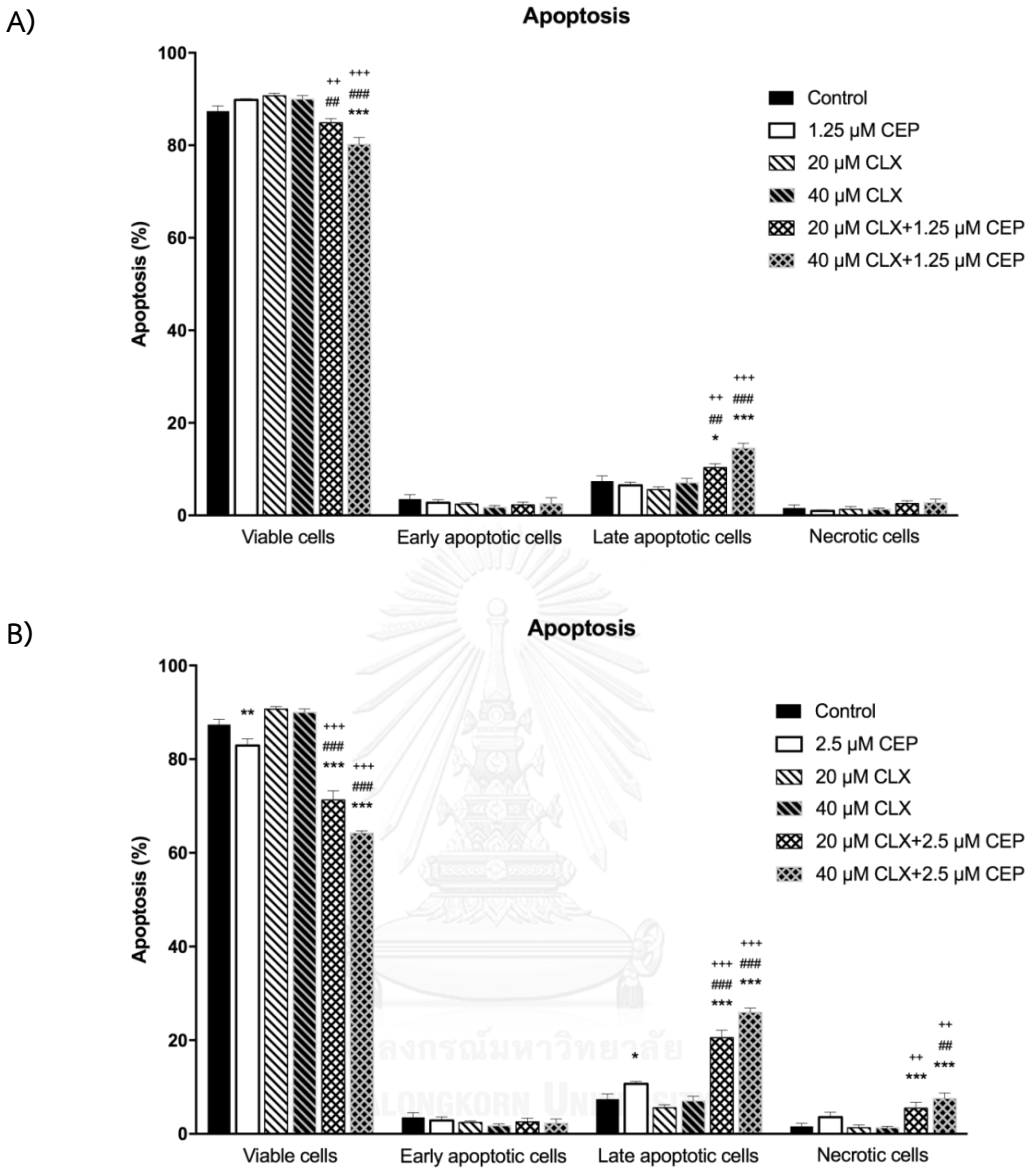


Figure 17 Effects of the celecoxib - cepharanthine combinations on HT-29 cell apoptosis. The cells were treated celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The patterns of cell death of the treated cells were determined by staining with annexin V – FITC/ PI and analyzing by fluorescence flow cytometer. The data are presented as mean ± SEM from three independent experiments (n=3). ** $P \leq 0.01$, *** $P \leq 0.001$ compared with 0.2% DMSO vehicle control; $^{++}$ $P \leq 0.01$, $^{+++}$ $P \leq 0.001$ compared with celecoxib alone; $^{\#\#}$ $P \leq 0.01$, $^{\#\#\#}$ $P \leq 0.001$ compared with cepharanthine alone.

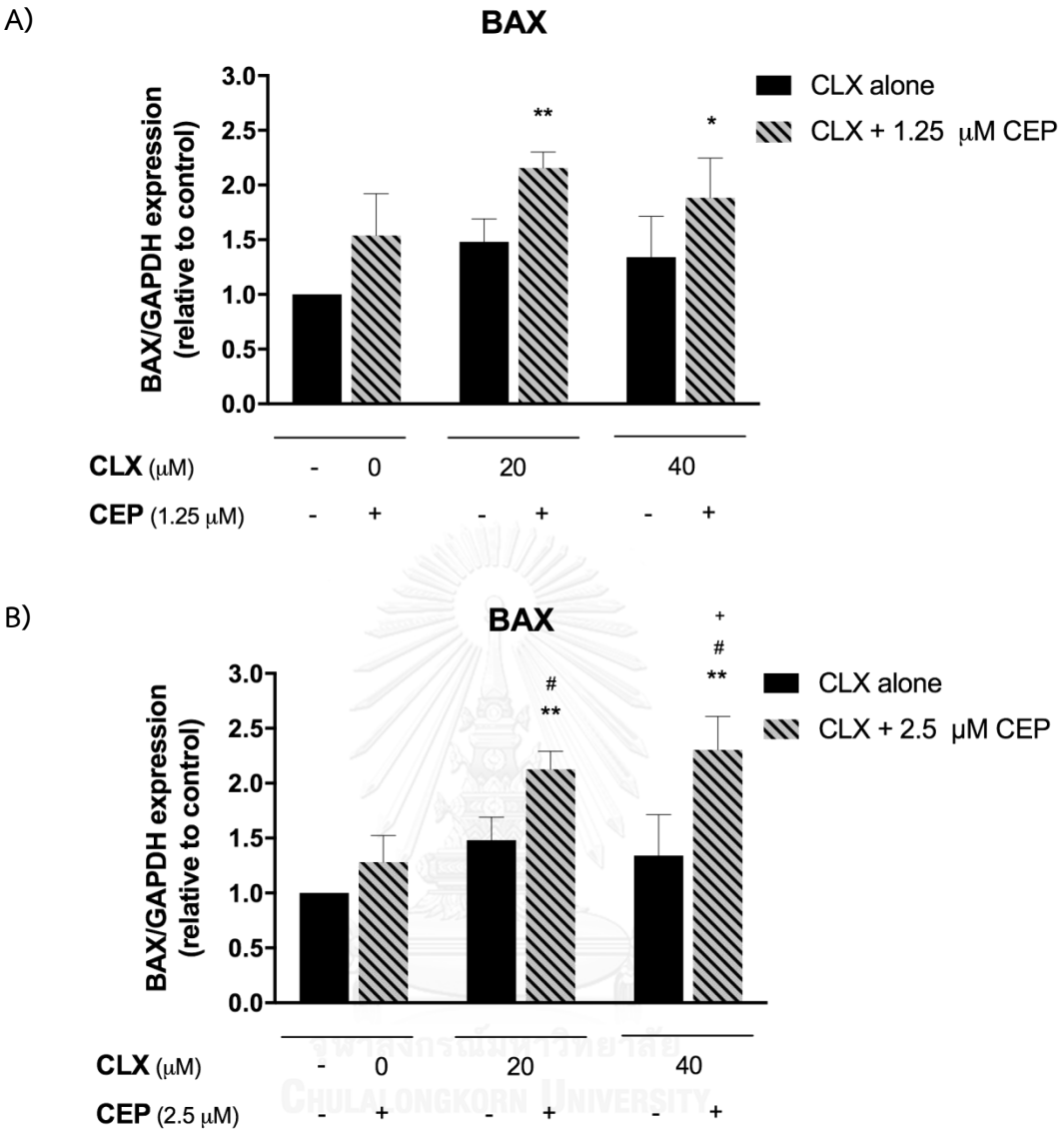


Figure 18 Effects of the celecoxib - cepharanthine combinations on the mRNA expression of BAX in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of pro-apoptotic BAX in the treated cells was determined by quantitative real time RT- PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3). * $P \leq 0.05$, ** $P \leq 0.01$ compared with 0.2% DMSO vehicle control; $^{+}$ $P \leq 0.05$ compared with celecoxib alone; $^{\#}$ $P \leq 0.05$ compared with cepharanthine alone.

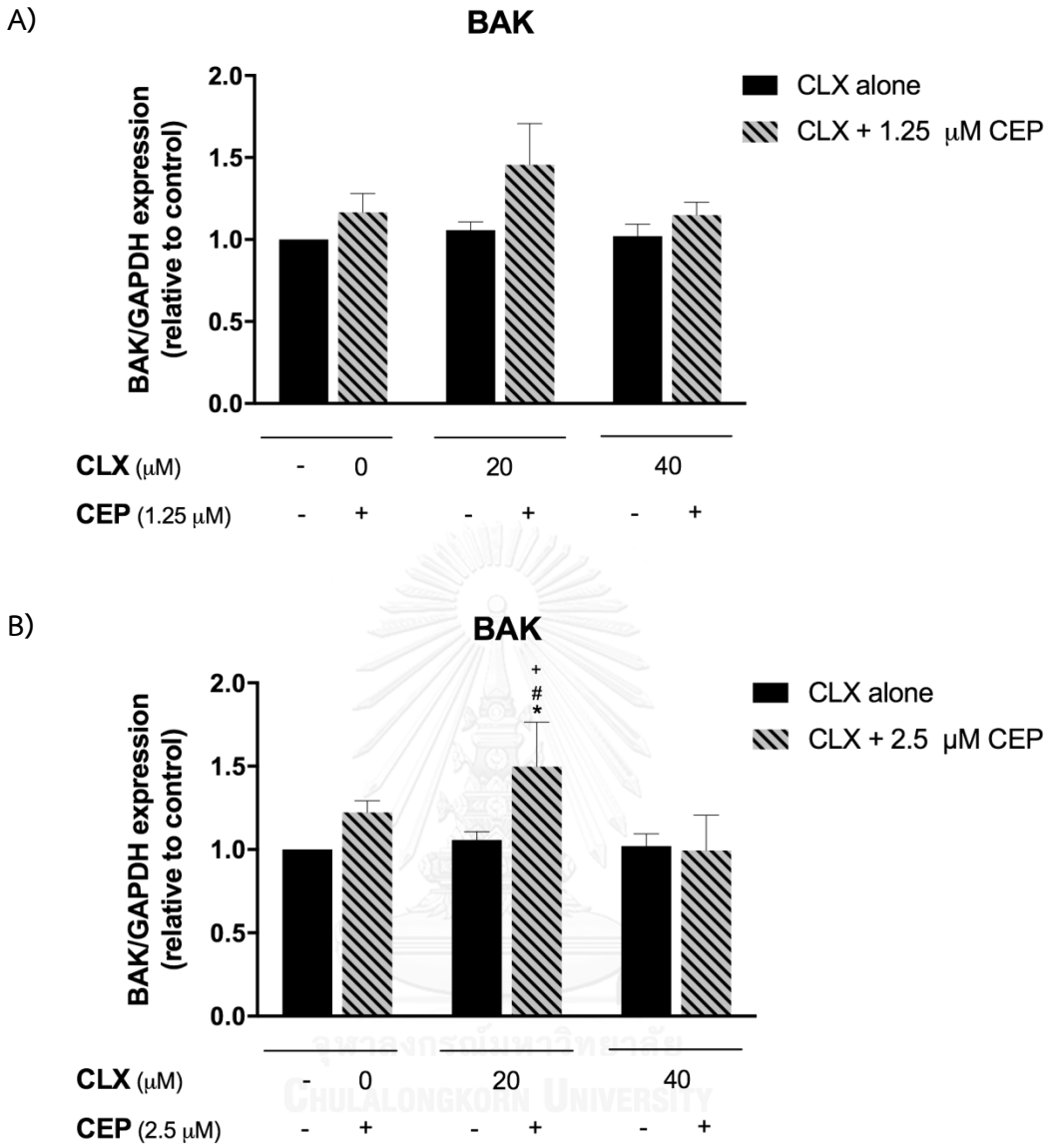


Figure 19 Effects of the celecoxib - cepharanthine combinations on the mRNA expression of BAK in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of pro-apoptotic BAK in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3). * $P \leq 0.05$ compared with 0.2% DMSO vehicle control; $^{+}$ $P \leq 0.05$ compared with celecoxib alone; $^{\#}$ $P \leq 0.05$ compared with cepharanthine alone.

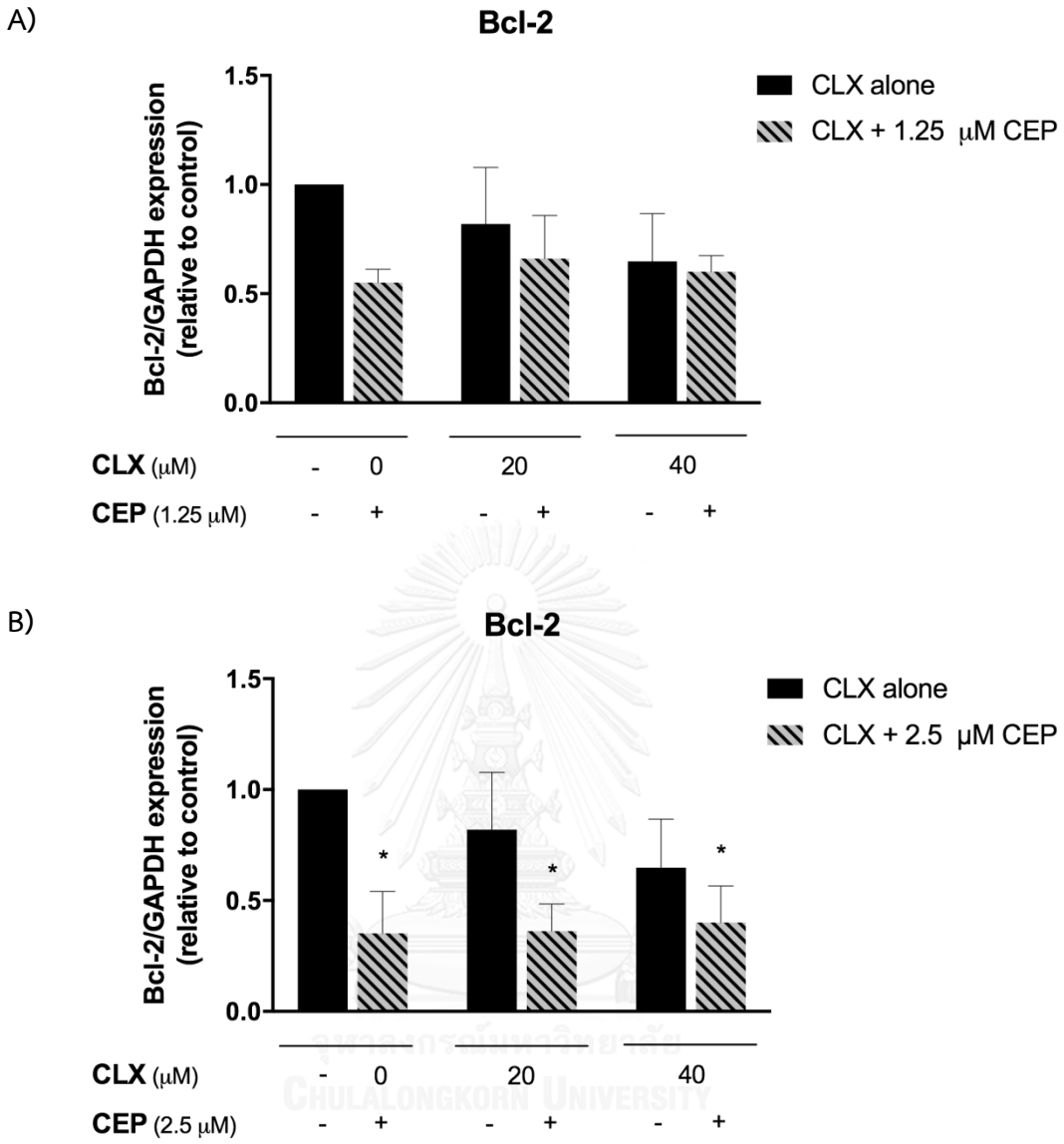


Figure 20 Effects of the celecoxib - cepharanthine combinations on the mRNA expression of Bcl-2 in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of anti-apoptotic Bcl-2 in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3). * $P \leq 0.05$ compared with 0.2% DMSO vehicle control.

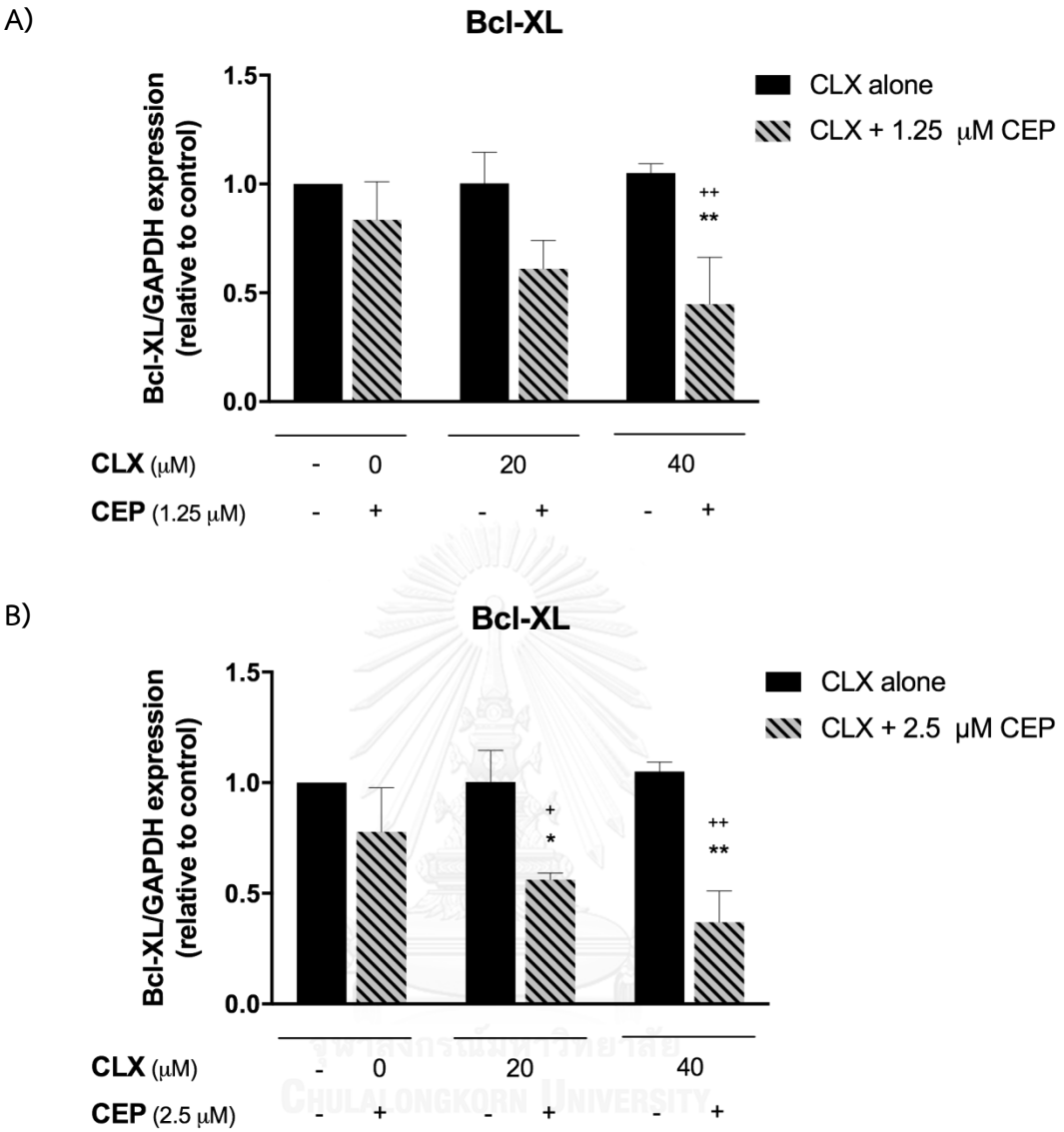


Figure 21 Effects of the celecoxib - cepharanthine combinations on the mRNA expression of Bcl-XL in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of anti-apoptotic Bcl-XL in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3). * $P \leq 0.05$, ** $P \leq 0.01$ compared with 0.2% DMSO vehicle control; $^{+}$ $P \leq 0.05$, $^{++}$ $P \leq 0.01$ compared with celecoxib alone.

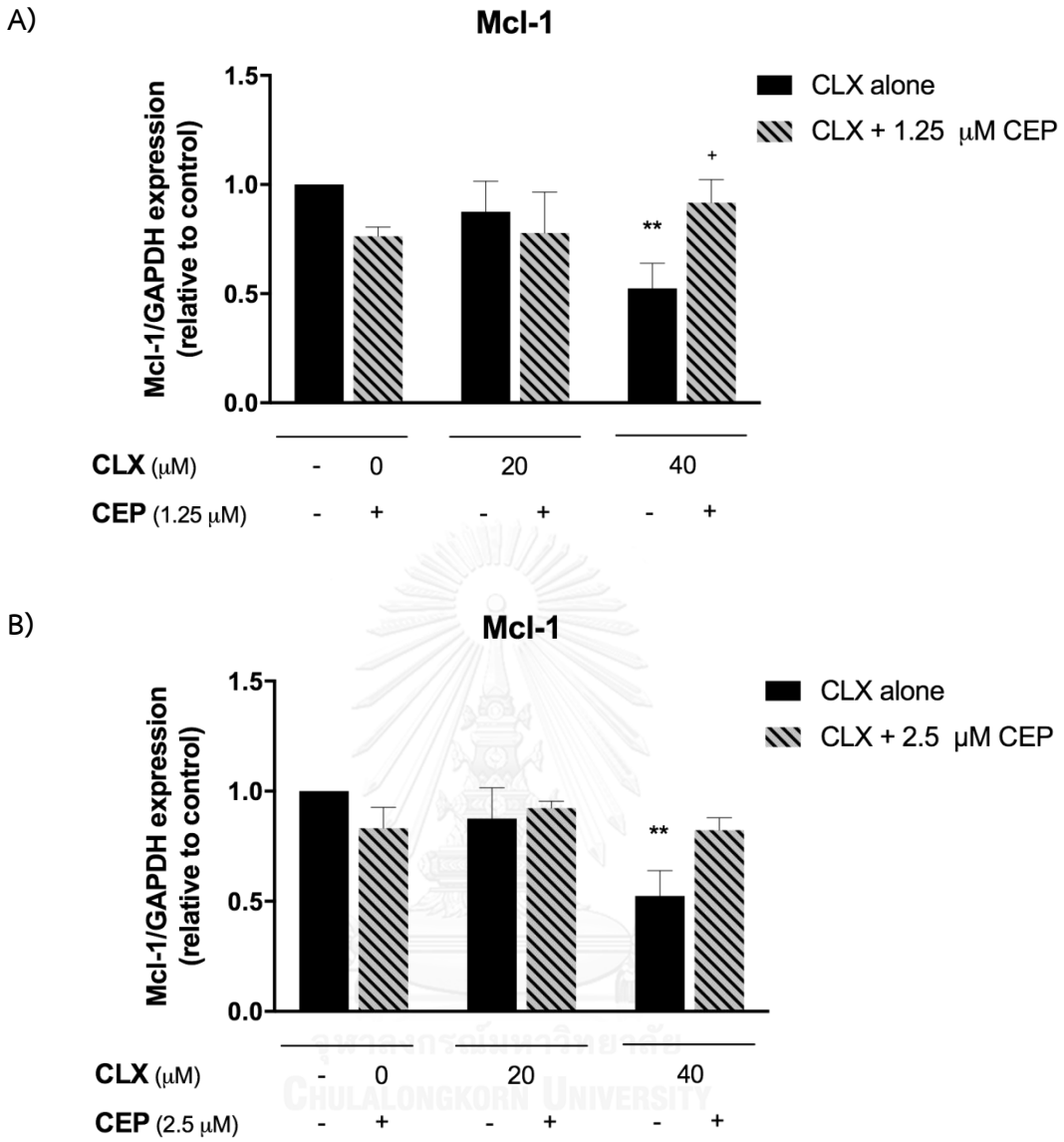


Figure 22 Effects of the celecoxib - cepharanthine combinations on the mRNA expression of Mcl-1 in HT-29 cells. The cells were treated with celecoxib at 20 and 40 μM in the presence and the absence of **A)** 1.25 μM and **B)** 2.5 μM of cepharanthine for 24 h. The mRNA expression of anti-apoptotic Mcl-1 in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean ± SEM of the fold changes from the vehicle control of three independent experiments (n=3). ** $P \leq 0.01$ compared with 0.2% DMSO vehicle control; $^{+}$ $P \leq 0.05$ compared with celecoxib alone.

CHAPTER V

Discussion and conclusion

Continuing investigations have been performed to search for novel targets and novel anticancer agents for CRC prevention and treatment. Epidemiological studies have revealed that the use of NSIADs, which inhibit both COX-1 and COX-2 enzymes, could decrease the risk of colorectal cancer (10). However, long-term use of NSAIDs associates with many gastrointestinal tract side effects which can progress to peptic or duodenal ulcers. COX-2 is known as a biomarker of several types of cancer, especially CRC. This expression is involved in increasing cell survival, inhibiting apoptosis, increasing angiogenesis, and inducing cancer metastasis. COX-2 is increasingly up-regulated during CRC progression (4, 5). COX-2 inhibitors are suggested to use for CRC prevention. Celecoxib is the most common COX-2 inhibitor for this indication. However, it is recommended to use at high dose (800 mg/day) for a long period of term, which increases the risk of cardiovascular side effects. This study therefore intended to investigate the combination effects of celecoxib and cepharanthine which demonstrated a potent anticancer agent against CRC in order to decrease side effects of celecoxib for CRC prevention and treatment. The results showed that celecoxib decreased the survival of HT-29 with its IC_{50} more than 50 μM and cepharanthine potently decreased HT-29 cell survival with its IC_{50} at 5.22 μM. At sub-IC_{50} of both drugs, the celecoxib - cepharanthine combinations had synergistic effects on HT-29 cell viability. Several studies have reported that celecoxib and cepharanthine could enhance anti-cancer effects of chemotherapeutic agents, radiation, other cytotoxic agents, and natural anticancer compounds (11-13, 27-32, 107-110, 120). However, the effect of celecoxib – cepharanthine combination on cancer cells has not been reported before. This study revealed for the first time that celecoxib - cepharanthine combination had synergistically cytotoxic activity on human colorectal HT-29 cells, at their sub-IC_{50} concentrations.

The cellular and molecular effects of celecoxib – cepharanthine combinations were further investigated. The combinations of celecoxib at 20 and 40 μM with cepharanthine at 1.25 and 2.5 μM, which were their sub-IC_{50} values, were used.

The combination effects on cell cycle arrest, apoptotic induction, and molecular parameters involved in the cell cycle progression and apoptosis were evaluated. These combinations significantly increased cell accumulation at G1 phase when compared to the effect of celecoxib or cepharanthine alone. Further mechanistic studies revealed that the combinations significantly decreased the mRNA expression of cyclin E which activates CDK2 as CDK2-cyclin E complex in late G1 phase of the cell cycle. They also dramatically down-regulated the expression of cyclin A, a cyclin for activation of CDK2 and CDK1 in S phase and G2 phase. Moreover, the combinations dramatically up-regulated the expression of a pan CKI p21. Similarly, treatment of HT-29 cells with celecoxib alone also up-regulated the expression of p21 and down-regulated cyclin E and cyclin A expression. Cepharanthine at sub-IC50 concentrations down-regulated only cyclin A expression. The combination of celecoxib 40 μM and cepharanthine 2.5 μM significantly up-regulated p21 and down-regulated cyclin A when compared to each drug. Several studies have demonstrated that celecoxib and cepharanthine arrested the cell cycle in several types of cancer cells, as well as colorectal cancer cells. It was reported that celecoxib arrested colon cancer cells at G1 phase (98, 124) and chronic myeloid leukemia cells at G1/S phase (7). It also up-regulated CKIs p21, p16 and 27, and down-regulated cyclin A, cyclin B1 in colorectal cancer cells (9). Likewise, cepharanthine arrested the cell cycle at G1 phase in adenosquamous carcinoma cells, myeloma cells as well as colorectal cancer HT-29 cells by up-regulating CKIs, especially p21, and down-regulating several cyclins (25). The effects of celecoxib – cepharanthine combinations on cell cycle arrest and its regulators were similar to the effect of each drug alone which demonstrated in this study and other previous studies. Therefore, it is likely that the more pronounce effects of the combinations on cell accumulation at G1 phase, up-regulation of p21, and down-regulation of cyclin A may associate with the synergistically cytotoxic effects of each individual drug on HT-29 cells.

It was previously reported that changes in cell cycle regulators could also trigger apoptosis induction. Binding of p21 with cyclin A/CDK2 complex led to activation of caspase 3, an executioner caspase in apoptosis pathway (125). The up-regulation of p21 expression also correlated with induction of pro-apoptotic protein BAX

expression. Moreover, the up-regulation of p21 was associated with cell cycle arrest and apoptosis in p53- wild type and p53- mutant cancer cell (127). The isoflavonoid genistein, a protein kinase inhibitor, was shown to induce p53 mutant breast cancer MDA-MB-231 cells to undergo apoptosis via induction of p21 and BAX expression (126). Taken together, it is possible that the apoptotic induction effects of the combination found in p53 mutant HT-29 cells may be mediated through modulation of cell cycle regulators. Therefore, the celecoxib – cepharanthine combinations effects on HT-29 apoptosis and on the expression of pro-apoptotic and anti-apoptotic proteins were investigated. At sub-IC_{50} concentrations, celecoxib and cepharanthine alone did not have cytotoxic effect on HT-29 cells after 24 h of treatment. However, the combinations significantly reduced HT-29 cell viability when compared to the effect of each drug alone. The decrease of cell viability was associated with the increase in late apoptotic cells, indicating that celecoxib and cepharanthine synergistically induced HT-29 apoptosis. This study also investigated the effects of the combinations on key Bcl-2 family proteins in the intrinsic apoptosis pathway. The expression of pro-apoptotic BAX and BAK, and anti-apoptotic Bcl-2, Bcl-XL, and Mcl-1 were evaluated. The combinations significantly up-regulated pro-apoptotic BAX expression and down-regulated anti-apoptotic Bcl-XL expression. Furthermore, the combinations of cepharanthine at 2.5 μM with celecoxib at 20 and 40 μM moderately down-regulated anti-apoptotic Bcl-2 expression whereas the combination of celecoxib at 40 μM– cepharanthine at 2.5 μM significantly up-regulated BAX when compared to the effect of each drug alone. Previous studies have demonstrated that celecoxib and cepharanthine induced apoptosis in many types of cancer cells by modulating the expression of proteins in the Bcl-2 family. Celecoxib was shown to down-regulate anti-apoptotic protein Mcl-1 and up-regulate BAX expression in breast cancer cells (99). Cepharanthine was also reported to up-regulate BAX expression but down-regulated Bcl-2 expression in non small cell lung cancer cells (24). The results of the combinations in this study on HT-29 apoptotic induction was correlated with the apoptotic-inducing effect of each drug alone, presented in this study and other previous studies. It also could be associated with the p53 independent p21 and BAX.

It was reported that anticancer activity of celecoxib was strongly associated with the reduction of PGE_2 production (89). Modulation of ROS production, both increasing and decreasing ROS level, was also a part of anticancer effect of this drug (71, 128, 129). Several studies have reported that ROS controlled the COX-2 expression (81-83). NOX1 plays an important role in modulating COX-2 expression in colorectal cancer cells (130). The effect of celecoxib – cepharanthine combinations on PGE_2 production, ROS generation, and COX-2 and NOXs expression were also determined in this study. The combinations decreased PGE_2 production, ROS generation, and NOX1 expression. Celecoxib also decreased PGE_2 production and NOX1 expression but did not have effect on the ROS generation. The result of celecoxib was similar to previous study demonstrating that celecoxib did not inhibit TPA-induced ROS in HT-29 cells (64). In contrast to celecoxib, cepharanthine did not have effect on these parameters. However, previous studies reported that cepharanthine reduced PGE_2 production by inhibiting COX-2 expression and modulating ROS production (123). The conflicting results could be associated with the concentration of this drug used in the experiment. Since there was no synergism between celecoxib and cepharanthine on these parameters, it is possible that the synergistic anticancer activity of celecoxib and cepharanthine, at their sub-IC_{50} concentrations, may be COX-2 and ROS-independent.

Inhibition of HT-29 cell proliferation by the combinations of celecoxib with cepharanthine may be mediated through induction of cell cycle arrest and apoptosis which were associated with up-regulation of cyclin dependent kinase inhibitor p21 correlated with down-regulation of cyclin A, and up-regulation of Bax and down-regulation of Bcl-XL, respectively. Taken together, the results of the celecoxib with cepharanthine combinations provide the rational for further study to investigate the anti-tumor effect of their combination in an animal models.

Conclusion

The results from this study demonstrated the synergistic anticancer effect of celecoxib – cepharanthine combinations at their sub-IC_{50} concentrations on human colorectal cancer HT-29 cells. The combinations arrested HT-29 cell cycle at G1 phase, mainly by up-regulating p21 and down-regulating cyclin A. These effects could also cause apoptotic induction in these cancer cells. These results may give useful information for reducing the dose and toxicities of celecoxib in colorectal cancer treatment. However, more investigations are needed to confirm these results.

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APPENDIX

APPENDIX I

Preparation of reagents

DMEM stock solution

DMEM powder	1	package
$NaHCO_3$	3.7	g
ddH_2O to	1,000	ml

Dissolve DMEM powder and $NaHCO_3$ with 1,000 ml ddH_2O, adjust pH 7.2 with 1 M HCl or 2 M NaOH and sterilize with 0.2 μm cellulose nitrate membrane filter.

1X Phosphate buffered saline (PBS) solution

NaCl	8.065	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g
ddH_2O to	1,000	ml

Dissolve NaCl, KCl, KH_2PO_4 and Na_2HPO_4 with 1,000 ml ddH_2O, adjust pH 7.4 with 1 M HCl or 2 M NaOH and sterilize with autoclave.

1X assay buffer solution

1 M HEPE solution	1	ml
0.1 M $CaCl_2$ solution	2.8	ml
5 M NaCl solution	2.5	ml
ddH_2O to	100	ml

Mix all solutions.

1X Hanks's balanced salt solution (HBSS)

Hank's balanced salt powder	1	package
$NaHCO_3$	0.35	g
ddH_2O to	1,000	ml

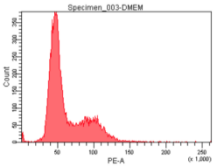
Dissolve Hank's balanced salt powder, $NaHCO_3$ in ddH_2O, adjust to 1000 ml, and sterilize with 0.2 μm cellulose nitrate membrane filter.

APPENDIX II

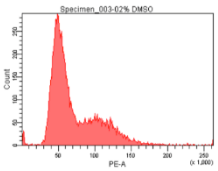
Results

Appendix II-1 Representative histograms of cell cycle analysis of HT-29 cells after treatment with celecoxib at 20 and 40 μM in the presence and the absence of cepharanthine at 1.25 μM and 2.5 μM for 48 h.

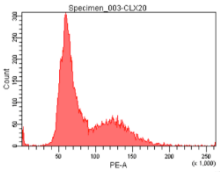
Untreated



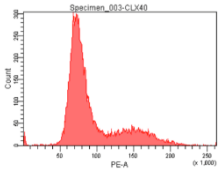
0.2%DMSO



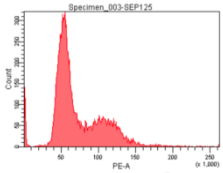
Celecoxib at 20 μM



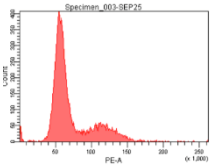
Celecoxib at 40 μM



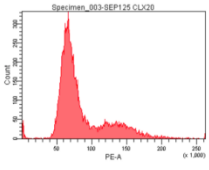
Cepharanthine at 1.25 μM



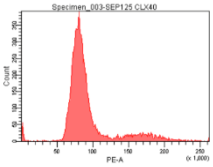
Cepharanthine at 2.5 μM



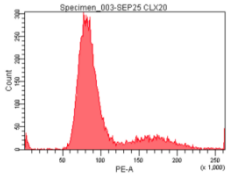
Celecoxib at 20 μM- cepharanthine at 1.25 μM combination



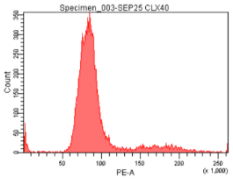
Celecoxib at 40 μM - cepharanthine at 1.25 μM combination



Celecoxib at 20 μM – cepharanthine at 2.5 μM combination

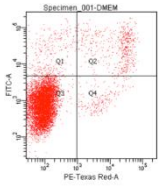


Celecoxib at 40 μM – cepharanthine at 2.5 μM combination

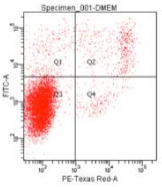


Appendix II-2 Representative cytograms of cell apoptosis analysis of HT-29 cells after treatment with celecoxib at 20 μM and 40 μM in the presence and the absence of cepharanthine 1.25 μM and 2.5 μM for 24 h.

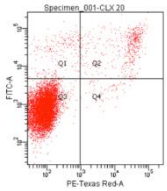
Untreated



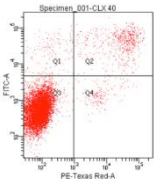
0.2% DMSO



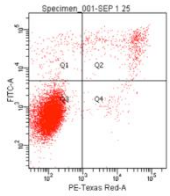
Celecoxib at 20 μM



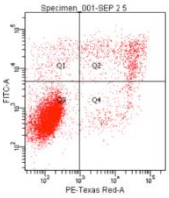
Celecoxib at 40 μM



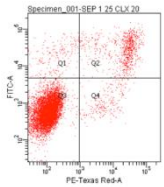
Cepharanthine at 1.25 μM



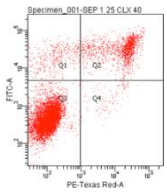
Cepharanthine at 2.5 μM



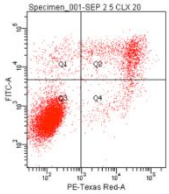
Celecoxib at 20 μM – cepharanthine at 1.25 μM combination



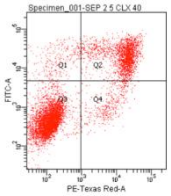
Celecoxib at 40 μM – cepharanthine at 1.25 μM combination



Celecoxib at 20 μM – cepharanthine at 2.5 μM combination



Celecoxib at 40 μM – cepharanthine at 2.5 μM combination



VITA

Miss Parawee Lerdwanangkun was born in Nakhon Sri Thammarat. She got a bachelor degree of Science in Biology from Kasetsart university. After the graduation, She start to study in master degree of Pharmacology at graduation school, Chulalongkorn university.

Poster presentation

1. Lerdwanangkun P., Wonganan P., and Limpanasitthikul W. 2016. SYNERGISTIC EFFECT OF CELECOXIB AND CEPHARANTHINE IN HUMAN COLORECTAL CANCER CELLS. The 42nd COngress on Science & Technology of Thailand (STT42). November 30, 2016 - December 2, 2016. Centara Grand at Central Plaza Ladprao, Bangkok, Thailand.

Publication

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