ฤทธิ์ต้านมะเร็งของเซฟราเรนทีนต่อเซลล์มะเร็งลำไส้ใหญ่และทวารหนักของมนุษย์

นายอากรณัท รัตนวงษ์

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

ANTICANCER EFFECTS OF CEPHARANTHINE ON HUMAN COLORECTAL CANCER CELLS

Mr. Arkornnut Rattanawong

A Thesis Submitted in Partial Fulfillment of the Requirements
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อากรณัท รัตนวงษ์ : ฤทธิ์ต้านมะเร็งของเซฟราเรนทีนต่อเซลล์มะเร็งลำไส้ใหญ่และทวารหนักของมนุษย์ (ANTICANCER EFFECTS OF CEPHARANTHINE ON HUMAN COLORECTAL CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. ปิยนุช วงศ์อนันต์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. วัชรี ลิมปนสิทธิกุล, 89 หน้า.

Cepharanthine (CEP) ซึ่งเป็นสารในกลุ่ม biscoclaurine alkaloid ที่พบได้ในรากของต้น *Stephania cepharantha* Hayata มีฤทธิ์ต้านมะเร็งหลายชนิดเช่น มะเร็งช่องปากและลำคอ มะเร็งเม็ดเลือดขาว มะเร็งตับ และมะเร็งท่อน้ำดี วัตถุประสงค์ของการทดลองนี้คือเพื่อศึกษาความเป็นพิษของ CEP รวมไปถึงกลไกการออกฤทธิ์ในเซลล์มะเร็งลำไส้ใหญ่และทวารหนักของมนุษย์ โดยใช้เซลล์ COLO 205 ซึ่งเป็นเซลล์ที่ไม่มีการแสดงออกของยีนค็อกซ์ 2 และ p53 ลักษณะปกติและ เซลล์ HT-29 ที่มีการแสดงออกของยีนค็อกซ์ 2 และ p53 ที่กลายพันธุ์ ในการศึกษานี้แสดงให้เห็นว่า CEP มีความเป็นพิษต่อเซลล์ COLO 205 และเซลล์ HT-29 แปรผันตามความเข้มข้นที่ใช้ทดสอบ โดยพบว่า CEP มีความเป็นพิษต่อเซลล์ HT-29 (IC_{50} = 6.12±1.68 ไมโครโมลาร์) มากกว่าเซลล์ COLO 205 (IC_{50} = 32.13±0.515 ไมโครโมลาร์) นอกเหนือจากนี้ CEP สามารถชักนำให้เซลล์มะเร็งลำไส้ใหญ่และทวารหนักตายแบบอะพอพโทซิสและชักนำให้วัฏจักรของเซลล์หยุดที่ระยะ G1/S การศึกษาในระดับกลไกพบว่า CEP สามารถลดการแสดงออกของยีนยับยั้งการตายแบบอะพอพโทซิส *Bcl-2*, *Mcl-1* ในเซลล์ COLO 205 และ *Bcl-xl* ในเซลล์ HT-29 นอกจากนี้ CEP สามารถเหนี่ยวนำให้เกิดการสร้างอนุมูลอิสระภายในเซลล์ทั้งสองชนิด และพบว่าความเป็นพิษของ CEP ต่อเซลล์ COLO 205 และเซลล์ HT-29 ลดลงโดย n-acetyl cysteine (NAC) อีกทั้ง CEP สามารถลดการแสดงออกของยีน *COX-2* ในเซลล์ HT-29 โดยสรุปจากผลการศึกษานี้แสดงให้เห็นฤทธิ์ต้านมะเร็งของ CEP โดยชักนำให้เกิดการตายแบบอะพอพโทซิส, หยุดวัฏจักรเซลล์, การสร้างอนุมูลอิสระ และยับยั้งการแสดงออกของยีน *COX-2* ซึ่งแสดงให้เห็นถึงความเป็นไปได้ของ CEP ในการรักษามะเร็งลำไส้ใหญ่และทวารหนัก

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ARKORNNUT RATTANAWONG: ANTICANCER EFFECTS OF CEPHARANTHINE ON HUMAN COLORECTAL CANCER CELLS. ADVISOR: PIYANUCH WONGANAN, Ph.D., CO-ADVISOR: ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., 89 pp.

Cepharanthine (CEP), a biscoclaurine alkaloid isolated from *Stephania cepharantha* Hayata, exhibits anticancer activity against several different types of cancer including oropharynx cancer, leukemia, hepatocarcinoma and cholangiocarcinoma. This study aimed to investigate the cytotoxic effect of CEP and its underlying molecular mechanisms of action in human colorectal cancer cells. Two colorectal cancer cell lines, COLO 205 (COX-2 negative and p53 wild-type) and HT-29 (COX-2 positive and p53 mutant) were used. In the present study, CEP was found to exhibit cytotoxicity in COLO 205 and HT-29 cells in a concentration-dependent manner. Notably, CEP was more toxic to HT-29 cells (IC_{50} = 6.12±1.68 µM) than COLO 205 cells (IC_{50} = 32.13±0.515 µM). Additionally, CEP could significantly trigger both colorectal cancer cell lines to undergo apoptosis and cell cycle arrest at G1/S phase. Mechanistic studies illustrated that CEP could down-regulate the expression of anti-apoptotic, *Bcl-2* and *Mcl-1* genes in COLO 205 cells and *Bcl-xl* gene in HT-29 cells. CEP also significantly increased reactive oxygen species (ROS) level in both COLO 205 and HT-29 cells and cytotoxic activity of CEP was abolished by N-acetylcysteine (NAC). Moreover, CEP was able to down-regulate the expression of *COX-2* gene in HT-29 cells. Taken together, the results of this study suggest that the anticancer effects of CEP involve induction of apoptosis, cell cycle arrest and ROS generation as well as inhibition of *COX-2* gene expression, illustrating a therapeutic potential of CEP in colorectal cancer treatment.

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LIST OF ABBREVAITIONS

%	= Percentage
µg/ml	= Microgram per milliliter
µl	= Microliter
µM	= Micromolar
15-PGDH	= 15-hydroxyprostaglandin dehydrogenase
5FU	= 5-fluorouracil
ANOVA	= Analysis of variance
AOM	= Azoxymethane
Apaf-1	= Apoptotic protease-activating factor-1
APC	= Adenomatous polyposis coli
ATCC	= American Type Culture Collection
BAD	= BCL-2 antagonist of cell death
BAK	= BCL-2-antagonist/killer-1
BAX	= BCL-2-associated X protein
BCL-2	= B-cell lymphoma-2
BID	= BH3-interacting domain death agonist
BIK	= BCL-2-interacting killer
BIM	= BCL-2-like-11
BMF	= BCL-2 modifying factor
Caspase	= Cysteine aspartic acid specific protease
CD	= Crohn's disease
cDNA	= Complementary DNA
CEP	= Cepharanthine
$CHCl_3$	= Chloroform
CO_2	= Carbon dioxide
COX	= Cyclooxygenases
CRC	= Colorectal cancer
CREB2	= cAMP-response element binding protein
CT	= Cycle threshold

DCF	= 2′, 7′- dichlorofluorescein
DCFH	= 2′, 7′- dichlorodihydrofluorescein
DCFH-DA	= 2′, 7′- dichlorodihydrofluorescein diacetate
DEPC	= Dietyl pyrocarbonate
DISC	= Death inducing signalling complex
DMEM	= Dulbecco's modified Eagle's medium
DMSO	= Dimethylsulfoxide
DNA	= Deoxyribonucleic acid
EDTA	= Ethylene diamine tetraacetic acid
EGF	= Epidermal growth factor
EGFR	= Epidermal growth factor receptor
ER	= Endoplasmic reticulum
ERK	= Extracellular signal-regulated kinase
EtOAc	= Ethyl acetate
FADD	= Fas-associated death domain
FAP	= Familial adenomatous polyposis
Fas	= Fibroblast associated antigen
FBS	= Fetal bovine serum
FDA	= Food and drug administration
GAPDH	= Glyceraldehyde 3-phosphate dehydrogenase
GSK-3β	= Glycogen synthase kinase 3β
h	= Hour
H_2O_2	= Hydrogen peroxide
HCl	= Hydrochloric acid
HEPES	= 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC50	= 50% Inhibition concentration
IL	= Interleukin
iNOS	= Inducible nitric oxide synthase
MAPK	= Mitogen-activated protein kinase
Mcl-1	= Myeloid cell leukemia 1
MeOH	= Methanol

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mg/ml	= Milligram per milliliter
MnSOD	= Manganese superoxide dismutase
mRNA	= Messenger RNA
MTT	= 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NaHCO3	= Sodium bicarbonate
NaOH	= Sodium hydroxide
NF-κB	= Nuclear factor kappa-light-chain-enhancer of activated B cells
nm	= Nanometer
NSAID	= Non-steroidal anti-Inflammatory drug
^{o}C	= Degree centigrade
$O_2^{\bullet -}$	= Superoxide anion
OD	= Optical density
$OH^{\bullet}$	= Hydroxyl radical
P53	= Tumor protein 53
PBS	= Phosphate buffer saline
PG	= Prostaglandin
pH	= The negative logarithm of hydrogen ion concentration
PI	= Propidium iodide
PI3K	= Phosphoinositide 3-kinase
PKC	= Protein kinase C
PUMA	= BCL-2 binding component-3
RNA	= Ribonucleic acid
RNase A	= Ribonuclease A
ROS	= Reactive oxygen species
Rpm	= Round per minutes
RPMI	= Roswell Park Memorial Institute medium
RQ	= Relative quantitation
RT-PCR	= Reverse transcription polymerase chain reaction

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Smac/DABLO	= Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein (IAP) with low pI
tBid	= Truncated Bid
TNFα	= Tumor necrosis factor alpha
TRADD	= TNFR1-associated death domain
TXA2	= Thromboxane A2
UDG	= Uracil-DNA glycosylase
UC	= Ulcerative colitis
UCP2	= Uncoupling protein 2
VEGF	= Vascular-endothelial growth factor
VEGFR	= Vascular-endothelial growth factor receptor

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Colorectal cancer (CRC) is the fourth leading cause of cancer death worldwide after lung, liver and stomach (1). In 2012, the National Cancer Institute of Thailand reported that the incidence of CRC is the second most commonly diagnosed cancer in man (14.80% of all cases) and the third in woman (9.48% of all cases) in Thailand (2). At present, surgery followed by adjuvant chemotherapy has been the standard treatment for CRC. Although chemotherapy such as 5-fluorouracil, oxaliplatin and capecitabine has been widely used, its application is often limited due to drug resistance and serious side effects. Therefore, a novel compound that has potent anticancer activity and minimal side effects is urgently needed.

Evidence has shown that patients with inflammatory bowel diseases including ulcerative colitis (UC) and Crohn's disease (CD) have a significant higher risk for developing colorectal cancer (3, 4). Cyclooxygenase (COX) enzyme has played a key role in the biosynthesis of prostaglandins and thromboxane from arachidonic acid. There are two major COX isoforms, including COX-1 and COX-2. COX-1 is constitutively expressed in many tissues and plays an important role in tissue homeostasis, while COX-2 is induced by inflammatory stimuli and involved in pathological processes (5). COX-2 overexpression has been detected in 84.9% of colon carcinoma and 57.9% of adenomas, highlighting a critical role of COX-2 in CRC development (6). Moreover, epidemiological studies have been demonstrated that long-term treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, or selective cyclooxygenase 2 (COX-2) inhibitors such as celecoxib could reduce the risk of CRC (7). Overexpression of COX-2 was found to increase accumulation of prostaglandins (PGs) particularly, PGE_2 which promotes colorectal tumor development by stimulating

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angiogenesis, cell proliferation and apoptosis evasion (8). Previous studies also showed that HT-29 colorectal cancer cells overexpressing COX-2 were resistant to apoptotic cell death (9), indicating that COX-2 may be an important target for colorectal cancer treatment.

Reactive oxygen species (ROS) play an important role in a variety of physiological and pathological processes. Excessive generation of ROS such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) can induce damage of several cellular components such as nucleic acids, lipids and proteins, leading to apoptosis or autophagy and in some cases necrosis of cancer cells (10, 11). In addition, it has been shown that various anticancer drugs including cisplatin, paclitaxel, doxorubicin and radiation induce apoptotic cell death through generation of ROS in several cancer cells (12, 13). Therefore, inducing ROS production is a very promising therapeutic strategy for cancer treatment.

Cepharanthine (CEP), a natural compound isolated from *Stephania cepharantha* Hayata, possess many pharmacological effects such as anti-inflammation, anti-retrovirus, anti-oxidant and anti-cancer (14). CEP could inhibit production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and nitric oxide by suppressing NF-κB (15). Several studies also revealed that CEP has anticancer activity against several types of cancer such as oropharynx cancer, leukemia, hepatocarcinoma and cholangiocarcinoma (16-19). CEP has been shown to inhibit tumor growth through multiple mechanisms, including increasing host immune response (20), inducing cancer cell to undergo apoptosis (21), stimulating cell cycle arrest (22) and activating ROS production (23). To my best knowledge, there have been only a few reports on anticancer activity of CEP against CRC and its molecular mechanism(s) have never been identified. In the present study, I therefore evaluated the anti-cancer activity of CEP on a COX-2 positive human colorectal cancer cell line,

HT-29 and a COX-2 negative human colorectal cancer cell line, COLO 205. Molecular mechanism(s) underlying its anti-cancer effect was also investigated.

1.2 Objectives

To determine the *in vitro* cytotoxic effect of cepharanthine on human colorectal cancer cells.

To investigate the molecular mechanism(s) of the anticancer effects of cepharanthine on human colorectal cancer cells.

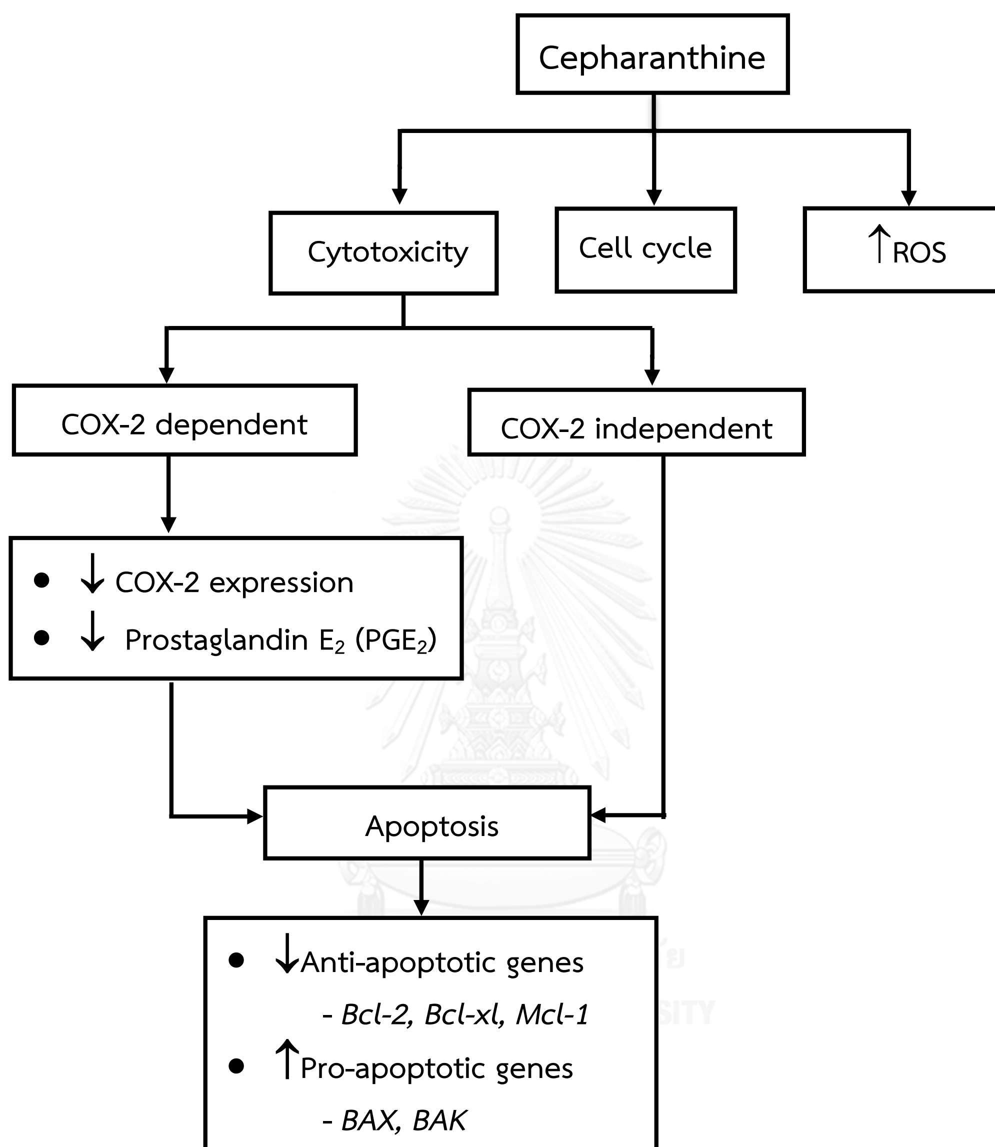
1.3 Hypothesis

Cepharanthine possess anti-cancer effect against human colorectal cancer cells through COX-2-dependent and -independent mechanisms.

1.4 Contribution of the study significance

The results from this study would provide preliminary data of whether cepharanthine has anticancer activity against COX-2 positive and/or COX-2 negative colorectal cancer cells and its underlying mechanism(s). Therefore, this information would be helpful in considering the possibility of using this compound as a novel treatment option for colorectal cancer.

1.5 Conceptual framework



CHAPTER II
LITERATURE REVIEWS

2.1 Colorectal cancer

Colorectal cancer (CRC) is a term for cancer that starts in the colon or rectum (Figure 1). It is the third most common cancer worldwide with 1.4 million new cases recorded in 2012. Mortality is approximately nearly 69,4000 deaths, making it the fourth most common cause of death from cancer (24). Countries with the highest incidence rates include Australia, New Zealand, Canada, the United States, and parts of Europe (25). In Thailand, CRC is the second most commonly diagnosed cancer in man (14.80% of all cases) and the third in woman (9.48% of all cases) (2). The survival rate of colorectal cancer patients primarily depends on stage of disease at diagnosis, which are typically ranges from 90% 5-year survival rate for cancers detected at the localized stage; 70% for patients with regional spread and 10% for patients diagnosed for distant metastatic cancer (26).

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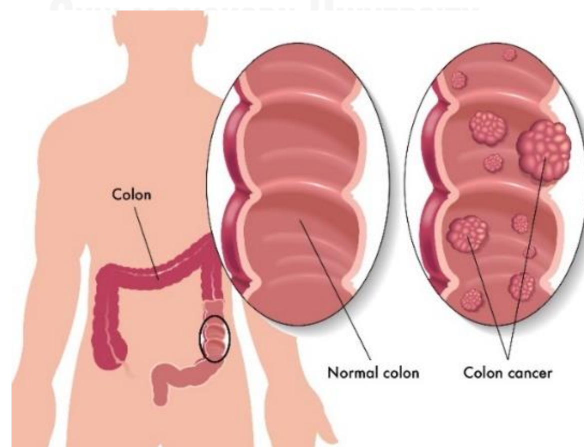

Figure 1. The difference between a normal colon and a cancerous colon *(27)*

Studies have found the following risk factors for CRC, including age over 50, colon polyps family history of colorectal cancer, genetic alterations, personal history of cancer, ulcerative colitis or Crohn's disease, diet and lifestyle, heavy alcohol use and cigarette smoking (28).

Genetic alterations have widely been identified in colorectal cancer patients and somatic mutations and deletions of tumor suppressor genes such adenomatous polyposis coli (*APC*) and tumor protein 53 (*TP53*) are the most common genetic changes in this disease. Previous clinical studies revealed that inactivation of *APC* is present in most sporadic colorectal adenomas and cancer while inactivation of *TP53* is often associated with the transition of adenomas into invasive carcinoma (29, 30). Furthermore, mutation of *p53* was detected in tumors of rectal cancer patients who were poor responders to radiotherapy (31). Similarly, *p53* deficiency was reported to lower response of cancer cells to apoptosis, increasing resistance to 5-FU in mice bearing human colon cancer xenografts (32). In addition to mutational inactivation of tumor suppressor genes, activation of mitogen-activated protein kinase (MAPK) signaling caused by oncogenic changes of *RAS*, primarily *KRAS*, and *BRAF* play important roles in the progression of CRC. Mutations of *RAS* and *BRAF* were detected in 37% and 13% of colorectal cancer patients, respectively (33-35). Apart from genetic changes, activation of growth factor pathways such as epidermal growth factor (EGF) and cyclooxygenase 2 (COX-2), was found to be involved colorectal cancer development (Figure 2) (27).

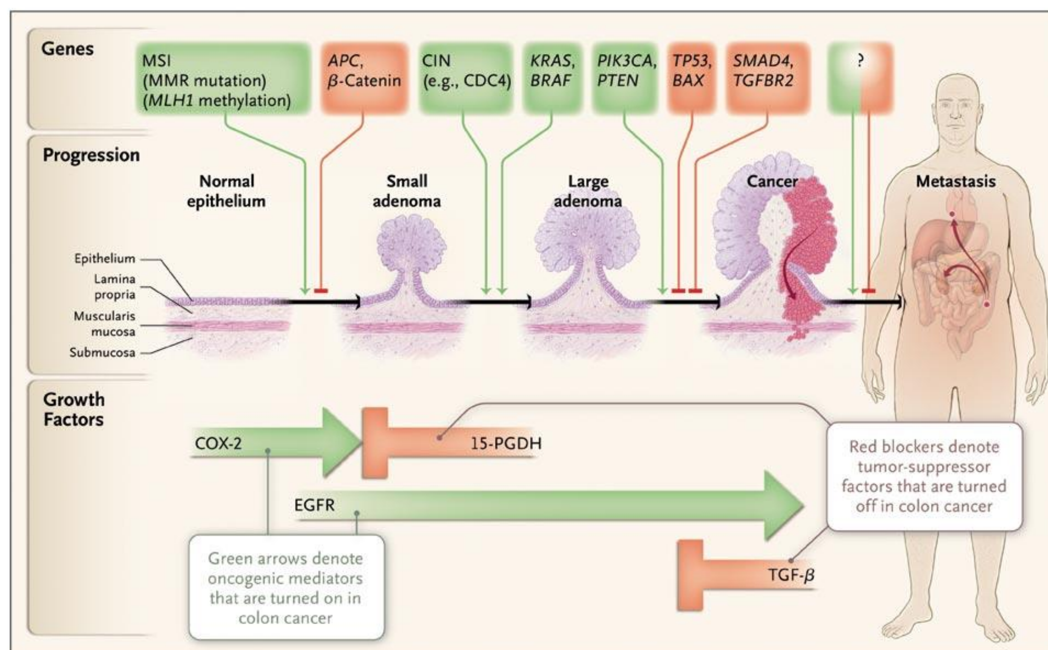

Figure 2. Alteration of genes and growth factor pathways in progression of colorectal cancer. (27)

2.2 Stages of colorectal cancer (36)

Stage of cancer is a critical factor determining prognosis and treatment. According to the TNM staging method, CRC can be categorized into five stages (0-IV), depending on three factors including tumor size, lymph node status and distant metastasis.

Colorectal cancer can therefore be stages as follows:

- Stage 0: The abnormal cells are found in the mucosa (innermost layer) of the bowel wall
- Stage I: Cancer have formed several layers in the mucosa of the colon or rectum
- Stage II: Cancer have infiltrated through the colon or rectum wall completely and may have begun to spread to nearby tissue
- Stage III: Cancer has spread to regional lymph nodes

- Stage IV: Cancer has spread to one organ that is not near the colorectal, such as the liver, lung, or ovary, or to a distant lymph node.

2.3 Treatment of colorectal cancer

There are many options including surgery, chemotherapy and radiation for CRC treatment depending on the stage of the disease. Surgery is the most common treatment option for early colorectal cancer. After surgical resection, adjuvant chemotherapy or radiotherapy may be used in patients with stage II or III to reduce a risk of recurrence and increase survival. For more advanced stages, chemotherapy has been the mainstay approach for treating patients with metastasis disease (25). The chemotherapeutic agents commonly used to treat colorectal cancer are 5-fluorouracil, oxaliplatin, irinotecan, and capecitabine which are normally given in combination in order to improve efficacy and decrease the possibility of drug resistance. In addition to chemotherapy, US FDA has approved three biologic drugs, which are bevacizumab (Avastin®), cetuximab (Erbitux®) and panitumumab (Vectibix®) for treatment of patients with metastatic CRC. Bevacizumab is a humanized monoclonal antibody targeting vascular endothelial growth factor (VEGF) whereas panitumumab and cetuximab are monoclonal antibody blocking ligand-induced epidermal growth factor receptor (EGFR) (37-39). Although the use of chemotherapy and targeted therapy has been shown to increase survival in metastatic CRC, adverse toxicity and resistance to chemotherapeutic drugs as well as high cost of biologic agents remain serious problems.

2.4 Cyclooxygenase-2 and its role in colorectal cancer

Cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase (PTGs) is a key enzyme in the production of bioactive prostanoids, including prostaglandins and thromboxane. COX enzymes catalyze the conversion of arachidonic

acid, released from membrane phospholipid by phospholipase A2 (PLA2), into prostaglandin G_2 (PGG_2), an unstable intermediate that is rapidly converted to PGH_2. A precursor for all prostaglandins, PGH_2, is then converted to different structurally-related PGs, including PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TXA_2) by specific enzyme prostaglandin synthases (40). In human, COX exists in three different isoforms including COX-1, COX-2 and COX-3, a third isoform which has recently been identified. COX-1, a constitutive enzyme found in most tissues, plays a key role in physiological homeostasis such as production of gastric mucosa and regulation of kidney and platelet function (41). Conversely, COX-2 is an inducible enzyme expressing in responsible to various stimuli such as pro-inflammatory cytokines, growth factors, endotoxin and tumor promoters and mainly responsible for pathophysiological processes such as inflammation and cancer (42). Previous studies reported that *COX-2* mRNA are found over expressed in 80% of the colorectal carcinomas and 70-40% of adenomas, compared with adjacent normal colorectal mucosa (42). It has been shown that transfection of COX-2-negative HCT-15 cells with *COX-2* gene led to their resistance to apoptosis induced by 5-fluorouracil and NSAIDs (43). Further mechanistic studies revealed that this was due to reduced cytochrome c release and caspase 3 activation and increased anti-apoptotic Bcl-2 mRNA and protein.

Several studies illustrated that transcription factors such as nuclear factor-kappaB (NF-κB), cAMP-response element binding protein (CREB2) and co-activators such as p300 could promote COX-2 expression in colorectal cancer cells (44, 45). Moreover, enhanced *COX-2* gene expression in colorectal cancer was detected following mutation of tumor suppressor gene such as *APC* and oncogene such as *RAS* and activation of EGF, HER2 and IGF-I receptor pathways (46). It was demonstrated that mutation of *COX-2* gene markedly reduces the number and size of intestinal polyps in $APC^{\Delta 716}$ knockout mice model (47). Moreover, many NSAIDs and selective COX-2 inhibitors have been extensively investigated for prevention and treatment of CRC (48).

In clinical study, treatment of familial adenomatous polyposis (FAP) patients with celecoxib 400 mg twice daily for 6 months could reduce number of duodenal and rectal polyps (49). Similarly, the patients with FAP receiving 25 mg/day of rofecoxib, showed a significant reduction number and size of rectal polyposis after 9 months of therapy (50). It is widely known that overexpression of COX-2 promotes colorectal tumorigenesis by increasing PGE_2 production (9). In male F344 rats, intraperitoneal PGE_2 injection could increase the incidence of azoxymethane (AOM)-induced colon tumor and reduce apoptotic index in this model (51). Moreover, celecoxib-induced radiosensitization was found to be mediated through inhibition of COX2-derived PGE_2, a survival factor for tumor (52). The action of PGE_2 are mediated by binding with E-type prostanoid receptors including EP_1, EP_2, EP_3 and EP_4, which are G protein-coupled receptors (GPCRs) (53). It was suggested that, in colon cancer DLD-1 cells, binding of PGE_2 to EP2 receptor leads to activation of phosphoinositide 3-kinase (PI-3K) and the protein kinase Akt by free G-protein $\beta\gamma$ subunits and association of Axin with G-protein α subunit. Activation of these two pathways promotes the release of glycogen synthase kinase-3β (GSK-3β) from its complex, thereby inhibiting phosphorylation and degradation of β-catenin. Following its accumulation in the cytoplasm, β-catenin can therefore translocate to the nucleus and activate genes involved in cell survival, proliferation and angiogenesis (Figure 3) (54). Moreover, COX-2/PGE_2 signaling might suppress apoptosis via the EP_4 receptor by activating the Ras/MAPK/ERK pathway, resulting enhanced expression of the anti-apoptotic protein Bcl-2 through activation of *CREB* transcription enhance expression of the anti-apoptotic protein Bcl-2 (55). Recent *in vitro* and *in vivo* studies illustrated that sulindac and celecoxib, anti-inflammatory drugs, could overcome tumorigenesis effects by increasing expression of GSK-3β and suppressing Akt and inducing apoptosis in colorectal cancer cells (56). In addition to GSK-3β, Shao et al. indicated that PGE_2 increased expression of cyclin D1

and VEGF and activated the β-catenin/T cell factor-dependent transcription through the cAMP/protein kinase A (PKA) pathway in colon cancer LS-174T cells (57).

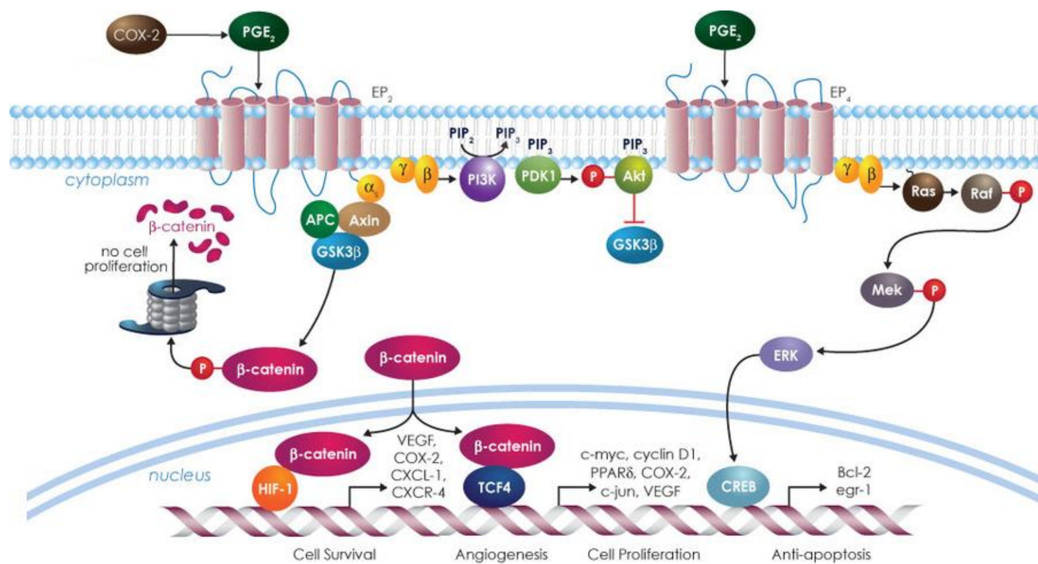

Figure 3. Signaling pathway of COX-2/PGE2 in colorectal cancer. (58)

2.5 Oxidative stress

Oxidative stress is defined as an imbalance between production of reactive oxygen species (ROS) and elimination of ROS. ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^•$), mainly produced by mitochondria, are highly reactive molecules that can react with nucleic acids, proteins and lipids (59). Therefore, overproduction of ROS in cells can lead to oxidative stress, causing cell damage and cell death (23, 60-62). Several lines of evidence indicated that increased level of ROS production can be detected in cancer cells and are responsible for the acquisition of several hallmarks of cancer including sustaining cell proliferation and survival, disrupting cell death, angiogenesis and metastasis (63). In addition to increased ROS, it is important to note that cancer cells also enhance many antioxidant proteins in order to avoid detrimental effects of ROS (64-66). Considering that cancer cells contain higher rates of ROS production, enhanced oxidative stress by further

increasing ROS levels may be a promising approach to induce cell death and cell cycle arrest. Previous studies illustrated that ROS generators exhibited anticancer effects in SP 2/0 mouse myeloma cells (67). Similarly, human melanoma cell line (A375) expressing high levels of manganese superoxide dismutase (MnSOD) by cDNA transfection was shown to have increased resistance to oxygen free radical damage-induced by mitomycin C and doxorubicin (68). H_2O_2 was shown to induce apoptosis by activating the c-Jun N-terminal kinase/stress activating protein kinase (JNK/SAPK) signaling pathway (69). Additionally, Simizu et al. demonstrated that activation of caspase-3-like proteases by various anticancer drugs such as camptothecin, vinblastine, inostamycin, and adriamycin caused generation of H_2O_2 likely through the activation of NADPH oxidase, thereby inducing DNA fragmentation and apoptosis of small cell lung cancer Ms-1 cells (70). It was also shown that treatment of lung cancer cells with paclitaxel could inhibit cell growth through increase O_2^- and H_2O_2 levels both *in vitro* and *in vivo* while antitumor activity of paclitaxel was abolished by N-acetyl cysteine (NAC) (13). Inhibition of uncoupling protein 2 (UCP2), a regulator of mitochondrial-derived ROS, was reported to suppress ROS production and cell growth and enhance sensitivity of cancer cells to tamoxifen, leading to apoptosis and autophagy in breast cancer MCF-7 cells (71).

2.6 Cell cycle

The cell cycle, a set of events responsible for cell division, can be divided into four phases including gap 1 (G_1), synthesis (S), gap 2 (G_2) and mitosis (M). G_1 phase is the interval between mitosis and initiation of DNA replication. During this phase, the cell is metabolically active and continuously grows. Once it passes through the restriction point, the cell can progress into S phase and commit to complete the cell cycle. However, under abnormal conditions, the cell can withdraw from further proliferation and enters a quiescent state, call G_0. The cell can remain in this phase for a long time unless called on to proliferate by appropriate stimuli such as growth

factors. S phase is defined as the stage in which DNA replication occurs. The completion of DNA synthesis is followed by the G_2 or pre-mitotic phase. During this phase, cell continues to grow and proteins are actively synthesis in preparation for mitosis. During M phase, the replicated chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells (72).

The progression through the phases of a cell cycle is mainly regulated by cyclin-dependent kinases (CDKs), a family of serine/threonine kinases including Cdk4, Cdk6, Ckd2 and Ckd1. Their kinase activity is further dependent on the presence of activating subunits called cyclins such as cyclin D, E, A and B (73). In fact, under normal conditions, cyclin D/Cdk4 and 6 are activated and the cells can progress from G_0 into early G_1 phase. At the end of G_1 phase, phosphorylation of retinoblastoma protein family (Rb) lead to further activation of E2F mediated transcription at the restriction point, causing the cells to enter S phase (74). During the G_1 to S phase alteration, cyclin E/Cdk2 are also activated. In addition to cyclin A, cyclin E can bind to Cdk2 between S phase or to Cdk1 in the G_2 to M phase transition. Finally, cyclin B/Cdk1 complexes can actively participate in and complete mitosis (75).

The activity of Cdk complexes is regulated by two families of Cdk-inhibitory subunits (CKIs) including the inhibitors of cdk4 (INK4) family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$), which inhibit cdk4 and cdk6, and the Cip/Kip family ($p21^{WAF1/cip1}$, $p27^{kip1}$, and $p57^{kip2}$), which inhibit Cdk2/cyclin E, Cdk2/cyclin A, Cdk1/cyclin A, as well as Cdk1/cyclin B activity (75, 76). It should be noted that the checkpoints can be activated by extreme conditions, such as stress, DNA damage and telomere dysfunction, inducing the cell to repair the damage. After damage repair, progression through the cell cycle resumes. However, if the damage cannot be repaired, the cell is eliminated through apoptosis process.

2.7 Apoptosis

Apoptosis plays a crucial role in both physiological and pathological processes. Most chemotherapeutic agents induce cancer cells to undergo apoptosis (76, 77). Apoptosis, also known as programmed cell death, is characterized by plasma

membrane blebbing, cell shrinkage and DNA fragmentation. There are two main apoptotic pathways: the extrinsic and the intrinsic pathways (78).

Extrinsic or death receptor pathway is initiated by binding of death receptors, which are members of the tumor necrosis factor (TNF) receptor family, with their cognate ligands, recruiting adaptor molecules such as Fas-associated death domain (FADD) and then procaspase-8. This leads to activation of caspase 8, which can subsequently activates downstream effector caspases such as caspase 3 and caspase 7 (79).

Intrinsic or mitochondrial pathway can be initiated by different stimuli such as oxidative stress, UV and anticancer drugs, opening the mitochondrial membrane (80). This results in the release of cytochrome c which can bind with apoptotic protease-activating factor 1 (APAF1), forming apoptosome, an activator of procaspase-9. Once activated, caspase 9 can cleave and activate executioner caspases such as caspase 3 and caspase 7, leading to apoptosis (81). It is well known that the mitochondrial membrane permeability is regulated by members of the Bcl-2 family proteins, which can be divided into three functional groups: pro-apoptotic activators, pro-apoptotic effectors and anti-apoptotic proteins. It was reported that pro-apoptotic activators such as Bid, Bim, Bad, PUMA and NOXA promotes oligomerization and membrane insertion of pro-apoptotic effectors such as BAX and BAK, resulting in formation of pores in the mitochondrial membrane (82). This leads to the release of apoptogenic substrates such as cytochrome c, apoptosis-inducing factor (AIF) and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein (IAP) with low pI (Smac/DABLO) from mitochondria, initiating the apoptotic program (83). On the other hand, the anti-apoptotic proteins such as Bcl-2, Bcl-xl and Mcl-1 can bind with pro-apoptotic proteins, preventing mitochondrial pore formation and suppressing apoptotic signaling cascade.

2.8 Cepharanthine

Cepharanthine (CEP) is a biscoclaurine alkaloid isolated from the root of *Stephania cepharantha* Hayata cultivated in Japan (Figure 4). It can also been extracted from *Stephania venosa* (Blume) Spreng and *Stephania erecta* Craib, which are commonly found in Thailand. This compound has been approved by the Japanese Ministry of Health to treat various diseases including allergic, alopecia areata, and leukopenia during radiation therapy. Additionally, CEP has been reported to have many pharmacological effects such as anti-inflammation, anti-retrovirus, anti-oxidant, anti-platelets, anti-cancer, and multidrug resistance reversal (14, 84, 85). Notably, CEP has long been widely used, however it serious side effect has never been reported. Sato revealed that out of a total 3556 clinical case, 28 case (approximately 0.79%) given 10-20 mg CEP per day experienced very mild side effects such as headaches, stomach discomfort and dizziness (85). Intravenous injection of 40-60 mg CEP daily for 2 months was found to be safe (86). Additionally, CEP has been shown to effectively prevent toxics caused by radiotherapy and chemoradiotherapy in patients with prostate and head and neck cancer (87, 88). Bun et al. also found that CEP lack of mutagenic activity in colon cancer (89). Due to its safety, this compound has gained big attention as a new therapeutic agent for cancer treatment.

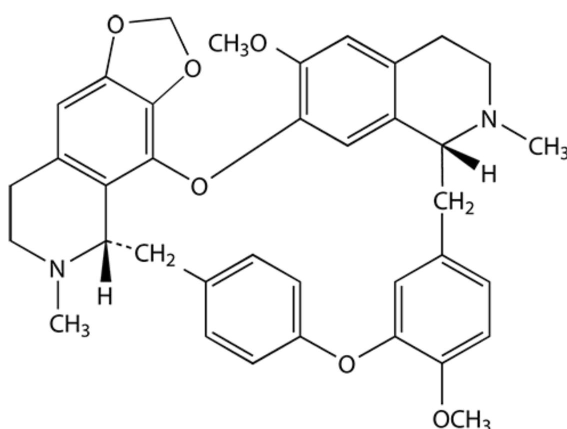

Figure 4. Chemical structure of cepharanthine.

2.7.1 The pharmacological effects of cepharanthine

2.7.1.1 Anti-cancer activity

Several lines of evidence demonstrated that CEP exhibits anticancer activity against several different types of cancer including osteosarcoma, oropharynx cancer, leukemia, hepatocarcinoma and cholangiocarcinoma both *in vitro* and *in vivo* (16, 19, 22, 90). Cytotoxicity studies of three alkaloids including cepharanthine, tetrahydropalmatine and xylopinine on hepatocellular carcinoma and colon adenocarcinoma cell lines (HepG2, HT-29, SW620, and LS174T) illustrated that only CEP exhibited marked cytotoxicity against all tested cell lines except SW620. Its IC_{50} values were between 2.4 and 5.3 μM for which type of cell lines, respectively (89). Moreover, CEP could inhibit cell growth in several human cancer cell lines including human lung carcinoma (A-549), human esophagus cancer (ECA109), human myeloid leukemia (HL-60), human breast cancer (MCF-7), hepatocellular carcinoma (SMMC-7721), and colon cancer (SW480) cells (91). Previously, it was reported that CEP exerts antitumor effect by increasing immunological competence of the host. Ebina et al. demonstrated that its antitumor effect in a double-grafted tumor system in mice was associated with a sequential immune mechanism in which cytotoxic macrophages were induced in the tumor (20). Furthermore, CEP could induce apoptosis both in a mouse leukemia P388 doxorubicin-sensitive (P388/S) and –resistant (P388/DOX) cells by inducing production of ROS and expression of Fas-antigen expression in tumor cells (92). Studies also showed that 1-10 μM CEP could induce apoptosis of a human leukemia T cell line, Jurkat, and a human chronic myelogenous leukemia cell line, K562, which expresses an anti-apoptotic p210 Bcr-Abl fusion protein (18). CEP treatment also induced activation of caspase-9 and -3 and cleavage of PARP, Bid, lamin B1, and DFF45/ICAD in both Jurkat and K562 cells, whereas caspase-8 activation, Akt cleavage and degradation Bcr-Abl protein were observed after CEP treatment in

K562 cells only (18). Further studies demonstrated that CEP at the concentration required for and at the time of apoptosis induction, could activate p38 kinase in both Jurkat and K562 cells whereas activation of ERK were observed only in K562 cells (90). Similarly, Biswas et al. reported that the compound induced apoptosis in HuH-7 cells through the generation of ROS, the activation of JNK1/2, MAPK p38, ERk p44/42 and the down-regulation of the protein kinase B (Akt) (16). Harada et al. also illustrated that CEP could activate caspase-3 as well as induce cell cycle G1 arrest in a human adenosquamous cell carcinoma (TYS) cell line and a human oral squamous cell carcinoma (OSCC) cell line by the induction of cell cycle regulators, p21WAF1 and p27Kip1, respectively (21, 93). It was revealed by the same group that CEP could inhibit angiogenesis and growth of OSCC cells by inhibiting NF-κB activity, suppressing expression of VEGF and IL-8 (94). Takahashi-Makise et al. demonstrated that CEP inhibited primary effusion lymphoma (PEL) cells growth via the suppression of NF-κB activity, particularly by blocking the phosphorylation of p65 NF-κB (95). Previous studies showed that CEP controlled the growth of cholangiocarcinoma (CCA) cells *in vitro* through inhibition of NF-κB nuclear translocation and effectively reduced tumor size in CCA-inoculated mice without showing serious side effects (19). Uthaisar et al. also reported that CEP exhibited anti-metastatic effects on human CCA cell lines by inhibiting expression of ICAM-1 and MMP-2, resulting in suppression of migration activity and invasion of KKU-M213 and KKU-M214 (96). Furthermore, CEP (5 and 10 μM) significantly inhibited the expression of STAT3 target genes, including the anti-apoptotic gene *Bcl-xL* and the cell cycle regulators *c-Myc* and *cyclin D1* and induced G1-phase cell cycle arrest of SaOS2 cells (22). In addition, the compound could induce apoptosis in non-small lung cancer cells H1299 and A549 via up-regulation of BAX, down-regulation of Bcl-2 and activation of caspase-3 and cleaved PARP which was mediated by ROS (23). Moreover, CEP was shown to enhance sensitivity of HeLa cells to radiation

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in vitro and *in vivo* by inducing apoptosis and reducing expression of STAT3, Bcl-2, c-Myc, and COX-2 (97). It has also been revealed that CEP could reverse multidrug resistant (MDR) of LLC-GA5-COL1-50 cells expressed P-glycoprotein (P-gp) to vinblastine and daunorubicin by directly interacting with P-gp which results in accumulation of anticancer drugs in cancer cells (98). Consistency, Nakajima et al. demonstrated that co-treatment of CEP significantly improved cytotoxicity of doxorubicin in DOX-resistant hepatocellular carcinoma cell lines (99). Moreover, this compound could reverse resistance of *MRP7*-tranfected HEK293 cells to paclitaxel by suppressing the MRP-7-mediated efflux (100). Han et al. also illustrated that the expression of *MDR1* mRNA was decreased by CEP in human chronic myeloid leukemia K562 cells and adriamycin-tolerance K562 cells (K562/ADR) through activation of JNK/c-Jun pathway (101).

2.7.1.2 Anti-inflammatory

Many previous studies have demonstrated CEP exerts anti-inflammatory by inhibiting production of pro-inflammatory cytokines. It was reported that this compound significantly inhibited the increase in plasma tumor necrosis factor-alpha (TNF-α) concentrations in LPS-induced acute pulmonary vascular injury in rats well as the production of TNF-α in LPS-stimulated human monocytes (102). CEP could also inhibit production of pro-inflammatory cytokines, including TNF-α, interleukin (IL)-1β, IL-6 and nitric oxide through suppression of NF-κB in macrophage RAW264.7 cells (15). Huang et al. has recently reported that CEP inhibit the release of TNF-α, IL-6, IL-1β in LPS-stimulated RAW264.7 cells by suppressing activation of NF-κB, degradation of IκBα, and phosphorylation of ERK, JNK, and p38 while the compound reduced lung histopathologic changes and decreased the level of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, in the mouse acute lung injury model (103). Similarly, CEP was found to reduce the levels of TNF-α, IL-1β, and IL-6, attenuate the infiltration

of neutrophils, inhibit myeloperoxidase activity, as well as inhibit the phosphorylation of NF-κB p65 subunit and the degradation of IκBα in LPS-induced mouse mastitis (104).

CHAPTER III
MATERIALS AND METHODS

3.1 Equipments

- Analytical balance (Mettler Toledo, Switzerland)
- Autopipette (Gilson, USA)
- Autoclave (Sanyo, Japan)
- Biohazard laminar flow hood (Science, Germany and Labconco, USA)
- Controller pipette (Gilson, USA)
- CO_2 incubator (Thermo, USA)
- Centrifuge (Hettich, USA and Eppendrof, Germany)
- Fluorescence flow cytometer (BD Biosciences, USA)
- Light microscope (Nikon, Japan)
- Microplate reader (Thermo, Finland)
- Fluorescence microplate reader (Thermo, Finland)
- PCR thermal cycler (Eppendorf, Germany)
- pH meter (Mettler Toledo, Switzerland)
- StepOnePlus^{TM} Real-Time PCR system (Applied Biosystems, USA)
- Vortex mixer (Scientific Industries, USA)

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3.2 Materials

- 0.1mL low profile polypropylene thin wall PCR tube strips (Corning Life Sciences, USA)
- 100 mm^{2}cell culture dish (Corning Inc., USA)
- 25 and 75 cm^2 rectangular cell culture flask (Corning Inc., USA)
- 6-well plate (Corning Inc., USA)
- 24-well plate (Corning Inc., USA)

- 96-well plate (Corning Inc., USA)
- 5 ml round bottom polystyrene test tube (Falcon, USA)

3.3 Reagents

- 0.25% trypsin-EDTA (Gibco, USA)
- 0.4% trypan blue dye (Sigma, USA)
- 2-Propanol (Merk, Germany)
- Annexin V, Fluorescein (FITC) (Gibco,USA)
- Celecoxib (Sigma, USA)
- Cepharanthine (Abcam, UK)
- Chloroform (Lab-scan, Thailand)
- DEPC-treated water (Ambion, USA)
- Dimethyl sulfoxide (DMSO) (Sigma, USA)
- Dichloro-dihydro-fluorescein diacetate (Sigma, USA)
- Dulbecco's modified eagle medium (DMEM) (Gibco, New Zealand)
- Ethanol (Merk, Germany)
- Express SYBER Green qPCR supermix universal (Invitrogen, USA)
- RPMI-1640 medium (Gibco, USA)
- Resazurin (Sigma, USA)
- Fetal bovine serum (Gibco, New Zealand)
- ImProm-II^{TM} Reverse Transcription system (Promega, USA)
- Penicillin-streptomycin (Gibco, New Zealand)
- Propidium iodide (Santa Cruz Biotechnology, USA)
- Prostaglandin E_2 (Sigma, USA)
- Prostaglandin E2 competitive ELISA Kit (Thermo, USA)
- Trizol reagent (Gibco, USA)

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3.4 Methods

3.4.1 Preparation of cepharanthine stock solution

A 100 mM cepharanthine stock solution was prepared in dimethyl sulfoxide (DMSO) and stored at 4 ^{O}C until use. In the experiments, the stock solution was diluted in culture medium to give appropriate final concentrations. The 0.2% DMSO was used as a vehicle control.

3.4.2 Cell culture

Human colorectal cancer cell lines HT-29 (COX-2 positive) and COLO 205 (COX-2 negative) were purchased from American Type Culture Collection (ATCC) (Rockville, MD). HT-29 and COLO 205 were cultured in Dulbecco's modified eagle medium (DMEM) and RPMI 1640 medium respectively, supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% CO_2 incubator. For routine passage, cultures were split 1:5 when they reached 80-90% confluence, using 0.25% trypsin solution containing 1 mM EDTA (Gibco, USA). Exponentially growing cells were used in all experiments.

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3.4.3 Determination of cell viability using resazurin assay

Cell viability was evaluated by the ability of mitochondrial reductase enzyme in living cells to reduce resazurin (blue) into resorufin (pink). Briefly, both cell lines were seeded in a 96-well plate at a density $5x10^3$ cells/well and incubated overnight at 37°C and 5% CO_2. Cells were then treated with 0.1, 1, 5, 10, 20, 30 or 40 µM cepharanthine or 0.2% DMSO (vehicle control) for 48 hours at 37°C and 5% CO_2. Five hours before the end of the treatment period, 15 µL of resazurin solution (0.05 mg/ml) was added to each well and incubated at 37°C. The colorimetric was quantified by

measuring the absorbance at 570 and 600 nm using a microplate reader (Thermo, Finland). The percent of cell viability was calculated using the following equation:

% cell viability = (Abs. sample/Abs. control) x100.

The values of half inhibitory concentration (IC_{50}) were determined using the fitted line by GraphPad prism software (GraphPad Software, USA).

3.4.4 Detection of apoptosis by flow cytometry

Phosphatidylserine is a negatively charged phospholipid that normally mainly presents in membrane leaflets facing the cytosol. The exposure of phosphatidylserine on the outer leaflet of the plasma membrane constitutes an early event in apoptosis. Therefore, phosphatidylserine can be readily detected by fluorescent-labelled Annexin V which specifically binds externalized phosphatidylserine while the cell membrane itself remains intact. Conversely, necrosis is accompanied by loss of cell membrane integrity and leakage of intracellular content into environment. Consequently, fluorescent-labelled Annexin V combined with cell-impermeable dyes, such as propidium iodide (PI) can be used to distinguish apoptotic cells from living and necrotic cells. HT-29 and COLO 205 cells were seeded in a 6-well plate at a density of $3x10^5$ cells/well and incubated overnight. HT-29 cells were then treated with 2.5, 5, 10 or 20 μM CEP or 0.2% DMSO for 24 hours. COLO 205 cells were treated with 10, 20 or 40 μM CEP or 0.2% DMSO for 12 or 24 hours. At the end of the treatment, floating cells were collected by centrifugation at 1500 rpm for 5 min while attached cells were washed with PBS, harvested by trypsinization and centrifuged at 1500 rpm for 5 min. The cell pellets were then combined, washed twice with cold PBS and re-suspended with 500 μl of assay buffer. The cells were then stained with 1 μl Annexin V FIT-C (Invitrogen, USA) and 1 μl of 0.05 μg/ml PI (Santa Cruz Biotechnology, USA) for 15 min at room temperature in dark. Four populations of stained cells, including viable cells (Annexin V^- , PI^-), early apoptotic cells (Annexin V^+, PI^-), late apoptotic cells

(Annexin V^+, PI^+) and necrotic cells (Annexin V^-, PI^+), can be distinguished using a fluorescence flow cytometer (BD LSR II, Biosciences), which are located in the lower left, lower right, upper right, and upper left quadrants of the cytograms, respectively.

3.4.5 Analysis of the cell cycle by flow cytometry

To measure DNA content, propidium iodide (PI), an intercalating fluorescent dye which can bind directly to DNA and double-stranded RNA is used. Therefore, RNase, an endoribonuclease, is added to remove double-stranded RNA in the samples. HT-29 and COLO 205 cells were seeded in a 6-well plate at a density of $3x10^5$ cells/well and incubated overnight. HT-29 cells were then treated with CEP at concentrations of 2.5, 5, 10 or 20 µM for additional 12, 24 or 36 hours while COLO 205 cells were also treated with CEP at concentrations of 10, 20 or 40 µM for 12, 24 or 36 hours. The 0.2% DMSO in medium was used as a vehicle control. Subsequently, the cells were washed with PBS harvested by trypsinization, and centrifuged at 1500 rpm for 5 min. The cell pellets were washed twice with cold PBS, and fixed in 70% ethanol for 20 min at -20 °C. After fixation, cells were washed twice with cold PBS, re-suspended with 500 µl of assay buffer and incubated with 5 µl of 4 mg/ml RNase A for 30 min at room temperature. The cells were then stained with 5 µl of 0.05 µg/ml PI for 30 min at room temperature in dark. The DNA content of 1×10^4 stained cells was evaluated using the BD LSR II flow cytometer. The cells with diploid DNA content (2n, G0–G1 phase), synthesizing DNA (>2n but<4n, S phase), and duplicated DNA (4n, G2/M phase) were analyzed using FCS Express 5 Image Cytometry software (De Novo Software, CA).

3.4.6 Evaluation of Bcl-2 family gene expression analysis using quantitative real-time RT-PCR

The effects of CEP on the mRNA expression levels of Bcl-2 protein family including, pro-apoptosis (*BAX, BAK*) and anti-apoptosis (*Bcl-2, Bcl-xl, Mcl-1*) were

analyzed by real-time RT-PCR using SYBR Green as a probe. The SYBR Green, a fluorescent dye specifically bind to the double-stranded DNA of the PCR products, will emit light upon excitation. The intensity of the fluorescence increases as the PCR products accumulate. HT-29 and COLO 205 cells were seeded in a 6-well plate at a density of 5 $\times 10^5$ cells/well and incubated overnight. The culture medium was then replaced with fresh complete medium containing 2.5, 5, 10 or 20 μM CEP (for HT-29 cells) and incubated for 6 or 16 hours. COLO 205 cells were treated with 10, 20 or 40μM CEP and incubated for 6 hours. At the end of the treatment, the total RNA of attached and floating cells was isolated using TRIzol (Invitrogen, USA) and then reversely transcribed using Improm-II™ Reverse Transcription system (Promega, USA) according to the manufacturer's instructions. Amplification of target genes was carried out using SYBR Green qPCR super mix universal (Invitrogen, USA) with the specific primers listed in Table 1. *GAPDH* was used as an internal control. Real-time reactions were run on StepOnePlus™ Real-Time PCR (Thermo Fisher Scientific, USA) with the following cycling conditions: 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 30s. The fold change in gene expression after CEP treatment normalized to *GAPDH* and relative to the expression in vehicle treatment was calculated using the $2^{-\Delta\Delta CT}$ method.

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Table I. Sequences of primers used for quantitative real-time RT-PCR

Target Gene	Primer sequences
GAPDH	Forward: 5'-AAGGTCGGAGTCAACGGATTTGGT-3' Reverse: 5'-ATGGCATGGACTGTGGTCATGAGT-3'
BAX	Forward: 5'-GACGAACTGGACAGTAACATG-3' Reverse: 5'-AGGAAGTCCAATGTCCAGCC-3'
BAK	Forward: 5'-AGCTGCCATGGTAATCTAACTCA-3' Reverse: 5'-GATGTGGAGCGAAGGTCACT-3'
Bcl-2	Forward: 5'-TCATGTGTGTGGAGAGCGTCAA-3' Reverse: 5'-CTACTGCTTTAGTGAACCTTTTGC-3'
Bcl-xl	Forward: 5'-TTGGACAATGGACTGGTTGA-3' Reverse: 5'-GTAGAGTGGATGGTCAGTG-3'
Mcl-1	Forward: 5'-TGCTGGAGTAGGAGCTGGTT-3' Reverse: 5'-CCTCTTGCCACTTGCTTTTC-3'

3.4.7 Analysis of COX-2 gene expression using quantitative real-time RT-PCR

The effects of CEP on the *COX-2* gene expression was determined in HT-29 cells. The cells were seeded in a 6-well plate at a density of 5 $\times 10^5$ cells/well and incubated overnight. The culture medium was then replaced with fresh complete medium containing 2.5, 5, 10 or 20 µM CEP and further incubated for 6 hours. The amount of *COX-2* mRNA after treatment was then assessed by quantitative real-time RT-PCR analysis as mentioned above using the following primers: 5'-CCCTGAGCATCTACGGTTTG-3' (forward) and 5'-TCGCATACTCTGTTGTGTTCC-3' (reverse). *GAPDH* was used as an internal control.

3.4.8 Quantitative determination of PGE_2 using enzyme linked immunoassay (ELISA kit)

In addition to COX-2 gene expression, the effects of CEP on PGE_2 level, a product of COX-2 reaction, were also evaluated. HT-29 cells were seeded in a 24-well plate at a density of 3 $\times 10^5$ cells/well and incubated overnight. The culture medium

was then replaced with fresh complete medium containing 2.5, 5, 10 or 20 μM CEP, 10 μM of celecoxib (positive control) or 0.2% DMSO (vehicle control) and further incubated for 6, 12 and 18 hours. At the end of the treatment, the cell-free supernatants were collected and stored at -20 °C until PGE_2 analysis. The PGE_2 concentration in the culture medium was determined by Prostaglandin E2 competitive ELISA kit (Thermo, USA) according to the manufacturer's instructions. The colorimetric was quantified by measuring the absorbance at 405 and 570 nm using a microplate reader and the concentration of PGE_2 was calculated using standard dilutions.

3.4.9 Determination of intracellular ROS using DCFH-DA assay

Dichloro-dihydro-fluorescein diacetate (DCFH-DA), a hydrophobic compound, can diffuse to the cells and be hydrolyzed by esterase to 2', 7'-dichlorodihydrofluorescein (DCFH) which can further be oxidized by intracellular reactive oxygen species (ROS) to 2', 7'- dichlorofluorescein, the highly fluorescent product. The fluorescence intensity indicated the ROS level in the cell. Briefly, HT-29 and COLO 205 cells were seeded in a 96-well plate at a density of $5x10^3$ cells/well and incubated overnight. Both cells were incubated with 100 μl of 10 μM DCFH-DA in Hank's buffered salt solution (HBSS) at 37°C for 30 min in dark. After incubation, the cells were removed and wash with PBS. The HT-29 cells were then exposed with 2.5, 5, 10 and 20 μM CEP whereas COLO 205 cells were treated with 10, 20 and 40 μM CEP for 1 h. At the end of treatment, the cells were washed twice with cold PBS and 200 μl of 1% triton-X in 0.3 M NaOH was added. The fluorescence intensity was measured by microplate reader (Thermo, Finland) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The fluorescence intensity in each groups were expressed in the percentage of control as calculated from following equation: % control = Fsample / Fcontrol × 100

3.4.10 Statistical analysis

All data are presented as mean ± standard error of mean (SEM) from at least three independent experiments performed in duplicate or triplicate. Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by LSD post hoc test and student's *t*-test were used to compare significant difference between two groups using SPSS statistics 21 software (IBM Corporation, USA). The difference is considered significant if $P < 0.05$.

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CHAPTER IV
RESULTS

4.1 Effect of cepharanthine (CEP) on cell viability of COLO 205, COX-2 negative human colorectal cancer cells

To determine the anticancer effect of CEP on human colorectal cancer cells, I first evaluated the cytotoxic effects of CEP on COX-2 negative human colorectal cancer cells, COLO 205 using resazurin assay. The cells were exposed to 0.01-100 μM of CEP for 24, 48 and 72 h. As shown in Figure 5A, 50 and 100 μM of CEP exhibited potent toxicity against COLO 205 cells at all-time points. In order to determine the value of half inhibitory concentration (IC_{50}), the cytotoxicity of CEP at a concentration of 0.1, 1, 5, 10, 20, 30 and 40 μM was determined. At 48 h after incubation, CEP significantly inhibited cell growth in a dose dependent manner (Figure 5B, $P<0.001$). The percentages of cell viability were approximately 90, 75, 60 and 10 of control group after treatment with 10, 20, 30 and 40 of CEP, respectively. The IC_{50} of this compound in COLO 205 was about 32.13±0.515 μM. Thus, three concentrations of CEP at 10, 20 and 40 μM were chosen for the rest of the experiments.

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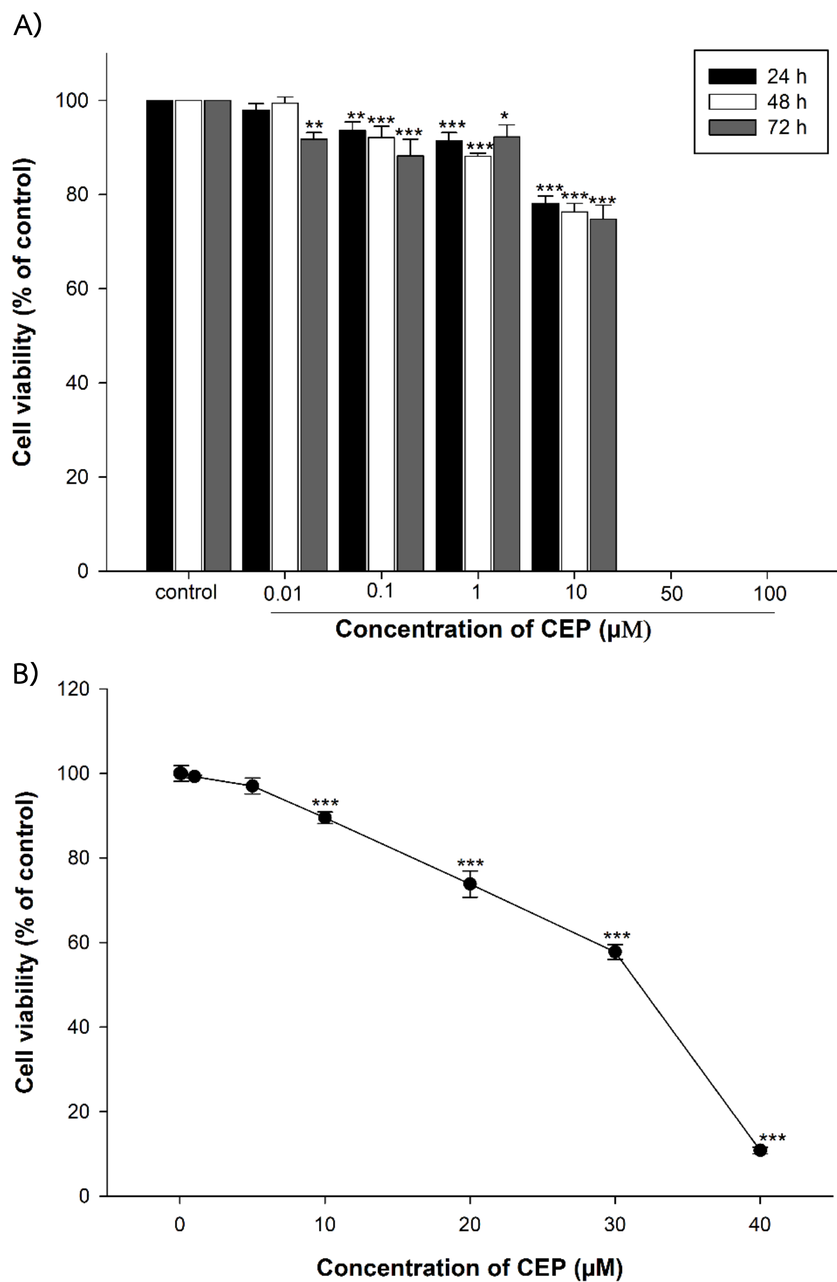

Figure 5. Effect of CEP on viability of COLO 205, COX-2 negative human colorectal cancer cells. A) The cells were treated with 0.01, 0.1, 1, 10, 50 and 100 μM for 24, 48 and 72 h. B) The cells were treated with 0.1, 1, 5, 10, 20, 30 and 40 μM for 48 h. Cell viability was determined using resazurin assay. Each value is expressed as the mean ± SEM. (n=3). *P< 0.05, **P< 0.01, ***P< 0.001 with respect to vehicle control (0.2% DMSO). Data are representative of three independent experiments.

4.2 Effect of CEP on apoptotic cell death of COLO205 cells

It is commonly known that chemotherapeutic agents induce cell death mainly through apoptotic mechanisms. Thus, the apoptotic induction effect of CEP on COLO 205 cells was explored using Annexin V-FITC/PI staining followed by flow cytometry analysis. Cells were then categorized into 3 groups, including living cells (Annexin V^-/ PI^-), early apoptotic cells (Annexin V^+/PI^-), and late apoptotic or necrotic cells (Annexin V^+/PI^+ or Annexin V^-/PI^+). As illustrated in Figure 6, CEP could significantly induce apoptotic cell death in a concentration and time-dependent manner. Treatment of COLO 205 cells with CEP at a concentration of 20 μM could significantly induce cells to undergo apoptosis by approximately 7.3% and 20.6% at 12 and 24 h, respectively. It however should be noted that exposure of CEP at a higher concentration stimulated COLO 205 cells to undergo late apoptosis rather than early apoptosis. CEP at 40 μM significantly caused 7.7% early apoptotic and 27.3% late apoptosis cell death at 12 h after incubation (Figure 6A, $P<0.05$). Similarly, exposure of the cells with CEP at the same concentration for 24 h resulted in approximately 90% cell death (Figure 6B), which is primarily late apoptosis [9.4% (early apoptotic cells) vs. 41.8% (late apoptotic cells)].

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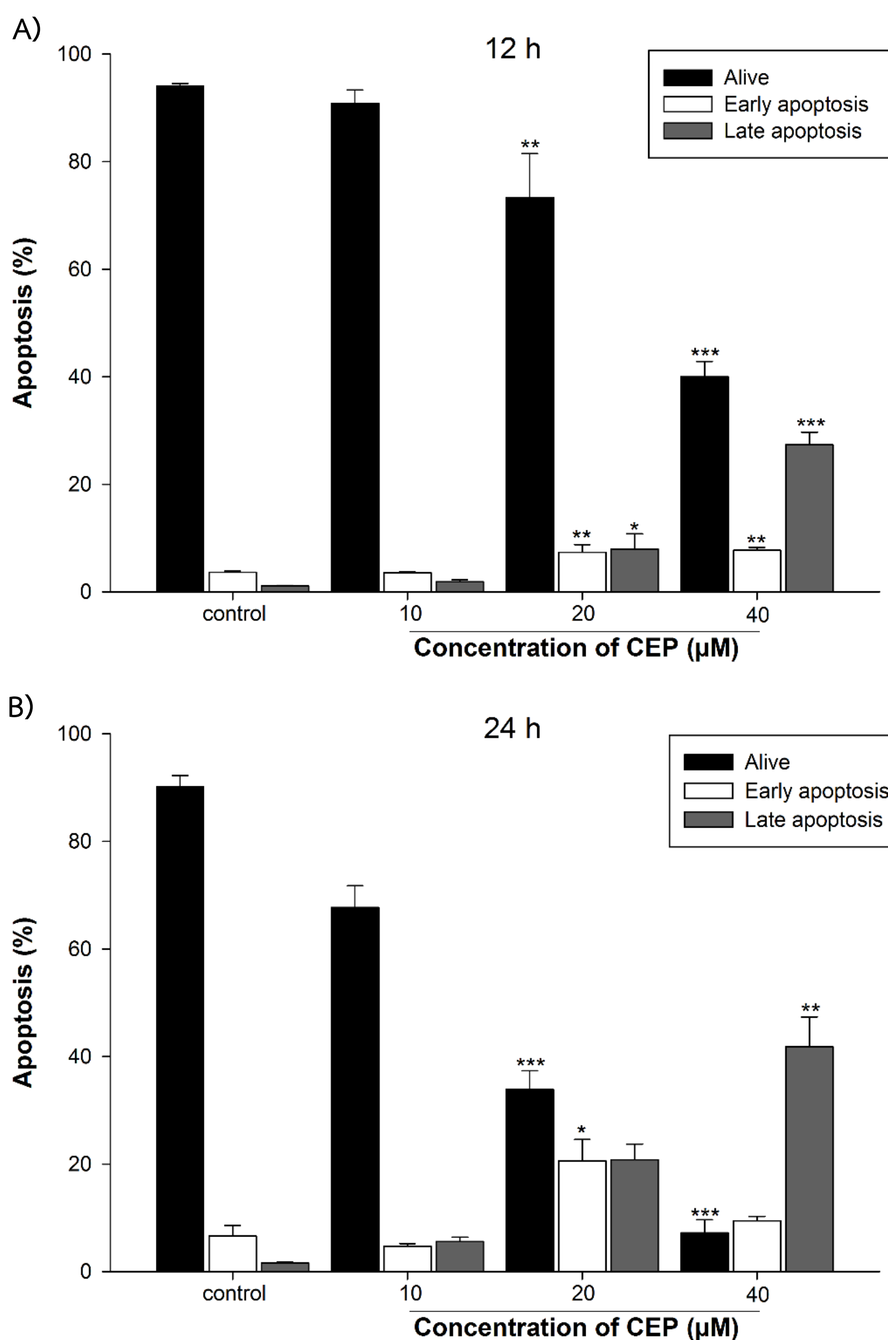

Figure 6. Effect of CEP on apoptotic cell death of COLO205 cells. The cells were exposed with 10, 20 and 40 µM CEP for 12 and 24 h and examined by flow cytometry after Annexin V-FITC/PI double staining. The percentages of different COLO 205 cell populations (alive, early and late apoptotic cells) after treatment with CEP for 12 (A) and 24 h (B) were plotted. Each value is expressed as mean ± SEM. (n=3). *P<0.05, **P<0.01, *** P<0.001 compared to the vehicle control (0.2% DMSO)

4.3 Effect of CEP on the expression of Bcl-2 family genes in COLO 205 cells

To evaluate the mechanism by which CEP induce apoptosis of COLO 205 cells, the mRNA levels of Bcl-2 family members, including anti-apoptotic genes (*Bcl-2, Bcl-xl* and *Mcl-1*) and pro-apoptotic genes (*BAX* and *BAK*) were quantified using real-time RT-PCR analysis. As shown in Figure 7, CEP significantly down-regulated 2 out of 3 tested anti-apoptotic genes. Treatment of CEP for 6 h significantly decreased *Bcl-2* mRNA levels in a concentration dependent manner (Figure 7A, $P<0.001$). The expression of *Bcl-2* was approximately 40, 30 and 20% of baseline level following incubation of CEP at 10, 20 and 40 μM, respectively. CEP at 20 and 40 μM also significantly down-regulated *Mcl-1* mRNA expression levels (Figure 7B, $P<0.05$). This compound, however, did not significantly alter the expression of *Bcl-xl* (Figure 7C). Similar to *Bcl-xl*, the expression of both tested pro-apoptotic genes, *BAX* and *BAK*, was significantly unaffected following CEP treatment (Figure 8A and B). These results suggest that CEP could induce COLO 205 cells to undergo apoptosis by down-regulating the expression of anti-apoptotic*, Bcl-2* and *Mcl-1* genes.

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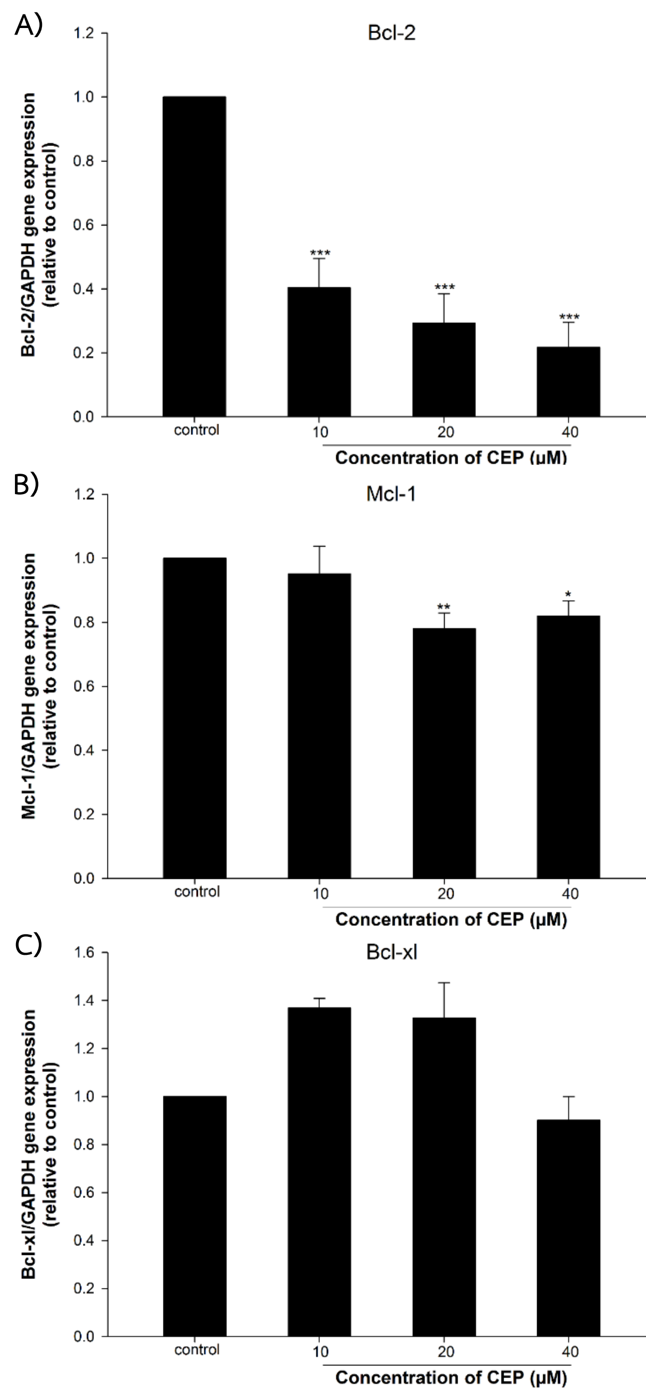

Figure 7. Effect of CEP on the expression of anti-apoptotic genes *Bcl-2, Mcl-1* and *Bcl-xl* in COLO 205 cells. The cells were treated with CEP 10, 20 and 40 μM for 6 h and the mRNA expression levels of *Bcl-2* (A), *Mcl-1* (B) and *Bcl-xl* (C) were analyzed by quantitative real-time RT-PCR. The values were normalized to *GAPDH* (the endogenous control) and shown as fold changes relative to vehicle control cells. Data are means ± SEM of results from at least three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared to the vehicle control (0.2% DMSO)

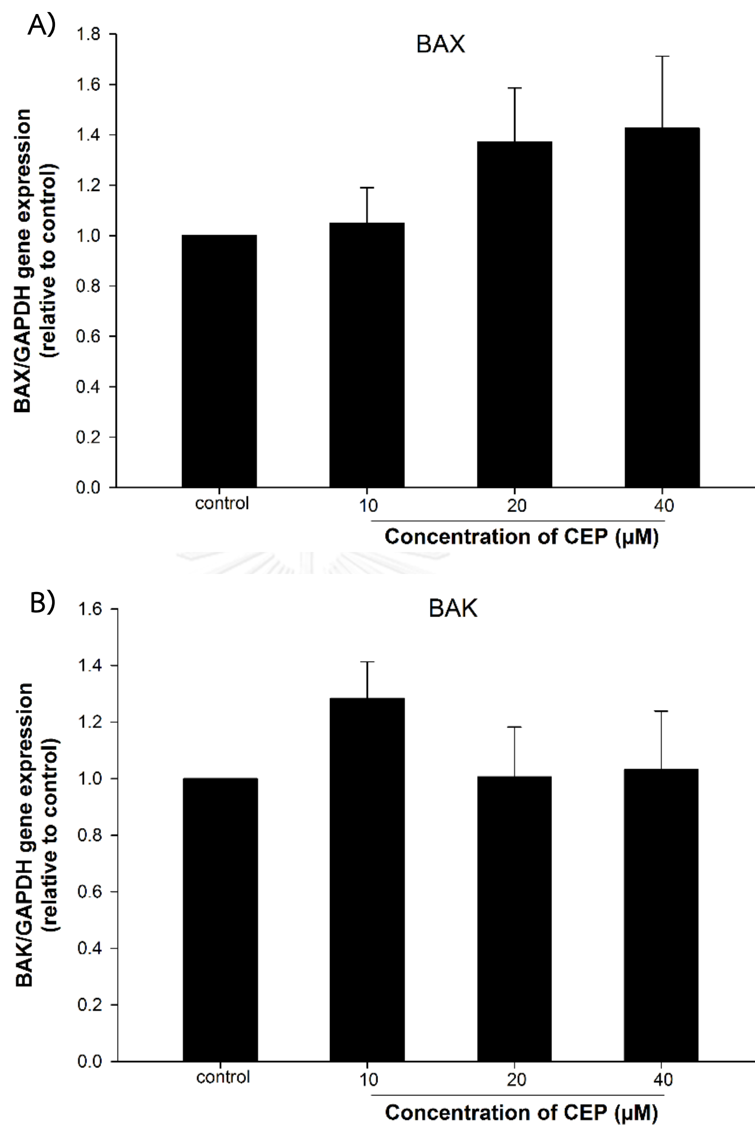

Figure 8. Effect of CEP on the expression of pro-apoptotic genes *BAX* and *BAK* in COLO 205 cells. The cells were treated with CEP 10, 20 and 40 µM for 6 h and the mRNA expression levels of *BAX* (A) and *BAK* (B) were analyzed by quantitative real-time RT-PCR. The values were normalized to *GAPDH* (the endogenous control) and shown as fold changes relative to vehicle control cells. Data are means ± SEM of results from at least three independent experiments.

4.4 Effect of CEP on cell cycle progression of COLO 205 cells

In addition to apoptosis induction, previous studies reported that CEP could induce cell cycle arrest of myeloma and osteosarcoma cells (22, 105). This study therefore determined the effect of CEP on cell cycle progression of COLO 205 cells using propidium iodide (PI) staining followed by flow cytometry analysis. At 12 h of incubation, CEP at 10 μM significantly induced accumulation of cells at G1/S phase whereas CEP at 20 μM resulted in an increase of cells in the G1 phase only [47.3 % (control) vs. 60.9% (CEP)] (Figure 9A). CEP at 40 μM, however, caused sub G1 cell cycle arrest along with a concomitant decrease of cell number in G1, S, and G2/M phase at all-time points ($P<0.01$). Interestingly, treatment of CEP at a 20 μM for longer incubation times, 24 and 36 h, also led to significant accumulation of cells at sub-G1 phase (Figure 9B and C, $P<0.01$). On the other hand, 10 μM CEP did not affect the cell numbers in the sub-G1 phase at any time.

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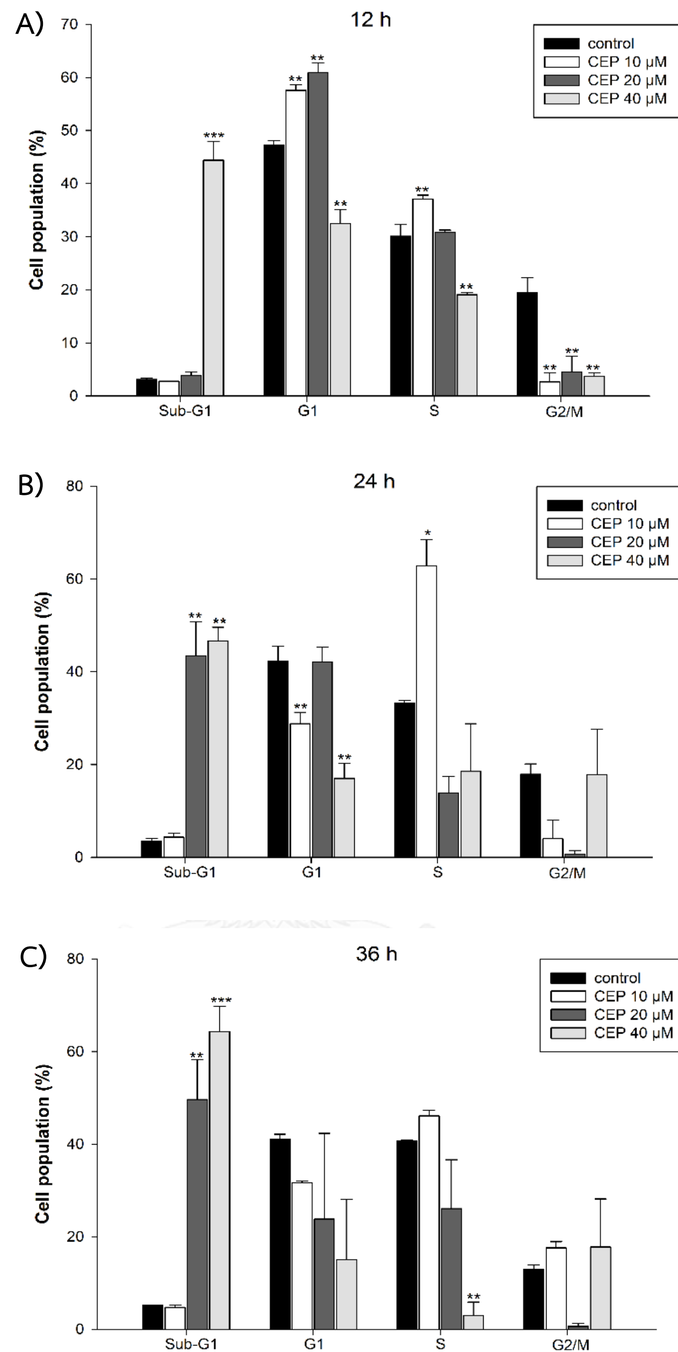

Figure 9. Effect of CEP on cell cycle progression of COLO 205 cells. The cells were exposed with CEP 10, 20 and 40 μM for 12 (A), 24 (B) and 36 (C) h. Cell cycle progression was performed by fixing and staining the cells with PI followed by fluorescence flow cytometry. The percentage of cells in sub-G1, G1, S and G2/M phases are given as means ± SEM. (n=2) **P*<0.05, ***P*<0.01, ****P*<0.001 compared to the vehicle control (0.2% DMSO)

4.5 Effect of CEP on ROS production in COLO 205 cells

High levels of reactive oxygen species (ROS) are normally toxic to cells. To investigate the effect of CEP on intracellular ROS of COLO 205, the cells were treated with CEP at 10, 20 and 40 µM or 200 µM of H_2O_2 (positive control) for 1 h and the levels of ROS were analyzed by measuring the DCF fluorescence intensity. As illustrated in Figure 10A, CEP at 20 and 40 µM and 200 µM of H_2O_2 significantly increased the level of ROS from 100% to approximately 120%, 130% and 175%, respectively. To further determine whether CEP induced colorectal cancer cell death through increased ROS production, we pre-treated cells with N-acetylcysteine (NAC, a ROS scavenger) for 2 h and then incubated with CEP for additional 24 h. The cytoxicity was evaluated using resazurin assay. Although NAC did not affect the viability of COLO 205 cells (data not shown), cell death was significantly attenuated by pretreatment with 5 mM NAC (Figure 10B). NAC could significantly prevent cell death following exposure of CEP at 10, 20 and 40 µM by 1.2, 1.4 and 8 folds, respectively. Taken together, these results suggest that CEP-induced cell death in COLO 205 cells is partly mediated through increased ROS production.

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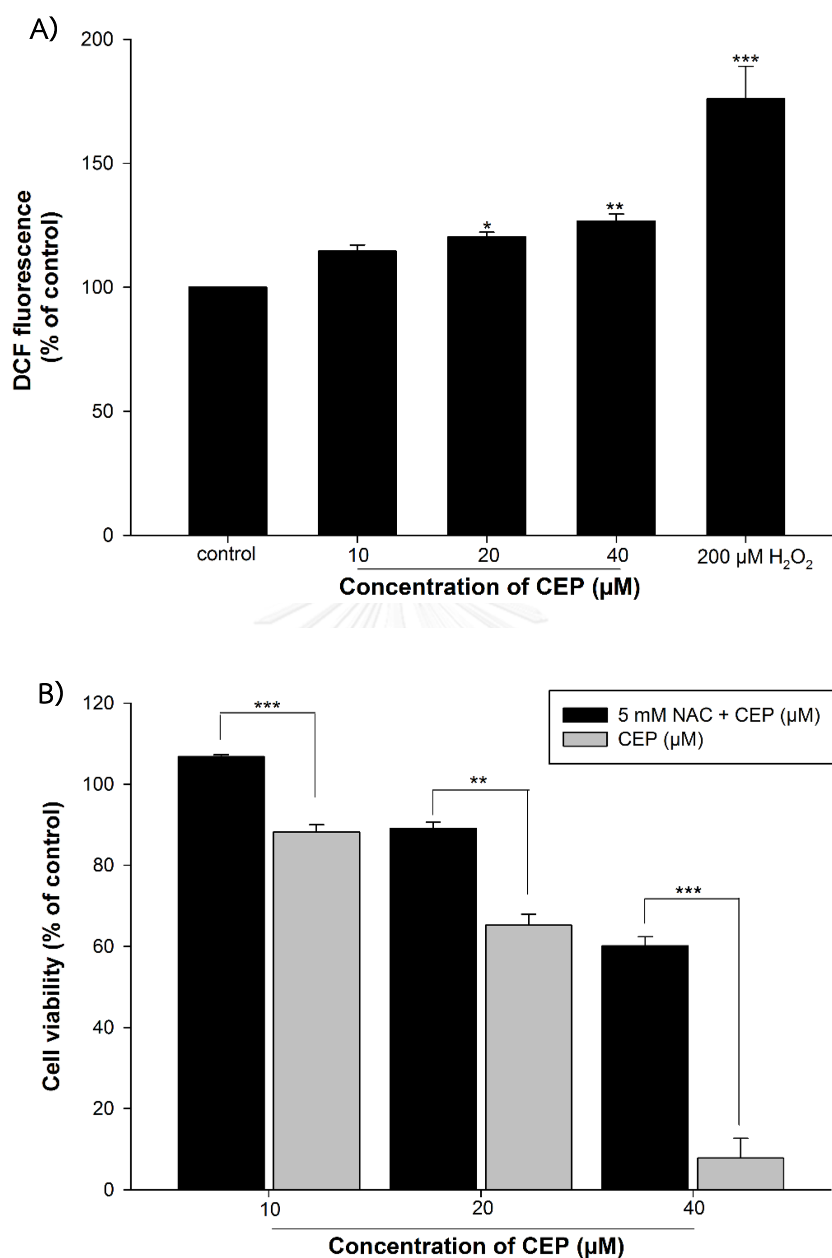

Figure 10. Effect of CEP on ROS production in COLO 205 cells. A) The cells were treated with CEP 10, 20 and 40 µM or 200 µM of H_2O_2 (positive control) for 1 h and ROS production was determined by measuring DCF fluorescence intensity using microplate reader. Data are the mean ± SEM. (n=3) **P*<0.05, ***P*<0.01, ****P*<0.001 compared to the vehicle control (0.2% DMSO). B) The cells were treated with CEP for 24 h, in the presence or absence of 5 mM NAC and the percentages of cell viability were measured by resazurin assay. Data are the mean ± SEM. (n=3) ***P*<0.01 and ****P*<0.001 with respect to CEP-treated cells.

4.6 Effect of CEP on cell viability of HT-29, COX-2 positive human colorectal cancer cells

Cyclooxygenase-2 (COX-2) was found to promote colorectal tumor development by inducing angiogenesis, cell proliferation and apoptosis evasion (8). Previous studies also showed that HT-29 colon cancer cells overexpressing COX-2 were resistant to apoptotic cell death (9), which is the major mechanism of action of most chemotherapeutic drugs. The present study therefore determined the anticancer activity of CEP against a COX-2 positive human colorectal cancer cell line, HT-29 using resazurin assay. As shown in Figure 11A, CEP at 1-100 μM significantly inhibited cell growth in a concentration and times-dependent manner. To determine the IC_{50} for HT-29 cells, cytotoxicity of CEP at concentrations of 0.1-40 μM was evaluated. At 48 h of incubation, 5-40 μM significantly inhibited the growth of HT-29 cells with respect to vehicle control (Figure 11B, $P < 0.001$). The IC_{50} value of CEP was 6.21± 1.68 μM, suggesting that CEP possesses anticancer activity against COX-2-positive human colon cancer cells. Therefore, the concentrations of CEP at 2.5, 5, 10 and 20 μM were chosen for the rest of the experiments.

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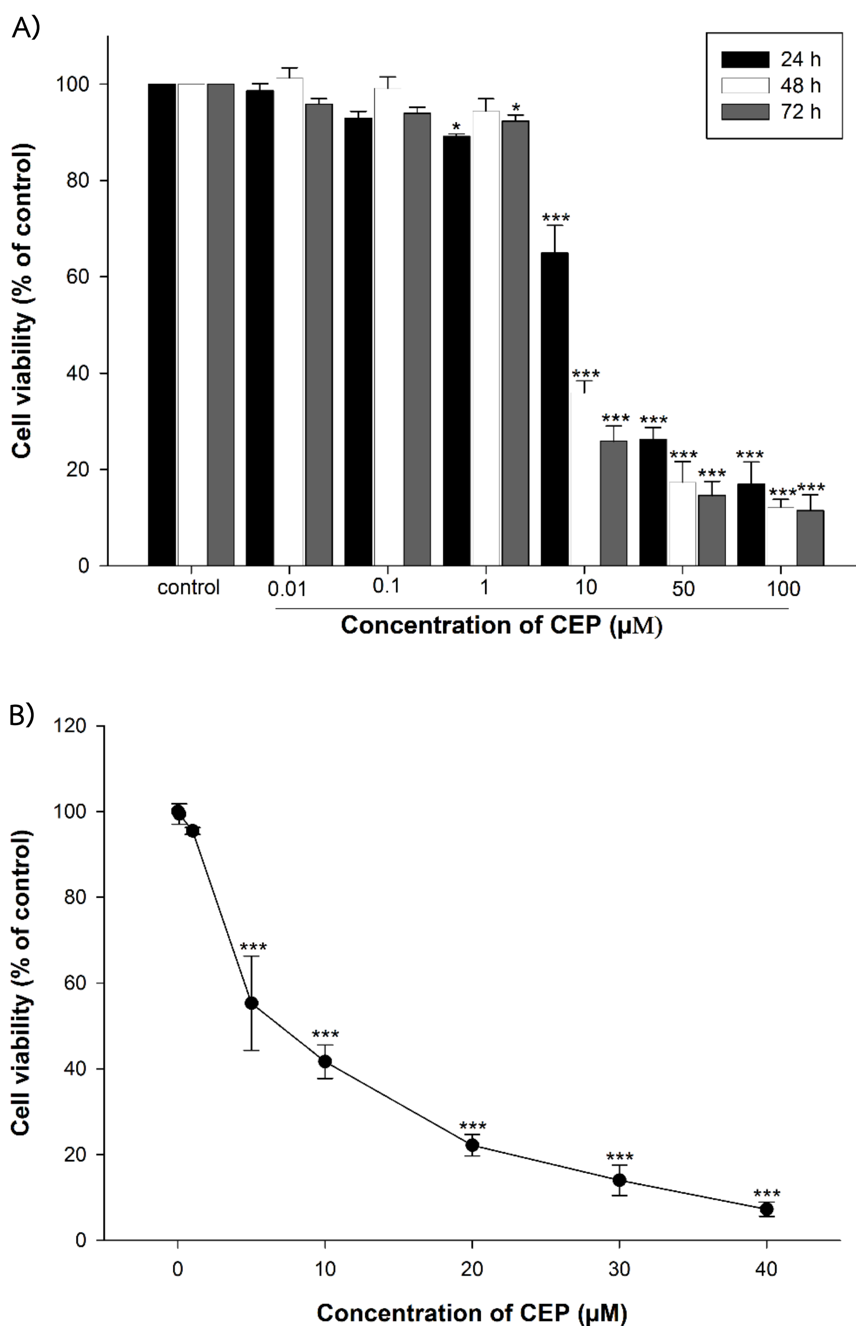
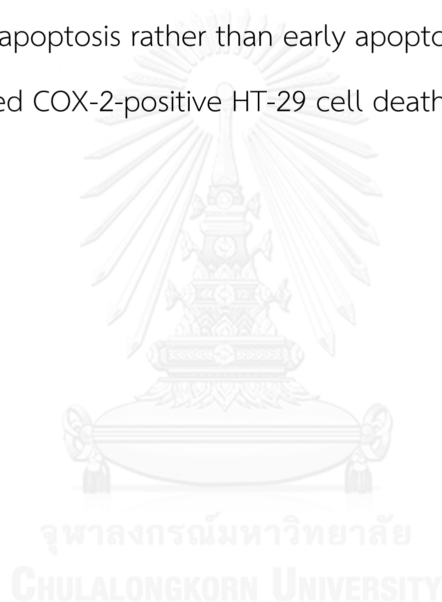


Figure 11. Effect of CEP on cell viability of HT-29, COX-2 positive human colorectal cancer cells. A) The cells were treated with 0.01, 0.1, 1, 10, 50 and 100 μM for 24, 48 and 72 h (A) or 0.1, 1, 5, 10, 20, 30 and 40 μM for 48 h (B). Cell viability was determined using resazurin assay. Each value is expressed as the mean ± SEM. (n=3). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to vehicle control (0.2% DMSO). Data are representative of three independent experiments.

4.7 Effect of CEP on apoptotic cell death of HT-29 cells

I then determined whether CEP can induce apoptotic cell death in COX-2 positive human colorectal cancer cells using Annexin V-FITC and PI double staining followed by flow cytometry analysis. At 24 h of incubation, CEP at 2.5, 5, 10 or 20 μM could significantly induce apoptotic cell death in a concentration-dependent manner (Figure 12). The number of viable cells was decreased 3 times compared to the vehicle along with the increase in apoptotic cells following treatment with CEP at 20 μM. It however should be noted that exposure of HT-29 cells with CEP for 24 h could induce cells to undergo late apoptosis rather than early apoptosis. These results suggest that CEP effectively induced COX-2-positive HT-29 cell death via apoptosis induction.



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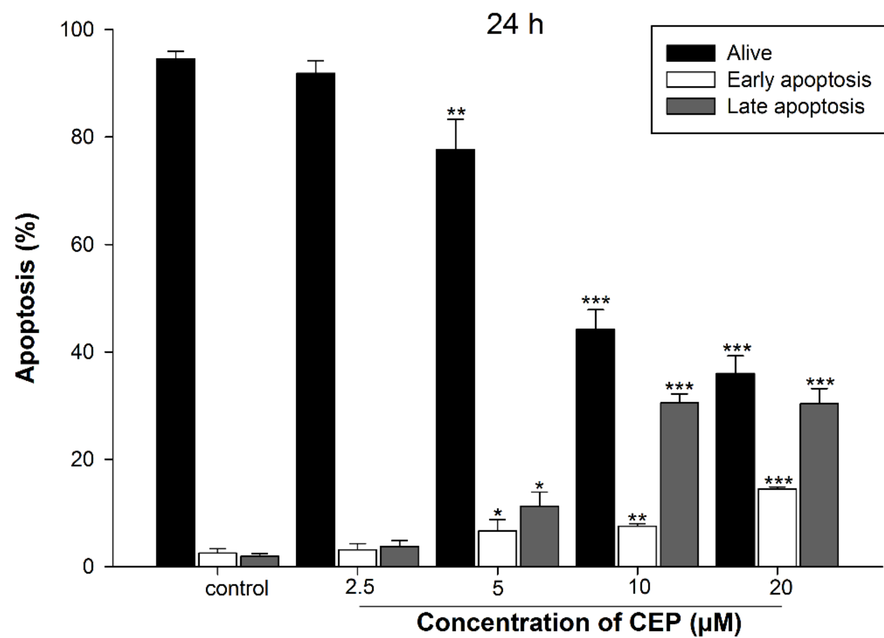

Figure 12. Effect of CEP on apoptotic cell death of HT-29 cells. Cells were incubated with CEP at 2.5, 5, 10 and 20 µM or vehicle control for 24 h. Apoptosis was assessed by Annexin V-FITC and PI staining follow by flow cytometry analysis. Cells were then categorized into three groups, including alive, early and late apoptotic cells. Each value is expressed as the mean ± SEM. (n=3). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to vehicle control (0.2% DMSO).

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4.8 Effect of CEP on the expression of Bcl-2 family genes in HT-29 cells

The gene expression of anti-apoptosis (*Bcl-2, Bcl-xl* and *Mcl-1*) and pro-apoptosis (*BAX* and *BAK*) were assessed to determine underlying mechanism of apoptotic induction effect CEP on HT-29 cells. As shown in Figure 13, treatment of the cells with CEP for 6 h did not significantly alter the expression of both anti- and pro-apoptotic genes. However, at 16 h of incubation. CEP at 10 and 20 μM significantly decreased the expression of anti-apoptosis *Bcl-xl* gene (Figure 14B, $P<0.05$) while the expression of *Bcl-2* and *Mcl-1* genes were not different with respect to the control (Figure 14A and C). The expression of pro-apoptotic genes, *BAX* and *BAK* in the cells was also unaffected following treatment with CEP at this time point (Figure 15). These results suggest that CEP could induce apoptosis in HT-29 cells is mediated through down regulation of *Bcl-xl* gene.

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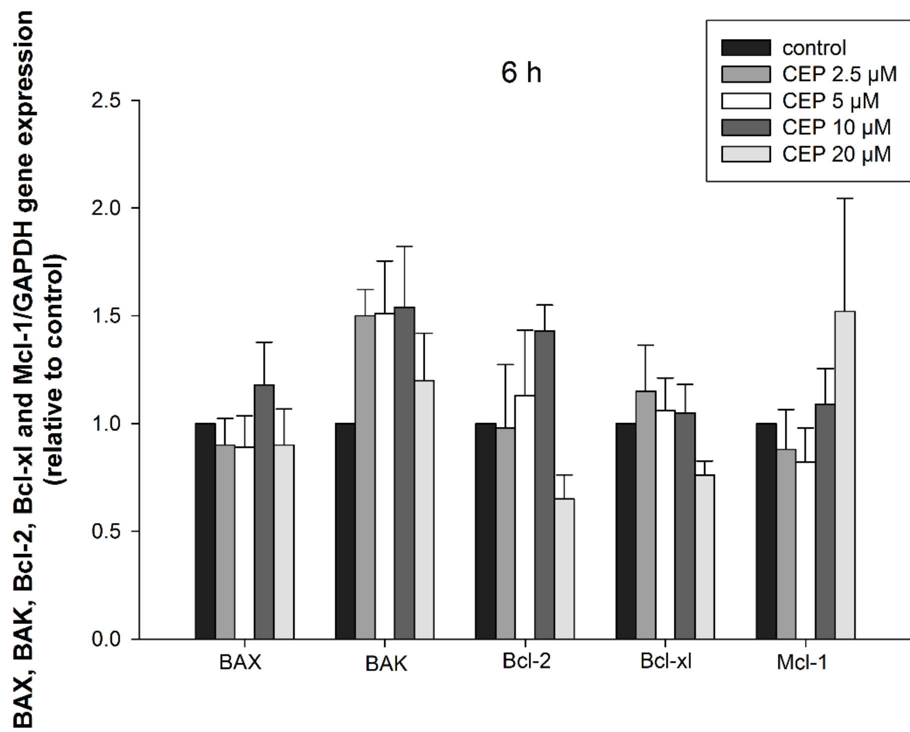

Figure 13. Effect of CEP on Bcl-2 family genes expression in HT-29 cells. The cells were treated with CEP 2.5, 5, 10 and 20 µM for 6 h and the expression of Bcl-2 family genes were analyzed by quantitative real-time RT-PCR. These data are shown as fold induction relative to control cells. Data are means ± SEM of results from three independent experiments.

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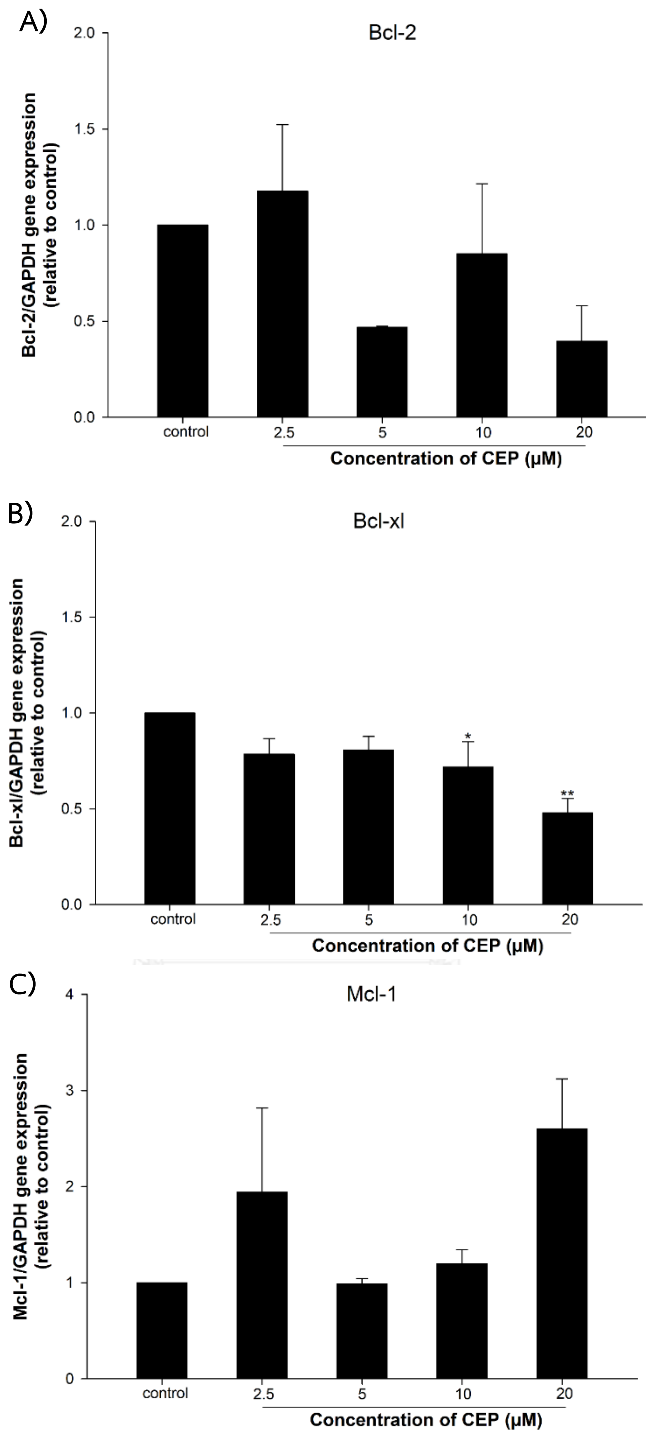


Figure 14. Effect of CEP on the expression of anti-apoptotic genes *Bcl-2*, *Bcl-xl* and *Mcl-1* in HT-29 cells. The cells were treated with CEP 2.5, 5, 10 and 20 μM for 16 h and the expression of *Bcl-2* (A), *Bcl-xl* (B), and *Mcl-1* (C) were analyzed by quantitative real-time RT-PCR. These data are shown as fold change relative to control cells. Data are means ± SEM. (n=3) **P*<0.05, ***P*<0.01 compared to the vehicle control (0.2% DMSO).

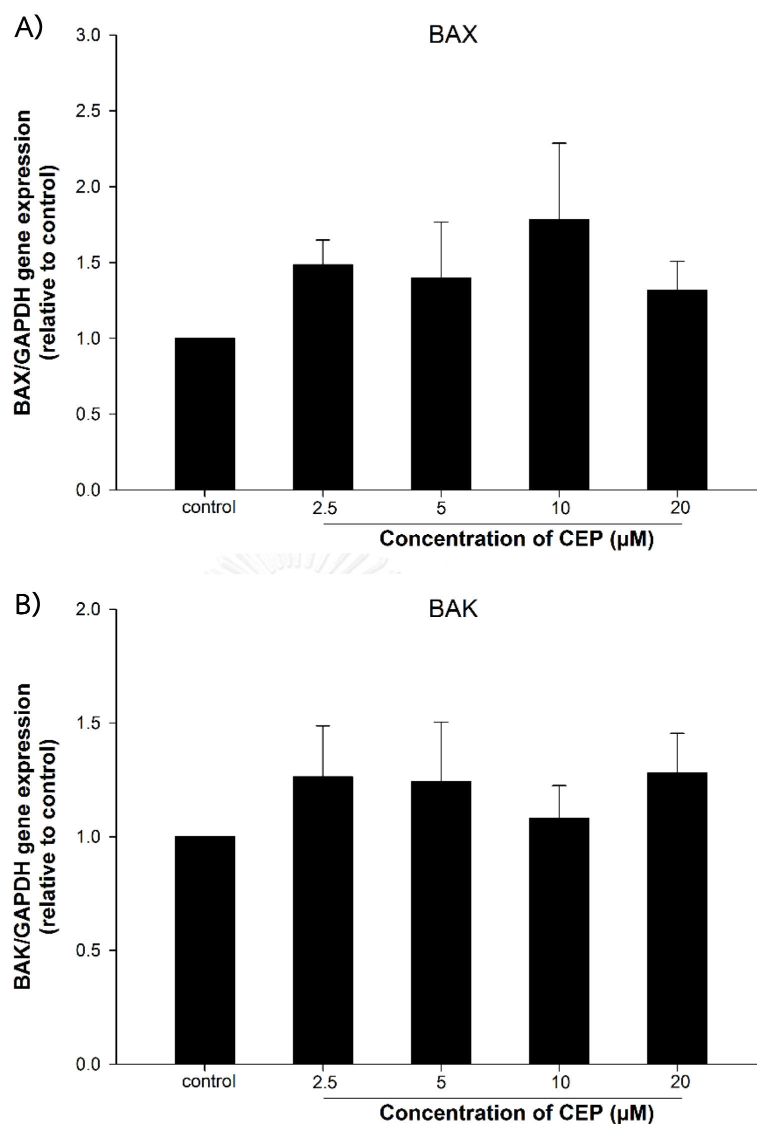
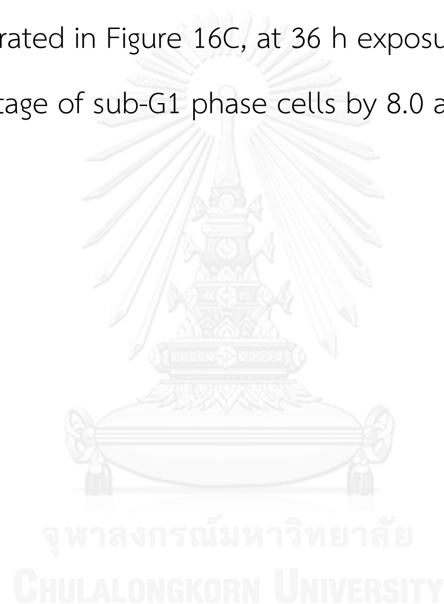


Figure 15. Effect of CEP on the expression of pro-apoptotic genes *BAX* and *BAK* in HT-29 cells. The cells were treated with CEP 2.5, 5, 10 and 20 μM for 16 h and the mRNA expression levels of *BAX* (A) and *BAK* (B) were analyzed by quantitative real-time RT-PCR. The values were normalized to *GAPDH* (the endogenous control) and shown as fold changes relative to vehicle control cells. Data are means ± SEM of results from at least three independent experiments.

4.9 Effect of CEP on cell cycle progression of HT-29 cells

This study found that the effects of CEP on cell cycle pattern of HT-29 cells were different depending on the concentrations and exposure times used. Treatment with CEP at 10 μM for 12 h could significantly induce cell accumulation in the G1 phase (39.6% for control vs. 55.4% for CEP, $P<0.01$) which was associated with a reduction of cell numbers in the S phase (Figure 16A, $P<0.01$). At 24 h of incubation, both 5 and 10 μM of CEP caused cell cycle arrest at the G1 phase whereas there was a significant decrease in numbers of cells at this phase following treatment of 20 μM CEP (Figure 16B, $P<0.01$). As illustrated in Figure 16C, at 36 h exposure time, CEP at 10 and 20 μM increased the percentage of sub-G1 phase cells by 8.0 and 20.6 folds, respectively.



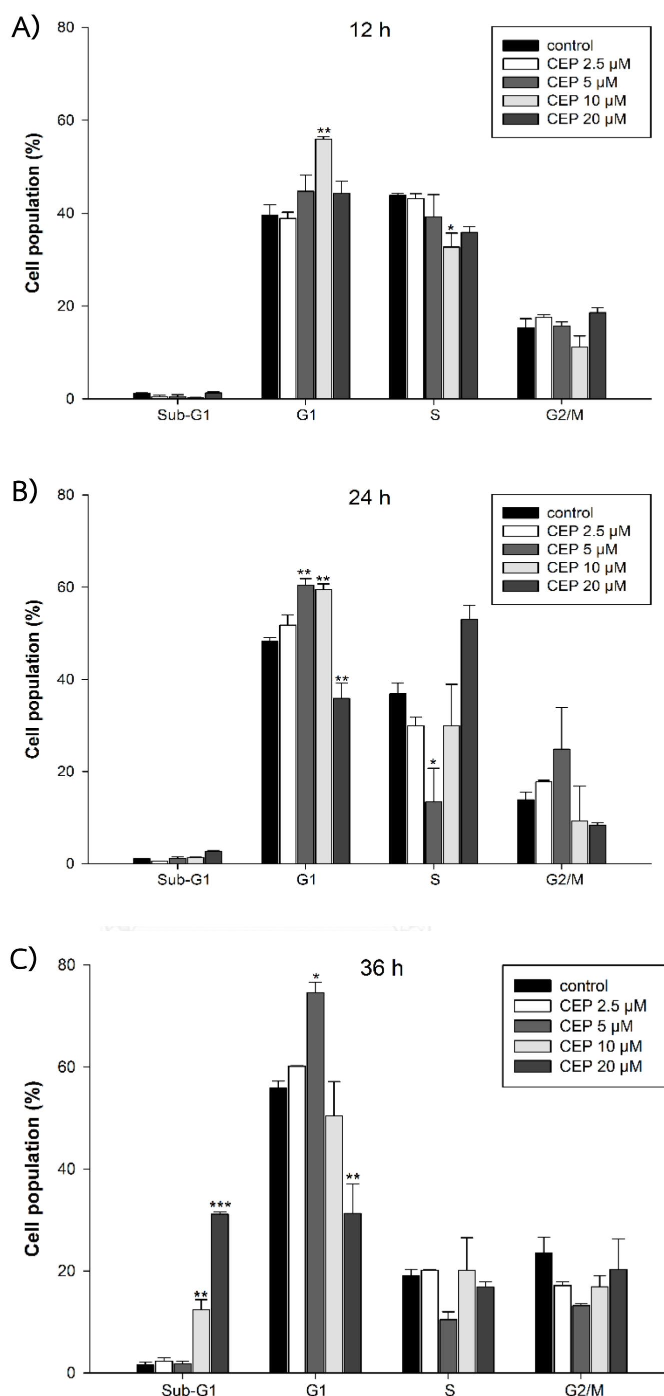
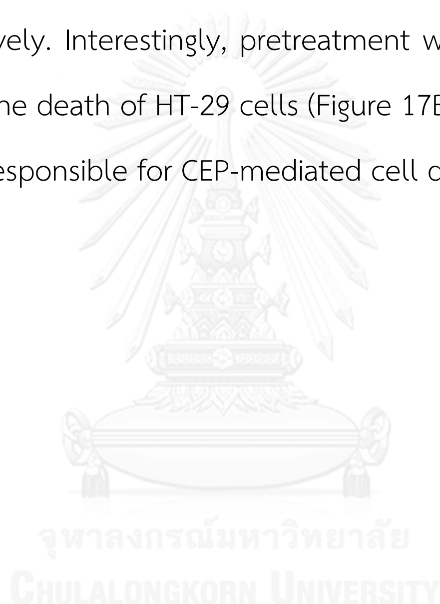


Figure 16. Effect of CEP on cell cycle progression of HT-29 cells. The cells were treated with CEP 2.5, 5, 10 and 20 μM for 12 (A), 24 (B) and 36 (C) h and analyzed using PI staining followed by flow cytometry analysis. The percentage of cells in sub-G1, G1, S and G2/M phases are given as means ± SEM. (n=2) *P<0.05, **P<0.01, ***P<0.001 compared to the vehicle control (0.2% DMSO)

4.10 Effect of CEP on ROS production in HT-29 cells

I then determined whether CEP-induced cell death is associated with ROS production in HT-29 cells. The cells were treated with CEP at various concentrations or 200 μM of H_2O_2 (positive control) for 1 h and the levels of ROS generation were analyzed by measuring the DCF fluorescence intensity. As shown in Figure 17A, treatment of HT-29 cells with CEP for 1 h significantly increased the levels of ROS compared with the control group (*P<0.05*). The levels of ROS were approximately 120%, 140% and 150% of control following exposure of 10 and 20 μM CEP and 200 μM of H_2O_2, respectively. Interestingly, pretreatment with 5 mM NAC for 2 h could significantly prevent the death of HT-29 cells (Figure 17B, *P<0.01*), suggesting that ROS production is partly responsible for CEP-mediated cell death in HT-29 cells.



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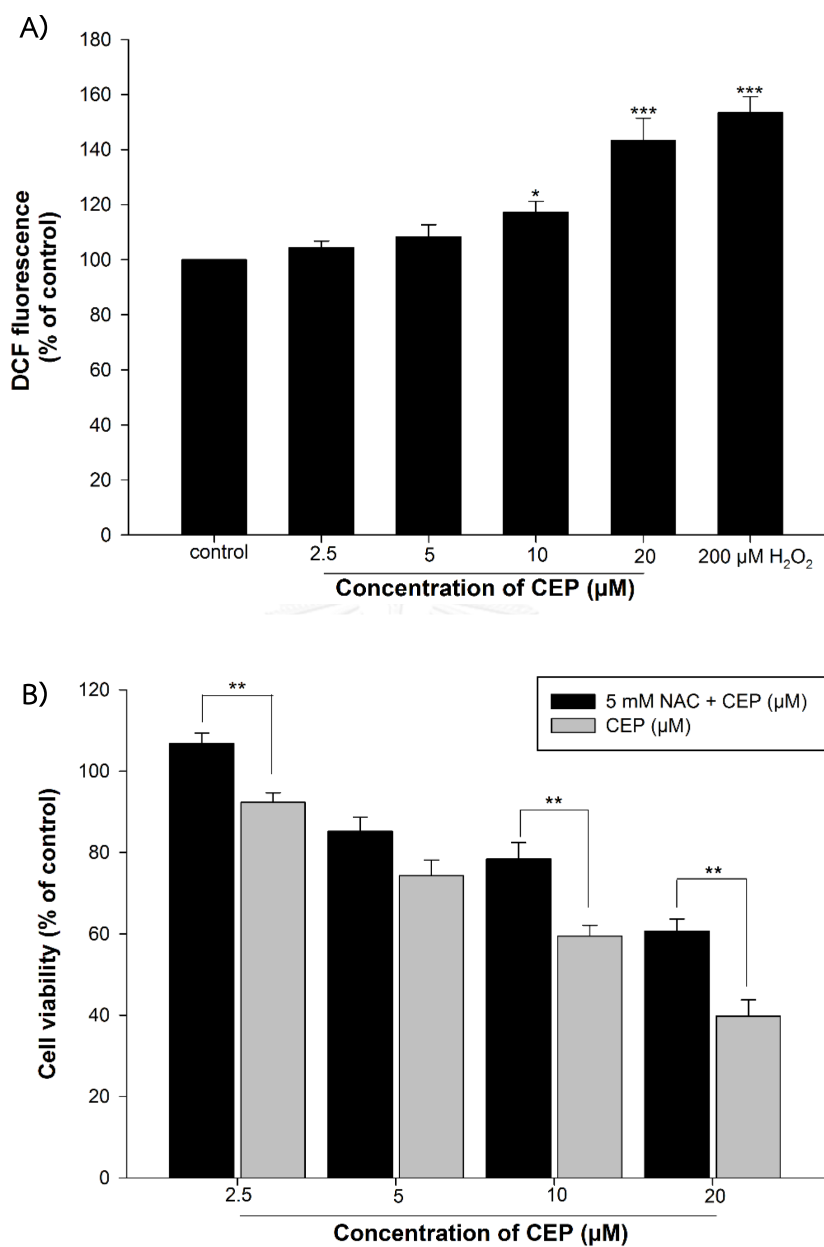

Figure 17. Effect of CEP on ROS production in HT-29 cells. A) The cell were treated with CEP 2.5, 5, 10 and 20 µM or 200 µM of H_2O_2 (positive control) for 1 h. ROS production was determined by measuring DCF fluorescence intensity using microplate reader B) The cells were treated with the concentration of CEP for 24 h, in the presence or absence of NAC, and the percentages of cell viability were measured by resazurin assay. Data are the mean ± SEM. (n=3) *$P<0.05$, **$P<0.01$, ***$P<0.001$compared to the vehicle control (0.2% DMSO)

4.11 Effect of CEP on COX-2 mRNA expression and PGE2 production in HT-29 cells

COX-2 has played an important role in inflammation-promoted cancer by inducing cell proliferation, angiogenesis and metastasis and inhibiting apoptosis (9), suggesting that agents that suppress COX-2 expression should have a potential in cancer treatment. CEP was found to exhibit anti-inflammatory activity both *in vitro* and *in vivo* (103). It has also been used to treat several inflammatory diseases such as septic shock and various cancers (106). Moreover, this alkaloid has been found to enhance radiation response by inhibiting COX-2 and STAT-3 (97). Previously, it was reported that *COX-2* mRNA level was only observed in HT-29 cells while it was undetected in COLO 205 (107). Using quantitative real-time PCR analysis, the results of present study were able to confirm their results. *COX-2* mRNA level in HT-29 cells was found to be 2000 fold higher than that in COLO 205 cells. I therefore used HT-29 cells as a model of colon cancer cells expressing COX-2 to determine the effect of CEP on *COX-2* mRNA expression in colon cancer cells. The cells were treated with 2.5, 5, 10 and 20 μM of CEP for 6 h and *COX-2* mRNA expression was assessed using quantitative real-time RT-PCR. As shown in Figure 18, treatment of HT-29 cells with 5 or 10 μM CEP significantly down-regulated COX-2 mRNA expression level ($P<0.05$). Studies have shown that PGE_2 is implicated in COX-2-mediated resistance to apoptosis of cancer cells (108). Thus, we evaluated the effect of CEP on PGE_2 production in HT-29 cells by treating cells with 2.5, 5, 10 and 20 μM of CEP or 10 μM celecoxib (positive control) for 6, 12 and 18 h and determined the PGE_2 level in the culture supernatants using Prostaglandin E2 competitive ELISA kit. As shown in Figure 19A, CEP treatment did not significantly affect the PGE_2 level with respect to the control whereas celecoxib, a selective COX-2 inhibitor, at a concentration of 10 μM significantly inhibited production of PGE_2. To assess whether PGE_2 is involved in CEP-induced cytotoxicity, I pre-treated cells with 1 μM of PGE_2 for 1 h and then incubated with CEP for additional 24 h. The

cytotoxicity was evaluated using MTT assay. Figure 19B demonstrated that the number of viable HT-29 cells following treatment with CEP alone was not significantly different from the number of living cells after exposure of HT-29 cells with CEP in the presence of PGE_2 , suggesting that growth inhibitory activity of CEP in HT-29 cells is unlikely to be mediated via PGE_2 dependent pathway.

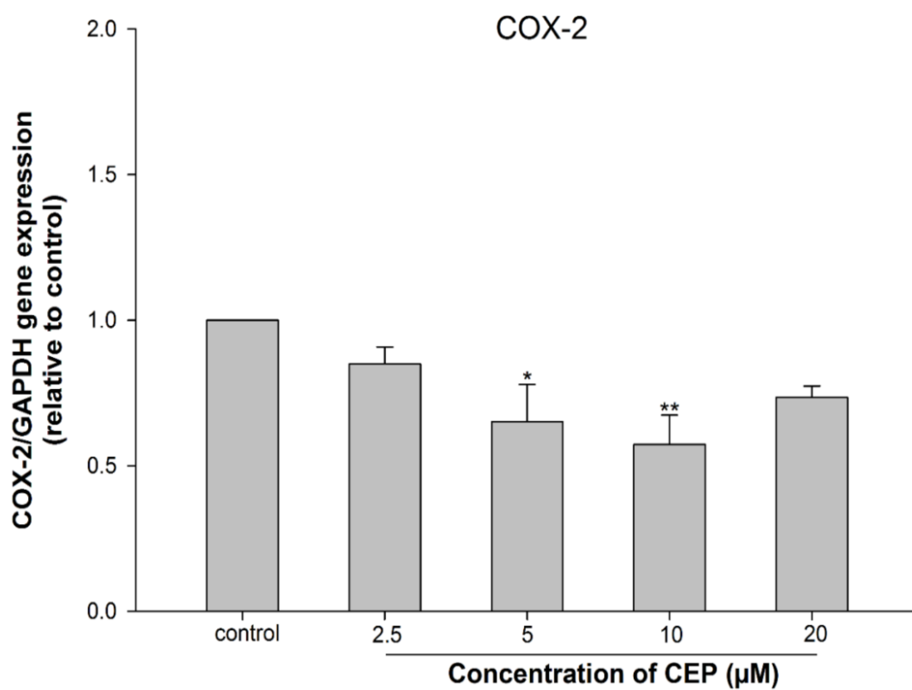

Figure 18. Effect of CEP on COX-2 mRNA expression in HT-29 cells. The cells were treated with at 2.5, 5, 10 and 20 μM of CEP for 6 h and COX-2 mRNA expression was determined using quantitative real-time RT-PCR. Data are the mean ± SEM. (n ≥ 3). *P<0.05 and **P<0.01 compared to the vehicle control (0.2% DMSO)

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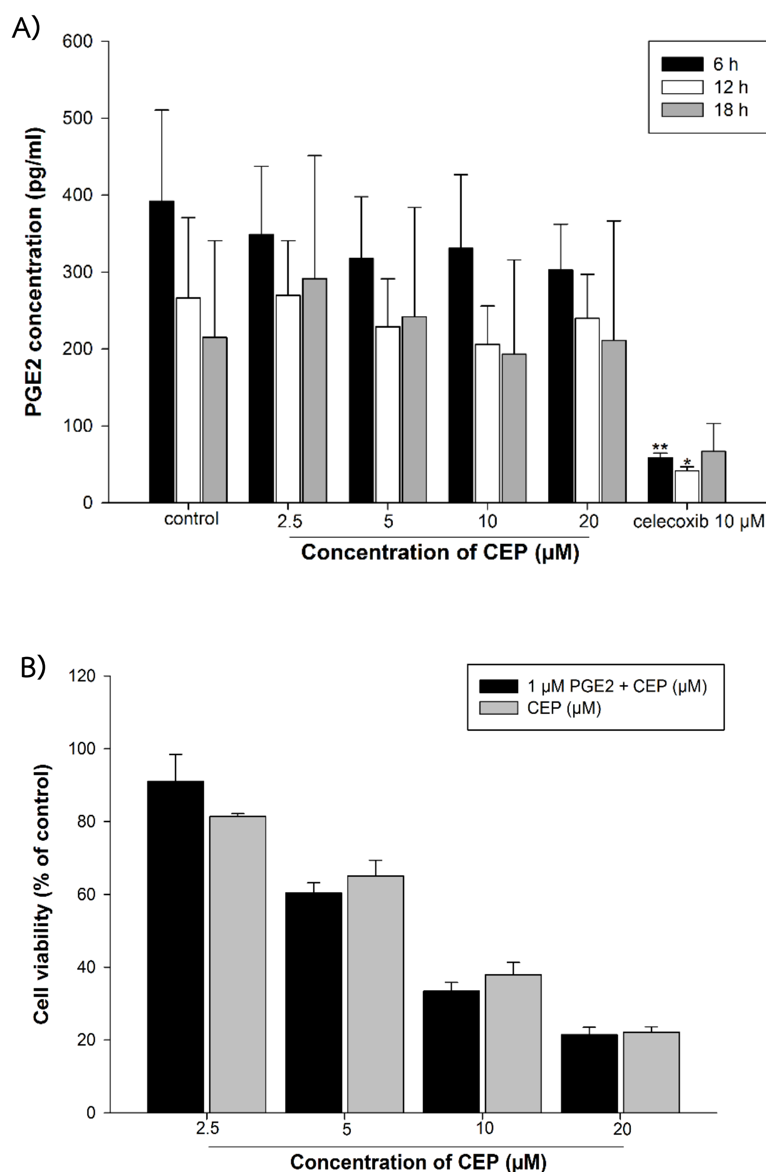

Figure 19. Effect of CEP on PGE2 production in HT-29 cells. A) The cells were treated with CEP 2.5, 5, 10 and 20 μM or 10 μM of celecoxib (positive control) for 6, 12 and 18 h. After incubation, the PGE_2 level in the culture supernatant was measured using Prostaglandin E2 competitive ELISA kit (Thermo, USA). B) The cells were treated with the concentration of CEP for 24 h, in the presence or absence of PGE_2, and the percentages of cell viability were measured by MTT assay. Data are the mean ± SEM. (n $\geq$ 3). **P*<0.05 and ***P*<0.01 compared to the vehicle control (0.2% DMSO)

CHAPTER V
DISCUSSION AND CONCLUSION

Colorectal cancer (CRC) is one of the most common cancers in both man and woman worldwide. Standard treatment option for CRC depends mainly on the size and location of the tumor. Chemotherapy has been used as adjuvant for stage III patients with resectable cancers while it is considered as standard treatment for advanced metastatic CRC (25). Four chemotherapeutic agents, including 5-fluorouracil, oxaliplatin, irinotecan, and capecitabine, are commonly used for patients with CRC. Although they are normally given in combination, severe side effects and poor clinical outcome remain significant problems. In addition to chemotherapy, three biologic drugs which are bevacizumab, cetuximab, and panitumumab, are currently approved for metastatic CRC (37, 38). The use of targeted therapy has been shown to delay progression and extend survival for patients with metastatic CRC, their uses have however been limited due to high cost (109). As a result, novel anticancer agents with less toxicity are urgently needed.

Cepharanthine (CEP) has been widely used in Japan to treat various diseases such as alopecia areata, alopecia pityrodes, radiation-induced leukopenia and venomous snakebites for more than 40 years and is considered pharmacologically safe (14, 106). CEP has also been shown to possess anticancer activity against several different types of cancer such as osteosarcoma, oropharynx, leukemia, hepatocarcinoma and cholangiocarcinoma both *in vitro* and *in vivo* (16, 19, 22, 90). In the present study, I investigated the anticancer activity of CEP on two human colorectal cancer cell lines, HT-29 and COLO 205 cells and found that CEP induced colorectal cancer cell death *in vitro* in a time- and dose-dependent manner (Figure 5 and 11). Interestingly, CEP was found to be more cytotoxic to HT-29 cells (IC_{50} = 6.21 µM) than COLO 205 cells (IC_{50} = 32.13 µM). These findings are; however, somewhat different

from many studies. It was previously demonstrated that COLO 205 cells were more sensitive than HT-29 cells to commonly used anticancer drugs such as 5-FU, oxaliplatin and irinotecan (110) as well as promising chemotherapeutic agents such as 3′-hydroxypterostillbene (111), 3,5,4′-trimethoxystilbene (112), and myricetin (113). It is important to note that there are genetic differences between these two cell lines. The HT-29 cells were reported to carry *APC*, *BRAF*, *PIK3CA* and *p53* mutations while the COLO 205 cells were found to contain mutations of only *BRAF* but not *APC* and *p53* (112, 114, 115). Pan et al. indicated that 3,5,4′-trimethoxystilbene was more potent against the growth of wild-type p53 COLO 205 cells than p53-mutant HT-29 cells, suggesting an important role of p53 in 3,5,4′-trimethoxystilbene-induced cell death in COLO 205 cells (112). Since this study showed that CEP was more toxic to HT-29 cells (p53 mutant) than COLO 205 cells (p53 wild type), it is possible that other factors besides p53 play a pivotal role in anticancer effect of CEP on human colorectal cancer cells. Previously, Harada et al. reported that CEP could inhibit the growth of human adenosquamous cell carcinoma TYS cells which have a *p53* gene mutant at codon $281^{Asp \rightarrow His}$ and induce cell cycle arrest at G1 phase *in vitro* and suppress tumor growth and induce apoptosis *in vivo* (21). Further mechanistic studies illustrated that, in p53 mutant cells, CEP induced cell cycle arrest at G1 phase and cell apoptosis by activating p21WAF1 and caspase 3, respectively (21).

Cyclooxygenase-2 (COX-2) is often found to be overexpressed in many types of cancers, including breast, prostate and colon (42, 116). Increased expression of COX-2 has played a critical role in development and progression of cancer by stimulating angiogenesis, cell proliferation, invasion and survival, as well as evasion of apoptosis (54). As COX-2 is a key player, suppression of its expression and function may be a promising approach in cancer treatment. It was previously shown that NSAIDs could inhibit COX-2 activity, resulting in cell cycle arrest in several cancer cells (117).

Enhanced *COX-2* gene expression was detected in colon cancer following mutation of tumor suppressor gene such as *APC* and oncogene such as *RAS* (46). Consistent with *APC* mutation, COX-2 is constitutively expressed in HT-29 cells (108). Treatment of HT-29 cells with CEP significantly decreased *COX-2* mRNA expression (Figure 18). NF-κB is a transcription factor regulating the expression of genes involved in inflammation and cancer including interleukins, TNF-α, COX-2, inducible nitric oxide synthase (iNOS), c-myc and cyclin D1 (118). Previous studies indicated that CEP could inhibit NF-κB activity in several types of cancer such as human oral squamous cell carcinoma, cholangiocarcinoma, and lymphoma (19, 94, 95). Therefore, it is possible that CEP suppresses NF-κB signaling pathway, resulting in COX-2 down-regulation in human colorectal cancer cells. The effect of CEP on the expression and function of iNOS, the other gene products of NF-κB, are currently being investigated. It has been reported that COX-2 promotes cancer cell proliferation, invasion and metastasis by increasing PGE_2 production (9). Recently, Li et al reported that anticancer effect of paeonol on human colorectal cancers was associated with a reduction in COX-2 expression and a decrease in the PGE_2 levels (119). In the present study, CEP only down-regulated COX-2 expression but did not inhibit PGE_2 production (Figure 19A), In addition, treatment of HT-29 cells with PGE_2 did not affect cytotoxic effect of CEP (Figure 19B). Taken together these results suggest that PGE_2 may not be involved in cytotoxic activity of CEP in human colorectal cancer HT-29 cells.

CEP has been reported to inhibit the growth of cancer cells by promoting apoptosis in human non-small cell lung cancer A549 and H1299 cells (23), human leukemia Jurkat and K562 cells (18), human oral squamous cell carcinoma B88 cells (94), human hepatocellular carcinoma HuU-7 cells (16) and human cholangiocarcinoma KKU-M213 cells (19). This study demonstrated that CEP could stimulate both HT-29 and COLO 205 cells undergo apoptosis in a dose- and time-dependent manner

(Figure 6 and 12). However, treatment of human colorectal cancer cells with CEP at high concentrations or for long periods of time resulted in late apoptotic cells. Bcl-2 family proteins, including anti-apoptotic proteins (Bcl-2, Bcl-xl and Mcl-1) and pro-apoptotic protein (BAX and BAK) are regulator of mitochondria or intrinsic apoptotic pathway (83). Anti-apoptotic proteins could interrupt various apoptotic signals whereas pro-apoptotic proteins are able to induce the release of apoptogenic factors such as cytochrome C or AIF from mitochondria into cytoplasm (120). The present study demonstrated that CEP decreased expression of *Bcl-xl* gene in HT-29 cells (Figure 14B) and down-regulated *Mcl-1* and *Bcl-2* genes in COLO 205 cells (Figure 7A and 7B). Previous studies revealed that CEP induced apoptosis through suppressing inflammation-related pathways, including NF-κB and STAT3 pathways (19, 95). It has also been reported that CEP inhibited Bcl-xl expression through down-regulation of STAT3 protein in human osteosarcoma SaOS2 cells (22). Moreover, CEP was recently found to enhance sensitivity of HeLa cells to radiation by inducing apoptosis and reducing expression of STAT3, c-myc, Bcl-2 and COX-2 (97). Therefore, apoptosis-inducing and growth inhibitory effects of CEP on human colorectal cancer cells are likely mediated through inhibition of NF-κB and STAT3 pathways, resulting in suppression of downstream gene products, including *COX-2, Bcl-2, Bcl-xl* and *Mcl-1*. However, the effects of CEP on NF-κB and STAT3 signaling pathways require further elucidation. It should also be noted that alteration of anti-apoptotic genes following CEP treatment was detected in COLO 205 cells as early as 6 h (Figure 7) while it was observed in HT-29 cells at 16 h (Figure 14). The tumor suppressor gene, *p53*, is an important regulator of the Bcl-2 family proteins (121) and mutations of p53 are capable of not only distrupting the antiproliferative properties, but also promoting various oncogenic responses to cell growth, metastasis, invasion, chemoresistance, genomic instability and apoptosis resistance (122). It was reported that mutant p53 could delay

fibroblast growth arrest (123). Thus, it is possible that modulation effect of CEP on expression of Bcl-2 family members may have been compromised by mutation of p53 in HT-29 cells.

In addition to apoptosis induction, many chemotherapeutic agents exert their anticancer activity via cell cycle arrest. Previous studies demonstrated that CEP could inhibit cell cycle progression in various types of cancer (21, 22, 105). Similarly, treatment of human colorectal cancer cells with CEP also resulted in cell cycle arrest at G1/S phase in the present study (Figure 9 and 16). Interestingly, cell cycle arrest effects was detected following CEP treatment at low concentrations (10 µM for COLO 205 and 5 µM for HT-29 cells) while its apoptotic-induction effect was noted at higher concentrations (20, 40 µM for COLO 205 and 10, 20 µM for HT-29 cells) in both HT-29 and COLO205 cells. Since the mechanisms by which CEP exerts anticancer effects are diverse, it is likely that different concentrations of CEP have different modes of action.

Oxidative stress is an overproduction of reactive oxygen species (ROS) more than the cellular antioxidant capacity. It was reported that oxidative stress can trigger p53-dependent and p53-independent apoptotic cell death pathway in cancer cells (124, 125). The production of ROS could increase mitochondrial permeability (MMP) leading to the release of apoptogenic substrates, which in turn activates the intrinsic apoptosis pathway (126). Numerous natural agents having potent anticancer activity such as epigallocatechin-3-gallate (EGCG), resveratrol, and curcumin were reported to induce apoptosis in cancer cells via a ROS-dependent pathway (121). Previous studies demonstrated that CEP triggered apoptosis in human hepatocellular carcinoma cells and non-small-cell lung cancer cells through ROS (16, 23) Similarly, the results of this study showed that CEP significantly increased ROS level in COLO 205 and HT-29 cells (Figure 10A and 17A). Moreover, the cytotoxicity of CEP was significantly abolished by N-acetylcysteine (NAC), a specific ROS inhibitor (Figure 10B and 17B), indicating that

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CEP's cytotoxic effect is mediated by ROS. Besides apoptosis, ROS was shown to induce autophagic cell death in various cancers such as colon (127), breast (128) and non-small-cell lung (129). Mechanistic studies illustrated that ROS induced expression of beclin-1, a key regulator of autophagy (130). Recent studies demonstrated that CEP could induce autophagic cell death in apoptosis-resistant cells through AMPK/mTOR signaling pathway (131). To determine autophagy-inducing effect of CEP, I plan to explore the effect of CEP on beclin-1 expression.

Several previous studies illustrated that CEP exerts potent antitumor activity against several types of cancer. The present study illustrated the anticancer activity of CEP and its underlying mechanisms on human colorectal cancer cells, highlighting therapeutic potential of CEP in cancer. Generally, anticancer drugs are given in combination in order to enhance therapeutic efficacy, however their use has been limited due to toxicities. CEP is a safe drug and it has also been approved to treat leukopenia caused by radiation therapy. Its safety and strong anticancer activity make CEP an attractive agent to be used in combination with commonly used chemotherapeutic drugs which may reduce undesirable side effects and enhance or maintain therapeutic effects. Taken together, these findings provide rational for further preclinical investigation of anticancer effect of CEP on human colorectal cancer cells.

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Conclusion

The results in the present study clearly demonstrated that cepharanthine (CEP) has a remarkable anticancer activity against HT-29 (COX-2 positive) and COLO 205 (COX-2 negative) human colorectal cancer cells. Mechanistic studies indicated that CEP effectively induced cell cycle arrest at G1/S phase and apoptosis through modulation of Bcl-2 family genes and accumulation of ROS level. Moreover, CEP down-regulated the expression of *COX-2* gene in HT-29 cells. These findings suggest that CEP could potentially be used as a novel anticancer agent for colorectal cancer cells which are often resistant to currently chemotherapeutic agent.

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APPENDIX

APPENDIX A
PREPARATION OF REAGENTS

DMEM stock solution 1 liter

DMEM powder	10.4 g
Sodium bicarbonate	3.7 g
ddH_2O	900 ml

Adjust pH to 7.4 with 1 N HCl and 1 N NaOH

Add ddH_2O to 1 liter and sterilized by filtering through a 0.2 sterile membrane filter

RPMI 1640 stock solution 1 liter

RPMI powder	10.4 g
$NaHCO_3$	1.5 g
Glucose	4.5 g
Sodium pyruvate	0.11 g
HEPES (1M)	10 ml
ddH_2O	900 ml

Adjust pH to 7.2 with 1 N HCl and 1 N NaOH

Add ddH_2O to 1 liter and sterilized by filtering through a 0.2 sterile membrane filter

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1x Phosphate Buffered Saline (PBS) 1 liter

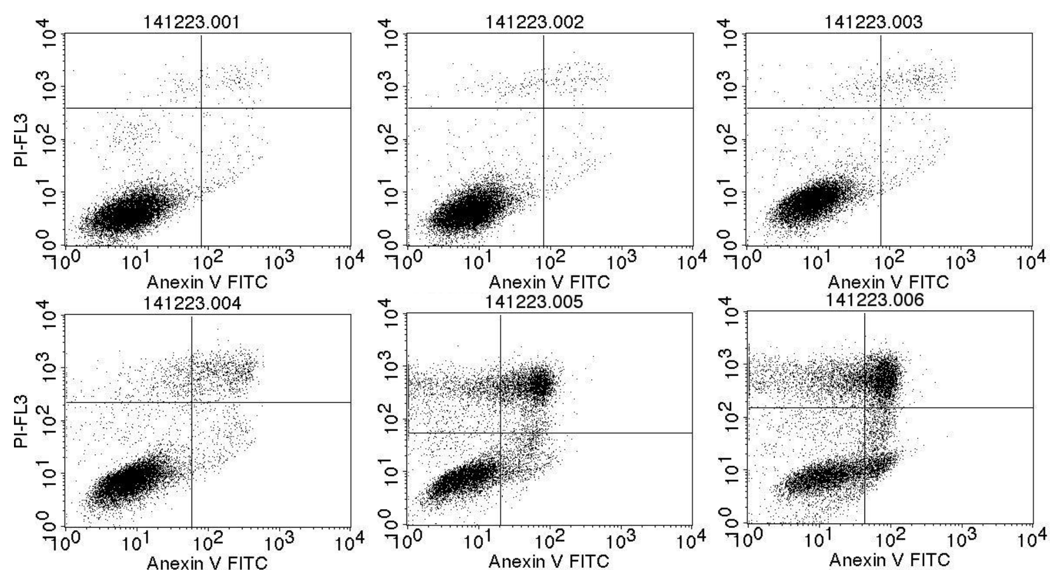
NaCl	8.065 g
KCl	0.2 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g
ddH2O	900 ml

1x Assay Buffer for Flow Cytometer 100 ml

HEPES (1M)	1.0 ml
$CaCl_2$ (0.1M)	2.8 ml
NaCl (5M)	2.5 ml
ddH2O	93.7 ml

APPENDIX B
RESULTS

Appendix B-1: Representative cytograms of cell apoptosis analysis of HT-29 cells after treatment with CEP (2.5-20 μM) for 24 h.



Appendix B-2: The apoptotic effect of CEP on HT-29 cells at 24 h.

Sample (μM)	Apoptosis (%)			
	Alive	Early	Late	Necrosis
Untreated	96.53 ± 0.56	1.48 ± 0.28	1.33 ± 0.48	0.65 ± 0.27
0.2% DMSO	94.54 ± 1.43	2.50 ± 0.84	1.91 ± 0.53	1.06 ± 0.28
CEP 2.5	91.84 ± 2.38	3.15 ± 1.14	3.80 ± 1.04	1.20 ± 0.26
CEP 5	77.67 ± 5.59	6.63 ± 2.15	11.25 ± 2.66	4.44 ± 1.02
CEP 10	44.18 ± 3.66	7.54 ± 0.44	30.47 ± 1.68	17.82 ± 2.38
CEP 20	35.95 ± 3.30	14.47 ± 0.38	30.36 ± 2.80	19.23 ± 0.55

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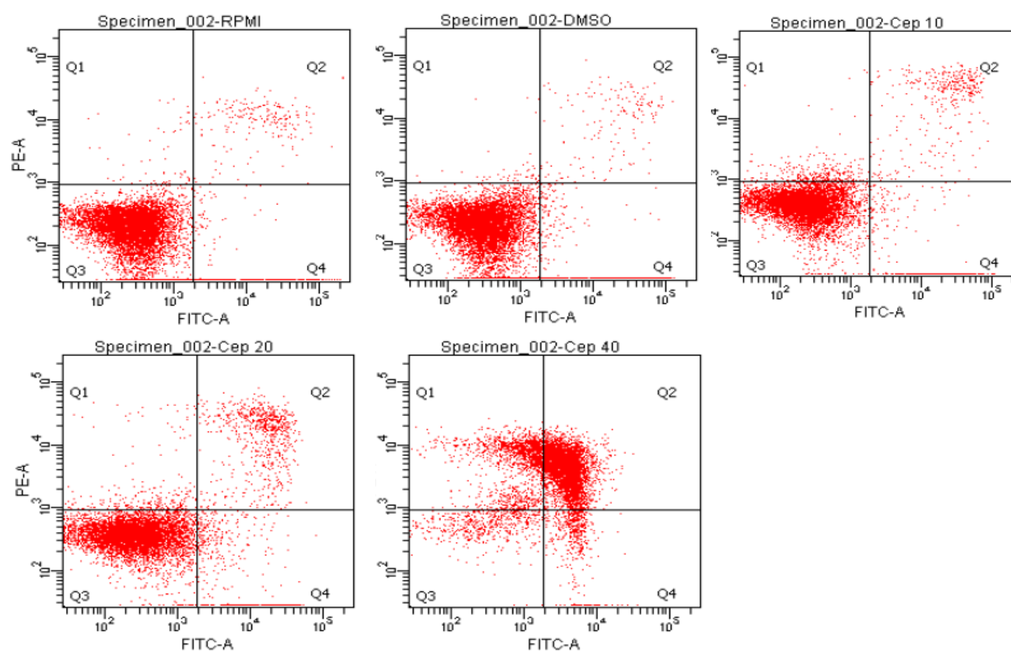
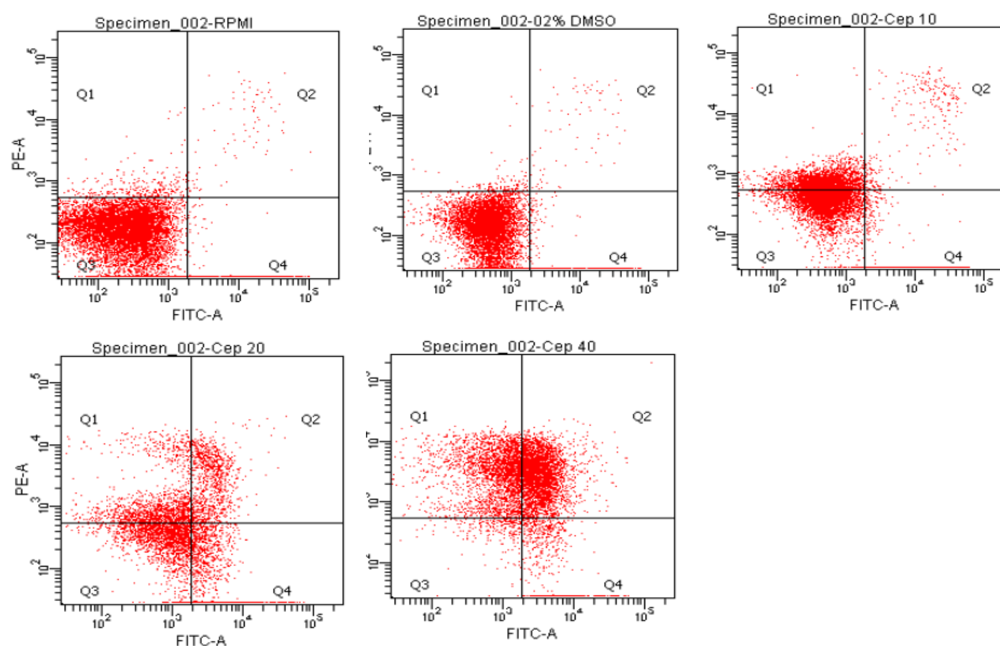
Appendix B-3: Representative cytograms of cell apoptosis analysis of COLO 205 cells after treatment with CEP (10-40 μM) for 12 h.



Appendix B-4: The apoptotic induction effect of CEP in COLO 205 cells at 12 h.

Sample (μM)	Apoptosis (%)			
	Alive	Early	Late	Necrosis
Untreated	93.80 ± 0.65	3.70 ± 0.24	1.10 ± 0.00	1.40 ± 0.40
0.2% DMSO	94.10 ± 0.45	3.63 ± 0.24	1.07 ± 0.12	1.20 ± 0.36
CEP 10	90.83 ± 2.47	3.57 ± 0.23	1.87 ± 0.37	3.77 ± 2.00
CEP 20	73.33 ± 8.12	7.33 ± 1.46	7.93 ± 2.85	11.43 ± 4.88
CEP 40	40.00 ± 2.81	7.70 ± 0.52	27.33 ± 2.33	24.97 ± 3.77

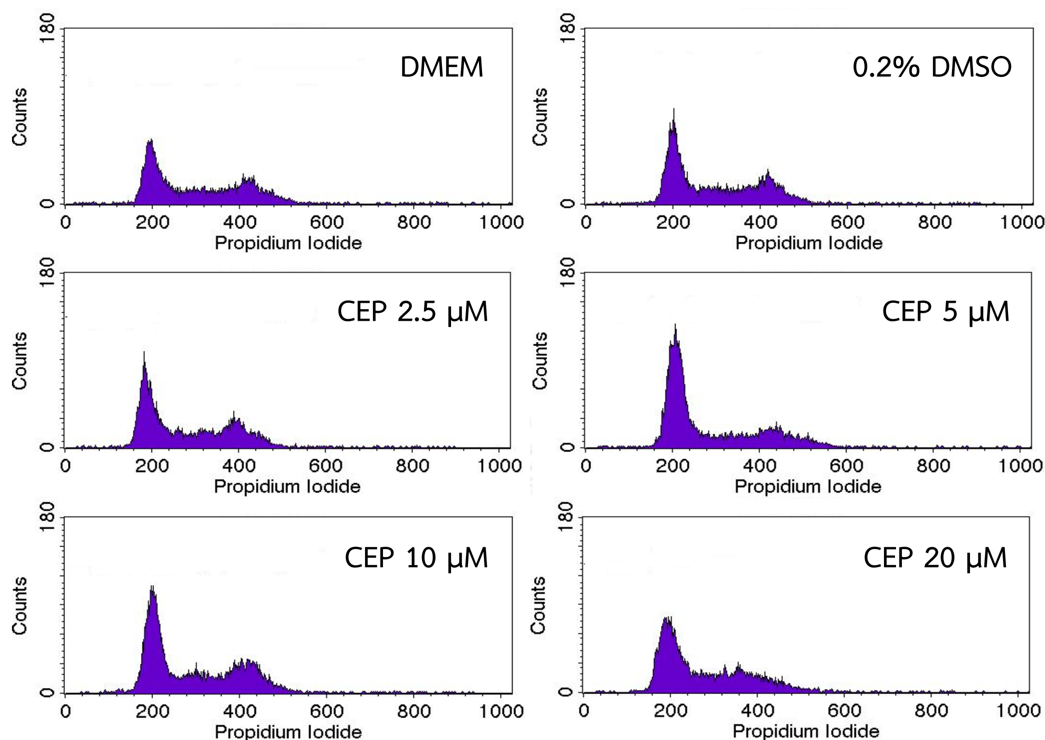
Appendix B-5: Representative cytograms of cell apoptosis analysis of COLO 205 cells after treatment with CEP (10-40 μM) for 24 h.



Appendix B-6: The apoptotic effect of CEP on COLO 205 cells at 24 h.

Sample (μM)	Alive	Apoptosis (%) Early	Late	Necrosis
Untreated	91.28 ± 0.91	4.15 ± 0.47	1.30 ± 0.17	3.23 ± 0.71
0.2% DMSO	90.15 ± 2.09	6.63 ± 1.95	1.58 ± 0.23	1.68 ± 0.06
CEP 10	67.70 ± 4.04	4.70 ± 0.50	5.58 ± 0.82	22.30 ± 3.83
CEP 20	33.88 ± 3.46	20.60 ± 3.95	20.78 ± 2.93	24.78 ± 0.73
CEP 40	7.18 ± 2.46	9.45 ± 0.79	41.83 ± 5.51	41.55 ± 3.14

Appendix B-7: Representative histogram from flow cytometric analysis of HT-29 cell cycle pattern after treatment with CEP (2.5-20 μM) for 12 h.



Appendix B-8: Distribution of HT-29 cells in the cell cycle phases after treatment with CEP at 12 h.

Sample (μM)	Cell population (%)			
	Sub-G1	G1	S	G2/M
Untreated	0.54 ± 0.05	38.05 ± 3.95	45.28 ± 1.77	16.12 ± 2.23
0.2% DMSO	1.25 ± 0.06	39.59 ± 2.23	43.86 ± 0.33	15.32 ± 1.96
CEP 2.5	0.46 ± 0.35	38.88 ± 1.32	43.12 ± 1.05	17.53 ± 0.62
CEP 5	0.45 ± 0.45	44.73 ± 3.39	39.19 ± 4.77	15.63 ± 0.94
CEP 10	0.20 ± 0.14	55.93 ± 0.50	32.67 ± 3.02	11.16 ± 2.39
CEP 20	1.30 ± 0.23	44.24 ± 2.61	35.88 ± 1.26	18.58 ± 1.11

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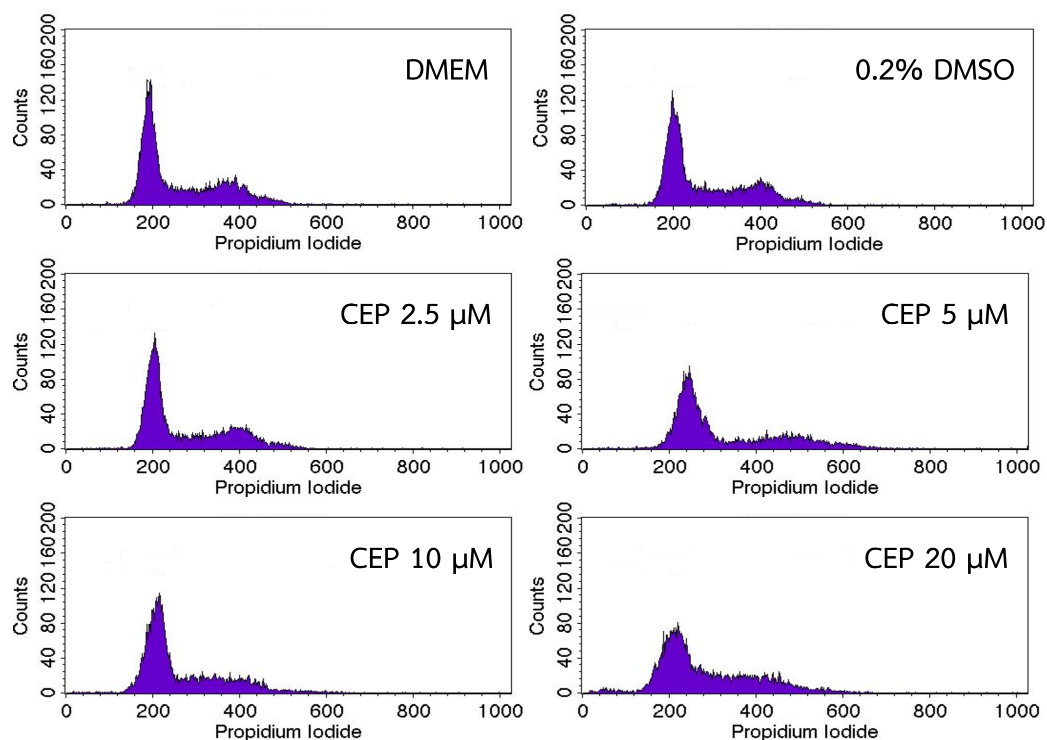
Appendix B-9: Representative histogram from flow cytometric analysis of HT-29 cell cycle pattern after treatment with CEP (2.5-20 μM) for 24 h.



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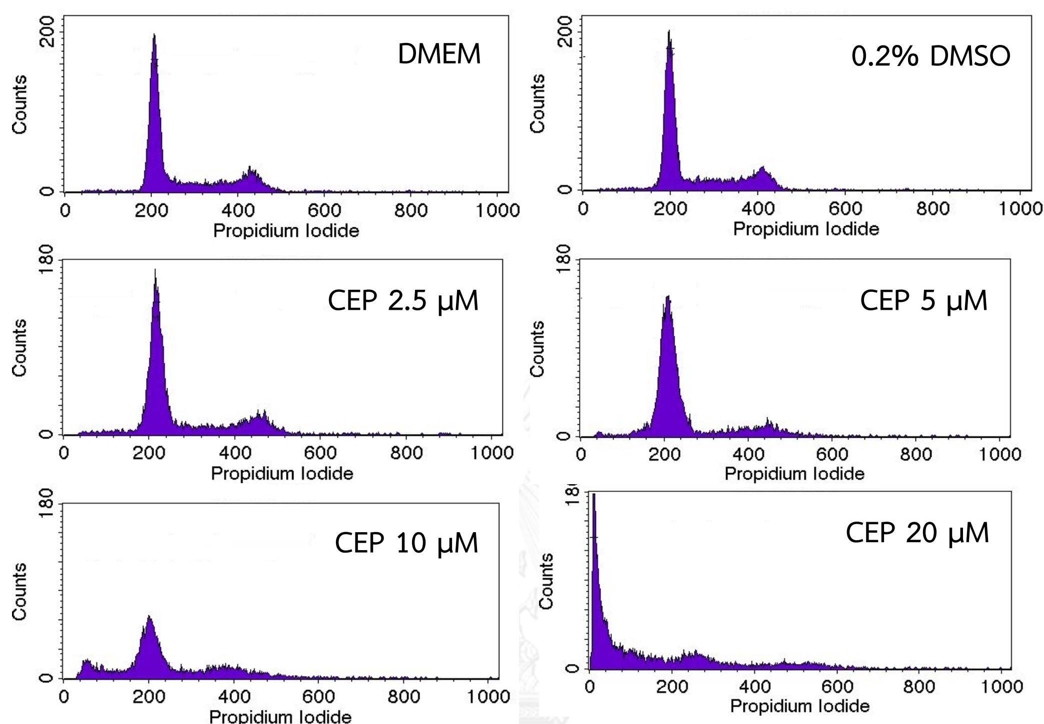
Appendix B-10: Distribution of HT-29 cells in the cell cycle phases after treatment with CEP at 24 h.

	Cell population (%)			
Sample (μM)	**Sub-G1**	**G1**	**S**	**G2/M**
Untreated	0.37 ± 0.37	46.22 ± 4.03	43.22 ± 11.12	10.14 ± 6.77
0.2% DMSO	1.06 ± 0.11	48.22 ± 0.81	36.84 ± 2.30	13.88 ± 1.61
CEP 2.5	0.57 ± 0.02	51.77 ± 2.21	29.92 ± 1.85	17.73 ± 0.37
CEP 5	1.21 ± 0.31	60.40 ± 1.44	13.39 ± 7.31	24.85 ± 9.02
CEP 10	1.35 ± 0.09	59.43 ± 1.27	29.94 ± 8.97	9.27 ± 7.59
CEP 20	2.72 ± 0.20	35.87 ± 3.27	53.00 ± 3.00	8.40 ± 0.44

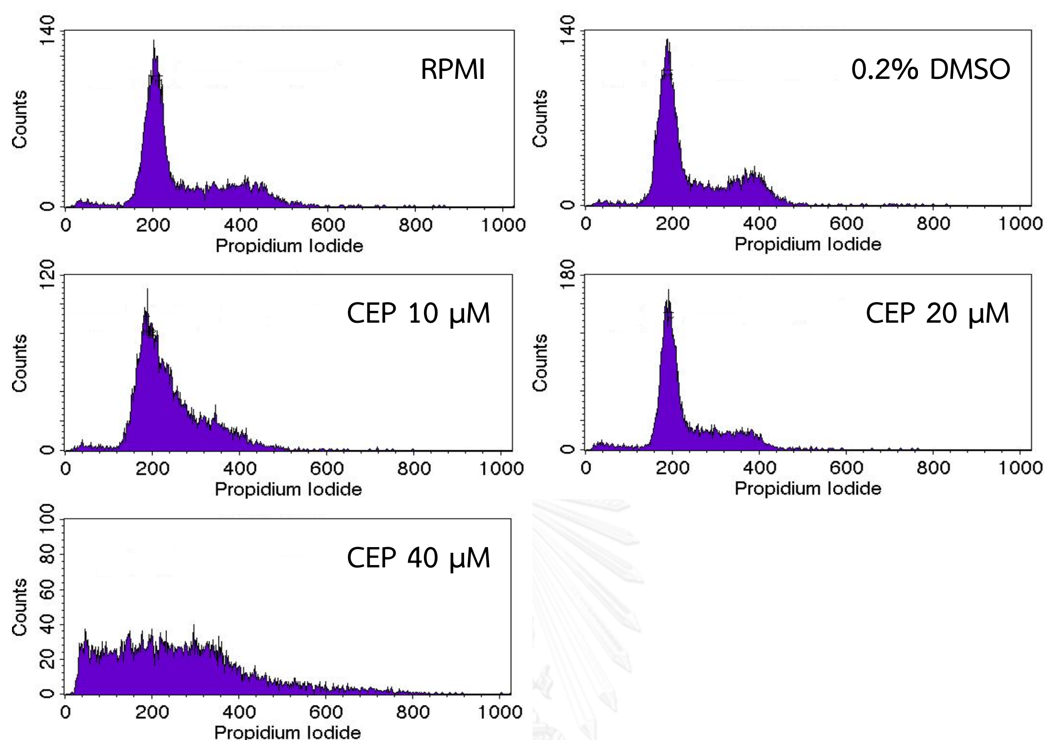
Appendix B-11: Representative histogram from flow cytometric analysis of HT-29 cell cycle pattern after treatment with CEP (2.5-20 μM) for 36 h.



Appendix B-12: Distribution of HT-29 cells in the cell cycle phases after treatment with CEP at 36 h.

	Cell population (%)			
Sample (μM)	Sub-G1	G1	S	G2/M
Untreated	1.31 ± 0.18	52.42 ± 4.61	22.07 ± 2.40	24.20 ± 2.50
0.2% DMSO	1.51 ± 0.61	55.81 ± 1.42	19.06 ± 1.18	23.48 ± 3.09
CEP 2.5	2.27 ± 0.75	60.13 ± 0.11	20.16 ± 0.06	17.13 ± 0.77
CEP 5	1.75 ± 0.60	74.52 ± 2.07	10.44 ± 1.56	13.21 ± 0.43
CEP 10	12.41 ± 1.96	50.39 ± 6.73	20.11 ± 6.39	16.91 ± 2.11
CEP 20	31.14 ± 0.42	31.28 ± 5.78	16.82 ± 1.03	20.26 ± 5.97

Appendix B-13: Representative histogram from flow cytometric analysis of COLO 205 cell cycle pattern after treatment with CEP (10-40 μM) for 12 h.



Appendix B-14: Distribution of COLO 205 cells in the cell cycle phases after treatment with CEP at 12 h.

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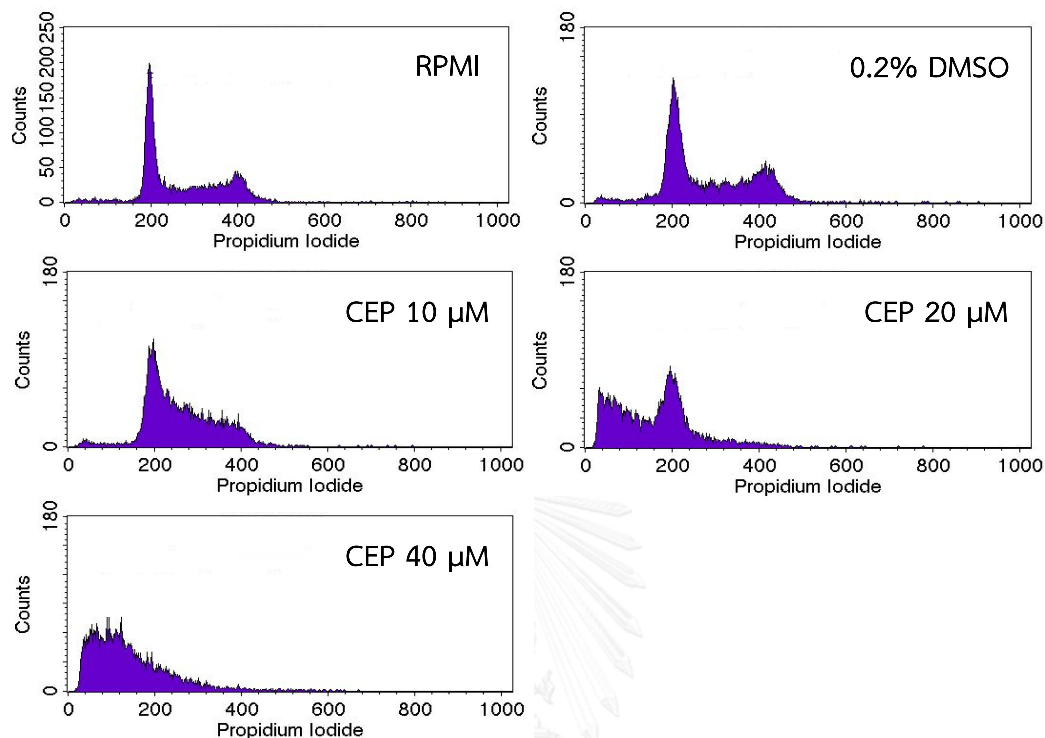
	Cell population (%)			
Sample (μM)	Sub-G1	G1	S	G2/M
Untreated	2.09 ± 0.25	45.44 ± 0.72	32.26 ± 0.50	20.21 ± 0.97
0.2% DMSO	3.11 ± 0.29	47.30 ± 0.80	30.09 ± 2.24	19.51 ± 2.75
CEP 10	2.76 ± 0.01	57.56 ± 1.01	37.06 ± 0.76	2.62 ± 1.76
CEP 20	3.90 ± 0.64	60.87 ± 1.85	30.77 ± 0.47	4.47 ± 2.97
CEP 40	44.41 ± 3.54	32.52 ± 2.61	19.07 ± 0.43	19.07 ± 0.57

Appendix B-15: Representative histogram from flow cytometric analysis of COLO 205 cell cycle pattern after treatment with CEP (10-40 μM) for 24 h.



Appendix B-16: Distribution of COLO 205 cells in the cell cycle phases after treatment with CEP at 24 h.

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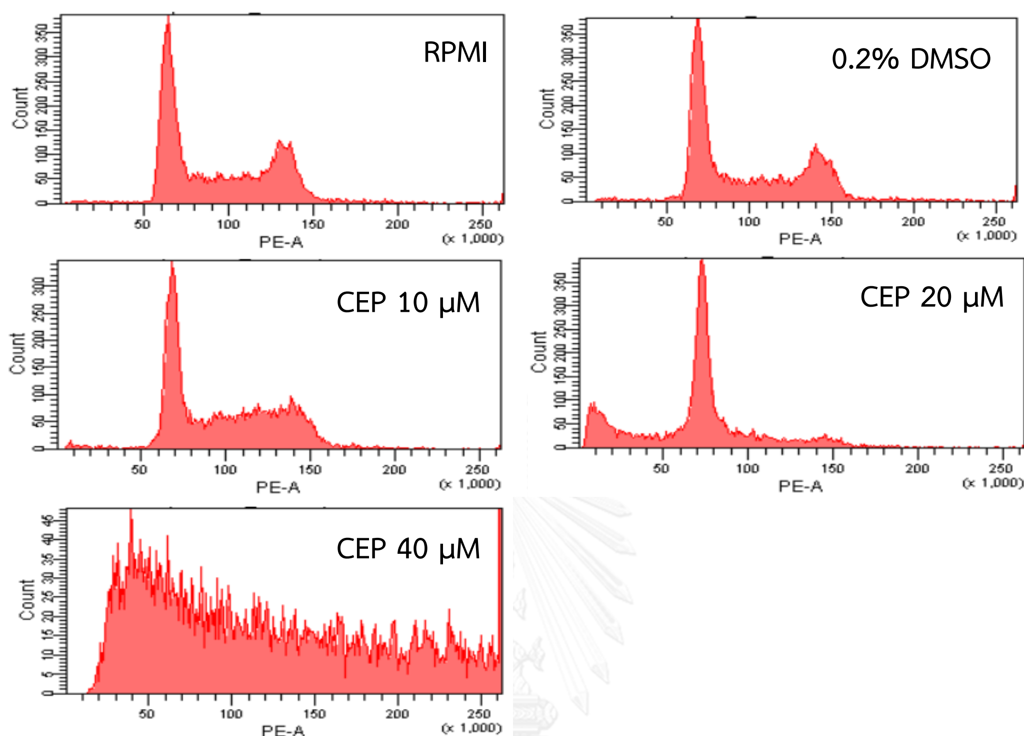
	Cell population (%)			
Sample (μM)	Sub-G1	G1	S	G2/M
Untreated	2.45 ± 0.56	44.22 ± 2.34	35.31 ± 1.19	18.00 ± 0.59
0.2% DMSO	3.50 ± 0.53	45.32 ± 3.21	33.30 ± 0.45	17.90 ± 2.23
CEP 10	4.34 ± 0.86	28.78 ± 2.47	62.86 ± 5.64	4.02 ± 4.02
CEP 20	43.37 ± 7.46	42.12 ± 3.13	13.85 ± 3.58	0.74 ± 0.74
CEP 40	46.65 ± 2.91	16.98 ± 3.31	18.51 ± 10.31	17.82 ± 9.80

Appendix B-17: Representative histogram from flow cytometric analysis of COLO 205 cell cycle pattern after treatment with CEP (10-40 μM) for 36 h.



Appendix B-18: Distribution of COLO 205 cells in the cell cycle phases after treatment with CEP at 36 h.

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	Cell population (%)			
Sample (μM)	Sub-G1	G1	S	G2/M
Untreated	4.40 ± 0.13	46.93 ± 2.21	33.32 ± 2.45	15.48 ± 0.02
0.2% DMSO	5.21± 0.03	41.14 ± 0.98	40.65 ± 0.18	13.01 ± 0.84
CEP 10	4.72 ± 0.51	31.64 ± 0.36	46.06 ± 1.20	17.58 ± 1.35
CEP 20	49.61 ± 8.62	23.80 ± 18.52	26.06 ± 10.58	0.62 ± 0.62
CEP 40	63.31 ± 5.46	15.07 ± 12.92	2.93 ± 2.93	17.78 ± 10.38

VITA

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Publication

1. Rattanawong A., Limpanasithikul W., and Wonganan P. 2015. Anti-cancer effects of cepharanthine in human colon cancer cells. KKU Journal Graduate studies. 15(3)

Poster Presentation

1. Rattanawong A., Limpanasithikul W., and Wonganan P. 2015. Anti-cancer effects of cepharanthine in human colon cancer cells. The 34th National Graduate Research Conference. March 27, 2015, Khon Kaen Unoversity, Thailand.

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