การศึกษาความเป็นพิษและกลไกการออกฤทธิ์ของสิ่งสกัดจากใบส้มชื่น
ต่อเซลล์มะเร็งลำไส้ใหญ่ของมนุษย์

นางณัฏฐพร บูรณะบุญวงศ์

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชวิทยา (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
เป็นแฟ้มข้อมูลของนิสิตเจ้าของวิทยานิพนธ์ ที่ส่งผ่านทางบัณฑิตวิทยาลัย
The abstract and full text of theses from the academic year 2011 in Chulalongkorn University Intellectual Repository (CUIR)
are the thesis authors' files submitted through the University Graduate School.

CYTOTOXIC ACTIVITY AND MECHANISM OF GLYCOSMIS PARVA LEAF EXTRACT ON HUMAN COLORECTAL CANCER CELLS

Mrs. Nattaporn Buranabunwong

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmacology
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2013

Copyright of Chulalongkorn University

Thesis Title	CYTOTOXIC ACTIVITY AND MECHANISM OF GLYCOSMIS PARVA LEAF EXTRACT ON HUMAN COLORECTAL CANCER CELLS
By	Mrs. Nattaporn Buranabunwong
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Wacharee Limpanasithikul, Ph.D.
Thesis Co-Advisor	Associate Professor Nijsiri Ruangrungsi, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Graduate School

(Associate Professor Amorn Petsom, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Assistant Professor Naowarat Suthamnatpong, D.V.M., Ph.D.)

.....Thesis Advisor

(Assistant Professor Wacharee Limpanasithikul, Ph.D.)

.....Thesis Co-Advisor

(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

.....Examiner

(Sireerat Sooampon, D.D.S., Ph.D.)

.....Examiner

(Wannarasmi Ketchart, M.D., Ph.D.)

.....External Examiner

(Pongpun Siripong, Ph.D.)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

ณัฏฐพร บูรณะบุญวงศ์ : การศึกษาความเป็นพิษและกลไกการออกฤทธิ์ของสิ่งสกัดจากใบส้มชื่นต่อเซลล์มะเร็งลำไส้ใหญ่ของมนุษย์. (CYTOTOXIC ACTIVITY AND MECHANISM OF GLYCOSMIS PARVA LEAF EXTRACT ON HUMAN COLORECTAL CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. วัชรี ลิมปนสิทธิกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นิจศิริ เรืองรังษี, 127 หน้า.

ต้นส้มชื่นเคยมีรายงานว่ามีความเป็นพิษและมีฤทธิ์ต้านการอักเสบโดยไปลดการแสดงออกของยีนค็อกซ์ 2 ในหลอดทดลอง การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของสิ่งสกัดเอทิลอะซีเตทจากใบส้มชื่น (จีพีอี) ต่อเซลล์มะเร็งลำไส้ใหญ่ของมนุษย์ที่มีการแสดงออกของยีนค็อกซ์ 2 (เซลล์เอชที 29) และไม่มีการแสดงออกของยีนค็อกซ์ 2 (เซลล์โคโล 205) รวมไปถึงกลไกการออกฤทธิ์ ความเป็นพิษของสิ่งสกัดจีพีอีต่อเซลล์เอชที 29 และเซลล์โคโล 205 แปรผันตามความเข้มข้นและระยะเวลาที่ใช้ทดสอบ โดยมีค่าความเข้มข้นที่ทำให้เซลล์ตายร้อยละ 50 ตาย เท่ากับ 69.49±2.04, 55.89±1.86 และ 48.94±2.99 ไมโครกรัม/มิลลิลิตร หลังจากได้รับสารนาน 24, 48 และ 72 ชั่วโมง ตามลำดับในเซลล์เอชที 29 ส่วนในเซลล์โคโล 205 ค่าความเข้มข้นที่ทำให้เซลล์ตายร้อยละ 50 ตาย เท่ากับ 59.92±4.34, 28.85±1.44 และ 25.42±1.65 ไมโครกรัม/มิลลิลิตร หลังจากได้รับสารนาน 24, 48 และ 72 ชั่วโมง ตามลำดับ สิ่งสกัดจีพีอีสามารถเหนี่ยวนำให้เซลล์เกิดการตายแบบเอพอพโตซิสในเซลล์ทั้งสองชนิด โดยดูได้จากการย้อมด้วย Annexin V-FITC/Propidium Iodide ผลการศึกษาความเป็นพิษและการเหนี่ยวนำให้เซลล์เกิดการตายแบบเอพอพโตซิสนั้น พบว่าสิ่งสกัดจีพีอีมีผลต่อเซลล์โคโล 205 มากกว่าเซลล์เอชที 29 สิ่งสกัดจีพีอีที่ความเข้มข้น 25-100 ไมโครกรัม/มิลลิลิตรมีผลยับยั้งการแบ่งตัวของเซลล์มะเร็งทั้งสองชนิด ซึ่งผลการยับยั้งการแบ่งตัวของสิ่งสกัดจีพีอีนี้มีผลต่อเซลล์เอชที 29 มากกว่าเซลล์โคโล 205 การศึกษาถึงผลของสิ่งสกัดจีพีอีต่อวัฏจักรเซลล์ พบว่าทำให้เกิดการลดลงของเซลล์ในระยะ S และเพิ่มการสะสมของเซลล์ในระยะ G0/G1 และ G2/M โดยมีผลคล้ายกันในเซลล์ทั้งสองชนิด การศึกษาในระดับกลไกของสิ่งสกัดจีพีอีพบว่าสิ่งสกัดน่าจะมีกลไกการออกฤทธิ์ทั้งที่ผ่านทางค็อกซ์ 2 และไม่ผ่านค็อกซ์ 2 กลไกที่ผ่านทางค็อกซ์ 2 เกี่ยวข้องกับการลดการแสดงออกของยีนค็อกซ์ 2 ซึ่งจะไปมีผลต่อการส่งสัญญาณภายในเซลล์ที่อยู่ระดับถัดจากค็อกซ์ 2 ลงไป ส่วนกลไกที่ไม่ผ่านค็อกซ์ 2 นั้นเกี่ยวข้องกับการเปลี่ยนแปลงการแสดงออกของยีนที่ควบคุมวัฏจักรเซลล์ และความสมดุลของยีนในกลุ่ม Bcl-2 ในเซลล์เอชที 29 พบว่าสิ่งสกัดจีพีอีมีผลลดการแสดงออกของยีน cyclin A, cyclin E, Bcl-2 และ COX-2 และเพิ่มการแสดงออกของยีน Bak ส่วนในเซลล์โคโล 205 สิ่งสกัดจีพีอีมีผลลดการแสดงออกของยีน cyclin A, Bcl-2 และ Bcl-XL และเพิ่มการแสดงออกของยีน p21 โดยสรุปสิ่งสกัดจีพีอีมีความเป็นพิษ, เหนี่ยวนำให้เกิดการตายแบบเอพอพโตซิส, ยับยั้งเพิ่มจำนวน, และหยุดวัฏจักรเซลล์ ในเซลล์มะเร็งลำไส้ใหญ่ของมนษย์ทั้งสองชนิด ซึ่งกลไกการออกฤทธิ์ผ่านทั้งทางค็อกซ์ 2 และไม่ผ่านค็อกซ์ 2 การศึกษาในครั้งนี้จึงสามารถเป็นพื้นฐานเพื่อที่จะพัฒนายาต้านมะเร็งจากสิ่งสกัดจีพีอีซึ่งมีสารออกฤทธิ์ที่มีความเป็นไปได้ในการวิจัยยาต้านมะเร็งลำไส้ใหญ่ต่อไป

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5287852320 : MAJOR PHARMACOLOGY
KEYWORDS: GLYCOSMIS PARVA / COLORECTAL CANCER / CYTOTOXICITY / APOPTOSIS / CELL CYCLE ARREST / COX-2 / BCL-2 / CYCLINS / HT-29 / COLO-205

NATTAPORN BURANABUNWONG: CYTOTOXIC ACTIVITY AND MECHANISM OF GLYCOSMIS PARVA LEAF EXTRACT ON HUMAN COLORECTAL CANCER CELLS. ADVISOR: ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D.,ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 127 pp.

Glycosmis parva Craib (Rutaceae) was reported to have cytotoxicity and anti-inflammatory activities with the reduction of COX-2 expression, in vitro. This study aimed to investigate the effect of ethyl acetate extract from leaves of G. parva (GPE) on human colorectal cancer expressing COX-2, HT-29, and not express COX-2, Colo-205 and its underlying mechanisms of action. Cytotoxicity of GPE against HT-29 and Colo-205 were exhibited in both dose- and time- dependent manner. The IC50 values of GPE against HT-29 were 69.49±2.04, 55.89±1.86 and 48.94±2.99 µg/ml at 24, 48 and 72 h, respectively. In Colo-205, the IC50 were 59.92±4.34, 28.85±1.44 and 25.42±1.65 µg/ml at 24, 48 and 72 h, respectively. GPE significantly induced apoptosis in both cell lines as evidenced by AnnexinV/FITC and PI staining. The effect of GPE on cytotoxicity and induction of apoptosis were higher in Colo-205 than HT-29. GPE at 25-100 µg/ml significantly inhibited cell proliferation in both cells. The effect was greater in HT-29 cells. Cell cycle analysis demonstrated that GPE caused a decrease in the cells in S phase which was associated with G0/G1 and G2/M accumulation. The changes in cell cycle pattern following GPE treatment were similar in both cell lines. Mechanistic studies suggested that the effects of GPE may be mediated through both COX-2 dependent and COX-2 independent pathway. The COX-2 dependent involved with reduction of COX-2 expression which affected downstream COX-2 pathway. The COX-2 independent pathway included the changes in the expression of cell cycle control genes and the alteration in the balance of Bcl-2 family gene expression. In HT-29, GPE down-regulated cyclin A and cyclin E expression and increased the expression of pro-apoptotic Bak while decreased the anti-apoptotic Bcl-2. It also significantly decreased COX-2 expression. In Colo-205, GPE decreased cyclin A and up-regulated p21 expression. It also decreased the expression of anti-apoptotic Bcl-2 and Bcl-XL. Taken together, GPE exhibited cytotoxic activity, induction of apoptosis, inhibition of cell proliferation and arrest cell cycle in both cells. The underlying mechanisms involved both COX-2 dependent and COX-2 independent pathway. These findings provide the fundamental knowledge of the anti-cancer effect of GPE which could be a potential compounds for the treatment of colorectal cancer.

Field of Study: Pharmacology
Academic Year: 2013

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

This dissertation is a milestone in my academic achievement. I am grateful to a number of people who have guided and supported me throughout the process and provided assistance for my venture.

I would like to express my deepest appreciation and gratitude to my advisor, Asst. Prof. Wacharee Limpanasithikul, for the patient guidance and dedication she provided to me, all the way from when I was first consider applying to the Ph.D. program in Pharmacology, through the completion of this degree.

I would like to thank my co-advisor Assoc. Prof. Nijsiri Ruangrungsi, College of Public Health Sciences, Chulalongkorn University for his valuable suggestion and his support of herbal extracts in this study.

I would also like to express my sincere appreciation to the dissertation committees for their constructive suggestions and crucial review of my dissertation.

I would like to give my special thanks to Dr. Supranee Buranapraditkun, Cellular Immunology Laboratory, Allergy and Clinical Immunology Unit, Department of Medicine, Faculty of Medicine, Chulalongkorn University for technical assistance on flow cytometry and Dr. Piyanuch Wonganan for helpful suggestion on manuscript writing. I wish to thank all of the staffs from the Department of Pharmacology, Faculty of Medicine, and also thank all my friends for their support and encouragement.

For the financial support, I am thankful to the Graduate school, Chulalongkorn University for providing the Chulalongkorn University Graduate Scholarship to commemorate the 72nd anniversary of His Majesty King Bhumibol Adulyadej and the 90th Anniversary of Chulalongkorn University Fund.

Finally, my family has supported and helped me along the course of this dissertation by giving encouragement and providing the moral and emotional support I needed to complete my dissertation. To them, I am eternally grateful.

CONTENTS

Page

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Page

Page

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF TABLES

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF FIGURES

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF ABBREVAITIONS

µg/ml	=	Microgram per milliliter
µl	=	Microliter
µM	=	Micromolar
15-PGDH	=	15-hydroxyprostaglandin dehydrogenase
5FU	=	5-fluorouracil
ADCC	=	Antibody-dependent cell-mediated cytotoxicity
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
CDKI	=	Cyclin-dependent kinase inhibitor
Cdks	=	Cyclin-dependent kinases
cDNA	=	Complementary DNA
CH_2THF	=	5,10-methylene tetrahydrofolate
$CHCl_3$	=	Chloroform
CO_2	=	Carbondioxide
COX	=	Cyclooxygenases
CRC	=	Colorectal Cancer
C_T	=	Cycle threshold

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

dATP	=	Deoxyadenosine Triphosphate
DEPC	=	Dietyl pyrocarbonate
DHFU	=	Dihydrofluorouracil
DISC	=	Death inducing signalling complex
DMEM	=	Dulbecco's modified Eagle's medium
DMSO	=	Dimethylsulfoxide
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide
DPD	=	Dihydropyrimidine dehydrogenase
dTMP	=	Deoxythymidine monophosphate
dUMP	=	Deoxyuridine monophosphate
dUTP	=	Deoxyuridine triphosphate
EC_{50}	=	50% Effective Concentration
EDTA	=	Ethylene diamine tetraacetic acid
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
FdUMP	=	Fluorodeoxyuridine monophosphate
FdUTP	=	Fluorodeoxyuridine triphosphate
FUDP	=	Fluorouridine diphosphate
FUMP	=	Fluorouridine monophosphate
FUR	=	Fluorouridine
FUTP	=	Fluorouridine triphosphate
GPE	=	Ethyl acetate extracts from the leaves of *Glycosmis parva*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

H	=	Hour
HCl	=	Hydrochloric acid
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRK	=	Harakiri (death protein-5)
HSV-2	=	Herpes simplex virus type 2
IC_{50}	=	50% Inhibition Concentration
IgG	=	Immunoglobulin G
IL	=	Interleukin
iNOS	=	Inducible nitric oxide synthase
LV	=	Leucovorin
MAPK	=	Mitogen-Activated Protein Kinase
MeOH	=	Methanol
mg/ml	=	Milligram per milliliter
$MgCl_2$	=	Magnesium Chloride
mRNA	=	Messenger RNA
$NaHCO_3$	=	Sodium bicarbonate
NaOH	=	Sodium hydroxide
NF-κB	=	Nuclear factor kappa B
nm	=	Nanometer
NSAID	=	Non-Steroidal Anti-Inflammatory Drug
°C	=	Degree centigrade
OD	=	Optical density
OPRT	=	Orotate phosphoribosyltransferase
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
PPAR	=	Peroxisome Proliferator Activated Receptor
PRPP	=	Phosphoribosyl pyrophosphate

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

PUMA	=	BCL-2 binding component-3
Rb	=	Retinoblastoma protein
RNA	=	Ribonucleic acid
Rpm	=	Round per minutes
RPMI	=	Roswell Park Memorial Institute medium
RQ	=	Relative Quantitation
RR	=	Ribonucleotide reductase
RT-PCR	=	Reverse transcription polymerase chain reaction
TBE	=	Tris-Borate-EDTA buffer
tBid	=	Truncated Bid
TK	=	Thymidine kinase
TNFα	=	Tumor necrosis factor α
Topo I	=	Topoisomerase I
TRADD	=	TNFR1-associated death domain
TS	=	Thymidylate synthase
TXA2	=	Thromboxane A2
UDG	=	Uracil-DNA glycosylase
UK	=	Uridine kinase
UP	=	Uridine phosphorylase
VEGF	=	Vascular-endothelial growth factor
VEGFR	=	Vascular-endothelial growth factor receptor

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER I
INTRODUCTION

Background and Rationale

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide. It ranks the third in males and the second in females with over 1.2 million new cancer cases (1). In Thailand, colorectal cancer is the second most commonly diagnosed cancers in male (14.8% of all cases) and the third in females (10.4% of all cases) (2). Despite the advances in novel cancer therapeutic agents, colorectal cancer remains the leading cause of malignancy-related death with approximately 608,700 deaths estimated to have occurred in 2008 worldwide (1).

Fluoropyrimidines base, 5-fluorouracil (5-FU), remains the first line therapy in colorectal cancer for over 40 years (3). Other standard cytotoxic drugs used in treating metastatic colorectal cancer are capecitabine, irinotecan and oxaliplatin (4). Recently, new targeted therapies that have been approved to use in addition to standard chemotherapy for treating advance colorectal cancer which are anti-vascular endothelial growth factor-bevacizumab and anti-epidermal growth factor receptor-cetuximab and panitumumab. However, these agents come with an increase in toxicity and treatment costs (5).

Over the past decade, epidemiologic studies have shown that patients taking nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, have approximately a 40% to 50% reduction in mortality from CRC (6). As elevated COX-2 expression was found in approximately 50% of adenomas and 85% of adenocarcinomas, it was hypothesized that NSAIDs exert some of anti-inflammatory and antitumor effects through inhibition of the COX-2 (7). Recently, cyclooxygenase-2 (COX-2) inhibitor has become a new candidate to the prevention and treatment of CRC.

The discovery of agents that regulate biological pathways related to COX-2 pathway may provide new avenues for the treatment of CRC. Major attention has been focused on identifying phytochemicals that can inhibit tumor development processes. *Glycosmis parva* CRAIB (Rutaceae) is a wild small shrub found in Thailand. Some plants in the genus *Glycosmis* have been used as Thai traditional medicines for

treatments of abscess, scabies, and snakebite. The genus *Glycosmis* is a rich source of acridone alkaloids and sulfur-containing propanamides. Many biological activities of acridone alkaloids have been reported such as antimalarial activity, anti-viral activity, inhibition of tumor cell growth, and antiproliferative effect (8). Recent works have shown that the ethyl acetate (EtOAc) extract from the leaves of *G. parva* exhibited potent cytotoxic activity in human B-cell lymphoma (9). Moreover, the hexane and EtOAc extract of *G. parva* exhibited anti-inflammatory activity with the reduction in mRNA expression of COX-2 in LPS-activated macrophage J774A.1 cells (10). Taken together, *G. parva* has interesting pharmacological properties that could be further explore in the area of chemotherapy and chemoprevention involved with COX-2 pathway. This study is, therefore, aimed to investigate the effect of ethyl acetate extract from the leaves of *Glycosmis parva* on human colorectal cancer cells and its underlying mechanism of action.

Research Objectives

1. To examine the *in vitro* effect of ethyl acetate extract from the leaves of *Glycosmis parva* on human colorectal cancer cells.
    1) Cytotoxic effect
    2) Induction of apoptosis
    3) Anti-proliferative effect
    4) Cell cycle analysis
2. To investigate the molecular mechanism(s) of ethyl acetate extract from the leaves of *Glycosmis parva* on human colorectal cancer cells.
    1) Expression of cell-cycle regulatory genes.
    2) Expression of genes involved in intrinsic apoptotic pathway (pro-apoptotic and anti-apoptotic of BCL-2 family)
    3) Expression of COX-2 gene.

Hypothesis

Ethyl acetate extract from the leaves of *Glycosmis parva* can induce apoptosis of human colorectal cancer cells by modulation of the expression of genes involved in cell-cycle regulation, induction of apoptosis and COX-2 pathway.

Expected benefit and application

The result of anti-cancer activity of *Glycosmis parva* leaf extract on human colorectal cancer cells and its underlying mechanism involved COX-2 dependent and COX-2 independent pathway could provide the basis for further development of the compounds that target COX-2, BCL-2, and cell cycle regulation pathways which are the potential cancer therapeutic and chemoprevention targets in colorectal cancer.

Key Words

Glycosmis parva, colorectal cancer, cytotoxicity, apoptosis, cell cycle arrest, COX-2, BCL-2, cyclins, HT-29, Colo-205.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER II
LITERATURE REVIEWS

1. Colorectal Cancer (CRC)

Colorectal cancer is the third most common cancer in men (663,000 cases, 10.0% of the total) and the second in women (570,000 cases, 9.4% of the total) worldwide. Almost 60% of the cases occur in developed regions (1; 11). However, the incidence rates are rapidly increasing in several areas historically at low risk including Eastern Asia. About 608,000 deaths from colorectal cancer are estimated worldwide, accounting for 8% of all cancer deaths, making it the fourth most common cause of death from cancer (1). In Thailand CRC is the second most commonly diagnosed cancers in male (14.8% of all cases) and third in females (10.4% of all cases) (2).

The etiology of CRC involves the complex interaction between dietary and genetic factors. Most cases (> 90%) are sporadic. Sporadic cancers arise within adenomatous polyps by the acquisition of a series of somatic genetic mutations following a stereotyped adenoma–carcinoma sequence. Carcinogenesis proceeds through the accumulation of series of genetic mutations involving several tumor-suppressor genes (*APC, P53*), oncogenes (*K-ras*), as well as epigenetic changes (methylation) (12). COX-2 is expressed early during these sequences, suggesting an important role in carcinogenesis as shown in Figure 1. Two major types of inherited (familial) cancer syndromes have been characterized. Familial adenomatous polyposis (FAP) (< 1% of cases) arises as a result of germ-line mutations in the *APC* gene (13). Individuals who are affected typically develop multiple colonic polyps in adolescence and, without treatment, invasive cancer in early adulthood. Hereditary non-polyposis CRC (3 – 5%) is characterized by an autosomal dominant mode of inheritance and early age of cancer diagnosis (14). Modifiable risk factors for CRC include smoking, physical inactivity, overweight and obesity, red and processed meat consumption, and excessive alcohol consumption (1).

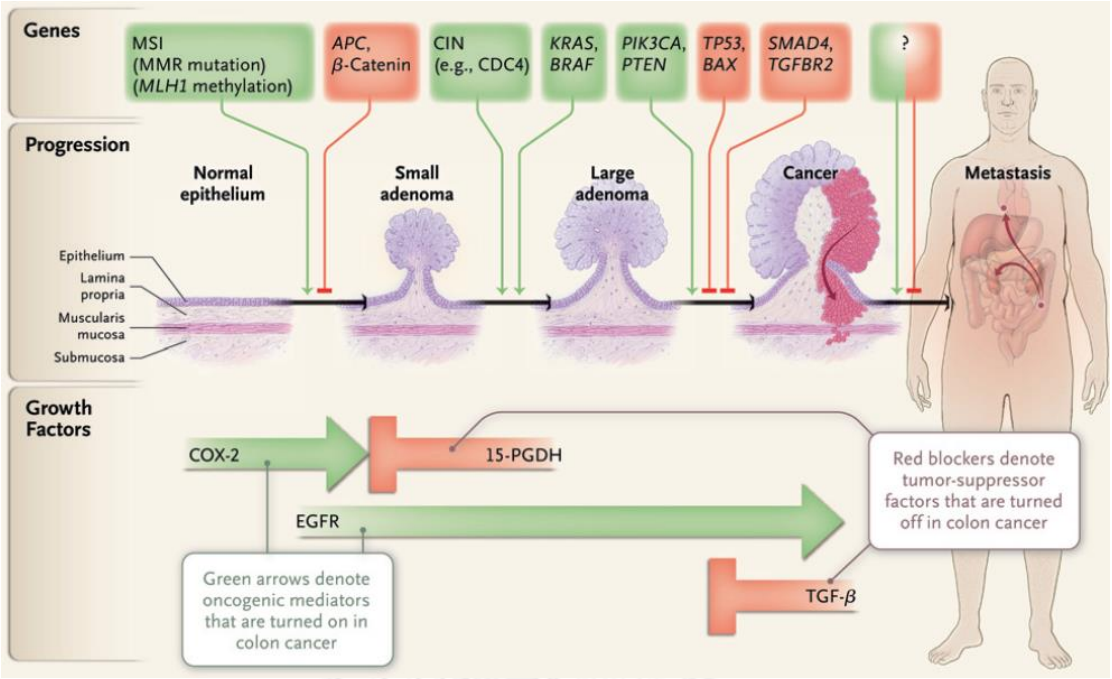


Figure 1: Genes and growth factor pathways that drive the progression of colorectal cancer (15).

The disease begins as a benign adenomatous polyp, which develops into an advanced adenoma and then progresses to an invasive cancer. Invasive cancers that are confined within the wall of the colon (stages I and II) are curable, but if untreated, they spread to regional lymph nodes (stage III) and then metastasize to distant sites (stage IV). Stage I and II tumors are curable by surgical excision, and up to 73% of cases of stage III disease are curable by surgery combined with adjuvant chemotherapy. Recent advances in chemotherapy have improved survival, but stage IV disease is usually incurable (15).

2. Management of colorectal cancer

Resectable colon cancer is recommended to do surgical resection with adequate lymphadenectomy. Adjuvant chemotherapy with FOLFOX, FLOX, CapeOx, 5FU/LV or capecitabine is recommended for patients with stage III disease. Adjuvant chemotherapy for patients with high-risk stage II disease is also an option with 5FU/LV with or without oxaliplatin (FOLFOX or FLOX) or capecitabine with or without oxaliplatin. Recommended initial therapy options for advanced or metastatic disease

depend on whether or not the patient is appropriate for intensive therapy. The more intensive therapy options include FOLFOX, FOLFIRI, CapeOx, and FOLFOXIRI. Addition of biological agents such as Bevacizumab, cetuximab, or panitumumab is either recommended or listed as an option in combination with some of these regimens (16).

Table 1: Chemotherapy regimen for the treatment of CRC

Name	Regimen
FOLFOX	Oxaliplatin 85 mg/m^2 IV over 2 hours, day 1 Leucovorin 400 mg/m^2 IV over 2 hours, day 1 5-FU 400 mg/m^2 IV bolus on day 1, then 1200 mg/m^2/day x 2 days (total 2400 mg/m^2 over 46 – 48 hours IV continuous infusion Repeat every 2 weeks
FLOX	5-FU 500 mg/m^2 IV bolus weekly x 6 + Leucovorin 400 mg/m^2 IV weekly x 6, each 8 week cycle x 3 with oxaliplatin 85 mg/m^2 IV administered on weeks 1, 3, and 5 of each 8 week cycle x 3.
CapeOx	Oxaliplatin 130 mg/m^2 IV over 2 hours, day 1 Capecitabine 850 – 1000 mg/m^2 twice daily PO for 14 days Repeat every 3 weeks
5-FU/ Leucovorin	Leucovorin 500 mg/m^2 IV over 2 hours and repeated weekly x 6 5-FU 500 mg/m^2 IV bolus given 1 hour after the start of leucovorin and repeated weekly x 6 Every 8 weeks for 4 cycles
Capecitabine	Capecitabine 1250 mg/m^2 twice daily PO days 1-14 every 3 weeks x 24 weeks
FOLFIRI	Irinotecan 180 mg/m^2 IV over 30-90 minutes, day 1 Leucovorin 400 mg/m^2 IV infusion to match duration of irinotecan infusion, day 1 5-FU 400 mg/m^2 IV bolus on day 1 then 1200 mg/m^2/day x 2 days (total 2400 mg/m^2 over 46 – 48 hours) IV continuous infusion Repeat every 2 weeks

Table 1: Chemotherapy regimen for the treatment of CRC (Cont.)

Name	Regimen
FOLFOXIRI	Irinotecan 165 mg/m^2 IV day 1, Oxaliplatin 85 mg/m^2 IV day 1, Leucovorin 400 mg/m^2 IV day 1, 5-FU 3200 mg/m^2 over 48 hour continuous infusion starting on day 1 Repeat every 2 weeks

3. Chemotherapeutic drugs used in CRC

The chemotherapeutic drugs that have been the gold standard for the treatment of advanced CRC for over 40 years are the combination of 5-fluorouracil/Leucovarin (5-FU/LV). It has been proven to provide a survival benefit over supportive care. The newer cytotoxic agents that come into regular practice are irinotecan and oxaliplatin due to improved response rates and survival when combine with 5FU/LV. The newest alternatives are the addition of biological agents, bevacuzimab and cetuximab, to the standard cytotoxic drugs which have a very promising result (17).

3.1 5-Fluorouracil/Leucovorin

The 5-fluorouracil (5-FU) is a fluoropyrimidine antimetabolite drugs. It acts specifically to the cell in S phase of the cell cycle. 5-FU is widely used in the treatment of a range of cancers, including colorectal and breast cancers. 5-FU-based chemotherapy improves overall and disease-free survival of patients with resected stage III colorectal cancer (18).

5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen. Due to the structure similarity, 5-FU rapidly enters the cell by the same facilitated transport mechanism as uracil. It is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The anticancer mechanisms of 5-FU exert through the inhibition of thymidylate synthase (TS) and

incorporation of its metabolites into RNA and DNA therefore disrupting RNA and DNA synthesis (18).

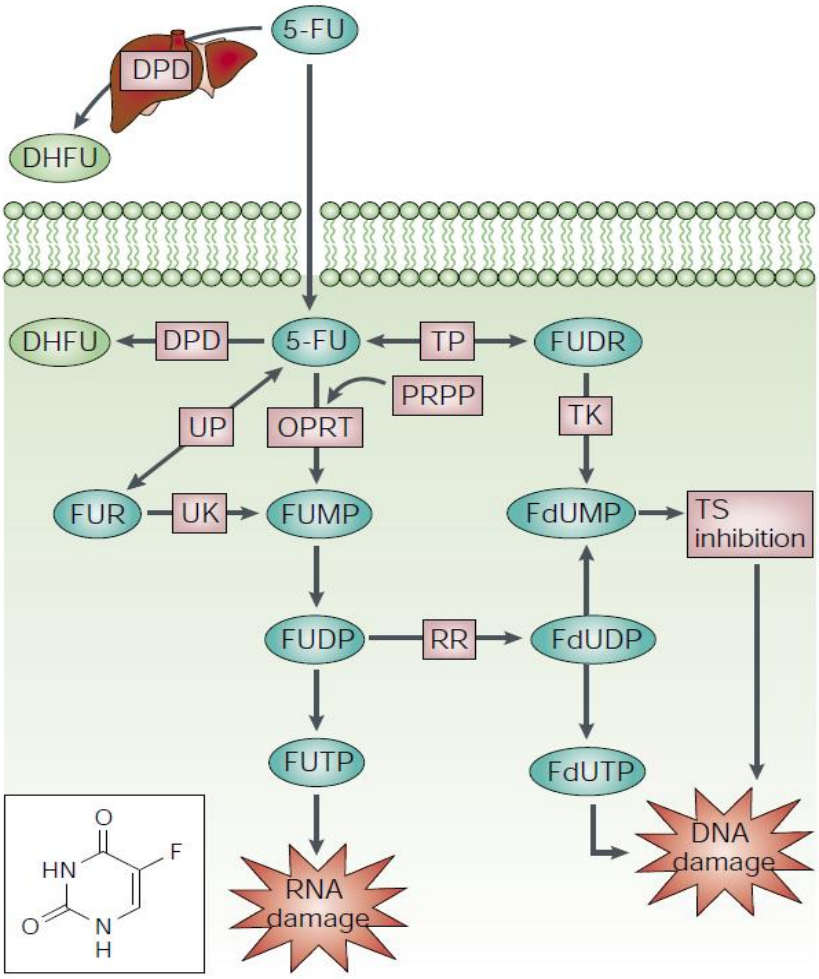


Figure 2: 5-Fluorouracil metabolism (18).

Chulalongkorn University

The anticancer activity of 5FU can be enhanced by combining with leucovorin (LV, 5′-formyltetrahydrofolate). LV enters the cell via the reduced folate carrier and is anabolized to reduced folate (CH_2THF). High intracellular levels of the CH_2THF are necessary for optimal binding of FdUMP to TS. Moreover, CH_2THF is polyglutamated by folylpolyglutamate synthetase. Polyglutamation results in increases the cellular retention of CH_2THF, and enhances the stabilization of its ternary complex with TS and FdUMP. LV has been used to increase the intracellular concentration of CH_2THF and has been shown to enhance the toxicity of 5-FU in many cancer cell lines (18).

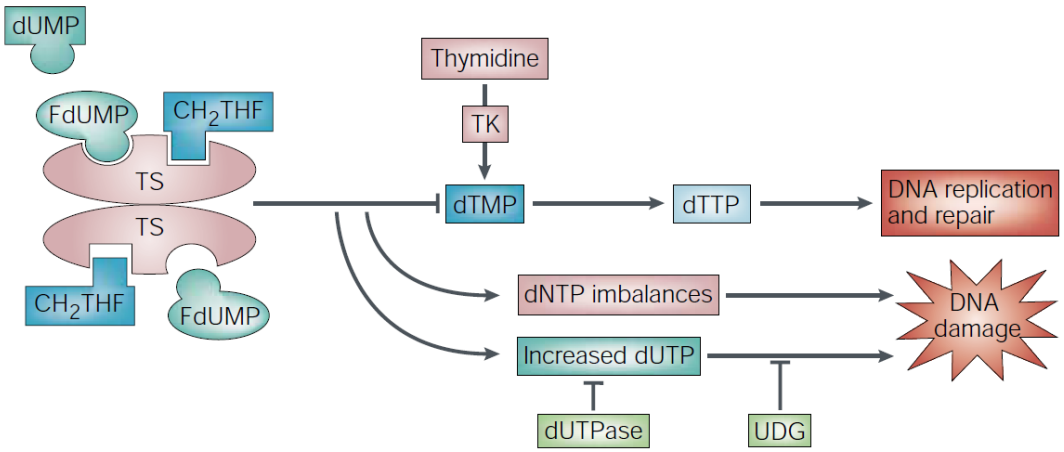
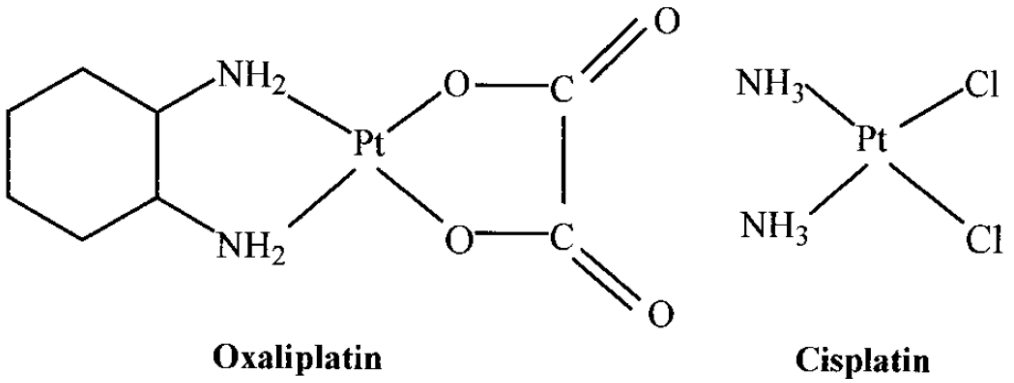


Figure 3: Mechanism of thymidylate synthase inhibition by 5-fluorouracil (18).

3.2 Oxaliplatin

Oxaliplatin is a third-generation platinum derivative, which, when combined with 5FU and leucovorin, is among the most effective chemotherapies for metastatic colorectal cancer (19). The mechanism of action of platinum compounds is similar to the alkylating agents. It covalently binds to nucleophilic sites on DNA to form a drug-DNA complex. The platinum compounds can form both intrastrand and interstrand cross-links with the DNA. DNA adducts inhibit DNA replication and transcription and lead to breaks and miscoding, and if recognized by p53 and other checkpoint proteins, induction of apoptosis (20).

Oxaliplatin, a diaminocyclohexane-containing platinum, has a spectrum of activity and mechanisms of action and resistance that different from those of other platinum-containing compounds (20).



Oxaliplatin **Cisplatin**

Figure 4: Chemical structures of oxaliplatin and cisplatin (20).

3.3 Irinotecan

Irinotecan is a semisynthetic analog of camptothecin, originally isolated from the Chinese/Tibetan ornamental tree *Camptotheca acuminata*. The prototype drug is topotecan (21). Irinotecan has shown activity against colorectal, esophageal, gastric, non-small-cell and small-cell lung cancers, leukemia and lymphomas, as well as central nervous system malignant gliomas (22).

The mechanism of action of irinotecan is by interacting with topoisomerase I (Topo I) in the cell. The topoisomerase I is the enzyme that unwind DNA strand to facilitate DNA replication. The Topo I cuts one strand of double-stranded DNA, relax the strand, and reanneal the strands. Irinotecan binds to the Top I-DNA complex, stabilizing it and preventing re-ligation of the complex. Collision with advancing replication forks results in the formation of double-stranded DNA breaks. These breaks activate cell cycle arrest and, if unrepaired, can cause cell death. It is a chemotherapy agent that acts in the S-phase of cell cycle (23).

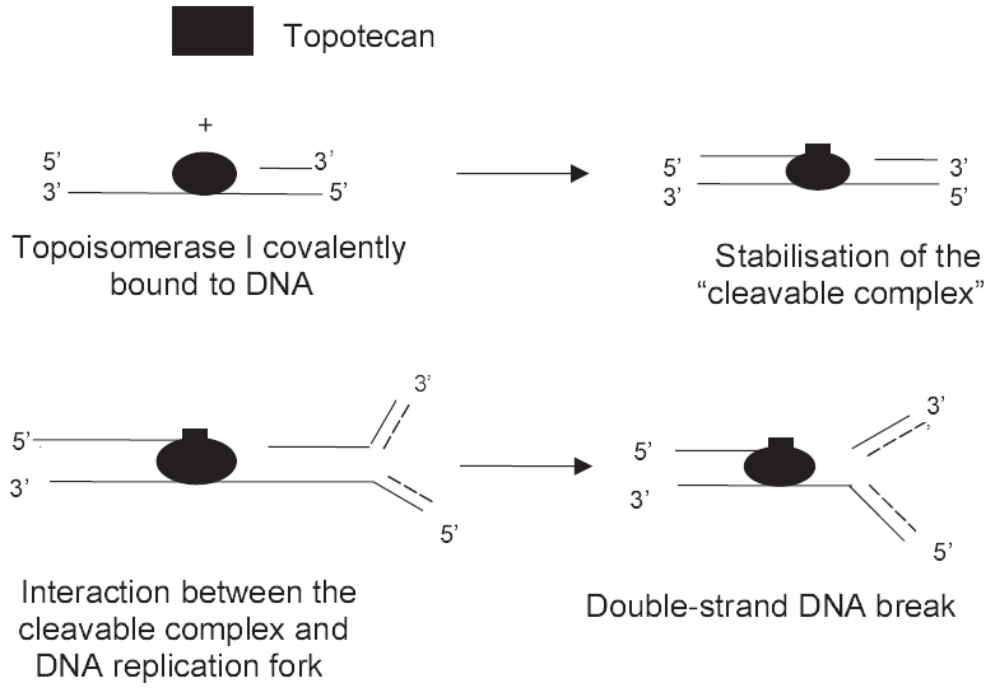


Figure 5: Mechanism of action of topotecan (24).

3.4 Bevacizumab

Bevacizumab is a humanized monoclonal antibody against the vascular-endothelial growth factor (VEGF). VEGF is a potent pro-angiogenic factor, the stimulator of the growth of vascular endothelial cells. Bevacizumab binds to VEGF-A, which prevents its interaction with the VEGF receptor tyrosine kinases VEGFR1 and VEGFR2 receptors (25).

VEGF expression is up-regulated in several tumor types, including breast, ovarian, non–small cell lung, and colorectal cancer. Its expression correlates with neovascularization within tumors. In colorectal cancer, micro-vessel density is associated with progression of adenomas to carcinomas and with metastatic potential and poor prognosis. Bevacizumab is FDA approved for metastatic colorectal cancer in combination with 5-FU based chemotherapy (26).

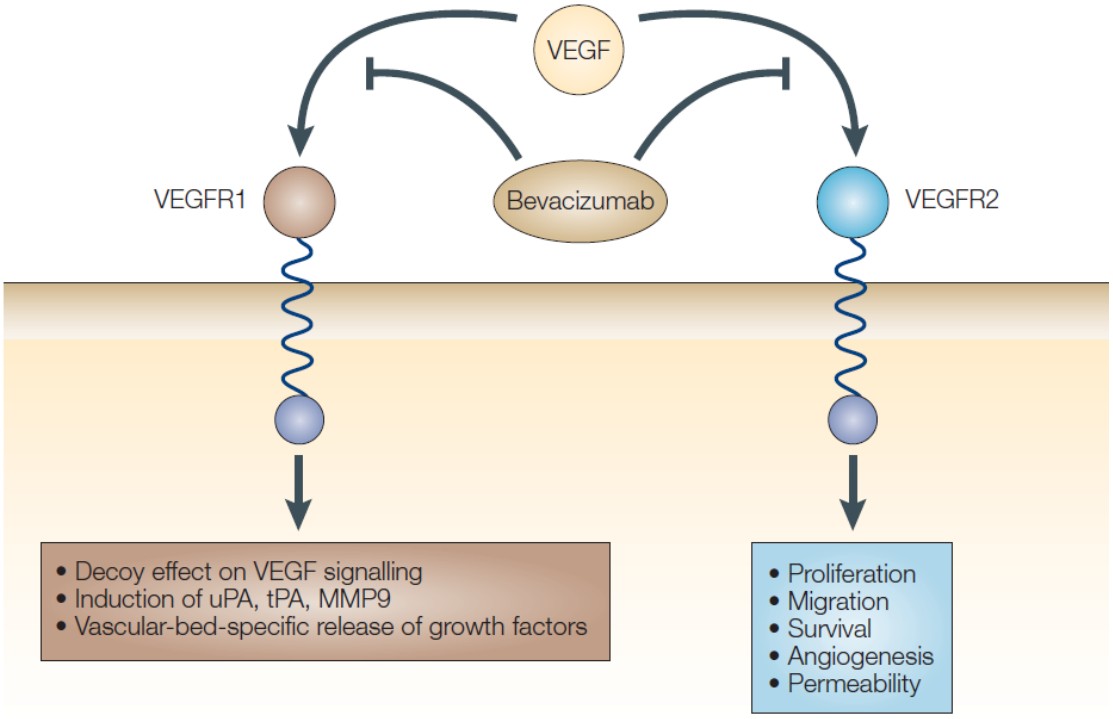


Figure 6: Mechanism of action of Bevacizumab (25).

3.5 Cetuximab

Cetuximab is a chimeric IgG_1 monoclonal antibody that recognizes the epidermal growth factor receptor (EGFR). Activation of EGFR tyrosine kinase receptor is associated with proliferation, survival, and angiogenesis. EGFR expression is found in 60–75% of colorectal cancers, where it has been linked to tumor progression and a poor prognosis. Multiple epithelial cancers, such as breast, lung, kidney, prostate, brain, pancreas, bladder, and head and neck malignancies, also express EGFR and are potential therapeutic targets of cetuximab (27).

Cetuximab binds to the extracellular domain of EGFR, blocks ligand induced receptor signaling and modulates tumor cell growth. Immune-mediated antitumor mechanisms, such as antibody-dependent cell-mediated cytotoxicity (ADCC), may also contribute to the activity of cetuximab (28).

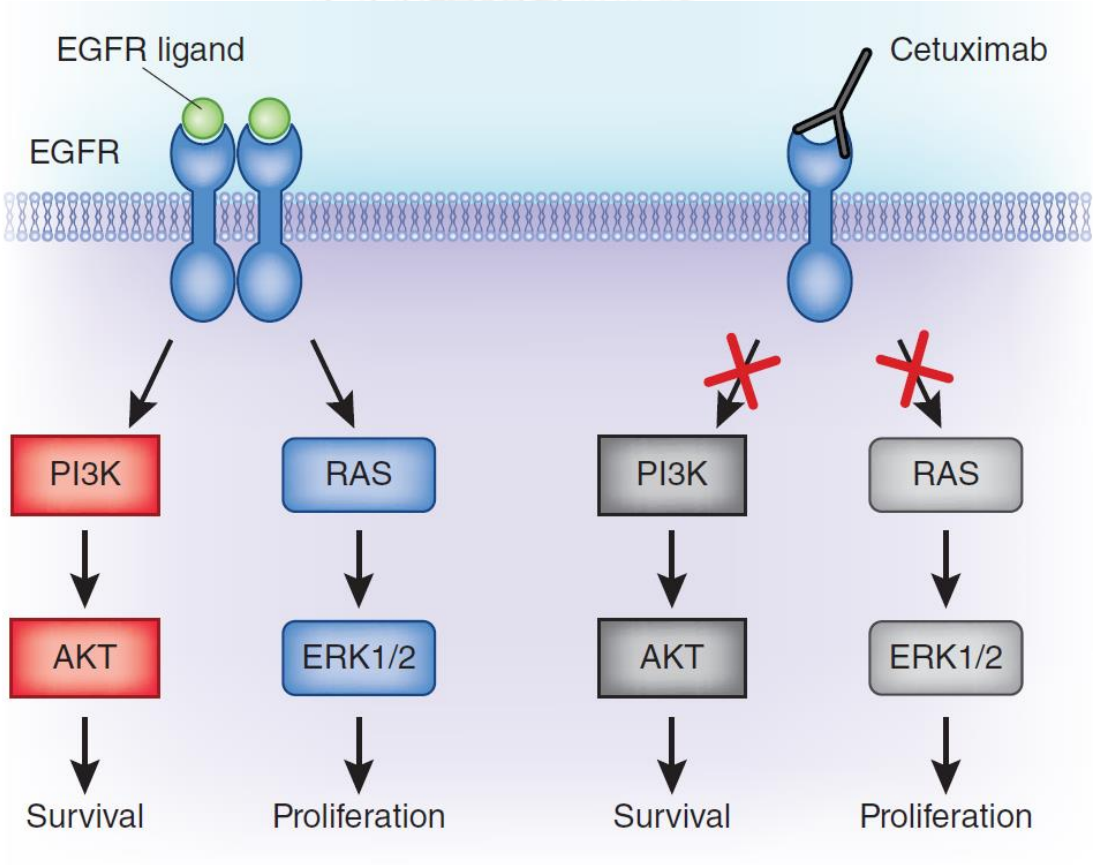


Figure 7: Mechanism of action of Cetuximab (29).

Cetuximab is FDA approved for the treatment of EGFR-positive metastatic colorectal cancer as a single agent in patients who were unable to tolerate irinotecan-based therapy, or in combination with irinotecan for refractory patients (27).

4. COX-2 and CRC

4.1 COX biology

Cyclooxygenases (or prostaglandin H synthases), commonly known as COXs, are a family of myeloperoxidases located at the luminal side of the endoplasmic reticulum and nuclear membrane, which catalyze the rate-limiting step of prostaglandin biosynthesis from arachidonic acid (30). These enzymes act by two coupled reactions. The first one is the conversion of arachidonic acid to prostaglandin G_2 by the cyclooxygenase activity. The second reaction is the conversion of prostaglandin (PG) G_2 to prostaglandin H_2 which mediated by the peroxidase activity. Then, different synthases convert prostaglandin H_2 to prostaglandin D_2, $F_{2}\alpha$, E_2, I_2, and thromboxane A_2.

Prostaglandins are immediately released from the cells to act locally in an autocrine and paracrine way through different receptors and activating different intracellular pathways (31). Important physiological functions of prostaglandins are vasodilatation (PGD_2, PGE_2, PGI_2), gastric cytoprotection (PGI_2), maintenance of renal homeostasis, and platelet aggregation (TXA_2). Besides, prostaglandins play a major role in mediating fever (PGE_2), pain sensitivity, and inflammation. Up to now, three isoforms of COXs have been identified.

Cyclooxygenase-1 (COX-1) is constitutively expressed in different tissues. COX-1 plays a role in tissue homeostasis by modulating several cellular processes ranging from cell proliferation to angiogenesis or platelet aggregation due to thromboxane production (30). Cyclooxygenase-2 (COX-2) is the inducible isoform, which is regulated by growth factors and different cytokines such as IL1β, IL6, or TNFα which overexpressed during inflammation (32). The COX-2 promoter displays an NF-kB response element as well as other cytokine-dependent (i.e., IL6) response elements

(30). Lastly, COX-3 has been identified as a splice variant of COX-1, and it is present mainly in brain and spinal cord. Currently, the role of COX-3 is not known (33).

4.2 COX-2 expression in CRC

The relationship between COX expression and tumor biology has shown that higher COX-2 expression is associated with larger tumor size, more advanced stage of disease, an increased likelihood of lymph node involvement and a reduced likelihood of patient survival (34). Five-year survival in the absence of COX-2 expression is 91.6% compared with a survival rate of 40.5% for those patients whose tumors express the enzyme (35). Animal models provide additional evidence of the role of COX-2 in colorectal carcinogenesis (36). The multiple intestinal neoplasia mice model resembles the familial adenomatous polyposis (FAP) syndrome in human. COX-2 levels are elevated in the polyps of these MIN mice (37). COX-2 is also detected in pre-cancerous lesions and is increased in the later stages of cancer development (38). The elevation of COX-2 early in the development of CRC, suggests a role of COX-2 in the carcinogenesis. Other evidence of the involvement of COX-2 in CRC was found by using gene knockout techniques. In the APC MIN mice model, the COX-2 gene was disrupted to investigate the role of COX-2 in the formation of polyps. The development of intestinal adenomas was less than six-fold in these COX-2 knockout MIN mice compared to the COX-2 wild-type MIN mice. Although polyp formation in COX-$2^{-/-}$ MIN mice was not totally prevented, this study demonstrated direct genetic evidence of the importance of COX-2 in the carcinogenesis in CRC (39). Taken together, the evidence from animal studies reinforces the concept that COX-2 plays an important role in tumor development.

Cyclooxygenase-2-derived PGs are involved in many of pathological processes include CRC. Multiple lines of evidence have demonstrated that PGE_2 mediates the tumor-promoting effects of COX-2 in CRC. The steady-state cellular levels of PGE_2 depend on the relative rates of COX-2/PGE synthase-dependent biosynthesis and 15-hydroxyprostaglandin dehydrogenase (15-PGDH)-dependent degradation (7). PGE_2 has a predominant role in promoting colorectal tumor growth. PGE_2 is the most abundant PG found in human CRC (40). In contrast, 15-PGDH is highly expressed in

normal colon mucosa but is lost in most human CRCs (41). Moreover, the loss of 15-PGDH leads to resistance of the antitumor effects of celecoxib in an azoxymethane (AOM) mouse model (42). Recent studies showed that PGE_2 treatment drastically increased both small and large intestinal adenoma burden in $Apc^{Min/+}$ mice and significantly enhanced AOM-induced colon tumor incidence and multiplicity (43). In contrast, inhibition of endogenous PGE_2 by genetic deletion of mPGES-1 suppressed intestinal tumorigenesis in $Apc^{Min/+}$ and AOM models (44).

Many reports have shown that PGE_2 promotes colorectal tumor growth by stimulating angiogenesis, cell invasion, cell growth and inhibiting apoptosis. These processes are regulated by PGE_2-activated signaling pathways, including EGFR, PI3K-Akt, Ras-MAPK, PPARδ, VEGF, BCL-2, chemokines and their receptors (45; 46). On the other hand, many downstream targets of PGE_2 can also up-regulate COX-2 expression. Such positive feedback loops may amplify the activity of the COX-2 pathway and magnify the potency of COXIBs (7).

4.3 NSAIDs and coxibs in colorectal cancer

NSAIDs are widely used as analgesics, anti-inflammations and anti-pyretics. Their anti-inflammatory actions result from the inhibition of COX activity and consequently PGs formation. Aspirin and many other NSAIDs inhibit non-specifically both isoforms of COX, while some newer generations, such as the coxibs, have higher selectivity on the COX-2 isoform. COX-inhibitors block the synthesis of PGs by directly competing with the substrate, arachidonic acid, for access to the active site of the COX enzyme. Aspirin, the prototype NSAID, has a distinct mechanism of action from other NSAIDs. It irreversibly inactivates COX by acetylating a serine residue within the active site of both COX-1 and COX-2 (12).

A multiple line of evidence from population-based studies, case–control studies and clinical trials indicate that regular use of NSAIDs including aspirin and COXIBs over a 10- to 15-year period reduces the relative risk of developing CRC by 40–50% (47-49). Moreover, aspirin use was associated with a risk reduction in patients whose colon tumors expressed higher levels of COX-2 (50). Besides prevention, regular aspirin use after the diagnosis of CRC at stage I-III improves overall

survival, especially among patients with tumors that overexpress COX-2 (51), suggesting the potential therapeutic use of NSAIDs in advanced CRC.

Furthermore, several large randomized double-blind placebo-controlled trials have demonstrated preventive effects of NSAIDs on patients with previous polyps by reducing the risk of colorectal (adenomatous) polyp recurrence (52; 53). These findings alerted investigators to evaluate the preventive effects and safety of COXIBs in patients with FAP or previous history of adenomas. Observational studies demonstrating that both celecoxib and rofecoxib prevented the occurrence and recurrence of CRC stimulated growing interest in the anti-neoplastic properties of this new generation of NSAIDs (54). Particularly, the evidence that treatment of FAP patients with celecoxib significantly reduced the polyp burden in a randomized controlled trial led to the Food and Drug Administration approval of celecoxib for use in patients with FAP at 400 mg twice a day (55). Initial enthusiasm for the use of selective COX-2 inhibitors waned following reports of an increased risk of cardiovascular events in patients taking these agents for long periods in CRC chemoprevention trials. The results of the APPROVe (Adenomatous Polyp Prevention on Vioxx™) trial showed a doubling in the relative risk of thrombotic events in patients receiving rofecoxib for > 18 months and led to the withdrawal of the drug by the manufacturer (56). Likewise, long-term treatment with celecoxib in the APC (adenoma prevention with celecoxib) trial was also associated with increased risk of death from cardiovascular causes (57). However, the APPROVe, APC and subsequent polyp prevention trials confirmed that both rofecoxib and celecoxib were effective in the reduction of colorectal adenoma recurrence (52; 58; 59). The mechanism of increased cardiovascular risk of COXIBs probably involves reduction in vascular production of the anti-thrombotic prostacyclin, without a change in the generation of the pro-thrombotic thromboxane (derived from COX-1 activity in platelets) (60).

5. Apoptosis pathway

Apoptosis or programmed cell death is an essential physiological process that plays a crucial role in controlling the number of cells in development and

throughout the life by removal of cells at the appropriate time. However, apoptosis is also involved in a broad range of pathological conditions include neurodegenerative diseases, cardiovascular diseases, immunological diseases, and cancer (61). There are 3 different pathways to initiated apoptosis: 1) extrinsic apoptotic pathway which can be trigger by binding of death ligands to death receptors and consequent caspase-8 activation; 2) intrinsic apoptotic pathway, which is initiated by cellular stress followed by activation of caspase-9; or 3) Perforin/granzyme pathway, where the cytotoxic T cell produces protease enzyme and delivers to sensitize target cells. Each of these pathways converges to a common execution step that requires proteolytic cleavage and activation of caspases-3 and/or -7 (62).

5.1 Intrinsic apoptotic pathway

The intrinsic apoptotic pathway or mitochondria pathway is triggered by various extra- and intracellular stimulus, including oxidative stress, irradiation, and cytotoxic drugs (62). This pathway is involved with the permeability of the mitochondrial membrane which is regulated by the BCL-2 family proteins. When induction of apoptosis, the pro-apoptotic BCL-2 proteins, Bax and Bak, insert into mitochondrial membrane and cause cytochrome c release from the mitochondrial inter-membrane space into the cytosol. Anti-apoptotic BCL-2 family proteins, BCL-2 and BCL-XL, prevent cytochrome c release by binding and inhibition of BAX and BAK. Cytochrome c then binds to the Apaf1 and together with dATP causes recruitment of pro-caspase-9 to the complex, called apoptosome (63; 64). Caspase-9 is then activated and in turn activates caspase-3 to initiate the proteolytic cascade (65).

5.2 Extrinsic apoptotic pathway

The extrinsic apoptotic pathway is initiated by extracellular ligands bind with death receptors. Typical death receptors are Fas (fibroblast associated antigen or Apo-1 or CD95) and tumour necrosis factor receptor 1 (TNFR1). Binding of ligands to death receptors induce receptor clustering and formation of a death inducing signalling complex (DISC) (66) in which the adaptor proteins FADD and/or TRADD bind

with their death domain (DD) to a death domain of the receptors in the cytoplasmic region. This leads to the recruitment of caspase-8 or -10 (initiator caspases) to the DISC (67). Activated caspase-8 then directly cleaves pro-caspase-3 or other executioner caspases, and finally leading apoptosis cell death. Caspase-8 can also cleave the BH3-only protein Bid. The resulting truncated Bid (tBid) then moves to the mitochondria and induces cytochrome c release, leading to activation of caspase-9 and caspase-3 (68).

5.3 Perforin/Granzyme pathway

Cytotoxic T cells can exert their cytotoxic effects on tumor cells and virus-infected cells via a pathway that involves secretion of the transmembrane pore-forming molecule perforin with a subsequent exophytic release of cytoplasmic granules through the pore and into the target cell (69). The serine proteases granzyme A and granzyme B are the most important component within the granules. Granzyme B will cleave proteins at aspartate residues and will therefore activate pro-caspase-10 (70). Moreover, granzyme B can utilize the mitochondrial pathway for amplification of the death signal by specific cleavage of Bid and induction of cytochrome c release (71). However, granzyme B can also directly activate caspase-3. In this way, the upstream signaling pathways are bypassed and there is direct induction of the execution phase of apoptosis.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

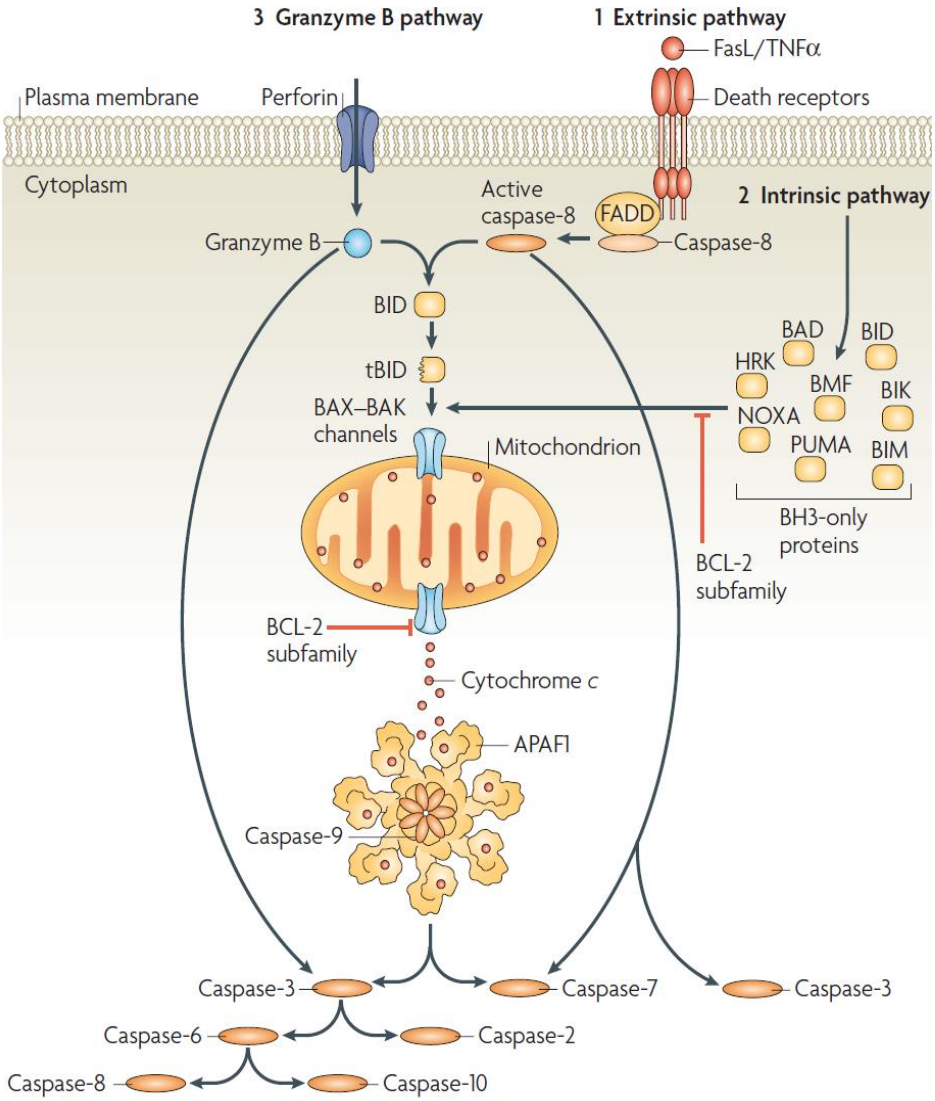


Figure 8: Apoptosis pathway (72).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

6. BCL-2 family protein and the regulation of apoptosis

The BCL-2 family proteins are key molecules which function as anti-apoptotic or pro-apoptotic in the intrinsic apoptotic pathway. All of BCL-2 family structure shares the same conserved regions termed BCL-2 homology (BH) domains. It can be divided into 3 sub group according to the function and structure.

1) Pro-apoptotic BH3 only proteins - BIM, PUMA, BID, BAD, BIK, BMF, HRK, NOXA
2) Pro-apoptotic BAX-like proteins – BAX, BAK
3) Anti-apoptotic Bcl-2-like proteins - BCL-2, BCL-XL, BCL-W, MCL-1, A1

The pro-apoptotic BH3-only proteins share only the BH3 region of homology and serve as upstream sensitizer, becoming activated in response to various forms of cellular stress, whereas pro-apoptotic Bax-like proteins contain multiple BH domains (BH1, BH2 and BH3) and are required downstream of BH3-only proteins to induce apoptosis. The anti-apoptotic proteins share up to four regions of sequence homology (BH1-4**)** (73).

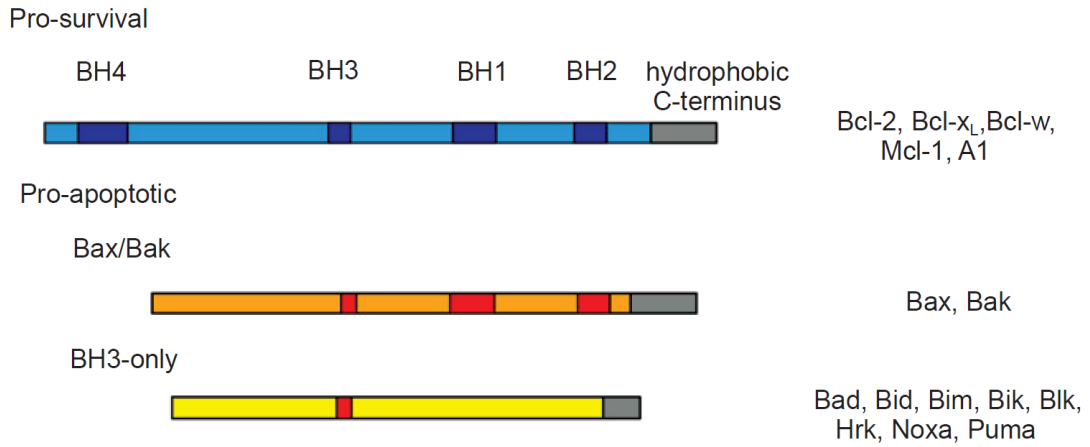


Figure 9: Sequence homology between proteins of the BCL-2 family (73).

Activating intrinsic apoptotic pathway requires both types of pro-apoptotic proteins. The BH3-only proteins act as damage sensors and direct antagonists of anti-apoptotic BCL-2-like proteins. whereas the Bax-like proteins, once activated, act further downstream, probably by permeabilizing the mitochondrial outer membrane and perhaps also by perturbing the endoplasmic reticulum (ER)/nuclear envelope (74).

Bax and Bak appear to be largely redundant in function. A biochemical difference between Bax and Bak is that, in healthy cells, Bax is largely cytosolic or loosely associated with mitochondria, whereas Bak is an integral membrane protein on the cytosolic face of the mitochondrion and ER. In response to cytotoxic signals, Bax translocates to those membranes, and both Bax and Bak change conformation and form membrane-associated homo-oligomers. These oligomers are thought to permeabilize the outer mitochondrial membrane (74).

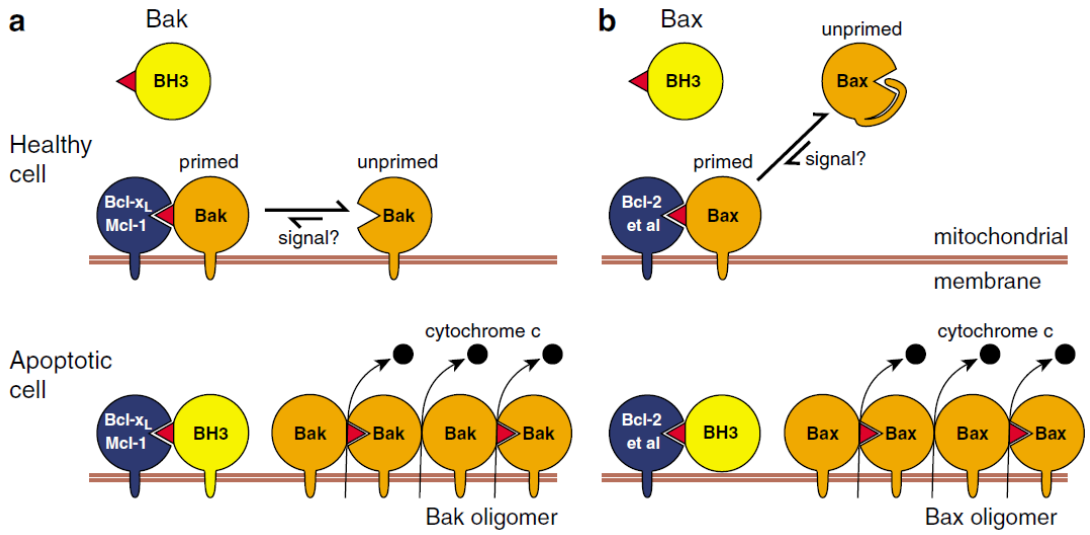


Figure 10: Model for the regulation of Bak and Bax by their pro-survival relatives (74).

The anti-apoptotic activity of BCL-2 has been demonstrated in both overexpression and gene targeting studies. Overexpression of any of the five pro-survival members protects cells against apoptosis induced by a variety of cytotoxic stimuli family (73). Anti-apoptotic BCL-2 proteins are generally integrated within the mitochondrial outer membrane, but may also be in the cytosol or ER membrane. They preserve mitochondrial outer membrane integrity by directly inhibiting the pro-apoptotic BCL-2 proteins (75).

7. The regulation of Cell cycle

The eucaryotic cell cycle is divided into four stages: G1, S, G2, and M. G1 is the gap phase during which cells prepare for the process of DNA replication. During the G1 phase, cell integrates mitogenic and growth inhibitory signals and makes the decision to proceed, pause, or exit the cell cycle. S phase is defined as the stage in which DNA synthesis occurs. G2 is the second gap phase during which the cell prepares for the process of division. M stands for mitosis, the phase in which the replicated chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells. In addition to G1, S, G2, and M, the term G0 is used to describe cells that have exited the cell cycle and become quiescent (76).

The ability of the cells to divide is mainly attributed to the presence of two classes of molecules, cyclin-dependent kinases (CDKs), a family of serine/threonine kinases and their binding partners, cyclins, which are synthesized and destroyed at specific times during the cell cycle, thus regulating kinase activity in a timely manner.. The discovery of CDKs and cyclins has increased considerably during evolution. However, only certain CDK–cyclin complexes are thought to control cell cycle progression. According to the 'classical' model for the mammalian cell cycle, specific CDK–cyclin complexes are responsible for driving the various events known to take place during interphase in a sequential and orderly fashion (77).

In early G1 phase, Cdk4 and/or Cdk6 are activated by D-type cyclins and initiate phosphorylation of the retinoblastoma protein (Rb) family. This leads to the release of E2F transcription factors and results in the activation and transcription of E2F responsive genes required for cell-cycle progression. Early E2F responsive genes include E- and A-type cyclins. In the late G1 phase, Cdk2 is activated by binding to cyclin E and completes the phosphorylation of Rb leading to further activation of E2F mediated transcription. This leads to passage through the restriction point at the boundary of the G1/S phase, and to S phase initiation. Later Cdk2 plays an important role in S phase progression by complexing with cyclin A. A-type cyclins are synthesized at the onset of the S phase and phosphorylate proteins involved in DNA replication. During the G2/M transition, Cdk1/cyclin A activity is required for the initiation of prophase. Finally, Cdk1/cyclin B complexes actively participate in and complete mitosis (78).

The activities and functions of Cdk/cyclin complexes under normal as well as extreme conditions, such as stress, DNA damage, telomere dysfunction and others, are regulated by two families of Cdk inhibitors; the INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$) specifically bind to Cdk4 and Cdk6 and prevent D-type cyclin activity and the Cip/Kip family ($p21^{Cip1/Waf1/Sdi1,}$ $p27^{Kip1}$, $p57^{Kip2}$) inhibits Cdk2/cyclin E, Cdk2/cyclin A, Cdk1/cyclin A, as well as Cdk1/cyclin B activity (78).

The basic concept put forward by this model, that is, that each phase of the cycle is driven by specific CDKs, has been recently challenged by genetic studies in mice. Extensive analyses of these models revealed that most of the Cdks and

cyclins, originally thought essential for cell cycle regulation, are in fact largely dispensable. Cyclin A2 and B1 emerged as the most non-redundant cyclins. CDK2, CDK4 and CDK6 are not essential for the mammalian cell cycle. Instead, they are only required for the proliferation of specific cell types. By contrast, Cdk1 emerged as the master regulator of mammalian cell-cycle regulation whereas and Cdk1 alone by complexing with all the cyclins can drive the cell cycle, at least until mid-gestation (77).

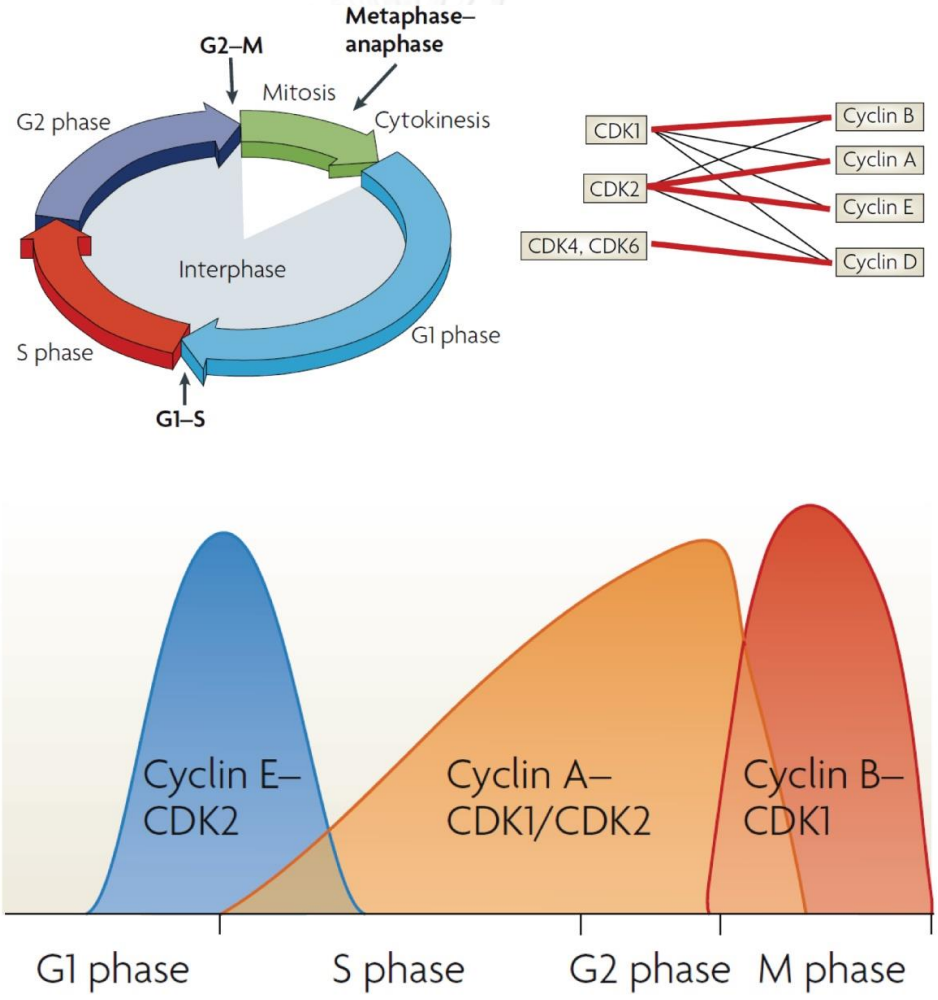


Figure 11: The control of cell cycle (79).

8. *Glycosmis parva*

8.1 Plant

Glycosmis parva Craib (Rutaceae) is wild small shrub distributed in Thailand with general name of Som-chuen or Prayon-Kluean. The botanical characteristics are leaves alternate, 1-5 foliate. Flowers are usually small, axillary panicles, calyx 4 or 5 partial imbricate. Petals 4 or 5 imbricate, stamen 8 to 10 free, filaments dilated below. Ovary 2 to 5 celled, the style very short, not jointed ovule 1 in each cell. Fruits globose, freshy, berry. Seed 1 to 3 oblong, testa membranous (80).

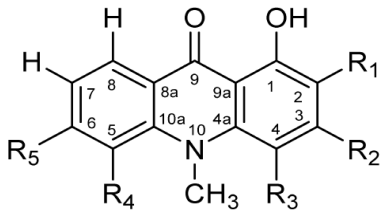
Figure 12: *Glycosmis parva* CRAIB

8.2 Compounds

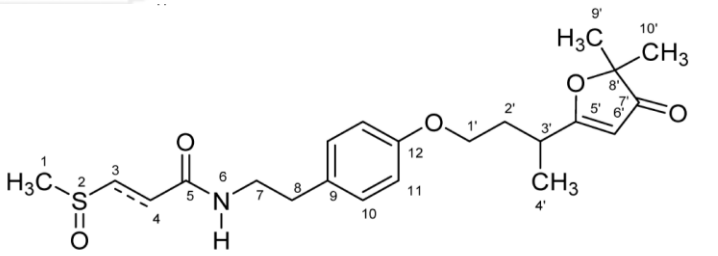
The genus *Glycosmis* is a rich source of acridone alkaloids and sulfur-containing propanamides especially in branches and leaves, respectively. Chemical examination of the branches and leaves of *G. parva* led to the identification of the following compounds (8);

Table 2: Chemical components from the branches and leaves of *G. parva*

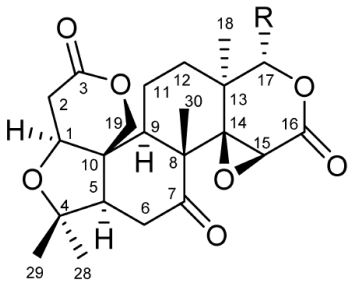
Part	Main Structure	Chemical name
Branches	Acridone alkaloids	1. Glycosparvarine 2. N-methylatalaphylline 3. N-methylcyclo-atalaphylline-A 4. Glycofolinine 5. Citramine
	Limonoids	1. Limonin 2. Limonexic acid 3. Isolimonexic acid
Leaves	Sulfur-containing propanamide derivatives	1. S-deoxydihydroglyparvin 2. S-deoxytetrahydroglyparvin 3. Glyparvin-A 4. Dihydroglyparvin
	Acridone alkaloids	1. Arborinine



Acridone alkaloids



Sulfur-containing propanamide derivatives



Limonoids

Figure 13: Structure of chemical constituents found in *G. parva* (8)

8.3 Pharmacological activities

Some plants in the genus *Glycosmis* have been used as Thai traditional medicines for treatments of abscess, scabies, and snakebite. Numbers of biological activities of the compound isolated from *Glycosmis* spp. have been reported. The majority of them are cytotoxicity, anti-tumor activity, antiviral activity and anti-malarial activity of acridone alkaloids. For sulfur-containing propanamides, antifungal activities were mainly reported.

Antimaralial effects

Many acridone alkaloids from *Glycosmis* spp. exhibited antimaralial activities that were comparable to anti-malarial drug-chloroquin. 5-hydroxynoracronycine at 10 μg/ml inhibited 90% of *Plasmodim yoelii*, *in vitro* (81).

Antiviral activities

Atalaphillidine, citracridone-I and 5-hydroxy-N-methylseverifoline possessed antiviral activity against herpes simplex virus type 2 (HSV-2) (82). Compounds isolated from leaves and branches of *G. parva* exhibited an antiviral activity against both HSV-1 and HSV-2. Glycosparvarine, glycofolinine and (+)-tetrahydroglyparvin showed moderate activities with EC_{50} if 151 μM, 348 μM and 229 μM, respectively. While (+)-S-deoxydihydroglyparvin exhibited more potent activities with EC_{50} of 29.8 μM and 44.6 μM, respectively, against HSV-1 and HSV-2 (8).

Antifungal activities

Many sulfur-containing propanamide compounds found in *Glycosmis spp.* exhibited antifungal activity. Methylillukumbin-A at very low concentration (10 μg/ml) showed antifungal activity against *Cladosporium cladosporioides* (83). Moreover, it could inhibit *Cladosporium herbarum* with an ED50 of 5.5 μg/ml (84).

Anti-inflammation

The hexane and EtOAc extracts from the branches and leaves of *G. parva* inhibited nitric oxide production, mRNA expression of iNOS, COX-2, and pro-inflammatory cytokines (IL-1, IL-6,TNF-α) in LPS-activated macrophage J774A.1 cells (10).

8.4 Anti-cancer activities

Several effects related to anti-cancer activities have been reported from the compounds found in Glycosmis spp.

Anti-proliferative effect

Acridone alkaloids have been reported to possess antiproliferative effect. Atalaphyllidine and des-*N*-methylnoracronycine inhibited clonal proliferation and induce cell differentiation in HL-60 (Human promyelocytic leukemia) (85). In addition, atalaphyllidine, 5-hydroxy-N-methylseverifoline and des-N-methylnoracronycine exhibited antiproliferative against several cancer cell lines include human lung carcinoma (A-549), mouse melanoma (B16-melanoma 4A5), T-cell leukemia (CCRF-HSB-2) and human gastric cancer cell and lymph-node metastasized (TGBC11TKB) (86).

Arborinine which is the main acridone alkaloids found in EtOAc extract from the leaves of *G. parva* showed potent antiproliferative effect against cervix adenocarcinoma (HeLa) with an IC_{50} of 1.84 μM which was lower than that of cisplatin (12.43 uM) (36).

Cancer prevention

Glycocitrine-II and O-methylglycocitrine-II had potent cancer prevention properties by inhibiting Epstein-Barr virus early antigen activation with IC_{50} of 280 and 281 mol ration/32 pmol TPA (87).

Inhibit cell cycle progression and induction of apoptosis

Glyfoline inhibited leukemic HL-60 cell growth in vitro with IC50 of 1.1 μM (88). Moreover, it induced nasopharyngeal carcinoma (NPC) cell growth arrest at G2/M and induced apoptosis (89). The EtOAc extract from the leaves of *G. parva* exhibited potent cytotoxic activity (IC50 15.68 μg/ml), induction of apoptosis (decrease BCL-XL, increase BAK), and caused G1/S phase accumulation (decreased cyclin D1 and cyclin E, increase p21) in human B-cell lymphoma (9).

CHAPTER III
MATERIAL AND METHOD

1. Material

1.1 Cell cultures

Human colorectal cancer cell lines, HT-29 and Colo-205, were obtained from American Type Culture Collection-ATCC (Rockville, MD). HT-29 was maintained in Dulbecco's modified Eagle's medium (DMEM) while Colo-205 was propagated in Roswell Park Memorial Institute medium (RPMI) 1640. Medium were supplemented with 10% fetal bovine serum and penicillin (100 units/ml), streptomycin (0.1 mg/ml) at 37°C in a humidified atmosphere with 5% CO_2.

1.2 Tested compounds

The leaves of *G. parva* were collected at Sakaerat, Wang Nam Khieo district, Nakorn Ratchasima province, Thailand in December 2007. A voucher (NSR 510209) has been deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The GPE is a part of solvent partitioned extraction method as mentioned before (8). Ethyl acetate extract from the leaves of *Glycosmis parva* (GPE) were prepared and identified by Associate Professor Nijsiri Ruangrungsi, College of Public Health Sciences, Chulalongkorn University, Bangkok, Thailand. The extract was prepared by partitioned extraction method. Briefly, the dried leaves of *Glycosmis parva* were grounded and macerated with methanol. Afterward, the methanol extract was partitioned with hexane to obtain the hexane extract. The remaining layers were added with water and further partitioned with ethyl acetate (EtOAc) to yield the EtOAc extract (8). All solvents were removed by evaporation. The extract in solid form was stored at -20°C prior to use. The percentage yield of EtOAc extract was 2.08% of dried *G. parva* leaves. Repeated chromatographies of the GPE using the solvent systems of MeOH–$CHCl_3$, acetone–hexane, and acetone–$CHCl_3$ led to the isolation of one acridone alkaloid and four sulfur-containing propanamide derivatives. 0.2% dimethylsulfoxide

(DMSO) was used as a negative control. Standard cytotoxic drugs including cisplatin, oxaliplatin, and 5-FU were used as positive controls.

Table 3: Chemical components found in the EtOAc extract from the leaves of *G. parva* (80)

Main Structure	Chemical name	% in GPE	% of Total
Acridone alkaloids	Arborinine	0.12	65.04
Sulfur-containing propanamide derivatives	S-deoxydihydroglyparvin	0.03	14.81
	S-deoxytetrahydroglyparvin	0.02	7.95
	Glyparvin-A	0.02	7.95
	Dihydroglyparvin	0.01	4.24

1.3 Chemicals

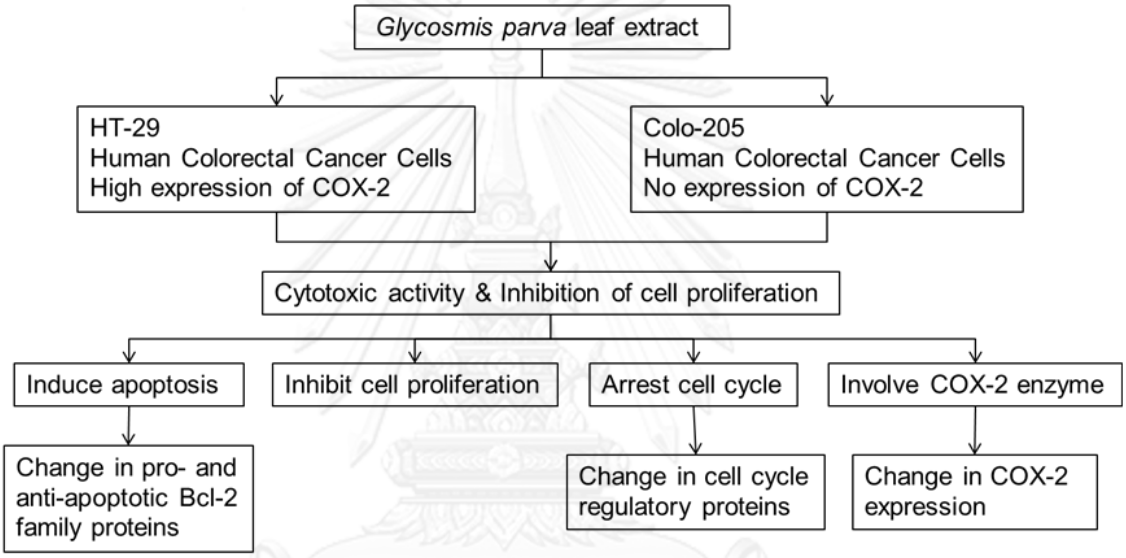
The following reagents and reagent kits were used in this study; DMEM medium (Gibco, USA), RPMI-1640 (Gibco, USA), fetal bovine serum (Gibco, USA), L-glutamine (Gibco, USA), penicillin/streptomycin (Gibco, USA), Trypsin/EDTA (Gibco, USA), 0.4% trypan blue dye (Sigma, USA), DMSO (Sigma, USA), Resazurin (Sigma, USA), annexin V apoptosis detection kit (Invitrogen, USA), Propidium Iodide (Santa Cruz Biotechnology, USA), TRIzol Reagent (Invitrogen, USA), dietyl pyrocarbonate (DEPC) (Molekula, UK), absolute ethanol (Merck, Germany), Improm-II reverse transcription system (Promega, USA), TaqMan® Gene Expression System (Applied Biosystems, USA).

1.4 Equipments and Instruments

The following equipments and instruments were used in this study; analytical balance (GMPH, Satorius, Germany and UMT2, Mettler Toledo, Switzerland), autopipette (Gilson, USA), biohazard laminar flow hood (Science, Germany and Labconco, USA), centrifuge (Hettich, USA and Eppendrof, Germany), autoclave (Hirayama, Japan), pH meter (Mettler Tuledo, Switzerland), refrigerator 4°C and -20°C (Sanyo, Japan), Incubator (Thermo, USA), Light microscope (Nikon, Japan), vortex mixer (Scientific Industries, USA), fluorescence flow cytometer (BD Biosciences, USA),

PCR thermal cycler (Eppendorf, Germany), StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA), T25 tissue culture flasks (Corning, USA), 96-well microplates (Corning, USA), and 6-well culture plates (Corning, USA). MicroAmp Fast 8-tube strip (0.1 ml) (Applied Biosystems, USA), MicroAmp optical 8-cap strip (Applied Biosystems, USA), Scepter™ Handheld Automated Cell Counter (Millipore, USA).

2. Conceptual Framework



3. Methods

All assays were done at least three independent experiments in duplication or triplication. 0.2% DMSO was used as a negative control. Cells in the exponential growth phase with over 95% viability were used in the experiments.

3.1 Preparation of the stock solutions of GPE

The stock solutions were prepared at the concentration of 50 mg/ml in 100% DMSO and stored at -20°C until use. Prior to use, the stock solutions were diluted with sterile double-distilled water to the final concentration of 1000 µg/ml in 2% DMSO. The solutions then were diluted with 2% DMSO to the desired concentration. They were used to treat cells at the ratio of 1:10 which gave in the final solvent concentration constantly at 0.2% DMSO.

3.2 Determination of baseline COX-2 mRNA expression

The level of baseline COX-2 mRNA expression in human colorectal cancer cells, HT-29 and Colo-205, were determined by reverse transcription polymerase chain reaction (RT-PCR) and followed by agarose gel electrophoresis. Briefly, the total RNA of each cell lines were extracted using Trizol® reagent and reverse transcribed to cDNA by Improm II™ reverse transcription system. PCR amplification of COX-2 gene were done using PCR thermal cycler and the house keeping gene, GAPDH, was used as a loading control. Then, the PCR products were analyzed by electrophoresis in 1.5% agarose gel, stained the gel with ethidium bromide and determined the band densities by gel documentation. The detailed method is the same as method described in section 3.8.

3.3 Determination of cytotoxic activities of GPE

The indicator dye resazurin is used to measure the metabolic capacity of cells—an indicator of cell viability. Viable cells have the ability to reduce resazurin (dark-blue, maximum absorption wavelength 605 nm) into resorufin (pink, maximum absorption wavelength 573 nm). Nonviable cells rapidly lose metabolic capacity thus do not reduce the indicator dye.

1. Human colorectal cancer cells, HT-29 and Colo-205, were harvested by trypsinization and re-suspended at a final concentration of 5×10^4 cells/ml in fresh medium with 10% FBS.
2. Aliquots of 90 µl cell suspension were seeded in 96-well plates and allowed to attach for 24 hours.
3. The cells were treated with a range of GPE concentrations (6.25, 12.5, 25, 50, 100 µg/ml) using standard cytotoxic drugs and 0.2% DMSO as positive and negative control, respectively.
4. After 24, 48 and 72 hours, 5 µl of a 1 mg/ml resazurin solution were added to each well and the plates were incubated for 6, 3, and 2 hours, respectively.
5. The absorbance of individual wells was determined at 570 and 600 nm using a microplate reader.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

6. The percentages of cytotoxicity of the tested compound were calculated using the following formula

$$\% \text{ Cytotoxic} = \frac{(\Delta \text{ OD}_{(\text{negative control})} - \Delta \text{ OD}_{(\text{sample})})}{\Delta \text{ OD}_{(\text{negative control})}} \times 100$$

Where Δ OD = Absorbance 570 nm - Absorbance 600 nm

The inhibition concentration 50 (IC_{50}) was determined for each time point. The 3 concentrations of GPE were chosen for the next experiments.

3.4 Determination of apoptotic induction

Induction of apoptosis represents a most potent cellular mechanism against cancer. One biochemical feature of apoptotic cell death is the externalization of the normal inward-facing phosphatidylserine of the cell's lipid bilayer to the outer layers of the plasma membrane which result in the early phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue.

Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used for the detection of apoptosis (90).

1. Human colorectal cancer cells, HT-29 and Colo-205, were harvested by trypsinization and re-suspended at a final concentration of 5×10^4 cells/ml in fresh medium with 10% FBS.
2. Aliquots of 3.6 ml cell suspension were seeded in 6-well plates and allowed to attach for 24 hours.
3. The cells were treated with a range of GPE concentrations (25, 50, 100 µg/ml) using standard cytotoxic drugs and 0.2% DMSO as positive and negative control, respectively, for 18 and 24 hours.
4. The floated cells in the supernatant were collected. The remaining attached cells were harvested by trypsinization. The media were removed by centrifugation at 1,500 rpm, 25ºC for 5 minutes.
5. Cell pellets were washed twice with 500 µl cold PBS, and collected the cells by centrifugation at 1,500 rpm, 4ºC for 5 minutes.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

6. Cell pellets were re-suspended in 100 µl assay buffer and transferred in to flow cytometer tube
7. Cells were stained with 1 µl annexin V-FITC and 1 µl of 0.05 µg/ml propidium iodide (PI).
8. The stained cells were incubated in the dark at room temperature for 15 minutes.
9. The 400 µl of assay buffer were added. Cells suspensions at 3×10^4 cells/sample were immediately analyzed using fluorescence flow cytometer.
10. The type of cells were determined as following; viable cells is annexin V-FITC$^-$/PI$^-$ cells, early apoptotic cells is annexin V-FITC$^+$/PI$^-$ cells, necrotic cells is annexin V-FITC$^-$/PI$^+$cells, and non-apoptotic cells, late apoptotic or secondary necrotic cells are annexin V-FITC$^+$/PI$^+$cells.

3.5 Determination of anti-proliferative effect of GPE

Anti-proliferative effects of the GPE against human CRC cells were determined using automated cell counter.

1. Human colorectal cancer cells, HT-29 and Colo-205, were harvested by trypsinization and re-suspended at a final concentration of 5×10^4 cells/ml in fresh medium with 10% FBS.
2. Aliquots of 3.6 ml cell suspension were seeded in 6-well plates and allowed to attach for 24 hours.
3. The cells were treated with a range of GPE concentrations (25, 50, 100 µg/ml) using standard cytotoxic drugs and 0.2% DMSO as positive and negative control, respectively, for 24 hours (91-93).
4. The floated cells in the supernatant were collected. The remaining attached cells were harvested by trypsinization. The media were removed by centrifugation at 1,500 rpm, 25ºC for 5 minutes.
5. Cell pellets were washed twice with 500 µl cold PBS, and collected the cells by centrifugation at 1,500 rpm, 4ºC for 5 minutes.
6. Cell pellets were re-suspended in 500 µl cold assay buffer. The number of cells in each well were then counted using Scepter™ Handheld Automated Cell Counter.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

7. The percentages of cell proliferation were calculated compared to the vehicle control using the following formula;

$$\% \text{ Proliferation} = \frac{\text{Total cell count}_{(\text{Sample})}}{\text{Total cell count}_{(\text{Negative control})}} \times 100$$

3.6 Determination of the effect of GPE on the expression of genes in BCL-2 family

The expressions of genes in BCL-2 family were determined by quantitative RT-PCR as following procedures. There were three main steps in this experiment.

1. Preparation of total RNA
2. Conversion of RNA to cDNA
3. Quantitative RT-PCR

Preparation of total RNA

Total RNA from HT-29 and Colo-205 treated cells were isolated using TRIzol® reagent according to the manufacturer's protocol.

1. Human colorectal cancer cells, HT-29 and Colo-205, were harvested by trypsinization and re-suspended at a final concentration of 5×10^4 cells/ml in fresh medium with 10% FBS.
2. Aliquots of 3.6 ml cell suspension were seeded in 6-well plates and allowed to attach for 24 hours.
3. The cells were treated with a range of GPE concentrations (25, 50, 100 µg/ml) using standard cytotoxic drugs and 0.2% DMSO as positive and negative control, respectively, for 24 hours.
4. The floated cells in the supernatant were collected. The media were removed by centrifugation at 1,500 rpm, 25°C for 5 minutes.
5. The remaining attached cells were added with 500 µl of TRIzol® reagent per well. Cells were lysed and homogenized by pipetting the cells up and down several times. The floated cells were also lysed with TRIzol® reagent.

6. All the cell lysate were transferred to 1.5 ml eppendorf tubes and incubate for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.
7. Phase separations were performed by adding 0.2 ml of chloroform per 1 ml of TRIzol® Reagent used for homogenization. The tubes must be cap securely and shaken vigorously by hand for 15 seconds. Then, the tubes were incubated for 2–3 minutes at room temperature.
8. The samples were centrifuged at 12,000 × g for 15 minutes at 4°C. The mixtures were separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is about 50% of the total volume.
9. The aqueous phase was removed of the sample by angling the tube at 45° and pipetting the solution out. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase. The aqueous phase was placed into a new tube and proceeds to the RNA Isolation Procedure.
10. Isopropanol was used to precipitate RNA. 0.5 ml of 100% isopropanol was added to the aqueous phase, per 1 ml of TRIzol® Reagent used for homogenization. The tubes were incubated at room temperature for 10 minutes and centrifuged at 12,000 × g for 10 minutes at 4°C. The RNA is often invisible prior to centrifugation, and forms a gel-like pellet on the side and bottom of the tube.
11. RNA pellet were subjected to RNA wash. The supernatant was removed from the tube, leaving only the RNA pellet. The pellets were washed with 1 ml of 75% ethanol per 1 ml of TRIzol® Reagent used in the initial homogenization.
12. The samples were briefly vortexed and centrifuged the tube at 7500 × g for 5 minutes at 4°C. The wash was discarded.
13. The RNA pellets were air dried for 5–10 minutes. Do not allow the RNA to dry completely, because the pellet can lose solubility.
14. The RNA pellets were resuspended in 0.1% DEP-C treated water 10–20 µl by passing the solution up and down several times through a pipette tip.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

15. The RNA concentration and possible contamination were determined by measuring the absorbance at 260 and 280 nm. The RNA samples should have ratio of $A_{260/280}$ more than 1.8.
16. The total RNA samples were stored at -70°C prior to converse to cDNA.

Conversion of RNA to cDNA

Total RNA samples were converted to cDNA using Improm-II™ reverse transcription system according to manufacturer's protocol.

1. Place sterile 0.2 ml PCR tube on ice. Thaw the experimental RNA on ice and return any unused portion to the freezer as soon as aliquots are taken.
2. On ice, combine the experimental RNA 1 µg, 1 µl of the Oilgo$(dT)_{15}$ primer 0.5 µg/µl and Nuclease-Free Water for a final volume of 5µl per reaction. Mix and spin each tube to maintain the original volume.
3. Close each tube of RNA tightly. Place the tubes into a water bath at 70°C for 5 minutes. Immediately chill on ice for at least 5 minutes.
4. Spin each tube for 10 seconds in a microcentrifuge to collect the condensate and maintain the original volume. Keep the tubes closed and on ice until the reverse transcription reaction mix is added.
5. Prepare the reverse transcription reaction mix by combining the following components of the ImProm-II™ Reverse Transcription System in a sterile 1.5 ml microcentrifuge tube on ice. Prepare sufficient mix to allow 15µl for each cDNA synthesis reaction to be performed. Determine the volumes needed for each component, and combine them in the order listed. Vortex gently to mix, and keep on ice prior to dispensing into the reaction tubes. The reverse transcription reaction mix contains the following components

1) Nuclease-Free Water (to a final volume of 15µl)	7.3	µl
2) ImProm-II™ 5X Reaction Buffer	4.0	µl
3) 25 mM $MgCl_2$ (final concentration 1.5 mM)	1.2	µl
4) dNTP Mix (final concentration 0.5mM each dNTP)	1.0	µl
5) Recombinant RNasin® Ribonuclease Inhibitor (20 u)	0.5	µl
6) ImProm-II™ Reverse Transcriptase	1.0	µl

Final volume	15.0	µl

6. Add 15 µl aliquots of the reverse transcription reaction mix to each reaction tube on ice for a final reaction volume of 20 µl per tube. Be careful to prevent cross-contamination.
7. Place the reaction tube in the PCR thermal cycler machine to generate cDNA using the following conditions; annealing at 25ºC for 5 minutes, extension at 42ºC for 1:30 hour, and inactivate Reverse Transcriptase at 70ºC for 15 minutes. The cDNA samples were stored at -20ºC to use as the template for determining gene expression.

Quantitative RT-PCR

Quantitative RT-PCR were carried out for *BCL-2* (Hs00608023_m1), *BCL-XL* (Hs00236329_m1), *BAK* (Hs00832876_g1) and *BAX* (Hs00180269_m1) genes in each cDNA sample using TaqMan® Gene Expression System (Applied Biosystem, USA).

1. Thaw on ice, completely resuspend by gently vortexing, then briefly centrifuge to bring liquid to the bottom of the tube of 20X TaqMan® Gene Expression Assays (containing specific primers and probes for genes of interest) and cDNA samples. Mix the 2X TaqMan® Gene Expression master mix reagent (containing Taq Polimerase and reaction buffer) by gently swirling the bottle.
2. The assays were done in 10 µl singleplex reactions. For each sample, pipet the following into a nuclease free 1.5-ml microcentrifuge tube:

1) 20X TaqMan® Gene Expression Assays	0.5	µl
2) 2X TaqMan® Gene Expression master mix	5	µl
3) cDNA template 50 ng	1	µl
4) RNase-free water	3.5	µl
Final volume	10.0	µl

3. Cap the tube and invert it several times to mix the reaction components. Centrifuge the tube briefly.
4. Load the microcentrifuge tubes into the Applied Biosystems StepOnePlus™ Real-Time PCR System.

5. Create an experiment for the run using the parameter values as following; standard run, reaction volume 10 µl, thermal cycling conditions: pre-incubation at 95ºC for 10 min, then cycling for 45 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Run the experiment.
6. The quantification of gene expression levels were used relative quantitation method where GAPDH was an endogenous control and 0.2% DMSO was a negative control as following equation;

$$\text{Relative Quantitation (RQ)} = 2^{-\Delta\Delta C_T}$$

Where C_T = cycle threshold

7. The ratios of pro-apoptotic/anti-apoptotic expression were calculated from the following equation;

$$\text{Ratio of Proapoptotic/Anti-apoptotic} = \frac{RQ_{BAK} + RQ_{BAX}}{RQ_{BCL\text{-}2} + RQ_{BCL\text{-}XL}}$$

3.7 Determination of the effect of GPE on the cell cycle

The nuclear DNA content of a cell can be quantitatively measured by flow cytometry. Initially, a fluorescent dye that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei. The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Thereafter, such fluorescence data are considered a measurement of the cellular DNA content (94). This method of DNA staining utilizes ethanol to fix the cells and permeabilize the membrane, which allows the dye (Propidium Iodide) to enter the cells. Propidium Iodide (PI) is a DNA-binding fluorochrome that intercalates in the double-helix. Ribonuclease-A is used to eliminate the staining of double-stranded RNA.

1. Human colorectal cancer cells, HT-29 and Colo-205, were harvested by trypsinization and re-suspended at a final concentration of 5×10^4 cells/ml in fresh medium with 10% FBS.

2. Aliquots of 3.6 ml cell suspension were seeded in 6-well plates and allowed to attach for 24 hours.
3. The cells were treated with a range of GPE concentrations (25, 50, 100 µg/ml) using standard cytotoxic drugs and 0.2% DMSO as positive and negative control, respectively, for 1 hour and further incubated for 24 hours in a fresh medium without GPE (95).
4. The floated cells in the supernatant were collected. The remaining attached cells were harvested by trypsinization. The media was removed by centrifugation at 1,500 rpm, 25ºC for 5 minutes.
5. Cell pellets were washed twice with 500 µl cold PBS, and the cells were collected by centrifugation at 1,500 rpm, 4ºC for 5 minutes.
6. The cells were re-suspended in 150 µl cold PBS and 350µl absolute ethanol was slowly added with continuously shaking to fix the cells and incubated at -20ºC for 15 minutes.
7. Fixed cells were washed twice with 500 µl cold PBS to remove the remaining ethanol and the cells were collected by centrifugation at 1,500 rpm, 4ºC for 5 minutes.
8. The cells were re-suspended in 500 µl assay buffer.
9. Five µl of 4 mg/ml RNAse was added to eliminate RNA and incubated for 30 min at room temperature.
10. Cells were stained with 5 µl of 0.05µg/ml PI and incubated in the dark for 15 min at room temperature.
11. Cell cycle patterns of 3×10^4 cells/sample were immediately analyzed using fluorescence flow cytometer.
12. The cell cycle analysis was evaluated using FCS Express 4 Image Cytometry software.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.8 Determination of the effect of GPE on the mRNA expression of cell cycle regulatory genes

The mRNA expression of cell cycle regulatory genes including cyclin A, cyclin B1, cyclin D1, cyclin E, p21 and p53 were performed in GPE treated HT-29 and Colo-205 cells using RT-PCR. There were four main steps in this experiment.

1. Preparation of total RNA
2. Conversion of RNA to cDNA
3. RT-PCR
4. Agarose gel electrophoresis

Preparation of total RNA

1. Human colorectal cancer cells, HT-29 and Colo-205, were harvested by trypsinization and re-suspended at a final concentration of 5×10^4 cells/ml in fresh medium with 10% FBS.
2. Aliquots of 3.6 ml cell suspension were seeded in 6-well plates and allowed to attach for 24 hours.
3. The cells were treated with a range of GPE concentrations (25, 50, 100 µg/ml) using standard cytotoxic drugs and 0.2% DMSO as positive and negative control, respectively, for 1 hour and further incubated for 24 hours in a fresh medium without GPE (95).
4. Repeat the *Preparation of total RNA* method in section 3.6 steps 4-16.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Conversion of RNA to cDNA

The method to converse the total RNA to cDNA was the same as section 3.6 steps 1-7 in the section *Conversion of RNA to cDNA*.

RT-PCR

1. Add the following components to a sterile 0.2-ml microcentrifuge tube. The reaction volumes were 25 µl. Prepare a master mix of common components for multiple reactions.
    1) 10X PCR Buffer, Minus Mg 2.5 µl

2)	10 mM dNTP mixture (final concentration 0.2 mM each)	0.5	µl
3)	50 mM $MgCl_2$ (final concentration 1.5 mM)	0.75	µl
4)	10 µM Forward Primer (final concentration 0.2 µM)	0.5	µl
5)	10 µM Reverse Primer (final concentration 0.2 µM)	0.5	µl
6)	cDNA Template DNA	1	µl
7)	Platinum® Taq DNA Polymerase (1.0 unit)	0.2	µl
8)	Autoclaved, distilled water	to 25	µl

2. Cap the tubes, mix, and centrifuge briefly to collect the contents.
3. Incubate tubes in a thermal cycler at 94°C for 2 minutes to completely denature the template and activate the enzyme.
4. Perform 35 cycles of PCR amplification as follows:

Denature	94°C	for 30 seconds
Anneal	Tm	for 30 seconds
Extend	72°C	for 1 minute

5. Perform a final extension at 72°C for 10 minutes.
6. Maintain the reaction at 4°C after cycling. After the cycle is complete, analyze the PCR products or store the amplifications at –20°C until use.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 4: Primers for RT-PCR and their annealing temperature.

Gene	Primer sequences	Tm °C	PCR product (bp)
GAPDH	Forward: 5'-AAG GTC GGA GTC AAC GGA TTT GGT-3' Reverse: 5'-ATG GCA TGG ACT GTG GTC ATG AGT-3'	60	530
P53	Forward: 5'-CAT GAG CGC TGC TCA GAT AG-3' Reverse: 5'-CTG AGT CAG GCC CTT CTG TC-3'	56	643
P21	Forward: 5'-GCG ATG GAA CTT CGA CTT TGT-3' Reverse: 5'-GGG CTT CCT CTT GGA GAA GAT-3'	54	352
Cyclin A	Forward: 5'-GTC ACC ACA TAC TAT GGA CAT G-3' Reverse: 5'-AAG TTT TCC TCT CAG CAC TGA C-3'	53	300
Cyclin B1	Forward: 5'-CGG GAA GTC ACT GGA AAC AT-3' Reverse: 5'-AAA CAT GGC AGT GAC ACC AA-3'	55	177
Cyclin D1	Forward: 5'-CTG GCC ATG AAC TAC CTG GA-3' Reverse: 5'-GTC ACA CTT GAT CAC TCT GG-3'	54	482
Cyclin E	Forward: 5'-AAT AGA GAG GAA GTC TGG-3' Reverse: 5'-AGA TAT GAC ACC TGC ATG-3'	55	440
COX-2	Forward: 5'- TTC AAA TGA GAT TGT GGG AAA AT-3' Reverse: 5'- AGA TCA TCT CTG CCT GAG TAT CTT-3'	50	305

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Agarose gel electrophoresis

1. Prepare 1.5% Agarose gel by weighing 1.2 gram of agarose and adding agarose to Erlenmeyer flask. Add 80 ml of 1X TBE to Erlenmeyer flask. Swirl vigorously to thoroughly mix agarose.
2. Put agarose and 1X TBE slurry into microwave. Heat on high for 30 seconds at a time. After 30 seconds, remove from microwave and swirl. Be careful as the mixture was hot. After 1 minute of heating, repeat heating and swirling procedure every 10 seconds until mixture is clear.
3. Place gel tray into casting chamber. Add casting combs into the appropriate slots.

4. Pour the gel when the agarose has cooled to about 55° C. When pouring the gel, avoid creating bubbles as this will prevent current from flowing through the gel.
5. The gel should be allowed to cool for at least 30 minutes until it has set (it will turn whitish and opaque when ready). Gels should be fairly thin, approximately 1/4 to 1/2 inch.
6. Carefully remove combs by pulling them upwards firmly and smoothly in a continuous motion.
7. Place gel and tray into gel rig, with wells on the cathode side. Then, fill gel rig with 1X TBE sufficient to cover the entire gel.
8. Cut a piece of parafilm and place a 2 µl drop of 6X loading dye onto the waxy side for each sample to be loaded.
9. Keeping PCR product samples on ice, pipette up 8 µl of a sample and add the sample to one of the drops of loading dye.
10. Mix the sample and loading dye by pipetting the mixture up and down several times then load the 9 µl of the mixture into a well.
11. Continue loading the rest of the samples, placing 3 µl of DNA ladder at the first well of each row as a marker.
12. Place the lid on the gel box, connecting the electrodes. Connect the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected.
13. Turn on the power supply to 100 volts. Run the gel for 50-60 minutes. Stop the run before the bromophenol blue loading dye front exits the gel.
14. Turn off the power pack, remove the gel and place it in a staining solution with 0.5 µg/ml ethidium bromide 400 ml in 1X TBE for 15 minutes. Then, destain the gel in 1X TBE for 15 minutes.
15. Determine the band densities by gel documentation using Gel Doc™ XR+ System and Image Lab™ software.

3.9 Determination of the effect of GPE on the COX-2 mRNA expression

The effect of GPE on the COX-2 expression was performed only in HT-29 cells which constitutively expressed COX-2. The level of COX-2 mRNA expression were

determined by reverse transcription polymerase chain reaction (RT-PCR) and followed by agarose gel electrophoresis.

Preparation of total RNA

1. Human colorectal cancer cells, HT-29, were harvested by trypsinization and re-suspended at a final concentration of 5×10^4 cells/ml in fresh medium with 10% FBS.
2. Aliquots of 3.6 ml cell suspension were seeded in 6-well plates and allowed to attach for 24 hours.
3. The cells were treated with a range of GPE concentrations (25, 50, 100 µg/ml) using standard cytotoxic drugs and 0.2% DMSO as positive and negative control, respectively, for 24 hours.
4. The total RNA from HT-29 treated cells were isolated using TRIzol® reagent according to the manufacturer's protocol as previously described in section 2.6.

The total RNA samples were subjected to converse to cDNA, RT-PCR, and agarose gel electrophoresis as described in section 3.8.

3.10 Statistical analysis

All data were presented as mean ± S.E. Statistical evaluations were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Student t test was used to compare the differences between control and the tested group in the RT-PCR experiment. The differences between the two studied cell lines were assessed by student t test. Any p-value less than 0.05 were considered statistically significant. All assays were performed in duplicate or triplicate of three independent experiments.

CHAPTER IV
RESULTS

1. Baseline COX-2 mRNA expression

COX-2 plays a pivotal role in CRC carcinogenesis and disease progression (34; 39). Human colorectal cancer cells, HT-29 and Colo-205, the two cell lines used in this study, were subjected to the determination of baseline COX-2 mRNA expression by RT-PCR. The result has shown that HT-29 cells are constitutively expressed COX-2 while Colo-205 cells lack of COX-2 expression (Figure 14).

The further studies were done using these 2 human colorectal cancer cell lines in order to compare the effect of GPE on different cell lines which carry different COX-2 expression status. Moreover, the involvement of COX-2 on GPE action was investigated in COX-2 expressing cells, HT-29.

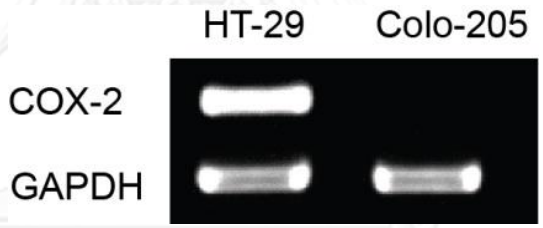


Figure 14: Baseline COX-2 expression in HT-29 and Colo-205 cells. HT-29 and Colo-205 cells were harvested and the total RNAs were extracted by TRIzol® Reagent. The RNAs were converted to cDNA by Improm-II™ reverse transcription system and amplified with the COX-2 primers by RT-PCR. The PCR products were identified by 1.5% agarose gel electrophoresis and analyzed by gel documentation. GAPDH was used as a loading control.

2. Effect of GPE on human colorectal cancer cell expressing COX-2, HT-29 cells.

The anti-cancer effects of GPE were determined on human colorectal cancer cell that constitutively expressed COX-2, HT-29 cells as following.

2.1 Cytotoxic activities of GPE

GPE was initially tested for its cytotoxic effects against HT-29 cells by resazurin reduction assay. The cells were exposed to 6.25 - 100µg/ml of GPE for 24, 48 and 72 h. Standard cytotoxic drugs recommended for the treatment of CRC, 5FU and Oxaliplatin were used as positive controls in this experiment.

As shown in Figure 15, 50 and 100µg/ml of the extract significantly exhibited cytotoxicity against HT-29 cells. The half maximum inhibition concentrations (IC_{50}) of GPE against HT-29 were 69.49±2.04, 55.89±1.86 and 48.94±2.99 µg/ml at 24, 48 and 72 h, respectively (Figure 16). The cytotoxic effect of the extract was seen in a dose-dependent and time-dependent manner. However, the dose-dependency was more pronounced. At 24 h treatment time, GPE showed higher cytotoxic activities against HT-29 when compared to that of standard cytotoxic agents, 5FU and Oxaliplatin. The IC_{50} of 5FU against HT-29 were >1,000, 45.06, and 7.22 µM at 24, 48 and 72 h, respectively. Oxaliplatin showed the IC_{50} of 849.34, 4.44, and 2.35 µM against HT-29 at 24, 48 and 72 h, respectively.

According to the IC_{50} of GPE, three concentrations at 25, 50 and 100µg/ml were chosen for the rest of the experiments.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

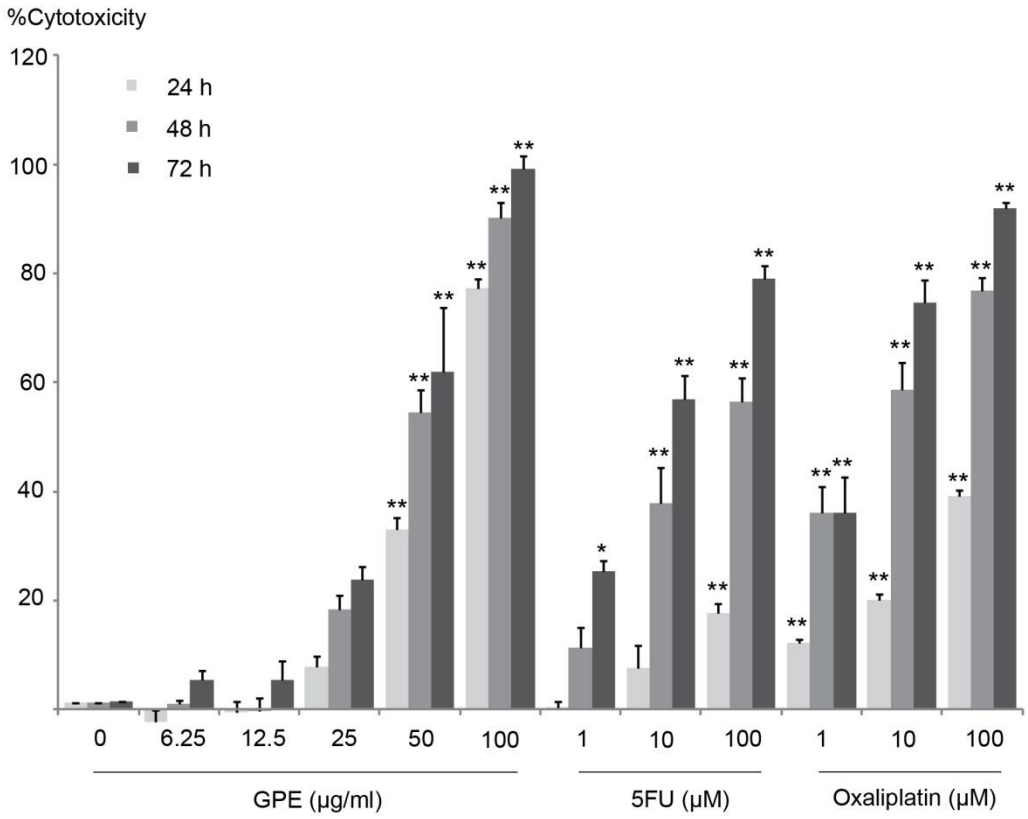


Figure 15: Cytotoxic effects of GPE against HT-29 cells. The cells were treated with GPE at 6.25 - 100µg/ml, 5FU at 1 – 100 µM and Oxaliplatin at 1 – 100 µM for 24, 48 and 72 hours. The cytotoxic effects were determined by resazurin reduction assay. The data represent the means ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

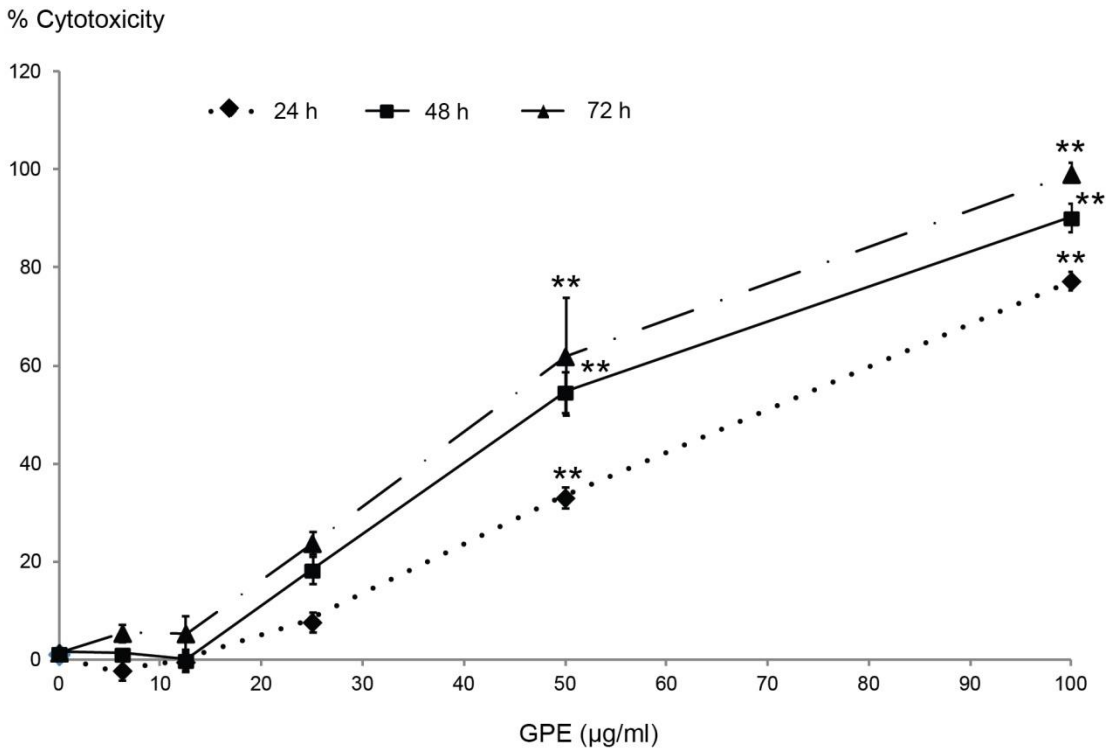


Figure 16: Line graphs show cytotoxicity of GPE against HT-29 cells. The cells were treated with GPE at 6.25 - 100µg/ml for 24, 48 and 72 h. The cytotoxic effects were determined by resazurin reduction assay. The data represent the means ± S.E. of three independent experiments. The percentages of cytotoxicity of GPE were plotted against GPE concentration. Linear trend lines of each treatment time were calculated. The linear equations were used to determine the IC50 of GPE at each treatment time. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.2 Apoptotic induction effect of GPE

Induction of apoptosis is the main effect of many cytotoxic drugs to induce cancer cell death (96). The apoptotic induction effect of GPE was evaluated against HT-29. The cells were treated with 25, 50 and 100 µg/ml of GPE for 18 and 24 h. Apoptotic cells death were determined by AnnexinV-FITC/PI staining and detected with fluorescence flow cytometer. The patterns of cell death can be categorized into viable cells (AnnexinV-FITC$^-$/PI$^-$), necrosis cells (AnnexinV-FITC$^-$/PI$^+$), late apoptosis and/or necrosis cells (AnnexinV-FITC$^+$/PI$^+$), and apoptosis cells (AnnexinV-FITC$^+$/PI$^-$). Oxaliplatin and 5FU have been reported to induce colorectal cancer cell death by apoptosis were used as positive controls in this experiment (97; 98).

GPE induced total cell death in a concentration- and time- dependent manner after 18 and 24 h exposures. GPE at 50µg/ml was significantly induced apoptotic cell death in HT-29 by 14.79±2.91% and 20.97±0.91% at 18 and 24 h, respectively. At this concentration of GPE, apoptotic cells death represented the majority type of cell death with 45.14% and 57.71% of total cell death at 18 and 24 h, respectively. On the other hand, 100µg/ml GPE caused majority of cell death as late apoptosis and/or necrosis stages while cells treated with 25µg/ml GPE remained viable (Figure 17). Oxaliplatin and 5FU showed a minimal apoptotic cells death. It was due to the short exposure time which caused the majority of cells remained viable. The results were correlated with the cytotoxicity study. The molecular mechanism of GPE on apoptotic induction was further evaluated at the same concentration at 24 h exposure.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

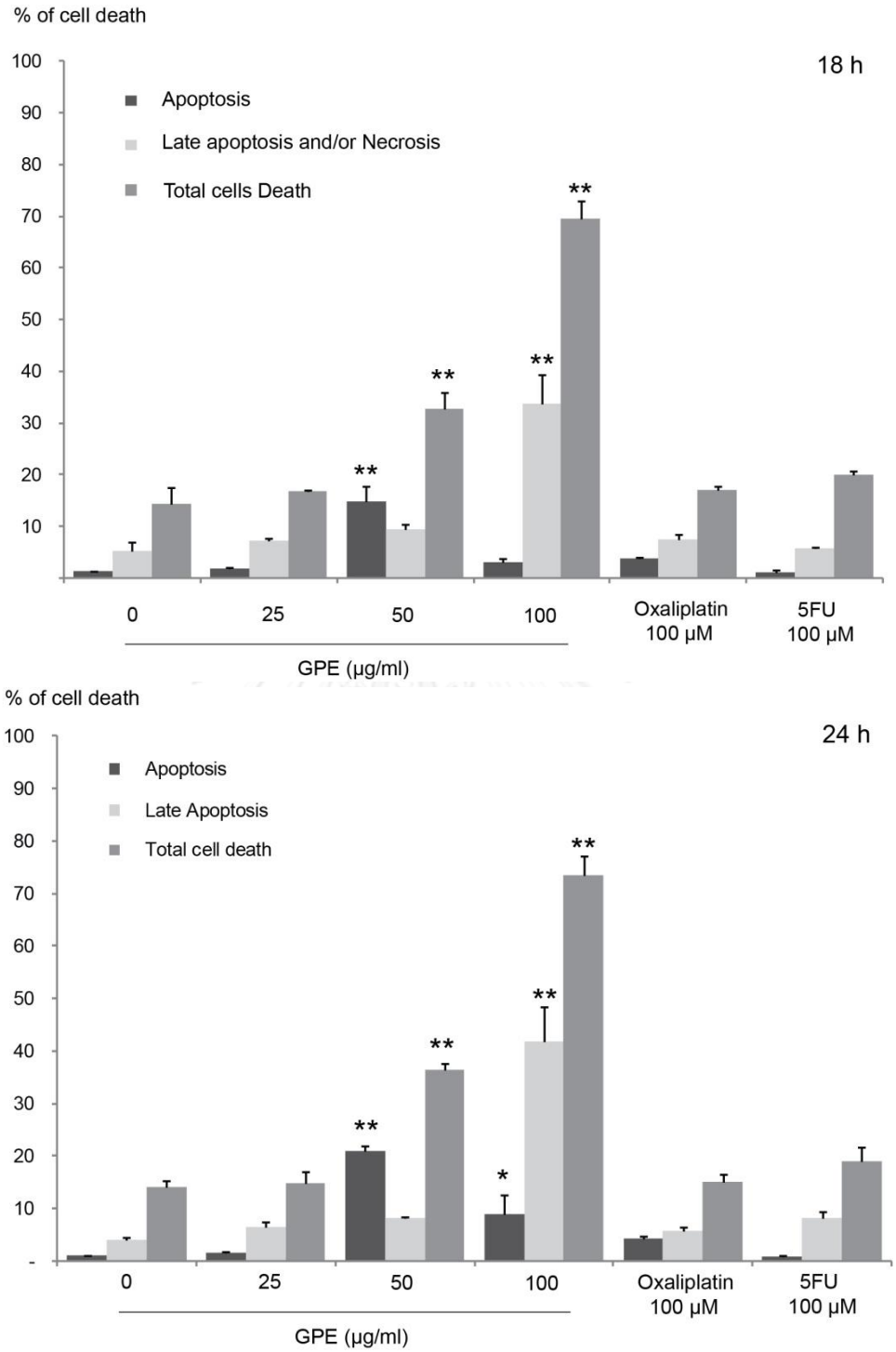


Figure 17: The apoptotic effect of GPE on HT-29 cells. The cells were treated with 25 – 100µg/ml of GPE for 18 (A) and 24 h (B). The percentage of cells labeled with annexin V+/PI-(early apoptotic cells), annexin V+/PI+ (late apoptotic cells/necrotic cells) and total cell death were plotted. Data are the mean values ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO)

2.3 Effect of GPE on the expression of genes in BCL-2 family

Apoptosis is regulated by a variety of pro-apoptotic and anti-apoptotic BCL-2 family proteins (90). To evaluate the mechanism by which GPE induced apoptosis in HT-29, the mRNA expression of BCL-2 family genes were assessed by quantitative RT-PCR.

HT-29 cells were treated with 25, 50 and 100 μg/ml of GPE for 24 h, and then the cells were subjected to RNA isolation, cDNA conversion, and PCR amplification of target genes. GPE at 25 and 50μg/ml increased the expression of pro-apoptotic BAK to 1.25±0.16 and 1.79±0.08, respectively while GPE at 100μg/ml tended to decrease BAK expression. The expression of anti-apoptotic BCL-2 was down-regulated to 0.68±0.2, 0.32±0.13, 0.55±0.28 following GPE 25, 50, and 100μg/ml treatment, respectively (Figure 18). Treatment with GPE also tended to decrease both anti-apoptotic BCL-XL and pro-apoptotic BAX. However, the ratio of pro-apoptotic/anti-apoptotic (BAK and BAX/BCL-2 and BCL-XL) were favor apoptotic cell deaths (ratio > 1) across the experimental concentrations with the ratio of 1.24±0.21, 2.66±0.48, and 1.37±0.17 following GPE 25, 50, and 100μg/ml treatment, respectively (Figure 19).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

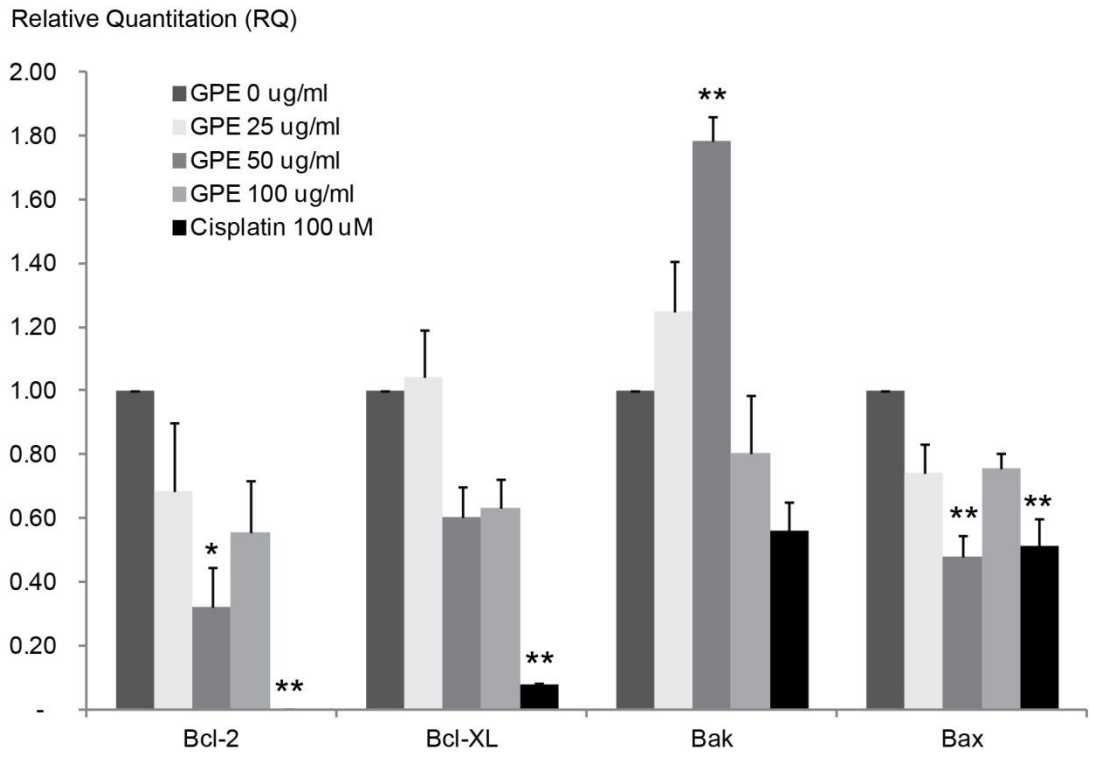


Figure 18: GPE alters the expression of BCL-2 family genes in HT-29 cells. The expressions of BCL-2 family genes were determined by quantitative RT-PCR. The cells were treated with GPE 25 - 100µg/ml for 24 h. The treated cells were subjected to RNA isolation, cDNA conversion and quantitative RT-PCR amplification of BAK, BAX, BCL-2 and BCL-XL genes. The value is given as relative copy number normalized to the endogenous control GAPDH and 0.2% DMSO solvent control. The data represent the means $\pm$ S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

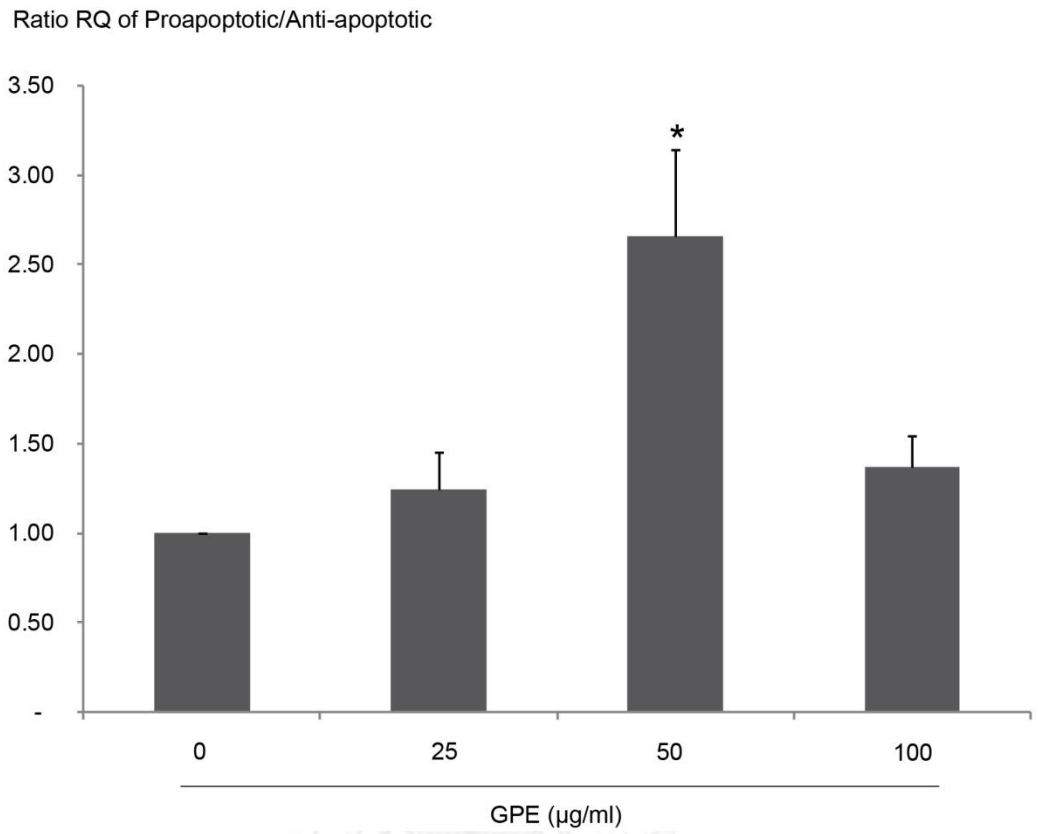


Figure 19: The ratio of pro-apoptotic/anti-apoptotic expression (BAK and BAX/BCL-2 and BCL-XL) in HT-29 treated cells. The cells were treated with GPE 25 - 100µg/ml for 24 h. The treated cells were subjected to RNA isolation, cDNA conversion and quantitative RT-PCR amplification of BAK, BAX, BCL-2 and BCL-XL genes. The ratios of pro-apoptotic/anti-apoptotic expression were calculated. The data represent the means $\pm$ S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2.4 Effect of GPE on the cell proliferation

Besides the ability of GPE to induce cancer cell death, its capability to inhibit cancer cell proliferation is also contributed to its cytotoxic activity. Inhibition of HT-29 cell proliferation by GPE was determined by cell counting. Standard cytotoxic drug, 5-FU, was used as a positive control in this experiment.

HT-29 cells were treated with 25, 50 and 100 µg/ml of GPE for 24 h afterward the total cells were counted. The results were expressed as a percentage of cells compared to the untreated control. GPE significantly inhibited HT-29 cell proliferation to 72.40±5.83%, 52.46±6.45%, and 26.68±6.12% at GPE 25, 50 and 100µg/ml, respectively when compared to the vehicle control. The effect was seen in a dose-dependent manner (Figure 20). The cell proliferation inhibition effect of GPE against HT-29 was similar to that of 5FU which 100 µM 5FU significantly inhibited cell proliferation to 44.47±0.07%.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

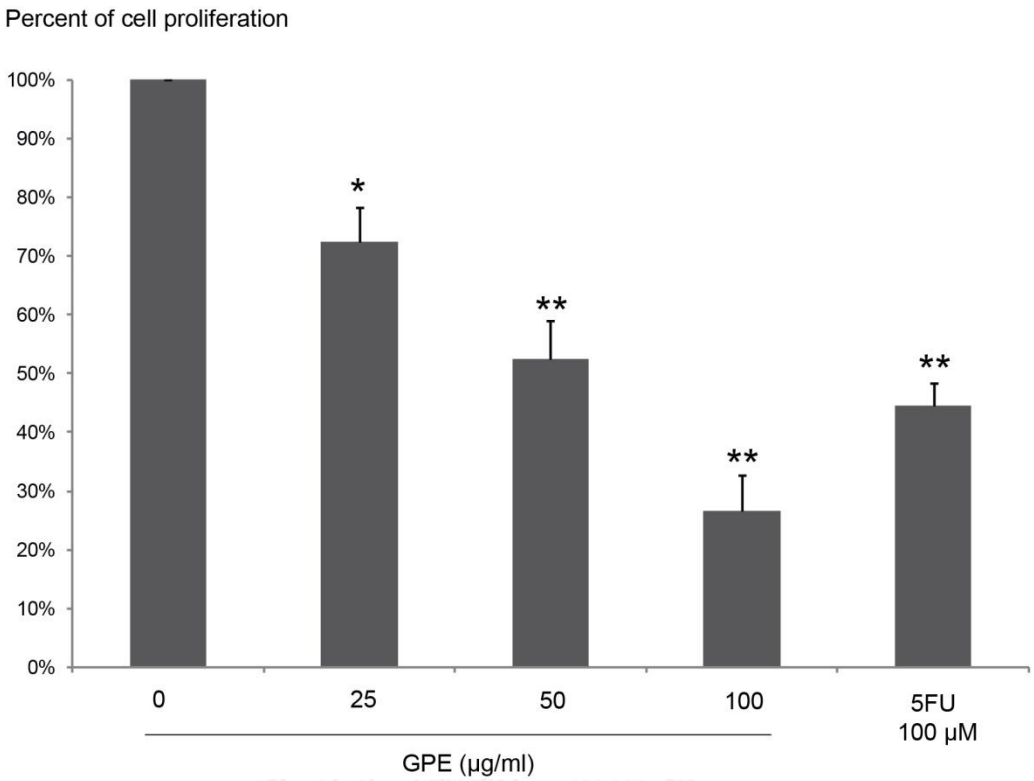


Figure 20: Anti-proliferative effects of GPE against HT-29 cells. The cells were treated with GPE 25 – 100µg/ml for 24 h. Cell proliferation was measured by cell counting using an automated cell counter. The data represent the means ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.5 Effect of GPE on the cell cycle progression

To determine whether the suppression of cell proliferation by GPE resulted from inhibition of cell cycle progression, HT-29 cells were treated with 25, 50 and 100 µg/ml GPE for 1 h and further incubated for 24 h in a fresh medium without GPE. The treated cells were subjected to fluorescence flow cytometric analysis for DNA content (Figure 21). 5FU which arrests the cell cycle at S phase and causes cell accumulation at G1/S phase was used as a positive control.

The effect of GPE on cell cycle pattern of HT-29 cells was difference which depended on the concentration used. GPE at 25µg/ml caused the accumulation of cells at S phase (32.00±0.51% to 44.85±2.59%) which was associated with a decrease in the cells in G0/G1 phase (50.13±1.28% to 40.95±1.33%). GPE at 50 and 100µg/ml caused a decrease in the proportion of cells in the S phase (from 32.0±0.51% to 26.78±1.76% and 18.72±3.55%, respectively). In cells treated with 50µg/ml GPE, this decrease was associated primarily with accumulation of cells in the G0/G1 phase (from 50.13±1.28% to 60.73±0.98%). At higher concentration, GPE (100µg/ml) resulted in a significant reduction in the S fraction (from 32.0±0.51% to 18.72±3.55%) with a shift of cells accumulation to the G2/M phases (from 17.86±1.04% to 33.53±0.85%) as shown in Figures 22. The positive control, 5FU, significantly accumulated cells population at S fraction with the reduction of the cells in G0/G1 and G2/M phases. The molecular mechanism of GPE on cell cycle pattern was further evaluated at the same concentration and treatment time.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

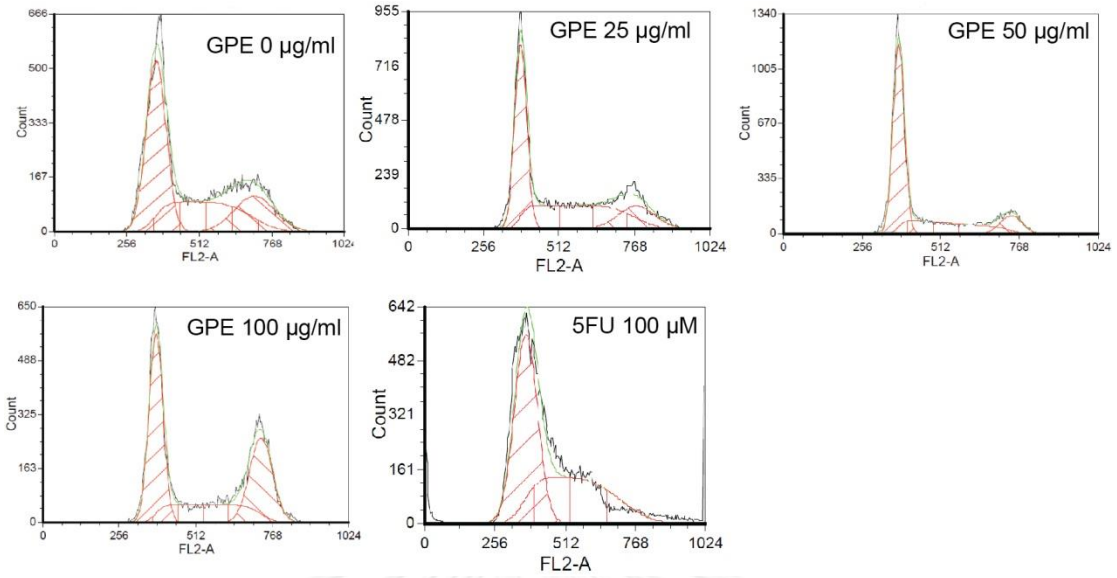


Figure 21: Representative histogram from flow cytometric analysis of HT-29 cell cycle pattern after treatment with GPE. The cells were treated with GPE at 25 - 100µg/ml for 1 h. The treated cells were washed and incubated in fresh medium for additional 24 h. Analysis of cells in the cell cycle was performed by fixing and staining the cells with PI. Cell cycle pattern of $3x10^4$ cells/sample were analyzed using fluorescence flow cytometer. The cell cycle distributions were evaluated using FCS Express 4 Image Cytometry.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

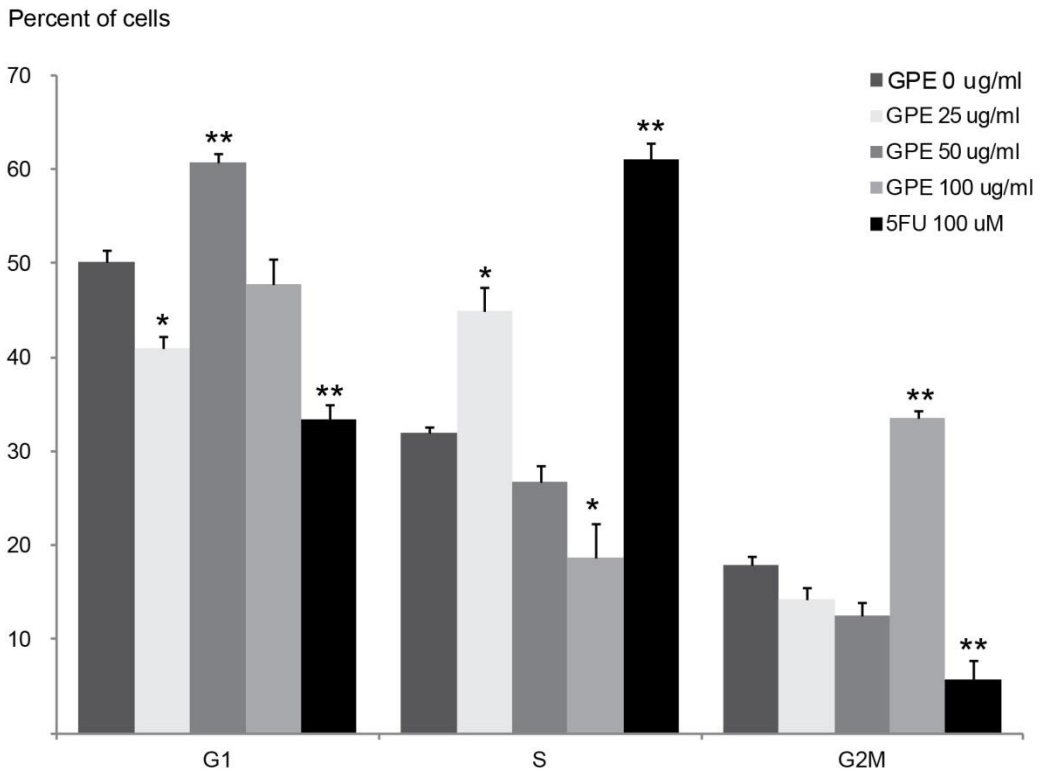


Figure 22: Distribution of HT-29 cells in the cell cycle phases after treatment with GPE. The cells were treated with GPE 25 - 100µg/ml for 1 h. The treated cells were washed and incubated in fresh medium for additional 24 h. Analysis of cells in the cell cycle was performed by fixing and staining the treated cells with PI followed by fluorescence flow cytometry. The percentage of cells in G1, S, and G2M phases are given as means ± S.E. of 3 independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.6 Effect of GPE on the mRNA expression of cell cycle regulatory genes

Since GPE changed the cell cycle pattern of HT-29 cells, the alterations in the expression of cell cycle regulatory genes might be involved. HT-29 cells were treated with 25, 50 and 100 µg/ml GPE for 1 h and further incubated for 24 h in a fresh medium without GPE. The treated cells were subjected to RNA isolation, cDNA conversion, PCR amplification of the genes involved in cell cycle progression including cyclin A, cyclin B1, cyclin D1, cyclin E, p21 and p53. PCR products were analyzed by agarose gel electrophoresis. The housing keeping genes, GAPDH, was used as a loading control.

As shown in Figure 23, GPE at the concentration of 50 and 100µg/ml significantly down-regulated cyclin A and cyclin E expression in a dose-dependent manner. However, the level of cyclin B1, cyclin D1, p21 and p53 remained unchanged.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

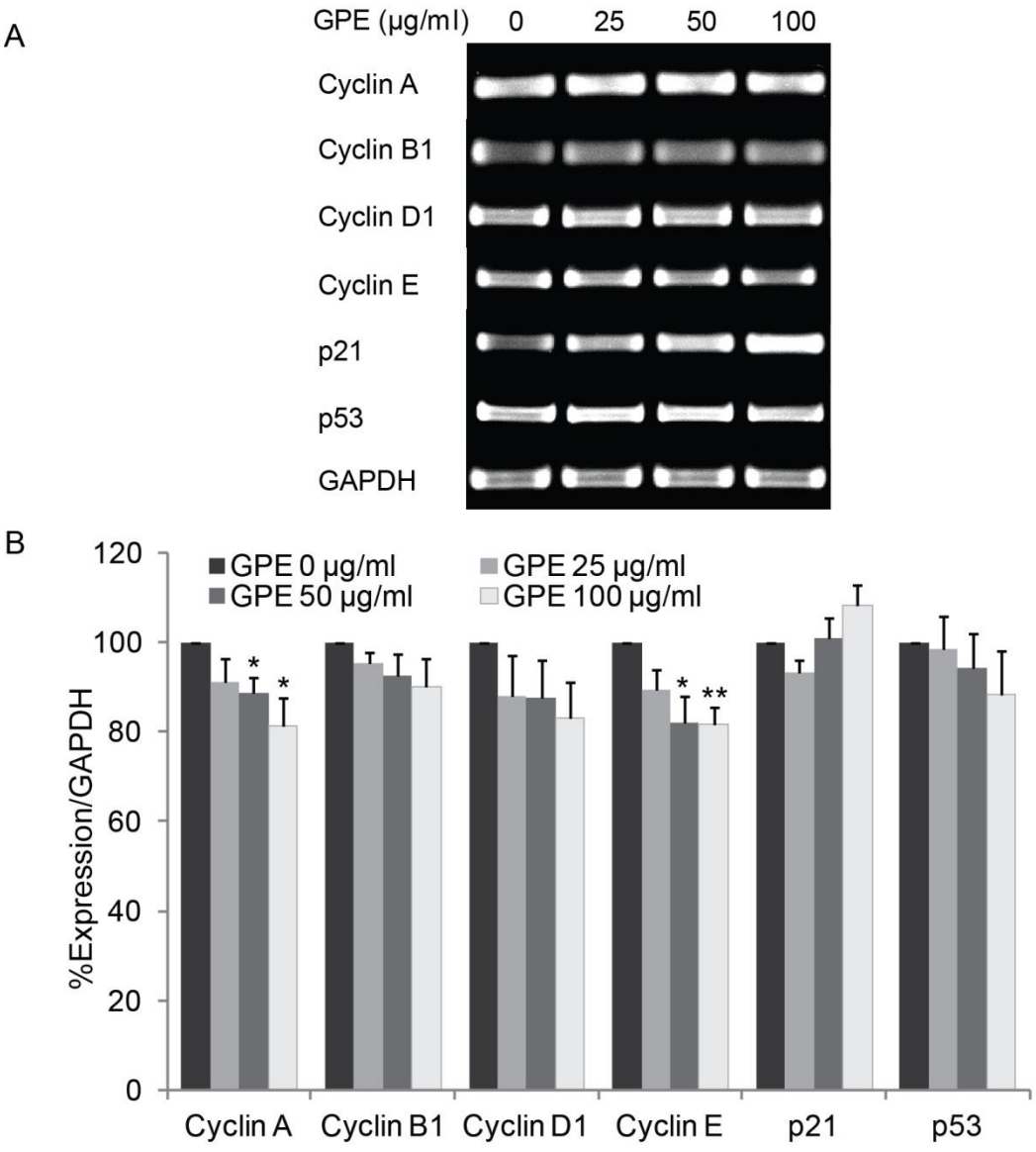


Figure 23: GPE alters the expression of cell cycle regulators genes in HT-29. The cells were treated with GPE 25 - 100µg/ml for 1 h and incubated with fresh medium without GPE for additional 24 h. Expression of cell cycle regulators were determined by RT-PCR. A) A representative of PCR products of the cell cycle regulatory genes. B) Densitometric analysis of the PCR products relative to GAPDH presented as % of control. The data represent the means ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

2.7 Effect of GPE on the COX-2 mRNA expression

COX-2 is an important growth factor involved the carcinogenesis of colorectal cancer (15). HT-29 cells were treated with 25, 50 and 100 µg/ml GPE for 24 h. The treated cells were subjected to RNA isolation, cDNA conversion, PCR amplification of the COX-2 genes. PCR products were analyzed by agarose gel electrophoresis. The housing keeping genes, GAPDH, was used as a loading control.

The effect of GPE on COX-2 mRNA expression was investigated in HT-29 cells which constitutively expressed COX-2. GPE at the concentration of 25 – 100µg/ml significantly inhibited COX-2 mRNA expression after 24 h of treatment (Figure 24).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

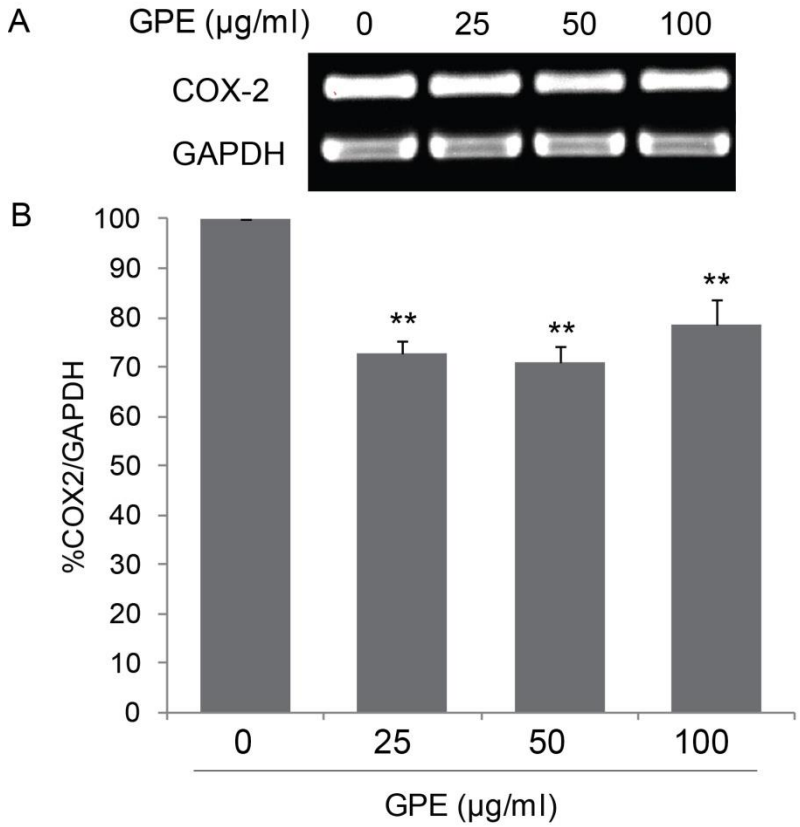


Figure 24: GPE alters the expression of COX-2 mRNA in HT-29. The cells were treated with GPE 25 - 100µg/ml for 24 h. Expressions of COX-2 genes. B) Densitometric analysis of the PCR products relative to GAPDH presented as % of control. The data represent the means ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3. Effect of GPE on human colorectal cancer cell not expressing COX-2, Colo-205

The anti-cancer effects of GPE were determined on human colorectal cancer cells that not expressing COX-2, Colo-205 cells as following.

3.1 Cytotoxic activities of GPE

Colo-205 cells were tested for cytotoxic effects of GPE by resazurin reduction assay. The cells were exposed to 6.25 - 100µg/ml of GPE for 24, 48 and 72 h. Standard cytotoxic drugs recommended for the treatment of CRC, 5FU and Oxaliplatin were used as positive controls in this experiment.

As shown in Figure 25 25, 50 and 100µg/ml of the extract significantly exhibited cytotoxicity against Colo-205 cells. The IC_{50} values of GPE against Colo-205 were 59.92±4.34, 28.85±1.44 and 25.42±1.65 µg/ml at 24, 48 and 72 h, respectively (Figure 26). The cytotoxic effect of the extract was seen in a dose-dependent and time-dependent manner. However, the dose-dependency was more pronounced. At 24 h treatment time, GPE showed higher cytotoxic activities against Colo-205 when compared to that of standard cytotoxic agents, 5FU and Oxaliplatin. The IC_{50} of 5FU against Colo-205 were >1,000, 29.52, and 10.12 µM at 24, 48 and 72 h, respectively. Oxaliplatin showed the IC_{50} of >1,000, 11.94, and 5.53 µM against Colo-205 at 24, 48 and 72 h, respectively.

According to the IC_{50} of GPE, three concentrations at 25, 50 and 100µg/ml were chosen for the rest of the experiments.

Chulalongkorn University

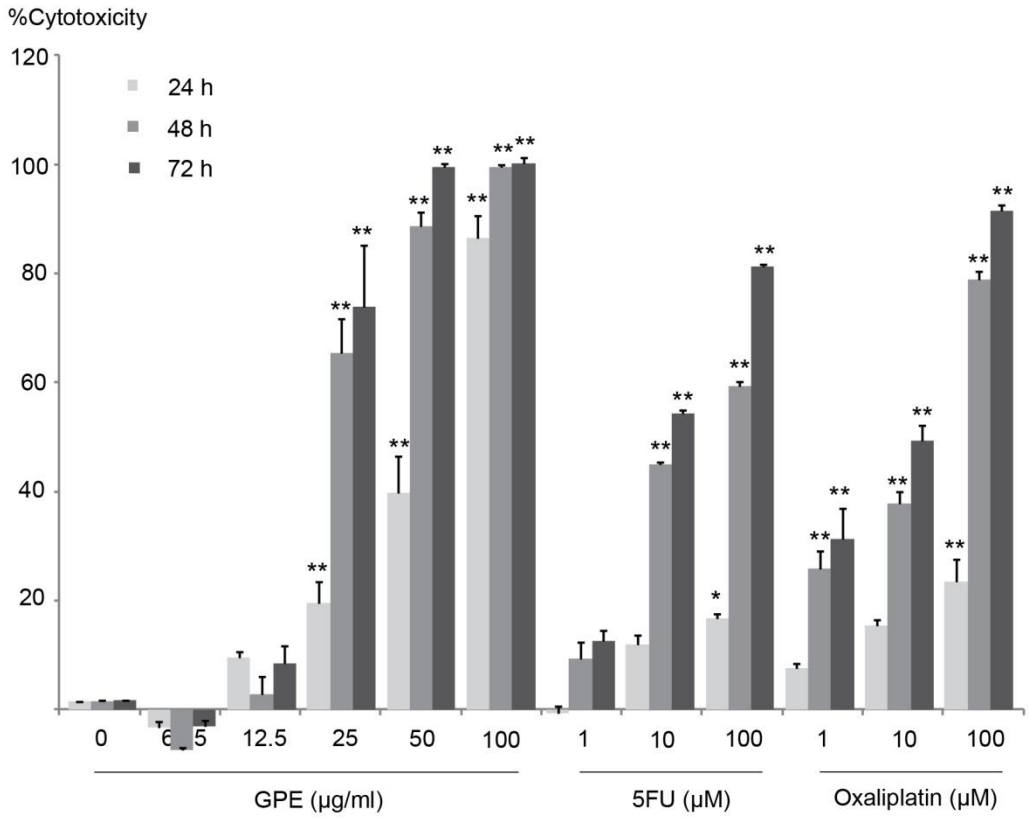


Figure 25: Cytotoxic effects of GPE against Colo-205 cells. The cells were treated with GPE at 6.25 - 100µg/ml, 5FU at 1 – 100 µM and Oxaliplatin at 1 – 100 µM for 24, 48 and 72 h. The cytotoxic effects were determined by resazurin reduction assay. The data represent the means ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

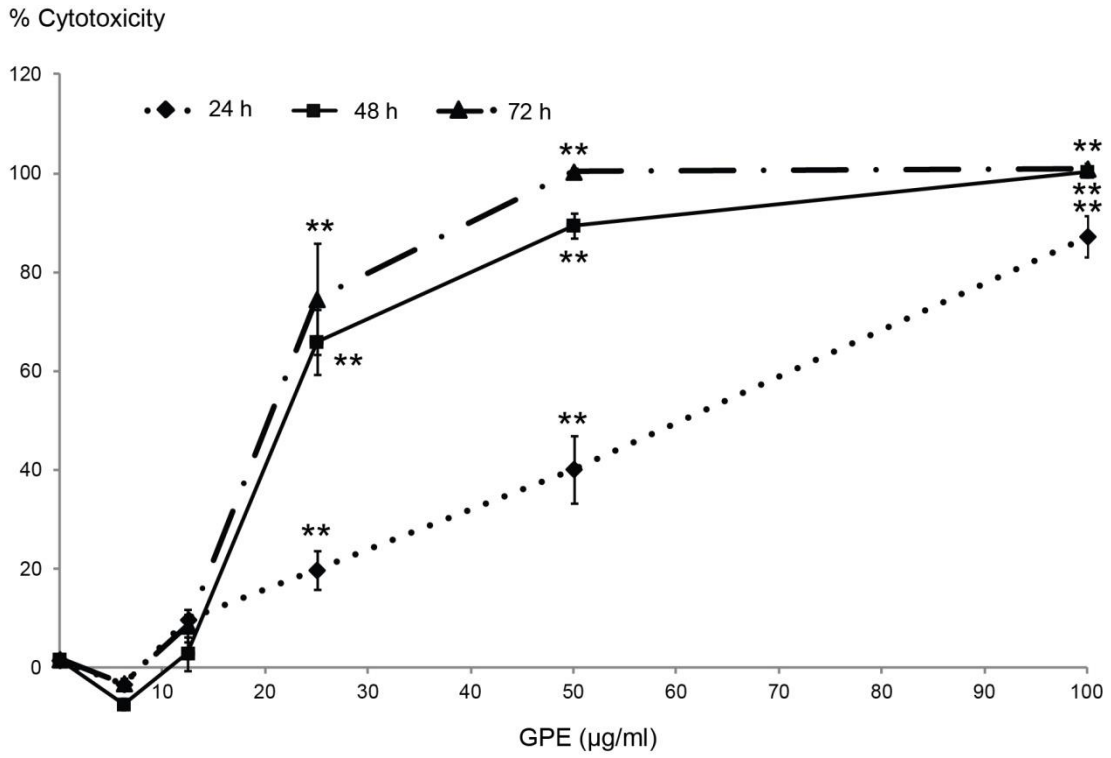


Figure 26: Line graphs show cytotoxicity of GPE against Colo-205 cells. The cells were treated with GPE at 6.25 - 100µg/ml for 24, 48 and 72 h. The cytotoxic effects were determined by resazurin reduction assay. The data represent the means + S.E. of three independent experiments. The percentages of cytotoxicity of GPE were plotted against GPE concentration. Linear trend lines of each treatment time were calculated. The linear equations were used to determine the IC50 of GPE at each treatment time. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.2 Apoptotic induction effect of GPE

The apoptotic induction effect of GPE was evaluated against Colo-205. The cells were treated with 25, 50 and 100 μg/ml of GPE for 18 and 24 h. Apoptotic cells death were determined by AnnexinV-FITC/PI staining and detected with fluorescence flow cytometer. Oxaliplatin and 5FU were used as positive controls in this experiment.

GPE induced total cell death in a concentration- and time- dependent manner after 18 and 24 h exposures. GPE at 50 and 100μg/ml were significantly induced apoptotic cell death in Colo-205 by 27.70±5.09% and 14.42±2.83% at 18h and 24.28±5.72% and 18.07±2.84% at 24 h, respectively. At 50μg/ml of GPE, apoptotic cells death represented the majority type of cell death with 59.45% and 49.52% of total cell death at 18 and 24 h, respectively. On the other hand, 100μg/ml GPE caused majority of cell death as late apoptosis and/or necrosis stages while cells treated with 25μg/ml GPE remained viable (Figure 27). Oxaliplatin and 5FU showed a minimal apoptotic cells death. It was due to the short exposure time which caused the majority of cells remained viable. The results were correlated with the cytotoxicity study. The molecular mechanism of GPE on apoptotic induction was further evaluated at the same concentration at 24 h exposure.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

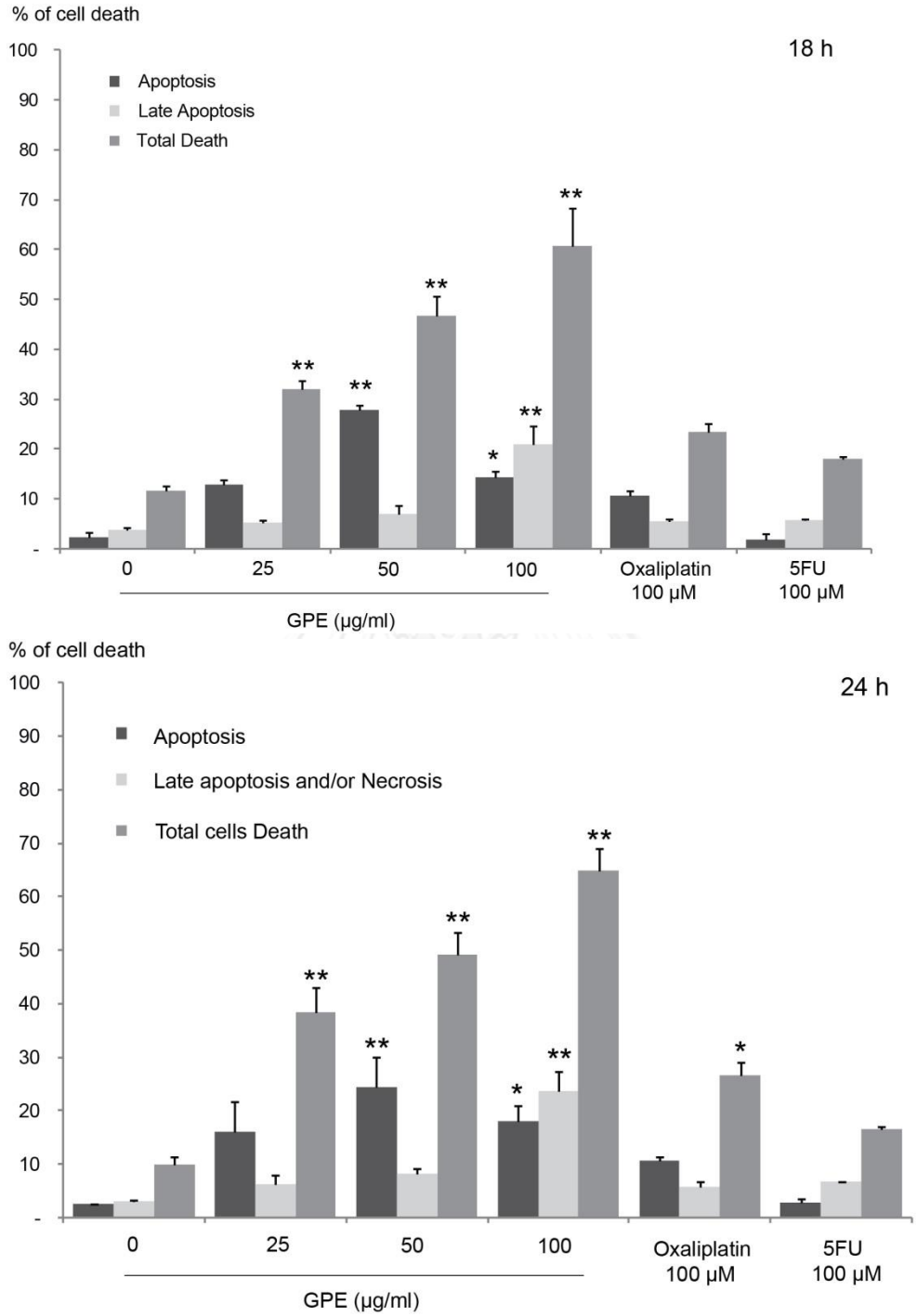


Figure 27: The apoptotic effect of GPE on Colo-205 cells. The cells were treated with 25 – 100µg/ml of GPE for 18 (A) and 24 h (B). The percentage of cells labeled with annexin V+/PI- (early apoptotic cells), annexin V+/PI+ (late apoptotic cells/necrotic cells) and total cell death were plotted. Data are the mean values ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

3.3 Effect of GPE on the expression of genes in BCL-2 family

To evaluate the mechanism by which GPE induced apoptosis in Colo-205, the mRNA expression of BCL-2 family genes were assessed by quantitative RT-PCR.

Colo-205 cells were treated with 25, 50 and 100 μg/ml of GPE for 24 h, and then the cells were subjected to RNA isolation, cDNA conversion, and PCR amplification of target genes. GPE at 25, 50 and 100μg/ml significantly decreased the expression of anti-apoptotic BCL-2 to 0.06±0.01, 0.39±0.07 and 0.09±0.05, respectively. Moreover, the expression of anti-apoptotic BCL-XL was down-regulated to 0.23±0.00 and 0.42±0.05 following GPE 50 and 100μg/ml treatment, respectively (Figure 28). On the other hand, treatment with GPE tended to decrease pro-apoptotic BAK and BAX. However, the ratio of pro-apoptotic/anti-apoptotic (BAK and BAX/BCL-2 and BCL-XL) were favor apoptotic cell deaths (ratio > 1) with the ratio of 1.68, 1.94, and 3.09 following GPE 25, 50, and 100μg/ml treatment, respectively (Figure 29).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

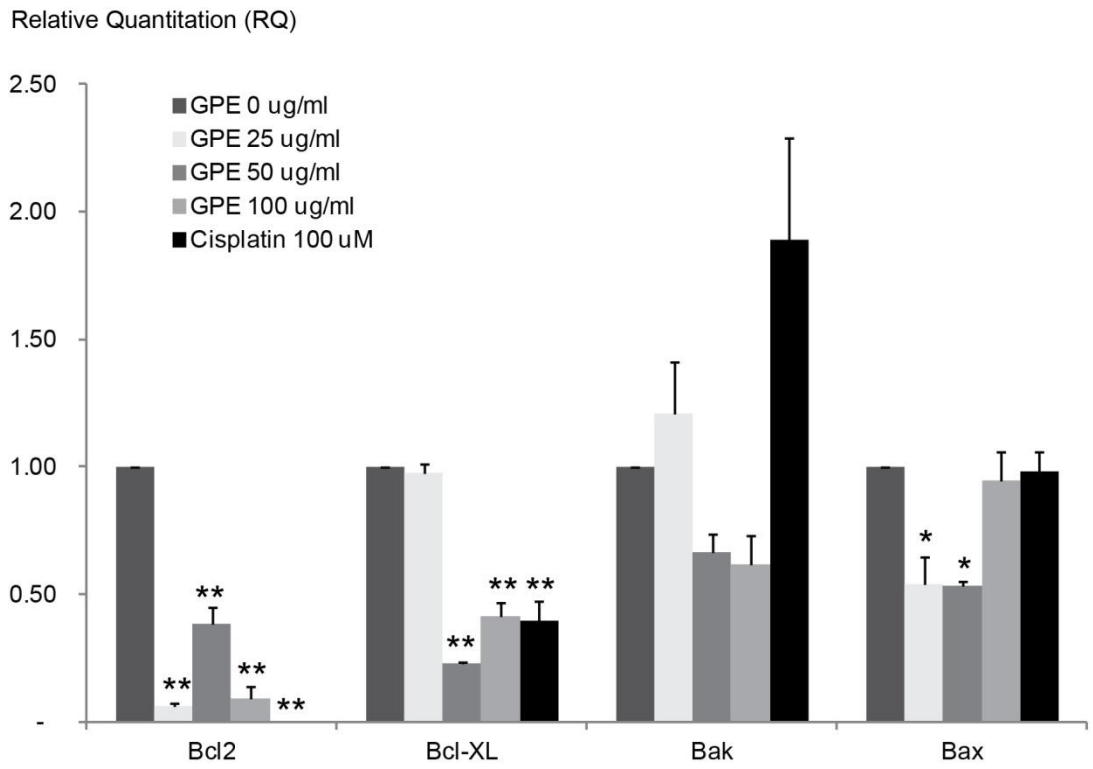


Figure 28: GPE alters the expression of BCL-2 family genes in Colo-205 cells. The expressions of BCL-2 family genes were determined by quantitative RT-PCR. The cells were treated with GPE 25 - 100µg/ml for 24 h. The treated cells were subjected to RNA isolation, cDNA conversion and quantitative RT-PCR amplification of BAK, BAX, BCL-2 and BCL-XL genes. The value is given as relative copy number normalized to the endogenous control GAPDH and 0.2% DMSO solvent control. The data represent the means ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

Chulalongkorn University

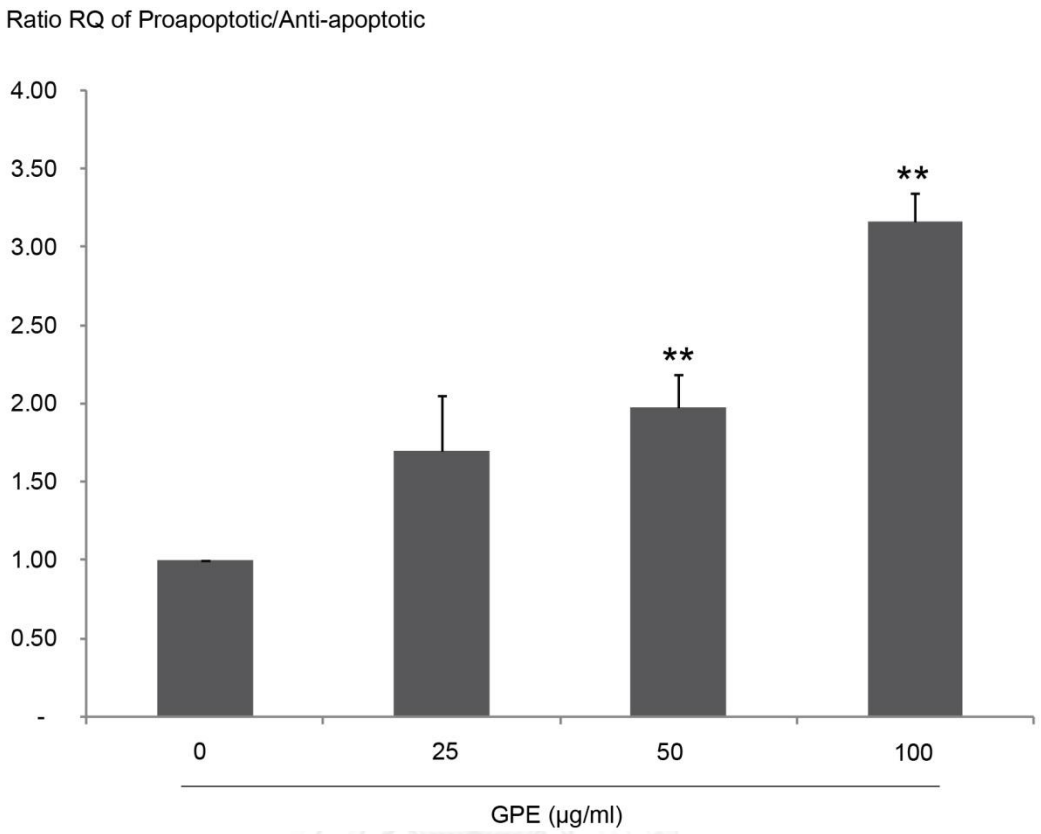


Figure 29: The ratio of pro-apoptotic/anti-apoptotic expression (BAK and BAX/BCL-2 and BCL-XL) in Colo-205 treated cells. The cells were treated with GPE 25 - 100µg/ml for 24 h. The treated cells were subjected to RNA isolation, cDNA conversion and quantitative RT-PCR amplification of BAK, BAX, BCL-2 and BCL-XL genes. The data represent the means $\pm$ S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

Chulalongkorn University

3.4 Effect of GPE on the cell proliferation

Inhibition of Colo-205 cell proliferation by GPE was determined by cell counting. Standard cytotoxic drug, 5-FU, was used as a positive control in this experiment.

Colo-205 cells were treated with 25, 50 and 100 µg/ml of GPE for 24 h, afterward the total cells were counted. The results were expressed as a percentage of cells compared to the untreated control. GPE significantly inhibited Colo-205 cell proliferation to 60.71±5.13%, 71.63±8.84%, and 59.91±2.44% at GPE 25, 50 and 100µg/ml, respectively when compared to the vehicle control. However, the effect was not seen in a dose-dependent manner (Figure 30). The cell proliferation inhibition effect of GPE against Colo-205 was slightly lower than that of 5FU which 100 µM 5FU significantly inhibited cell proliferation to 53.91±5.08%.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

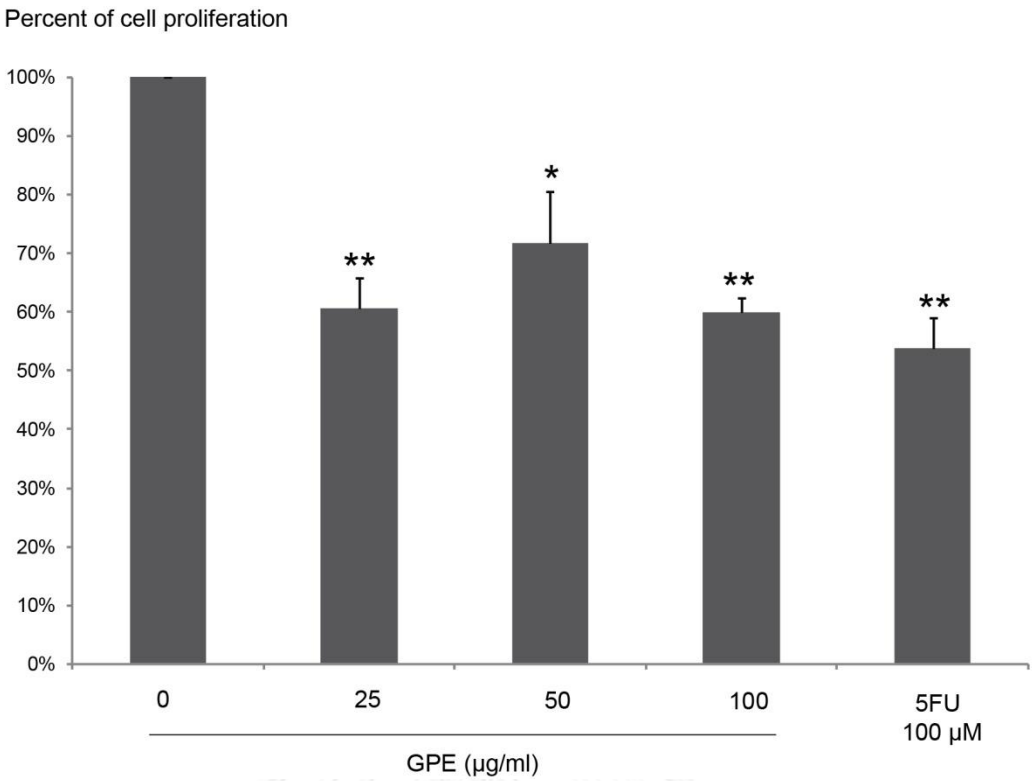


Figure 30: Anti-proliferative effects of GPE against Colo-205 cells. The cells were treated with GPE 25 – 100µg/ml for 24 h. Cell proliferation was measured by cell counting using an automated cell counter. The data represent the means ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.5 Effect of GPE on the cell cycle progression

Colo-205 cells were treated with 25, 50 and 100 µg/ml GPE for 1 h and further incubated for 24 h in a fresh medium without GPE. The treated cells were subjected to fluorescence flow cytometric analysis for DNA content. 5FU which arrests the cell cycle at S phase and causes cell accumulation at G1/S phase was used as a positive control (Figure 31).

The effect of GPE on cell cycle pattern of Colo-205 cells was difference which depended on the concentration used. GPE at 25 and 50µg/ml caused a decrease in the proportion of cells in the S phase (from 36.37±0.83% to 25.49±2.00% and 16.62±1.79%, respectively). In cells treated with 25µg/ml GPE, this decrease was associated primarily with accumulation of cells in the G0/G1 phase (from 51.49±1.73% to 65.39±1.04%). At 50µg/ml, GPE resulted in a significant reduction in the S fraction with a shift of cells accumulation to the G2/M phases (from 12.14±0.95% to 30.07±1.73%) as shown in Figures 32. The positive control, 5FU, significantly accumulated cells population at S fraction with the reduction of the cells in G2/M phases. The molecular mechanism of GPE on cell cycle pattern was further evaluated at the same concentration range and treatment time.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

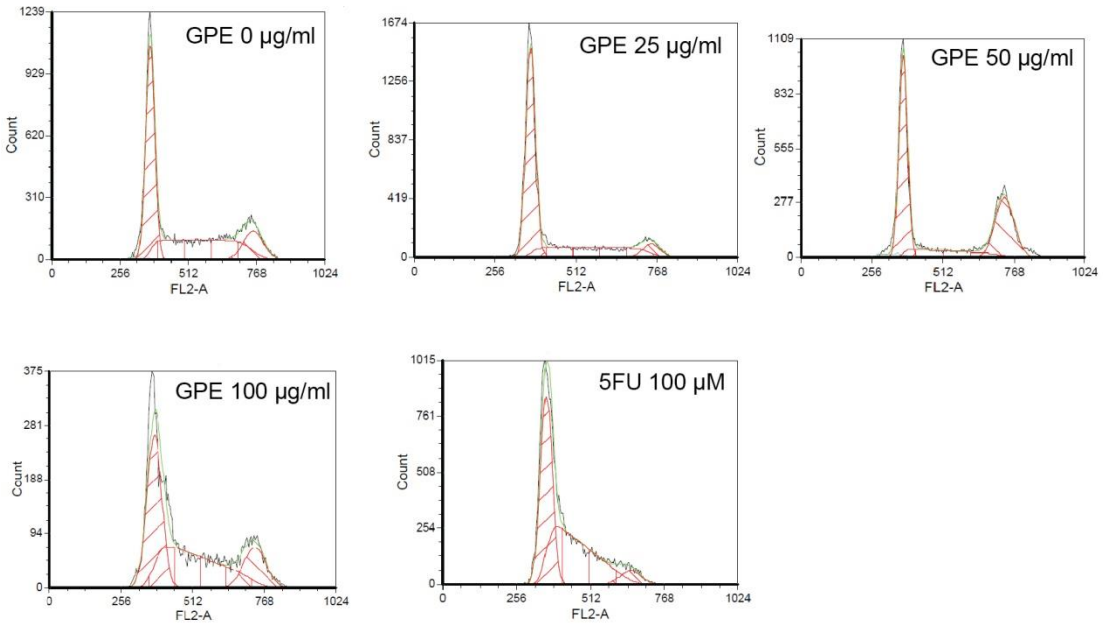


Figure 31: Representative histogram from flow cytometric analysis of Colo-205 cell cycle pattern after treatment with GPE. The cells were treated with GPE at 25 – 100 µg/ml for 1 h. The treated cells were washed and incubated in fresh medium for additional 24 h. Analysis of cells in the cell cycle was performed by fixing and staining the cells with PI. Cell cycle pattern of $3x10^4$ cells/sample were analyzed using fluorescence flow cytometer. The cell cycle distributions were evaluated using FCS Express 4 Image Cytometry.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

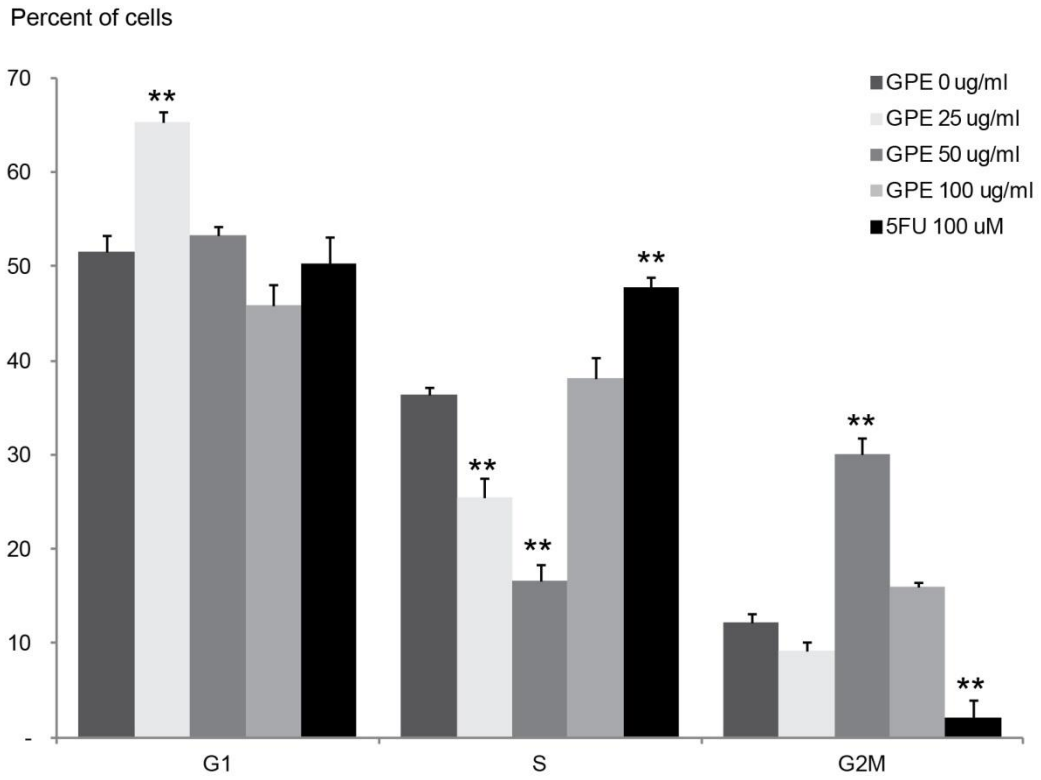


Figure 32: Distribution of Colo-205 cells in the cell cycle phases after treatment with GPE. The cells were treated with GPE 25 - 100µg/ml for 1 h. The treated cells were washed and incubated in fresh medium for additional 24 h. Analysis of cells in the cell cycle was performed by fixing and staining the treated cells with PI followed by fluorescence flow cytometry. The percentage of cells in G1, S, and G2M phases are given as means ± S.E. of 3 independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

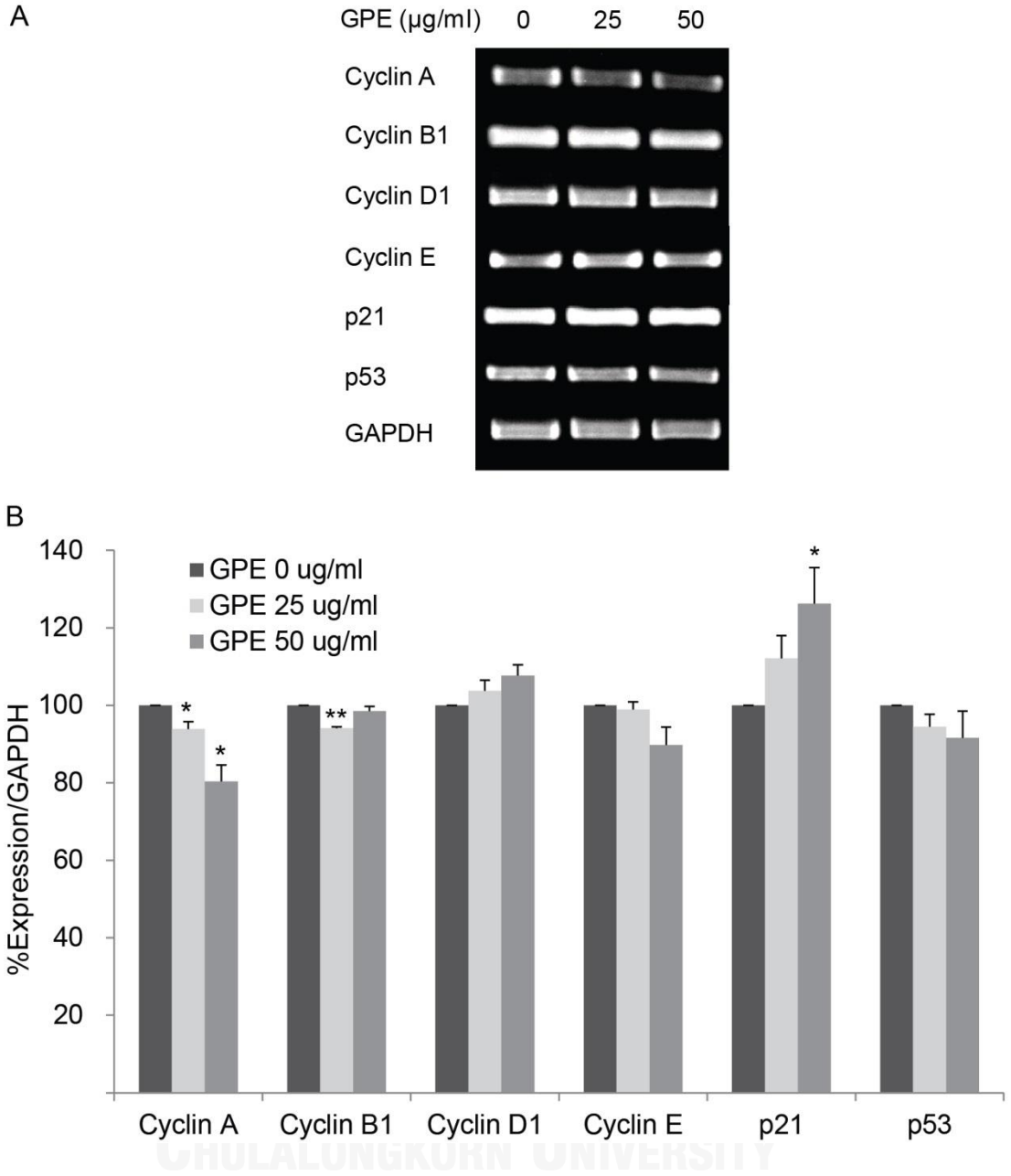
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.6 Effect of GPE on the mRNA expression of cell cycle regulatory genes

Since GPE changed the cell cycle pattern of Colo-205 cells, the alterations in the expression of cell cycle regulatory genes might be involved. Colo-205 cells were treated with 25, 50 and 100 µg/ml GPE for 1 h and further incubated for 24 h in a fresh medium without GPE. The treated cells were subjected to RNA isolation, cDNA conversion, PCR amplification of the genes involved in cell cycle progression including cyclin A, cyclin B1, cyclin D1, cyclin E, p21 and p53. PCR products were analyzed by agarose gel electrophoresis. The housing keeping genes, GAPDH, was used as a loading control.

As shown in Figures 33, GPE at the concentration of 25 and 50 µg/ml significantly down-regulated cyclin A expression in a dose-dependent manner. The cyclin E expression was likely to decrease following GPE treatment. However, the change was not statistically significant. The expression on CDKI p21 tended to decrease following GPE treatment though it reached statistically significant only at 50µg/ml concentration. Moreover, the level of cyclin B1, cyclin D1, and p53 remained unchanged. As the GPE at 100 µg/ml caused majority of Colo-205 cell death which affected the expression of the housing keeping gene, GAPDH, the effects of 100 µg/ml GPE on Colo-205 mRNA expression of cell cycle regulatory genes were excluded from the analysis.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University



CHULALONGKORN UNIVERSITY

Figure 33: GPE alters the expression of cell cycle regulators genes in Colo-205. The cells were treated with GPE 25 and 50µg/ml for 1 h and incubated with fresh medium without GPE for additional 24 h. Expression of cell cycle regulators were determined by RT-PCR. A) A representative of PCR products of the cell cycle regulatory genes. B) Densitometric analysis of the PCR products relative to GAPDH presented as % of control. The data represent the means $\pm$ S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO).

4. Compare the effect of GPE on human colorectal cancer cell expressing COX-2, HT-29 cells and not expressing COX-2, Colo205 cells.

4.1 Cytotoxic activities of GPE

The cytotoxic effect of GPE against HT-29 and Colo-205 was compared using independent sample student t test. At 24 h of treatment, the IC_{50} of GPE against HT-29 and Colo-205 was not significantly difference. However, with the increased treatment time to 48 h and 72 h, GPE was significantly more cytotoxic against Colo-205 than HT-29. The cytotoxic activity of GPE against both HT-29 and Colo-205 were seen at 25 – 100 µg/ml of GPE (Figure 34).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

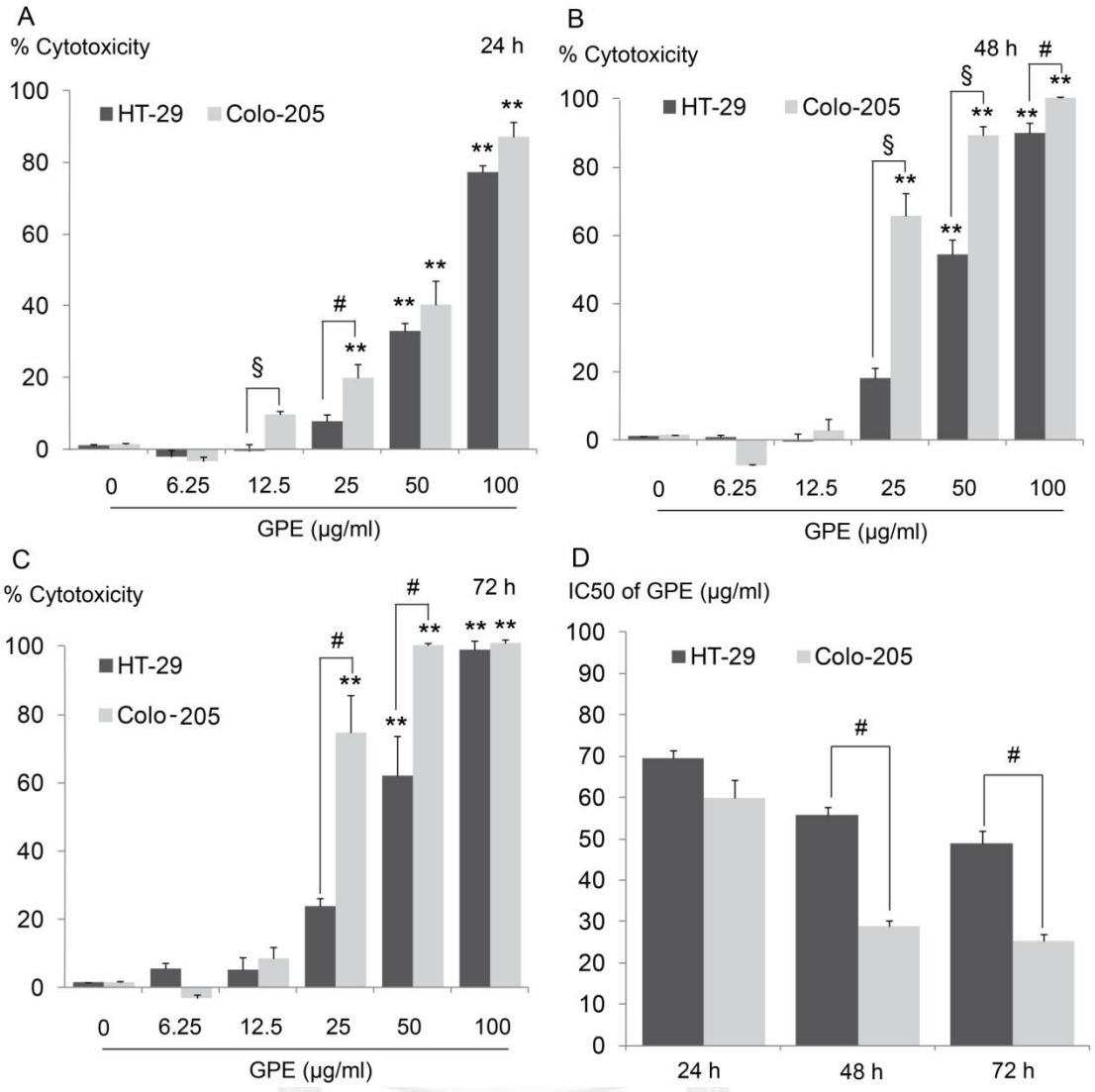


Figure 34: Cytotoxic effects of GPE against HT-29 and Colo-205 cells. The cells were treated with GPE at 6.25 - 100µg/ml for 24 (A), 48 (B) and 72 (C) h. The cytotoxic effects were determined by resazurin reduction assay. The percentages of cytotoxicity of GPE were plotted against GPE concentration. Linear trend lines of each treatment time were calculated. The linear equations were used to determine the IC_{50} of GPE at each treatment time (D). The data represent the means ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO). # $p<0.05$ and § $p<0.01$ indicate statistically significant difference between the two cell lines.

4.2 Apoptotic induction effect of GPE

GPE can induce apoptotic cells death in both HT-29 and Colo-205 cells. At 18 h of treatment, GPE at 25 and 100 µg/ml significantly induced apoptotic cells death in Colo-205 cells higher than HT-25 cells. At 24 h, there was no significant difference in the induction of apoptosis between the two cell lines (Figure 35). The total cells death was also significantly higher in Colo-205 treated cells.

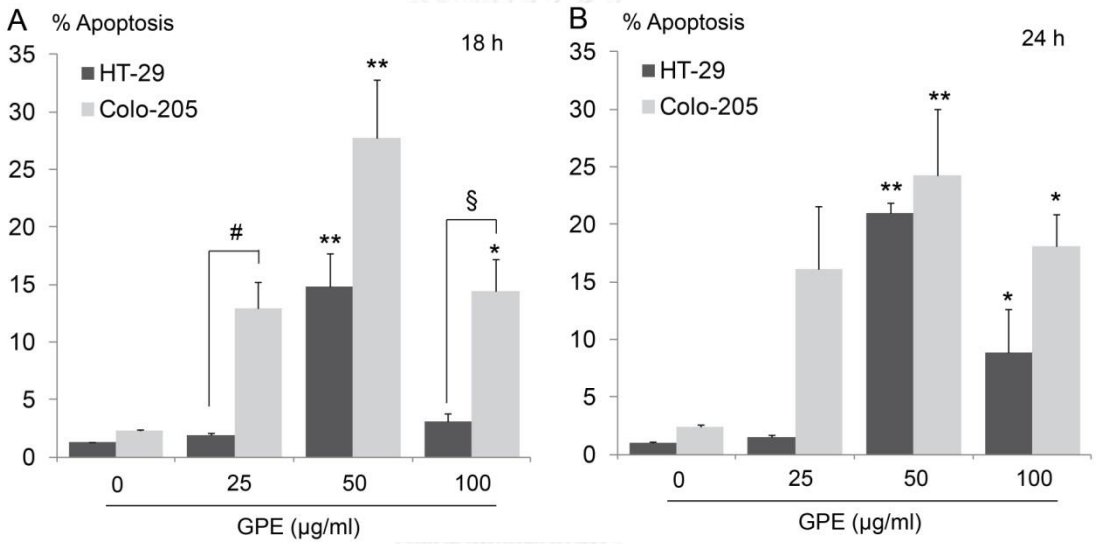


Figure 35: The apoptotic effect of GPE on HT-29 and Colo-205 cells. The cells were treated with 25 – 100µg/ml of GPE for 18 (A) and 24 h (B). The percentage of cells labeled with annexin V^+/PI^-(early apoptotic cells) were plotted. Data are the mean values ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO). # $p<0.05$ and § $p<0.01$ indicate statistically significant difference between the two cell lines.

4.3 Effect of GPE on the expression of genes in BCL-2 family

The expressions of genes in BCL-2 family in HT-29 and Colo-205 cells after treatment with GPE were both altered in the favor of apoptotic cells death. However, there were some differences in the expression of these genes in the two cell lines. Anti-apoptotic BCL-2 gene was down-regulated in both HT-29 and Colo-205 cells following GPE treatment. GPE at 25μg/ml significantly decreased BCL-2 gene expression in Colo-205 more than HT-29 cells. Anti-apoptotic BCL-XL gene also decreased in both cell lines after exposed to GPE. The difference in the reduction in BCL-XL expression was significantly seen at GPE 50μg/ml which affected Colo-205 more than HT-29. GPE tended to increase pro-apoptotic BAK in HT-29 cells. In contrast, the expression of BAK was down-regulated in Colo-205 after treated with GPE. The expression of pro-apoptotic BAX was likely to decrease following GPE treatment in both HT-29 and Colo-205 cells (Figure 36).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

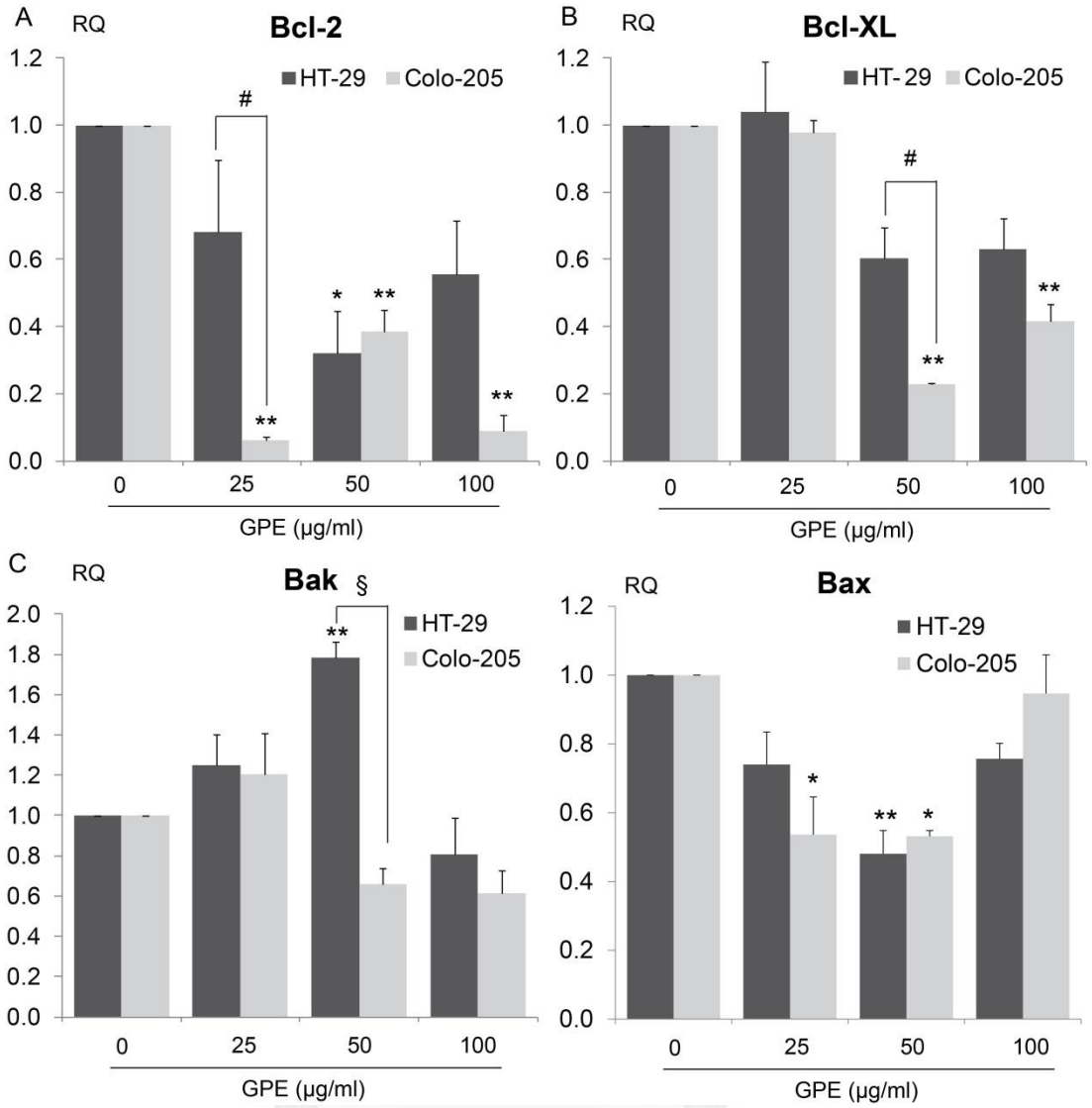


Figure 36: GPE alters the expression of BCL-2 family genes in HT-29 and Colo-205 cells. The expressions of BCL-2 family genes were determined by quantitative RT-PCR. The cells were treated with GPE 25 - 100µg/ml for 24 h. The treated cells were subjected to RNA isolation, cDNA conversion and quantitative RT-PCR amplification of BCL-2 (A), BCL-XL (B), BAK (C) and BAX (D) genes. The value is given as relative copy number normalized to the endogenous control GAPDH and 0.2% DMSO solvent control. The data represent the means ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO). # $p<0.05$ and § $p<0.01$ indicate statistically significant difference between the two cell lines.

4.4 Effect of GPE on the cell proliferation

GPE at 25-100 µg/ml significantly inhibited HT-29 and Colo-205 cells proliferation. However, the intensity was different in the two cells line. GPE at 100µg/ml significantly inhibit HT-29 cells proliferation more than Colo-205 cells. The concentration dependent of the cell proliferation inhibition was seen only in HT-29 treated with GPE (Figure 37).

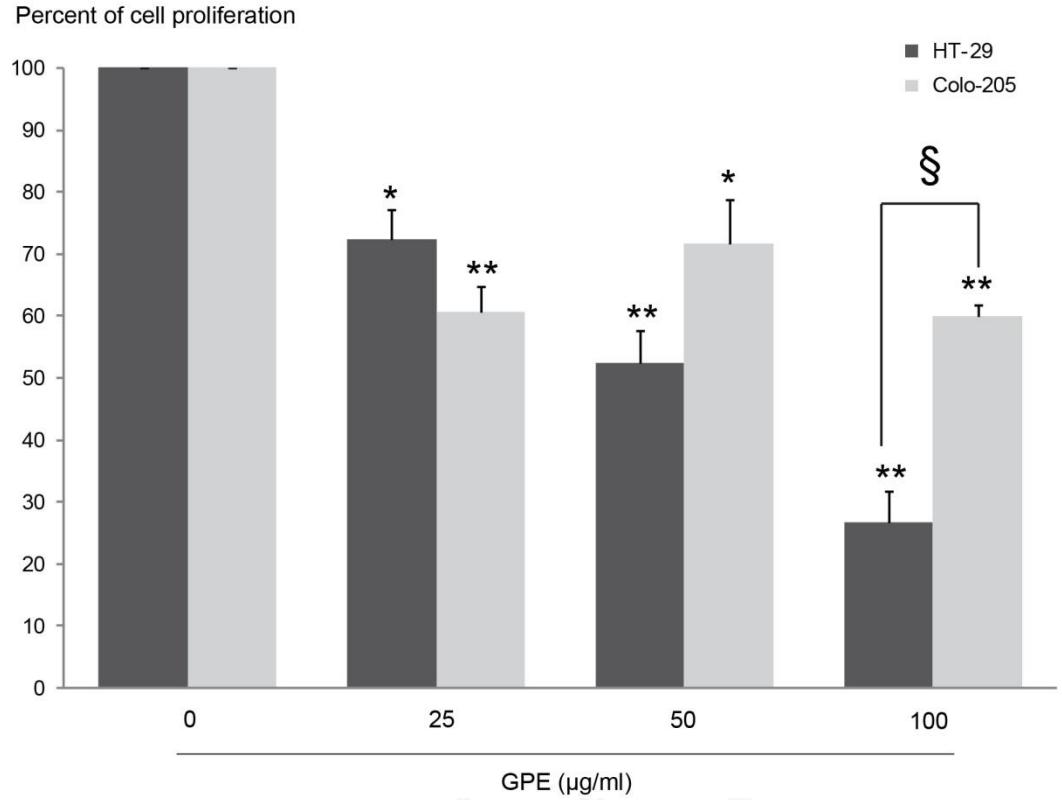


Figure 37: Anti-proliferative effects of GPE against HT-29 and Colo-205 cells. The cells were treated with GPE 25 – 100µg/ml for 24 h. Cell proliferations were measured by cell counting using an automated cell counter. The data represent the means ± S.E. of three independent experiments. * $p<0.05$ and ** $p<0.01$ indicate statistically significant difference when compare to the solvent control (0.2% DMSO). # $p<0.05$ and § $p<0.01$ indicate statistically significant difference between the two cell lines.

4.5 Effect of GPE on the cell cycle progression

The effect of GPE on the cell cycle pattern of HT-29 and Colo-205 cells was difference depended on the concentration of GPE. Cell cycle pattern had shifted to the decrease in S phase and increase in G1 cells population following GPE 50 and 25μg/ml in HT-29 and Colo-205, respectively. The pattern had changed to the decrease in S phase with the cell accumulation in G2/M phase after treated with GPE 100 and 50μg/ml in HT-29 and Colo-205, respectively (Figure 38).

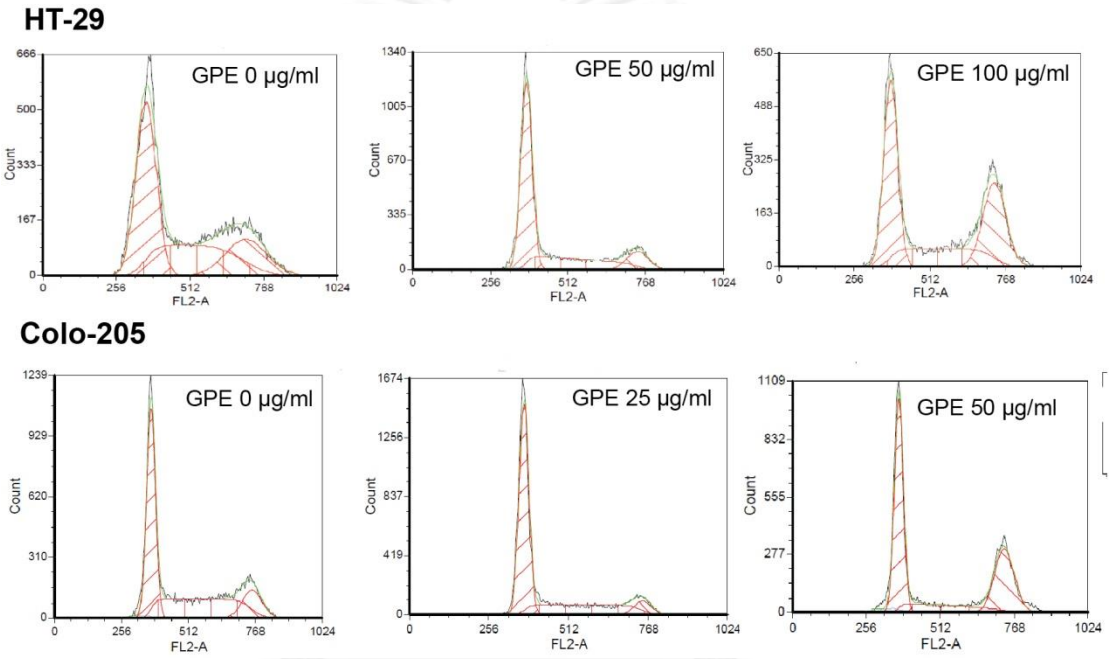


Figure 38: Representative histogram from flow cytometric analysis of HT-29 and Colo-205 cell cycle pattern after treatment with GPE. The cells were treated with GPE at 25 – 100 μg/ml for 1 h. The treated cells were washed and incubated in fresh medium for additional 24 h. Analysis of cells in the cell cycle was performed by fixing and staining the cells with PI. Cell cycle pattern of $3x10^4$ cells/sample were analyzed using fluorescence flow cytometer. The cell cycle distributions were evaluated using FCS Express 4 Image Cytometry.

4.6 Effect of GPE on the mRNA expression of cell cycle regulatory genes

GPE affected the expression of cell cycle regulatory genes in both HT-29 and Colo-205. The expression of cyclin A was down-regulated following GPE treatment in both cell lines. GPE decreased the expression of cyclin E gene significantly only in HT-29 cells. The expression of CDKI, p21, was significantly up-regulated only in Colo-205 treated cells at GPE 50μg/ml. The level of cyclin B1, cyclin D1, and p53 remained unchanged in both cell lines.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER V
DISCUSSION AND CONCLUSION

In this study, the effects of EtOAc extract from the leaves of *G. parva* (GPE) were examined on human colorectal cancer expressing COX-2, HT-29, and non-expressing COX-2, Colo-205 cells. The GPE exhibited cytotoxic activity, inhibited cell proliferation, and induced apoptosis as well as cell cycle arrest in both cell lines but with some differences intensity. The effect of GPE on cytotoxicity and induction of apoptosis were higher in Colo-205 cells than HT-29 cells. However, the anti-proliferative effects of GPE were superior in HT-29 cells. The changes in cell cycle pattern following GPE treatment were similar in both cell lines with the reduction of cells in S phases which accompanied with the accumulation of cells in G0/G1 and G2M phases. Further studies suggested that the effects of GPE may be mediated through both COX-2 dependent and COX-2 independent pathway. The COX-2 independent pathway included the changes in the expression of cell cycle control genes and the alteration in the balance of BCL-2 family gene expression. While the COX-2 dependent involved with reduction of COX-2 expression which could affect downstream COX-2 pathway. However, these two pathways may have overlapping or crosstalk mechanisms.

Throughout medical history, plant products have been shown to be valuable sources of novel anti-cancer drugs discovery (99). *Glycosmis parva* is a wild small shrub found in Thailand. Chemical examination of the EtOAc extract from the leaves of *G. parva* led to the identification of the acridone alkaloid and sulfur-containing propanamides as its major active compounds. Arborinine, a main acridone alkaloid found in EtOAc of the leaves of *G. parva*, has been reported to have potent anti-proliferative effect against cervix adenocarcinoma HeLa cells with an IC_{50} of 1.84 μM, which was lower than that of cisplatin (12.43 μM) (36), indicating arborinine possesses higher anti-proliferative activity than cisplatin. Additionally, antifungal activities of sulfur-containing propanamides derivative, S-deoxydihydroglyparvin, S-deoxytetrahydroglyparvin, glyparvin-A, and dihydroglyparvin, (8) were reported (83). In the present study, the results showed that GPE possessed moderate cytotoxic

activity against HT-29 and Colo-205 with the IC_{50} of 69.49 and 59.92μg/ml, respectively, after 24 h exposure. The cytotoxicity of the extract was in a dose- and time-dependent manner in both cell lines. GPE was more cytotoxic to Colo-205 than HT-29 cells especially with higher exposure time. The cytotoxicity study by resazurin reduction assay demonstrated both the ability of the tested compound to kill the cells and the effect of cell proliferation inhibition. Therefore, the effects of the GPE on cell death and cell proliferation would further clarify the different effects on the two cell lines.

Treatment of cancer with ionizing radiation and several anticancer drugs kills target cells primarily by induction of apoptosis (100). The results showed that GPE at 50μg/ml significantly induced cell death mainly by apoptosis in both HT-29 and Colo-205 cells. The proportion of apoptotic cell death to total cell death was higher than that of currently used cytotoxic drugs, oxaliplatin and 5FU which were used as positive controls in this study. However, at higher concentration, 100μg/ml of GPE, the late apoptotic and/or necrotic cell death was more prominent than early apoptotic cells. The apoptotic induction effects of GPE were higher in Colo-205 than HT-29 cells.

Activation of apoptosis signaling following treatment with cytotoxic drugs has been shown to lead to activation of the mitochondrial (intrinsic) pathway of apoptosis (96). BCL-2 family proteins play a pivotal role in the regulation of the mitochondrial pathway of apoptosis. They comprise both anti-apoptotic members-e.g., BCL-2, BCL-XL, and MCL-1-as well as pro-apoptotic molecules such as BAX, BAK, and BAD (101). GPE decreased the expression of anti-apoptotic BCL-2 significantly in both HT-29 and Colo-205 cells while anti-apoptotic BCL-XL was significantly decreased only in Colo-205. However, GPE at 50μg/ml significantly increased the expression of pro-apoptotic BAK and BAX only in HT-29. Although, some concentrations of GPE decreased pro-apoptotic BAK and BAX expression in both cells, the ratios of pro-apoptotic/anti-apoptotic (BAK and BAX/BCL-2 and BCL-XL) were designated cancer cell death.

Cell proliferation is a key character of cancer progression (102). The results showed that GPE significantly inhibited both HT-29 and Colo-205 cells proliferation.

The dose dependence was only seen in HT-29 cells. The effects were found significantly across the experimental dose-range (25-100µg/ml) in both cell lines but the proliferation inhibition effects were higher in HT-29 cells.

The ability of anticancer agents to suppress the growth of cancer cells can also associated with blocking the cell cycle progression (103). Based on the significant inhibition of HT-29 and Colo-205 cell proliferation by GPE, it was postulated that the extract might modulate the cell cycle progression of these cancer cells. Cell cycle distribution analysis showed that GPE caused a reduction in S phase population which was associated with accumulation of cells in the G0/G1 phase. At higher concentration of GPE, the treated cells accumulated at the G2/M phases. The cell cycle pattern changes were similar in both cell lines but the effects were seen in different concentration of GPE. This result may in part due to more than one active compounds presented in the extract affected on the cell cycle but at different phase of the cycle. Additional studies to identify active compounds presented in the EtOAc extract are warranted.

Cyclin dependent kinases (CDKs) and cyclins are important determinants in the regulation of cell cycle progression. As the cells progress from G1 to mitosis, a series of CDKs is expressed along with that of cyclins (104). Cell cycle arrest involves down-regulation of cyclins and CDKs as well as up-regulation of cyclin dependent kinase inhibitors (CDKIs) (105). Cyclin E/CDK2 and cyclin A/CDK2 complexes play an important role in G1-S transition and DNA replication, respectively. Moreover, cyclin A can form complex with CDC2, which is essential for mitosis (104). The CDKI p21 mediates p53-dependent cell cycle arrest through inhibition of CDK2–cyclin E (progression into and through S phase), CDK2–cyclin A (progression through S phase) and CDK1–cyclin A (progression into G2), and CDK1–cyclin B1 (progression through G2 and G2/M) which lead to growth arrest at specific stages in the cell cycle (106). The results indicated that GPE down-regulated mRNA expression of cyclin A and cyclin E in a dose-dependent manner in HT-29 cells. It also decreased cyclin A expression and up-regulated p21 expression in Colo-205 cells. This may explain why GPE could induce cell cycle arrest and the changes in the cell cycle pattern in both cell lines.

Cyclooxygenase enzymes are required for the conversion of arachidonic acid to prostaglandins. COX-2 mediates the inflammatory effects of COX activity. It is induced by a wide spectrum of growth factors and pro-inflammatory cytokines and overexpressed in numerous premalignant and malignant lesions, including CRC. Treatment with the selective COX-2 inhibitor has shown promising results in the prevention of CRC (6). However, the dose-related cardiovascular toxicity of selective COX-2 inhibitors remains a concern (56). Discovery of agents that regulate biological pathways related to COX-2 pathway may provide new avenues for the treatment of CRC. Previous study has shown that GPE inhibited mRNA expressions of COX-2 in LPS stimulated-macrophage J774A.1 cells (10). Similarly, the inhibitory effect of GPE on COX-2 expression was detected in human colorectal cancer cells constitutively expressed COX-2, HT-29 cells.

The overall effects of GPE on HT-29 and Colo-205 cells showed that regardless of the COX-2 expression status, GPE possessed an anti-cancer activity against human colorectal cancer cells. The mechanisms engaged both COX-2 dependent and COX-2 independent pathway. The COX-2 dependent mechanisms mainly involved the action of PGE_2, the most abundant COX-2-derived prostaglandins found in human CRC (7). PGE_2 stimulates proliferation and suppresses apoptotic cell death of cancer cells (45). PGE_2 activates multiple intracellular signaling pathways, including epidermal growth factor receptor (EGFR), protein kinase C (PKC)-ERK-c-Myc, and ß-catenin/TCF pathways (107). However, further mechanistic study on the action of GPE needed to be done in order to confirm its action. The COX-2 independent mechanisms may involve the alteration of the expression of cell cycle regulators, arrested cell cycle, and induced apoptosis in the colon cancer cells that do not express COX-2 (108). These were in line with our findings that GPE arrested cell cycle, changed the expression of cell cycle regulators, and induced apoptosis in Colo-205 which did not express COX-2.

Nevertheless, there are some limitations from this study. Although HT-29 and Colo-205 are both human colorectal cancer cells which have different COX-2 expression status, they came from different origin. HT-29 is a colorectal adenocarcinoma from primary site while Colo-205 is a colorectal adenocarcinoma

derived from ascites metastatic site. They have comparable population doubling time but different growth pattern. HT-29 is an adherent cell while Colo-205 grows as mixed adherent and suspension cells. They have similar oncogenes expression including c-myc, H-ras, K-ras, N-ras, myb, fos, and p53 (109). The mutational statuses of the two cell lines are somewhat different. HT-29 carries APC, BRAF, and PIK3CA mutations while Colo-205 has BRAF mutation and APC wild type. Both cells carry SMAD4 and TP53 wild type. These differences in the two cell lines needed to be taking into consideration when interpret the result from the study.

The promising anti-cancer action of GPE against human colorectal cancer cells with the interesting mechanism of actions involved both COX-2 dependent and COX-2 independent pathway deserve a further investigation of its possibility to be a candidate for colorectal cancer treatment. With the understanding of chemical constituents found in GPE, together with the confirmed anti-cancer activity, this could be a valuable source of information that benefits researchers in the anti-cancer drug development field.

Conclusions

The anti-cancer activity of the EtOAc extract from leave of *Glycosmis parva* on human colorectal cancer cells and its underlying mechanism could provide the basis for further development of the compounds from this plant which could be a potential compounds for cancer therapeutics and chemoprevention in colorectal cancer.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics. *CA: A Cancer Journal for Clinicians* 61:69-90
2. NCI. 2011. 2010 Hospital-Based Cancer Registry, National Cancer Institute, Department of Medical Service, Ministry of Public Health, Bangkok, Thailand
3. de Gramont A, Figer A, Seymour M, Homerin M, Hmissi A, et al. 2000. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *Journal of Clinical Oncology* 18:2938-47
4. Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJM, et al. 2009. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. *New England Journal of Medicine* 360:563-72
5. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, et al. 2006. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Research* 66:3992
6. Koehne C-H, Dubois RN. 2004. COX-2 inhibition and colorectal cancer. *Seminars in Oncology* 31, Supplement 7:12-21
7. Wang D, Dubois RN. 2010. The role of COX-2 in intestinal inflammation and colorectal cancer. *Oncogene* 29:781-8
8. Chansriniyom C, Ruangrungsi N, Lipipun V, Kumamoto T, Ishikawa T. 2009. Isolation of acridone alkaloids and N-[(4-monoterpenyloxy)phenylethyl]-substituted sulfur-containing propanamide derivatives from *Glycosmis parva* and their anti-herpes simplex virus activity. *Chemical and Pharmaceutical Bulletin (Tokyo)* 57:1246-50
9. Rosena A. 2010. *Cytotoxic activity and mechanism of Glycosmis parva leaf extracts on human B-lymphoma cells.* Chulalongkorn University, Bangkok,Thailand
10. Chumseng S. 2009. *Activity of the extracts from glycosmis parva craib in macrophage cell J774A.1*. Chulalongkorn University, Bangkok,Thailand
11. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer* 127:2893-917
12. Doherty GA, Murray FE. 2009. Cyclooxygenase as a target for chemoprevention in colorectal cancer: lost cause or a concept coming of age? *Expert Opinion on Therapeutic Targets* 13:209-18
13. Groden J, Thliveris A, Samowitz W, Carlson M, Gelbert L, et al. 1991. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* 66:589

14. Liu B, Parsons R, Papadopoulos N, Nicolaides NC, Lynch HT, et al. 1996. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nature Medicine* 2:169-74
15. Markowitz SD, Bertagnolli MM. 2009. Molecular basis of colorectal cancer. *New England Journal of Medicine* 361:2449-60
16. Benson III A, Amoletti J, Bekaii-Saab T. 2011. NCCN Clinical Practice Guidelines in Oncology: Colon Cancer. Version 3, 2012.
17. Goyle S, Maraveyas A. 2006. Chemotherapy for colorectal cancer. *Digestive Surgery* 22:401-14
18. Longley DB, Harkin DP, Johnston PG. 2003. 5-fluorouracil: mechanisms of action and clinical strategies. *Nature Reviews Cancer* 3:330-8
19. André T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, et al. 2004. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *New England Journal of Medicine* 350:2343-51
20. Raymond E, Faivre S, Chaney S, Woynarowski J, Cvitkovic E. 2002. Cellular and Molecular Pharmacology of Oxaliplatin. *Molecular Cancer Therapeutics* 1:227-35
21. Xu Y, Villalona-Calero M. 2002. Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. *Annals of Oncology* 13:1841-51
22. Rothenberg ML. 2001. Irinotecan (CPT-11): recent developments and future directions–colorectal cancer and beyond. *The Oncologist* 6:66-80
23. Gilbert D, Chalmers A, El-Khamisy S. 2011. Topoisomerase I inhibition in colorectal cancer: biomarkers and therapeutic targets. *British Journal of Cancer* 106:18-24
24. Arun B, Frenkel EP. 2001. Topoisomerase I inhibition with topotecan: pharmacologic and clinical issues. *Expert Opinion on Pharmacotherapy* 2:491-505
25. Muhsin M, Graham J, Kirkpatrick P. 2004. Bevacizumab. *Nature Reviews Drug Discovery* 3:995-6
26. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, et al. 2004. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *New England Journal of Medicine* 350:2335-42
27. Jonker DJ, O'Callaghan CJ, Karapetis CS, Zalcberg JR, Tu D, et al. 2007. Cetuximab for the treatment of colorectal cancer. *New England Journal of Medicine* 357:2040-8
28. Mendelsohn J, Baselga J. 2000. The EGF receptor family as targets for cancer therapy. *Oncogene* 19:6550-65
29. Bardelli A, Jänne PA. 2012. The road to resistance: EGFR mutation and cetuximab. *Nature Medicine* 18:199-200
30. Chandrasekharan N, Simmons DL. 2004. The cyclooxygenases. *Genome biology* 5

31. Narumiya S, FitzGerald GA. 2001. Genetic and pharmacological analysis of prostanoid receptor function. *Journal of Clinical Investigation* 108:25-30
32. Ramsay R, Ciznadija D, Vanevski M, Mantamadiotis T. 2003. Transcriptional regulation of cyclo-oxygenase expression: three pillars of control. *International Journal of Immunopathology and Pharmacology* 16:59
33. Kis B, Snipes JA, Isse T, Nagy K, Busija DW. 2003. Putative cyclooxygenase-3 expression in rat brain cells. *Journal of Cerebral Blood Flow & Metabolism* 23:1287-92
34. Sheehan KM, Sheahan K, O'Donoghue DP, MacSweeney F, Conroy RM, et al. 1999. The relationship between cyclooxygenase-2 expression and colorectal cancer. *JAMA: The Journal of the American Medical Association* 282:1254-7
35. Sheehan KM, O'Connell F, O'Grady A, Conroy RM, Leader MB, et al. 2004. The relationship between cyclooxygenase-2 expression and characteristics of malignant transformation in human colorectal adenomas. *European Journal of Gastroenterology & Hepatology* 16:619
36. Rethy B, Zupko I, Minorics R, Hohmann J, Ocsovszki I, Falkay G. 2007. Investigation of cytotoxic activity on human cancer cell lines of arborinine and furanoacridones isolated from *Ruta graveolens*. *Planta Medica-Natural Products and Medicinal Plant Research* 73:41-8
37. Williams C, Luongo C, Radhika A, Zhang T, Lamps L, et al. 1996. Elevated cyclooxygenase-2 levels in Min mouse adenomas. *Gastroenterology* 111:1134-40
38. DuBois R, Radhika A, Reddy B, Entingh A. 1996. Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. *Gastroenterology* 110:1259-62
39. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, et al. 1996. Suppression of intestinal polyposis in Apc [Delta] 716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 87:803-9
40. Rigas B, Goldman I, Levine L. 1993. Altered eicosanoid levels in human colon cancer. *The Journal of Laboratory and Clinical Medicine* 122:518
41. Backlund MG, Mann JR, Holla VR, Buchanan FG, Tai HH, et al. 2005. 15-Hydroxyprostaglandin dehydrogenase is down-regulated in colorectal cancer. *Journal of Biological Chemistry* 280:3217-23
42. Yan M, Myung SJ, Fink SP, Lawrence E, Lutterbaugh J, et al. 2009. 15-Hydroxyprostaglandin dehydrogenase inactivation as a mechanism of resistance to celecoxib chemoprevention of colon tumors. *Proceedings of the National Academy of Sciences* 106:9409

43. Kawamori T, Uchiya N, Sugimura T, Wakabayashi K. 2003. Enhancement of colon carcinogenesis by prostaglandin E2 administration. *Carcinogenesis* 24:985-90
44. Nakanishi M, Montrose DC, Clark P, Nambiar PR, Belinsky GS, et al. 2008. Genetic deletion of mPGES-1 suppresses intestinal tumorigenesis. *Cancer Research* 68:3251
45. Wang D, DuBois RN. 2006. Prostaglandins and cancer. *Gut* 55:115-22
46. Wang D, Wang H, Brown J, Daikoku T, Ning W, et al. 2006. CXCL1 induced by prostaglandin E2 promotes angiogenesis in colorectal cancer. *The Journal of Experimental Medicine* 203:941-51
47. Flossmann E, Rothwell PM. 2007. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. *The Lancet* 369:1603-13
48. Rostom A, Dubé C, Lewin G, Tsertsvadze A, Barrowman N, et al. 2007. Nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors for primary prevention of colorectal cancer: a systematic review prepared for the US Preventive Services Task Force. *Annals of Internal Medicine* 146:376-89
49. Chan AT, Giovannucci EL, Meyerhardt JA, Schernhammer ES, Wu K, Fuchs CS. 2008. Aspirin dose and duration of use and risk of colorectal cancer in men. *Gastroenterology* 134:21-8
50. Chan AT, Ogino S, Fuchs CS. 2007. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. *New England Journal of Medicine* 356:2131-42
51. Chan AT, Ogino S, Fuchs CS. 2009. Aspirin use and survival after diagnosis of colorectal cancer. *JAMA: The Journal of the American Medical Association* 302:649-58
52. Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, et al. 2003. A randomized trial of aspirin to prevent colorectal adenomas. *New England Journal of Medicine* 348:891-9
53. Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, et al. 2003. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *New England Journal of Medicine* 348:883-90
54. Rahme E, Barkun AN, Toubouti Y, Bardou M. 2003. The cyclooxygenase-2-selective inhibitors rofecoxib and celecoxib prevent colorectal neoplasia occurrence and recurrence. *Gastroenterology* 125:404-12
55. Steinbach G, Lynch PM, Phillips RKS, Wallace MH, Hawk E, et al. 2000. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *New England Journal of Medicine* 342:1946-52

56. Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, et al. 2005. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *New England Journal of Medicine* 352:1092-102
57. Solomon SD, McMurray JJV, Pfeffer MA, Wittes J, Fowler R, et al. 2005. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *New England Journal of Medicine* 352:1071-80
58. Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, et al. 2006. Celecoxib for the prevention of sporadic colorectal adenomas. *New England Journal of Medicine* 355:873-84
59. Arber N, Eagle CJ, Spicak J, Rácz I, Dite P, et al. 2006. Celecoxib for the prevention of colorectal adenomatous polyps. *New England Journal of Medicine* 355:885-95
60. McAdam B, Catella-Lawson F, Mardini I, Kapoor S, Lawson J, FitzGerald G. 1999. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. *Proceedings of the National Academy of Sciences* 96:272
61. Zimmermann KC, Bonzon C, Green DR. 2001. The machinery of programmed cell death. *Pharmacology & Therapeutics* 92:57-70
62. Ghavami S, Hashemi M, Ande SR, Yeganeh B, Xiao W, et al. 2009. Apoptosis and cancer: mutations within caspase genes. *Journal of Medical Genetics* 46:497-510
63. Ghobrial IM, Witzig TE, Adjei AA. 2005. Targeting apoptosis pathways in cancer therapy. *CA: A Cancer Journal for Clinicians* 55:178-94
64. Ly J, Grubb D, Lawen A. 2003. The mitochondrial membrane potential ($\Delta\psi$ m) in apoptosis; an update. *Apoptosis* 8:115-28
65. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, et al. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479-89
66. Ashkenazi A, Dixit VM. 1998. Death receptors: signaling and modulation. *Science* 281:1305-8
67. Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, et al. 2000. TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nature cell biology* 2:241
68. Li J, Yuan J. 2008. Caspases in apoptosis and beyond. *Oncogene* 27:6194-206
69. Trapani JA, Smyth MJ. 2002. Functional significance of the perforin/granzyme cell death pathway. *Nature Reviews Immunology* 2:735-47
70. Sakahira H, Enari M, Nagata S. 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 391:96-9

71. Barry M, Bleackley RC. 2002. Cytotoxic T lymphocytes: all roads lead to death. *Nature Reviews Immunology* 2:401-9
72. Taylor RC, Cullen SP, Martin SJ. 2008. Apoptosis: controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology* 9:231-41
73. van Delft MF, Huang DC. 2006. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Research* 16:203-13
74. Adams J, Cory S. 2007. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 26:1324-37
75. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. 2010. The BCL-2 family reunion. *Molecular Cell* 37:299-310
76. Johnson D, Walker C. 1999. Cyclins and cell cycle checkpoints. *Annual Review of Pharmacology and Toxicology* 39:295-312
77. Malumbres M, Barbacid M. 2009. Cell cycle, CDKs and cancer: a changing paradigm. *Nature Reviews Cancer* 9:153-66
78. Satyanarayana A, Kaldis P. 2009. Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. *Oncogene* 28:2925-39
79. Hochegger H, Takeda S, Hunt T. 2008. Cyclin-dependent kinases and cell-cycle transitions: does one fit all? *Nature Reviews Molecular Cell Biology* 9:910-6
80. Chansriniyom C. 2009. *Chemical constituents and anti-herpes simplex virus activity of Micromelum hirsutum and Glycosmis parva*. Chulalongkorn University, Bangkok,Thailand
81. Fujioka H, Nishiyama Y, Furukawa H, Kumada N. 1989. In vitro and in vivo activities of atalaphillinine and related acridone alkaloids against rodent malaria. *Antimicrobial Agents and Chemotherapy* 33:6-9
82. Yamamoto N, Furukawa H, Ito Y, Yoshida S, Maeno K, Nishiyama Y. 1989. Anti-herpesvirus activity of citrusinine-I, a new acridone alkaloid, and related compounds. *Antiviral Research* 12:21-36
83. Greger H, Zechner G, Hofer O, Hadacek F, Wurz G. 1993. Sulphur-containing amides from *Glycosmis* species with different antifungal activity. *Phytochemistry* 34:175-9
84. Greger H, Zechner G, Hofer O, Vajrodaya S. 1996. Bioactive amides from *Glycosmis* species. *Journal of Natural Products* 59:1163-8
85. Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M, et al. 1999. Acridones as inducers of HL-60 cell differentiation. *Leukemia Research* 23:263-9
86. Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M, et al. 1999. The antiproliferative effect of acridone alkaloids on several cancer cell lines. *Journal of Natural Products* 62:587-9

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

87. Itoigawa M, Ito C, Wu TS, Enjo F, Tokuda H, et al. 2003. Cancer chemopreventive activity of acridone alkaloids on Epstein–Barr virus activation and two-stage mouse skin carcinogenesis. *Cancer Letters* 193:133-8
88. Su TL, Dziewiszek K, Wu TS. 1991. Synthesis of glyfoline, a constituent of *glycosmis citrifolia* (Willd.) Lindl. and a potential anticancer agent. *Tetrahedron Letters* 32:1541-4
89. Su TL, Huang HM, Wang CK, Wu HC, Lin CT. 2005. Cytochrome C release induces apoptosis of nasopharyngeal carcinoma (NPC) by antitumor glyfoline. *Planta Medica-Natural Products and Medicinal Plant Research* 71:28-32
90. Elmore S. 2007. Apoptosis: a review of programmed cell death. *Toxicologic Pathology* 35:495-516
91. Qiao L, Koutsos M, Tsai L-L, Kozoni V, Guzman J, et al. 1996. Staurosporine inhibits the proliferation, alters the cell cycle distribution and induces apoptosis in HT-29 human colon adenocarcinoma cells. *Cancer Letters* 107:83-9
92. Cheng Y-L, Chang W-L, Lee S-C, Liu Y-G, Chen C-J, et al. 2004. Acetone extract of *Angelica sinensis* inhibits proliferation of human cancer cells via inducing cell cycle arrest and apoptosis. *Life Sciences* 75:1579-94
93. Huschtscha L, Bartier W, Ross C, Tattersall M. 1996. Characteristics of cancer cell death after exposure to cytotoxic drugs in vitro. *British Journal of Cancer* 73:54
94. Nunez R. 2001. DNA measurement and cell cycle analysis by flow cytometry. *Current issues in molecular biology* 3:67-70
95. Traganos F, Juan G, Darzynkiewicz Z. 2001. Cell-cycle analysis of drug-treated cells. In *DNA Topoisomerase Protocols*:229-40: Springer. Number of 229-40 pp.
96. Debatin K-M. 2004. Apoptosis pathways in cancer and cancer therapy. *Cancer Immunology, Immunotherapy* 53:153-9
97. Arango D, Wilson A, Shi Q, Corner G, Aranes M, et al. 2004. Molecular mechanisms of action and prediction of response to oxaliplatin in colorectal cancer cells. *British Journal of Cancer* 91:1931-46
98. Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. 2001. PUMA induces the rapid apoptosis of colorectal cancer cells. *Molecular Cell* 7:673-82
99. Mans DR, da Rocha AB, Schwartsmann G. 2000. Anti-cancer drug discovery and development in Brazil: targeted plant collection as a rational strategy to acquire candidate anti-cancer compounds. *The Oncologist* 5:185-98
100. Fisher DE. 1994. Apoptosis in cancer therapy: crossing the threshold. *Cell* 78:539-42
101. Antonsson B, Martinou J. 2000. The Bcl-2 protein family. *Experimental Cell Research* 256:50-7

102. Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. *Cell* 144:646-74
103. Agarwal C, Singh RP, Dhanalakshmi S, Tyagi AK, Tecklenburg M, et al. 2003. Silibinin upregulates the expression of cyclin-dependent kinase inhibitors and causes cell cycle arrest and apoptosis in human colon carcinoma HT-29 cells. *Oncogene* 22:8271-82
104. Park M-T, Lee S-J. 2003. Cell cycle and cancer. *Journal of Biochemistry and Molecular Biology* 36:60-5
105. Ahmad A, Wang Z, Ali R, Bitar B, Logna FT, et al. 2012. Cell cycle regulatory proteins in breast cancer: molecular determinants of drug resistance and targets for anticancer therapies. *Breast Cancer Cells*:113-30
106. Abbas T, Dutta A. 2009. p21 in cancer: intricate networks and multiple activities. *Nature Reviews Cancer* 9:400-14
107. Wu WKK, Yiu Sung JJ, Lee CW, Yu J, Cho CH. 2010. Cyclooxygenase-2 in tumorigenesis of gastrointestinal cancers: an update on the molecular mechanisms. *Cancer Letters* 295:7-16
108. Grösch S, Tegeder I, Niederberger E, Bräutigam L, Geisslinger G. 2001. COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. *The FASEB Journal* 15:2742-4
109. Trainer DL, Kline T, McCabe FL, Faucette LF, Feild J, et al. 1988. Biological characterization and oncogene expression in human colorectal carcinoma cell lines. *International Journal of Cancer* 41:287-96

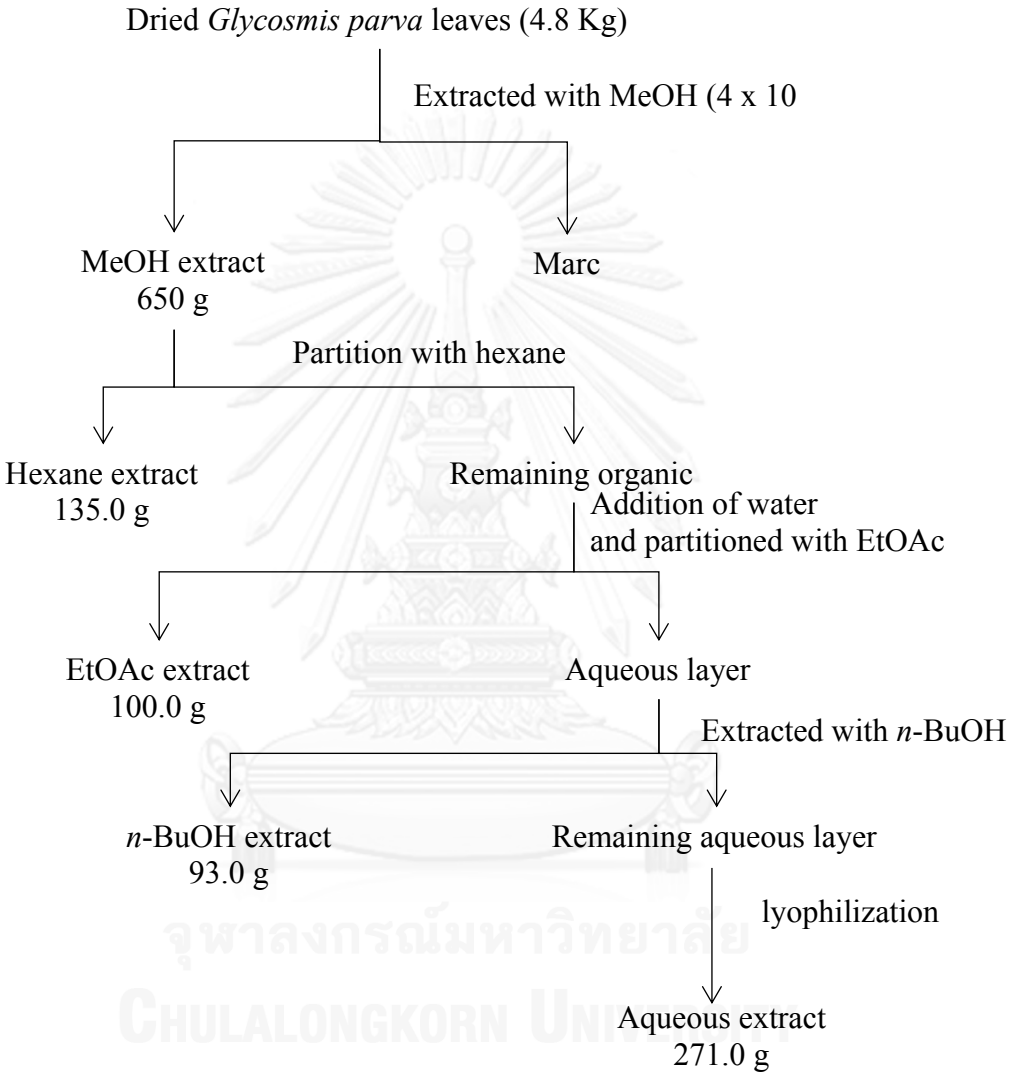
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX A

APPENDIX A-1: Extraction of *Glycosmis parva* leaves



จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX B

Buffers and Reagents

1. 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
Penicillin/Streptomycin	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

2. DMEM stock solution 1 liter

DMEM powder	10.4	g
Sodium bicarbonate	3.7	g
Penicillin/Streptomycin	10	ml
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

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3. 10x Phosphate Buffered Saline (PBS) 1 liter

NaCl	80.65	g
KCl	2	g
KH_2PO_4	2	g
Na_2HPO_4	11.5	g
ddH_2O	900	ml

Adjust pH to 7.4 with 1M HCl

Add ddH_2O to 1 liter and sterilize by autoclaving

4. 10x Assay Buffer for Flow Cytometer 100 ml

HEPES (1M)	10	ml
$CaCl_2$ (0.1M)	28	ml
NaCl (5M)	25	ml
ddH_2O	37	ml

5. EDTA 0.5M pH 8.0 100 ml

EDTA	18.612	g
ddH_2O	80	ml

Adjust pH to 8.0 with NaOH

Add ddH_2O to 100 ml and sterilize by autoclaving

6. 5x Tris-Borate-EDTA (TBE) Buffer 1 liter

Tris-base	54	g
Boric acid	27.5	g
EDTA 0.5M pH 8.0	20	ml

Sterilize by autoclaving

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX C

Results

Appendix C-1: Cytotoxic effects of GPE against HT-29 cells at 24, 48 and 72 h.

HT29 Cytotoxicity Study at 24 h	N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
				Lower Bound	Upper Bound		
0.2% DMSO	3	1.22	0.13	0.65	1.78	1.05	1.48
GPE 6.25 µg/ml	3	-2.12	2.07	-11.03	6.80	-5.32	1.76
GPE 12.5 µg/ml	3	-0.38	1.83	-8.24	7.48	-3.87	2.31
GPE 25 µg/ml	3	7.75	1.98	-0.75	16.26	4.07	10.85
GPE 50 µg/ml	3	33.10	2.10	24.07	42.14	29.59	36.85
GPE 100 µg/ml	3	77.31	1.81	69.50	85.11	73.80	79.87
5FU 1 µM	3	0.41	1.05	-4.10	4.91	-1.04	2.44
5FU 10 µM	3	7.57	4.18	-10.43	25.57	0.14	14.61
5FU 100 µM	3	17.70	1.78	10.03	25.38	14.32	20.38
Oxaliplatin 1 µM	3	12.14	0.77	8.84	15.44	10.94	13.57
Oxaliplatin 10 µM	3	20.23	1.00	15.94	24.52	18.91	22.19
Oxaliplatin 100 µM	3	39.23	0.94	35.21	43.26	37.59	40.82

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix C-1: Cytotoxic effects of GPE against HT-29 cells at 24, 48 and 72 h. (Cont.)

HT29 Cytotoxicity Study at 48 h	N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
				Lower Bound	Upper Bound		
0.2% DMSO	3	1.29	0.06	1.04	1.53	1.20	1.40
GPE 6.25 µg/ml	3	1.12	0.59	-1.42	3.65	0.11	2.15
GPE 12.5 µg/ml	3	-0.15	2.20	-9.63	9.33	-3.87	3.76
GPE 25 µg/ml	3	18.27	2.82	6.12	30.42	14.60	23.82
GPE 50 µg/ml	3	54.57	4.12	36.82	72.31	46.52	60.16
GPE 100 µg/ml	3	90.11	2.91	77.58	102.64	84.29	93.21
5FU 1 µM	3	11.40	3.62	-4.20	26.99	4.16	15.27
5FU 10 µM	3	37.83	6.67	9.15	66.52	24.55	45.45
5FU 100 µM	3	56.52	4.36	37.77	75.27	48.21	62.94
Oxaliplatin 1 µM	3	36.14	4.79	15.51	56.76	26.62	41.92
Oxaliplatin 10 µM	3	58.61	5.08	36.75	80.47	48.45	63.82
Oxaliplatin 100 µM	3	76.78	2.55	65.81	87.74	72.02	80.73

HT29 Cytotoxicity Study at 72 h	N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
				Lower Bound	Upper Bound		
0.2% DMSO	3	1.52	0.01	1.46	1.58	1.50	1.55
GPE 6.25 µg/ml	3	5.51	1.77	-2.09	13.10	2.27	8.34
GPE 12.5 µg/ml	3	5.42	3.48	-9.53	20.37	-0.22	11.76
GPE 25 µg/ml	3	23.85	2.31	13.89	33.81	19.29	26.83
GPE 50 µg/ml	3	61.94	11.91	10.69	113.19	39.02	79.03
GPE 100 µg/ml	3	99.11	2.45	88.58	109.65	94.22	101.78
5FU 1 µM	3	25.48	1.98	16.99	33.98	22.72	29.31
5FU 10 µM	3	56.87	4.37	38.06	75.69	48.18	62.04
5FU 100 µM	3	78.99	2.55	68.03	89.94	74.11	82.69
Oxaliplatin 1 µM	3	36.14	6.52	8.10	64.19	23.11	42.84
Oxaliplatin 10 µM	3	74.58	4.34	55.92	93.23	65.94	79.59
Oxaliplatin 100 µM	3	91.97	1.10	87.23	96.71	89.78	93.30

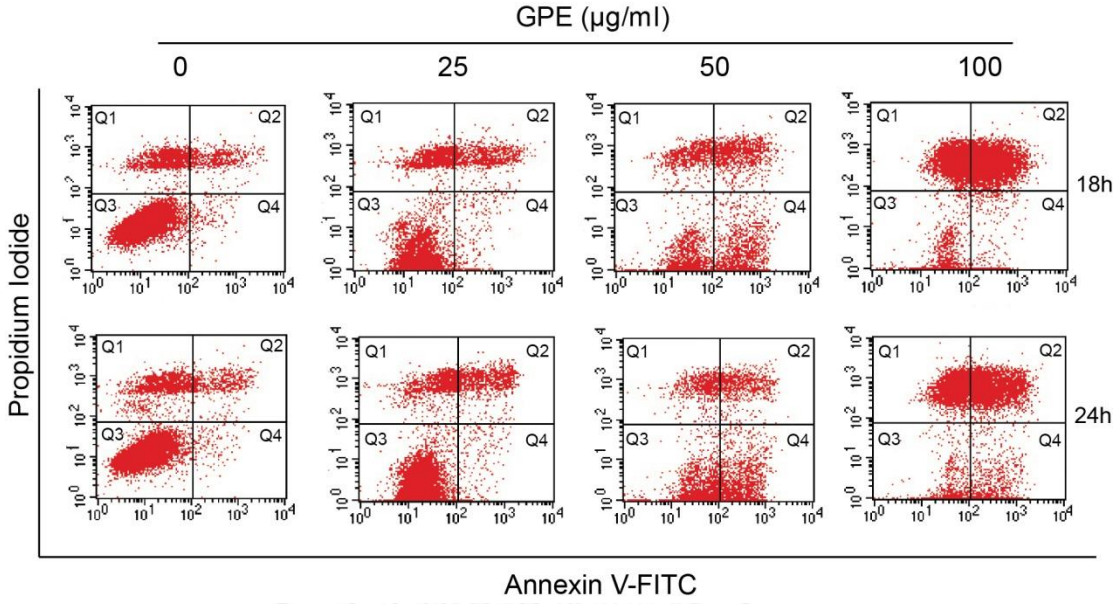
Appendix C-2: The apoptotic effect of GPE on HT-29 cells at 18 and 24.

HT-29 Apoptosis Study at 18 h		N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
Viable	Untreated, non-stained	3	99.86	0.03	99.75	99.98	99.81	99.89
	DMEM	3	83.03	1.85	75.06	91.00	80.91	86.72
	0.2% DMSO	3	87.28	1.69	80.00	94.56	84.71	90.47
	GPE 25 µg/ml	3	83.29	0.42	81.50	85.08	82.47	83.82
	GPE 50 µg/ml	3	67.24	3.16	53.63	80.85	61.22	71.94
	GPE 100 µg/ml	3	30.39	3.39	15.79	44.99	23.86	35.25
	Oxaliplatin 100 µM	3	83.01	0.72	79.92	86.10	82.20	84.44
	5FU 100 µM	3	80.10	0.85	76.43	83.77	78.77	81.69
Necrosis	Untreated, non-stained	3	0.08	0.04	-0.09	0.26	0.01	0.15
	DMEM	3	12.47	1.76	4.90	20.03	9.03	14.83
	0.2% DMSO	3	7.98	1.69	0.72	15.24	4.68	10.25
	GPE 25 µg/ml	3	7.60	0.44	5.71	9.49	6.73	8.13
	GPE 50 µg/ml	3	8.51	0.59	5.95	11.06	7.33	9.24
	GPE 100 µg/ml	3	32.79	9.38	-7.58	73.16	22.31	51.51
	Oxaliplatin 100 µM	3	5.74	1.08	1.08	10.40	4.18	7.82
	5FU 100 µM	3	13.05	1.29	7.50	18.59	10.88	15.34
Late apoptosis/ Necrosis	Untreated, non-stained	3	0.03	0.01	-0.01	0.06	0.01	0.04
	DMEM	3	3.49	0.19	2.68	4.29	3.16	3.81
	0.2% DMSO	3	5.14	1.88	-2.94	13.21	2.94	8.87
	GPE 25 µg/ml	3	7.19	0.41	5.43	8.95	6.38	7.70
	GPE 50 µg/ml	3	9.47	0.92	5.51	13.43	7.76	10.92
	GPE 100 µg/ml	3	33.74	5.65	9.44	58.04	22.87	41.83
	Oxaliplatin 100 µM	3	7.49	1.03	3.07	11.91	5.50	8.92
	5FU 100 µM	3	5.71	0.22	4.75	6.66	5.27	5.99
Early apoptosis	Untreated, non-stained	3	0.00	0.00	0.00	0.00	0.00	0.00
	DMEM	3	1.02	0.13	0.47	1.57	0.77	1.19
	0.2% DMSO	3	1.27	0.06	1.02	1.52	1.18	1.38
	GPE 25 µg/ml	3	1.92	0.15	1.27	2.56	1.69	2.20
	GPE 50 µg/ml	3	14.79	2.91	2.26	27.32	11.07	20.53
	GPE 100 µg/ml	3	3.08	0.66	0.22	5.94	1.75	3.81
	Cisplatin 100 µM	3	4.24	0.39	2.54	5.94	3.71	5.01
	5FU 100 µM	3	1.15	0.28	-0.07	2.36	0.61	1.57
Total death	Untreated, non-stained	3	0.11	0.05	-0.10	0.32	0.02	0.19
	DMEM	3	16.97	1.85	9.02	24.93	13.29	19.09
	0.2% DMSO	3	14.39	3.15	0.82	27.96	9.53	20.30
	GPE 25 µg/ml	3	16.71	0.41	14.93	18.48	16.18	17.52
	GPE 50 µg/ml	3	32.77	3.16	19.17	46.37	28.07	38.78
	GPE 100 µg/ml	3	69.61	3.39	55.03	84.19	64.75	76.13
	Oxaliplatin 100 µM	3	16.99	0.72	13.89	20.09	15.55	17.80
	5FU 100 µM	3	19.90	0.85	16.24	23.56	18.31	21.22

Appendix C-2: The apoptotic effect of GPE on HT-29 cells at 18 and 24. (Cont.)

HT-29 Apoptosis Study at 24 h		N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
Viable	Untreated, non-stained	3	99.90	0.02	99.81	100.00	99.86	99.93
	DMEM	3	86.05	3.64	70.39	101.70	81.50	93.24
	0.2% DMSO	3	86.05	1.42	79.93	92.17	83.43	88.32
	GPE 25 µg/ml	3	85.16	2.25	75.47	94.85	80.74	88.11
	GPE 50 µg/ml	3	63.67	1.31	58.04	69.29	61.66	66.12
	GPE 100 µg/ml	3	24.31	5.78	-0.56	49.18	13.90	33.87
	Oxaliplatin 100 µM	3	84.80	1.44	78.62	90.98	81.94	86.44
	5FU 100 µM	3	79.78	3.16	66.17	93.40	76.00	86.07
Necrosis	Untreated, non-stained	3	0.07	0.01	0.01	0.14	0.05	0.10
	DMEM	3	8.63	2.83	-3.54	20.81	3.20	12.72
	0.2% DMSO	3	9.03	1.79	1.32	16.73	5.65	11.75
	GPE 25 µg/ml	3	6.85	1.26	1.43	12.28	5.48	9.37
	GPE 50 µg/ml	3	7.21	0.19	6.37	8.04	6.93	7.58
	GPE 100 µg/ml	3	22.56	3.17	8.92	36.20	16.22	25.78
	Oxaliplatin 100 µM	3	5.07	0.39	3.39	6.75	4.43	5.78
	5FU 100 µM	3	9.97	1.23	4.69	15.25	7.52	11.34
Late apoptosis/ Necrosis	Untreated, non-stained	3	0.02	0.01	-0.01	0.06	0.01	0.04
	DMEM	3	4.52	0.92	0.55	8.49	2.82	5.99
	0.2% DMSO	3	3.94	0.50	1.77	6.10	3.16	4.88
	GPE 25 µg/ml	3	6.45	1.12	1.64	11.25	4.42	8.27
	GPE 50 µg/ml	3	8.16	0.23	7.15	9.17	7.76	8.57
	GPE 100 µg/ml	3	41.89	6.54	13.74	70.04	33.28	54.73
	Oxaliplatin 100 µM	3	5.81	0.55	3.44	8.18	5.04	6.88
	5FU 100 µM	3	8.20	1.28	2.69	13.72	5.97	10.41
Early apoptosis	Untreated, non-stained	3	0.00	0.00	0.00	0.00	0.00	0.00
	DMEM	3	0.80	0.11	0.32	1.29	0.64	1.02
	0.2% DMSO	3	0.99	0.11	0.50	1.48	0.77	1.15
	GPE 25 µg/ml	3	1.54	0.16	0.85	2.23	1.23	1.76
	GPE 50 µg/ml	3	20.97	0.91	17.05	24.89	19.19	22.20
	GPE 100 µg/ml	3	8.89	3.77	-7.34	25.13	2.79	15.79
	Oxaliplatin 100 µM	3	4.18	0.67	1.29	7.06	3.09	5.40
	5FU 100 µM	3	0.79	0.17	0.06	1.52	0.45	0.97
Total death	Untreated, non-stained	3	0.10	0.02	0.00	0.19	0.07	0.14
	DMEM	3	13.96	3.64	-1.69	29.61	6.77	18.50
	0.2% DMSO	3	13.95	1.42	7.83	20.07	11.68	16.57
	GPE 25 µg/ml	3	14.84	2.26	5.14	24.54	11.89	19.27
	GPE 50 µg/ml	3	36.34	1.31	30.70	41.97	33.88	38.35
	GPE 100 µg/ml	3	73.34	3.81	56.97	89.71	66.13	79.05
	Oxaliplatin 100 µM	3	15.06	1.52	8.51	21.60	13.13	18.06
	5FU 100 µM	3	18.96	2.61	7.72	30.20	13.94	22.72

Appendix C-3: Representative flow cytometric analysis of GPE induces apoptosis in HT-29 cells.

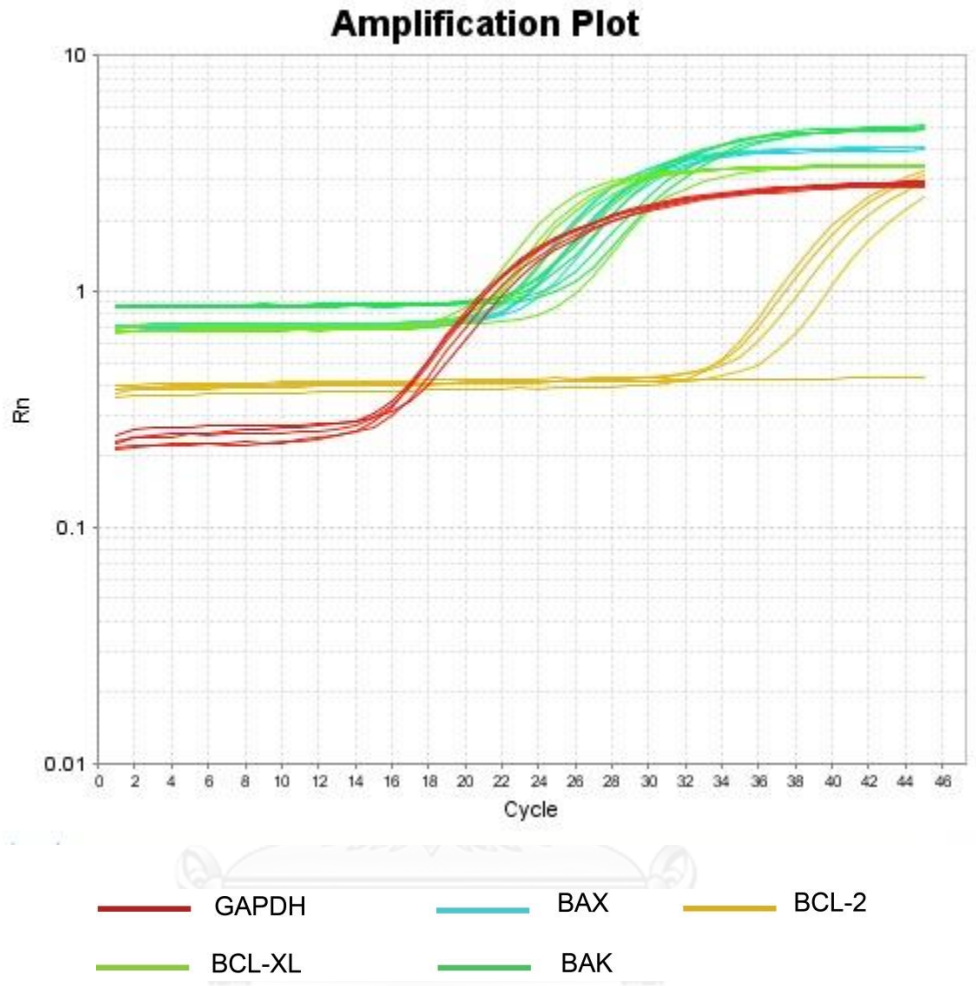


จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix C-4: Effect of GPE on the expression of BCL-2 family genes in HT-29 cells.

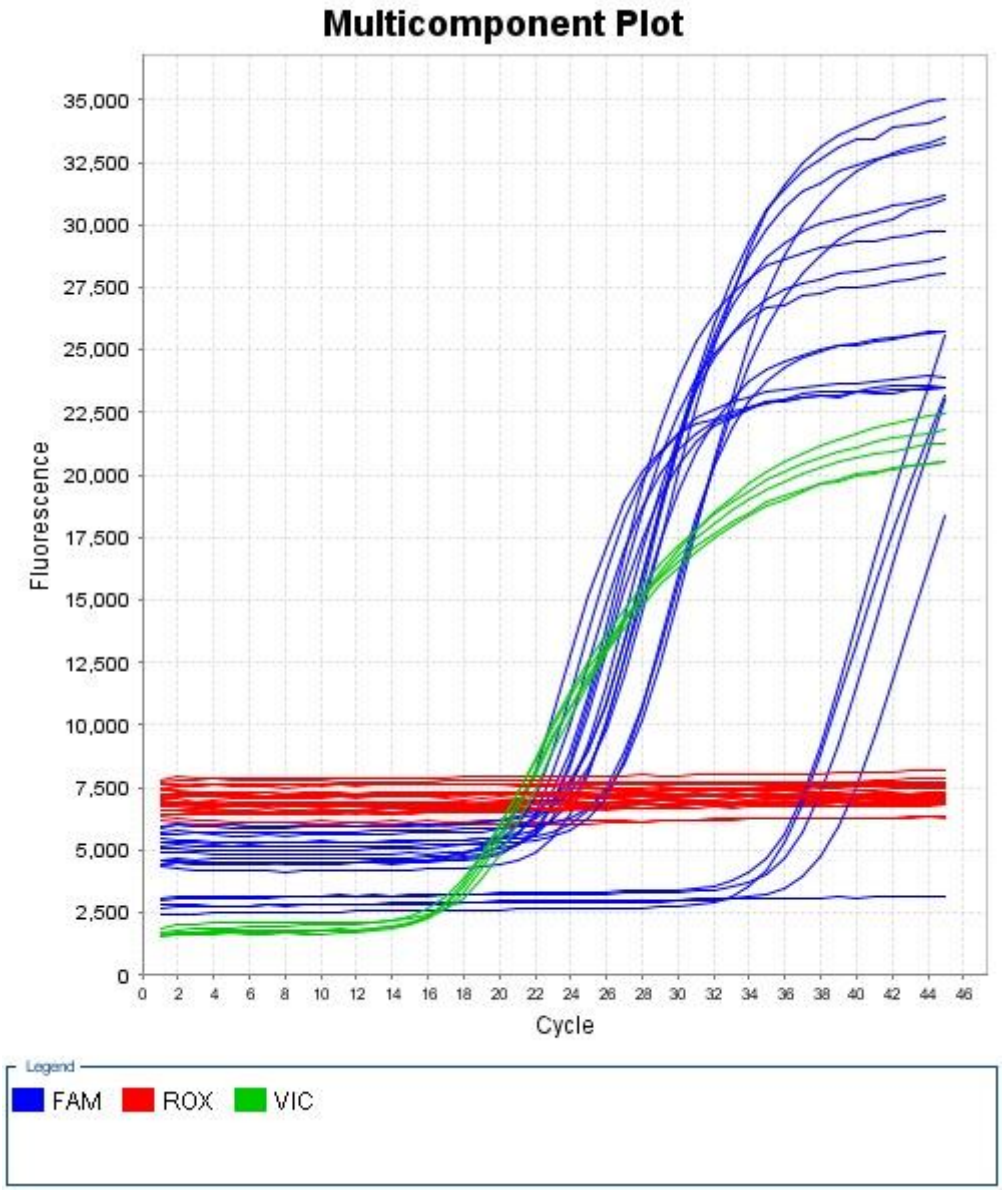
BCL-2 family gene expression in HT-29		N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
BCL-2	0.2% DMSO	3	1.00	0.00	1.00	1.00	1.00	1.00
	Cisplatin 100 μM	3	0.00	0.00	0.00	0.00	0.00	0.00
	GPE 25 μg/ml	3	0.68	0.21	-0.24	1.61	0.47	1.11
	GPE 50 μg/ml	3	0.32	0.13	-0.23	0.87	0.12	0.56
	GPE 100 μg/ml	3	0.55	0.16	-0.14	1.25	0.26	0.81
BCL-XL	0.2% DMSO	3	1.00	0.00	1.00	1.00	1.00	1.00
	Cisplatin 100 μM	3	0.08	0.00	0.06	0.10	0.07	0.09
	GPE 25 μg/ml	3	1.04	0.15	0.40	1.68	0.85	1.34
	GPE 50 μg/ml	3	0.60	0.09	0.21	1.00	0.44	0.76
	GPE 100 μg/ml	3	0.63	0.09	0.24	1.03	0.51	0.81
BAK	0.2% DMSO	3	1.00	0.00	1.00	1.00	1.00	1.00
	Cisplatin 100 μM	3	0.56	0.09	0.16	0.95	0.38	0.67
	GPE 25 μg/ml	3	1.25	0.16	0.57	1.92	1.06	1.56
	GPE 50 μg/ml	3	1.79	0.08	1.46	2.11	1.64	1.90
	GPE 100 μg/ml	3	0.81	0.18	0.02	1.59	0.61	1.17
BAX	0.2% DMSO	3	1.00	0.00	1.00	1.00	1.00	1.00
	Cisplatin 100 μM	3	0.51	0.09	0.14	0.88	0.37	0.67
	GPE 25 μg/ml	3	0.74	0.09	0.33	1.15	0.58	0.90
	GPE 50 μg/ml	3	0.48	0.07	0.19	0.77	0.38	0.61
	GPE 100 μg/ml	3	0.76	0.04	0.56	0.95	0.67	0.83

Appendix C-5: Representative amplification plot and from real-time RT-PCR experiment on the expression of genes in BCL-2 family in HT-29 cells.



จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix C-6: Representative multicomponent plot from real-time RT-PCR experiment on the expression of genes in BCL-2 family in HT-29 cells.



FAM = Fluorescence dye labeled with studied genes.
(BCL-2, BCL-XL, BAK, BAX)

ROX = Passive reference dye functioned as loading control.

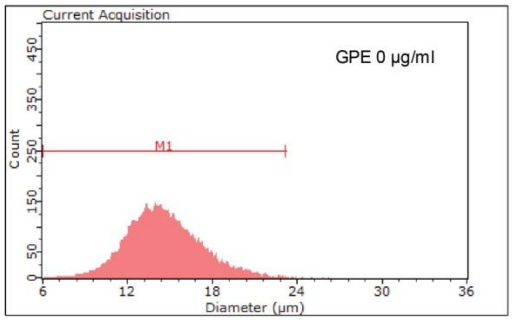
VIC = Fluorescence dye labeled with housekeeping genes (GAPDH).

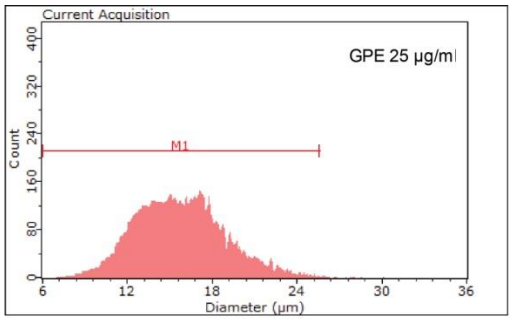
Appendix C-7: Anti-proliferative effect of GPE in HT-29 cells.

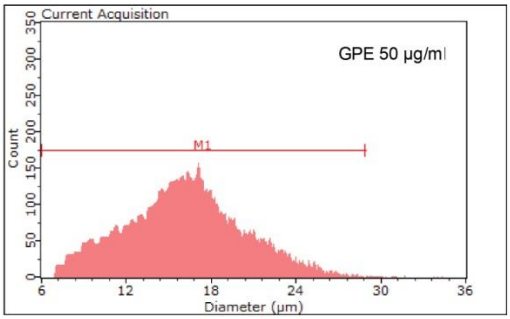
Cell Proliferation in HT-29	N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
				Lower Bound	Upper Bound		
0.2% DMSO	3	100.00%	0.00%	100.00%	100.00%	100.00%	100.00%
GPE 25 µg/ml	3	72.40%	5.83%	47.30%	97.50%	65.28%	83.96%
GPE 50 µg/ml	3	52.46%	6.45%	24.72%	80.20%	39.58%	59.47%
GPE 100 µg/ml	3	26.68%	6.12%	0.36%	53.00%	16.07%	37.26%
5FU 100 µM	3	44.47%	3.83%	27.98%	60.95%	37.74%	51.01%

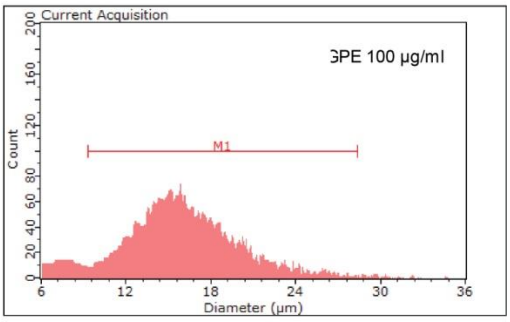
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

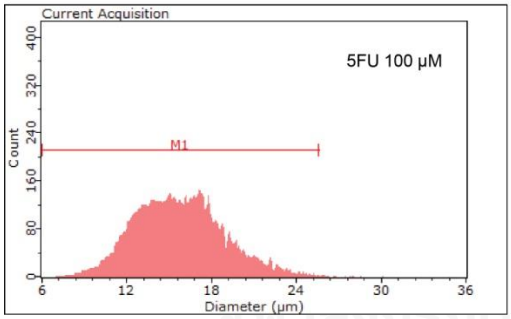
Appendix C-8: Representative histogram of cell counting from Scepter™ automate cell counter for determination of anti-proliferative effect of GPE in HT-29 cells.











จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix C-9: Distribution of HT-29 cells in the cell cycle phases after treatment with GPE.

Cell cycle analysis in HT-29		N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
G1	0.2% DMSO	3	50.13	1.28	44.61	55.66	47.73	52.12
	5FU 100 µM	3	33.32	1.67	26.15	40.49	30.80	36.47
	GPE 25 µg/ml	3	40.95	1.33	35.22	46.67	38.74	43.34
	GPE 50 µg/ml	3	60.73	0.98	56.52	64.94	59.04	62.43
	GPE 100 µg/ml	3	47.75	2.71	36.09	59.41	43.56	52.82
S	0.2% DMSO	3	32.00	0.51	29.80	34.21	31.00	32.68
	5FU 100 µM	3	61.00	1.81	53.24	68.77	58.85	64.59
	GPE 25 µg/ml	3	44.85	2.59	33.71	55.98	40.83	49.68
	GPE 50 µg/ml	3	26.78	1.76	19.22	34.33	24.02	30.04
	GPE 100 µg/ml	3	18.72	3.55	3.44	34.00	12.16	24.36
G2M	0.2% DMSO	3	17.86	1.04	13.39	22.33	16.78	19.94
	5FU 100 µM	3	5.68	2.05	-3.16	14.52	2.73	9.63
	GPE 25 µg/ml	3	14.21	1.33	8.50	19.92	11.58	15.84
	GPE 50 µg/ml	3	12.49	1.38	6.54	18.44	10.92	15.25
	GPE 100 µg/ml	3	33.53	0.85	29.88	37.18	32.08	35.02

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix C-10: Effect of GPE on the expression of cell cycle regulatory genes in HT-29 cells.

Expression of cell cycle regulators in HT-29		N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
CyclinA	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	90.58	3.73	74.55	106.61	84.25	97.15
	GPE 25 µg/ml	3	91.05	5.29	68.31	113.79	85.54	101.62
	GPE 50 µg/ml	3	88.54	3.75	72.40	104.69	81.07	92.87
	GPE 100 µg/ml	3	81.44	6.03	55.47	107.40	70.33	91.08
CyclinB1	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	94.25	3.66	78.50	110.01	87.20	99.49
	GPE 25 µg/ml	3	95.13	2.55	84.17	106.09	91.86	100.15
	GPE 50 µg/ml	3	92.64	4.70	72.42	112.87	83.28	98.05
	GPE 100 µg/ml	3	90.14	6.29	63.09	117.19	77.82	98.47
CyclinD1	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	95.93	3.75	79.80	112.07	89.09	102.01
	GPE 25 µg/ml	2	88.07	9.11	-27.68	203.82	78.96	97.18
	GPE 50 µg/ml	3	87.60	8.43	51.32	123.89	70.90	97.98
	GPE 100 µg/ml	3	83.15	7.90	49.16	117.14	67.35	91.21
CyclinE	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	96.24	0.63	93.53	98.95	95.01	97.10
	GPE 25 µg/ml	3	89.46	4.34	70.77	108.14	80.80	94.40
	GPE 50 µg/ml	3	82.02	5.93	56.50	107.54	70.21	88.86
	GPE 100 µg/ml	3	81.55	3.87	64.91	98.18	75.38	88.67
p21	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	107.94	1.80	100.20	115.68	104.47	110.49
	GPE 25 µg/ml	3	93.36	2.71	81.72	105.00	89.07	98.36
	GPE 50 µg/ml	3	100.79	4.72	80.48	121.09	92.85	109.18
	GPE 100 µg/ml	3	108.24	4.64	88.30	128.18	99.63	115.52
p53	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	101.32	6.14	74.92	127.73	92.23	113.01
	GPE 25 µg/ml	3	98.38	7.26	67.13	129.64	85.73	110.89
	GPE 50 µg/ml	3	94.14	7.85	60.37	127.91	78.58	103.70
	GPE 100 µg/ml	3	88.30	9.83	46.00	130.60	69.28	102.13

Appendix C-11: Cytotoxic effects of GPE against Colo-205 cells at 24, 48 and 72 h.

Colo-205 Cytotoxicity Study at 24 h	N	Mean	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
0.2% DMSO	3	1.57	0.01	1.53	1.61	1.55	1.59
GPE 6.25 µg/ml	3	-3.29	1.17	-8.31	1.73	-5.18	-1.16
GPE 12.5 µg/ml	3	9.79	1.02	5.41	14.17	8.09	11.61
GPE 25 µg/ml	3	19.81	3.82	3.36	36.26	15.08	27.38
GPE 50 µg/ml	3	40.22	6.80	10.97	69.46	31.52	53.62
GPE 100 µg/ml	3	87.25	4.09	69.65	104.84	80.28	94.44
5FU 1 µM	3	-0.51	1.18	-5.60	4.57	-1.83	1.85
5FU 10 µM	3	12.18	1.74	4.68	19.68	9.74	15.56
5FU 100 µM	3	16.97	0.84	13.36	20.58	15.37	18.21
Oxaliplatin 1 µM	3	7.76	0.79	4.36	11.16	6.66	9.29
Oxaliplatin 10 µM	3	15.62	1.10	10.89	20.36	13.55	17.29
Oxaliplatin 100 µM	3	23.67	4.16	5.77	41.58	18.61	31.92

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix C-11: Cytotoxic effects of GPE against Colo-205 cells at 24, 48 and 72 h. (Cont.)

Colo-205 Cytotoxicity Study at 48 h	N	Mean	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
0.2% DMSO	3	1.68	0.08	1.35	2.00	1.53	1.77
GPE 6.25 µg/ml	3	-7.42	0.49	-9.51	-5.33	-7.99	-6.46
GPE 12.5 µg/ml	3	2.90	3.34	-11.48	17.27	-3.40	7.98
GPE 25 µg/ml	3	65.88	6.50	37.92	93.85	56.34	78.30
GPE 50 µg/ml	3	89.40	2.57	78.33	100.47	85.61	94.31
GPE 100 µg/ml	3	100.25	0.44	98.36	102.15	99.39	100.83
5FU 1 µM	3	9.55	3.02	-3.43	22.52	6.38	15.57
5FU 10 µM	3	45.32	0.48	43.26	47.39	44.52	46.18
5FU 100 µM	3	59.74	0.92	55.77	63.70	57.91	60.84
Oxaliplatin 1 µM	3	26.13	3.29	11.96	40.30	22.20	32.67
Oxaliplatin 10 µM	3	38.15	2.11	29.06	47.25	35.64	42.36
Oxaliplatin 100 µM	3	79.54	1.60	72.64	86.44	76.49	81.92

Colo-205 Cytotoxicity Study at 72 h	N	Mean	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
0.2% DMSO	3	1.77	0.05	1.54	2.00	1.66	1.83
GPE 6.25 µg/ml	3	-3.09	1.15	-8.05	1.88	-4.60	-0.82
GPE 12.5 µg/ml	3	8.57	3.24	-5.37	22.51	4.58	14.99
GPE 25 µg/ml	3	74.58	11.22	26.30	122.85	58.30	96.09
GPE 50 µg/ml	3	100.33	0.64	97.55	103.10	99.16	101.38
GPE 100 µg/ml	3	100.95	1.17	95.92	105.97	98.69	102.59
5FU 1 µM	3	12.69	1.96	4.24	21.14	10.43	16.60
5FU 10 µM	3	54.74	0.80	51.29	58.19	53.29	56.06
5FU 100 µM	3	82.02	0.31	80.68	83.36	81.65	82.64
Oxaliplatin 1 µM	3	31.54	5.77	6.72	56.36	25.74	43.08
Oxaliplatin 10 µM	3	49.71	2.93	37.10	62.31	46.05	55.50
Oxaliplatin 100 µM	3	92.19	1.22	86.94	97.43	89.78	93.73

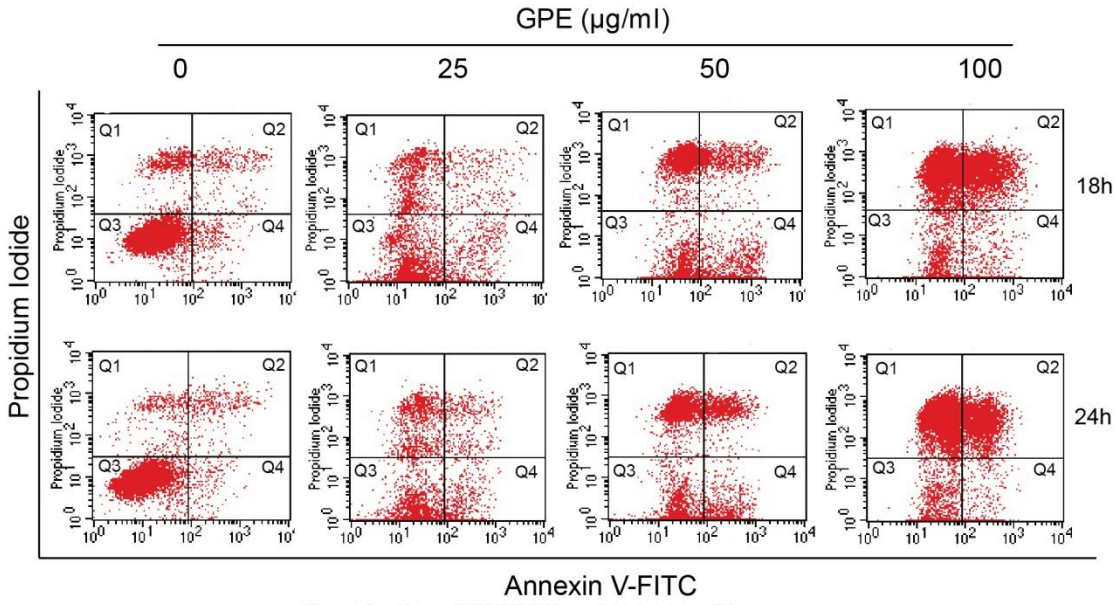
Appendix C-12: The apoptotic effect of GPE on Colo-205 cells at 18 and 24.

Colo-205 Apoptosis Study 18 h		N	Mean	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Viable	Untreated, non-stained	3	99.68	0.12	99.18	100.17	99.45	99.83
	DMEM	3	87.82	0.43	85.97	89.68	86.97	88.36
	0.2% DMSO	3	88.45	0.96	84.32	92.59	86.82	90.15
	GPE 25 µg/ml	3	67.96	1.54	61.33	74.59	65.10	70.38
	GPE 50 µg/ml	3	53.40	4.15	35.57	71.24	48.72	61.67
	GPE 100 µg/ml	3	39.27	7.63	6.46	72.09	26.35	52.75
	Oxaliplatin 100 µM	3	76.63	1.69	69.38	83.88	73.33	78.88
	5FU 100 µM	3	82.04	0.45	80.09	83.99	81.25	82.82
Necrosis	Untreated, non-stained	3	0.29	0.12	-0.22	0.81	0.14	0.53
	DMEM	3	6.61	0.32	5.24	7.98	6.07	7.17
	0.2% DMSO	3	5.57	0.61	2.95	8.20	4.88	6.79
	GPE 25 µg/ml	3	13.81	1.13	8.96	18.66	11.59	15.25
	GPE 50 µg/ml	3	11.93	4.22	-6.23	30.09	6.81	20.30
	GPE 100 µg/ml	3	25.47	7.03	-4.77	55.72	16.45	39.32
	Oxaliplatin 100 µM	3	7.34	0.59	4.80	9.87	6.23	8.24
	5FU 100 µM	3	10.21	0.36	8.68	11.74	9.57	10.80
Late apoptosis/ Necrosis	Untreated, non-stained	3	0.03	0.01	0.01	0.05	0.02	0.04
	DMEM	3	3.47	0.21	2.55	4.39	3.10	3.84
	0.2% DMSO	3	3.71	0.47	1.67	5.74	2.81	4.42
	GPE 25 µg/ml	3	5.35	0.35	3.84	6.85	4.91	6.04
	GPE 50 µg/ml	3	6.97	1.80	-0.76	14.70	3.75	9.96
	GPE 100 µg/ml	3	20.83	3.70	4.92	36.75	13.54	25.56
	Oxaliplatin 100 µM	3	5.36	0.57	2.91	7.81	4.58	6.47
	5FU 100 µM	3	5.84	0.02	5.73	5.94	5.79	5.87
Early apoptosis	Untreated, non-stained	3	0.00	0.00	0.00	0.00	0.00	0.00
	DMEM	3	2.09	0.04	1.91	2.28	2.02	2.17
	0.2% DMSO	3	2.27	0.12	1.75	2.79	2.14	2.51
	GPE 25 µg/ml	3	12.88	2.30	2.97	22.80	9.47	17.27
	GPE 50 µg/ml	3	27.70	5.09	5.82	49.58	19.93	37.27
	GPE 100 µg/ml	3	14.42	2.83	2.26	26.58	8.77	17.26
	Oxaliplatin 100 µM	3	10.68	1.79	2.99	18.36	7.85	13.98
	5FU 100 µM	3	1.92	0.08	1.60	2.24	1.83	2.07
Total death	Untreated, non-stained	3	0.32	0.12	-0.17	0.82	0.17	0.55
	DMEM	3	12.18	0.43	10.32	14.03	11.64	13.03
	0.2% DMSO	3	11.55	0.96	7.40	15.70	9.85	13.19
	GPE 25 µg/ml	3	32.04	1.54	25.43	38.66	29.63	34.90
	GPE 50 µg/ml	3	46.60	4.15	28.76	64.44	38.33	51.28
	GPE 100 µg/ml	3	60.73	7.63	27.91	93.54	47.25	73.65
	Oxaliplatin 100 µM	3	23.37	1.69	16.11	30.64	21.12	26.68
	5FU 100 µM	3	17.96	0.45	16.04	19.89	17.19	18.74

Appendix C-12: The apoptotic effect of GPE on Colo-205 cells at 18 and 24. (Cont.)

Colo-205 Apoptosis Study 24 h		N	Mean	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Viable	Untreated, non-stained	3	99.77	0.10	99.35	100.19	99.58	99.91
	DMEM	3	89.32	0.82	85.77	92.86	87.81	90.65
	0.2% DMSO	3	90.03	1.40	84.02	96.04	88.28	92.79
	GPE 25 µg/ml	3	61.57	4.60	41.78	81.36	53.64	69.57
	GPE 50 µg/ml	3	50.96	4.32	32.38	69.54	46.21	59.58
	GPE 100 µg/ml	3	35.20	4.22	17.05	53.35	27.38	41.85
	Oxaliplatin 100 µM	3	73.31	2.37	63.12	83.49	68.70	76.55
	5FU 100 µM	3	83.46	0.59	80.93	85.99	82.35	84.35
Necrosis	Untreated, non-stained	3	0.21	0.09	-0.17	0.59	0.08	0.38
	DMEM	3	4.67	0.69	1.71	7.64	3.96	6.05
	0.2% DMSO	3	4.54	1.33	-1.17	10.24	1.89	6.01
	GPE 25 µg/ml	3	16.22	2.69	4.66	27.78	11.37	20.65
	GPE 50 µg/ml	3	16.64	2.43	6.20	27.08	13.58	21.43
	GPE 100 µg/ml	3	23.05	3.29	8.90	37.19	17.68	29.02
	Oxaliplatin 100 µM	3	10.32	1.37	4.43	16.21	8.09	12.81
	5FU 100 µM	3	7.25	0.40	5.51	8.99	6.57	7.97
Late apoptosis/ Necrosis	Untreated, non-stained	3	0.02	0.01	-0.01	0.05	0.01	0.03
	DMEM	3	3.99	0.28	2.79	5.18	3.61	4.53
	0.2% DMSO	3	2.99	0.20	2.14	3.84	2.69	3.36
	GPE 25 µg/ml	3	6.14	1.77	-1.48	13.76	3.28	9.38
	GPE 50 µg/ml	3	8.12	1.12	3.29	12.95	6.14	10.03
	GPE 100 µg/ml	3	23.68	3.64	8.00	39.35	16.80	29.20
	Oxaliplatin 100 µM	3	5.69	0.99	1.43	9.96	3.85	7.25
	5FU 100 µM	3	6.62	0.08	6.28	6.95	6.48	6.75
Early apoptosis	Untreated, non-stained	3	0.00	0.00	0.00	0.00	0.00	0.00
	DMEM	3	2.02	0.28	0.83	3.22	1.57	2.53
	0.2% DMSO	3	2.45	0.11	1.99	2.90	2.29	2.65
	GPE 25 µg/ml	3	16.07	5.52	-7.66	39.81	6.50	25.61
	GPE 50 µg/ml	3	24.28	5.72	-0.32	48.88	12.85	30.18
	GPE 100 µg/ml	3	18.07	2.84	5.84	30.31	14.40	23.67
	Oxaliplatin 100 µM	3	10.68	0.65	7.87	13.50	9.38	11.43
	5FU 100 µM	3	2.66	0.85	-0.99	6.32	1.06	3.95
Total death	Untreated, non-stained	3	0.23	0.10	-0.18	0.64	0.09	0.41
	DMEM	3	10.68	0.82	7.14	14.23	9.35	12.19
	0.2% DMSO	3	9.97	1.40	3.96	15.99	7.21	11.72
	GPE 25 µg/ml	3	38.43	4.60	18.65	58.22	30.43	46.36
	GPE 50 µg/ml	3	49.04	4.32	30.46	67.62	40.42	53.79
	GPE 100 µg/ml	3	64.80	4.22	46.65	82.95	58.15	72.62
	Oxaliplatin 100 µM	3	26.69	2.37	16.51	36.88	23.45	31.30
	5FU 100 µM	3	16.53	0.59	14.00	19.07	15.65	17.65

Appendix C-13: Representative flow cytometric analysis of GPE induces apoptosis in Colo-205 cells.

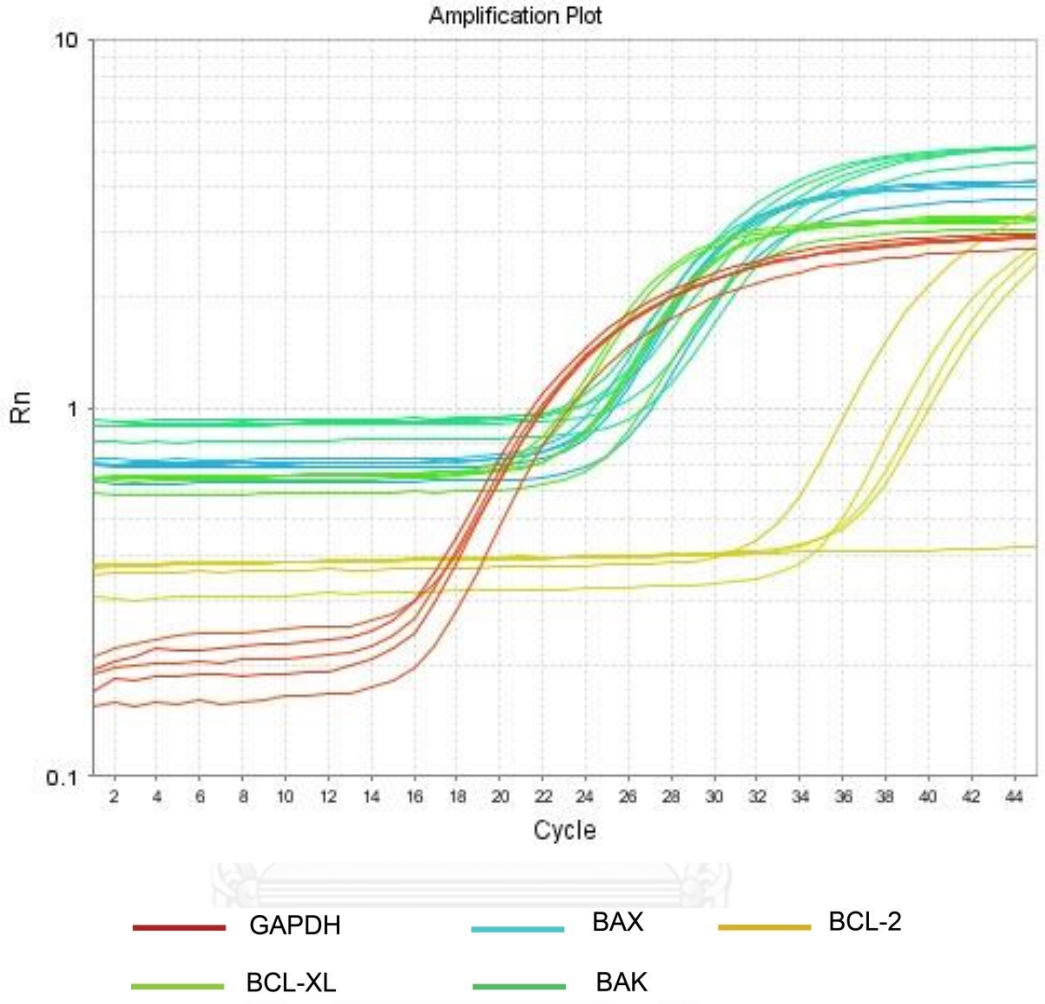


จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix C-14: Effect of GPE on the expression of BCL-2 family genes in Colo-205 cells.

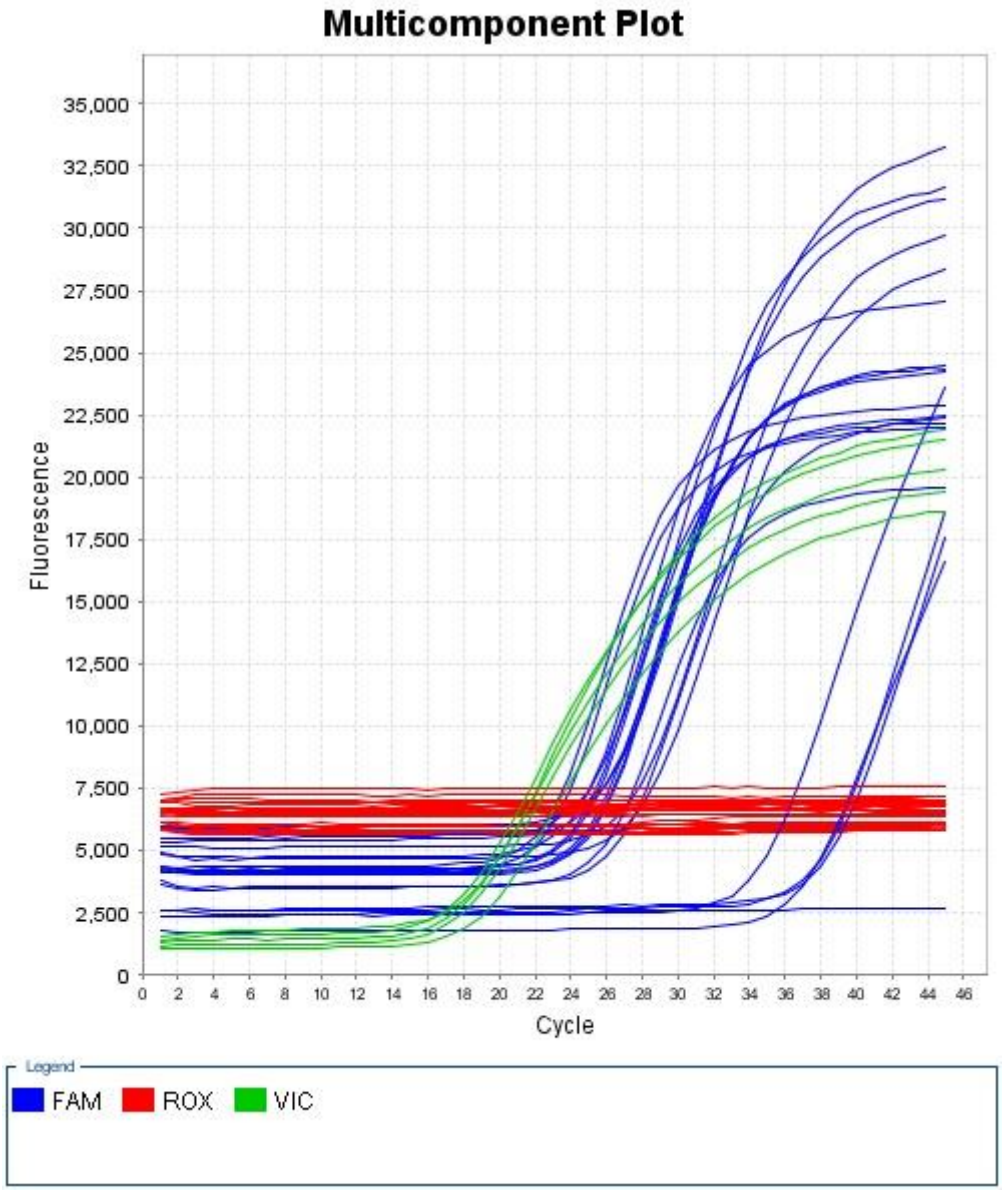
BCL-2 family gene expression in Colo-205		N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
BCL-2	0.2% DMSO	3	1.00	0.00	1.00	1.00	1.00	1.00
	Cisplatin 100 μM	3	0.00	0.00	0.00	0.00	0.00	0.00
	GPE 25 μg/ml	3	0.06	0.01	0.03	0.10	0.05	0.07
	GPE 50 μg/ml	3	0.39	0.07	0.10	0.67	0.25	0.46
	GPE 100 μg/ml	3	0.09	0.05	-0.11	0.29	0.00	0.16
BCL-XL	0.2% DMSO	3	1.00	0.00	1.00	1.00	1.00	1.00
	Cisplatin 100 μM	3	0.39	0.08	0.06	0.73	0.30	0.55
	GPE 25 μg/ml	3	0.98	0.04	0.82	1.14	0.91	1.04
	GPE 50 μg/ml	3	0.23	0.00	0.21	0.24	0.22	0.23
	GPE 100 μg/ml	3	0.42	0.05	0.20	0.63	0.34	0.51
BAK	0.2% DMSO	3	1.00	0.00	1.00	1.00	1.00	1.00
	Cisplatin 100 μM	3	1.89	0.40	0.18	3.60	1.45	2.68
	GPE 25 μg/ml	3	1.21	0.20	0.32	2.09	0.98	1.62
	GPE 50 μg/ml	3	0.66	0.08	0.34	0.98	0.58	0.81
	GPE 100 μg/ml	3	0.62	0.11	0.13	1.10	0.47	0.84
BAX	0.2% DMSO	3	1.00	0.00	1.00	1.00	1.00	1.00
	Cisplatin 100 μM	3	0.98	0.08	0.65	1.32	0.85	1.11
	GPE 25 μg/ml	3	0.54	0.11	0.07	1.01	0.37	0.74
	GPE 50 μg/ml	3	0.53	0.02	0.46	0.60	0.51	0.57
	GPE 100 μg/ml	3	0.95	0.11	0.46	1.43	0.72	1.09

Appendix C-15: Representative amplification plot from real-time RT-PCR experiment on the expression of genes in BCL-2 family in Colo-205 cells.



จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix C-16: Representative multicomponent plot from real-time RT-PCR experiment on the expression of genes in BCL-2 family in Colo-205 cells.



FAM = Fluorescence dye labeled with studied genes.
(BCL-2, BCL-XL, BAK, BAX)

ROX = Passive reference dye functioned as loading control.

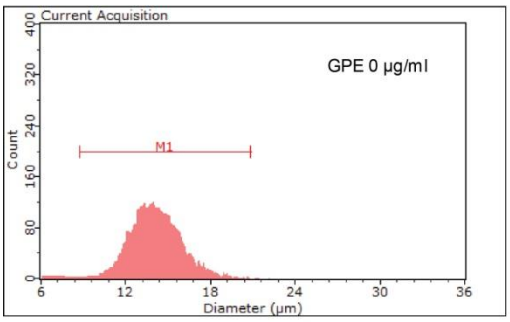
VIC = Fluorescence dye labeled with housekeeping genes (GAPDH).

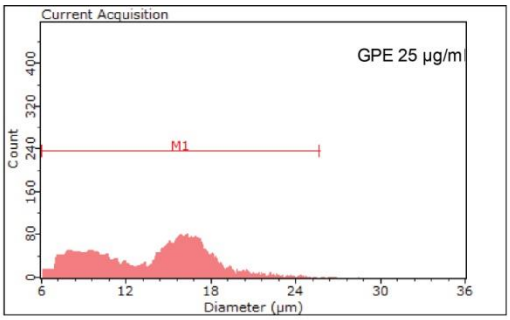
Appendix C-17: Anti-proliferative effect of GPE in Colo-205 cells.

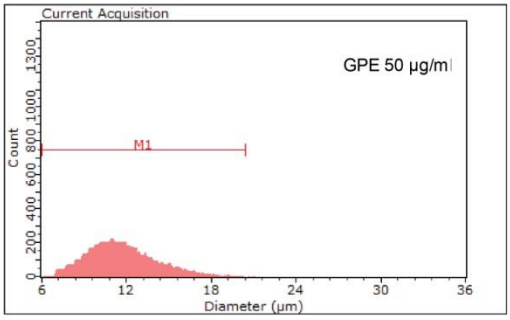
Cell Proliferation in Colo-205	N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
				Lower Bound	Upper Bound		
0.2% DMSO	3	100.00%	0.00%	100.00%	100.00%	100.00%	100.00%
GPE 25 µg/ml	3	60.71%	5.13%	38.64%	82.78%	51.75%	69.52%
GPE 50 µg/ml	3	71.63%	8.84%	33.59%	109.66%	62.01%	89.29%
GPE 100 µg/ml	3	59.91%	2.44%	49.42%	70.40%	57.07%	64.76%
5FU 100 µM	3	53.91%	5.08%	32.04%	75.78%	43.80%	59.90%

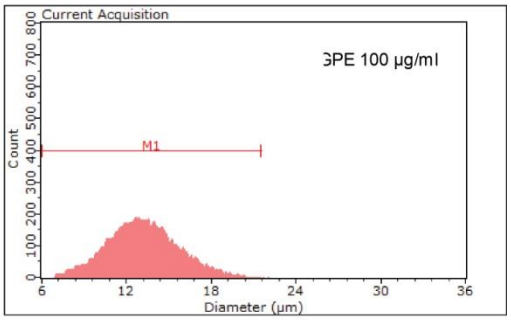
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

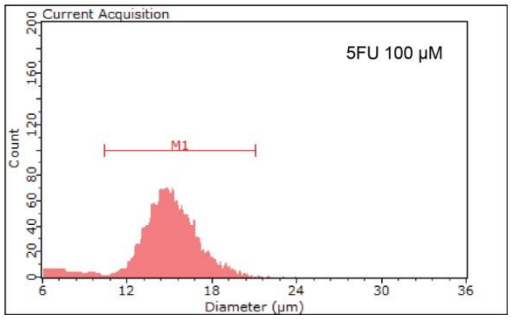
Appendix C-18: Representative histogram of cell counting from Scepter™ automate cell counter for determination of anti-proliferative effect of GPE in Colo-205 cells.











Appendix C-19: Distribution of Colo-205 cells in the cell cycle phases after treatment with GPE.

Cell cycle analysis in Colo-205		N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
G1	0.2% DMSO	3	51.49	1.73	44.03	58.95	48.03	53.39
	5FU 100 µM	3	50.24	2.86	37.95	62.52	44.79	54.45
	GPE 25 µg/ml	3	65.39	1.04	60.91	69.87	64.04	67.44
	GPE 50 µg/ml	3	53.31	0.89	49.50	57.12	52.36	55.08
	GPE 100 µg/ml	3	45.85	2.22	36.30	55.40	41.42	48.34
S	0.2% DMSO	3	36.37	0.83	32.81	39.93	35.18	37.96
	5FU 100 µM	3	47.75	1.12	42.94	52.56	45.55	49.18
	GPE 25 µg/ml	3	25.49	2.00	16.89	34.10	21.51	27.80
	GPE 50 µg/ml	3	16.62	1.79	8.91	24.32	14.35	20.15
	GPE 100 µg/ml	3	38.15	2.15	28.91	47.40	35.52	42.41
G2M	0.2% DMSO	3	12.14	0.95	8.07	16.22	10.98	14.02
	5FU 100 µM	3	2.01	2.01	-6.65	10.68	0.00	6.04
	GPE 25 µg/ml	3	9.12	1.03	4.66	13.57	7.51	11.05
	GPE 50 µg/ml	3	30.07	1.73	22.63	37.52	27.36	33.29
	GPE 100 µg/ml	3	15.99	0.46	14.02	17.96	15.13	16.69

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix C-20: Effect of GPE on the expression of cell cycle regulatory genes in Colo-205 cells.

Expression of cell cycle regulators in Colo-205		N	Mean	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
CyclinA	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	100.73	0.87	96.98	104.48	99.42	102.38
	GPE 25 µg/ml	3	93.90	1.92	85.62	102.18	90.08	96.23
	GPE 50 µg/ml	3	80.35	4.28	61.91	98.78	72.60	87.39
	GPE 100 µg/ml	3	66.45	4.18	48.45	84.45	59.13	73.62
CyclinB1	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	96.31	2.12	87.21	105.41	92.08	98.58
	GPE 25 µg/ml	3	94.09	0.34	92.62	95.56	93.51	94.69
	GPE 50 µg/ml	3	98.51	1.20	93.34	103.68	97.02	100.89
	GPE 100 µg/ml	3	95.49	4.80	74.83	116.16	88.19	104.55
CyclinD1	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	100.73	2.30	90.83	110.63	96.53	104.46
	GPE 25 µg/ml	3	103.77	2.73	92.03	115.51	99.01	108.46
	GPE 50 µg/ml	3	107.66	2.80	95.60	119.72	102.40	111.97
	GPE 100 µg/ml	3	99.73	3.37	85.25	114.22	95.86	106.44
CyclinE	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	125.59	1.92	117.35	133.84	121.81	128.01
	GPE 25 µg/ml	2	98.93	1.98	73.77	124.09	96.95	100.91
	GPE 50 µg/ml	3	89.81	4.56	70.17	109.44	83.69	98.73
	GPE 100 µg/ml	3	96.89	10.01	53.83	139.96	77.86	111.78
p21	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	125.34	9.03	86.47	164.21	112.23	142.66
	GPE 25 µg/ml	3	112.12	5.88	86.82	137.41	100.36	118.07
	GPE 50 µg/ml	3	126.29	9.25	86.49	166.10	110.85	142.84
	GPE 100 µg/ml	3	109.72	9.49	68.89	150.56	97.10	128.31
p53	0.2% DMSO	3	100.00	0.00	100.00	100.00	100.00	100.00
	5FU 100 µM	3	105.85	5.55	81.97	129.74	95.00	113.31
	GPE 25 µg/ml	2	94.48	3.20	53.88	135.07	91.28	97.67
	GPE 50 µg/ml	3	91.62	6.93	61.81	121.43	79.29	103.26
	GPE 100 µg/ml	3	49.62	9.61	8.25	90.99	36.65	68.40

VITA

Mrs. Nattaporn Buranabunwong was born on November 19, 1979 in Bangkok, Thailand. She received her Bachelor's degree of Pharmaceutical Science (2nd class honor) from the Faculty of Pharmaceutical Sciences, Chulalongkorn Uniersity, Bangkok, Thailand in 2002. She achieved her Master's degree Program in Marketing (International Program) from the Faculty of Commerce and Accountancy, Thammasat University, Bangkok, Thailand in 2007.

Publication

1. Buranabunwong N., Ruangrungsi N., and Limpanasithikul W. 2013. Anti-cancer activity of Glycosmis parva leaf extract on human colorectal cancer HT29 cell. Acta Pharmacologica Sinica. 34 supp:4-4.

Poster Presentation

1. Buranabunwong N., Ruangrungsi N., and Limpanasithikul W. 2013. Anti-cancer activity of Glycosmis parva leaf extract on human colorectal cancer HT29 cell. The 12th Meeting of the Asia Pacific Federation of Pharmacologists. July 9-13, 2013, Shanghai, China.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University