ANTI-CANCER EFFECTS OF THE ETHANOL EXTRACT FROM *Kaempferia parviflora* ON OVARIAN CANCER CELL LINE

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MASTER OF SCIENCE
IN PHARMACOLOGY

GRADUATE SCHOOL
CHIANG MAI UNIVERSITY
DECEMBER 2017

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A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PHARMACOLOGY

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MASTER OF SCIENCE
IN PHARMACOLOGY

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I would like to give this thesis to my beloved family who have always been my encouragement, supports and guidance for all my life.

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Suthasinee Paramee

หัวข้อวิทยานิพนธ์ ฤทธิ์ต้านมะเร็งของสารสกัดเอทานอลจากกระชายดำต่อเซลล์มะเร็งรังไข่เพาะเลี้ยง

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บทคัดย่อ

มะเร็งรังไข่เป็นปัญหาทางคลินิกที่สำคัญสำหรับผู้หญิงเนื่องจากมีผลกระทบสูงต่อคุณภาพชีวิต ผู้ป่วยส่วนใหญ่มักจะได้รับการวินิจฉัยหลังจากที่มะเร็งอยู่ในระยะลุกลามไปแล้ว เพราะไม่มีการแสดงอาการของโรคที่ชัดเจนในระยะเริ่มต้น เนื่องจากไม่มีวิธีการรักษาและการคัดกรองที่ได้ผลจึงทำให้มะเร็งรังไข่มีอัตราการเสียชีวิตสูงสุดในบรรดามะเร็งทางนรีเวชอื่นๆ การรักษาด้วยวิธีผ่าตัด หรือยาเคมีบำบัดและการฉายรังสีเป็นทางเลือกในการรักษาสำหรับผู้ป่วย อย่างไรก็ตามแต่ละวิธีมีผลข้างเคียงและมีค่าใช้จ่ายสูงมาก นอกจากนี้ยังเกิดการกำเริบของโรคมะเร็งหลังจากที่ได้รับการรักษา ดังนั้นการรักษาโดยการแพทย์ทางเลือกเป็นอีกหนึ่งทางเลือกสำหรับผู้ป่วยบางราย กระชายดำที่พบได้ในภาคเหนือของประเทศไทยมีศักยภาพในการรักษาโรคต่างๆ อย่างไรก็ตามยังไม่เคยมีใครศึกษาเกี่ยวกับฤทธิ์ต้านมะเร็งรังไข่ของสารสกัดจากกระชายดำ ดังนั้นผู้วิจัยจึงสนใจที่จะศึกษาผลของสารสกัดจากกระชายดำต่อ เซลล์รังไข่เพาะเลี้ยง ผลการทดลองพบว่าสารสกัดจากกระชายดำมีฤทธิ์ความเป็นพิษต่อเซลล์รังไข่เพาะเลี้ยง ที่ระดับ IC50 เท่ากับ 0.53 ± 0.08 mg/ml ในภาวะที่ไม่มี EGF และ 0.63 ± 0.08 มิลลิกรัมต่อมิลลิลิตรในภาวะที่มี EGF สารสกัดจากกระชายดำที่ความเข้มข้น 0.05 มิลลิกรัมต่อมิลลิลิตรเพิ่มเวลาในการแบ่งตัวของเซลล์เป็นสองเท่า (doubling time) จาก 24 ชั่วโมงเป็น 31.7 ชั่วโมง และทำให้การแบ่งตัวของเซลล์ลดลงประมาณ 50% เมื่อศึกษาด้วยวิธี wound healing จากผลการศึกษาเหล่านี้แสดงให้เห็นว่าสารสกัดจากกระชายดำช่วยยับยั้งการเจริญเติบโตของเซลล์ และยังมีผลต่อการยับยั้งการเคลื่อนที่ของเซลล์เมื่อศึกษาด้วยวิธี Transwell migration assay จากการวิเคราะห์ผลด้วยวิธี

zymography สารสกัดลดการทำงานของเอนไซม์ MMP-2 และ MMP-9 อย่างมีนัยสำคัญทั้งในภาวะที่มี EGF และไม่มี EGF ซึ่งแสดงให้เห็นว่าสารสกัดกระชายดำอาจมีบทบาทในการยับยั้งการแพร่กระจายของมะเร็งรังไข่ นอกจากนี้สารสกัดกระชายดำยังชักนำให้เกิดการตายของเซลล์แบบ apoptotic เมื่อตรวจสอบด้วยการย้อมสี AnnexinV/PI และวิเคราะห์ผลด้วย flow cytometry อีกทั้งการย้อมสีนิวเคลียสของเซลล์รังไข่เพาะเลี้ยงยังพบว่าเยื่อหุ้มของเซลล์มีลักษณะที่โป่งออก และมีการแตกหักและการอัดแน่นของดีเอ็นเอซึ่งเป็นลักษณะของการเกิดการตายแบบ apoptosis นอกจากนี้การวิเคราะห์โปรตีนด้วย western blot assay ของเอนไซม์ caspase-3, -7, -9 และโปรตีน PARP ซึ่งเป็นโมเลกุลที่เกี่ยวข้องกับการเกิด apoptosis พบว่าโปรตีนเหล่านี้ได้รับการกระตุ้นเมื่อให้สารสกัดจากกระชายดำ ผลการยับยั้งของสารสกัดกระชายดำต่อการเจริญเติบโตและความอยู่รอดของเซลล์รังไข่เพาะเลี้ยง อาจควบคุมผ่านทางสัญญาณ PI3K/Akt และ MAPK เนื่องจากมีการลดลงของการเติมฟอสเฟต Akt และ Erk1/2 ผลจากการศึกษาครั้งนี้ชี้ให้เห็นว่ากระชายดำอาจเป็นยาทางเลือกตัวใหม่ในการรักษามะเร็งรังไข่

Thesis Title	Anti-cancer Effects of the Ethanol Extract From *Kaempferia parviflora* on Ovarian Cancer Cell Line
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Degree	Master of Science (Pharmacology)
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ABSTRACT

Ovarian cancer is a major clinical problem for women due to its high impact on the quality of life. Most patients are diagnosed when the cancer is in an advanced stage because there is no specific sign or symptom at the early stage of the disease. Due to the poor treatment and inadequate screening, ovarian cancer has the highest mortality rate among gynecologic cancers. Surgery, chemotherapy, and radiation are treatment choices for patients. However, each method has many side effects and is highly expensive. Moreover, a cancer relapse often occurs even after aggressive treatments. Thus, alternative medicine is an option for some patients. *Kaempferia parviflora* (KP) which is found in the north of Thailand, has potential to treat various diseases. However, the effect of KP on ovarian cancer has not yet been investigated. We, therefore, examined the effect of KP on ovarian cancer SKOV3 cell line. The results showed that KP extract has cytotoxic effects on SKOV3 cells at IC50 of 0.53 ± 0.08 mg/mL with the absence of EGF and 0.63 ± 0.08 mg/mL with the presence of EGF. KP extract at 0.05 mg/mL increased the doubling time from 24 h to 31.7 h and showed approximately 50% reduction in wound healing. These results suggest that KP strongly suppresses cell proliferation. KP also exhibited inhibitory effects on cell migration evaluated by Transwell migration assay. From zymography

assay, the extract significantly reduced MMP-2 and MMP-9 activities in both absence and presence of EGF. This suggests that KP might play a role in suppressing tumor metastasis. Moreover, KP induced apoptotic cell death demonstrated by AnnexinV/PI staining and flow cytometry. Moreover, nuclear labelling of cell treated with KP extract showed plasma membrane blebling, DNA fragmentation and condensation which are apoptotic characteristics. Furthermore, western blot analysis of caspase-3, -7, -9 and PARP which are molecules involved in apoptosis were activated upon treatment with KP extract. The inhibitory effect of KP on SKOV3 cells on cell growth and survival may regulate through PI3K/Akt and MAPK pathways since the phosphorylation of Akt and Erk1/2 was reduced. The results from this study suggest that KP might be a new candidate for being an ovarian cancer chemotherapeutic agent.

CONTENT

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

5-H-TeMF	5-hydroxy-3,7,3′,4′-tetramethoxyflavone
AchE	Acetylcholine-esterase
Akt	Akt (Protein kinase B)
Apaf-1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
BchE	Butyrylcholinesterase
BRCA1	Breast cancer susceptibility gene 1
CDKs	Cyclin dependent kinases
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EOC	Epithelial ovarian cancer
ERKs	Extracellular signal-regulated kinases
EtOH	Ethanol
FADD	Fas-associated protein with death domain
FBS	Fetal Bovine Serum
FGFRs	Fibroblast growth factor receptors
GSK-3	Glycogen synthase kinase 3
GTPase	Guanosine triphosphate
HCl	Hydrochloric acid
HER2	Hydrogen chloride
HGFRs	Hepatocyte growth factor receptors
IAPs	Inhibitors of apoptosis proteins
IC50	The half maximal inhibitory concentration
JNKs	c-Jun N-terminal kinases

KP	*Kaempferia parviflora*
LSD	Least-significant difference
MAPK	Mitogen-activated protein kinase
MIC	Minimum inhibitory concentrations
MMPs	Matrix metalloproteinases
MRP	Multidrug resistance associated proteins
MSI	Microsatellite instability
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
OV	Ovarian cancer
pAkt	Phosphorylated Akt
pERK1/2	Phosphorylated ERK1/2
PARP	Poly ADP ribose polymerase
PBS	Phosphate-buffered saline
PI	Propidium iodide
PI3K	Phosphatidylinositide-3-kinase
PIK3CA	Phosphatidylinositol 3-kinase catalytic subunit
PMFs	Polymethoxyflavones
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
Raf	Raf (a family of three serine/threonine-specific protein kinases)
Ras	Ras (a family of three serine/threonine-specific protein kinases)
RPMI	Roswell Park Memorial Institute
RTKs	Receptor tyrosine kinases
S473	Serine 473
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFM	Serum-free media
SOS	Son of seven
T308	Threonine 308
TBST	Tris buffer containing tween-20
TeMF	5,7,4′-tetramethoxyflavone

TMF	5,7,4′-trimethoxyflavone
TNF	Tumor necrotic factor
TNF-β	Tumor necrosis factor -β
TP53	p53 tumor suppressor gene
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain
TRAIL	TNF-related apoptosis- Induction ligand
VEGFRs	Vascular endothelial growth factor receptors

CHAPTER 1

Introduction

1.1 Statement and significance of the problem

Ovarian cancer is the fifth most frequent cause of gynecologic malignancies. There are about 1,600 new cases each year in Thailand (1). Compared to others, ovarian cancer is the poorest prognosis with five-year survival rates of 44% (2). This may be due to the advance of the disease once diagnosed and cell types. Ovarian cancer is divided into two types; type 1 (low-grade) and type 2 (high-grade) in which cell morphology, pathogenesis, genotype, rate of growth, and response to treatment are different (3). Up to 70% of all ovarian cancer cases are high-grade carcinomas which grow aggressively, metastasize rapidly, and have high chromosomal instability(4). Since there is no specific sign and symptom at the initial stages of cancer development, most patients are therefore diagnosed at very late stages with distant metastasis. Conventional treatments are surgery plus platinum-based chemotherapies such as cisplatin or carboplatin. Moreover, additional therapeutic like taxol (paclitaxel) is regularly in used (5). Traditional therapies are less effective as seen from the majority of patients who eventually experience drug resistance, especially in recurrent cases. Moreover, chemotherapeutic agents have many side effects, including severe nausea/vomiting, hair loss, and bone marrow suppression. For these reasons, new effective drugs or alternative medicine with minimal adverse effects are urgently required.

Kaempferia parviflora (KP) is the plant in a family of Zingiberaceae known as Thai black ginger or krachai dum. It has many pharmacological effects such as sexual enhancement, anti-gastric ulcer, anti-allergic, and anti-plasmodial properties. Specifically, for anti-cancer effect, several studies have shown that KP suppresses multidrug resistance associated proteins (MRP) in A549 cells (lung carcinoma cell line) (6), induces apoptotic cell death, and enhances doxorubicin or paclitaxel treatment in promyelocytic leukemic cancer cell line (7). However, the effect of KP on ovarian cancer has not yet been

explored. Therefore, this present study aims to evaluate the anti-cancer properties of KP on high-grade ovarian cancer cell line (SKOV3). The effects for KP on cell viability assay, cell cycle analysis, zymography, apoptotic assay, cell migration assay, and immunofluorescence study in SKOV3 were investigated. Furthermore, the molecular signaling related to cell proliferation (MAPK pathway), cell survival (Akt) and cell death (caspases) were also performed to find the mechanism of KP on SKOV3 cell suppression. We hypothesized that KP has a potential to suppress ovarian cancer cell growth and induce cell death which may be a good candidate for alternative treatment of ovarian cancer.

1.2 Literature Review

1.2.1 Ovarian cancer

Ovarian cancer (OV cancer) is a malignant tumor in the ovaries, one of the female organ in reproductive system. The ovaries produce eggs and hormones estrogen and progesterone which control the menstrual cycle. In a lifetime, a woman has about 1 in 55 risk of getting ovarian cancer without a family history (8). There are many risk factors including elder age, inherited faulty genes (*BRCA1* and *BRCA2*), history of breast cancer, hormone replacement therapy, talcum powder, and obesity (9) that contribute to OV cancer development. In general, early stage of OV cancer in most patients are asymptomatic, however, some symptoms such as nonspecific abdominal discomfort such as bloating and abdominal pain, eating less, and pelvic pain could be noticeable (10).

OV cancer cells can be originated from supportive tissues called sex-cord stromal cells that produce hormones. This cancer type is very rare (7% of all primary OV cancers) (11). The germ cell type occurs approximately 10% of all OV cancers. More than 90% of OV cancer is developed from epithelial cells. Epithelial ovarian cancer (EOC) is divided into 2 major types which are high-grade (type I tumor) and low-grade (type II tumor) carcinomas. High-grade tumor has a high frequency of mutations in the epidermal growth factor receptor *(EGFR)* and *HER2* genes (12). They present unregulated inhibition of apoptosis signaling molecules,

particularly Akt which are strongly associated with the preference of high grade tumor. Moreover, high-grade tumor is also associated with aggressive tumor invasion and distant metastasis. The ability of tumor invasion is linked to many factors including the matrix metalloproteinases (MMPs) which are zinc-dependent endopeptidases that have proteolytic activity to degrade proteins especially basement membrane and extracellular matrix (ECM). These enzymes promote tumor invasion and progression (13, 14).

1.2.2 Human epithelial ovarian cancer cell line (SKOV3)

SKOV3 cells are ovarian adenocarcinoma cells which have epithelial-like morphology and closely similar to high grade serious ovarian carcinomas (15) that are resistant to tumor necrotic factor (TNF) and to several cytotoxic drugs including diphtheria toxin, cisplatin, and adriamycin (16). Therefore, SKOV3 cells are considered to be an aggressive type among OV cancer cell lines.

SKOV3 cells show a *TP53*, *PIK3CA*, and *BRCA1* mutations (15, 16). SKOV3 cell lines contain microsatellite instability (MSI) which is more often seen in the mucinous and endometrioid subtypes (16)

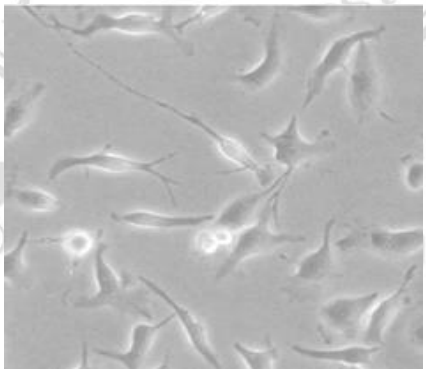


Figure 1.1 Morphology of SKOV3 cell line (17) observed by phase contrast microscopy

1.2.3 Signal transductions involved in ovarian cancer

Cellular signal transduction is the process by which cells recognize external stimulation and then communicate through several downstream signaling

molecules within the cell. The signals can be transferred via many mechanisms such as phosphorylation or dephosphorylation by kinase or phosphatase enzymes, respectively (18). These signal transductions are responsible for cell proliferation, cell growth, cell migration, cell survival, and cell death. These signal transduction pathways are regulated under certain condition to have normal function within the cell. However, imbalance or dysregulation in some signaling molecules finally causes the development of many diseases including OV cancer.

A family of cell surface receptors which are associated with ovarian cancer is the receptor tyrosine kinases (RTKs) that can be activated by growth factors, hormones, cytokines, and other extracellular signaling molecules. The RTK family encloses various subfamilies that consist of hepatocyte growth factor receptors (HGFRs), proto-oncogene c-KIT, fibroblast growth factor receptors (FGFRs), vascular endothelial growth factor receptors (VEGFRs) and epidermal growth factor receptors (EGFRs) (19, 20). EGFRs or erbB receptor family are pronounce in ovarian cancer. It is expressed in 10-70% of ovarian carcinomas and plays important role in regulating the growth and cell division, cell survival, motility, invasion and tumor angiogenesis (21). There are four types of erbB1 (EGFR / HER1), erbB2 (HER2 / neu), erbB3 (HER3) and erbB4 (HER4) (22). Binding of EGF to EGFR leads to stimulation of the mitogen-activated protein kinase (MAPK) pathways through the Ras effector, signal transducer and activators of transcription, and the phosphatidylinositide-3-kinase (PI3K)/AKT pathway, which eventually results in activation of proteins involved in cell cycle, cell survival, and cell death (23)(24). Mutations occurring in signal molecules of RTK pathway in particular PI3K/Akt and MAPK often lead to a variety of carcinomas (20).

Signals of MAPK in mammals are divided into 4 groups; Extracellular signal-regulated kinases (ERKs), C-Jun N-terminal kinases (JNKs), p38, and ERK5. Activation of growth factors and mitogens ERK signaling is involved to many types of carcinomas (25). Upon RTK activation, Son of seven (SOS) is recruited to the plasma membrane which further activates the Ras which has GTPase

activity. Ras-GTP provides the binding sites for Raf, which is a serine/threonine kinase. Raf then activates the phospholipase C which further phosphorylates and activates ERK1/2 (Fig. 1.2) (12, 20). ERK2 plays a role in Ras-dependent signaling in cell division while ERK1 affects to all signaling of the cell. The activation of ERK also plays a role in cell-division cycle which is composed of G_0, G_1, S, G_2, and M phases. The G_0 phase is a period of cells that is in resting state before moving to G_1 phase. Cells start to prepare mRNA and protein at this stage in order to have materials ready for S phase. The synthesis or S phase is a period of DNA synthesis for cell duplication. The mitosis or M phase is the stage of cells that has double amount of DNA which is ready to separate from one to two cells. It has been known that ERK stimulates cell activity from G1 to S and works with PI3K to stimulate cells from G2 to M (Fig.1.3). Besides MAPK, the cycle of cell is controlled by the kinase protein called cyclin dependent kinases (CDKs) which are activated by specific cyclins by phosphorylation process. There are several types of cyclins involving in cell cycle. Cyclin D, cyclin E, cyclin A, and cyclin B control G_1 phase, G_1 to S phase, S phase to G_2 phase, and G_2 to M phase, respectively (26, 27).

In addition to MAPK pathway, the PI3-kinase/AKT pathway is also overactivated in OV cancers. Upregulation of AKT in cancer enhances neovascularization, increases invasion, and raises resistance to chemotherapeutic agents. Moreover, activated AKT can suppress apoptosis (20). The activation of this pathway starts with binding of specific ligands to the tyrosine kinase receptor on the cell surface that causes in matching and phosphorylation at the amino acid position of the receptor (Fig. 1.2). The PI3K is then recruited to the plasma membrane. After PI3K is activated, it further phosphorylate PIP2 to PIP3. PIP3 then pulls the AKT to the plasma membrane via the PH domain. AKT is activated by adding two phosphate groups at threonine 308 (T308) of the kinase domain and serine 473 (S473) of the regulatory domain. Activated AKT acts as a mediator for stimulating and inhibiting downstream targets such as the glycogen synthase kinase 3 (GSK-3) enzyme. After phosphorylation, the GSK-3 becomes inactive. The AKT/GSK-3 pathway is involved in cell survival and in the regulation of apoptosis (28) (29).

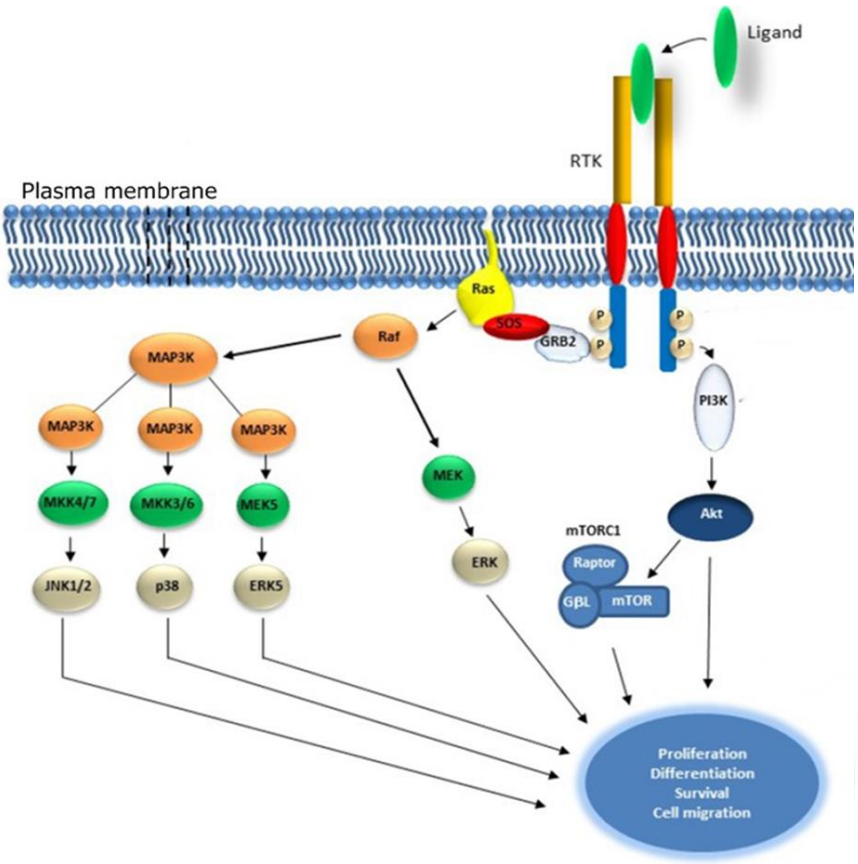


Figure 1.2. Ovarian signaling pathway (20)

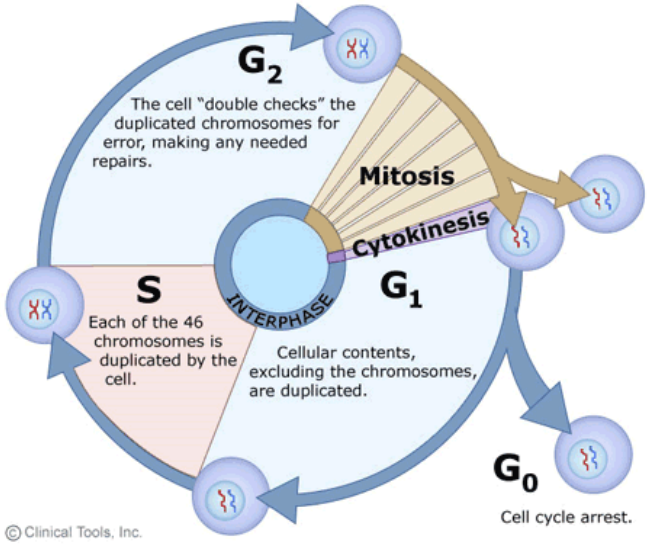


Figure 1.3. Cell cycle (27)

1.2.4 Apoptotic signal transduction pathway

Apoptosis is a programmed cell death that plays an important role in the development and maintenance of cellular balance in the higher organisms. Abnormalities in apoptotic pathway lead to many diseases or pathologies especially the suppression of apoptosis which results in development of cancer or autoimmune diseases. Apoptotic cells are manifested as membrane blebbing, chromatin condensation, DNA and nuclear fragmentation. Apoptotic cells can be caught by macrophage without causing inflammation. In contrast, necrosis shows massive inflammation due to cytokines leakage. Therefore, apoptosis is a preferential process for inducing cancer cell death. Apoptotic pathway is composed of two main pathways; extrinsic and intrinsic pathways. The extrinsic or death receptor pathways are activated through the binding of external ligands such as tumor necrosis factor -β (TNF-β), Fas ligand, TNF-related apoptosis-Induction ligand (TRAIL) to the death receptors (Fig.1.4). Binding of the death ligand to its receptor results in the aggregation of FADD, TRADD receptor molecules which further stimulates caspase-8. Caspase-8 activation leads to the production of enzyme caspases-3, -6, and -7 enzymes which digest their substrates and eventually cause apoptosis. Another pathway is the intrinsic pathway or mitochondrial pathway which is caused by the release of cytochrome c protein from mitochondria into the cytoplasm. The cytochrome c form a complex with Apaf-1 protein, ATP and procasepase-9 called apoptosome that further stimulates caspase-3, -6, and -7 activation. Some of the mitochondrial proteins, such as Smac, are inhibited by inhibitors of apoptosis proteins (IAPs) which inhibit caspase-3, -6, and -7 (Fig.1.4) (30). In addition, survival signals have been shown to increase the expression of apoptotic-resistant proteins or antiapoptotic proteins such as Bcl-2, Bcl-xL. These proteins inhibit the release of cytochrome c whereas proapoptotic proteins, such as Bid, Bax, and Bak stimulate releasing of cytochrome c from the mitochondria (Fig. 1.4)(31). Caspase is able to cleave many major cellular substrates such as PARP, poly ADP ribose polymerase which is normally involved in DNA repair process. Cleavage of PARP normally results in inactivated PARP which can cause DNA degradation (32, 33).

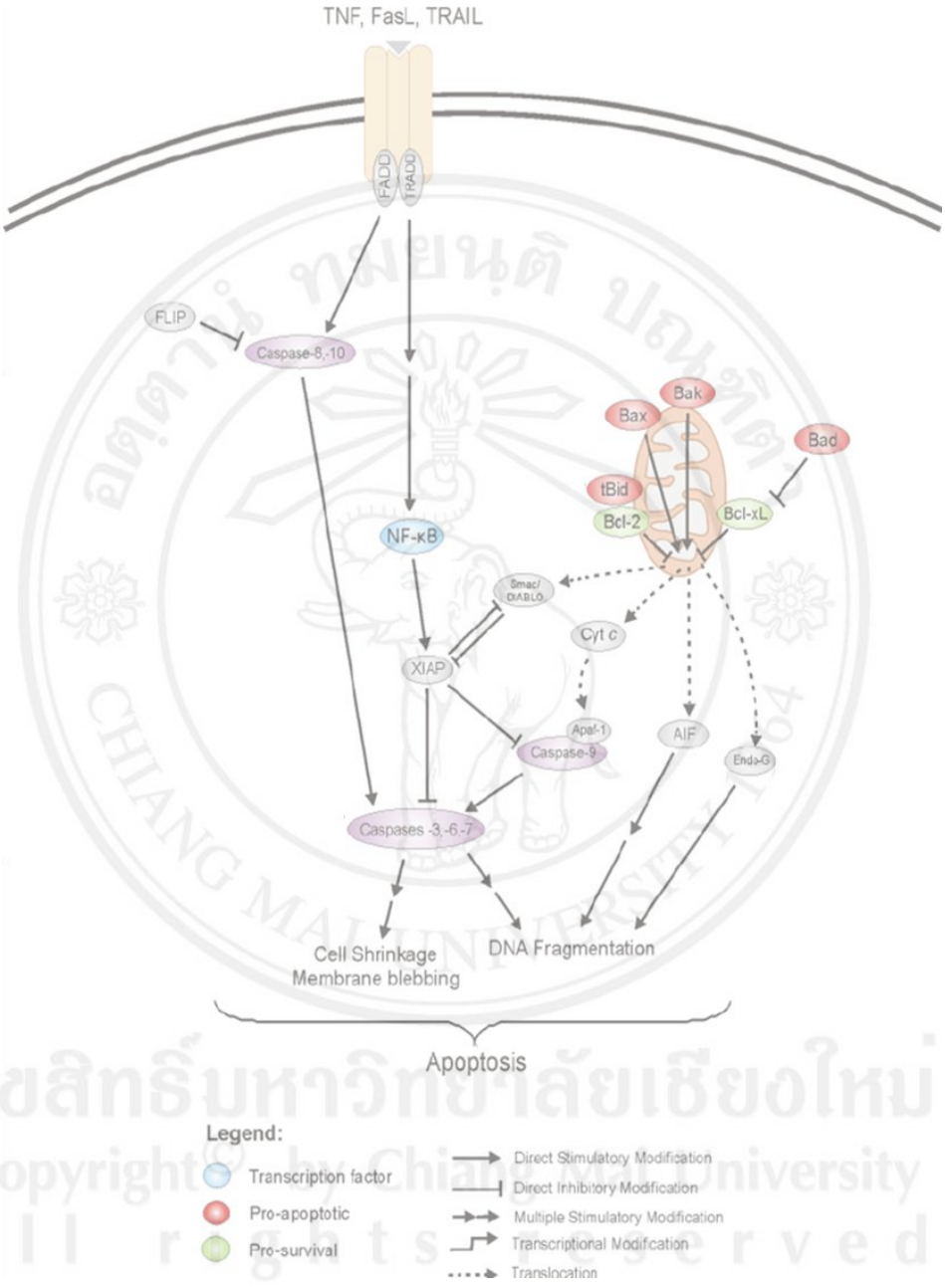


Figure 1.4. Apoptosis signaling (31)

1.2.5 Anti-ovarian cancer agents

Chemotherapeutic drugs are agents used for cancer treatment. The agents used for OV cancer are summarized as follow (Fig. 1.5):

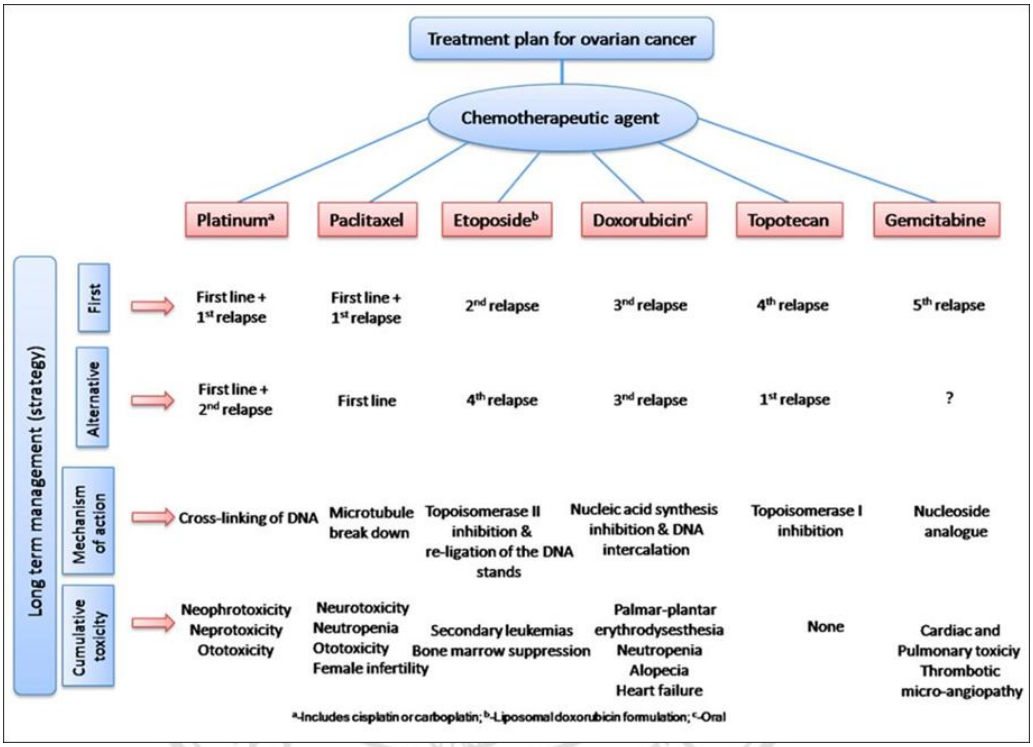


Figure 1.5. Long term treatment strategy for OV cancer using various anti-cancer drug combinations (34)

1.) Platinum agents

Cisplatin (Platinol®; Bristol-Myers Squibb; Princeton, NJ) is a metallic (platinum) coordination compound with a square planar geometry (35). This agent is used as a first-line drug or second-line therapy for sensitive OV cancer and drug-resistant OV cancer, respectively. Side effects of cisplatin are nephrotoxicity, ototoxicity, gastrotoxicity, and neurotoxicity (36). The mechanisms of cisplatin are induction of DNA damage, inhibition of DNA synthesis and mitosis, and induction of apoptosis (35).

2.) Paclitaxel

Paclitaxel (Taxol®; Bristol-Myers Squibb Princet, NJ) is a nonplatinum-based cytotoxic agent which has been used as the first-line treatment for advanced OV cancer (36). Adverse effects of this drug consist of hypersensitivity, diarrhea, alopecia, nausea, vomiting, and mucositis (37). Additionally, The cumulative peripherals nerve are neurotoxicity, peripheral neuropathy, myalgia and arthralgia (36). The drug has a specific binding site on the microtubule polymer and blocks cells in the G2/M phase of the cell cycle and the prophase of mitosis (38, 39).

3.) Oral etoposide

Etoposide (VePesid®; Bristol-Myers Squibb Princet, NJ) is attributed to break cellular DNA by interactions with DNA topoisomerase II or through the formation of reactive free radicals. The drug which is the second-line therapy for advanced OV cancer is a derivative of the plant lignin podophyllotoxin. The most common adverse effects are severe hematologic toxicities for long term use and anaphylaxis, mucositis, and acute hypo- and hypertensive (36).

4.) Liposomal Doxorubicin

Doxorubicin (Rubex®; Bristol-Myers Squibb) is an anthracycline drug extracted from *Streptomyces peucetius* var. *caesius*. This agent intercalates into the DNA structure and breaks topoisomerase II-mediated DNA repair and generates free radicals causing damage to cellular membranes, DNA and proteins (40). Doxorubicin therapy is associated with cumulative cardiotoxicity, and hematologic toxicity (36).

5.) Topotecan

Topotecan (Hycamtin®; GlaxoSmithKline; Philadelphia, PA) a water-soluble derivative of camptothecin which plays a role as an inhibitor of topoisomerase I, an enzyme essential for DNA replication (41). The drug prevents re-ligation of the single-stranded breaks (36).

6.) Gemcitabine

Gemcitabine (Gemzar®; Eli Lilly; Indianapolis, IN) acts by inhibiting DNA synthesis which eventually prevents the progression of cells to the G1/S-phase of the cell cycle (42). This agent is a synthetic nucleoside analogue and used in combination with platinum as the second-line therapy. Its adverse effects include cardiac and pulmonary toxicities.

Most chemotherapy drugs exhibit many adverse effects, and they are less effective in the treatment of OV cancer (34). Moreover, resistant to these chemotherapies are commonly observed. Therefore, it is always a good idea to seek for alternative and complementary therapies from herbs which may be less toxic and effective for OV cancer treatment.

1.2.6 *Kaempferia parviflora* (**KP**)

KP is an herb in the Zingiberaceae family found in India, Myanmar, Thailand (Loei, Tak, Kanchana Buri, and other northern provinces). It is commonly known as "Thai black ginger, or Krachai dum". Its rhizome is normally used in traditional medicine. This plant has simple leaf with deep violet color, oval, or elliptical shape. The flower is inflorescence appearing and can be white or pink (Fig. 1.6).

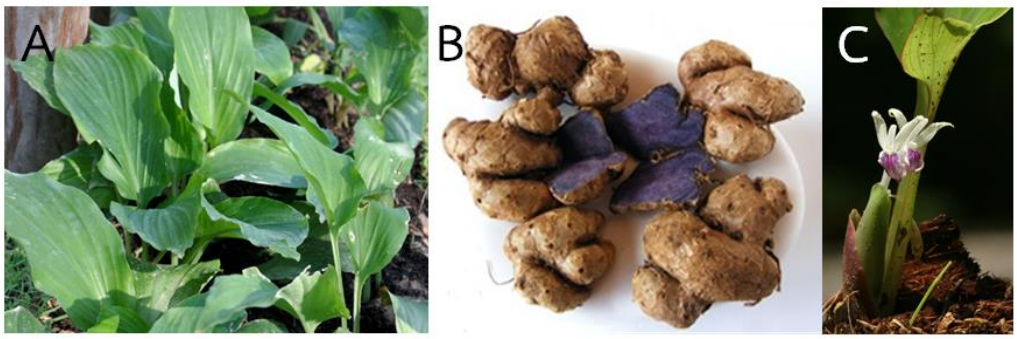
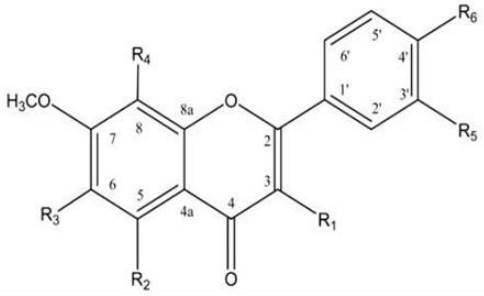


Figure 1.6. Leaves (A), rhizomes (B), and flowers (C) of *Kaempferia parviflora* (43)

KP is famous in enhancing sexual activity in male (44). In addition, it has been previously demonstrated to have several pharmacological effects including anti-plasmodial (45), anti-fungal (45), anti-mycobacterial (45), anti-gastric ulcer (45),

anti-allergic (46), anti-acetylcholine-esterase (AChE) activities (47), and anti-cancer (6, 45, 48). It has been reported that major bioactive flavonoids of KP, called "polymethoxyflavones (PMFs)" possesses inhibitory biological activities (Fig. 1.7). (45, 48) PMFs of KP have been studied for its anticancer activity in may cancer cell lines; for example, human cervical cancer cell line (HeLa) (48), human gastric adenocarcinoma cell line (AGS) (48), lung carcinoma cell line (NCI-H187, A549) (6, 45), breast cancer cell line (BC) (45), oral human epidermoid carcinoma cell line (KB)(45), promyelocytic leukemic cell line (HL-60, U937) (7, 49), and colorectal carcinoma cell line (HCT-15) (50). However, there has been no report for the anticancer activity of KP in OV cancer cell line (SKOV3).



Compound	R_1	R_2	R_3	R_4	R_5	R_6
5,7-Dimethoxyflavone	H	OCH_3	H	H	H	H
5,7,4'-Trimethoxyflavone	H	OCH_3	H	H	H	OCH_3
5,7,3',4'-Tetramethoxyflavone	H	OCH_3	H	H	OCH_3	OCH_3
5-Hydroxy-7-methoxyflavone	H	OH	H	H	H	H
5-Hydroxy-7,4'-dimethoxyflavone	H	OH	H	H	H	OCH_3
5-Hydroxy-3,7-dimethoxyflavone	OCH_3	OH	H	H	H	H
5-Hydroxy-3,7,4'-trimethoxyflavone	OCH_3	OH	H	H	H	OCH_3
5-Hydroxy-3,7,3',4'-tetramethoxyflavone	OCH_3	OH	H	H	OCH_3	OCH_3
3,5,7-Trimethoxyflavone	OCH_3	OCH_3	H	H	H	H
3,5,7,4'-Tetramethoxyflavone	OCH_3	OCH_3	H	H	H	OCH_3
3,5,7,3',4'-Pentamethoxyflavone	OCH_3	OCH_3	H	H	OCH_3	OCH_3

Figure 1.7. The major structures of PMFs in KP (48)

1.2.7 Pharmacological effects of KP

1.) Sexual activity

There is a study showing that the KP extract at 240 mg/kg reduced the time in the first 10 minutes of rat courting behavior. All dosages have no effect on kidney and liver function, but the histopathological study showed a

morphological change in the liver. Therefore, safety in human needs to be verified before use (44).

2.) Anti-fungal activity

3,5,7,4′-tetramethoxyflavone and 5,7,4′-trimethoxyflavone active flavonoids of KP have been shown to have antifungal activity against *Candida albicans* with IC50 values (determined by formazan assay) of 39.71 and 17.63 µg/ml, respectively (45).

3.) Anti-plasmodial activity

Micro-dilution radioisotope technique revealed that the active flavonoid of KP named 5,7,3′,4′-tetramethoxyflavone and 5,7,4′-trimethoxyflavone exhibit anti-plasmodial activity against *Plasmodium falciparum* with IC50 values of 3.70 and 4.06 µg/ml, respectively (45).

4.) Anti-mycobacterial activity

The study using Micro-Alamar Blue assay for anti-mycobacterial activity demonstrated that 5,7,4′ - trimethoxyflavone and 3,5,7,4′ - tetramethoxyflavone" ulcer activity. Oral administration of the KP extract at 30, 60, and 120 mg/kg inhibited significant gastric ulcer formation activated by indomethacin, HCl/EtOH and water immersion restraint-stress in rats. In ethanol-induced ulcerated rats, gastric wall mucus was fermented significantly by the KP extract pretreatment at doses of 60 and 120 but not at 30 mg/kg. Moreover, the ethanolic extract of KP inhibited gastric acid secretion (51).

5.) Anti-allergic activity

Previous studies reported that the powder of KP can be used to treat allergy and asthma. The isolated active compound 5- hydroxy- 3,7,3',4'-tetramethoxyflavone, had the highest anti-allergic activity against antigen-induced beta- hexosaminidase release. The 5- hydroxy- 3,7,3',4'-tetramethoxyflavone and 5- hydroxy- 7- methoxyflavone inhibited cell degranulation which is involved in Ca^{2+} influx to the cells (46)

6.) Anti-acetylcholine-esterase (AChE)

The rhizome of KP significantly showed acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities. The 5,7,4'-trimethoxyflavone and 5,7-dimethoxyflavone exhibited the highest potential inhibition of AChE and BChE with the percentage inhibitory activity of 43-85%. However, only 5,7-dimethoxyflavone exerts strong selectivity for AChE inhibition which is preferred in the treatment of Alzheimer's disease (47).

7.) Anti-cancer activity

8.1.) Lung carcinoma cell line

The effects of KP extracts on multidrug resistance associated-proteins (MRP) - mediated transport in A549 cells were evaluated. The accumulation of calcein and doxorubicin by KP ethanol extract for MRP function was better than that of the KP aqueous extract. The 5,7-Dimethoxyflavone isolated from KP had a maximal induced effect on the accumulation of doxorubicin in A549 cells. This study examined that KP extracts suppressed MRP function which may be helpful for improving multidrug resistance in cancer cells (6).

8.2.) Promyelocytic leukemia

The study showed that KP extract has cytotoxicity and apoptotic effects on HL-60 cells in a concentration- and time-dependent manner (49). Combination of KP extract with chemotherapeutic drugs, doxorubicin or paclitaxel, showed synergistic effect for reducing mitochondrial transmembrane potential in the human promonocytic leukemic U937 cell line (7).

8.3.) Human colorectal carcinoma

This study focused on anticancer activity of the three KP compounds; 5,7,4′ - trimethoxyflavone (TMF) , 5- hydroxy- 3,7,3′ ,4′ - tetramethoxyflavone (5- H- TeMF) and 5,7,4′ - tetramethoxyflavone

(TeMF). The authors showed that the compounds were toxic to human colorectal carcinoma (HCT-15) cells. Induction of caspase-3 which are the executor for cell death were shown in the cells treated with KP compounds (50).

1.3 Objective of this study/Hypothesis

1.3.1 To investigate whether *Kaempferia parviflora* ethanol extract is cytotoxic to ovarian cancer cell line, SKOV3.

1.) KP ethanol extract has cytotoxicity to ovarian cancer cell line, SKOV3.

1.3.3 To further investigate the effect of *Kaempferia parviflora* ethanol extract on cell proliferation, cell metastasis, and cell death in ovarian cancer cell line, SKOV3.

1.) KP ethanol extract decreases cancer cell proliferation, reduces metastasis, and increases cell death in ovarian cancer cell line, SKOV3.

1.3.3 To determine signal transduction pathways and underlying mechanisms of inhibitory effects of KP to cell growth, survival, and motility in SKOV3.

1.) The inhibitory effect of KP ethanol extract is via suppression of AKT and MAPK signal molecules.

CHAPTER 2

Materials and methods

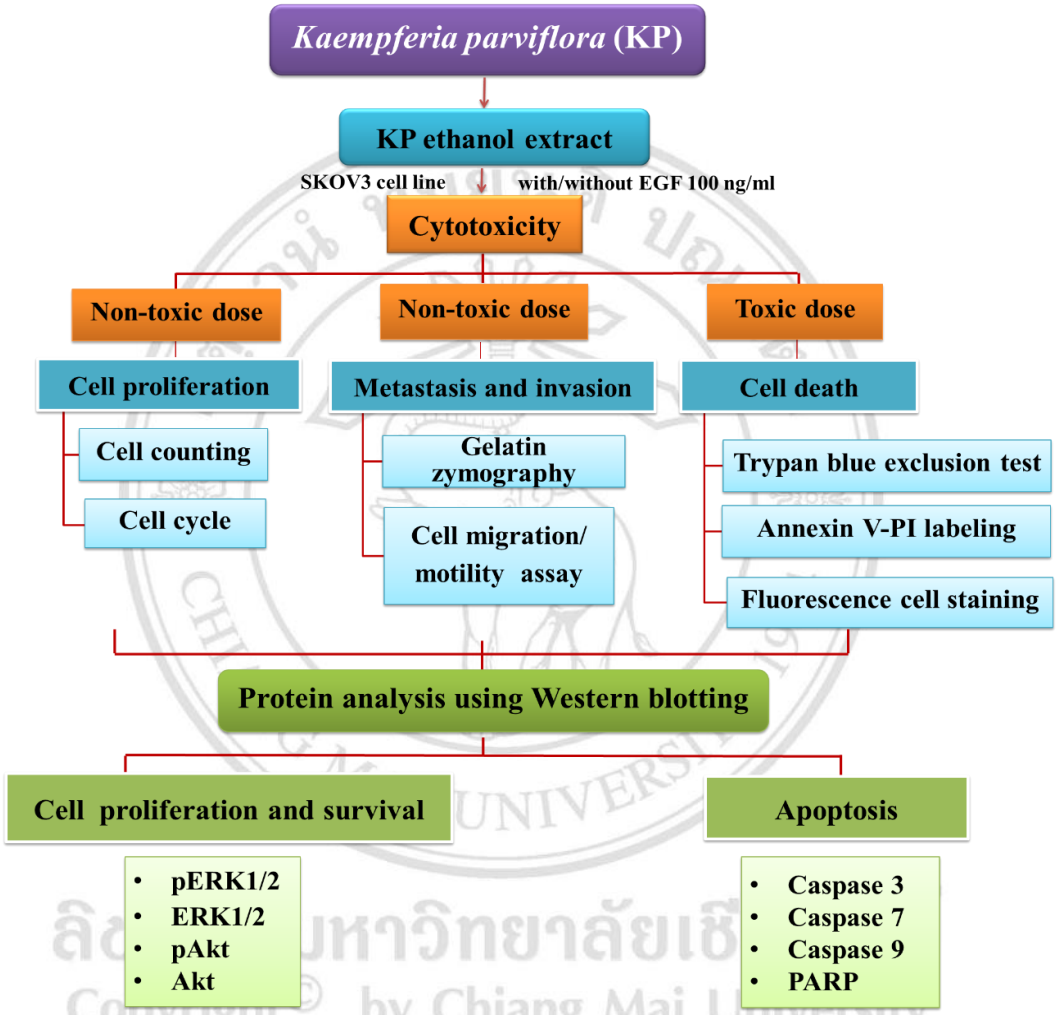


Figure 2.1. Methodology of the study

2.1 Preparation of KP crude extract

The plant used in the current study was from the area of Chiang Dao District of Chiang Mai. The plant has been identified using plant taxonomy methodology performed by Associate Professor Dr. Somporn Putiyananta, Faculty of Pharmacy, Chiang Mai University. The voucher specimen of KP has been identified by Assistant Professor Dr.

Angkana Inta. The dried plant samples were kept at the herbarium at the Faculty of Pharmacy, Chiang Mai University. Moreover, techniques in molecular biology were used to verify the species of the plant by Assistant Professor Dr. Siriporn Rotarayanont and Assistant Professor Dr. Masalin Osathanunkul from Department of Biology, Faculty of Science, Chiang Mai University. The rhizomes of the plant were extracted using ethanol, and the extracted chemicals were quantified by Assistant Professor Dr. Pitchaya Mungkornasawakul, Department of Chemistry, Faculty of Science, Chiang Mai University. Briefly, the rhizomes of KP were cut into small pieces and extracted with 95% ethanol at room temperature for 3 days. Then, the ethanolic extract was filtered. The extract was evaporated to eliminate the solvent under reduced pressure. The KP extraction had yielded 9.85% dry weight of KP rhizomes. The plant ethanolic extraction was prepared by dissolving 1 g of KP ethanolic extract with 1 ml of 100% DMSO (dimethyl sulfoxide) to make a 1 g/ml stock concentration. The KP stock was pre-diluted in the medium prior to each treatment. Each experiment was performed with three independent batches of KP extract. The final concentration of DMSO was maintained below 0.5% v/v throughout the experiment.

2.2 Cell culture

The human ovarian cancer SKOV3 cell line (ATCC, USA) maintained in complete media which composed of RPMI-1640 medium (Gibco, USA), 10% fetal bovine serum (FBS) (Gibco BRL, USA) and 100 U/ml penicillin-100 µg/ml streptomycin (Caisson, USA) and incubated at 37°C in a humidified 5% CO_2 atmosphere. The cells were sub-cultured at 70%-80% confluent by 0.1%trypsin. The complete media were changed every 2-3 days.

2.3 Cell viability assay

The cytotoxicity of KP on SKOV3 cells was performed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide). Cells were seeded at a density of $1x10^4$ cells per well in 96-well plates in complete media overnight at 37°C in a humidified 5% CO_2 atmosphere. Then, cells were divided into two groups; vehicle control group and treatment group. For treatment group, cells were incubated with complete media

containing different concentrations of KP extract ranging from 0-1 mg/ml with or without the presence of 100 ng/ml of EGF in quadruplicate for 24 h at 37°C in a humidified 5% CO_2 atmosphere. Since DMSO was used to solubilize KP extract, we therefore used DMSO as a vehicle control. DMSO was diluted in medium to obtain the concentrations corresponding to those DMSO concentrations (0.006-0.1%) present in the KP treatment groups with or without the presence of EGF. The condition of cell incubation was similar to that in the treatment groups. After 24 h of incubation, 12.5 µl of 0.5 mg/ml MTT reagent (Applichem GmbH, Germany) was added to each well containing 100 µl of complete cells media, and the plate was incubated for 1-3 h at 37 °C until the purple color of formazan crystal is visible. The suspension was aspirated, and 100 µl of DMSO was added to stop the reaction and dissolved the formazan crystal. Finally, the intensity of MTT reaction was measured by Synergy™ H4 Hybrid Multi-Mode Microplate Reader at 570 nm of absorbance. Cell viability assay was performed in 3 individual experiment.

2.4 Determination of the duplication time of SKOV3 cells

Cells were plated in 24-well plates at a density of 5 x 10^4 cells/well in complete media. KP extract at non-toxic concentrations (0, 0.01, 0.025, and 0.05 mg/ml) obtained from the MTT assay was used to treat the cells. Cells were counted for the total number of cells using a hemocytometer at different time points including 0, 24, 48, 72, and 96 h. A specific time for cell duplication was calculated as the following formula;
Doubling time = (Time×log2)/log (final number)-log (initial number).

2.5 Gelatin zymography

MMP-2 and MMP-9 levels were evaluated by gelatin zymography. Cells were seeded at a density of 3 x 10^5 cells/ml and cultured in complete media in 3-cm culture dishes for 24 h at 37°C, 5% CO_2. Then, cells were washed with PBS and treated with different concentrations of KP extract (0, 0.01, 0.05, and 0.1 mg/ml) with or without EGF (100 ng/ml) in complete media for 24 h at 37°C, 5% CO_2. Next, the supernatants were collected and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1 mg/mL of gelatin B (Bio-Rad Laboratories, Hercules, California, USA) under a non-reducing condition in a cold condition. After electrophoresis, the gels

were washed twice in 2.5% TritonX-100 for 60 min at room temperature and then rinsed with 10 mM Tris, pH 8.0 for 2 min. Then, the gels were incubated overnight at 37°C with gelatinase buffer (50 mM Tris, pH 8, 5 mM $CaCl_2$ and 10^6 M $ZnCl_2$). After incubation, the gels were stained with 0.5% (w/v) Coomassie brilliant blue R250 (Bio-Rad Laboratories) in 50% methanol and 10% glacial acetic acid and destained with destaining solution (10% acetic acid and 50% methanol). The bands of gelatinolytic activity were detected as clear zone bands on a blue background, indicating the digestive activity of MMP-2 or MMP-9 and analyzed using ImageJ software.

2.6 Cell cycle analysis

Percent of cells in G0/G1, S, and G2/M phases of the cell cycle using cell cycle analysis was performed. Cells were seeded in 3-cm culture dishes at a density of 5 x 10^5 cells/ml in complete media for 24 h at 37°C, 5% CO_2. Before treating cells with the KP extract, media was changed to serum-free media (SFM, RPMI without FBS) to acquire homogenous cells at the same starting point in the cell cycle. Cells were then treated with KP extract at different concentrations (0, 0.01, 0.025, and 0.05 mg/ml) in complete media and incubated for at least its duplication time at 37°C, 5% CO_2. Then, cells were harvested into centrifuge tubes and washed twice with cold PBS. Cells were centrifuged at 300 g for 5 min and then fixed with 70% ethanol on ice for at least 1-2 h at 4°C. Cells were subsequently centrifuged for 5 min at 300g and washed one time with cold PBS. Finally, the cell pellet was resuspended in 200 µl of cell cycle guava reagent (Merck), kept in the dark at room temperature for 30 min, and measured by flow cytometry.

2.7 Cell migration

A 24-multiwell insert plate (8 µm) (SPL Life Sciences, South Korea) was used to confirm the effect of KP on suppressing cell migration. Cells were seeded on the upper chambers at a density of 3x10^5 cells/ml with complete media in a 24-well plate for 24 h. Then, cells in the upper chamber were treated with non-cytotoxic concentrations of KP (0, 0.01, 0.05 and 0.1 mg/ml) in serum-free media, and the upper chambers were put into the (lower) wells containing RPMI with 5% FBS and incubated for 24 h (Fig. 2.2). The invading or migrating cells were fixed with absolute methanol for 5 min and then stained with 0.5%

crystal violet for 15 min. The upper chambers were washed for 3 times with water, and cells attached to the surface of the chamber were removed with a cotton swab and the stained cells attached at the other site of the chamber were captured under microscope. The amount of migrated cells were determined by ImageJ free software.

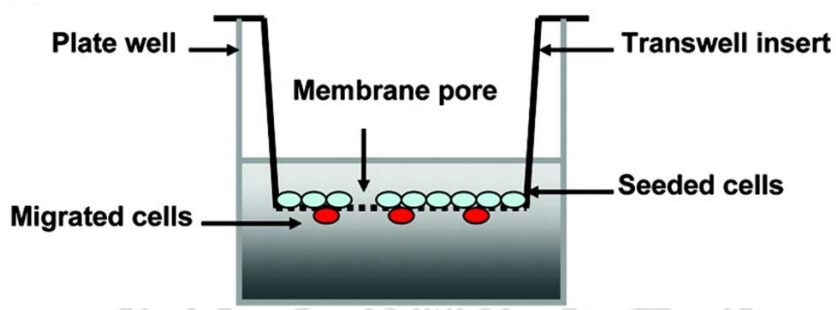


Figure 2.2. Cell migration was performed by Trans well plate (52)

2.8 Wound healing assay

Cell migration was confirmed by wound healing assay. Cells were seeded at a density of 3 x 10^5 cells/ml and cultured in 24-well plates for 24 h in complete media in 3-cm culture dishes for 24 h at 37°C, 5% CO_2. A scratch wound was made by using 200 µL pipette tip. Then, cells were treated with different concentrations of KP extract (0.01, 0.05, and 0.1 mg/mL) for 24 h. Images of the scratched wounds were captured at different time points (0, 12, and 24 h). The closing of scratched wounds was considered to be the completion of the migration process. The migrated areas were analyzed and determined using the ImageJ software.

2.9 Trypan blue exclusion test

Cells were plated in 24-well plates at a density of 5 x 10^4 cells/well in complete media and incubated for 24 h at 37°C, 5% CO_2. Cells were incubated with different cytotoxic concentrations (0, 0.05, 0.1, and 0.25 mg/mL) of KP extract and harvested after 3, 6, 12, 24, and 48 h of incubation. Cells were trypsinized and washed. Trypan blue solution (Gibco, USA) was added to the cell suspensions in a ratio of 1:1. Then the average of total cell number was counted from 4 areas of a hemocytometer. Dead cells stained in blue were counted. The percentage of living cells and dead cells was calculated.

2.10 Cell apoptosis assay

SKOV3 cells were seeded at a density of 3 x 10^5 cells/ml in 3-cm culture dishes and cultured in complete media for 24 h at 37°C, 5% CO_2. Then, cells were washed with PBS and incubated with different concentrations of KP extract (0, 0.1, 0.3, 0.5 mg/ml) for 12 h at 37°C, 5% CO_2. Then, cells were harvested and centrifuged at 300g for 2 min. Cells were washed with PBS and resuspended in 100 µl 1X annexin V-FITC binding buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5 mM DTT, 50% glycerol). Then, 7 µl of annexin V-FITC and 1 µl (ImmunoTools, Germany) of propidium iodide (PI) (Sigma Aldrich) was added and incubated for 15 min in the dark at room temperature. Finally, 400 µl of 1X annexin V-FITC binding buffer was added before the evaluation by flow cytometer.

2.11 Nuclear staining

Cells were seeded at a density of 5 x 10^5 cells/well on glass cover slips in 3-mm cell culture dishes and cultured in complete media for 24 h at 37°C, 5% CO_2. Then, cells were treated with KP extract at different cytotoxic concentrations (0.1, 0.3, and 0.5 mg/ml) in complete media. Cells were then fixed with 4% paraformaldehyde/PBS at room temperature for 15 min and then rinsed with PBS for 3 times. Then cells were incubated with 5 µg/ml of Hoechst 33342 (Thermo Fisher Scientific. Thailand) in PBS for 1 h. The sample slides were washed twice with PBS for 5 min and then with distilled water for 5 min. Finally, 8 µl of the fluoromount media (SouthernBiotech, United States) was added before observed by a fluorescent microscope, AX70 Olympus R, Japan, with 40x magnification, and micrographs were captured with the DP-BSW Basic Software for the DP71 microscope digital camera.

2.12 Western blot analysis

SKOV3 cells were seeded at a density of 3 x 10^5 cells/ml in 3-cm cell culture dishes and cultured in complete media for 24 h at 37°C, 5% CO_2. Cells were serum-starved with serum free media (SFM) overnight before treating cells. Then, cells were treated with KP extract at non-toxic concentrations (0.01 and 0.05 mg/mL for evaluating the relevant

signaling) for 6 h and 100 ng/mL EGF was added to the wells 15 min before harvesting cells. For evaluating apoptotic signaling molecules, cells were treated with KP extract at toxic concentrations (0.1, 0.3, and 0.5 mg/mL for caspase activation). Cells were lysed in 300 µL of 1X lysis buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% ß-mercaptoethanol, 12.5 mM EDTA, and 0.02 % bromophenol blue). The proteins were separated in 10% SDS-PAGE for 90 min at 140 V and transferred to PVDF (polyvinylidene difluoride) membranes for 120 min at 100 V (Immobilon-P; Millipore, Bedford, MA) in a cold condition. The membranes were blocked with 5% skim milk in PBS for 1 h at room temperature. Then, the membranes were incubated with primary antibodies (1:10000 of anti-ß-actin, 1:5000 of anti-pERK1/2, 1:5000 of anti-pAkt, 1:5000 of anti-ERK1/2, 1:5000 of anti-Akt, 1:3000 of caspase-3, or caspase-7, or caspase-9, or PARP) at 4°C overnight. Anti-ß-actin was obtained from US biological (USA) and the remaining antibodies were purchased from Cell Signaling Technology (USA). After washing 3 times with Tris buffer containing 0.05% tween-20 (TBST), the blots were followed by the incubation with an 1: 5000 of anti-mouse Ig conjugated with IRDye®800CW or an 1:5000 of anti-rabbit Ig conjugated with IRDye®680RT for 2-3 h, at room temperature. Finally, the protein bands were captured by Odyssey® CLx Imaging System (LI-COR Biosciences, NE, USA) and the immunoreactive band intensity was quantified by Image Studio Lite software.

2.13 Data Analysis and Statistical Methods

All data from the experiment were expressed as the mean ± standard deviation (SD). One-way ANOVA was used to determine the statistical significance of the difference between the values of various experimental and control groups. Then, post hoc least-significant difference (LSD) test with *p* values less than 0.05 was considered significant.

CHAPTER 3

Results

3.1 The cytotoxic effect of KP ethanol extract on human ovarian cancer cell line (**SKOV3**)

The cytotoxicity effects of KP ethanol extract on SKOV3 cell line were evaluated using the MTT assay, as shown in figure 3.1. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay is a colorimetric based-method for measuring cellular metabolic activity. In viable cells, the mitochondrial reductase can convert a yellow tetrazole of MTT into a purple formazan product which can be further quantified by spectrophotometer with an absorbance of 540-570 nm (53). When cells die, they lose an ability to convert MTT into purple formazan. Therefore, the intensity of purple color reflexes the amount of living cells.

In the present study, various concentrations of KP ethanol extract (0.006-1 mg/ml) were applied to determine its cytotoxic effect on SKOV3 cell for 24 h. We found that KP ethanol extract significantly decreased cell viability in a concentration-dependent manner when the concentration was in the range of 0.09 mg/ml - 1 mg/ml (Fig.3.1A). The half maximal inhibitory concentration (IC50) value was at 0.53 ± 0.08 mg/ml. Since the epidermal growth factor receptor (EGFR) is often exhibited in human carcinomas and associated with the progression and pathogenesis of ovarian cancer (54), we therefore incubated cells with KP extract with the presence of 100 ng/ml EGF and determined cytotoxic effect of KP extract. We demonstrated that with the presence of EGF, KP ethanol extract still significantly inhibited SKOV3 cell viability in a concentration-dependent manner which was in the range of 0.2 mg/ml to 1 mg/ml (Fig.3.1B). The IC50 of KP extract with the presence of EGF was 0.63 ± 0.08 mg/mL which is similar to IC50 of KP ethanol extract alone. These results indicates that KP ethanol extract has an ability to decease ovarian cancer cell proliferation in both presence and absence of EGF induction. Since DMSO was used as a solvent to dissolve KP ethanol extract, we also

evaluated the cytotoxic effect of DMSO in SKOV3 and showed that DMSO at all concentrations relevant to the doses of KP did not affect cell viability.

From MTT assay, we chose non-toxic concentrations of KP ethanol extract (≤ 0.1 mg/ml) for cell counting, gelatin zymography, cell migration assay, and protein analysis of signal transduction molecules involved in cell proliferation and survival pathway, whereas toxic concentrations (> 1 mg/ml) were chosen for cell death evaluation including nuclear staining, apoptosis, and caspase induction.

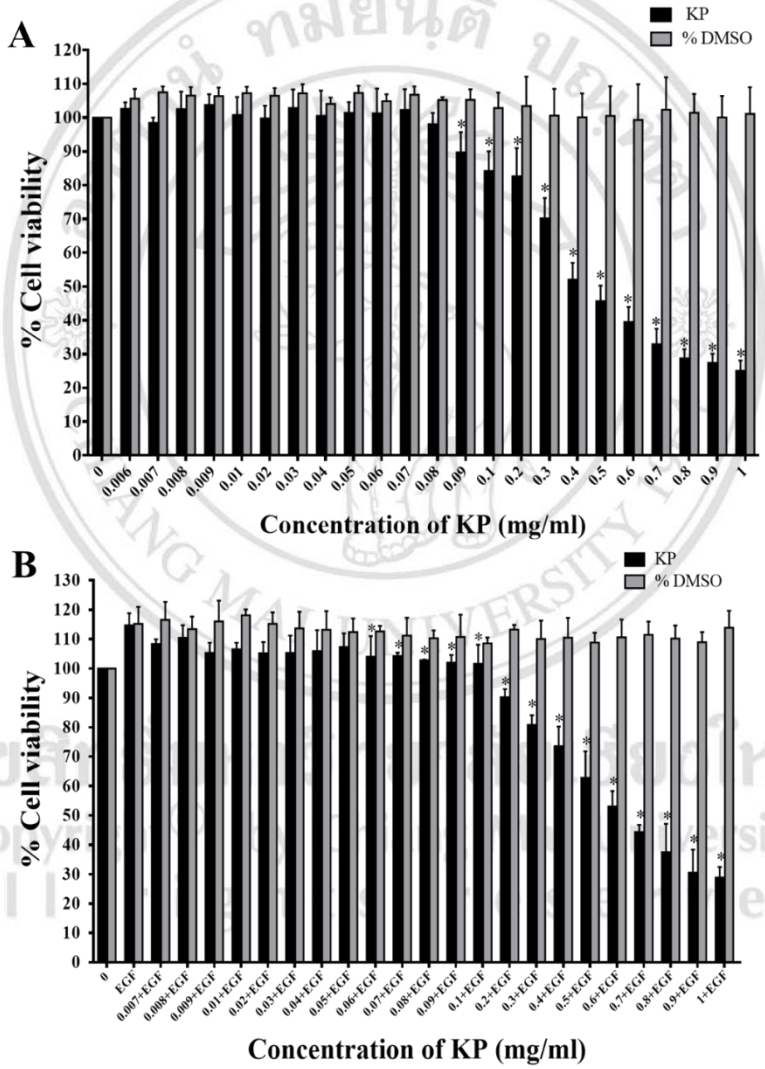


Figure 3.1 The cytotoxicity effect of different concentrations of KP ethanol extract on SKOV3 cells without EGF (A) and with 100 ng/ml EGF (B). All data were from 3 independent experiments and reported as means ± SD of each quadruplicate *$p<0.05$ compared to the control (untreated and EGF).

3.2 The proliferative effect of KP ethanol extract on SKOV3 cells

The doubling time is the period of time necessary for cells to increase in double amount which reflexes cell proliferation. It can be used to predict growth rate and the progression of ovarian cancer.

To study the growth rate of SKOV3 cells, we therefore performed cell counting assay at 24, 48, 72 and 96 h. In the absence of EGF, KP ethanol extract reduced cell growth in a concentration-dependent manner at all time points (Fig. 3.2). The number of the cells at 48 and 72 h was used to calculate the doubling time. In the control condition, SKOV3 spent 24 h to double amount of cell number. The doubling time of KP at 0.01, 0.025 and 0.05 mg/ml was 27.5 h, 30.9 h, and 31.7 h, respectively. This indicates that KP extract slows down the growth rate of SKOV3 in a concentration-dependent manner. Cells treated with KP at 0.01, 0.025 and 0.05 mg/ml with the presence of EGF increased doubling time to 26.5 h, 26.0 h, and 26.3 h, respectively.

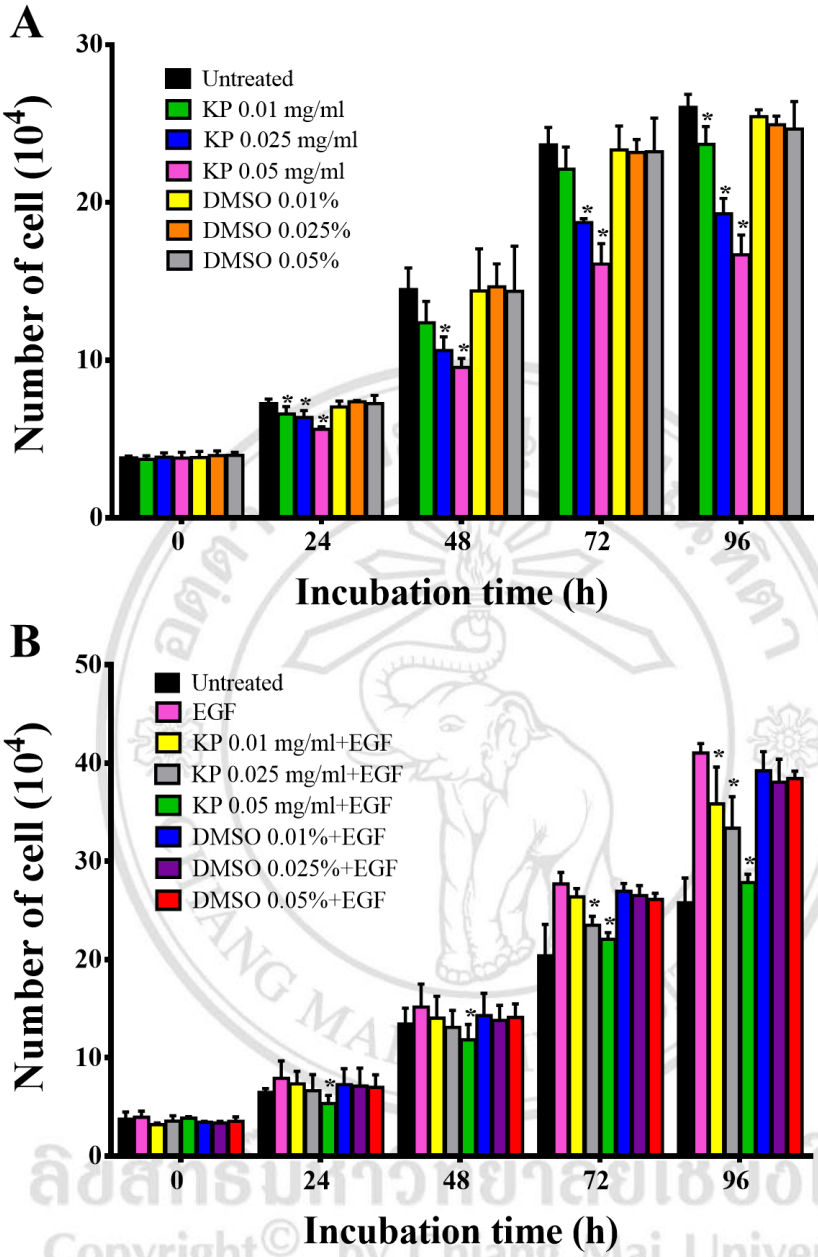
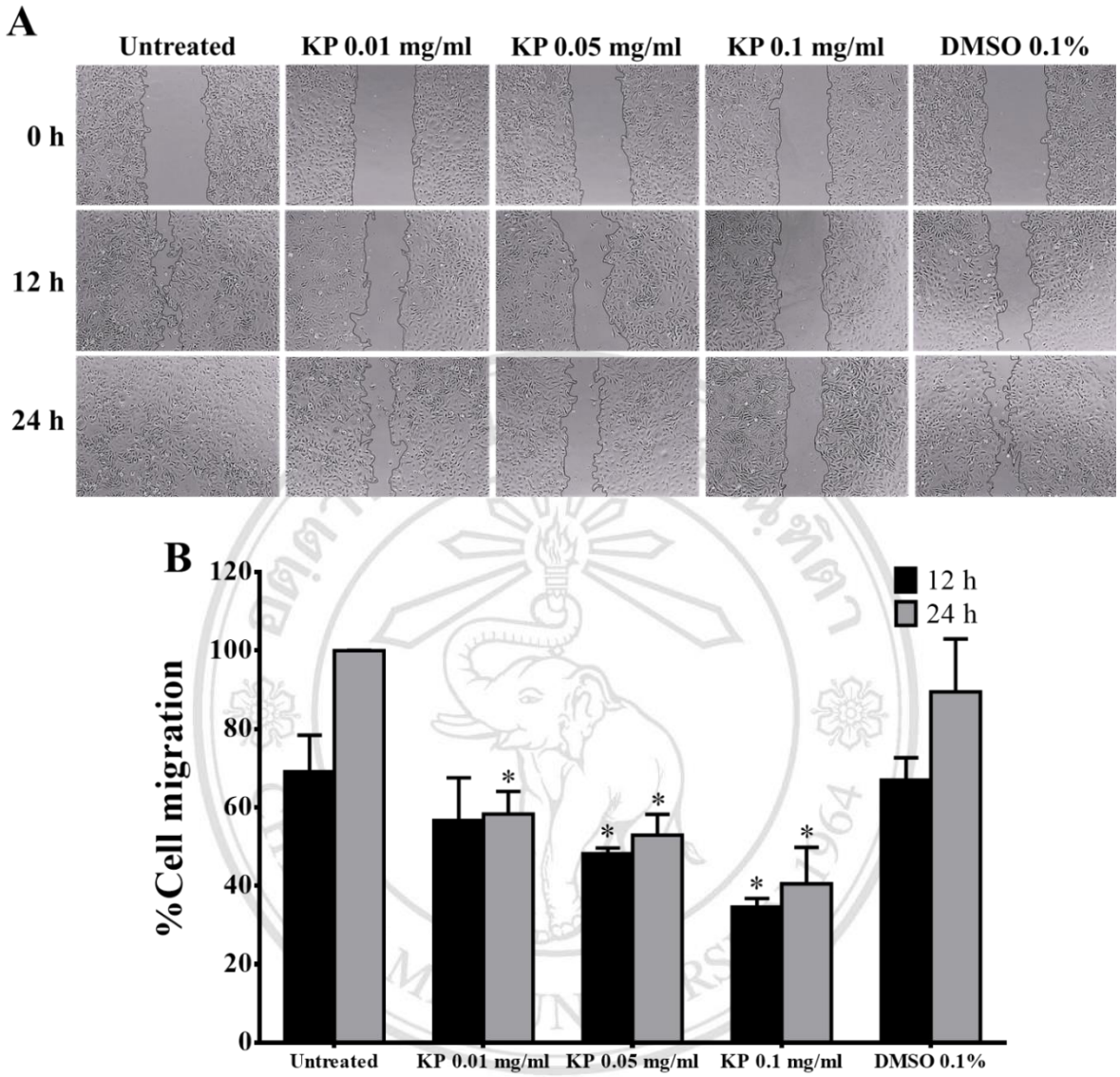


Figure 3.2 The number of SKOV3 cells treated with non-toxic concentrations of KP (0.01, 0.025, 0.05 mg/ml) without EGF (A) and with 100 ng/ml EGF (B) at 24, 48, 72 and 96 h. Data are expressed as mean ± SD (n = 3). *$p<0.05$ as compared to untreated (A) or EGF (B).

3.3 The effect of KP ethanol extract on cell migration inhibition

The effect of KP ethanol extract on cell metastasis was determined by the wound healing assay (Fig. 3.3 A), and Transwell migration assay (Fig.3.3C). In the wound healing assay, SKOV3 cells were scratched with the pipette tip and the areas between scratch margins were measured at 0, 12 and 24 h. As shown in Figure 3.3A, KP ethanol extract shows a significant reduction in cell migration at all three different time points. In control group, SKOV3 cells migrated approximately 60 % of the wound area after 12 h and the wound completely closed after 24 h of incubation. Cells treated with KP ethanol extract at concentration of 0.01, 0.05 and 0.1 mg/ml significantly reduced the percentage of cell migration to 56.6 ± 10.9 %, 48.2 ± 1.45 %, and 34.56 ± 2.18 %, respectively at 12 h and 58.30 ± 5.8 %, 52.91 ± 5.32 %, and 40.50 ± 9.27 %, respectively at 24 h. We next confirmed the results of wound healing assay with Transwell migration assay. The number of cells that migrated through the membrane filter were visualized and counted as migratory cells (Fig. 3.3C). We found that the amount of migratory cells was significantly reduced in cells treated with KP 0.01, and 0.05 mg/ml to 33.92 ± 4.6 % and 21.9 ± 2.7 %, respectively (Fig.3.3D). The vehicle control, DMSO at the comparable concentrations dissolved in KP ethanol extract did not suppress cell migration. The results showed that KP ethanol extract could suppress the migration of SKOV3 cell line.



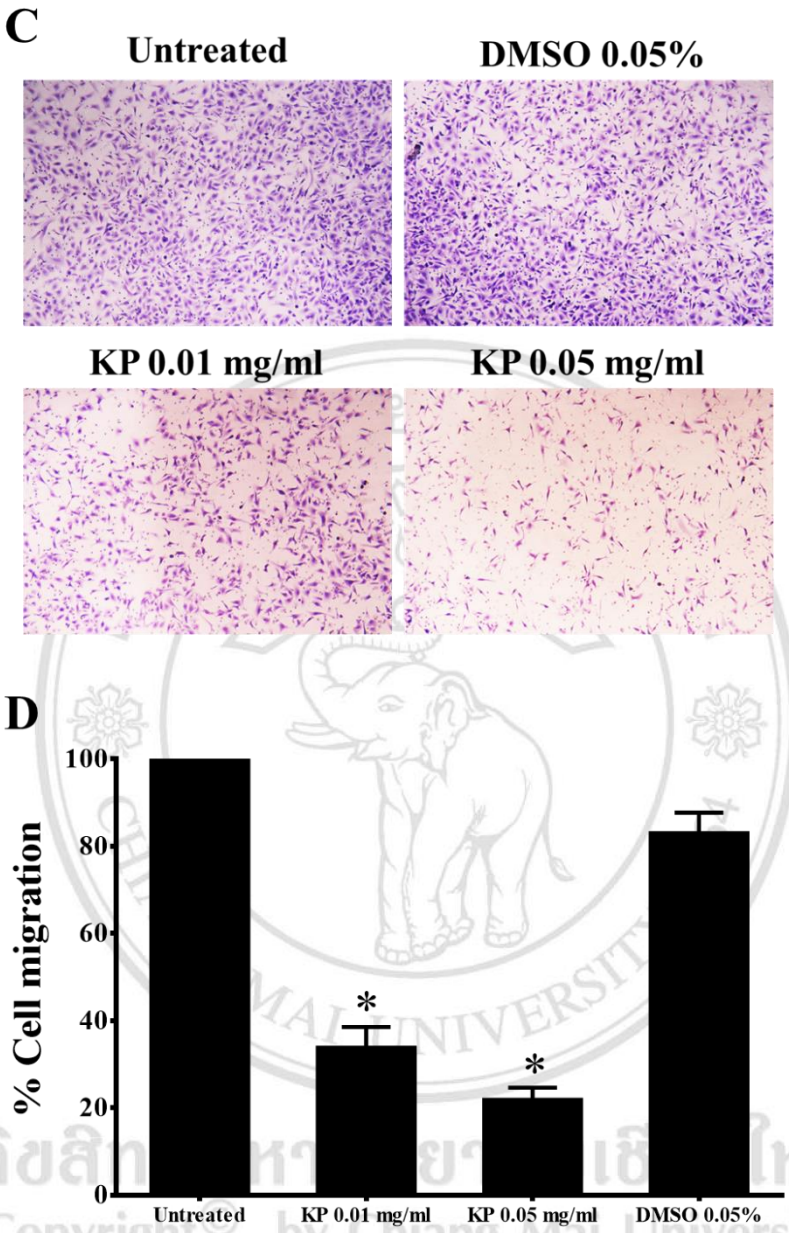


Figure 3.3 The effect of KP on SKOV3 cell migration. Wound-healing assay of SKOV3 cells treated with KP ethanol extract at 0, 12, and 24 h after performing the scratch (A). Histogram represents the percentage of cell migration (B). Transwell migration assay and represented histogram are shown in C and D. All data were from 3 independent experiments and reported as means ± SD for measurements in quadruplicate *$p<0.05$ as compared to the control.

3.4 The effect of KP ethanol extract on inhibition of MMP-9 and MMP-2 activities

Enzyme matrix metalloproteinases (MMPs) are calcium-dependent and zinc-dependent endopeptidases which are secreted from many cell types. These enzymes are involved in the degradation of extracellular matrix (18). MMPs such as MMP-2 and MMP-9 are founded in many cancers including ovarian cancer. These proteins have been reported to be associated with ovarian cancer cell migration, metastasis and progression. Based on gelatinase activity of MMP-2 and MMP-9, gelatin B was used as their substrates for gelatin zymography. The clear bands on the electrophoresis gels are areas in which gelatin B is degraded. MMP-2 and MMP-9 are secreted as protein of 72-kDa, and 92-kDa, respectively (55, 56).

The represented zymogram in figure 3.4A shows that the KP ethanol extract at non-toxic concentrations (0.01 and 0.05 mg/mL) could decrease MMP-2 and MMP-9 activity in a concentration-dependent manner. MMP-2 activity was decreased to 88.66 ± 6.17 and 68.83 ± 6.17 % after cells were treated with KP extract at 0.01 and 0.05 mg/mL, respectively (Fig.3.4B). Similarly, MMP-9 activity also reduced, but in a lesser extent, to 92.52 ± 8.55 and 81.92 ± 5.18 % after cells were treated with KP extract at 0.01 and 0.05 mg/mL, respectively (Fig.3.4C). In addition, cells treated with EGF at 100 ng/ml drastically increased MMP-2 and MMP-9 activities to 140.43 ± 5 and 128.95 ± 11.54 % , respectively. Interestingly, in the presence of EGF, KP treated cells at 0.01 and 0.05 mg/ml showed a reduction of MMP-2 activity to 121.4 ± 4.7 % and 104.01 ± 10.12 %, respectively, while, MMP-9 activity was reduced to 113.97 ± 10.7 % and 106.64 ± 9.9 %, respectively. The immunoreactive band of β-actin detected by western blot indicated an equal amount of cell in all group.

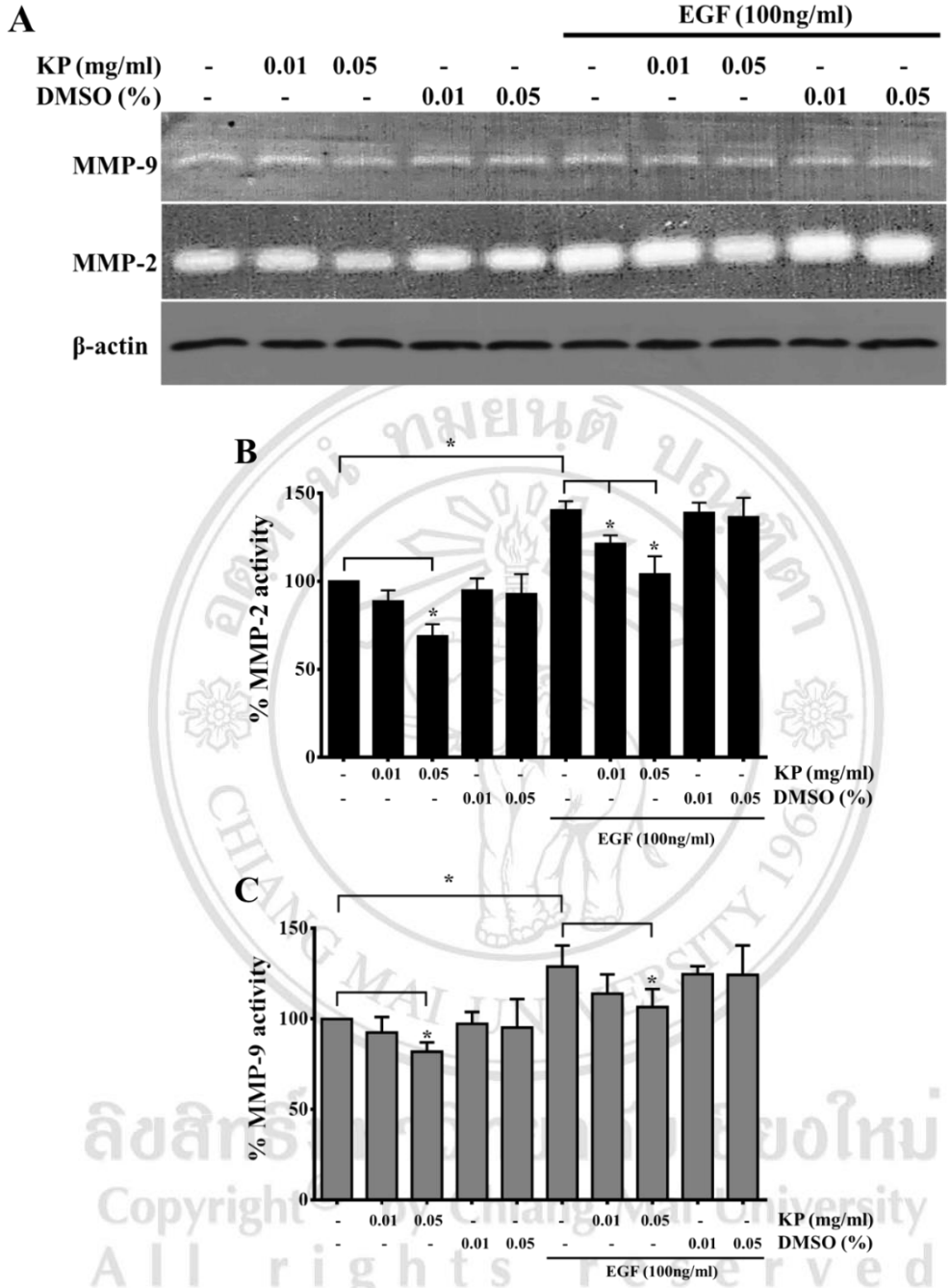


Figure 3.4 The effect of KP ethanol extract on MMP-9 and MMP-2 activity. Gelatin zymogram showing MMP9 and MMP2 activities (A), Immunoreactive bands of β-actin was used as a loading control. Histogram of MMP-2 and MMP-9 activity is presented as percent of activity (B-C). All data were from 3 independent experiments. *$p < 0.05$

3.5 The effect of KP ethanol extract on cell cycle

To examine the inhibitory effect of KP on cell proliferation, we next performed cell cycle analysis using propidium iodide which binds to the DNA content and then evaluated a group of cell by using flow cytometer. In general, the cell cycle is composed of 3 major phases; G0/G1, S, and G2/M. Sub G1 is considered as death or apoptotic cells (orange color in Fig.3.5A). As shown in figure 3.5, SKOV3 cells are in G0/G1 phase more than 60%. Cells treated with KP extract did not show any difference compared to untreated cells or DMSO-treated cells. These results indicate that KP does not inhibit cell proliferation via cell cycle arrest mechanism in SKOV3 cells.

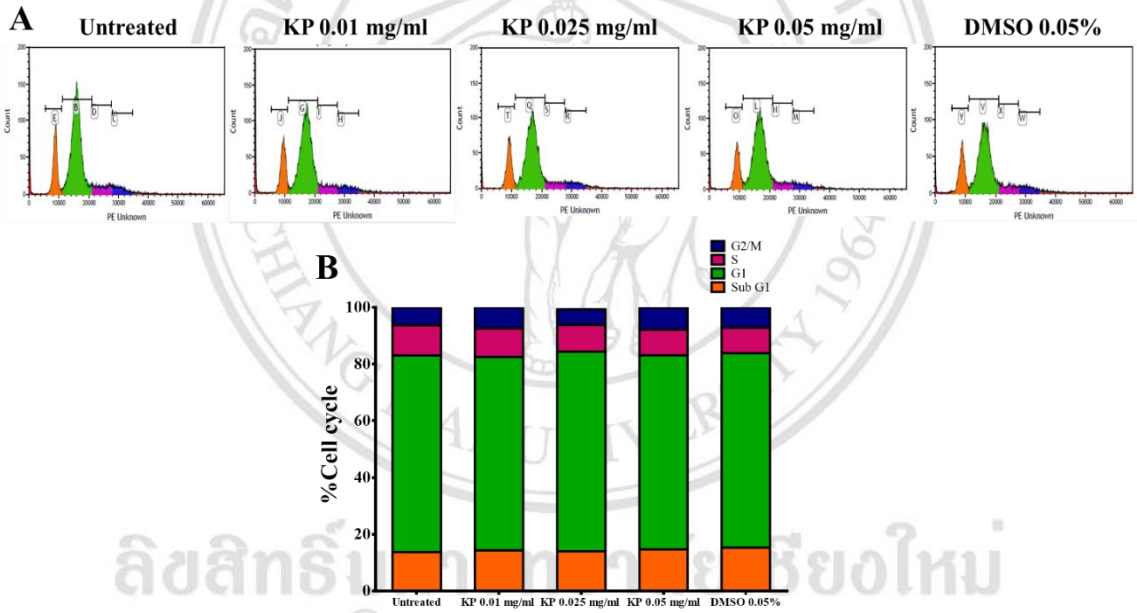


Figure 3.5 The effect of KP ethanol extract on cell cycle analysis of SKOV3 cells. Flow cytometry data (A) and histogram of percent of cell in sub G1, G1, S, and G2/M phases (B). Data represented as means ± SD, n = 3. *$p < 0.05$.

3. 6 The effect of KP ethanol extract on inhibiting growth and survival signal transduction pathways

Several transduction signaling molecules are involved in growth and survival pathways including the PI3K/AKT and ERK1/2 MAPK. We therefore determined whether inhibitory effect of KP acts via the suppression of theses signal molecules by evaluating the level of phosphorylation of AKT and ERK1/2 molecules. We showed that 100 ng/ml of EGF strongly stimulated ERK1/2 and AKT phosphorylation to 2.6 fold and 1.144 folds, respectively (figure 3.6). Interestingly, cells treated with KP ethanol extract decreased phosphorylation of Akt and ERK1/2 in both absence and presence of EGF. In the absence of EGF, the phosphorylation of Akt was reduced to 0.87 ± 0.04 and 0.58 ± 0.03 fold in cells treated with KP ethanol extract at 0.01 and 0.05 mg/ml, respectively. Moreover, the phosphorylation of ERK1/2 was decreased to 0.85 ± 0.02 and 0.64 ± 0.01 fold in cells treated with KP ethanol extract at 0.01 and 0.05 mg/ml, respectively. In addition, in the presence of EGF, SKOV3 cells treated with KP ethanol extract at 0.01 and 0.05 mg/ml also showed the suppression of Akt phosphorylation to 0.89 ± 0.07 and 0.70 ± 0.07, respectively and the reduction of ERK1/2 phosphorylation to 2.38 ± 0.22 and 2.21 ± 0.23, respectively. These results indicate that reduction of PI3K/AKT and EKR1/2 MAPK phosphorylation by KP extract is at least involved in growth and survival suppression of in SKOV3 ovarian cancer cells.

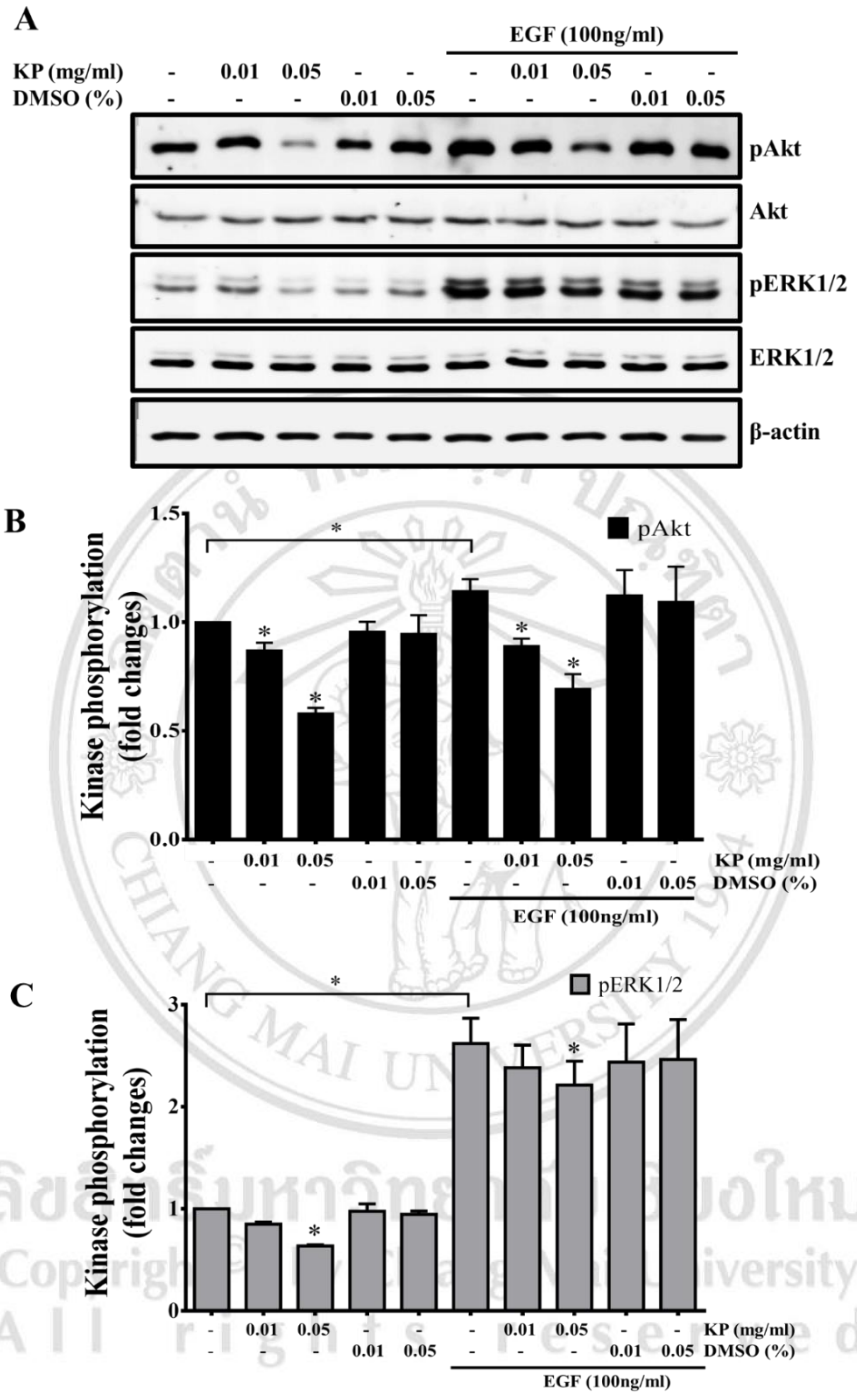


Figure 3.6 The effect of KP on the PI3K/AKT and EKR1/2 MAPK signal transduction in SKOV3 cells. The immunoreactive bands of pAKT, AKT, pERK1/2 and ERK1/2 (A). Histogram of phosphorylation level of AKT (B) and ERK1/2 (C). β-actin was used as a loading control. Data expressed as mean ± SD (n = 3). *$p < 0.05$.

3.7 The effect of KP on induction of cell death

3.7.1 Trypan blue exclusion test

According to the MTT assay, the toxic concentrations (0.1 and 0.25 mg/mL) of KP ethanol extract was applied to induce cell death. Trypan blue exclusion test was used to evaluate dead cells. Since live cells are excluded from trypan blue solution because of intact plasma membrane, dead cells in which membrane is permeable to the dye can be counted by hemocytometer under a light microscope. We demonstrated that cell treated with KP 0.1 and 0.25 mg/ml significantly increased cell death at 24 h of treatment. The percentage of dead cells was 15.67 ± 2% and 26.33 ± 3.5% in cells treated with KP at 0.1 and 0.25 mg/ml, respectively (Fig. 3.7.1). KP extract at 0.05 mg/ml was used to confirm its non-cytotoxicity. Importantly, in the presence of EGF 100 ng/mL, KP at 0.1 and 0.25 mg/mL extract was still able to increase cell death to 13.17 ± 1.8 % and 21.25 ± 2 %, respectively.

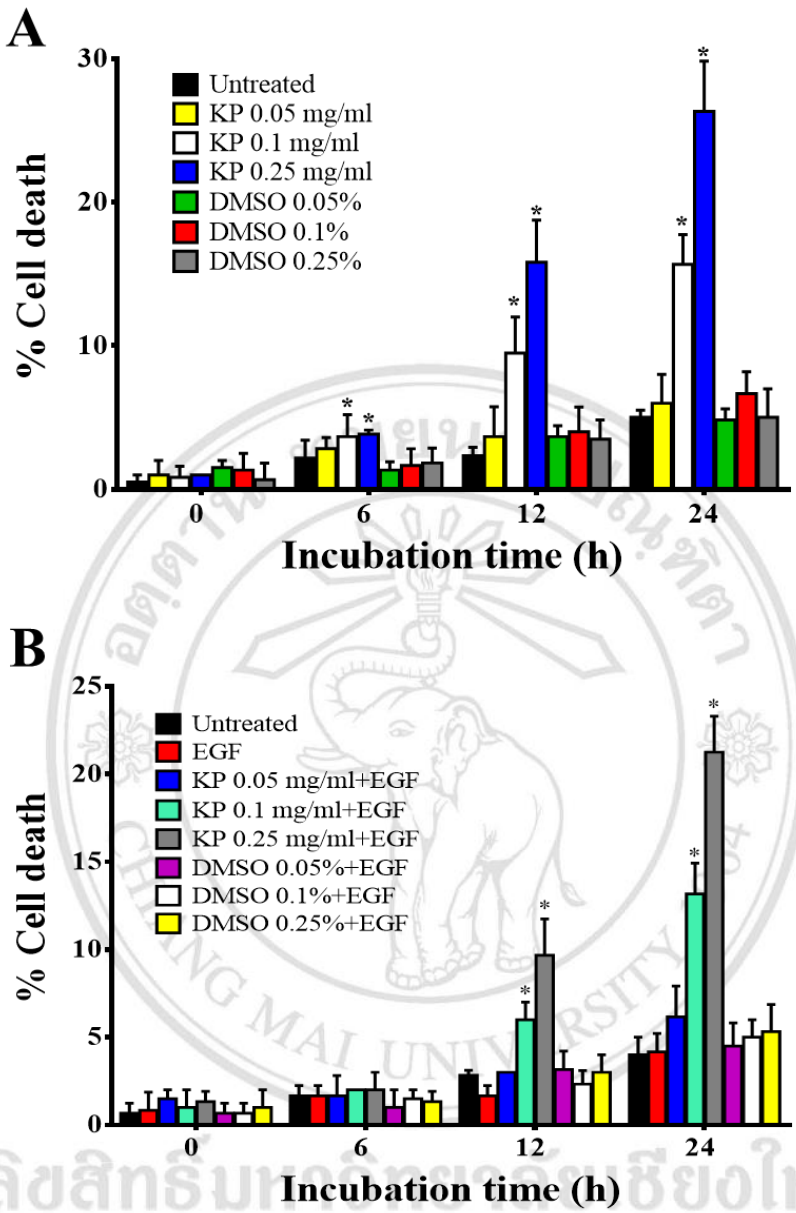


Figure 3.7.1 The effect of KP on cell death in SKOV3 cells by trypan blue exclusion assay. Percent of cell death of cells treated with KP without EGF (A) and with 100 ng/ml EGF (B). Data presented as means ± SD, n=3, *$p<0.05$ compared to untreated or DMSO control.

3.7.2 Flow cytometry of apoptotic cells

Apoptosis is a process of programmed cell death without inflammatory process, unlike necrosis. Substances that can induce cancer cell death without inflammation should be ideal chemotherapeutic agents. Therefore, we would like to investigate whether KP extract induces cell death via apoptosis manner. In fact, a hallmark of apoptotic cell death is the translocation of phosphatidylserine (PS) from the inside to outside leaflets of the plasma membrane or so called PS externalization (57). Since annexin V binds to PS, we can detect apoptotic cells by using green fluorescent or FITC-conjugated annexin V and measuring by flow cytometry. Propidium iodide that binds to the DNA content is also detected with flow cytometry. Living cells (left lower quadrant) have the intact plasma membrane therefore no dye was detected (FITC negative/PI negative) (Fig. 3.7.2A). Early apoptotic cell whose PS is externalized but the plasma membrane is still intact, can be detected as FITC positive/PI negative cells which are presented in the right lower quadrant. Late apoptotic cells whose membrane integrity is reduces are in the right upper quadrant which is positive for PI and annexin V. We found that cells treated with KP ethanol extract at 0.3 and 0.5 mg/mL increase apoptosis to 22.13 ± 7.6 % and 41.13 ± 19.15 %, respectively, compared to the vehicle control (DMSO) or untreated cells which showed less than 5% of apoptotic cells.

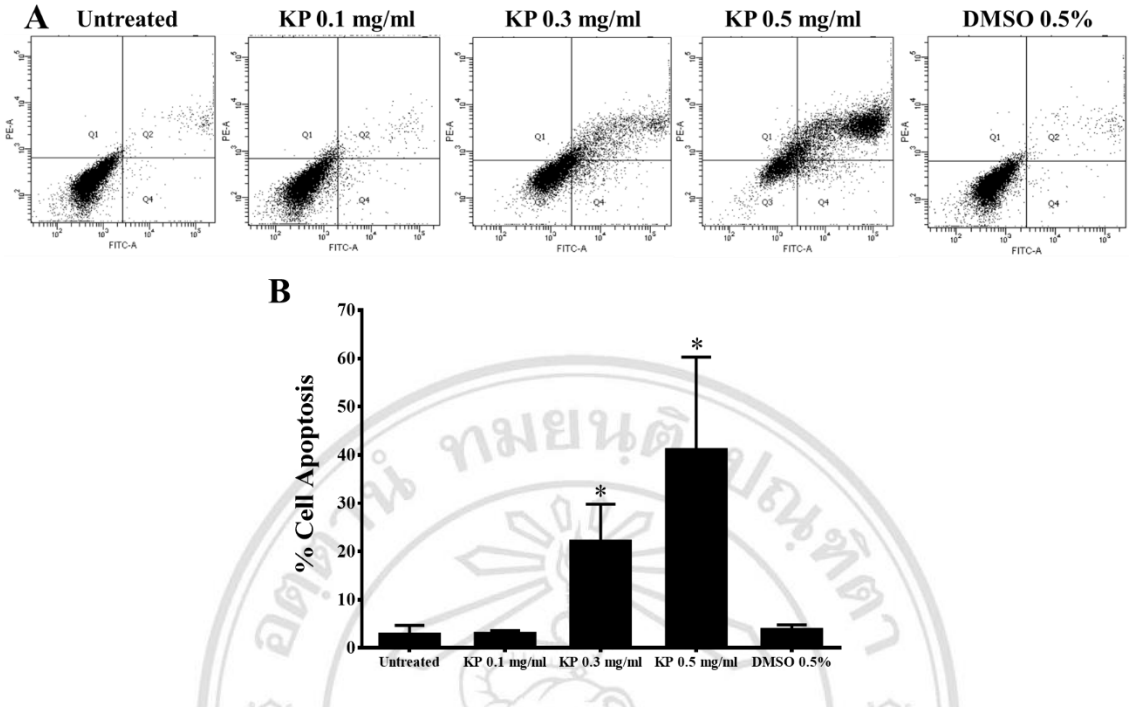


Figure 3.7.2 The effect of KP ethanol extract on apoptosis in SKOV3 cells. Annexin V-FITC and PI labeling in KP treated cells was measured by flow cytometer (A). Histogram of percent of apoptotic cells is shown in B. Data were presented as mean ± SD of three replicates. *$p < 0.05$.

3.7.3 Fluorescence nuclear staining

We next confirmed apoptosis with nuclear staining. Since the nucleus of apoptotic cells exhibits the specific morphology such as chromatin condensation, loss of plasma membrane asymmetry, cell shrinkage, plasma membrane blebbing and DNA fragmentation, we therefore used Hoechst 33342 for fluorescence nuclear staining. Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a cell-permeable DNA staining dye that is activated by ultraviolet light and illuminates blue fluorescence. The stain binds into the minor groove of DNA, and this dye is an adenine-thymine–specific fluorescent dye for evaluating apoptosis by microscopy (58). After incubating cells with KP at 0.3-0.5 mg/ml for 6 h and performing

nuclear staining, we found that KP induced DNA condensation, shrinking of nucleus and nuclear membrane blebbing (red arrows in Fig. 3.7.3). The nuclei of cells treated with DMSO control showed shaped and round nuclear membrane without condensation of nucleus. This indicates that KP indeed is able to induce apoptosis in SKOV3 cells.

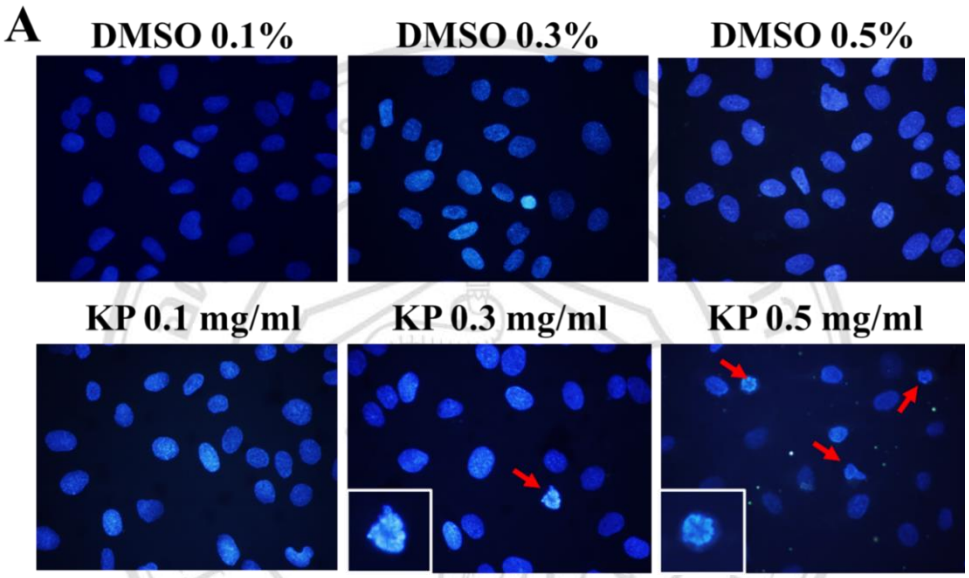


Figure 3.7.3 Effect of KP on nuclear deformity assayed by DNA staining (Hoechst 33342). The condensation of the nucleus was observed in KP ethanol extract treatments compared to the vehicle control DMSO. Original magnification,400x.

3.7.4 Apoptotic signaling pathway using western blot analysis

Since the Annexin-V/PI flow cytometry data and nuclear staining point the direction to cell apoptosis induction by KP. We also would like to further explore the intracellular signaling of apoptosis by detecting caspase-3, -7, -9, and poly ADP ribose polymerase (PARP) molecules. Caspases are a family of protease enzymes involved in programed-cell death signal transduction. Full-length caspases (inactive forms) are cleaved to active forms which finally causes DNAse activation and DNA-degradation. PARP is a family of proteins that plays a role in many cellular processes including DNA repair and apoptosis. PARP is inactivated by

caspase-3 or caspase-7 cleavage. Inactivated PARP eventually leads to DNA degradation (58, 59). By using western blot analysis, we demonstrated that cells treated with KP ethanol extract significantly reduced immunoreactive bands of full length caspase-3, -7, -9 and PARP in a concentration-dependent manner. Reducing of full length caspases indirectly indicates an increase of active-caspase forms which lead to apoptosis.

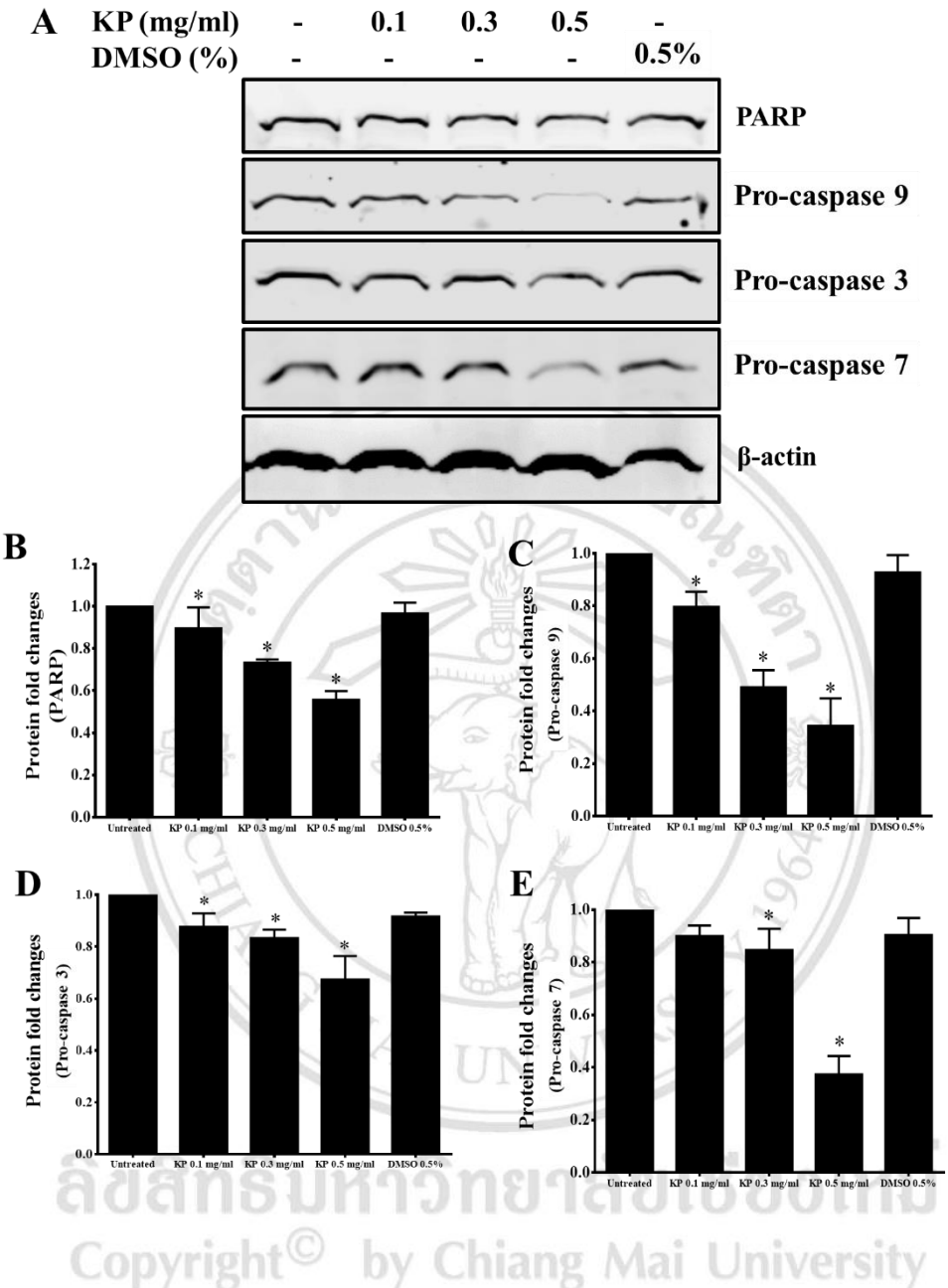


Figure 3.7.4 Effect of KP on caspase and PARP activation in SKOV3 ovarian cancer cells using western blot analysis (A). Histogram of relative intensity of full-length of PARP, caspase-9, -3, and -7 is shown in B, C, D and E, respectively. β-actin is used as a protein loading control. These data are represented as mean ± SD of three replicates. *$p<0.05$.

CHAPTER 4

Discussions

In Thailand, ovarian cancer is the fifth leading cause of cancer-related death (1). This is because most cancers are asymptomatic until metastasis begins in the advanced stage of the disease. High grade epithelial ovarian cancer is found to be particularly aggressive (3, 4, 12). In addition, it is largely resistant to chemotherapy. There is a growing interest in the use of traditional Thai medicine as an alternative to traditional treatment of ovarian cancer. KP, which is found in the north of Thailand and which has been used for a long time, has potential as an anti-cancer treatment as well as being an anti-fungal and anti-plasmodial. This medication is inexpensive and has been demonstrated to be safe , and to reduce resistance to other drugs as well as reducing the side effects of chemotherapy (45).

In this study, we used SKOV3 cells, ovarian cancer cells with epithelial-like morphology, which are a type 2 or high grade serious ovarian cancer (12). We first investigated the cytotoxic effect of KP ethanol extract on SKOV3 ovarian cancer cells. Our results showed that KP slowed the cancer cell doubling time, inhibited cancer cell migration, reduced production of MMP enzymes, and decreased phosphorylation of Akt and ERK1/2 proteins in the survival and growth signaling pathways. It also induced cell apoptosis via the caspase and PARP pathway.

Previous studies have shown that KP has a cytotoxic effect on oral human epidermoid carcinoma (KB cell line), breast cancer (BC cell line), and lung cancer (NCI-H187 cell line). KP is composed of at least nine flavonoids; 5-Hydroxy-3,7-dimethoxyflavone, 5-hydroxy-7- methoxyflavone, 5- hydroxy- 3,7,4'- trimethoxyflavone, 5- hydroxy- 7,4'-dimethoxyflavone, 5-hydroxy-3,7,3',4'-tetramethoxyflavone, 3,5,7-trimethoxyflavone, 3,5,7,4'- tetramethoxyflavone, 5,7,4'- trimethoxyflavone and 5,7,3',4'-tetramethoxyflavone. This study found that the cytotoxic effect of the rhizomes of KP appears to be safe and to reduce the need for chemotherapy(45).

EGF is a growth factor that stimulates the growth of SKOV3 human ovarian cancer cells. EGFR signaling regulates the advance of induced epithelial ovarian cancer cell migration and cell survival. Binding of EGF to EGFR activates many signal transduction pathways such as PI3K/AKT and MAPK. Patients in with high expression of EGFR result in aggressive ovarian cancer and a poor prognosis (60). To test the effect of KP on EGF, we added EGF 100 ng/ml to the cells to stimulate the growth and survival signaling. We found that with KP treatment, cell viability was decreased in a concentration-dependent manner. Even with EGF stimulation, cell viability still significantly decreased at a rate similar to that of cells which had not been stimulated by EGF. This suggests that KP has strong cytotoxic effect to SKOV3 cells. In a cell counting assay, we measured SKOV3 ovarian cancer cell proliferation using a Transwell plate and wound healing assay. We found that KP ethanol extract reduced cell growth in a concentration-dependent manner.

MMP-2 and -9 are a family of zinc-dependent endopeptidases which are associated with ovarian carcinoma cells, and which promote invasion, angiogenesis, and metastasis (55, 61). Previous studies have investigated MMP-2 and -9 involvement in the movement of ovarian cancer cells and found that MMP-2 and -9 enhance metastasis of SKOV3 cells. MMP-2 and -9 increase proteolytic activity that is related to high-grade ovarian cancer such as SKOV3 (56). We found that KP treated SKOV3 cells have less MMP-2 and MMP-9 activity in both with and without EGF. This result suggests that KP might be a great substance to suppress invasion and reduce metastasis in ovarian cancer.

The phosphoryration level of PI3K/Akt and MAPK is important for SKOV3 cell growth. These two signaling pathways frequently induce cell growth and survival. Many studies have suggested that PI3K plays a role in the invasion and metastasis pathway of ovarian cancer (62, 63). EGF is involved in the activation of PI3K/Akt and MAPK in human ovarian cancer (64). Interestingly, we showed that KP ethanol extract decreased phosphorylation of PI3K/Akt-and MAPK(ERK1/2) in the treated cells with and without the presence of EGF.

It has been previously demonstrated that *K. parviflora* Wall. ex. Baker rhizome ethanolic extract induces caspase-3 through a mitochondria-mediated pathway by supplementing

inhibitors of Akt, PI3K and MEK (MAPK) that are involved in a death signaling pathway in human promyelocytic leukemic HL-60 cells. Reports have stated that cell viability is reduced in a dose- and time-dependent manner with the treatment of KP Wall. ex. Baker rhizome ethanolic extract. Those studies confirmed the apoptosis pathway by demonstrating changes in cell morphology such as chromatin condensation and externalization of phosphatidylserine on the cell surface using fluorescence cell staining. Fluorescence intensity of the KP extract in HL-60 cells increased after treatment with inhibitors of Akt, PI3K and MEK, indicating that KP enhances apoptotic cell death via the suppression of PI3K/Akt and MAPK pathways (49, 63).

Md. Alamgir Hossain (2012) showed that when 3,5,7,4'-tetramethoxyflavone (TeMF), 5,7,4'-trimethoxyflavone (TMF), and 5-hydroxy-3,7,3',4'-tetramethoxyflavone (5-H-TeMF) purified from KP rhizome extracts were applied to HCT-15 cells (human colorectal carcinoma), the cells exhibited chromatin condensation and DNA fragmentation. Furthermore, purified KP strongly enhanced caspase-3 activity in a dose-dependent manner via the apoptosis pathway.

A trypan blue exclusion test in the present study found that KP at a concentration of 0.25 mg/ml resulted in an increased almost 30% cell death at 24 h. In addition, a cell death study using annexin V/PI labelling showed that KP treatment increased the percentage of cell apoptosis, especially the population in late apoptosis. These results were confirmed by the observation of the cell nuclear morphology using fluorescence nuclear staining. We found obvious DNA fragmentation on SKOV3 cells with 0.3 and 0.5 mg/ml KP ethanol extract.

Caspases are cysteine proteases that play a critical role in apoptosis progression and regulation of cell death (58). Two distinct pathways of apoptosis have been classified as the death receptor pathway and the mitochondrial pathway. Death-inducing signaling stimulates caspase-8 which activates the production of enzyme caspases-3 and -7. Moreover, the mitochondrial pathway is involved in the release of cytochrome c which activates caspase-9 activity. Caspase-9 further activates caspase-3 and -7 which cause

apoptosis as well as PARP, which has been considered indicative of functional caspase activation (59).

Using western blot analysis, KP ethanol extract could significantly increase the activation of caspase-3,-7, and -9 compared with the control in SKOV3 cells. Moreover, PARP was inactivated by KP treatment which indicates apoptosis induction. These data indicate that KP may play roles in cell death induction via the activation of caspase-3,-7,-9 and PARP inactivation.

In conclusion, the present study found that KP ethanol extract can inhibit the molecular signaling involved in cell proliferation by reducing the phosphorylation of Akt, ERK1/2, inhibiting the activity of MMP-2, -9, and inducing apoptotic cell death. Moreover, SKOV3 cells undergo morphological changes including DNA fragmentation of cells treated with KP ethanol extract. The results of this study support previous reports that KP ethanol extract has anti-cancer effects and is a potentially good alternative therapeutic agent for ovarian cancer.

LIST OF PUBLICATIONS

1. **Suthasinee Paramee,** Siriwoot Sookkhee, Choompone Sakonwasun3, Mingkwan Na Takuathung1, Pitchaya Mungkornasawakul, Wutigri Nimlamool1, Saranyapin Potikanond. Anti-cancer effects of Kaempferia parviflora on ovarian cancer SKOV3 cells. Submitted to BMC complementary & alternative medicine (23 November 2017)

REFERENCES

1. Wilailak S. Epidemiologic report of gynecologic cancer in Thailand. Journal of gynecologic oncology. 2009;20(2):81-3.

2. Toss A, De Matteis E, Rossi E, Casa LD, Iannone A, Federico M, et al. Ovarian cancer: Can proteomics give new insights for therapy and diagnosis? International journal of molecular sciences. 2013;14(4):8271-90.

3. Robert C. Bast IRaGBM. Molecular basis of cancer, The, Fourth edition. 2015:531-48.

4. Jones PM, Drapkin R. Modeling high-grade serous carcinoma: How converging insights into pathogenesis and genetics are driving better experimental platforms. frontiers in oncology. 2013;3:217.

5. McGuire WP, Markman M. Primary ovarian cancer chemotherapy: current standards of care. British Journal of Cancer. 2003;89(Suppl 3):S3-S8.

6. Patanasethanont D, Nagai J, Matsuura C, Fukui K, Sutthanut K, Sripanidkulchai BO, et al. Modulation of function of multidrug resistance associated-proteins by *Kaempferia parviflora* extracts and their components. Eur J Pharmacol. 2007;566(1-3):67-74.

7. Banjerdpongchai R, Chanwikruy Y, Rattanapanone V, Sripanidkulchai B. Induction of apoptosis in the human Leukemic U937 cell line by *Kaempferia parviflora* Wall.ex.Baker extract and effects of paclitaxel and camptothecin. Asian Pacific journal of cancer prevention : APJCP. 2009;10(6):1137-40.

8. Ries LAG. SEER Survival Monograph: Cancer Survival Among Adults: U.S. SEER Program patient and tumor characteristics national cancer institute. 2007.

9. McLemore MR, Miaskowski C, Aouizerat BE, Chen L-m, Dodd MJ. Epidemiologic and genetic factors associated with ovarian cancer. Cancer nursing. 2009;32(4):281-90.

10. Cooper CP, Gelb CA, Trivers KF, Stewart SL. Intended care seeking for ovarian cancer symptoms among U.S. women. Preventive medicine reports. 2016;3:234-7.

11. Haroon S, Zia A, Idrees R, Memon A, Fatima S, Kayani N. Clinicopathological spectrum of ovarian sex cord-stromal tumors; 20 years' retrospective study in a developing country. Journal of ovarian research. 2013;6:87-.

12. Landen CN, Jr., Birrer MJ, Sood AK. Early events in the pathogenesis of epithelial ovarian cancer. Journal of clinical oncology : official journal of the american society of clinical oncology. 2008;26(6):995-1005.

13. Löffek S, Schilling O, Franzke C-W. Biological role of matrix metalloproteinases: a critical balance. European respiratory journal. 2011;38(1):191-208.

14. Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. Cold spring harbor perspectives in biology. 2011;3(12):10.1101.

15. Anglesio MS, Wiegand KC, Melnyk N, Chow C, Salamanca C, Prentice LM, et al. Type-specific cell line models for type-specific ovarian cancer research. PloS one. 2013;8(9):e72162.

16. Beaufort CM, Helmijr JCA, Piskorz AM, Hoogstraat M, Ruigrok-Ritstier K, Besselink N, et al. Ovarian cancer cell line panel (OCCP): Clinical importance of in vitro morphological subtypes. PloS one. 2014;9(9):e103988.

17. Sumigama S, Ito T, Kajiyama H, Shibata K, Tamakoshi K, Kikkawa F, et al. Suppression of invasion and peritoneal carcinomatosis of ovarian cancer cells by overexpression of AP-2alpha. Oncogene. 2004;23(32):5496-504.

18. Lim W, Song G. Inhibitory effects of delphinidin on the proliferation of ovarian cancer cells via PI3K/AKT and ERK 1/2 MAPK signal transduction. Oncology letters. 2017;14(1):810-8.

19. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010;141(7):1117-34.

20. Regad T. Targeting RTK signaling pathways in cancer. Cancers. 2015;7(3):1758-84.

21. Zeineldin R, Muller CY, Stack MS, Hudson LG. Targeting the EGF receptor for ovarian cancer therapy. Journal of oncology. 2010;2010:414676.

22. Henson ES, Gibson SB. Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. Cellular signalling. 2006;18(12):2089-97.

23. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell. 1990;61(2):203-12.

24. Chen GJ, Karajannis MA, Newcomb EW, Zagzag D. Overexpression and activation of epidermal growth factor receptor in hemangioblastomas. Journal of neuro-oncology. 2010;99(2):195-200.

25. Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. Oncogene. 2007;26(22):3279-90.

26. Takenaka K, Moriguchi T, Nishida E. Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. Science (New York, NY). 1998;280(5363):599-602.

27. D'Andrilli G, Giordano A, Bovicelli A. Epithelial Ovarian Cancer: The role of cell cycle genes in the different histotypes. Open clinical cancer journal. 2008;7:7-12.

28. Cheaib B, Auguste A, Leary A. The PI3K/Akt/mTOR pathway in ovarian cancer: therapeutic opportunities and challenges. Chinese journal of cancer. 2015;34(1):4-16.

29. Cai J, Xu L, Tang H, Yang Q, Yi X, Fang Y, et al. The role of the PTEN/PI3K/Akt pathway on prognosis in epithelial ovarian cancer: a meta-analysis. The oncologist. 2014;19(5):528-35.

30. Oberst A, Bender C, Green DR. Living with death: The evolution of the mitochondrial pathway of apoptosis in animals. Cell death and differentiation. 2008;15(7):1139-46.

31. Silva JP, Coutinho OP. Free radicals in the regulation of damage and cell death - basic mechanisms and prevention. Drug discoveries & therapeutics. 2010;4(3):144-67.

32. Chaitanya GV, Alexander JS, Babu PP. PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. Cell communication and signaling : CCS. 2010;8:31-.

33. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. Cancer research. 1993;53(17):3976.

34. Yallapu MM, Jaggi M, Chauhan SC. Scope of nanotechnology in ovarian cancer therapeutics. Journal of Ovarian Research. 2010;3:19-.

35. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. European journal of pharmacology. 2014;0:364-78.

36. Dunton CJ. Management of treatment-related toxicity in advanced ovarian cancer. The oncologist. 2002;7.

37. Szebeni J, Muggia FM, Alving CR. Complement activation by Cremophor EL as a possible contributor to hypersensitivity to paclitaxel: an in vitro study. J Natl Cancer Inst. 1998;90(4):300-6.

38. Chen J-G, Horwitz SB. Differential mitotic responses to microtubule-stabilizing and -destabilizing Drugs. Cancer research. 2002;62(7):1935.

39. Kampan NC, Madondo MT, McNally OM, Quinn M, Plebanski M. Paclitaxel and its evolving role in the management of ovarian cancer. Biomed research international. 2015;2015:413076.

40. Thorn CF, Oshiro C, Marsh S, Hernandez-Boussard T, McLeod H, Klein TE, et al. Doxorubicin pathways: pharmacodynamics and adverse effects. Pharmacogenetics and Genomics. 2011;21(7):440-6.

41. Kollmannsberger C, Mross K, Jakob A, Kanz L, Bokemeyer C. Topotecan - A novel topoisomerase I inhibitor: pharmacology and clinical experience. Oncology. 1999;56(1):1-12.

42. Mutch DG, Orlando M, Goss T, Teneriello MG, Gordon AN, McMeekin SD, et al. Randomized phase III trial of gemcitabine compared with pegylated liposomal doxorubicin in patients with platinum-resistant ovarian cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007;25(19):2811-8.

43. สุดารัตน์ หอมหวล. (2553). กระชายดำ. ฐานข้อมูลเครื่องยาสมุนไพร คณะเภสัชศาสตร์ มหาวิทยาลัยอุบลราชธานี. ค้นเมื่อ 9 พฤศจิกายน 2560. จาก http://www.thaicrudedrug.com/main.php?action=viewpage&pid=4

44. Sudwan P, Saenphet K, Saenphet S, Suwansirikul S. Effect of *Kaempferia parviflora* Wall. ex. Baker on sexual activity of male rats and its toxicity. The Southeast Asian journal of tropical medicine and public health. 2006;37 Suppl 3:210-5.

45. Yenjai C, Prasanphen K, Daodee S, Wongpanich V, Kittakoop P. Bioactive flavonoids from *Kaempferia parviflora*. Fitoterapia. 2004;75(1):89-92.

46. Tewtrakul S, Subhadhirasakul S, Kummee S. Anti-allergic activity of compounds from *Kaempferia parviflora*. Journal of ethnopharmacology. 2008;116(1):191-3.

47. Sawasdee P, Sabphon C, Sitthiwongwanit D, Kokpol U. Anticholinesterase activity of 7-methoxyflavones isolated from *Kaempferia parviflora*. Phytotherapy research : PTR. 2009;23(12):1792-4.

48. Wongsrikaew N, Kim H, Vichitphan K, Cho SK, Han J. Antiproliferative activity and polymethoxyflavone composition analysis of *Kaempferia parviflora* extracts. Journal of the korean society for applied biological chemistry. 2012;55(6):813-7.

49. Banjerdpongchai R, Suwannachot K, Rattanapanone V, Sripanidkulchai B. Ethanolic rhizome extract from *Kaempferia parviflora* Wall. ex. Baker induces

apoptosis in HL-60 cells. Asian Pacific journal of cancer prevention : APJCP. 2008;9(4):595-600.

50. Hossain MA, Wongsrikaew N, Yoo G-W, Han J, Shin C-G. Cytotoxic effects of polymethoxyflavones isolated from *Kaempferia parviflora*. Journal of the korean Society for applied biological chemistry. 2012;55(4):471-6.

51. Rujjanawate C, Kanjanapothi D, Amornlerdpison D, Pojanagaroon S. Anti-gastric ulcer effect of *Kaempferia parviflora*. Journal of ethnopharmacology. 2005;102(1):120-2.

52. Shen J, DiCorleto PE. ADP Stimulates human endothelial cell migration via P2Y1; Nucleotide receptor–mediated mitogen-activated protein kinase pathways. circulation research. 2008;102(4):448.

53. Van Meerloo J, Kaspers GJL, Cloos J. Cell sensitivity assays: The MTT assay. In: Cree IA, editor. Cancer cell culture: Methods and protocols. Totowa, NJ: Humana press; 2011. p. 237-45.

54. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, et al. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene. 2006;366(1):2-16.

55. Belotti D, Paganoni P, Manenti L, Garofalo A, Marchini S, Taraboletti G, et al. Matrix metalloproteinases (MMP9 and MMP2) induce the release of vascular Endothelial growth Factor (VEGF) by ovarian carcinoma cells. Implications for ascites formation. 2003;63(17):5224-9.

56. Rabinovich A, Medina L, Piura B, Segal S, Huleihel M. Regulation of ovarian carcinoma SKOV-3 cell proliferation and secretion of MMPs by autocrine IL-6. Anticancer research. 2007;27(1a):267-72.

57. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell death differ. 1998;5(7):551-62.

58. Rahman MA, Ramli F, Karimian H, Dehghan F, Nordin N, Mohd Ali H, et al. Artonin E induces apoptosis via mitochondrial dysregulation in SKOV3 ovarian cancer cells. PLoS one. 2016;11(3):e0151466.

59. Bressenot A, Marchal S, Bezdetnaya L, Garrier J, Guillemin F, Plenat F. Assessment of apoptosis by immunohistochemistry to active caspase-3, active caspase-7, or cleaved PARP in monolayer cells and spheroid and subcutaneous xenografts of human carcinoma. The journal of histochemistry and cytochemistry : official journal of the histochemistry society. 2009;57(4):289-300.

60. Shao G, Wang J, Li Y, Liu X, Xie X, Wan X, et al. Lysine-specific demethylase 1 mediates epidermal growth factor signaling to promote cell migration in ovarian cancer cells. Scientific reports. 2015;5:15344.

61. Zhjang X, Liu P, Zhang B, Mao H, Shen L, Ma Y. Inhibitory effects of STAT3 decoy oligodeoxynucleotides on human epithelial ovarian cancer cell growth in vivo. International journal of molecular medicine. 2013;32(3):623-8.

62. Li Y, Jiang Y, Wan Y, Zhang L, Tang W, Ma J, et al. Medroxyprogestogen enhances apoptosis of SKOV-3 cells via inhibition of the PI3K/Akt signaling pathway2013. 43-50 p.

63. Dobbin ZC, Landen CN. The Importance of the PI3K/AKT/MTOR pathway in the progression of ovarian cancer. International journal of molecular sciences. 2013;14(4):8213-27.

64. Xia L, Wen H, Han X, Tang J, Huang Y. Luteinizing hormone inhibits cisplatin-induced apoptosis in human epithelial ovarian cancer cells. Oncology letters. 2016;11(3):1943-7.

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