



รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษาฤทธิ์ของสารโพลีเมทอกซีฟลาโวน ที่สกัดจาก *Kaempferia parviflora* ในการยับยั้งสัญญาณการเจริญและการมีชีวิตรวมถึงฤทธิ์ที่กระตุ้นให้เซลล์มะเร็งรังไข่และเซลล์มะเร็งปากมดลูกตาย

โดย

ผศ.ดร.วุฒิไกร นิมละมูล

ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์

มหาวิทยาลัยเชียงใหม่

มกราคม 2562

สัญญาเลขที่ MRG6080193

รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษาฤทธิ์ของสารโพลีเมทอกซีฟลาโวน ที่สกัดจาก *Kaempferia parviflora* ในการยับยั้งสัญญาณการเจริญและการมีชีวิตรวมถึงฤทธิ์ที่กระตุ้นให้เซลล์มะเร็งรังไข่และเซลล์มะเร็งปากมดลูกตาย

ผศ.ดร.วุฒิไกร นิมละมูล
ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์
มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
มหาวิทยาลัยเชียงใหม่

(ความเห็นในรายงานนี้เป็นของผู้วิจัย
สกว.และต้นสังกัดไม่จำเป็นต้องเห็นด้วยเสมอไป)

(รหัสโครงการ)

Project Title : Mechanisms of action of polymethoxyflavones from *Kaempferia parviflora* in suppressing growth and survival signaling and inducing apoptosis in ovarian and cervical cancer cells

(ชื่อโครงการ): การศึกษาฤทธิ์ของสารโพลีเมทอกซีฟลาโวน ที่สกัดจาก *Kaempferia parviflora* ในการยับยั้งสัญญาณการเจริญและการมีชีวิตรวมถึงฤทธิ์ที่กระตุ้นให้เซลล์มะเร็งรังไข่และเซลล์มะเร็งปากมดลูกตาย

Investigator : Dr.Wutigri Nimlamool, Department of Pharmacology, Faculty of Medicine, Chiang Mai University

(ชื่อนักวิจัย): ผศ.ดร.วุฒิไกร นิมละมูล ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

E-mail Address : wutigri.nimlamool@cmu.ac.th, wutigrinimlamool@gmail.com

Project Period : April 3, 2017 to April 3, 2019

(ระยะเวลาโครงการ): 3 เมษายน 2560 ถึง 3 เมษายน 2562

Abstract:

Kaempferia parviflora (KP) has been traditionally used as a folk remedy to treat several diseases including cancer. However, the ability of KP to regulate cancer cell growth and survival signaling is still largely unexplored. The current study aimed to investigate the effects of KP on cell viability, cell migration, cell invasion, cell apoptosis, and on signaling pathways related to growth and survival of cervical and ovarian cancer cells. We discovered that KP reduced the viability of HeLa and Skov-3 cells in a concentration-dependent manner. The potent cytotoxicity of KP was associated with the induction of cell apoptosis which is mediated through the activation of caspase 9 and caspase 7. We further investigated the effects of KP at non-cytotoxic concentrations on suppressing signal transduction pathways relevant to cell growth and survival. We found that KP suppressed the MAPK and PI3K/AKT signaling pathways in cells activated with EGF, as observed by a significant decrease in phosphorylation of ERK1/2, Elk1, PI3K, and AKT. Consistent with the inhibitory effect on EGF-stimulated signaling, KP potently suppressed the migration and invasion. The ability of KP in suppressing the migration and invasion was associated with the suppression of matrix metalloproteinase-2 and 9 production. These data strongly suggest that KP may slow tumor progression and metastasis. Taken together, the present report provides accumulated evidence revealing the potent anti-cancer activities of *Kaempferia parviflora* against cervical and ovarian cancer cells, and suggests its potential use as an alternative way for cervical and ovarian cancer prevention and therapy.

บทคัดย่อ

Kaempferia parviflora (KP) หรือกระชายดำ ได้ถูกนำมาใช้แบบแพทย์แผนโบราณเพื่อรักษาโรคหลายชนิดรวมถึงโรคมะเร็ง อย่างไรก็ตามหลักฐานในเชิงลึกที่แสดงให้เห็นถึงฤทธิ์ของกระชายดำในแง่

เกี่ยวกับสัญญาณการเจริญและการมีชีวิตของเซลล์มะเร็งยังไม่เป็นที่ทราบแน่ชัด โครงการวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของสารสกัดกระชายดำต่อการมีชีวิต การเคลื่อนที่ การแพร่กระจายและการกระตุ้นให้เซลล์ตายแบบอพอพโทซิสในเซลล์มะเร็งปากมดลูก และเซลล์มะเร็งรังไข่ ผู้วิจัยค้นพบว่ากระชายดำสามารถลดอัตราการมีชีวิตของ HeLa และ Skov-3 ได้โดยการตายของเซลล์จะเพิ่มขึ้นเมื่อความเข้มข้นของกระชายดำเพิ่มขึ้น ความสามารถของกระชายดำในการลดอัตราการมีชีวิตของเซลล์มะเร็งนั้นเกิดขึ้นจากการที่กระชายดำสามารถเหนี่ยวนำให้เซลล์มะเร็งตายแบบอพอพโทซิสได้ โดยผ่านทางกระบวนการกระตุ้นเอนไซม์ caspase 9 และ caspase 7 นอกจากนี้เมื่อผู้วิจัยศึกษาฤทธิ์ของกระชายดำที่ความเข้มข้นที่ไม่เป็นพิษต่อเซลล์พบว่ากระชายดำสามารถยับยั้งสัญญาณการเจริญและการมีชีวิตของเซลล์มะเร็งนั้นคือสัญญาณ MAPK และ PI3K/AKT ที่ถูกกระตุ้นด้วย EGF ได้อย่างมีประสิทธิภาพ ข้อสรุปนี้ได้มาจากการสังเกตว่ากระชายดำสามารถยับยั้งกระบวนการเติมหมู่ฟอสเฟตให้แก่โมเลกุล ERK1/2, Elk1, PI3K, และ AKT ยิ่งกว่านั้นกระชายดำยังสามารถยับยั้งการเคลื่อนที่และการแพร่กระจายของเซลล์มะเร็งที่ถูกกระตุ้นด้วย EGF ได้อย่างมีประสิทธิภาพ โดยผู้วิจัยค้นพบว่าความสามารถของกระชายดำดังกล่าวนี้เป็นเหตุมาจากการที่กระชายดำลดการสร้างและหลั่งเอนไซม์ matrix metalloproteinase-2 และ 9 ผลการศึกษานี้จึงแสดงความน่าเชื่อถือว่ากระชายดำอาจช่วยชะลอการพัฒนาและการแพร่กระจายของเซลล์มะเร็งปากมดลูกและมะเร็งรังไข่ได้ โดยสรุปแล้วโครงการวิจัยนี้ช่วยสร้างองค์ความรู้พื้นฐานเกี่ยวกับฤทธิ์ต้านมะเร็งปากมดลูกและมะเร็งรังไข่ของกระชายดำ ความรู้นี้ดังกล่าวนั้นนับว่าเป็นประโยชน์อย่างยิ่งสำหรับการพัฒนาไปเป็นวิธีการป้องกันและรักษามะเร็งปากมดลูกและมะเร็งรังไข่ในอนาคต

Keywords : anti-cancer; cancer; *Kaempferia parviflora*; MAPK pathway; PI3K/AKT pathway

(คำหลัก) : ฤทธิ์ต้านมะเร็ง; มะเร็ง; กระชายดำ (*Kaempferia parviflora*); สัญญาณ MAPK; สัญญาณ PI3K/AKT

เนื้อหางานวิจัย 1: *Kaempferia parviflora* Extract Exhibits Anti-cancer Activity against HeLa Cervical Cancer Cells

1.1 Introduction

Cervical cancer is still one of the most common causes of all cancer deaths in women, especially in developing countries (McGuire, 2016). Human papillomaviruses (HPVs) can subvert cellular mechanism of growth control (Moody and Laimins, 2010) and activate the PI3K/AKT/mTOR signaling (Surviladze et al., 2013). Like most cancers, cervical cancer does not show any signs during early disease development (Canavan and Doshi, 2000). However, symptoms usually appear when the tumor causes vaginal discharge and bleeding, and other symptoms including pain or backache may occur in patients with metastasis (Petignat and Roy, 2007). Therefore, most patients who notice symptoms typically have later stages of tumor development that have frequently progressed too far for curative treatment. Communication through signal transduction pathways is very important for regulating the balance between cell proliferation and cell death. The activation of cell growth and survival signal transduction pathways is stimulated via a growth factor binding to a specific growth

factor receptor (Margolis, 1992). Epidermal growth factor receptor (EGFR) is an important regulator of cell proliferation (Olayioye et al., 2000) and upon growth receptor activation, diverse downstream pathways are further stimulated. Those crucial pathways include Ras/Raf/MEK/MAPK, phospholipase C, and STAT (Yarden and Sliwkowski, 2001). Moreover, stimulation of the EGF receptor causes strong activation of the phosphatidylinositol-3 kinase (PI3K)/AKT pathway for maintaining cell metabolism and enhancing cell survival and proliferation (Cantley, 2002; Vivanco and Sawyers, 2002). The commonest characteristics of most cancers are the amplification of growth and survival signaling, and the inhibition of apoptosis (Hanahan and Weinberg, 2011). Furthermore, elevated levels of matrix metalloproteinase (MMP) is considered to be an important hallmark of many cancers, and MMP expression has been demonstrated to be associated with tumor invasion in many different tumors (Liotta et al., 1980; Fishman et al., 1997; Stetler-Stevenson, 2001; Roomi et al., 2010). The actual cause of cancer is not well-understood. However, over activation and aberrant cancer cell signal transduction mediated by factors such as mutations in key kinases that cause constitutive activation of growth and survival signaling pathways, may contribute to cancer development and metastasis (Huang et al., 1997; Moscatello et al., 1998; Chakravarti et al., 2002). The most effective way to decrease the burden of cervical cancer and the associated death rate is to prevent and screen for HPV lesions through HPV testing and Pap smears (Safaeian and Solomon, 2007). Vaccination is a better way to reduce and eventually prevent death from cervical cancer but this management option is currently limited to young people (Saslow et al., 2007). Moreover, in many underdeveloped countries routine screening may not be widely available due to limited resources (Denny et al., 2006). Although new chemotherapeutic agents have been developed to slow down the progression of the disease, the number of cancer related deaths is still high because of drug-resistance and metastasis (Liu et al., 2016; Siegel et al., 2016). Also, conventional treatments for cervical cancer are expensive (Subramanian et al., 2010). In particular, the direct cost per patient for cisplatin, paclitaxel, and topotecan treatments ranges from 2,000 to 10,000 USD (Geisler et al., 2012). Thus, many patients may not be able to afford these options. Alternative medicine has emerged as an interesting means for treating or curing diseases, and recently several plants and their constituents have been approved to be safe, effective, and less expensive for managing the development and progression of various cancers (Yin et al., 2013). Several medicinal plants have been discovered to contain active compounds that are able to disrupt homeostasis of cancer cells (Yin et al., 2013). *Kaempferia parviflora* (KP) is a plant in the family Zingiberaceae commonly known as Thai black ginger (or Krachai Dam in Thai). Its rhizome is used in traditional medicine for many purposes including anti-gastric ulcer, anti-allergic, anti-plasmodial, and anti-cancer, as well as for enhancing sexual activity (Saokaew et al., 2016). Specifically, for the anti-cancer effects of KP, studies have shown that KP suppressed multidrug resistance associated proteins (MRP) in A549 (lung cancer) cells (Patanasethanont et al., 2007). Moreover, KP induced apoptotic cell death and enhanced paclitaxel or doxorubicin treatment in a promyelocytic leukemic cancer cell line (Banjerdpongchai et al., 2009). However, the anti-cancer effects of KP against cervical cancer cells have not yet been investigated. Several aspects, especially the molecular mechanisms of action to understand how KP interferes with growth and survival functions of cancer

cells, remain largely unknown. Thus, this present study aimed to evaluate the anti-cancer properties of KP against HeLa cervical cancer cells. We particularly investigated effects of KP on inducing apoptotic cell death, suppressing cell migration and invasion, and inhibiting major molecular signal transduction pathways related to cancer cell growth and survival. Our study provides convincing evidence that KP possesses anti-cancer properties and may be a good candidate as a new therapeutic agent for cervical cancer.

1.2 Materials and methods

Cell Culture

The human HeLa cell line [HeLa 229 (ATCC CCL-2.1TM)] used in this study was obtained from ATCC (ATCC, Manassas, VA, United States). The cells were cultured in complete medium, which is Dulbecco's modified Eagle's medium (DMEM) (Gibco, United States), supplemented with 10% fetal bovine serum (Merck KGaA, Germany), and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) (Gibco, United States) and maintained under a humidified atmosphere of 37C, 5% CO₂. The cells were sub-cultured every 2–3 days. Plant Material and Extraction of *Kaempferia parviflora* Rhizomes Fresh rhizomes of KP were harvested from the CMU-RSPG Kaempferia housing at Chiang Dao, Chiang Mai Province, Thailand. Voucher specimen number, R-CMUKP002, was authenticated and deposited at the Faculty of Science, Chiang Mai University, Thailand. The rhizomes of the plant were weighed, chopped, and extracted with 95% ethanol at room temperature (RT) for 3 days. Then the ethanolic extract was filtered, concentrated using a rotary evaporator, and then lyophilized. The extraction process yielded residues of 9.85% dry weight of KP rhizomes for ethanolic extraction. The crude extract was kept in an air-tight, light protected container, and stored at -20C until used. The KP extract stock solution was freshly prepared using DMSO prior to each assay. One gram of ethanolic KP crude extract was dissolved in 1 ml of 100% DMSO to make a stock

solution of 1 g/ml, and the stock was pre-diluted in medium prior to each treatment. Each experiment was performed with three independent batches of KP extract, each assayed in triplicate (n = 9). The final concentration of DMSO was maintained below 0.5% v/v throughout the experiment.

Cell Viability Assay

The effect of KP on cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT assay was performed according to a previously published protocol (Mosmann, 1983). HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells per well for 24 h in complete medium. Cells were then treated with KP extract at various concentrations (0–1 mg/mL) or with vehicle (DMSO at 0.001–0.1%) for 24 h, then cells were exposed to the MTT reagent (0.5 mg/mL in PBS) for 2 h at 37C, 5% CO₂. After aspirating the culture supernatants, 200 μ L of DMSO was added to each well, and the plates were incubated in the dark for 10 min. The absorbance at 590 nm was measured using a microplate reader (BioTek

Instruments, United States). Cell viability assay was performed three times, and each assay was done in triplicate

(n D 9 in three individual experiments).

Apoptosis Assay by Flow Cytometry

Cell apoptosis was determined using FITC-annexin V (ImmunoTools, Germany) and propidium iodide (PI) (Sigma, United States). After treatment with KP extract at different concentrations (0.1, 0.3, and 0.5 mg/mL) for 6 h, cells (approximately 1×10^6 cells/mL) were harvested and washed in PBS one time by centrifugation. The supernatants were discarded and cells were resuspended in 1x annexin-binding buffer. Then, annexin V and PI were added to the cell suspension, and cells were incubated at RT for 15 min. Flow cytometry was performed using a BD FACSCANTO II flow cytometer (Becton Dickinson, United States) to determine the percentage of apoptotic cell death.

Immunofluorescence Study

HeLa cells were grown on glass cover slips for 24 h, and were then treated with KP extract at different concentrations (0.01, 0.05, and 0.1 mg/mL) for 24 h in serum-free medium. Fifteen minutes before harvesting cells, 100 ng/mL of EGF was added to KP-treated HeLa cells to activate growth and survival signaling, following which cells were fixed with 4% paraformaldehyde dissolved in PBS for 15 min. After washing three times with PBS, cells were permeabilized using 0.3% TritonX-100 in PBS for 5 min. Then cells were incubated with 1% BSA in PBS solution for 1 h, and with 1:100 of a phosphospecific (Thr 202/Tyr 204) rabbit anti-ERK1/2 antibody (Cell Signaling Technology, United States) at 4C overnight. Cells were washed three times with PBS for 5 min each time, and incubated with a 1:500 dilution of an appropriate secondary antibody (Alexa 594-conjugated goat anti-rabbit) (Life Technologies, United States) for 2 h, in the dark, at RT. After washing three times with PBS and one time with distilled water, cells were mounted using Fluoromount-G (0100-01; SouthernBiotech, United States). Observations were performed on a fluorescence microscope, AX70 Olympus R, Japan, with 40x magnification, and micrographs were captured with the DP-BSW Basic Software for the DP71 microscope digital camera. To determine the ability of the KP extract to induce nuclear fragmentation, the nuclei of HeLa cell samples were stained with 5 mg/mL of Hoechst 33342 for 1 h. Cells were washed three time with PBS and one time with distilled water, and mounted as described above.

Cell Migration Assay

A wound healing assay was performed to examine the effect of KP on cell migration. HeLa cells (0.5×10^6 cells/well) were seeded and cultured in 24-well plates for 24 h. A scratch wound was made with a sterile 200 mL pipette tip. Detached cells were removed and complete medium was added. Cells were treated with DMSO as a vehicle control or KP extract (0.01, 0.05, and 0.1 mg/mL) for 40 h. Images of the scratched wounds were recorded at different time points (0, 24, and 40 h). The closing of scratched wounds is an indicator of the completion of the migration process. The migrated areas were analyzed and determined using the ImageJ software.

Cell Invasion Assay

The effects of KP on HeLa cell invasion were determined using a Cell Culture Insert (SPL Life Sciences, South Korea). The polycarbonate invasion chambers (8 mm pore size) were coated with

15 mg of matrigel (Corning, NY, United States) per well and incubated at RT for 4 h. Cells at a density of 0.3×10^6 cells per well were seeded onto the matrigel and cultured in serum free medium for 24 h. On the next day cells in the invasion (upper) chambers were treated with different concentrations (0–0.1 mg/mL) of KP in serum-free media, and the invasion chambers were put into the (lower) wells containing DMEM with 5% FBS and incubated for 40 h. Cells were then fixed with absolute methanol for 5 min at RT and stained with 0.5% crystal violet for 30 min. After three washes with water, cells in the invasion chambers were removed with a cotton swab and the pictures of the stained cells attached at the other site of the invasion chamber were taken and analyzed with the ImageJ software.

Gelatinase Zymography

Cells were seeded at a density of 0.3×10^6 cells per well and cultured for 24 h. After KP treatment for 24 h, culture supernatants were collected and mixed with non-reducing sample buffer, and samples were separated by SDS-PAGE under cold running conditions. Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min at RT. The gels were then incubated with substrate buffer (50 mM, Tris HCL, and 10 mM CaCl₂ pH 8) overnight. The gels were stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min, and then destaining was performed. The intensity of each band was evaluated using the ImageJ software.

Western Blotting

For caspase and BID detection, HeLa cells were treated with 0.1, 0.3, and 0.5 mg/mL of KP extract for 6 h. For detecting the phosphorylation status of ERK1/2, Elk1, AKT, and PI3K, HeLa cells were treated with 0.01, 0.05, and 0.1 mg/mL of KP extract for 6 h. Cells were stimulated with EGF (100 ng/mL) for 15 min before harvesting cells. HeLa cell lysates were prepared by adding 1x reducing Laemmli buffer into the sample dishes. Samples were collected, heated at 95°C for 5 min, separated by SDS-PAGE, and electroblotted onto PVDF membranes (GE Healthcare Life Sciences, Germany). Membranes were blocked with 5% skim milk in TBS-T (0.02 M Tris-HCl, pH 7.6, 0.0137 M NaCl, and 0.1% (wt/v) Tween 20) at RT for 1 h. Membranes were then incubated with an appropriate primary antibody (Cell Signaling Technology, United States) at 4°C overnight. Primary antibodies used included a 1:1,000 dilution of a phosphospecific rabbit anti-PI3 kinase p85 (Tyr 458)/p55 (Tyr 199) antibody, or a phosphospecific rabbit anti-AKT (Ser 473) (D9E) antibody, or a phosphospecific rabbit anti-ERK1/2 (Thr 202/Tyr 204) antibody, or a phosphospecific rabbit anti-Elk-1 (Ser 383), or a mouse anti-caspase-7 (C7) antibody, or a rabbit anti-caspase-9 antibody, or a rabbit anti-BID antibody, and a 1:10,000 dilution of an anti- β -actin antibody. After three washes with TBS-T, membranes were incubated with 1:5000 dilution of an appropriate horseradish peroxidase-conjugated secondary antibody (KPL, United States) for 2 h, at RT. Immune complexes were detected using enhanced chemiluminescence reagent. The intensity of the immunoreactive bands was analyzed and quantified using the ImageJ software.

Statistical Analysis

Data were analyzed by one-way ANOVA. Data are presented as mean $\pm$ SD. In all analyses, a p-value ($p < 0.05$) was considered statically significant. NS indicates a non-significant difference

1.3 Results

The Effects of KP on HeLa Cell Viability

To determine the effects of KP on cell viability, cells were treated with the KP extract at different concentrations ranging from 0.01 to 1 mg/mL for 24 h. The results from MTT assay (Figure 1) showed that the KP extract had a strong cytotoxic effect on HeLa cells in a concentration-dependent manner. The IC₅₀ value of KP extract was 0.22 mg/mL. KP extract at 0.5 mg/mL showed maximum cytotoxic effect, where approximately 90% reduction of HeLa cell viability was observed. HeLa cells treated with higher concentrations of KP extract (0.6–1 mg/mL) showed effects on cell viability similar to those treated with KP at 0.5 mg/mL. However, the KP extract at concentrations below 0.07 mg/mL did not affect HeLa cell viability. DMSO (vehicle control), at all concentrations (0.001–0.1%) relevant to the treatment group showed no apparent cytotoxicity to HeLa cells.

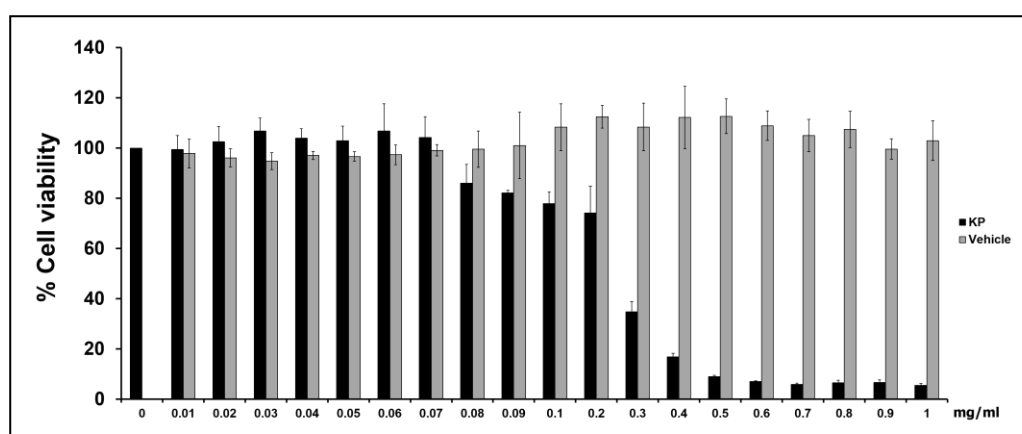


FIGURE 1 | The effects of KP on HeLa cell viability. The bars indicate percent cell viability of HeLa cells treated with different concentrations of KP extract (0–1 mg/mL) for 24 h with cell viability measured by the MTT assay. Data represent mean $\pm$ SD of three independent experiments. * $p < 0.05$.

The Effects of KP on Inducing Cell Apoptosis

To investigate whether KP induces apoptotic cell death, we first observed for morphological aberrations of HeLa cells in response to KP extract exposure. Since KP extract at 0.5 mg/mL showed maximum cytotoxic effect, HeLa cells were exposed to 0.5 mg/mL of KP extract and phase-contrast images of cells were taken at different time points (0–24 h). Data showed that KP extract caused HeLa cell morphological changes in a time-dependent manner. Figure 2A shows that at 0 h of KP treatment, HeLa cells were observed to exhibit normal morphology of epithelial cells with discrete cell-cell-contact, and most cells were tightly attached to the surface of the dish. After 3 h of treatment, even though cells were still attached to the dish, the vast majority of cells showed distinct morphological changes. Those cells changed from the normal characteristic elongated shape and become spherical. After 6 h, all cells exhibited spherical morphology, and cell detachment from the dish was observed in some areas. Eventually, most cells detached from the surface after 24 h of KP treatment, and the remaining cells with aberrant morphology were likely to be dead or dying cells.

Since one of the characteristics of apoptotic cell death is nuclear fragmentation, we performed nuclear staining using Hoechst 33342 of HeLa cells treated with KP extract at various concentrations (0.1, 0.3, and 0.5 mg/mL) for 6 h. We found that KP extract at potent cytotoxic concentrations (0.3 and 0.5 mg/mL) induced nuclear deformity and nuclear fragmentation of HeLa cells after 6 h of incubation, and these effects were independent of DMSO used as the vehicle control (Figure 2B).

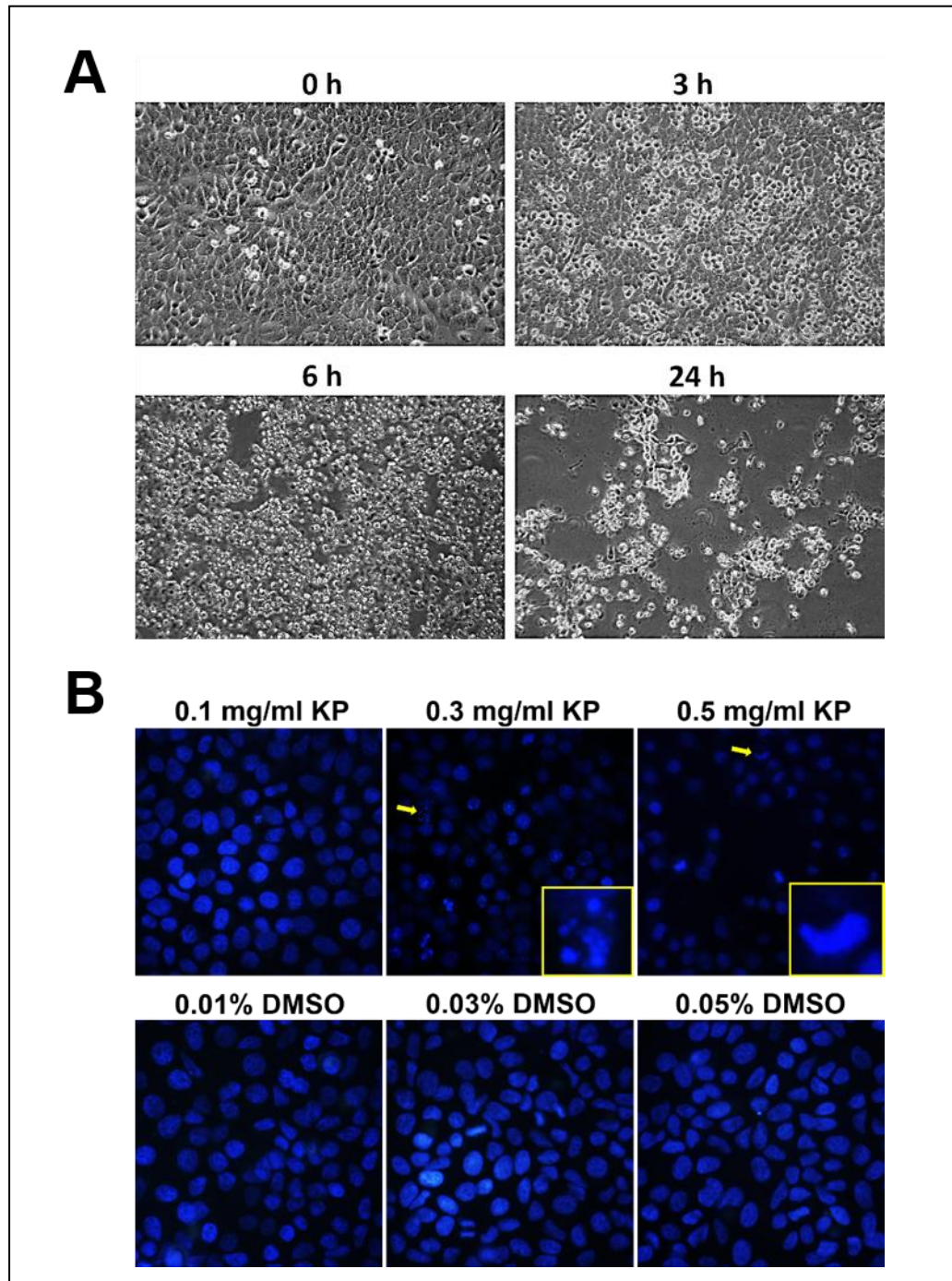


FIGURE 2 | Morphological changes and nuclear fragmentation of HeLa cells exposed to KP extract. (A) Phase-contrast images of HeLa cells treated with 0.5 mg/mL of KP extract taken at different time points (0 , 3 , 6 , and 24 h). (B) The nuclei of HeLa cells treated with KP extract at different concentrations (0.1–0.5 mg/mL) or with DMSO (0.01–0.05%), stained with Hoechst33342, and

visualized using a fluorescent microscope. Arrows indicate cells with nuclear fragmentation or nuclear deformity, and magnified views of cells indicated with arrows are shown at the bottom right corners. Data are representative of three replicates.

Based on these observations, we hypothesized that KP has the potential ability to induce programmed cell death. We therefore performed flow cytometry to determine whether KP induces HeLa cell apoptosis. As expected, KP extract induced apoptotic cell death in a concentration-dependent manner. We found that HeLa cell apoptosis was significantly increased to $39.8 \pm 2.40\%$ for cells treated with 0.3 mg/mL KP and to $69.85 \pm 3.04\%$ for cells treated with 0.5 mg/mL of KP extract, whereas DMSO at all concentrations relevant to those present in KP treatment groups did not induce apoptosis in HeLa cells (Figures 3A,B). We further confirmed the effects of KP on inducing apoptosis in HeLa cells by performing western blot analysis to determine the activation of caspase 9, caspase 7, and the pro-apoptotic protein BID, using specific antibodies that can recognize both full length and cleaved forms of the proteins. The results showed that the KP extract induced cleavage of caspase 7 and 9 in a concentration-dependent manner, however, the extract at all concentrations tested did not increase the cleaved form of BID (Figure 3C).

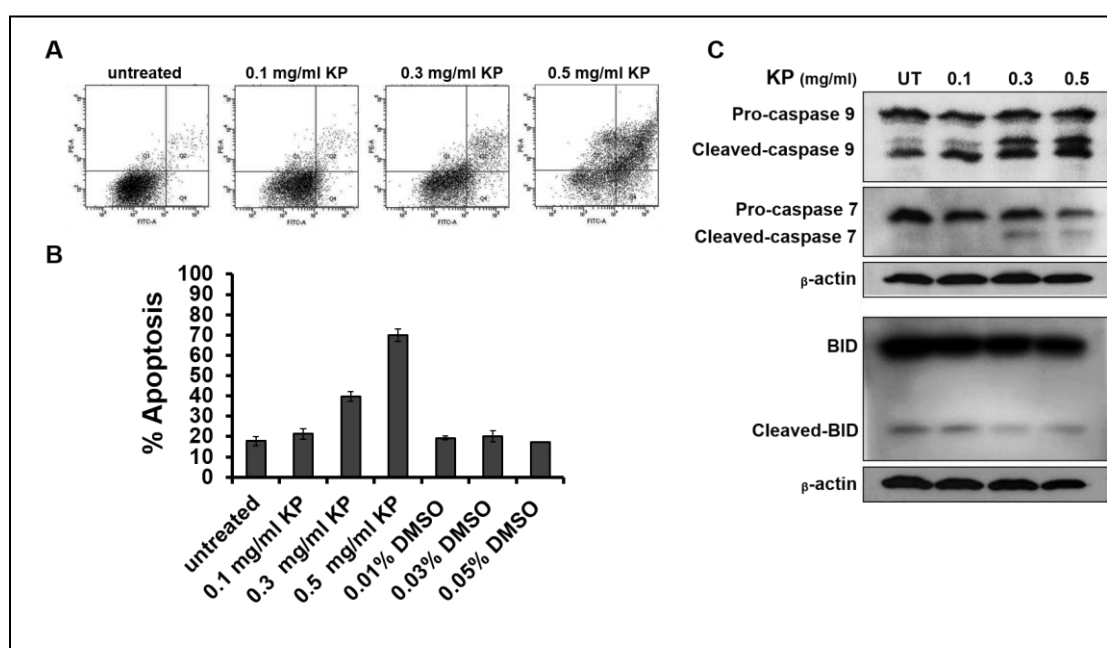


FIGURE 3 | The effects of KP on inducing HeLa cell apoptosis. (A) Representative figures from flow cytometry showing HeLa cells undergoing apoptotic cell death upon incubation with different concentrations (0–0.5 mg/mL) of KP extract for 6 h. (B) Quantitative analysis of percentage cell apoptosis from flow cytometry. Data are representative of three replicates and are expressed as mean $\pm$ SD. (C) A representative western blot of caspase 9, caspase 7, and BID from HeLa cells treated with different concentrations (0–0.5 mg/mL) of KP extract for 6 h. Two immunoreactive bands of cleaved-caspase 9 indicate a p35 subunit and a p37 subunit of active caspase 9. Beta-actin was used as a loading control.

The Effects of KP on Inhibiting Cell Migration and Invasion

One of the important characteristics of malignant tumors is enhanced cell migration. We determined whether the KP extract can inhibit migration of HeLa cells by performing a wound healing assay. Results showed that the KP extract significantly suppressed the migration of HeLa cells at both 24 and 40 h, and the suppression was seen in a concentration-dependent manner (Figure 4 A). Compared to untreated HeLa cells which exhibited $86.4 \pm 77.22\%$ cell migration at 40 h, cells treated with 0.01, 0.05, and 0.1 mg/mL of KP extract showed decreases in the percent cell migration to approximately $52.53 \pm 21\%$, $36.77 \pm 18.8\%$, and $15.46 \pm 9.13\%$, respectively (Figure 4B). The percent cell migration in cells treated with 0.01, 0.05, and 0.1% of DMSO (vehicle control) was $78.53 \pm 21.46\%$, $75.35 \pm 27.71\%$, and $76.76 \pm 27.58\%$, respectively. Based on the observation that KP extract significantly inhibited HeLa cell migration, it is reasonable to hypothesize that KP also inhibits cell invasion and so a cell invasion test was performed using the Transwell invasion assay. We found that KP extract significantly reduced cell movement through the matrigel-coated chamber in a concentration-dependent manner (Figure 4C). The percentage of cell invasion compared to that of the vehicle control (0.1% DMSO) was reduced to $47.79 \pm 9.21\%$, $57.83 \pm 4.97\%$, and $21.88 \pm 3.21\%$ for HeLa cells treated with 0.01, 0.05, and 0.1 mg/mL of KP extract, respectively (Figure 4D).

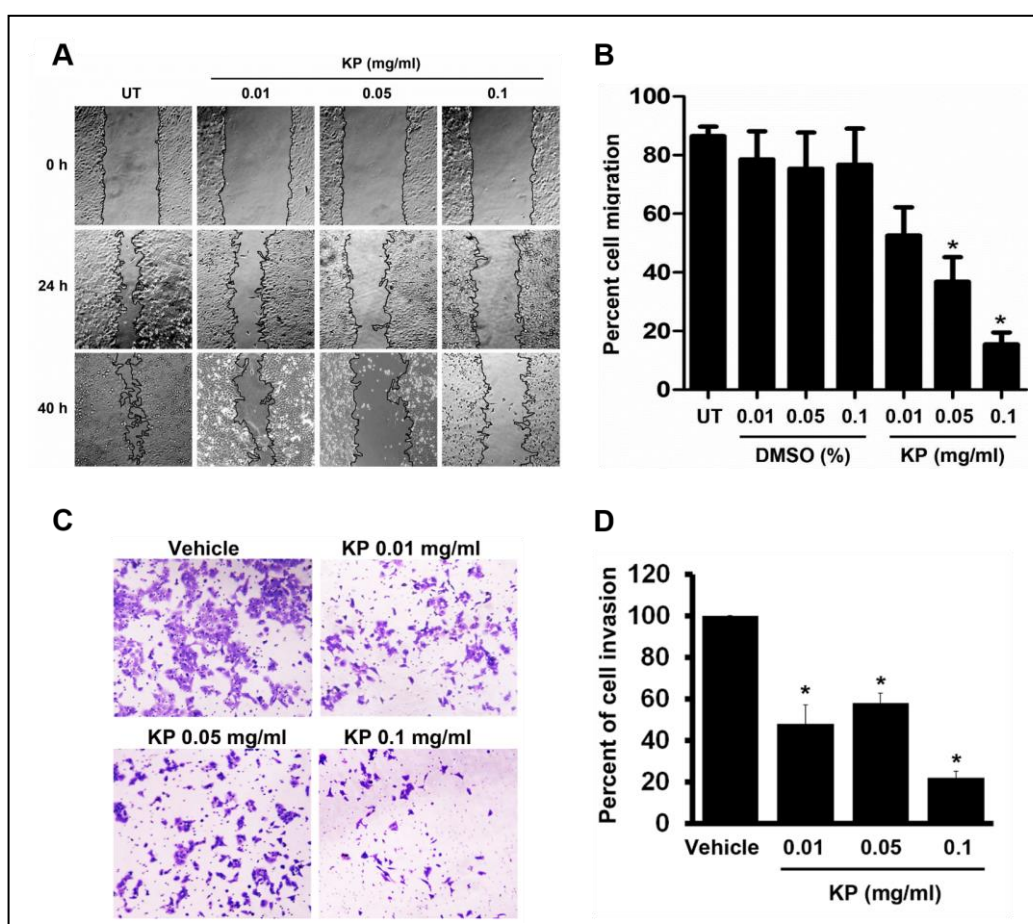


FIGURE 4 | The effects of KP on HeLa cell migration and invasion. (A) Scratch wounds of monolayers HeLa cells treated with KP extract (0.01, 0.05, and 0.1 mg/mL) for 40 h. Cell migration was monitored with 4x magnification, and phase-contrast images of cell migration were taken at the

time of the scratch and at 24 and 40 h post-scratch. (B) Quantitative analysis of cell migration into the scratch wound at 40 h post-scratch. Data are expressed as mean $\pm$ SD. Asterisks indicate significantly different from the control groups (untreated groups) (* $p < 0.05$). (C) Representative images of HeLa cell invasion treated with KP extract (0.01, 0.05, and 0.1 mg/mL) and examined by the Transwell invasion assay. Vehicle is the control group where cells were treated with the highest concentration of DMSO (0.01%) which corresponded to the concentration present in 0.1 mg/mL of KP. (D) Quantitative analysis of percent of cell invasion in KP-treated cells compared to the vehicle control. Data are representative of three replicates and are expressed as mean $\pm$ SD. * $p < 0.05$ compared with the vehicle control.

The Effect of KP on Inhibiting Metalloproteinase-2 Activity

Since we observed that KP inhibited HeLa cell migration and invasion, we then tested our hypothesis that KP may suppress the activity of MMP-2, which is a major metalloproteinase expressed by HeLa cells. Data from gelatinase zymography shown in Figures 5A,B demonstrated that the KP extract significantly reduced MMP-2 activity in a concentration-dependent manner. The immunoreactive bands of actin detected by western blot showed the approximately equal loading in each group. Approximately 30, 50, and 90% inhibition of MMP-2 activity was observed in HeLa cells treated with 0.01, 0.05, and 0.1 mg/mL of KP extract, respectively. When the treatment was performed in the presence of EGF at 100 ng/mL, we found that EGF slightly increased the production of MMP-2 by HeLa cells. Nevertheless, KP extract was still able to potently inhibit MMP-2 activity in a concentration-dependent manner.

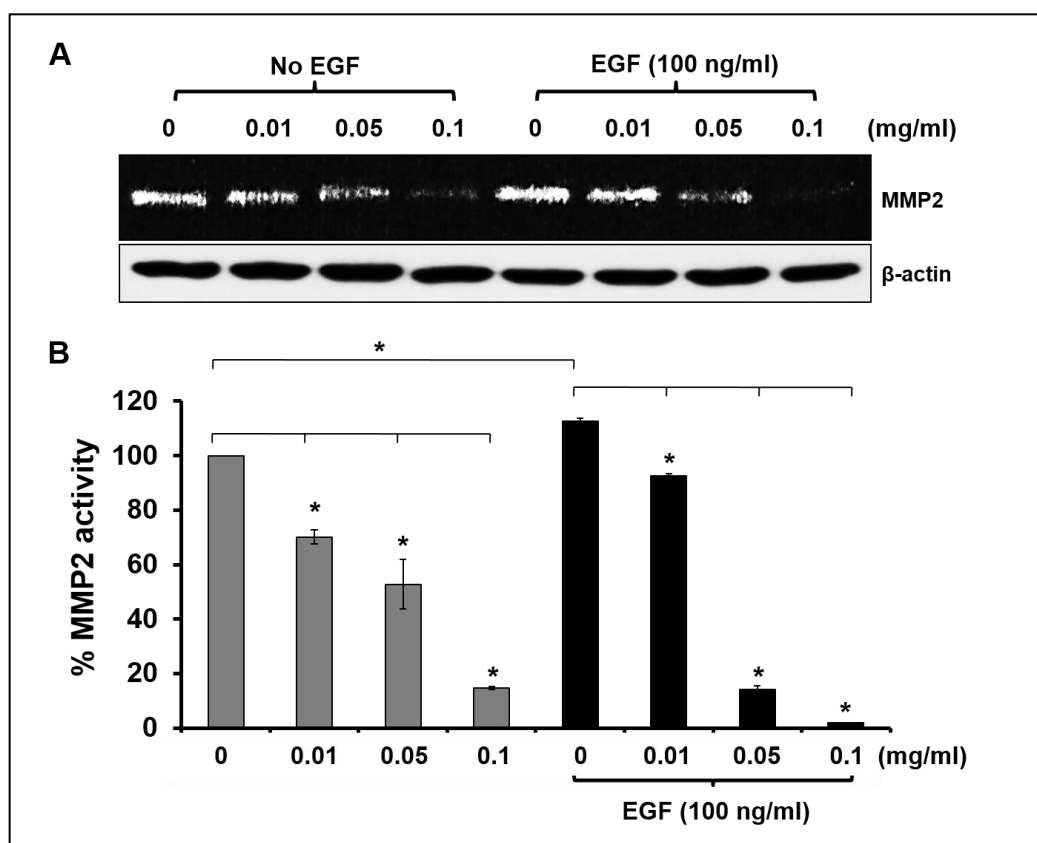


FIGURE 5 | Effects of KP extract on suppressing MMP-2 activity. (A) Zymographic analysis for MMP-2 activity in HeLa cells treated with KP extract at various concentrations (0–0.1 mg/mL) with or without the presence of 100 ng/mL of EGF. Western blot for beta actin was used as a loading control. (B) Quantitative analysis of MMP-2 level using ImageJ software. Beta actin from the western blot was used as an internal control for normalization. Data are expressed as mean $\pm$ SD. * $p < 0.05$ compared with untreated cells.

The Effects of KP on Suppressing Growth and Survival Signal Transduction Pathways

To further investigate the effects of KP extract on growth of HeLa cells, the phosphorylation status of several major protein kinases, including PI3K, AKT, ERK1/2, and Elk1, in response to KP extract treatment was determined. We initially undertook immunofluorescent staining of an important member of the MAPK signaling pathway, pERK1/2, in HeLa cells treated with KP extract at different concentrations, to examine whether KP extract can inhibit growth factor mediated signaling. Results from the immunofluorescence study showed that serum-starved HeLa cells showed a very low basal level of ERK1/2 phosphorylation (Figure 6A). When EGF at 100 ng/mL was added to serum-starved HeLa cells, phosphorylation of ERK1/2 was markedly increased. However, EGF-treated cells exposed to KP extract at 0.01 and 0.05 mg/mL showed obvious reductions of ERK1/2 phosphorylation. Based on these results, we further defined whether KP extract can affect phosphorylation status of other crucial players in growth and survival signal transduction pathways. We therefore undertook western blot analysis of a number of signal transduction kinases, and the results showed that KP extract could effectively suppress the EGF-dependent phosphorylation of PI3K, AKT, ERK1/2, and Elk1 in a concentration-dependent manner. Figures 6B,C shows that HeLa cells stimulated with EGF alone for 15 min exhibited an increase in phosphorylation of PI3K (2.82 ± 0.06 fold), AKT (10.28 ± 0.72 fold), ERK1/2 (4.27 ± 0.11 fold), and Elk1 (1.74 ± 0.08 fold). Interestingly, pretreatment of HeLa cells with 0.01 mg/mL of KP extract for 6 h before the addition of EGF dramatically reduced stimulated phosphorylation of PI3K to 1.64 ± 0.36 fold, AKT to 3.98 ± 0.57 fold, ERK1/2 to 2.84 ± 0.12 fold, and Elk1 to 1.67 ± 0.05 fold. Moreover, EGF-stimulated cells treated with 0.05 mg/mL KP extract significantly reduced phosphorylation of PI3K to 0.74 ± 0.06 fold, AKT to 1.81 ± 0.57 fold, ERK1/2 to 2.24 ± 0.14 fold, and Elk1 to 1.19 ± 0.02 fold. Finally, EGF-treated cells treated with 0.1 mg/mL KP extract significantly reduced phosphorylation of PI3K to 0.54 ± 0.03 fold, AKT to 0.24 ± 0.14 fold, ERK1/2 to 0.56 ± 0.11 fold, and Elk1 to 1.01 ± 0.04 fold. Beta actin was used as an internal control and for normalization. The immunoreactive bands of actin indicated approximately equal protein loading in each well.

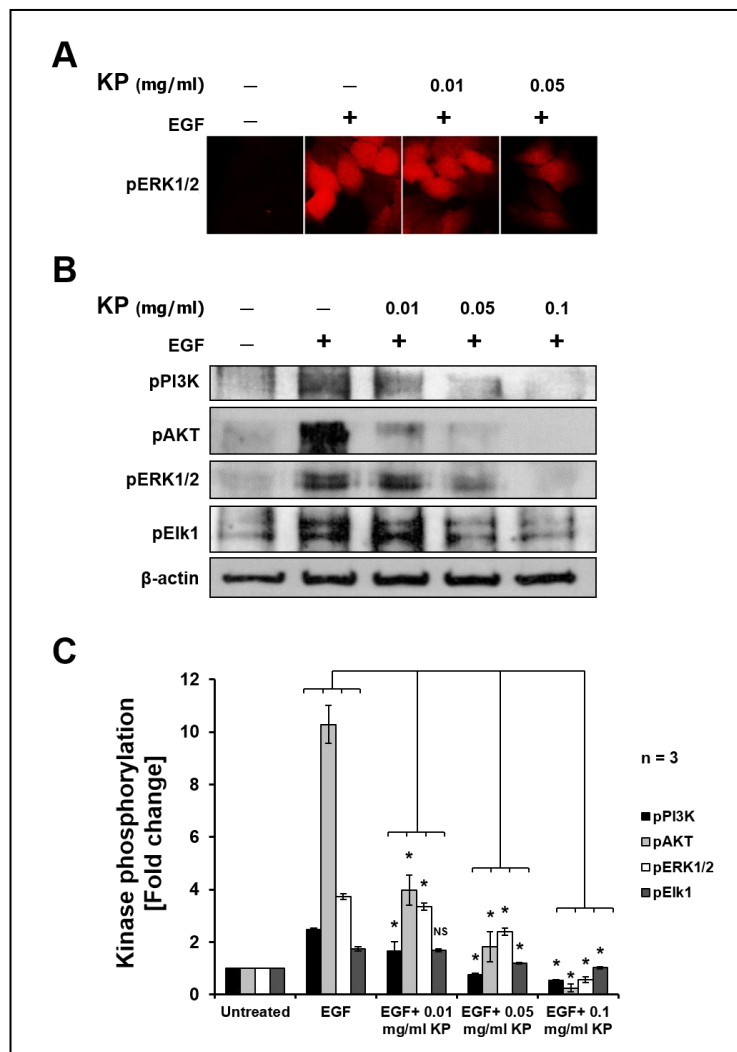


FIGURE 6 | The effects of KP extract on suppressing growth and survival signal transduction pathways. (A) Immunofluorescence of pERK1/2 in HeLa cells treated with KP extract. (B) Western blot showing immunoreactive bands of pPI3K, pAKT, pERK1/2, pElk1, and b-actin of HeLa cells stimulated with 100 ng/mL EGF and treated with different concentrations of KP extract. (C) Quantitative analysis of phosphorylation status of PI3K, AKT, ERK1/2, and Elk1 of HeLa cells treated with 100 ng/mL EGF and different concentrations of KP extract. Beta actin was used as an internal control and for normalization. Data are representative of three independent replicates and are expressed as mean $\pm$ SD. * $p < 0.05$ compared with untreated cells.

1.4 Discussion

Although the 5-year overall survival for women diagnosed with early-stage cervical cancer is more than 90% (Quinn et al., 2006), cervical cancer is currently the fourth most common cancer in women worldwide (Zigras et al., 2017). Conventionally, chemotherapy, radiation, and surgery are common treatments for all patients, however, most of these conventional therapeutic strategies have severe adverse effects to the patients (Petignat and Roy, 2007). Alternative medicines have emerged as one of potential treatments with less side effects and a more affordable cost (Wang et al., 2013), and in particular medicinal herbs are major sources of anti-cancer drug discovery and development (Desai et al., 2008). In the current study, we investigated KP, which has been reported to possess anti-cancer properties against a number of different cancer cell lines (Banjerdpongchai et al., 2008). However, evidence for mechanism of action of KP is very limited, and in particular data about its

roles in modulating molecular signal transduction in cervical cancer is not available. In this study, we investigated the anti-cancer activities of KP against the cervical cancer cell line HeLa. We found that KP extract reduced cervical cancer cell viability in a concentration dependent manner. Our results are consistent with previous studies showing that KP exhibited cytotoxicity to human colorectal carcinoma (HCT-15) cells and to human leukemic (U937) cells (Banjerdpongchai et al., 2008, 2009). Moreover, it has been reported that 5,7,4-trimethoxyflavone (KP.8.10), which is one of the major constituents of KP, inhibited proliferation of human cholangiocarcinoma cell lines (HuCCA-1 and RMCCA-1) (Leardkamolkarn et al., 2009). Results from our study highlight the genuine cytotoxic activity of KP against different cancers. Based on the results from the MTT assay, we further proved that KP reduces viability of HeLa cells via the induction of programmed cell death. We first monitored changes in morphology of HeLa cells exposed to a cytotoxic concentration of KP extract (0.5 mg/mL) at different time points and observed that KP extract gradually induced morphological changes over time and eventually caused HeLa cell detachment from the surface of the culture dish at 24 h of incubation. The rounding and detachment of cells is one of the classic hallmarks of programmed cell death. To further verify our hypothesis that KP induces apoptosis in HeLa cells, we stained KP-treated cells with annexin V/PI and performed flow cytometry analysis. We found that KP significantly induced apoptosis in HeLa cells after 6 h of incubation and this induction was in a concentration-dependent manner. Our observations are similar to those reported in 2008 where an ethanolic extract of KP significantly induced apoptosis in HL-60 cells as evaluated by flow cytometry (Banjerdpongchai et al., 2008). Consistent with our results obtained from flow cytometry, when KP-treated HeLa cells were stained with Hoechst 33342, the aberration of the nuclei was observed. Specifically, we clearly showed that KP induced nuclear deformity and nuclear fragmentation in HeLa cells. Nuclear fragmentation is known to be one of major characteristics of cells undergoing apoptosis where the nuclear lamina is destabilized by active caspases (Elmore, 2007). Therefore, nuclear fragmentation observed in our study confirms that KP kills HeLa cells via the induction of apoptosis. We next defined which specific apoptotic signaling pathways are activated in response to KP treatment. Western blot analysis clearly demonstrated that KP induced caspase 7 and 9 activation in a concentration-dependent manner, indicating that the intrinsic apoptotic pathway is stimulated in response to KP extract exposure. However, KP did not induce any change in the level of full-length or cleaved BID indicating that caspase 8 in the Fas signaling pathway (extrinsic pathway) was not activated in HeLa cells treated with KP extract. Hence, we proposed that the activation of the intrinsic apoptotic pathway is an underlying mechanism of action of KP in inducing HeLa cell death. The observation that KP extract at cytotoxic concentrations could potentially induce apoptosis in HeLa cells is interesting. However, we additionally explored the effects of KP at noncytotoxic concentrations in modulating growth and survival signaling of cervical cancer cells, and major signal transduction pathways relevant to cell growth and cell survival including the MAPK and PI3K/AKT pathways were investigated and in particular the inhibitory effects of KP on growth and survival signaling over the influence of EGF was explored. EGF is a growth factor that stimulates cell growth, proliferation, and differentiation of target cells by binding and activating the specific EGFR receptor (She et al., 2005). The activation of the tyrosine kinase EGFR leads to further stimulation of

several different downstream signal transduction cascades, including the MAPK and PI3K/AKT pathways, which eventually lead to changes in cellular activity (She et al., 2005). One of important events after EGF activation is an increase in cell division which is a hallmark of tumors (Normanno et al., 2006). Furthermore, constitutive activation of the PI3K/AKT pathway in most cancers is typical, and has been shown to promote cancer cell survival (Zhang et al., 2015). Therefore, agents or therapeutic strategies that can adequately inhibit the over activation of EGF or the downstream effectors in the signaling cascade would be interesting targets for developing as effective anti-cancer therapies (Harari, 2004). We hypothesized that KP extract at low and non-cytotoxic concentrations may suppress the activation of the MAPK and AKT signaling. The results confirmed our hypothesis since KP extract significantly suppressed phosphorylation of PI3K, AKT, ERK1/2, and Elk1. The reduction in phosphorylation status observed in PI3K and AKT suggests that survival signaling in HeLa cells is interrupted even though EGF was present. Similar observations were seen in ERK1/2 in cells exposed to KP extract at non-cytotoxic concentrations, suggesting that cell growth and proliferation signaling in HeLa cells is suppressed. MAPKs are among the central elements that transduce extracellular stimuli into cellular responses, and are known to play a crucial role in cell growth (Seger and Krebs, 1995). In general, when ERK1/2 is activated by phosphorylation, it translocates into the nucleus and further phosphorylates and activates several different transcription factors involved in cell cycle progression (Chambard et al., 2007). One of those pERK1/2 targets is Elk1 (Mebratu and Tesfagzi, 2009). A noticeable decrease in Elk1 phosphorylation in KP-treated HeLa cells along with that of ERK1/2 verifies that KP has a potent property to inhibit EGF-dependent activation of HeLa cell growth and survival signaling. It is reasonable to conclude that KP extract can not only induce cell apoptosis but also suppresses growth and survival of cervical cancer cells. Besides effects of KP extract on growth and survival signaling, there are other interesting aspects to be explored. The observation that phosphorylation of Elk1 is significantly reduced after incubation with KP extract led us to believe that KP also has a role in regulating the remodeling of the extracellular matrix of HeLa cells via interfering with the expression of metalloproteinase 2. It has been reported that the activated Elk1 controls the expression of molecules engaged in the proteolysis of the extracellular matrix, such as MMP-2 and MMP-9 (Choi et al., 2011). Consequently, Elk1 is able to control cell migration and invasion (Odrowaz and Sharrocks, 2012). Interestingly, consistent with previous reports, we found that KP inhibited the activity of MMP-2 in a concentration dependent manner and this effect was still observed in HeLa cells treated with KP extract in the presence of EGF. This means that KP has a potent effect over the effect of EGF to suppress MMP-2 production. MMP expression has been shown to be linked to tumor invasion in many different tumors (Liotta et al., 1980; Cottam et al., 1992; Garzetti et al., 1995; Fidler, 1997; Fishman et al., 1997; Gohji et al., 1998; John and Tuszynski, 2001). In addition, many clinical studies have emphasized the association of MMP expression with progression of cervical cancer (Zhou et al., 2002; Asha Nair et al., 2003), and many types of human tumor have been reported to be associated with increased expression of MMP-2 (Di Nezza et al., 2002; Sato et al., 2004; Berube et al., 2005). Our current study reporting that KP extract can effectively reduce MMP-2 activity provides valuable information to support that KP may inhibit migration and invasion of cervical cancer cells. Therefore, we performed

cell migration and invasion assays and found that KP extract at non-cytotoxic concentrations significantly inhibited migration and invasion of HeLa cells in a concentration-dependent manner. This observation elicits a role for KP extract in suppressing remodeling of the extracellular matrix and inhibiting migration and invasion. This statement is supported by our finding that KP inhibited the PI3K/AKT signaling which is a signal transduction pathway reported to be associated with cell motility and invasion (Zhou and Wong, 2006). Since KP extract at the concentrations that did not kill cells could successfully suppress migration and invasion of HeLa cells, we strongly believe that KP possesses its authentic anticancer property against cervical cancer, at least in part, through inhibition of migration and invasion.

1.5 Conclusion

This study presents accumulated evidence that *Kaempferia parviflora* extract possesses anti-cancer properties including the suppression of growth and survival signaling pathways, inhibition of metalloproteinase 2 activity, inhibition of cell migration and invasion, and induction of apoptosis in the HeLa cervical cancer cell line. Our report strongly suggests that *Kaempferia parviflora* contains active compounds which may directly or indirectly inhibit EGF-dependent signal transduction pathways and subsequently suppress tumor progression and induce cancer cell death. Identification of potential active KP compounds along with determination of their interaction with EGF receptor or specific downstream effectors is worth further investigation since the obtained information would be beneficial for verifying that *Kaempferia parviflora* can be used as a valid chemopreventive and chemotherapeutic agent in cervical cancer treatment.

1.6 References

- Asha Nair, S., Karunakaran, D., Nair, M. B., and Sudhakaran, P. R. (2003). Changes in matrix metalloproteinases and their endogenous inhibitors during tumor progression in the uterine cervix. *J. Cancer Res. Clin. Oncol.* 129, 123–131. doi: 10.1007/s00432-002-0411-9
- Banjerdpongchai, R., Chanwikruy, Y., Rattanapanone, V., and Sripanidkulchai, B. (2009). Induction of apoptosis in the human Leukemic U937 cell line by *Kaempferia parviflora* Wall.ex.Baker extract and effects of paclitaxel and camptothecin. *Asian Pac. J. Cancer Prev.* 10, 1137–1140.
- Banjerdpongchai, R., Suwannachot, K., Rattanapanone, V., and Sripanidkulchai, B. (2008). Ethanolic rhizome extract from *Kaempferia parviflora* Wall. ex. Baker induces apoptosis in HL-60 cells. *Asian Pac. J. Cancer Prev.* 9, 595–600.
- Berube, M., Deschambeault, A., Boucher, M., Germain, L., Petitclerc, E., and Guerin, S. L. (2005). MMP-2 expression in uveal melanoma: differential activation status dictated by the cellular environment. *Mol. Vis.* 11, 1101–1111.
- Canavan, T. P., and Doshi, N. R. (2000). Cervical cancer. *Am. Fam. Physician* 61, 1369–1376.
- Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science* 296, 1655–1657. doi: 10.1126/science.296.5573.1655
- Chakravarti, A., Chakladar, A., Delaney, M. A., Latham, D. E., and Loeffler, J. S. (2002). The epidermal growth factor receptor pathway mediates resistance to sequential administration of

radiation and chemotherapy in primary human glioblastoma cells in a RAS-dependent manner. *Cancer Res.* 62, 4307–4315.

Chambard, J.-C., Lefloch, R., Pouyssegur, J., and Lenormand, P. (2007). ERK implication in cell cycle regulation. *Biochim. Biophys. Acta* 1773, 1299–1310. doi: 10.1016/j.bbamcr.2006.11.010

Choi, B. D., Jeong, S. J., Wang, G., Park, J. J., Lim, D. S., Kim, B. H., et al. (2011). Secretory leukocyte protease inhibitor is associated with MMP-2 and MMP-9 to promote migration and invasion in SNU638 gastric cancer cells. *Int. J. Mol. Med.* 28, 527–534. doi: 10.3892/ijmm.2011.726

Cottam, D. W., Rennie, I. G., Woods, K., Parsons, M. A., Bunning, R. A., and Rees, R. C. (1992). Gelatinolytic metalloproteinase secretion patterns in ocular melanoma. *Invest. Ophthalmol. Vis. Sci.* 33, 1923–1927.

Denny, L., Quinn, M., and Sankaranarayanan, R. (2006). Chapter 8: screening for cervical cancer in developing countries. *Vaccine* 24(Suppl. 3), S71–S77. doi: 10.1016/j.vaccine.2006.05.121

Desai, A. G., Qazi, G. N., Ganju, R. K., El-Tamer, M., Singh, J., Saxena, A. K., et al. (2008). Medicinal plants and cancer chemoprevention. *Curr. Drug Metab.* 9, 581–591. doi: 10.2174/138920008785821657

Di Nezza, L. A., Misajon, A., Zhang, J., Jobling, T., Quinn, M. A., Östör, A. G., et al. (2002). Presence of active gelatinases in endometrial carcinoma and correlation of matrix metalloproteinase expression with increasing tumor grade and invasion. *Cancer* 94, 1466–1475. doi: 10.1002/cncr.10355

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* 35, 495–516. doi: 10.1080/01926230701320337

Fidler, I. J. (1997). “Molecular biology of cancer: invasion and metastasis,” in *Cancer: Principles and Practice of Oncology*, 5th Edn, eds V. T. De Vita, S. Hellman, and S. A. Rosenberg (Philadelphia, PA: Lippincott-Raven), 135–152.

Fishman, D. A., Bafetti, L. M., Banionis, S., Kearns, A. S., Chilukuri, K., and Stack, M. S. (1997). Production of extracellular matrix-degrading proteinases by primary cultures of human epithelial ovarian carcinoma cells. *Cancer* 80, 1457–1463. doi: 10.1002/(SICI)1097-0142(19971015)80:8<1457::AID-CNCR13>3.0.CO;2-4

Garzetti, G. G., Ciavattini, A., Lucarini, G., Goteri, G., de e Nicolis, M., Garbisa, S., et al. (1995). Tissue and serum metalloproteinase (MMP-2) expression in advanced ovarian serous cystadenocarcinomas: clinical and prognostic implications. *Anticancer Res.* 15, 2799–2804.

Geisler, J. P., Swathirajan, J., Wood, K. L., and Manahan, K. J. (2012). Treatment of advanced or recurrent cervical cancer with cisplatin or cisplatin containing regimens: a cost effective analysis. *J. Cancer* 3, 454–458. doi: 10.7150/jca.4807

Gohji, K., Fujimoto, N., Hara, I., Fujii, A., Gotoh, A., Okada, H., et al. (1998). Serum matrix metalloproteinase-2 and its density in men with prostate cancer as a new predictor of disease extension. *Int. J. Cancer* 79, 96–101. doi: 10.1002/(SICI)1097-0215(19980220)79:1<96::AID-IJC18>3.0.CO;2-F

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Harari, P. M. (2004). Epidermal growth factor receptor inhibition strategies in oncology. *Endocr. Relat. Cancer* 11, 689–708. doi: 10.1677/erc.1.00600

Huang, H. S., Nagane, M., Klingbeil, C. K., Lin, H., Nishikawa, R., Ji, X. D., et al. (1997). The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J. Biol. Chem.* 272, 2927–2935. doi: 10.1074/jbc.272.5.2927

John, A., and Tuszynski, G. (2001). The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. *Pathol. Oncol. Res.* 7, 14–23. doi: 10.1007/BF03032599

Leardkamolkarn, V., Tiemyuyen, S., and Sripanidkulchai, B. O. (2009). Pharmacological activity of *Kaempferia parviflora* extract against human bile duct cancer cell lines. *Asian Pac. J. Cancer Prev.* 10, 695–698.

Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., and Shafie, S. (1980). Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* 284, 67–68. doi: 10.1038/284067a0

Liu, H., Wang, H., Li, C., Zhang, T., Meng, X., Zhang, Y., et al. (2016). Spheres from cervical cancer cells display stemness and cancer drug resistance. *Oncol. Lett.* 12, 2184–2188. doi: 10.3892/ol.2016.4893

Margolis, B. (1992). Proteins with SH2 domains: transducers in the tyrosine kinase signaling pathway. *Cell Growth Diff.* 3, 73–80.

McGuire, S. (2016). World cancer report 2014. Geneva, Switzerland: world health organization, international agency for research on cancer, WHO press, 2015. *Adv. Nutr.* 7, 418–419. doi: 10.3945/an.116.012211

Mebratu, Y., and Tesfagzi, Y. (2009). How ERK1/2 activation controls cell proliferation and cell death is subcellular localization the answer? *Cell Cycle* 8, 1168–1175.

Moody, C. A., and Laimins, L. A. (2010). Human papillomavirus oncoproteins: pathways to transformation. *Nat. Rev. Cancer* 10, 550–560. doi: 10.1038/nrc2886

Moscato, D. K., Holgado-Madruga, M., Emlet, D. R., Montgomery, R. B., and Wong, A. J. (1998). Constitutive activation of phosphatidylinositol 3-kinase by a naturally occurring mutant epidermal growth factor receptor. *J. Biol. Chem.* 273, 200–206. doi: 10.1074/jbc.273.1.200

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63. doi: 10.1016/0022-1759(83)90303-4

Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M. R., et al. (2006). Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene* 366, 2–16. doi: 10.1016/j.gene.2005.10.018

Odrowaz, Z., and Sharrocks, A. D. (2012). The ETS transcription factors ELK1 and GABPA regulate different gene networks to control MCF10A breast epithelial cell migration. *PLOS ONE* 7:e49892. doi: 10.1371/journal.pone.0049892

Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.* 19, 3159–3167. doi: 10.1093/emboj/19.13.3159

Patanasethanont, D., Nagai, J., Matsuura, C., Fukui, K., Sutthanut, K., Sripanidkulchai, B. O., et al. (2007). Modulation of function of multidrug resistance associated-proteins by *Kaempferia parviflora* extracts and their components. *Eur. J. Pharmacol.* 566, 67–74. doi: 10.1016/j.ejphar.2007.04.001

Petignat, P., and Roy, M. (2007). Diagnosis and management of cervical cancer. *BMJ* 335, 765–768. doi: 10.1136/bmj.39337.615197.80

Quinn, M. A., Benedet, J. L., Odicino, F., Maisonneuve, P., Beller, U., Creasman, W. T., et al. (2006). Carcinoma of the cervix uteri. FIGO 26th annual report on the results of treatment in gynecological cancer. *Int. J. Gynaecol. Obstet.* 95(Suppl. 1), S43–S103. doi: 10.1016/S0020-7292(06)60030-1

Roomi, M. W., Monterrey, J. C., Kalinovsky, T., Rath, M., and Niedzwiecki, A. (2010). In vitro modulation of MMP-2 and MMP-9 in human cervical and ovarian cancer cell lines by cytokines, inducers and inhibitors. *Oncol. Rep.* 23, 605–614. doi: 10.3892/or_00000675

Safaeian, M., and Solomon, D. (2007). Cervical cancer prevention – cervical screening: science in evolution. *Obstet. Gynecol. Clin. North Am.* 34, 739–799. doi: 10.1016/j.ogc.2007.09.004

Saokaew, S., Wilairat, P., Raktanyakan, P., Dilokthornsakul, P., Dhippayom, T., Kongkaew, C., et al. (2016). Clinical effects of *Krachaidum* (*Kaempferia parviflora*): a systematic review. *J. Evid. Based Complement. Altern. Med.* doi: 10.1177/2156587216669628 [Epub ahead of print].

เนื้อหาบทวิจัย 2: **Anti-cancer effects of *Kaempferia parviflora* on ovarian cancer SKOV3 cells**

1.1 Introduction

Ovarian cancer is one of the three common gynecological cancers worldwide after cervical and uterine cancers [1]. However, it is the most leading cause of death among these three gynecologic cancers [2]. Compared to others, ovarian cancer has the poorest prognosis, with the five-year survival rate of 44% for all stages [3]. Up to 70% of all ovarian cancer cases are high-grade carcinomas which grow aggressively, metastasize rapidly, and have high chromosomal instability [4, 5]. Asymptomatic or non-specific symptoms at an early stage together with poor screening method makes ovarian cancer a late diagnostic tumor. Chemotherapeutic drugs are treatment choices for unresectable tumor. However, they have many side effects including hair loss, fatigue, bone marrow suppression, and bleeding which can lower the quality of patient life [6]. Even though many new chemotherapeutics have been developed, the drugs are less accessible for many patients due to their high cost. We hope that our findings of effective medicinal plant tested in vitro may be an important step valuable for pacing into the next level of drug discovery and to be a complementary option with reasonable cost for patients with ovarian cancer. *Kaempferia parviflora* (KP) is a Thai traditional plant in the Zingiberaceae family. It is commonly known as Thai black ginger or in Thai as “Krachai dum”. KP has been previously demonstrated to have several pharmacological effects including anti-plasmodial, anti-fungal, anti-mycobacterial [7], and anti-cancer properties [7–9]. We

previously described the anti-cancer property of KP against cervical cancer HeLa cells showing the promising possibility that KP may be used as a potential agent for cervical cancer treatment [10]. However, the anti-cancer effects of KP against ovarian cancer have not yet been reported. This leads us to investigate anti-cancer properties of KP against a high-grade ovarian cancer cell line, SKOV3, which is highly resistant to many cytotoxic agents. Since epidermal growth factor receptor (EGFR), is strongly expressed in ovarian cancer [11] and involved in cell proliferation, cell migration, cell survival, and metastasis, we therefore examined the effects of KP on SKOV3 alone and under the influence of EGF to verify whether KP can overcome the EGF-dependent growth and survival signal transduction pathways. Nevertheless, the molecular mechanisms of how KP suppresses tumor growth and survival were also explored. In particular, the effects of KP on the PI3K/AKT and MAPK pathways which are important signal transduction pathways for tumorigenesis [12, 13] were defined.

1.2 Materials and methods

Cell culture

Human ovarian cancer SKOV3 cells were obtained from ATCC (ATCC, Manassas, VA, United States) and maintained in (Roswell Park Memorial Institute) RPMI-1640 medium (Gibco, BRL, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, USA) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) (Caisson, USA) and incubated at 37 °C in a humidified atmosphere, 5% CO₂. The cells were sub-cultured every 2–3 days. Extraction of *Kaempferia parviflora* rhizomes The rhizomes of *Kaempferia parviflora* with voucher specimen number (R-CMUKP002) authenticated by Dr. Angkhana Inta and deposited at the Faculty of Science, Chiang Mai University, Thailand, were harvested from the CMU-RSPG *Kaempferia* housing at Chiang Dao, Chiang Mai Province, Thailand. For the extraction, chopped rhizomes of the plant were extracted with 95% ethanol at room temperature (RT) for 3 days and filtered before concentrated using a rotary evaporator. After solvent evaporation, the plant ethanolic extraction yielded 9.85% dry weight of KP rhizomes. One milliliter of DMSO was used to dissolve 1 g of KP extract to make a 1 g/mL stock solution. The KP stock was pre-diluted in medium prior to each treatment. Each experiment was performed with three independent batches of KP extract, each assayed in triplicate. The final concentration of DMSO was maintained below 0.5% v/v throughout the experiment.

Cell viability assay

The cytotoxicity of KP on SKOV3 cells was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide). Cells were seeded at a density of 1×10^4 cells per well in 96-well plates overnight and treated with KP or DMSO (vehicle control 0.006–0.1%) in quadruplicate. For the treatment group, cells were incubated with complete media containing different concentrations of KP extract, ranging from 0 to 10 mg/mL with or without the presence of 100 ng/ml of EGF. After 24 h, cells were incubated with 0.5 mg/mL MTT reagent (Appllichem GmbH, Germany) for 1–3 h. The culture supernatant was aspirated and 100 µl of DMSO was added to each well. The absorbance was measured at 570 nm using Synergy™ H4 Hybrid Multi-Mode Microplate Reader. Cell viability assay was performed in 3 individual experiments.

Cell counting

Cells were seeded in 24-well plates at a density of 0.05×10^6 cells/well in culture media and incubated for 24 h at 37 °C, 5% CO₂. Cells were treated with KP extract at non-toxic concentrations (0.01, 0.025, and 0.05 mg/mL). The total number of cells at different time points (0, 24, 48, 72 and 96 h) was counted using a haemocytometer. The doubling time of the cell was calculated according to the following formula: Doubling time = (Time $\times$ log2)/(log(final number)-log(initial number)).

Gelatin zymography

The activity of MMP-2 and MMP-9 was examined using gelatin zymography. The sample culture supernatants of SKOV3 cells (1×10^6 cells in a 3-cm dish) incubated with different concentrations of KP extract (0, 0.01, 0.05, and 0.1 mg/mL) with or without the presence of EGF (100 ng/mL) for 24 h were collected. The sample culture supernatants were separated in 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 cold running conditions. After electrophoresis, the gels were incubated with 2.5% Triton X-100 twice (for 30 min each), at RT and washed with 10 mM Tris buffer, pH 8.0 for 2 min. The gels were incubated with 1% gelatinase buffer (50mMTris HCl, 10 mMCaCl₂, pH 8) overnight at 37 °C. The gels were stained with 0.5% (w/v) Coomassie brilliant blue R250 (Bio-Rad Laboratories) in 50% methanol and 10% glacial acetic acid for 30 min and destained with a destaining solution (10% acetic acid and 50% methanol). Proteolytic activities of MMP-2 and MMP-9 were visualized as clear zone bands on a blue background and analyzed using ImageJ software.

Wound healing assay

SKOV3 cells (0.5×10^6 cells/well) were seeded and cultured in 24-well plates for 24 h. A scratch wound was made by using 200 μ L pipette tip. 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.

Cell migration

A Cell Culture Insert (8 μ m) (SPL Life Sciences, South Korea) was used to confirm the effect of KP on suppressing cell migration. Cells at a density of 0.3×10^6 cells/well were seeded in the upper chambers and cultured in serum-free media for 24 h. The next day cells in the upper chamber were treated with different concentrations (0, 0.01, 0.05, and 0.1 mg/mL) of KP in serum free media (SFM), and the upper chambers were put into the (lower) wells containing RPMI with 5% FBS and incubated for 24 h. Absolute methanol was used to fix cells for 5 min at RT, and cells were then stained with 0.5% crystal violet for 30 min. The upper chambers were washed for 3 times with water, and cells attached to the surface inside the chamber were removed with a cotton swab and the stained cells attached at the other site of the chamber were captured and analyzed with the ImageJ software.

Cell invasion assay

The effects of KP on SKOV3 cell invasion were determined using Cell Culture Inserts (SPL life sciences, Korea). The polycarbonate invasion chambers (8 μ m pore size) were coated with Matrigel® Matrix (356,234, Lot 4,272,006, Corning, Bedford, USA) per well and incubated at RT for 1–4 h. Cells, at a density of 0.25×10^6 cells per well, were seeded on Matrigel with 0.01 and 0.05 mg/mL of KP in serum-free media and the invasion chambers were put into the wells (the lower) containing RPMI with 10% FBS and incubated for 20 h. Cells were then fixed with absolute methanol for 5 min at RT and stained with 0.5% crystal violet for 15 min. After three washes with water, cells in the invasion chambers were removed with cotton swab and the pictures of the stained cells attached at the other site of the invasion chamber were taken and analyzed with ImageJ software.

Trypan blue exclusion test

Cells were seeded at a density of 0.05×10^6 cells/well in 24-well plates and incubated with different cytotoxic concentrations (0, 0.05, 0.1, and 0.25 mg/mL) of KP extract. Cells were harvested after 3, 6, 12, 24, and 48 h of incubation. Trypan blue solution (Gibco, USA) was added to the cell suspensions in a ratio of 1:1. Total cells and dead cells (stained in blue) were counted using haemocytometer. The percentage of living cells and dead cells was calculated.

Cell apoptosis assay

Cell apoptosis was assessed by annexin-V-FITC/propidium iodide (PI) staining. Cells were seeded at a 0.3×10^6 cells/well density in 3-cm cell culture dishes and cultured for 24 h. Cells were treated with different concentrations of KP extract (0, 0.1, 0.3, and 0.5 mg/mL) for 12 h. Cells were harvested and resuspended in 1X annexin-V binding buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5 mM DTT, 50% glycerol). Cells were incubated with annexin V-FITC (ImmunoTools, Germany) and propidium iodide (PI) (Sigma Aldrich) for 15 min in the dark at RT before performing flow cytometry.

Nuclear staining

SKOV3 cells were seeded at a density of 0.5×10^6 cells/well on glass coverslips for 24 h. Cells were treated with KP extract at different concentrations (0.1, 0.3, and 0.5 mg/mL) and incubated for 7 h. Cells were fixed with 4% paraformaldehyde/PBS at RT for 15 min. Then, cells were washed thrice and incubated with 5 μ g/mL of Hoechst 33342 in PBS (Thermo Fisher Scientific, Thailand) for 1 h. Following staining, the sample slides were washed twice with PBS for 5 min each time, and the sample slides were mounted using Fluoromount media (SouthernBiotech, United States). Cells were 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.

Western blot analysis

SKOV3 cells were seeded in 3-cm dishes at a density of 0.3×10^6 cells/well for 24 h. The next day, media were changed to SFM and cells were cultured for 24 h. Cells were treated with KP extract at non-toxic concentrations (0.01 and 0.05 mg/mL) for 6 h and 100 ng/mL of EGF was added to the wells 15 min before harvesting cells. Cell lysates were prepared by adding 300 μ L of 1X reducing Laemmli buffer and heating at 95 °C for 5 min. Cell lysates were separated by SDS-PAGE for 90 min at 140 V and transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, MA) for 120 min at 100 V. After electrophoresis, membranes were blocked with 5% skim milk in TBS containing 0.1%

tween-20 (TBST) at RT for 1 h. The blots were incubated with primary antibodies (1:10000 of anti- β -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) at 4 °C overnight. Anti- β -actin was obtained from US biological (USA) and the remaining antibodies were purchased from Cell Signaling Technology (USA). The membranes were washed and incubated with an anti-mouse Ig conjugated with IRDye®800CW (1:5000) or an anti-rabbit Ig conjugated with IRDye®680RT (1:5000) at RT for 2 h. The immunoreactive bands were visualized by Odyssey ® CLx Imaging System - LI-COR Biosciences (USA). The bands were analyzed using Image Studio Lite.

Statistical analysis

Data are presented as mean $\pm$ SD. Data were analyzed by one-way ANOVA and P-value < 0.05 was considered statically significant.

1.3 Results

The effect of KP on SKOV3 cell viability and cell proliferation

To investigate antitumor properties of KP, we first evaluated its cytotoxicity to SKOV3 by using MTT assay. We found that cells treated with KP extract at different concentrations (0.006–1 mg/mL) for 24 h showed significant reduction in cell viability in a concentration-dependent manner from the range of 0.09 mg/mL to 1 mg/mL as shown in Fig. 1a. The half maximal inhibitory concentration (IC₅₀) of KP extract was 0.53 ± 0.08 mg/mL. Since epidermal growth factor receptor (EGFR), which is highly expressed in ovarian cancer cells, is a very important factor for tumor growth [11], we therefore stimulated SKOV3 cells with EGF and performed MTT assay to evaluate whether KP still be able to suppress cell viability. As shown in Fig. 1b, EGF significantly increased cell viability approximately 15%. Interestingly, in the presence of EGF, KP still exhibited strong growth suppression in a concentration-dependent manner. The vehicle control, DMSO, at all concentrations, did not show any cytotoxic effect. The IC₅₀ of KP extract in the presence of EGF was 0.63 ± 0.08 mg/mL which is similar to the IC₅₀ of KP treatment without EGF.

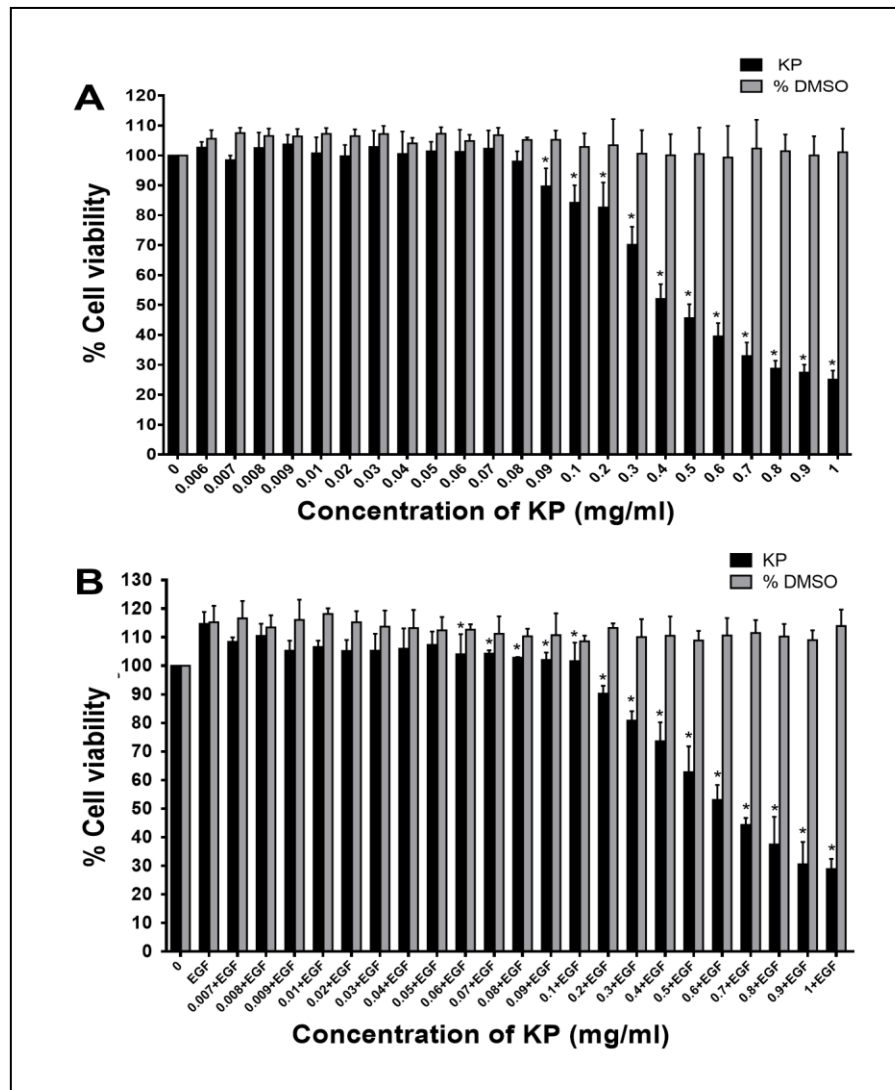


Fig. 1 The cytotoxicity effect of different concentrations of KP ethanol extract on SKOV3 cells without EGF (a) and with 100 ng/mL EGF (b). All data were from 3 independent experiments and reported as means $\pm$ SD of each quadruplicate *P < 0.05 compared to the control (untreated and EGF)

We further performed cell counting at 24, 48, 72, 96 h after KP treatment and found that KP extract significantly reduced the number of cells in a concentration-dependent manner (Fig. 2). These observations were still seen in the treatment with the presence of EGF. The number of cell from different time points were used to calculate the doubling time which is the time required for cell dividing from one to two cells. The doubling time of SKOV3 cell was approximately 24 h. Interestingly, cells treated with KP at 0.025 and 0.05 mg/mL significantly increased cell doubling time to 32.6 h, and 31.5 h, respectively.

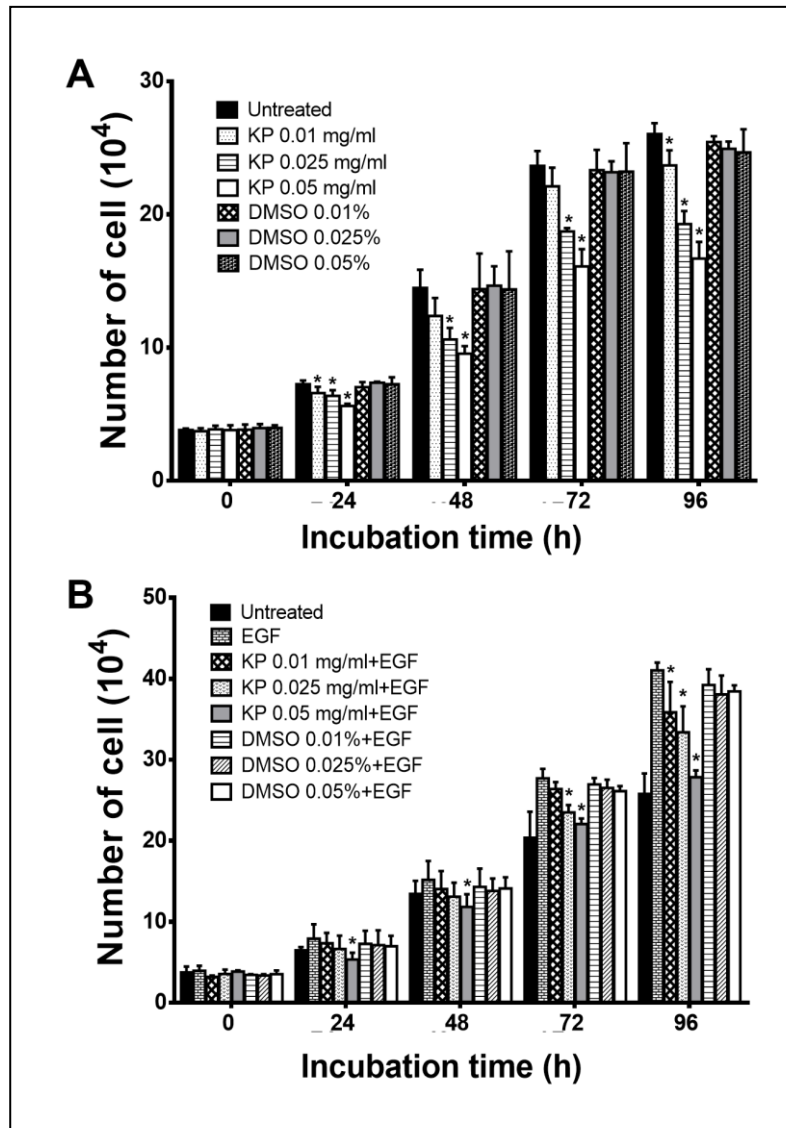


Fig. 2 The number of SKOV3 cells treated with non-toxic concentrations of KP (0.01, 0.025, 0.05 mg/mL) without EGF (a) and with 100 ng/mL EGF (b) at 24, 48, 72 and 96 h. Data are expressed as mean $\pm$ SD (n = 3). *P < 0.05 as compared to untreated (a) or EGF (b)

The effect of KP on inhibiting MMP-9 and MMP-2 activities

We next determined whether KP extract suppresses MMP-9 and MMP-2 activities. Data from zymographic analysis showed that cells treated with KP extract at 0.01 and 0.05 mg/mL reduced MMP-9 activity to $92.52 \pm 8.55\%$ and $81.92 \pm 5.18\%$ and MMP-2 activity to 88.66 ± 6.17 and $68.83 \pm 6.17\%$, respectively (Fig. 3a and b). EGF at 100 ng/mL strongly increased MMP-2 and MMP-9 activities over 140%. As we expected, KP extract with the presence of EGF was still able to suppress MMP-9 and MMP-2 activities. The percent reduction of MMP-9 activity was 113.97 ± 10.7 and 106.64 ± 9.9 mg/mL and MMP-2 activity was 121.4 ± 4.7 and 104.01 ± 10.12 mg/mL for cell treated with KP at 0.01 and 0.05 mg/mL, respectively. The immunoreactive bands of β -actin detected by western indicated the equal amount of cells in all treated groups. The effect of KP on inhibiting cell migration and invasion.

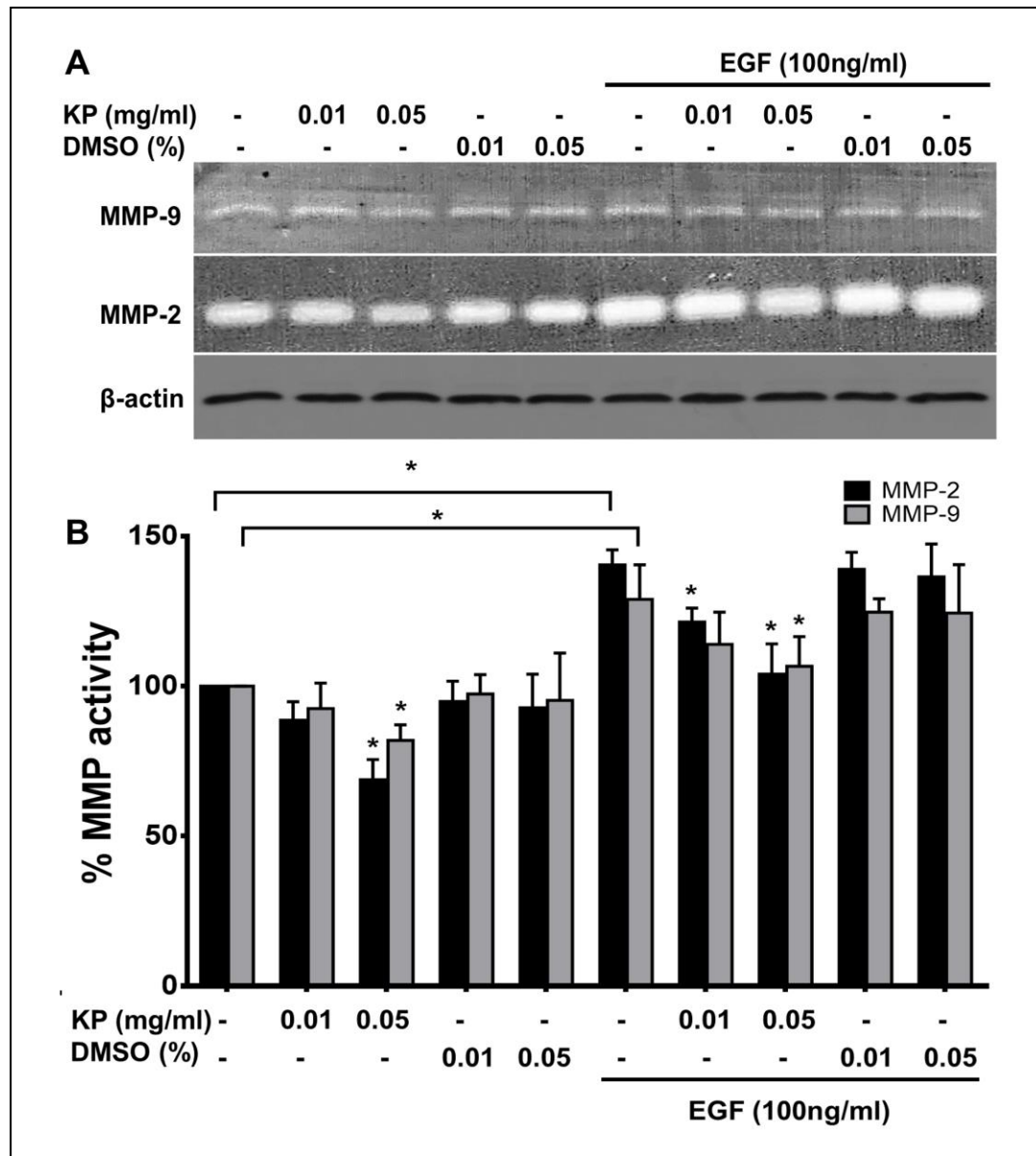


Fig. 3 The effect of KP ethanol extract on MMP-9 and MMP-2 activity. Gelatin zymogram showing MMP9 and MMP2 activities (a), Immunoreactive bands of β -actin was used as a loading control. Histogram of MMP-2 and MMP-9 activity is presented as percent of activity (b). All data were from 3 independent experiments.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

Based on the fact that MMP-9 and MMP-2 are crucial factors for tumor migration and metastasis, we therefore performed wound healing assay to examine cell migration and found that cells treated with KP at 0.01, 0.05, and 0.1 mg/mL effectively reduced the percent of cell migration to 58.30 ± 5.8 , 52.91 ± 5.32 , and $40.50 \pm 9.27\%$, respectively (Fig. 4a and b). Moreover, we confirmed the ability of KP in inhibiting cell migratory function of SKOV3 cells with Transwell migration assay. The results showed that SKOV3 cells without any treatment could migrate through the upper well to the lower chamber. However, the number of migrated cells was drastically decreased in cells treated with KP

extract whereas the vehicle control did not show any inhibitory effect on SKOV3 cell migration (Fig. 4c and d).

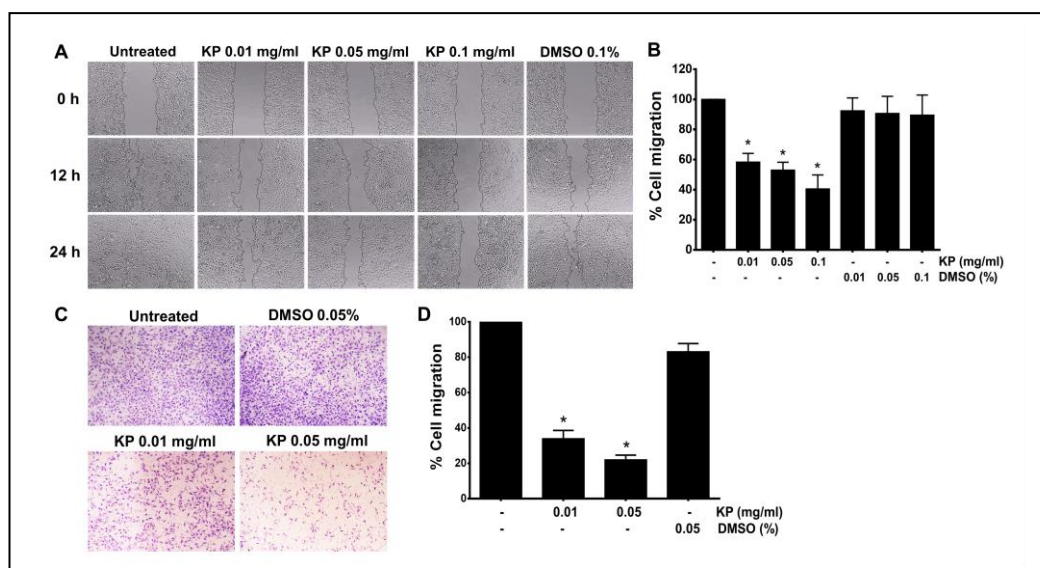


Fig. 4 The effect of KP on SKOV3 cell migration. Wound-healing assay of SKOV3 cells treated with KP ethanol extract at 0, 12, and 24 h after performing the scratch (a). Histogram represents the percentage of cell migration (b). Transwell migration assay and represented histogram are shown in c and d.

The effect of KP on inhibiting growth and survival signal transduction pathways

Several signaling molecules are involved in cell growth and survival processes in response to EGF stimulation. Those molecules include ERK1/2 and AKT proteins. We therefore investigated the possible underlying mechanism of KP that suppresses growth in SKOV3 cells. As shown in Fig. 5, we found that cells treated with KP at 0.01 and 0.05 mg/mL exhibited reduction in ERK1/2 phosphorylation to 0.85 ± 0.02 and 0.64 ± 0.031 , respectively. Even though EGF strongly activated ERK1/2 phosphorylation (2.6 fold), KP at 0.01 and 0.05 mg/mL was able to reduce the phosphorylation of ERK1/2 to 2.38 ± 0.22 and 2.21 ± 0.23 , respectively. Moreover, KP extract at 0.01 and 0.05 mg/mL reduced the phosphorylation of AKT to 0.87 ± 0.04 and 0.58 ± 0.03 without the presence of EGF and to 0.89 ± 0.04 and 0.7 ± 0.07 with the presence of EGF, respectively.

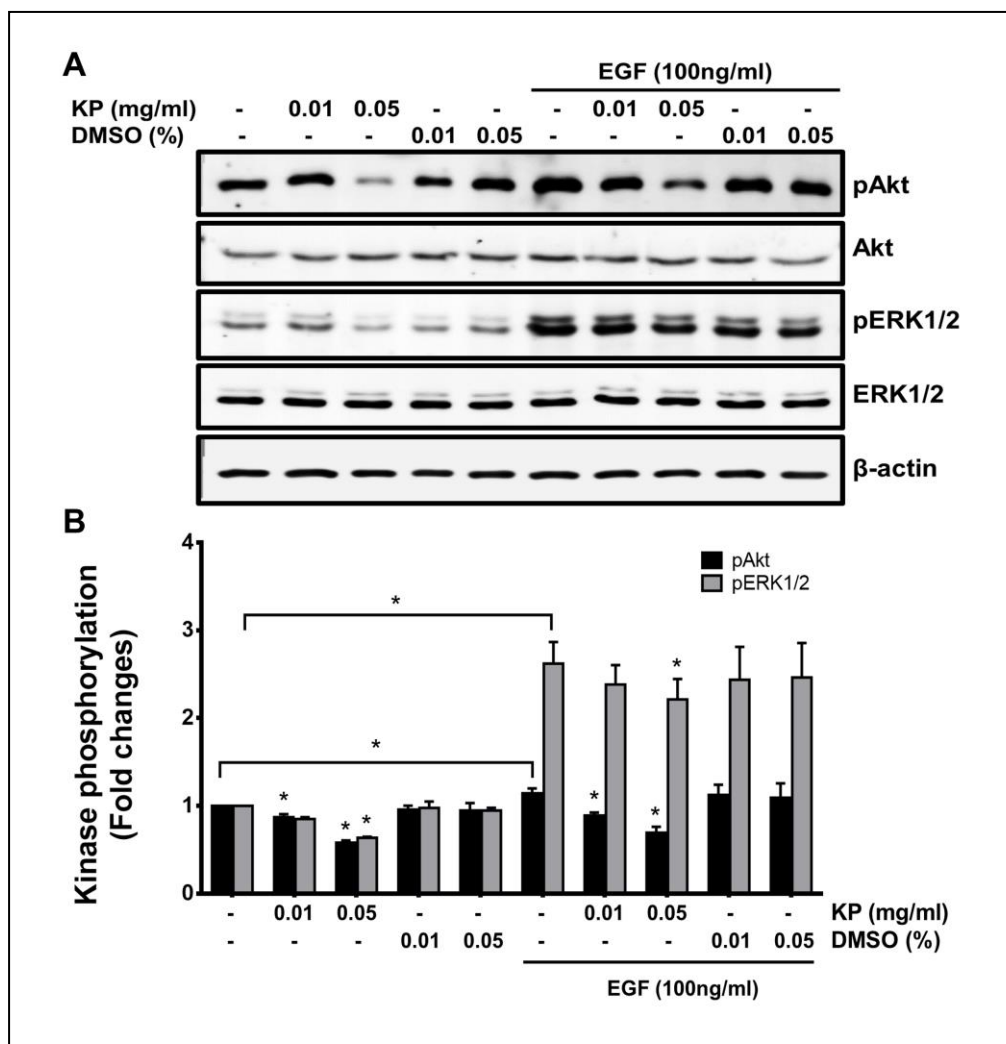


Fig. 5 The effect of KP on the PI3K/AKT and EKR1/2 MAPK signal transduction in SKOV3 cells. The immunoreactive bands of pAKT, AKT, pERK1/2 and ERK1/2 (a). Histogram of phosphorylation level of AKT and ERK1/2 (b). β -actin was used as a loading control. Data expressed as mean $\pm$ SD (n = 3). *P < 0.05

The effect of KP on inducing apoptotic cell death

Since cell viability assay showed cytotoxicity of KP extract at the concentrations over 0.1 mg/mL. We thus examined whether KP extract increases cell death by using trypan blue exclusion test. We found that cells treated with KP extract at 0.1 and 0.25 mg/mL significantly increased cell death after 24 h of incubation. The percentage of cell death was $15.67 \pm 2\%$ and $26.33 \pm 3.5\%$ with KP treatment at 0.1 and 0.25 mg/mL, respectively (Fig. 6a). Importantly, with the presence of EGF 100 ng/mL, KP extract at 0.1 and 0.25 mg/mL was able to induce cell death to $13.17 \pm 1.8\%$ and $21.25 \pm 2\%$. In order to confirm whether dead cells were apoptotic cell, fluorescence nuclear staining using Hoechst 33342 was performed after treating cells with KP for 6 h. Figure 6c showed nuclear fragmentation of SKOV3 cells treated with KP extract at 0.3 and 0.5 mg/mL. This effect was seen in cells treated with DMSO.

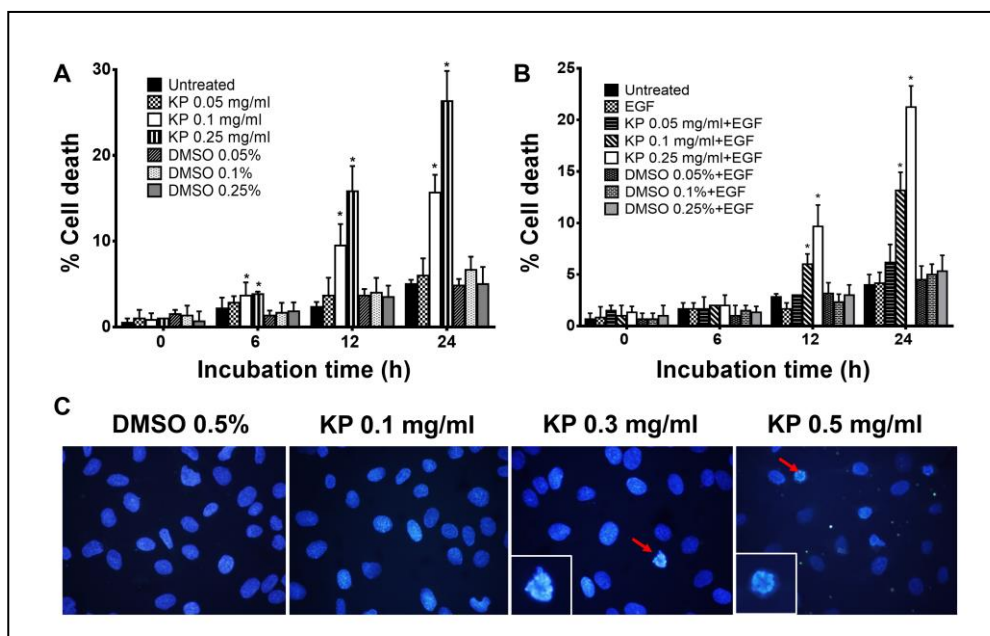


Fig. 6 The effect of KP on cell death in SKOV3 cells by trypan blue exclusion assay. Percent of cell death of cells treated with KP without EGF (a) and with 100 ng/mL EGF (b). Data presented as means $\pm$ SD, $n = 3$, * $P < 0.05$ compared to DMSO control. The DNA staining (Hoechst 33342) of KP treated cells shows nuclear deformity (c). The condensation of the nucleus was observed in KP ethanol extract treatments compared to the vehicle control DMSO. Original magnification, 400X

Based on this observation, we speculated that KP extract induces cell death via apoptosis machinery. We next determined apoptotic event by performing AnnexinV and PI fluorescent staining and detecting with flow cytometer. We found that cells treated with KP extract at 0.3 and 0.5 mg/mL increased apoptosis to $22.13 \pm 7.6\%$ and $41.13 \pm 19.15\%$, respectively (Fig. 7a, b). We further examined the activation of caspase-9, caspase-3, and caspase-7 by western blot analysis and found that the full length of all caspases was significantly reduced in a concentration-dependent manner (Fig. 7c, d). These data strongly suggest that KP induces cell death via the activation of apoptotic cell death.

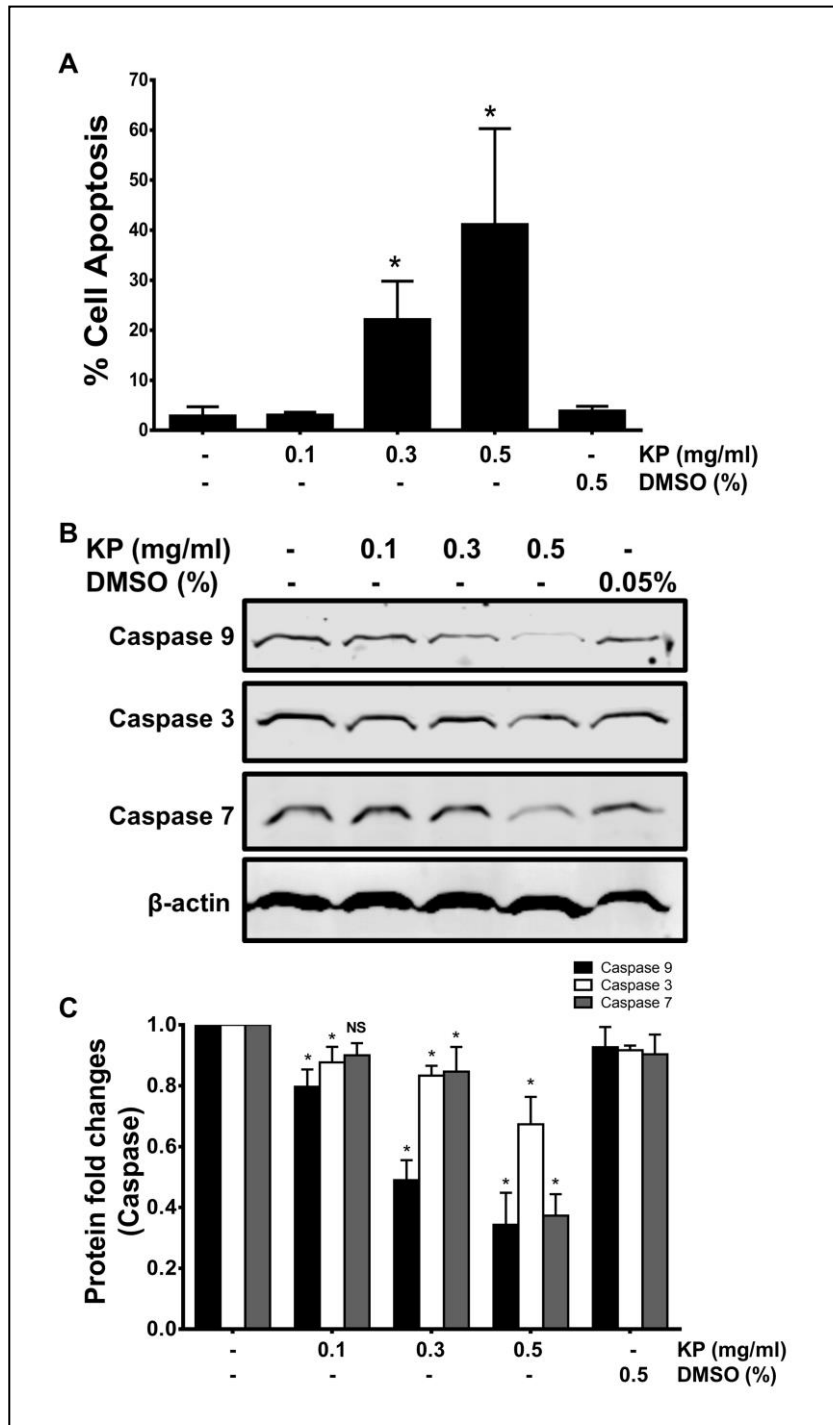


Fig. 7 The effect of KP ethanol extract on apoptosis and caspases in SKOV3 cells. Annexin V-FITC and PI labeling in KP treated cells was measured by flow cytometer and histogram of percent of apoptotic cells is shown in (a). The level of caspase-3, -9, and -7 by western blotting are shown in (b). Histogram of relative intensity of full-length of caspase-3, -9, and -7. β -actin is used as a protein loading control (c). These data are represented as mean $\pm$ SD of three replicates. * $P < 0.05$ indicates significant difference compared to control

1.4 Discussion

Ovarian cancer is the most common cause of cancer death among other gynecologic cancers [14]. No obvious symptoms are present at the early stage, thus, most of patients are diagnosed when cancer is in an advanced stage which gives rise to poor response to chemotherapies [15]. Staging is an important factor determining prognosis and clinical outcome. Stage I, is defined as tumor is confined in the ovary. This stage shows overall survival of approximately 84 months. In particular, stage IV has much less overall survival rate of only 10 months [15]. The survival rate is not only related to stages of the disease but also associated with ovarian cell types. Several cell types have been identified in ovarian cancer. The most common histologic subtype is high-grade serous adenocarcinoma which has the worst prognosis [16, 17]. We are particularly interested in using a high-grade serous adenocarcinoma, SKOV3, cell line as a model for investigating anticancer activity of *Kaempferia parviflora* in ovarian cancer. We first performed cell viability assay to evaluate the cytotoxicity of KP extract in SKOV3 cells. It was found that KP extract decreased cell viability of ovarian cancer in a concentration-dependent manner with IC₅₀ of approximately 0.5 mg/mL, which was slightly higher than the previously reported IC₅₀ of KP in cervical cancer cell line, HeLa [10]. Interestingly, KP extract also strongly exhibited the reduction of ovarian cancer cell viability in the presence of EGF, suggesting that KP has potent cytotoxic effects, which overcome the influence of EGF in maintaining cell viability. We further discovered that treating cells with KP extract at non-toxic concentrations (with or without the presence of EGF) at various time points could significantly inhibit number of cells in a concentration-dependent manner. Therefore, the doubling time was increased to approximately 1.3–1.4 folds compared to untreated SKOV3 cells. This observation suggests that KP extract may suppress SKOV3 cell proliferation. According to cell viability results, the non-toxic concentrations were chosen for further experiments. Since SKOV3 cells are known to be a high grade serous adenocarcinoma which has been reported to have high metastatic rate [16, 18]. One major factor that plays important roles in cell invasion and metastasis is matrix metalloproteinase (MMP). Extensive evidence has been shown that the increased MMP level correlates with tumor progression and metastasis, especially in advanced ovarian serous cancers [19, 20]. MMP expression, particularly MMP-2 and MMP-9, has been shown to have clinical association with progression of ovarian cancer [21, 22]. MMPs degrade various components of the extracellular matrix and play a crucial role in tumorigenesis, migration, invasion, and metastasis [23], and inhibition of MMPs by specific inhibitors has been demonstrated to markedly suppress tumor invasion and metastasis [24, 25]. Based on these previous reports, we hypothesized that KP extract may be able to modulate the expression of MMPs. Undoubtedly, our zymographic study revealed that KP extract dramatically inhibited the activity of MMP-2 and MMP-9 in a concentration-dependent manner in SKOV3 cells. The ability of KP extract in suppressing the activity of MMPs was independent on the presence of EGF, since KP extract could be able to strongly overcome the effects of EGF. Our findings are in line with our previous studies showing that KP suppressed MMP-2 production in cervical cancer, HeLa cells [10]. Similar observation was reported in colorectal carcinoma cells, where a flavonoid, myricetin, inhibited MMP-2 activity and cell invasion [26]. These let us to believe that KP extract may also be able to reduce cancer cell migration and invasion. We,

therefore, further investigated the effects of KP extract on cell migration and invasion. Generally, cancer cell migration is involved in altering the cell-matrix interface on the cell surface [27]. The overexpression of MMPs could enhance cell migration [28], whereas the inhibition of MMP activity or overexpression of tissue inhibitor of metalloproteinases (TIMPs) resulted in a decrease in cancer cell migration [29]. Our results from wound healing assays showed that KP extract suppressed cell migration of SKOV3 cells in a concentration-dependent manner. Furthermore, the migratory function of cells was confirmed by Transwell migration showing that KP extract drastically inhibited migration and invasion of SKOV3 cells. The results from invasion assay with the presence of matrigel definitively verified that KP extract could be able to suppress invasion of SKOV3 cells. Together, these results strongly suggest that KP possesses the inhibitory effect on migration and invasion of SKOV3. These observations are consistent with the zymography results, suggesting that the reduced activity of MMPs may greatly contribute to the reduction of cancer cell invasion and migration. Besides the ability of KP extract on an aspect of suppressing ovarian cancer cell metastasis, we would also like to explore its effect on ovarian cancer cell growth and survival. In particular, since SKOV3 cells apparently express EGF receptor (EGFR) [30], we therefore examined whether KP extract can overcome the influence of EGF on activating molecular signal transduction pathways relevant to cell growth and survival. EGFR is involved in cell proliferation, motility, adhesion, angiogenesis, and survival via the activation of phosphatidylinositol-3 kinase (PI3K/AKT) pathway, and the extracellular signal-regulated kinase (ERK) pathway [31]. EGFR is widely expressed in 33–75% of ovarian cancer and has been implicated in the growth and progression of this cancer [32–34]; therefore, EGFR is important to represent a potential target for anticancer drug development. An example of EGFR-directed monoclonal antibody is cetuximab, which inhibits cell growth in OVCAR-2 cells, whereas the growth of SKOV3 cells is not affected [35]. Another class of EGFR inhibitor is a group of small molecule tyrosine kinase inhibitors that target the receptor catalytic domain of EGFR. Those include gefitinib and erlotinib [36]. AKT and ERK are major downstream signaling molecules of EGFR [37]. Previous evidence demonstrated that KP extract significantly suppressed the phosphorylation of PI3K, AKT, ERK1/2, and Elk1 in HeLa cells [10], thus we hypothesized whether KP extract can suppress the activation of ERK1/2 and AKT signaling in high grade serous ovarian cancers. Our study clearly showed that KP extract markedly suppressed phosphorylation of ERK1/2 and AKT. This observation was still seen when the experiment was performed with the presence of EGF. The present results indicate that KP extract suppresses ERK1/2 pathway which is normally involved in cell proliferation, and the extract suppressed AKT pathway which plays roles in cell survival. A study in *Drosophila* showed that a gain-of-function mutation that results in enhanced ERK1/2 signaling capabilities could support ERK1/2 activation in the cancer cells [38]. Our findings are supported by recent studies showing that the use of RNA interference to silence ERK1/2 phosphorylation led to the complete suppression of tumor cell proliferation [39]. Since various cancers have aberrant regulation of AKT pathway that leads to prolonged survival of tumor [40], and previous studies showed that the inhibition of AKT activity is useful as a therapeutic approach for the therapy of cisplatin-resistant ovarian cancer because an activation of AKT promotes cisplatin-

resistance [41, 42], we hope that KP may be a novel and effective agent that has some potential targets in AKT signaling and therefore may be beneficial for developing a cancer therapeutic means. One of our key observations was an increase in cell death at 24 h after treatment with KP extract at toxic concentrations. Nuclear fragmentation, which is a result of the cleavage of chromosomal DNA into oligonucleosomal size fragments, is an integral part of apoptosis [43]. The cell apoptosis leads to deformity of nuclear lamina, and consequently increases active caspases [44]. Therefore, to confirm whether these dead cells were apoptotic cells, we stained SKOV3 cells with Hoechst 33342 and observed nuclear fragmentation of SKOV3 cells treated with KP extract. This finding is consistent with Potikanond et al. (2017) indicating that nuclear deformity and nuclear fragmentation were induced by KP treatment in HeLa cells. In addition, the apoptotic event of KP-treated SKOV3 was further determined by Annexin V/PI and flow cytometry analysis. Clearly, our results showed that KP significantly induced apoptosis in SKOV3 cells in a concentration-dependent manner. To confirm our hypothesis that KP extract induces apoptosis in ovarian cancer cells, we specifically analyzed the key apoptosis execution enzymes, caspase-3, caspase-7, and caspase-9 in SKOV3 cells treated with KP extract. The results showed that the full-length structure of all caspases was reduced in a concentration-dependent manner in SKOV3 cells treated with KP extract, implicating that SKOV3 cell death in KP treatment was possessed through programmed cell death signaling pathway.

1.5 Conclusions

The current study demonstrated that KP extract has anti-cancer properties against a high grade serous adenocarcinoma, SKOV3. Specifically, even though SKOV3 cells are very aggressive and resistant to many chemotherapeutic agents, our results showed that KP extract was able to suppress the activity of MMP-2 and MMP-9, migration and invasion, activation of growth and survival signal transduction pathways, and induction of apoptotic cell death. These observations convince us to believe that KP extract is a potential agent to be further developed as an effective therapy for ovarian cancer.

1.6 References

1. Waldmann A, Eiseemann N, Katalinic A. Epidemiology of Malignant Cervical, Corpus Uteri and Ovarian Tumours - Current Data and Epidemiological Trends. *Geburtshilfe Frauenheilkd*. Thieme Medical Publishers; 2013 [cited 2017 Nov 14];73:123–9.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin*. 2017 [cited 2018 May 10];67:7–30.
3. Jelovac D, Armstrong DK. Recent progress in the diagnosis and treatment of ovarian cancer. *CA Cancer J Clin*. NIH Public Access. 2011;61:183–203.
4. Vang R, Shih I-M, Kurman RJ. Ovarian low-grade and high-grade serous carcinoma: pathogenesis, clinicopathologic and molecular biologic features, and diagnostic problems. *Adv Anat Pathol*. NIH Public Access. 2009;16:267–82.
5. Kurman RJ. Origin and molecular pathogenesis of ovarian high-grade serous carcinoma. *Ann Oncol*. 2013;24:x16–21.

6. Mukhtar E, Adhami VM, Mukhtar H. Targeting microtubules by natural agents for cancer therapy. *Mol Cancer Ther.* NIH Public Access. 2014;13:275–84.
7. Yenjai C, Prasanth K, Daodee S, Wongpanich V, Kittakoo P. Bioactive flavonoids from *Kaempferia parviflora*. *Fitoterapia.* 2004;75:89–92.
8. Patanasethanont D, Nagai J, Matsuura C, Fukui K, Sutthanut K, Sripanidkulchai B, et al. Modulation of function of multidrug resistance associated-proteins by *Kaempferia parviflora* extracts and their components. *Eur J Pharmacol.* 2007;566:67–74.
9. 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 Pac J Cancer Prev.* 2009;10:1137–40.
10. Potikanond S, Sookkhee S, Na Takuathung M, Mungkornasawakul P, Wikan N, Smith DR, et al. *Kaempferia parviflora* extract exhibits anti-cancer activity against HeLa cervical Cancer cells. *Front Pharmacol.* 2017;8:630.
11. Hudson LG, Zeineldin R, Silberberg M, Stack MS. Activated epidermal growth factor receptor in ovarian cancer. *Cancer Treat Res.* NIH Public Access. 2009;149:203–26.
12. Catasús L, Bussaglia E, Rodríguez I, Gallardo A, Pons C, Irving JA, et al. Molecular genetic alterations in endometrioid carcinomas of the ovary: similar frequency of beta-catenin abnormalities but lower rate of microsatellite instability and PTEN alterations than in uterine endometrioid carcinomas. *Hum Pathol.* 2004;35:1360–8.
13. Mandai M, Konishi I, Kuroda H, Komatsu T, Yamamoto S, Nanbu K, et al. Heterogeneous distribution of K-ras-mutated epithelia in mucinous ovarian tumors with special reference to histopathology. *Hum Pathol.* 1998;29:34–40.
14. Wilailak S. Epidemiologic report of gynecologic cancer in Thailand. *J Gynecol Oncol.* Korean Society of Gynecologic Oncology and Colposcopy. 2009;20:81.
15. Cristea M, Han E, Salmon L, Morgan RJ. Review: practical considerations in ovarian cancer chemotherapy. *Ther Adv Med Oncol.* 2010;2:175–87.
16. 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:e72162.
17. Reid BM, Permuth JB, Sellers TA. Epidemiology of ovarian cancer: a review. *Cancer Biol Med.* Chinese Anti-Cancer Association. 2017;14:9–32.
18. 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. Pearson R, editor. *PLoS One.* 2014;9:e103988.
19. Garzetti GG, Ciavattini A, Lucarini G, Goteri G, De Nictolis M, Garbisa S, et al. Tissue and serum metalloproteinase (MMP-2) expression in advanced ovarian serous cystadenocarcinomas: clinical and prognostic implications. *Anticancer Res.* 1995;15:2799–804.
20. Fishman DA, Bafetti LM, Banionis S, Kearns AS, Chilukuri K, Stack MS. Production of extracellular matrix-degrading proteinases by primary cultures of human epithelial ovarian carcinoma cells. *Cancer.* 1997;80:1457–63.

21. Lopata A, Agresta F, Quinn MA, Smith C, Ostor AG, Salamonsen LA. Detection of endometrial cancer by determination of matrix metalloproteinases in the uterine cavity. *Gynecol Oncol. Academic Press.* 2003;90:318–24.
22. Torng P-L, Mao T-L, Chan W-Y, Huang S-C, Lin C-T. Prognostic significance of stromal metalloproteinase-2 in ovarian adenocarcinoma and its relation to carcinoma progression. *Gynecol Oncol.* 2004;92:559–67.
23. Bauvois B. New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: outside-in signaling and relationship to tumor progression. *Biochim Biophys Acta. Rev. Cancer.* 2012;1825:29–36.
24. Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature. Nature Publishing Group.* 1980;284:67–8.
25. Stetler-Stevenson WG. The role of matrix metalloproteinases in tumor invasion, metastasis, and angiogenesis. *Surg Oncol Clin N Am.* 2001;10:383–92.
26. Ko C-H, Shen S-C, Lee TJF, Chen Y-C. Myricetin inhibits matrix metalloproteinase 2 protein expression and enzyme activity in colorectal carcinoma cells. *Mol. Cancer Ther. American association for. Cancer Res.* 2005;4:281–90.
27. Kim YH, Kwon H-J, Kim D-S. Matrix metalloproteinase 9 (MMP-9)-dependent processing of β ig-h3 protein regulates cell migration, invasion, and adhesion. *J Biol Chem. American Society for Biochemistry and Molecular Biology.* 2012;287:38957–69.
28. Deryugina EI, Luo GX, Reisfeld RA, Bourdon MA, Strongin A. Tumor cell invasion through matrigel is regulated by activated matrix metalloproteinase-2. *Anticancer Res.* 1997;17:3201–10.
29. George SJ, Johnson JL, Angelini GD, Newby AC, Baker AH. Adenovirusmediated gene transfer of the human TIMP-1 gene inhibits smooth muscle cell migration and Neointimal formation in human saphenous vein. *Hum Gene Ther.* 1998;9:867–77.
30. Sewell JM, Macleod KG, Ritchie A, Smyth JF, Langdon SP Targeting the EGF receptor in ovarian cancer with the tyrosine kinase inhibitor ZD 1839 ("Iressa"). *Br J Cancer. Nature Publishing Group;* 2002;86:456–62.
31. Tanaka Y, Terai Y, Tanabe A, Sasaki H, Sekijima T, Fujiwara S, et al. Prognostic effect of epidermal growth factor receptor gene mutations and the aberrant phosphorylation of Akt and ERK in ovarian cancer. *Cancer Biol Ther.* 2011;11:50–7.
32. Berchuck A, Rodriguez GC, Kamel A, Dodge RK, Soper JT, Clarke-Pearson DL. Epidermal growth factor receptor expression in normal ovarian epithelium and ovarian cancer: II. Relationship between receptor expression and response to epidermal growth factor. *Am J Obstet Gynecol. Mosby.* 1991; 164:745–50.
33. Psyrri A, Kassar M, Yu Z, Bamias A, Weinberger PM, Markakis S, et al. Effect of epidermal growth factor receptor expression level on survival in patients with epithelial ovarian Cancer. *Clin Cancer Res.* 2005;11:8637–43.

34. Stadlmann S, Gueth U, Reiser U, Diener P-A, Zeimet AG, Wight E, et al. Epithelial growth factor receptor status in primary and recurrent ovarian cancer. *Mod Pathol*. Nature Publishing Group. 2006;19:607–10.
35. Bijman MNA, van Berkel MPA, Kok M, Janmaat ML, Boven E. Inhibition of functional HER family members increases the sensitivity to docetaxel in human ovarian cancer cell lines. *Anti-Cancer Drugs*. 2009;20:450–60.
36. Harari PM. Epidermal growth factor receptor inhibition strategies in oncology. *Endocr Relat Cancer*. 2004;11:689–708.
37. Bunn PA Jr and Franklin W. Epidermal growth factor receptor expression, signal pathway, and inhibitors in non-small cell lung cancer. *Semin Oncol*. 2002;29(5Suppl 14):38-44.
38. Bott CM, Thorneycroft SG, Marshall CJ. The sevenmaker gain-of-function mutation in p42 MAP kinase leads to enhanced signaling and reduced sensitivity to dual specificity phosphatase action. *FEBS Lett*. 1994;352:201–5.
39. Steinmetz R, Wagoner HA, Zeng P, Hammond JR, Hannon TS, Meyers JL, et al. Mechanisms Regulating the Constitutive Activation of the Extracellular Signal-Regulated Kinase (ERK) Signaling Pathway in Ovarian Cancer and the Effect of Ribonucleic Acid Interference for ERK1/2 on Cancer Cell Proliferation. *Mol. Endocrinol*. [Internet]. Oxford University Press; 2004;18:2570–82.
40. Steelman LS, Stadelman KM, Chappell WH, Horn S, Bäsecke J, Cervello M, et al. Akt as a therapeutic target in cancer. *Expert Opin Ther Targets*. 2008; 12:1139–65.
41. Yang X, Fraser M, Abedini MR, Bai T, Tsang BK. Regulation of apoptosis-inducing factor-mediated, cisplatin-induced apoptosis by Akt. *Br J Cancer*. 2008;98:803–8.
42. Peng D-J, Wang J, Zhou J-Y, Wu GS. Role of the Akt/mTOR survival pathway in cisplatin resistance in ovarian cancer cells. *Biochem Biophys Res Commun*. 2010;394:600–5.
43. Zhang JH, Ming M. DNA fragmentation in apoptosis. *Cell Res*. [Internet]. Nature Publishing Group. 2000;10:205–11.
44. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol*. 2007;35:495–516.

Output ที่ได้จากโครงการ

ผลงานวิจัยได้รับการตีพิมพ์ในวารสารทางเภสัชวิทยาระดับนานาชาติ เป็นจำนวน 2 เรื่อง ดังนี้

