Thesis for the Degree of Master of Science

Anti-cancer effect of *Meliosma myriantha* flower extract on Human Cervical Cancer HeLa cells

by

Seung Hyun Yoo

Department of Microbiology

The Graduate School

Pukyong National University

February 21, 2020

Anti-cancer effect of *Meliosma myriantha* flower extract on Human Cervical Cancer HeLa cells

(나도밤나무 메탄올 추출물의 인간 자궁경부암 세포에서의 항암 작용)

Advisor: Prof. Gun-Do Kim

by

Seung Hyun Yoo

A thesis submitted in partial fulfillment of the requirements

for the degree of

Master of Science

in Department of Microbiology, The Graduate School,

Pukyong National University

February 2020

Anti-cancer effect of *Meliosma myriantha* flower extract on Human Cervical Cancer HeLa cells

A dissertation

by

Seung Hyun Yoo

Approved by:

(Chairman) Young Tae Kim

(Member) Young Jae Jeon

(Member) Gun-Do Kim

February 21, 2020

CONTENTS

Anti-cancer Effect of *Meliosma myriantha* flower extract

on Human Cervical Cancer HeLa cells

Seung Hyun Yoo

Department of Micobiology, The Graduate School

Pukyong National University

ABSTRACT

Cervical cancer is a cancer arising from the cervix. It is due to the abnormal growth of cells that have the ability to invade or spread to other parts of the body. HeLa cells are used in research as a cervical cancer model cells. In present research we used methanol extract of flower from *Meliosma Myriantha* (MEMM) which is native tree of Korea. Best of our knowledge this is first report on anti-cancer effect and molecular mechanism of MEMM on HeLa cells. MEMM of 300 μg/ml shows cytotoxicity on HeLa cells while, the same concentration has no significant cytotoxicity on non-cancerous cells (HEK-293 and HaCaT). In addition, MEMM induced a morphological change and nucleic disruption as well as sub-G1 phase cell cycle arrest in HeLa cells. Molecular mechanism was analyzed by western blot assay. In results it is observed that protein expression of intrinsic apoptosis transcription factors, such as Bim, Bak, Apoptotic protease factor-1 (Apaf-1) and cytochrome *c* were upregulated by MEMM treatment. Furthermore, the caspase cascade was activated by MEMM from caspase-9 to caspase-3 and from caspase-8 to caspase-3. In immunofluorescence staining, the increased intensity of cleaved Poly ADP-ribose polymerase (PARP) and cleaved caspase-3 were observed. In addition, anti-proliferation activity of HeLa cells was inhibited by MEMM via down-regulation of PI3K/Akt signal pathway. Thus, these results demonstrate that MEMM has healing potential against HeLa cells *via* mitochondria and PI3K/Akt-dependent caspase apoptosis pathways.

1. INTRODUCTION

Cancer is the leading cause of death in the world. Cervical cancer is one of the malignant tumors that is a significant health concern for women (1, 2). Especially cervical cancer is the seventh leading cause of death in Korean women (3). Recently, the mortality and incidence rate of cervical cancer have been decreased since the cervical cancer screening program. However, its incidence of cervical cancer in young women has been remained as a health problem (4). Conventional cancer treatment is carried out mainly through surgical operations, radiation therapy and chemotherapy. Among them, chemotherapy is one of effective treatments for anticancer activity but damages normal cells along with cancer cells, causing side effects such as anemia, white blood cell reduction and vomiting (5). Therefore, a safe and effective therapeutic chemotherapy should be developed for the treatment of cervical cancer.

Apoptosis is one of the essential physiological processes for maintaining normal development and homeostasis of tissues by removing aging, damaged or mutated cells. However, abnormal control of this process causes a wide range of diseases, including cancer (6). Therefore, the development of a new anti-cancer therapeutic drugs that works selectively in cancer cells that can solve these problems, is necessary. A typical characteristic of apoptosis is severe morphological change and decomposition of cellular components (7), including two main apoptotic pathways, extrinsic and intrinsic pathway (8).

In the intrinsic pathway, mitochondria play an important role in mediating apoptosis, which is caused by the release of cytochrome *c* (8). Mitochondrial cytochrome *c* is regulated by the Bcl-2 family proteins, anti-apoptotic and pro-apoptotic proteins (9). In addition, cytochrome *c* released into the cytosol results in the cell death by activating from caspase-9 to caspase-3 (10).

Caspase activation is a well-known apoptosis pathway. Caspases also cleave a various substrates involved in activities that result in cell decomposition, such as the destruction of organelle function, cytoskeletal, and nuclear disruption, resulting in representative hallmark features of apoptotic cell death (11, 12). The activated caspase-8 executes the apoptotic signal by activating the effector caspase-3 (10). Caspase-3 activation is an important step in apoptosis execution (13).

Natural products have long been recognized for their role as a source of remedies. Despite the progressive development of science and technology, drugs derived from natural products still make tremendous contribution to drug discovery today (14). *Meliosma myriantha* is a native Korean plant belonging to the family Sabiaceae. Previous studies for MEMM have been confirmed for anti-oxidative activity and the inhibitory activity of α-amylase (15). However, *in vitro* apoptotic effects of methanol extract of flower from *M. myriantha* on human cervical cancer cell HeLa cells have not been reported.

Therefore, in this study, we investigated *in vitro* apoptotic effects of methanol extract from *Meliosma myriantha* and its underlying mechanisms on the apoptotic cell death on human cervical cancer HeLa cells was our research focus.

2. MATERIALS AND METHODS

2.1 Sample preparation

The methanol (99.9%, HPLC grade) extracts from flower of *Meliosma myriantha* (MEMM) were provided by the Korea Plant Extract Bank (Cheongju, Korea). Stock solution (100 mg/ml) of MEMM was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich., St. Louis, MO, USA) and was dilluted in fresh media for further experiments. The final concentration of DMSO was not exceeded 0.1% in all assays.

2.2 Cell culture

Human cervical cancer HeLa cells, Human epithelial kidney HEK293 cells were purchased from Korean Collection for Type Cultures (KCTC, Jeongeup, Korea), Human colon cancer HT-29, Human gastric cancer AGS, Human liver cancer Hep3B, Human lung cancer A549 were purchased from American Tissue Culture Collection (ATCC, Manassas, VA, USA). Human skin HaCaT cells were purchased from Cell Lines Service (CLS, Eppelheim, Germany). HeLa cells, HEK293 cells were incubated with Minimum Essential Medium (MEM), HT-29 cells, Hep3B cells and HaCaT cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM), AGS cells and A549 cells were incubated in RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum (FBS) (Corning Inc., Corning, NY, USA) and 1% antibiotic-antimycotic solution (Corning Inc., Corning, NY, USA), respectively. The cells were incubated in a humidified atmosphere with 5%

CO_2 at 37℃. The cells were sub-cultured every four to five days and morphological observations performed using inverted microscope.

2.3 Cell viability assay

The cytotoxicity of HeLa cells treated with or without MEMM was determined using the WST-1 assay. The cells were seeded in 96-well cell culture plate (5×10^3 cells/well) and incubated for 24 h. Then, the cells were treated with various concentrations (50, 100, 150, 200, 250 and 300 μg/ml) of MEMM and incubated for 24 h. After treatment, the media was replaced with 100 μl fresh media and 10 μl of EZ-cytox Cell Viability Assay Solution WST-1® (Daeil Lab Service, Gyeonggi, Korea) was added to each well. Then, the cells were further incubated at 37℃ for 150 min. The cell viability was measured by an ELISA reader (Thermo Fisher Scientific, MA, USA) at 460 nm.

2.4 Protein extractions and western blot analysis

HeLa cells were treated with MEMM. Treated cells were washed with ice-cold 1X phospho-buffered saline (PBS) and collected in cell lysis buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate and 0.1% SDS (Intron biotechnology, Gyenoggi, Korea) on ice for 30 min. For collecting the supernatants, the cell lysates were centrifuged at 13,200 rpm for 25 min. The protein concentration in the cell lysates was determined by Bradford reagent (Biosesang, Seongnam, Korea) using bovine serum albumin (BSA) as a standard. Equal volume of

proteins was boiled for 5 min and then separated by 12% SDS-polyacrylamide gel electrophoresis. Then proteins were electro-transferred to nitrocellulose membranes (Pall Life Sciences, Pensacola, MI, USA). The membranes were blocked in 1X PBST buffer (135 μM NaCl, 2.7mM KCl, 4.3 mM $NaPO_4$, 1.4 mM KH_2PO_4 and 0.5% Tween-20) containing 5% skim milk. After blocking, membranes were washed with 1X PBST and then incubated with primary antibodies (Cell Signaling Technology Inc.) for overnight at 4℃. Then, membranes were washed with 1X PBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology Inc.) for 70 min. After incubation, the membranes were washed with 1X PBST and then visualized using enhanced chemiluminescent (ECL®) detection solution (Pierce, Rockford, IL, USA).

2.5 Immunofluorescence (IF) staining

HeLa cells were cultured on cover-glass bottom dishes (SPL Lifesciences, Pocheon, Korea) with treatment of MEMM for 24 h. After incubation, the cells were washed with 1X PBS and pre-treated with 1 μg/ml DAPI (4`,6-diamidino-2-phenylindoledihydrochloride hydrate) in methanol for 20 min at 37℃ incubator. Then, the cells were fixed with 4% formaldehyde (Junsei Chemical Co., JAPAN) for 10 min at 25℃ and blocked for overnight at 4℃ including 5% rabbit and mouse normal serum (Santa Cruz Biotechnology Inc., Dallas, TX, USA) with 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). After blocking, the cells were incubated with the primary antibodies (β-actin, cleaved caspase3, cleaved PARP) at 4℃ for overnight. Then, cells were washed three

times with 1X PBS and incubated with 0.1 μg/ml anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor®488 conjugate) and anti-mouse IgG (H+L), F(ab')2 fragment (Alexa Fluor®555 conjugate) for 1 h at 25℃. Stained cells were moved on the slide with Prolong Gold® Anti-fade Reagent (Invitrogen, Grand Island, NY, USA) and visualized by Carl Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

2.6 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNAs from HeLa cells with or without treatment of MEMM were extracted using RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany) following the supplier's manual. Two-microgram of each total RNA was reverse transcribed to cDNA using SuPrimeScript RT-premix (GeNet Bio, Daejeon, Korea) according to manufacturer's instruction. Then, equal concentration of cDNAs were amplified (94℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s for 32 cycle) using Prime Taq Premix (GeNeT Bio, Daejeon, Korea) with specific primers, such as cleaved caspase-9, Cleaved caspase-3 and GAPDH (Table. 1). The amplified PCR products were separated by 1.5% agarose gel.

Table 1. The primer sequence used for RT-PCR

Genes	Forward Primers	Reverse Primers
Cleaved caspase-9	GCTCTTCCTTTGTTCATCTCC	CATCTGGCTCGGGGTTACTGC
Cleaved caspase-3	GGTATTGAGACAGACAGTGG	CATGGGATCTGTTTCTTTGC
GAPDH	GGAGCCAAAAGGGTCATC	CCAGTGAGTTTCCCGTTC

2.7 Flow cytometry analysis

HeLa cells were cultured without or with different concentrations (100, 200 and 300 μg/ml) of MEMM for 24h. The cells were harvested by trypsinization and fixed in 70% ethanol at 4℃ for overnight. After fixation, cells were resuspended in 1.12% sodium citrate buffer containing 20 μg/ml RNase A and incubated for 30 min at 37℃. The cells were stained with 20 μg/ml propidium iodide for 10 min in dark conditions. Cell cycle arrest was analyzed using a flow cytometry (Becton Dickinson, Mountain View, CA, USA).

2.8 Statistical analysis

The Statistical significance of data was presented using a Graph Pad Prism 6.0 software (Graph Pad, La Jolla, CA, USA). Normally distributed data are shown as mean ± standard deviation (SD). Differences between the mean value of each experimental group were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test. P values were considered to indicate statistical significance for the experiments performed at least three times independently.

3. RESULTS

3.1 Effect of MEMM on cell viability

To investigate cytotoxicity of MEMM, we performed the cell viability on human cervical cancer HeLa cells, human colorectal cancer HT29 cells, human gastric cancer AGS cells, human liver cancer Hep3B cells, human lung cancer A549 cells, non-cancerous human kidney HEK293 and human skin HaCaT cells using WST-1® assay. As shown in Figure 1 (A), MEMM induced cell death in human cervical cancer HeLa cells in a dose-dependent manner. In these cell lines, MEMM particularly induced the cytotoxicity of HeLa cells than any other cancer cell lines. The IC_{50} of MEMM on HeLa cells was about 155 μg/ml. However, MEMM did not showed significant cytotoxic effects on non-cancerous HEK293 and HaCaT cells. (Figure 1 (B)).

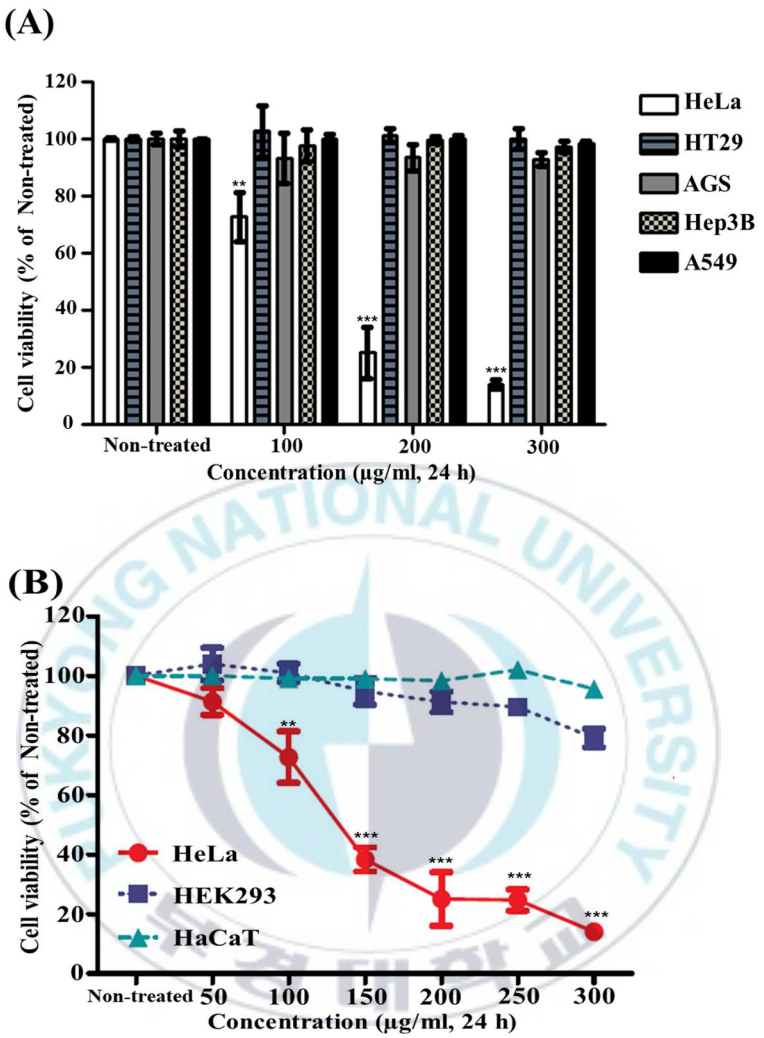


Figure 1. The cytotoxicity of MEMM on cell viability. (A) HeLa, HT29, AGS, Hep3B and A549 cancer cells were treated various concentrations of MEMM (100, 200 and 300 μg/ml) for 24 h. (B) The cell viability was compared between HeLa cancer cells and non-cancerous (HEK293, HaCaT) cells were treated with MEMM. The cell viability was measured using WST-1® assay kit. The experiments were performed three times independently and all values were shown as mean ± SD. Asterisks indicate difference from non-treated cells by ANOVA, **P<0.01 ***P<0.001.

3.2 MEMM induces morphological change and nucleic disruption on HeLa cells

As shown in Figure 1 (A), MEMM has anti-cancer effects in cancer cell lines which exhibited most effectively induced cell death on HeLa cells (Figure 1 (B)). HeLa cells treated with or without MEMM were visualized under a phase inverted microscope the ×100 and ×400 to investigate morphological change (Figure 2 (A)). To determine whether MEMM affects nucleic disruption in HeLa cells, DAPI staining was performed. The results of Figure 2 (B), indicated that HeLa cells treated with 200 μg/ml of MEMM showed morphological changes at nucleus. A typical hall marker of apoptosis, nucleic disruption (apoptosome formation), was observed in HeLa cells treated with 300 μg/ml MEMM for 24 h.

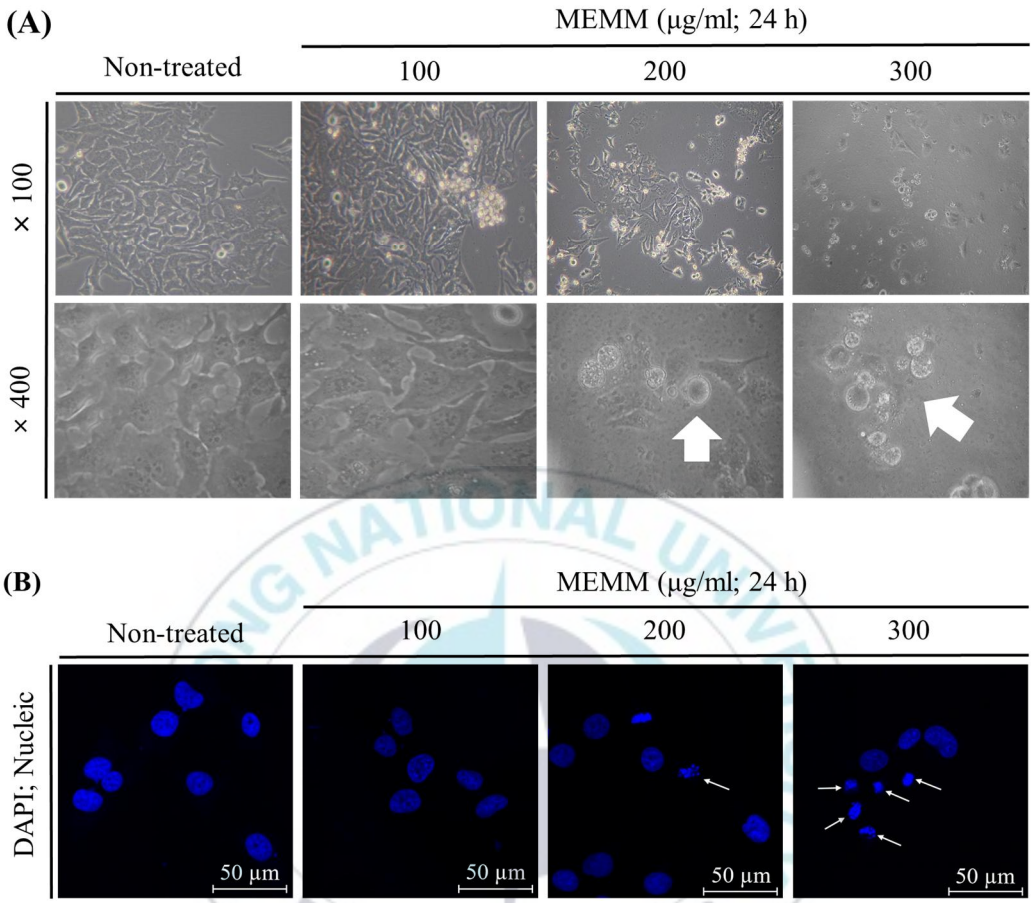
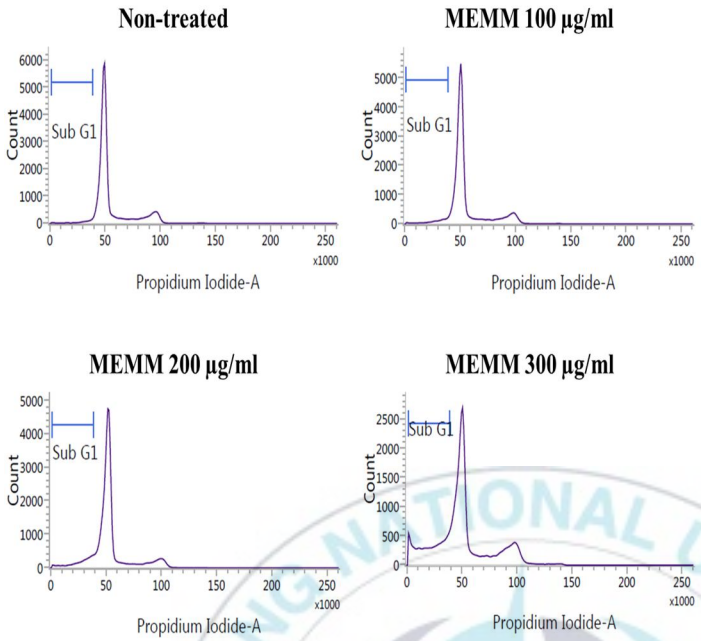


Figure 2. The effects of MEMM on morphological changes and nucleic disruption. (A) HeLa cells were treated with MEMM in a dose-dependent manner for 24 h and visualized the cell morphology under a phase inverted microscope at ×100 and ×400 magnifications. (B) The morphological changes and condensed chromatins in the cells observed by DAPI staining. Arrows in figure show morphological change in the MEMM treated cells.

3.3 MEMM induces sub-G1 cell cycle arrest on HeLa cells

To investigate the nucleic fragmentation of HeLa cells in response to MEMM treatment, cell cycle distribution was performed by flow cytometry analysis. The results showed that MEMM treated cells were accumulated progressively in the sub-G1 phase (Figure 3). As Compared with the non-treated, the treatment with MEMM has resulted an increased proportion of sub-G1 phase cells in a dose-dependent manner 5.43%, 11.27% and 26.97%. Therefore, MEMM induce the accumulation of sub-G1 phase was established and demonstrated by flow cytometry analysis.



	Sub G1 phase
Non-treated	3.10 %
MEMM 100 µg/ml	5.43 %
MEMM 200 µg/ml	11.27 %
MEMM 300 µg/ml	26.97 %

Figure 3. MEMM induces cell cycle arrest in HeLa cells. Cells were treated with 100, 200 and 300 µg/ml MEMM for 24 h and then the proportion of cell cycle phase was evaluated using flow cytometry.

3.4 Effect of MEMM on expression of mitochondrial apoptosis related proteins

To determine whether the anti-cancer effect of MEMM is related to the induction of apoptosis, HeLa cells were exposed to MEMM and the modulation of apoptosis-related proteins was investigated by western blot. As shown in Figure 4, MEMM treatment substantially increased the expression levels of apoptotic proteins related in mitochondrial apoptosis pathway, such as Bim, Apaf-1, Bak and cytochrome *c*. On the other hand, the expression levels of Bid and Bcl-2 was decreased. These results showed that the increased protein expressions of Bim, Apaf-1, Bak and cytochrome *c*, down-regulated protein expressions of Bid and Bcl-2 by MEMM. The results suggested the induction of mitochondrial apoptosis in HeLa cells.

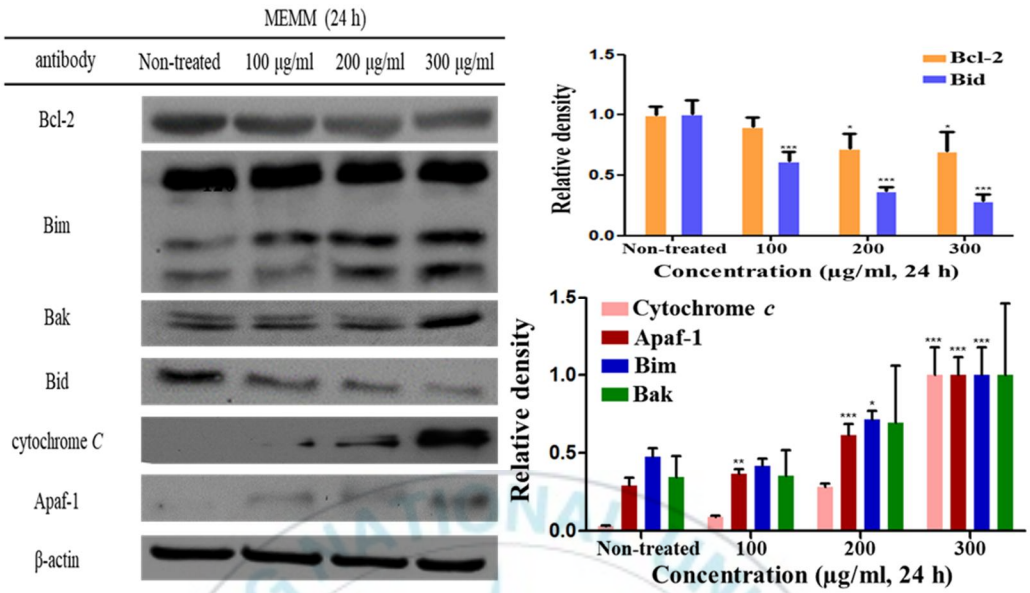


Figure 4. Effect of MEMM on the expression of mitochondrial apoptotic proteins on HeLa cells. Cells were treated with 100, 200 and 300 μg/ml of MEMM for 24 h. The expression levels of protein were determined by western blot. β-actin was used as internal control. The experiments were performed three times independently and all values were shown as mean ± SD. Asterisks indicate difference from non-treated cells by ANOVA, *P<0.05, **P<0.01 and ***P<0.001.

3.5 Effect of MEMM on expression of caspase cascade signal transduction

To investigate the effects of MEMM on the expression of caspase apoptotic factors at transcriptional and translational levels, we performed the RT-PCR and western blot analysis respectively. The protein expression of caspase family (caspase-9, caspase-8, caspase-7, caspase-3) and PARP were activated by MEMM in a dose-dependent manner. (Figure 5 (A)). As presented in Fig. 5 (B), both mRNA levels of cleaved caspase-9, and cleaved caspase-3 significantly increased by MEMM. Furthermore, to elucidate the nuclear translocation of cleaved PARP and cleaved caspase-3 under 300 μg/ml of MEMM treatment, we performed immunofluorescence staining. As a result, the fluorescent intensity of cleaved caspase-3 and cleaved PARP were increased by MEMM treatment (Figure 6). These results indicated that MEMM can induced caspase cascade apoptosis in HeLa cells.

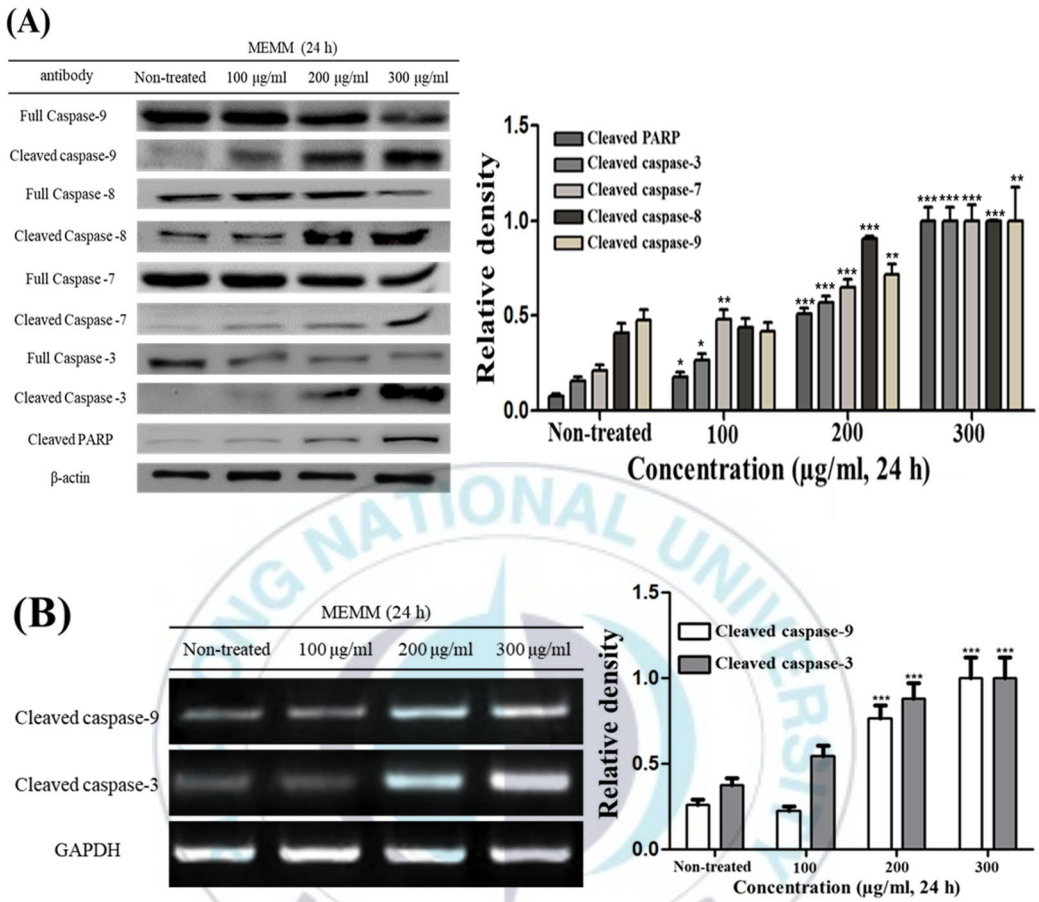


Figure 5. Effect of MEMM on Protein and mRNA expression of caspase cascade apoptosis related proteins on HeLa cells. The cells were treated with 100, 200 and 300 μg/ml MEMM for 24 hours. (A) Western blot results showing cleavage forms of caspase-9, caspase-8, caspase-7, caspase-3 and cleaved PARP expression and (B) RT-PCR results showing cleavage forms of caspase-9 and caspase-3 activity. β-actin and GAPDH were used as loading control. The experiments were performed three times independently and all values were shown as mean ± SD. Asterisks indicate difference from non-treated cells by ANOVA, *P<0.05, **P<0.01 and ***P<0.001.

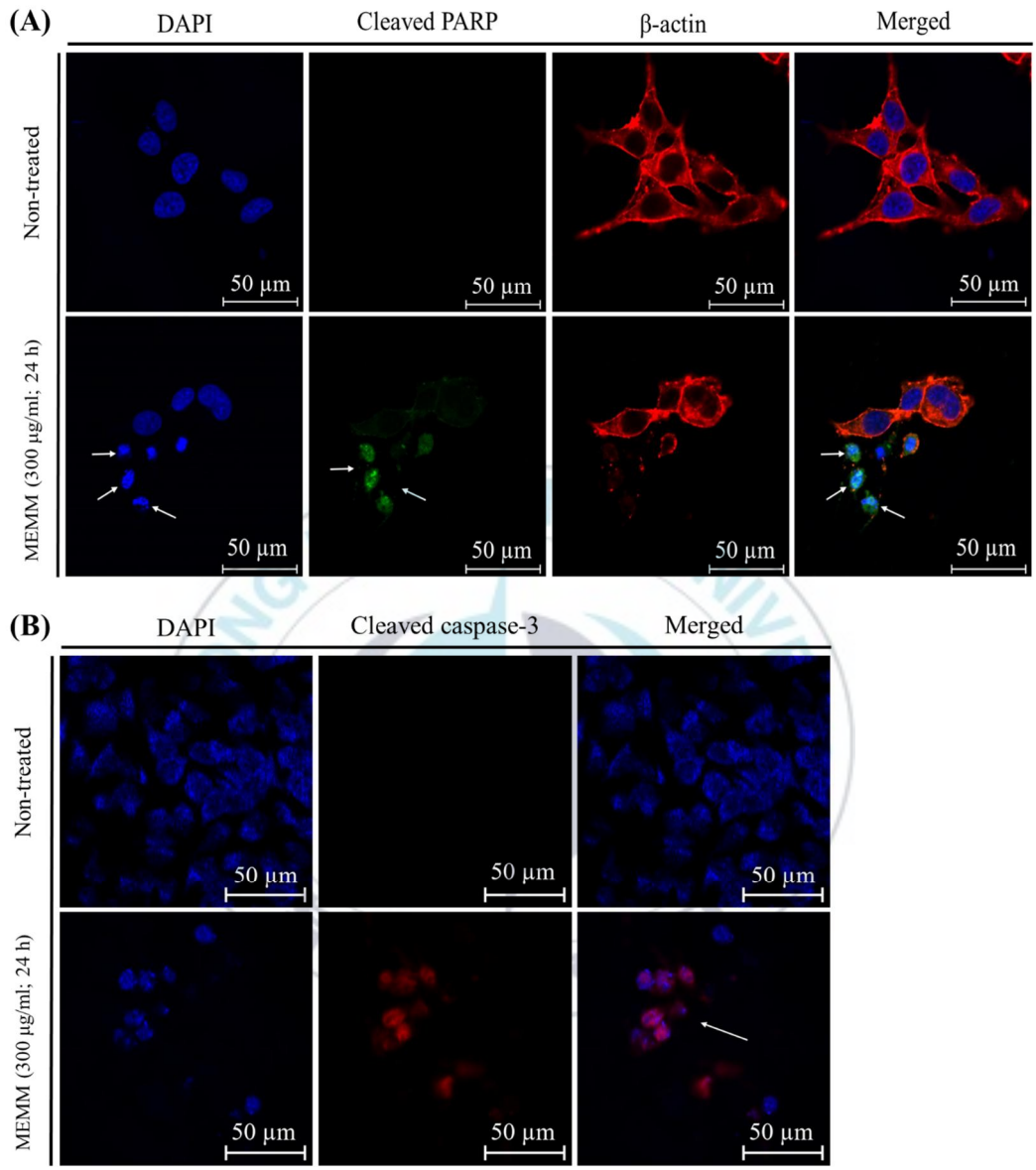


Figure 6. Effect of MEMM on the expression of cleaved PARP, β-actin and cleaved caspase-3. (A) (B) HeLa cells were treated with 300 μg/ml MEMM, and examined by immunofluorescence staining to detect the level of cleaved PARP (green), cleaved caspase-3 (red) with DAPI (blue) and β-actin (red) using confocal microscopy. Scale bars = 50 μm.

3.6 Effect of MEMM on the PI3K/Akt signaling

To investigate further mechanisms of MEMM, the PI3K/Akt signaling pathways was determined in HeLa cell lines. As shown in Figure 7, the protein expression of phosphatidylinositol 3-kinase (PI3K) p110α subunit and phospho-Akt (Ser 473) were significantly decreased in MEMM treated cells, whereas the expression level of total Akt remained constant during all MEMM treatment.

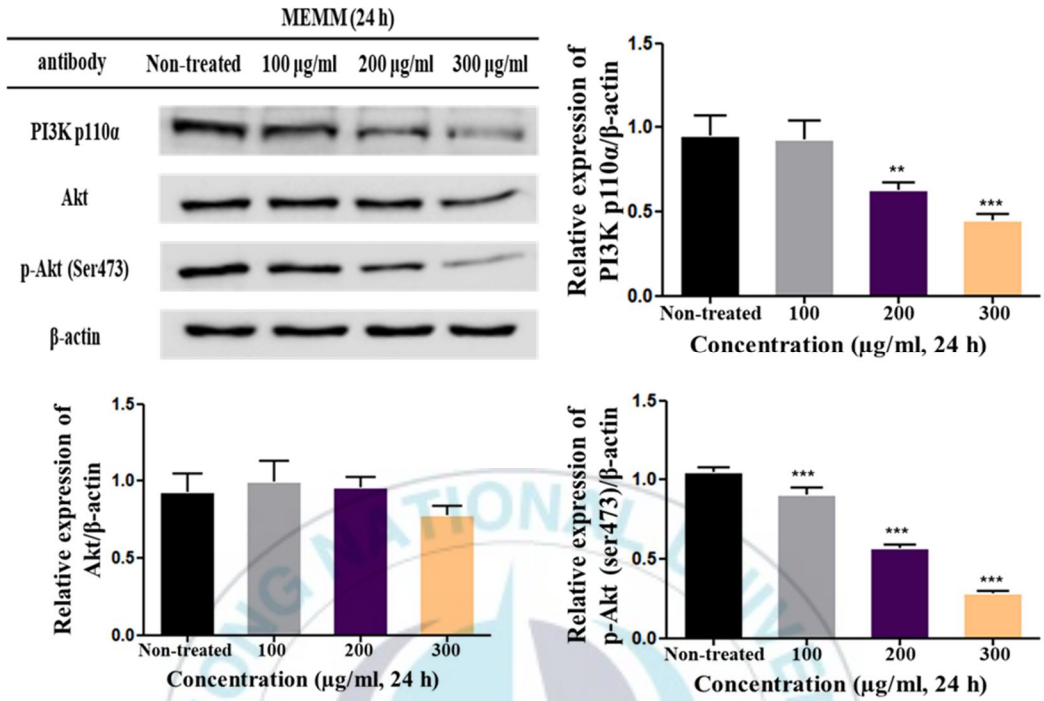


Figure 7. Effect of MEMM on the PI3k/Akt signaling pathway. HeLa cells were treated with dose-dependent manner (100, 200 and 300 μg/ml) for 24 h MEMM, followed by western blot assay for PI3K p110α, Akt and p-Akt (Ser473). β-actin was used as loading control. The experiments were performed three times independently and all values were shown as mean ± SD. Asterisks indicate difference from non-treated cells by ANOVA, **P<0.01 and ***P<0.001.

4. DISCUSSION

Currently, natural products have been valued as an important source as therapeutic agents for human diseases. Many extracts of natural plant have shown to have anti-cancer potential and considered to be a novel cancer therapeutic agents (16-19). This study demonstrates that MEMM induces down-regulation of the PI3K/Akt pathway, apoptosis *via* the mitochondrial pathway, leading to anti-cancer effects in human cervical HeLa cells.

The treatment of MEMM showed the highest anti-proliferation effect on HeLa cells without significant anti-proliferation effect on non-cancerous HaCaT and HEK293 cells (Figure 1 (B)). Especially, MEMM inhibited the proliferation of HeLa cells in a dose-dependent manner but not cancerous HT29, AGS, Hep3B and A549 cells (Figure 1 (A)). All kinds of human cancer cell lines were distinguished by several genomic data such as DNA copy number. Genetic variation in cancer cell lines is associated with sensitivity of cancer therapeutic agents (20, 21). Therefore, genetic differences in selected cell lines appear to have affected the MEMM sensitivity, which exhibited the anti-proliferation activity against HeLa cells. Furthermore, MEMM induced alteration of cell shape and nucleic disruption in HeLa cells (Figure 2 (A) and (B)). Several studies have reported that apoptosis in HeLa cells presented apoptotic morphological change with nuclei blebbing, formation of apoptotic bodies, cell shrinkage and chromatin condensation (22, 23). In this regards, the occurrence of apoptotic bodies and nucleic disruption is regarded as the apoptotic hall markers (24), the results suggested that MEMM induced the cell death in HeLa cells mainly through apoptosis. In addition, DNA fragmentation of MEMM treated

HeLa cells was analyzed using flow cytometry. Many studies have indicated that the result of cell cycle arrest could be triggered by apoptosis (25, 26). When cells occur apoptosis, cell cycle progression is accumulated in sub-G1 phase (27). In MEMM-treated cell population the G0/G1 phase was decreased, while cell population in the sub-G1 phase was progressively increased when compared with that of untreated cells (Figure 3). Thus, MEMM induced cell cycle arrest in the sub-G1 phase in HeLa cells. These results indicate that MEMM induces apoptotic cell death on human cervical cancer HeLa cells. Therefore, the molecular mechanism of MEMM was further detected by the expression of apoptosis related proteins.

Apoptosis is a necessary process for maintaining the homeostasis of cellular organisms and is a main types of programmed cell death (28). Many anti-cancer agents try to induce apoptosis to improve therapeutic efficiency (29). Apoptotic cell death is conducted by two molecular signaling pathways, mitochondrial intrinsic pathway and death receptor extrinsic pathway (8). The mitochondrial intrinsic pathway includes Bcl-2 family proteins, anti-apoptotic (Bcl-2 and Bcl-XL) and pro-apoptotic proteins (Bak, Bid, Bim, Bad, and Bax) that regulated by permeabilizing the mitochondrial membrane (30, 31). The caspase-8-mediated cleavage of the Bid is relation with two main apoptosis pathway (32). Under the apoptosis signal transduction, overexpression of pro-apoptotic protein induces mitochondrial malfunction through a release of cytochrome *c* from mitochondria. Cytochrome *c* released from mitochondria regulates Apaf-1 and caspase-9 to form apoptotic bodies (apoptosome) that induces cleavage of caspase-3 (33, 34). To reveal the effect of MEMM on mitochondrial apoptosis related proteins, western blotting was

performed. It was observed that MEMM was in the up-regulation of Bak, Bim, cytochrome *c*, Apaf-1 and down-regulation of Bcl-2 expression in a dose-dependent manner (Figure 4). This observation suggested that MEMM induced mitochondria dependent apoptosis in HeLa cells.

Cysteine proteases (caspases) family (caspase-3, -7, -8) play as a central role in the execution of apoptosis through the extrinsic pathway (10, 35). When cells undergo apoptosis, procaspase-8 promotes the activation of caspase-8 by cleavage (36). Downstream effectors caspase-7 and caspase-3 are sequentially cleaved after activation of caspase-8 (37). Although caspases normally located in the cells as pro-enzymes, it is cleaved and activated by apoptotic stimulation (10). Following activation of caspase-7 and caspase-3, poly ADP-ribose polymerase (PARP) is cleaved. In the process of apoptosis, the cleavage of PARP induces apoptotic cell death by interfering with DNA repair (35, 38). Our finding indicate that MEMM up-regulated the expression of down-stream proteins, cleaved caspase-8, -7, -3 and PARP (Figure 5). In addition, the expression level of key effector protein, cleaved PARP and caspase-3 were increased in immunofluorescence staining (Figure 6).

Phosphatidylinositol 3-kinase/Akt is one of the important intracellular pathways that are frequently overexpressed in various epithelial malignancies (39). Overexpression of Akt is correlated with poor prognosis, resistance to systematic therapy and tumor progress in many kinds of human cancer including cervix tumors (40, 41). Phosphorylation of Akt is able to significantly increase the expression of Bcl-2, and decreased the expression of Bak

in cervical cancer (42). PI3K/Akt pathway is regarded as potential targets for anti-cancer agents. Many researchers have studied the PI3K/Akt pathway as a potential target for cancer therapeutic strategy (43). Thus, the effect of MEMM on PI3K/Akt pathways was evaluated by measuring PI3K p110α, Akt, and phospho-Akt proteins using western blot analysis. It was observed that MEMM down-regulated the expression of PI3K p110α and phospho-Akt in a dose-dependent manner after 24 h, whereas expression levels of total Akt remained constant during all treatment (Figure 7). These results indicated that the MEMM induced anti-proliferative effects *via* down regulation of PI3K/Akt signaling pathway in HeLa cells.

In conclusion, we found that the MEMM induced apoptotic cell death in cervical cancer HeLa cells through induction of apoptosis as well as sub-G1 phase arrest. Investigate of apoptosis-related proteins in HeLa cells demonstrated that MEMM triggered the mitochondria-dependent apoptosis pathway as shown by increased the ratio of pro-apoptotic/anti-apoptotic proteins, sequential activation of caspase family and inhibition of PI3K/Akt signal pathway (Figure 8). Therefore, our results suggest that the methanol extract of flower from *Meliosma myriantha* is new natural products for the treatment of human cervical cancer and might be a potential as a developing therapeutic agent.

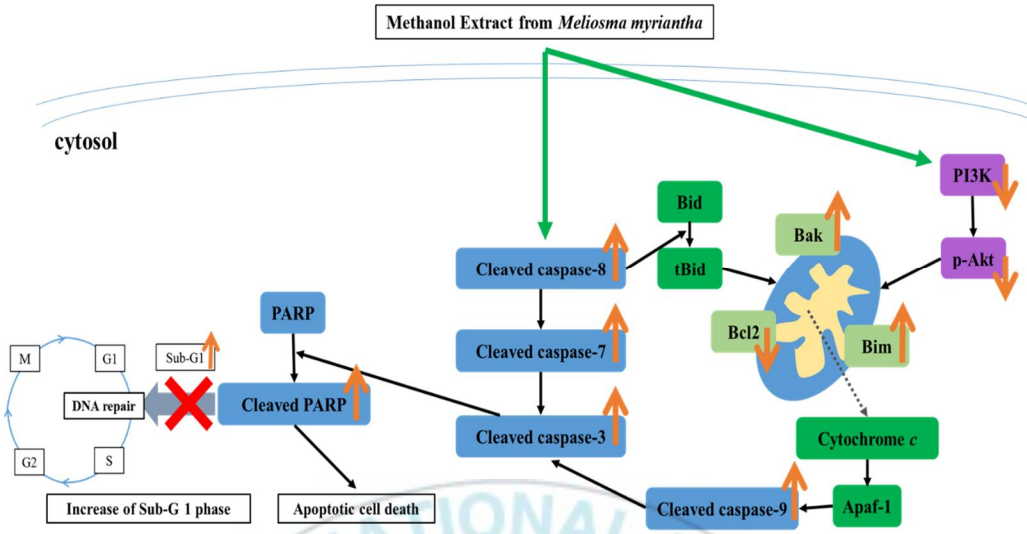


Figure 8. Proposed signaling pathway for the anti-cancer effects of MEMM in HeLa cells.

5. 국문초록

전 세계에서 암은 높은 사망의 원인으로 알려져 있다. 자궁경부암은 악성종양으로서, 분열이 빠르며 주위 세포에 쉽게 전이 되어 생명을 위협하게 된다. 기존 암에 대한 치료 방법으로는 외과적 수술, 방사선치료, 항암제와 같은 화학요법이 있다. 이 중 화학요법이 기본적인 치료방법이며, 항암제의 원천이 되는 천연물을 이용하여 암세포의 세포활성을 억제하는 것이 항암의 방법 중 하나로 정의된다. 나도밤나무는 한국의 자생식물이며 현재까지 항산화효과와 α-amlyase 저해 효능이 있다는 것이 밝혀진 상태이나 항암 효능에 대해서는 아직 까지 구체적으로 알려진 바가 없다. 본 연구에서는 인간 자궁경부암세포주인 HeLa 세포를 이용하여 나도밤나무 꽃의 메탄올 추출물이 가지는 항암 활성과 분자적 작용기전을 분석하였다. 우선, 나도밤나무 추출물을 이용하여 여러 암세포(위암, 대장암, 간암, 자궁경부암, 폐암)에 세포독성테스트를 실시한 결과 자궁경부암세포에서 가장 높은 세포 독성이 나타났으며, 세포자멸사를 유도함을 확인하였다. 그리고 세포자멸사의 intrinsic pathway 중 Bak 과 Bim 의 발현 증가와 cytochrome *c* 의 방출을 확인하였고, cleaved caspase-8, cleaved caspase-7, cleaved caspase-3 의 연쇄적인 발현 증가로 인한 caspase cascade activation 이 진행됨을 확인하였다. 그리고 세포 생존에 관여하는 PI3K/Akt pathway 를 억제시키며, sub-G1 기에서 세포 주기 진행의 억제를 확인하였다. 따라서 본 연구를 통하여 나도밤나무 추출물이 HeLa 세포에 특이적인 항암 효과를 확인하였고, 새로운 자궁경부암 치료제로서의 개발 가능성과 추가적인 연구를 통하여 항암 약제의 기초 자료로서 사용할 수 있을 것으로 사료된다.

6. REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca-Cancer J. Clin.* 2018;68(6):394-424.

2. Jemal A, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol Biomarkers Prev.* 2010;19(8):1893-907.

3. Fang CY, Ma GX, Tan Y, Chi N. A multifaceted intervention to increase cervical cancer screening among underserved Korean women. *Cancer Epidemiol Biomarkers Prev.* 2007 ;16(6):1298-302.

4. Moon E, Oh C, Won Y, Lee J, Jung K, Cho H, et al. Trends and age-period-cohort effects on the incidence and mortality rate of cervical cancer in Korea. *Cancer Res. Treat.* 2017;49(2):526.

5. Tan ML, Ooi JP, Ismail N, Moad AIH, Muhammad TST. Programmed cell death pathways and current antitumor targets. *Pharm Res.* 2009;26(7):1547-60.

6. Fuchs Y, Steller H. Programmed cell death in animal development and disease. *Cell.* 2011;147(4):742-58.

7. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell.* 2004;116(2):205-19.

8. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol.* 2007;35(4):495-516.

9. Desagher S, Martinou J. Mitochondria as the central control point of apoptosis. *Trends Cell Biol.* 2000;10(9):369-77.

10. Nuñez G, Benedict MA, Hu Y, Inohara N. Caspases: the proteases of the apoptotic pathway. *Oncogene.* 1998;17(25):3237.

11. Bari K, Petrik J, Rumora L. Biochemistry of apoptotic cell death. *Acta pharm.* 2003;53:151-64.

12. Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell.* 2002;9(3):459-70.

13. Yang W, Gong X, Zhao X, An W, Wang X, Wang M. Capsaicin induces apoptosis in HeLa cells via Bax/Bcl-2 and caspase-3 pathways. *Asian Pac. J. Trop. Med.* 2006;1(3-4):159-65.

14. Da Rocha AB, Lopes RM, Schwartsmann G. Natural products in anticancer therapy. *Curr. Opin. Pharmacol*. 2001;1(4):364-9.

15. Oh S, Koh S. Screening of antioxidative activity and α-amylase inhibitory activity in angiosperm plants native to Jeju island. *Korean J. Plant Res*. 2009;22(1):71-7.

16. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science.* 1997;275(5297):218-20.

17. Gullett NP, Amin AR, Bayraktar S, Pezzuto JM, Shin DM, Khuri FR, et al. Cancer prevention with natural compounds. *Semin. Oncol.* 2010;37:258-281

18. Nobili S, Lippi D, Witort E, Donnini M, Bausi L, Mini E, et al. Natural compounds for cancer treatment and prevention. *Pharmacol. Res.* 2009;59(6):365-78.

19. Qiao L, Wong BC. Targeting apoptosis as an approach for gastrointestinal cancer therapy. *Drug resist.* 2009;12(3):55-64.

20. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature.* 2012;483(7391):603.

21. Macconaill LE, Garraway LA. Clinical implications of the cancer genome. *J Clin Oncol.* 2010;28(35):5219-28.

22. Krajarng A, Nakamura Y, Suksamrarn S, Watanapokasin R. α-Mangostin induces apoptosis in human chondrosarcoma cells through downregulation of ERK/JNK and Akt signaling pathway. *J Agric Food Chem.* 2011;59(10):5746-54.

23. Li H, Nie F, Liu W, Dai Q, Lu N, Qi Q, et al. Apoptosis induction of oroxylin A in human cervical cancer HeLa cell line in vitro and in vivo. *Toxicology.* 2009;257(1-2):80-5.

24. Ziegler U, Groscurth P. Morphological features of cell death. *Physiology.* 2004;19(3):124-8.

25. Ye M, Liu J, Lu Z, Zhao Y, Liu S, Li L, et al. Grifolin, a potential antitumor natural product from the mushroom Albatrellus confluens, inhibits tumor cell growth by inducing apoptosis in vitro. *FEBS Lett.* 2005;579(16):3437-43.

26. Krishnan A, Hariharan R, Nair SA, Pillai MR. Fluoxetine mediates G0/G1 arrest by inducing functional inhibition of cyclin dependent kinase subunit (CKS) 1. *Biochem Pharmacol.* 2008;75(10):1924-34.

27. Tounekti O, Belehradek Jr J, Mir L. Relationships between DNA fragmentation, chromatin condensation, and changes in flow cytometry profiles detected during apoptosis. *Exp Cell Res.* 1995;217(2):506-16.

28. Safarzadeh E, Sandoghchian Shotorbani S, Baradaran B. Herbal medicine as inducers of apoptosis in cancer treatment. *Adv Pharm Bull*. 2014;4:421-7.

29. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. *Eur J Pharmacol.* 2014;740:364-78.

30. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev.* 1999;13(15):1899-911.

31. Danial NN. BCL-2 family proteins: critical checkpoints of apoptotic cell death. *Clin Cancer Res.* 2007;13(24):7254-63.

32. Kantari C, Walczak H. Caspase-8 and bid: caught in the act between death receptors and mitochondria. *Biochim. Biophys. Acta, Mol. Cell Res.* 2011;1813(4):558-63.

33. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell.* 1997;91(4):479-89.

34. Scorrano L, Korsmeyer SJ. Mechanisms of cytochrome *c* release by proapoptotic BCL-2 family members. *Biochem Biophys Res Commun.* 2003;304(3):437-44.

35. Cohen GM. Caspases: the executioners of apoptosis. *Biochem J.* 1997;326:1-16.

36. Tourneur L, Chiocchia G. FADD: a regulator of life and death. *Trends Immunol.* 2010;31(7):260-9.

37. Boatright KM, Salvesen GS. Mechanisms of caspase activation. *Curr Opin Cell Biol.* 2003;15(6):725-31.

38. Wang ZQ, Stingl L, Morrison C, Jantsch M, Los M, Schulze-Osthoff K, et al. PARP is important for genomic stability but dispensable in apoptosis. *Genes Dev.* 1997;11(18):2347-58.

39. Han Z, Hong L, Han Y, Wu K, Han S, Shen H, et al. Phospho Akt mediates multidrug resistance of gastric cancer cells through regulation of P-gp, Bcl-2 and Bax. *J Exp Clin Cancer Res.* 2007;26(2):261-8.

40. HU H, YANG Y, XU X, SHEN H, SHU Y, Ren Z, et al. Oridonin induces apoptosis via PI3K/Akt pathway in cervical carcinoma HeLa cell line 1. *Acta Pharmacol Sin.* 2007;28(11):1819-26.

41. Altomare DA, Testa JR. Perturbations of the AKT signaling pathway in human cancer. *Oncogene.* 2005;24(50):7455.

42. Kim MJ, Lee TH, Kim SH, Choi Y, Heo J, Kim Y. Triptolide inactivates Akt and induces caspase-dependent death in cervical cancer cells via the mitochondrial pathway. *Int J Oncol.* 2010;37(5):1177-85.

43. Li D, Guo J, Huang J, Wang L, Deng R, Liu J, et al. Rhabdastrellic acid-A induced autophagy-associated cell death through blocking Akt pathway in human cancer cells. *PLoS One.* 2010;5(8):12176.