MECHANISM OF APOPTOSIS INDUCTION BY *COLOCASIA ESCULENTA* VAR. *AQUATILIS* HASSK EXTRACT IN CERVICAL CANCER CELLS

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กลไกการชักนำการตายแบบ apoptosis ของสารสกัดหยาบจากพืช *Colocasia esculenta* var. *aquatilis* Hassk ในเซลล์มะเร็งปากมดลูก

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BY

NATHARIKA CHOMLAMAY

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Title	MECHANISM OF APOPTOSIS INDUCTION BY *COLOCASIA ESCULENTA* VAR. *AQUATILIS* HASSK EXTRACT IN CERVICAL CANCER CELLS
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In Thailand, it was reported that the incidence and mortality of cervical carcinoma was in the top five of cancers. Natural products had an overwhelming success for cancer therapy. In this study, the aim was to study the effects of *Colocasia esculenta* var. *aquatilis* Hassk, Elephant ear (EE) extract on ER stress responses and apoptosis induction in the cervical cancer HeLa cell line. In this study, the effects of EE extract on cell cytotoxicity by MTT assay, chromatin condensation and apoptosis were assessed by Hoechst 33342 dye and flow cytometric method, respectively. The loss of mitochondrial membrane potential was measured by JC-1 dye, and the expression of proteins was evaluated by Western blot analysis. The results found that EE extract could inhibit cell proliferation with IC_{50} values of 187.2 ± 0.3368 μg/ml. Moreover, it was found that EE extract induced apoptotic bodies and sub-G1 DNA content in HeLa cells as well as suppressing the expression of anti- apoptotic proteins Bcl-2, Bcl-xL, and Mcl-1, whereas enhanced pro-apoptotic protein, Bax. Remarkable, EE extract showed a potent increase in the accumulation of ER stress intermediary, increasing CHOP protein levels in EE-treated cells, which lead to disrupt the imbalance of Bcl-2 family proteins and mitochondria function resulting in an increase cleaved caspase-7, and cleaved PARP. The data indicated that EE extract induced ER stress associated apoptosis pathway in cervical cancer HeLa cells. This study provided a basic knowledge on bioactivities of stem extract of elephant ear in HeLa cell line for the development as an anticancer drug in the future.

Keyword : Colocasia esculenta var. aquatilis Hassk, elephant ear, anticancer, ER stress, apoptosis

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CHAPTER 1
INTRODUCTION

Cancer is a kind of diseases involving cells with loss of growth control. Human cervical carcinoma is a type of cancer found in the cervix and presented as the fourth most common cancer amongst women worldwide, the incidence showed about 570,000 new cases and 311,000 death cases in 2018 [1, 2]. In Thailand, it was reported that the incidence and death rates of cervical cancer was in the top five of cancers. Human papillomavirus (HPV) infection is a main cause of cervical that is passed through sexual contact [3, 4]. The HPV affects healthy cell growth without control and invade other tissue [5]. Currently, the treatments of cervical cancer are surgery, radiation therapy, and chemotherapy depending on several factors such as the stage of cancer and other health problem [6]. However, these treatments are not completely effective against the cancer growth without side effects.

Natural products had an overwhelming success for cancer therapy in the 20th century. Previous studies have found the phytochemicals in plant extracts presented anti-cancer activities via endoplasmic reticulum (ER) stress and apoptosis induction [7]. As well-known, ER plays an important role in controlling calcium storage and synthesis, degradation, and transport of cellular proteins as well as triggering apoptosis in cancer cells [8]. Interestingly, elephant ear (*Colocasia esculenta* var. *aquatilis* Hassk), the local name called “Bon” was widely found in Thailand and especially abundance in Northern Thailand. Previous studies demonstrated that the elephant ear extract enhanced phagocytosis ability of macrophages, suppressed cell growth and induced apoptosis in human colon cancer [9], and decreased metastasis in breast cancer and lung cancer [10]. Therefore, in this study, we aimed to study the effects of elephant ear extract on ER stress responses and apoptosis induction in the cervical cancer HeLa cell line. This study will provide a basic knowledge on bioactivities of stem extract of elephant ear in HeLa cell line for the development as an anticancer drug in the future.

Objective

1. To study the cytotoxicity of elephant ear (EE) extract on the cervical cancer HeLa cell line.

2. To study the effects of EE extract on endoplasmic reticulum stress associated apoptosis induction in the cervical cancer HeLa cell line.

Conceptual framework

We hypothesized that EE extract affects cell growth inhibition through endoplasmic reticulum stress associated apoptosis induction in cervical cancer HeLa cell line. The conceptual framework is shown in Figure 1.

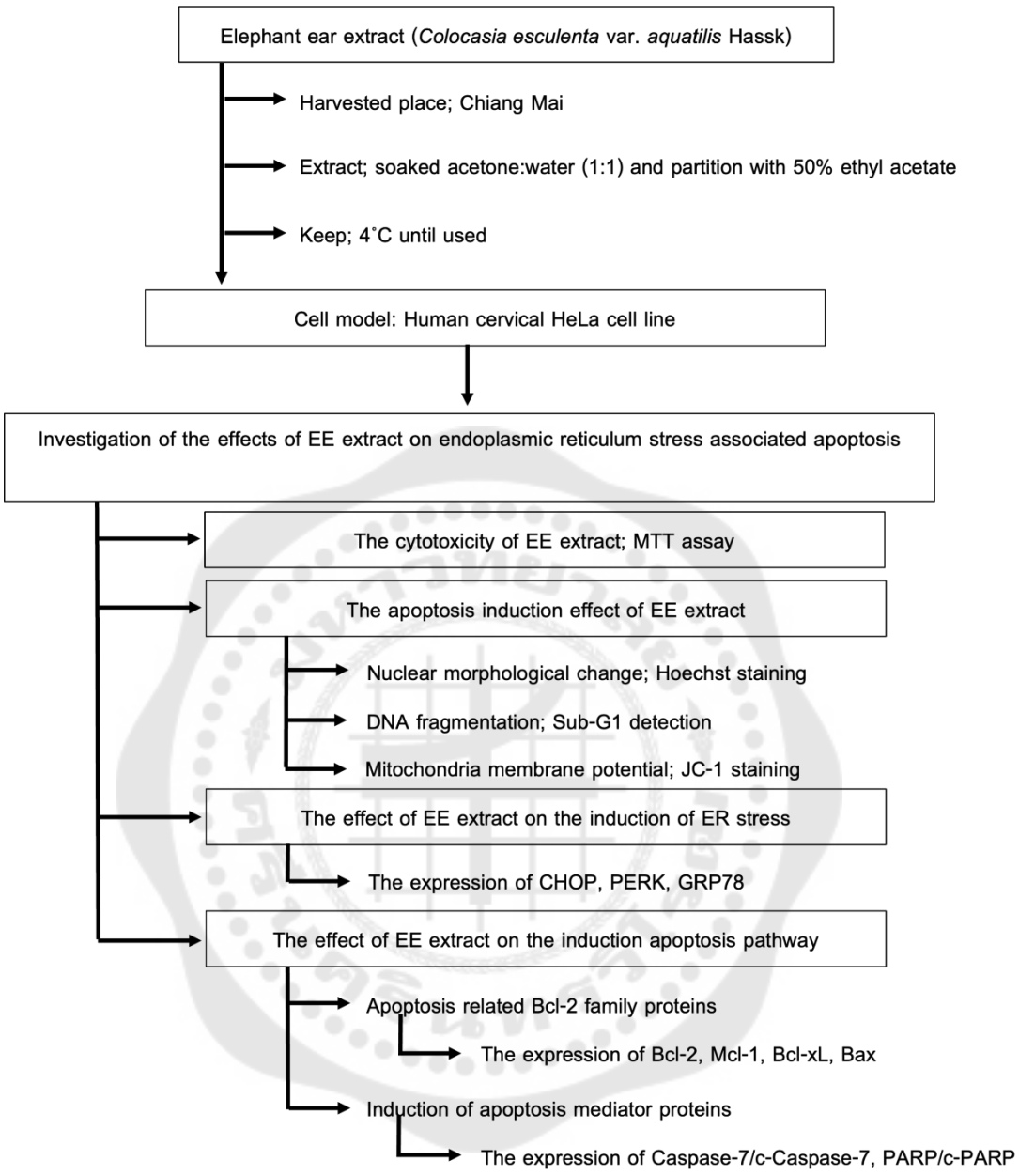


Figure 1 Conceptual framework

CHAPTER 2
REVIEW LITERATURE

Cervical cancer

Cervical cancer is the fourth common cancer in women, followed by breast cancer, colorectal cancer, and lung cancer respectively with an approximated 570,000 new cases and 311,000 deaths in 2018 [1, 2]. Moreover, the Asia, especially China, there is the highest incidence occurred a third of the global cervical cases [1]. Focusing on Thailand rank of cervical cancer is at 16.4 per 100,000 incidence cases and 7.4 per 100,000 mortality cases women, all age, per year in 2020 [1, 3].

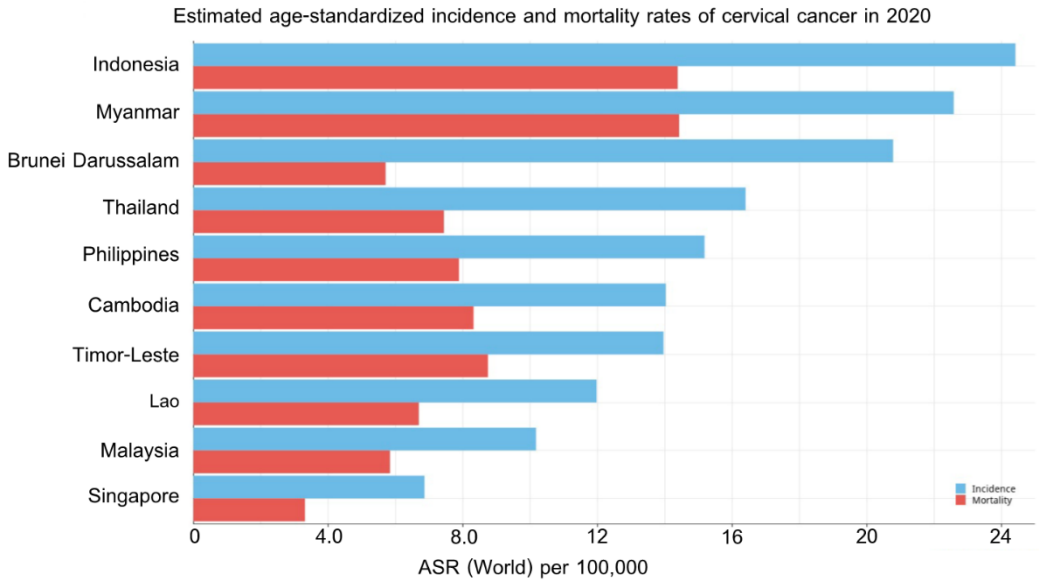


Figure 2 Estimated age-standardized new cases and mortality rates of cervical cancer of South-Eastern Asia in 2020, females (all ages) [3]. [Modified from World Health Organization. Health topics. Cancer [Internet]. 2021 Mar 3. Available from: https://www.who.int/news-room/fact-sheets/detail/cancer]

The most common cause of cervical cancer is HPV infection. Approximately 95% of woman was diagnosed with HPV infection which has various subtype such as HPV 16 and 18 mostly found to cause cervical cancer [4]. Moreover, two groups of HPV subtypes causing cervical cancer are high risk and low risk of lesion including high risk (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59, 61, 66, 67, and 68), and low risk (HPV 6, 11, 42, 43, 44, 53, 54, 55, 62, and 70) [11]. In Thailand, approximately 70% cervical cases are HPV subtype 16 and 18 [12]. Nonetheless, another cause of cervical cancer is rarely found such as tobacco smoking [13-15].

HPV pathology and cervical carcinoma

HPV infection begins when sexual intercourse or have many sexual partners, the virus infects the epithelium cells in the cervix and binds heparin on basement membrane surface. Then HPV genomes were delivered to host nucleus and induced proliferation resulted in squamous intraepithelial lesions and lastly invasive cancer to other organs [11].

The HPV belongs to *Papillomaviridea* family, which consists of 8,000 base pairs circular dsDNA virus genome. The genomes contain only eight open reading frames encoded for early (E) or late (L) in the HPV lifecycle [16, 17]. Integration of the eight HPV viral genome into nucleus of host cell, the genomes were expressed as autonomous replication and segregation into progeny cells. The early (E) genes encoded controlled proteins such as DNA replication, transcription, cell transformation, viral assembly, and release [12] including E1 and E2. The late (L) genes encode capsid proteins [12, 16] including L1 and L2. Other genes like E4, E5, E6, and E7 encode accessory proteins of each papillomavirus type. In addition, the HPV genome comprises transcriptional regulatory sequences and the viral origin of replication [17].

The function of E6 and E7 nuclear proteins lead to cell proliferation and tumor formation, E6 binds p53 tumor suppressor protein [12], which is degraded by a cellular ubiquitin ligase resulting in apoptosis, G1 arrest, and DNA repair are dismissed. E7 binds retinoblastoma (pRb) tumor suppressor protein releasing free E2F for transcription of S phase in host cells [16].

L1 and *L2* genes encode L1 and L2 capsid proteins which bind to receptor on cell surface and encapsulation of viral DNA, respectively [16].

E1 protein binds specifically to DNA sequence then bind to E2 protein forming hexameric complex as helicase, which controls E6/E7 expression. E4 protein is mostly found in cytoplasm to control the maturation and the HPV release. E5 protein is a small molecule and membrane-bound protein associated with E6/E7 induced tumor formation [18, 19]. Therefore, all processes result in the formation of malignant cells.

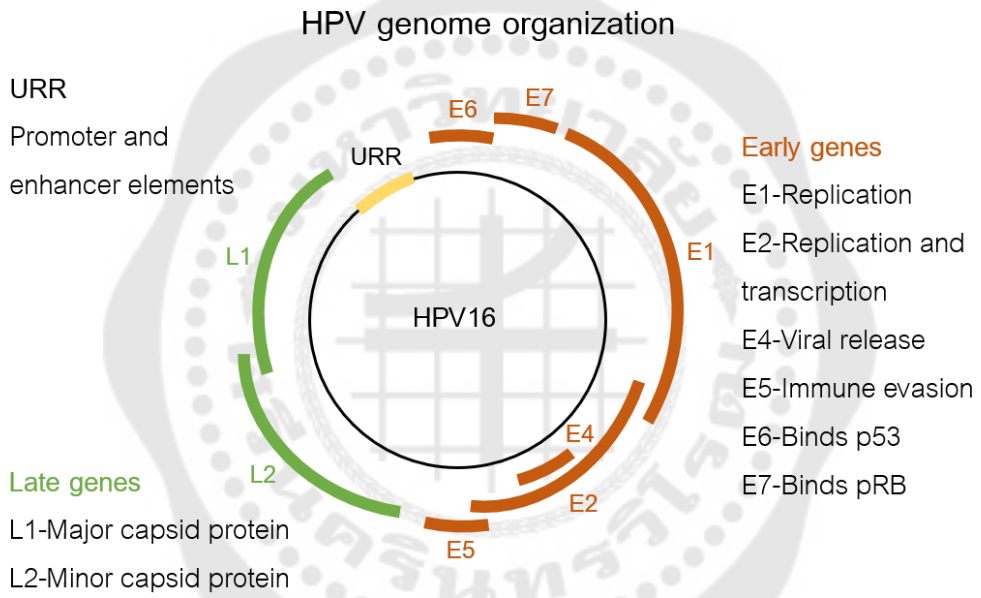


Figure 3 HPV genome organization. [Modified from Margaret S, et al. 2012. [19]]

Cervical cancer symptoms

Cervical cancer is slowly developed over a period of many years. In general, cervical cancer is asymptomatic, detected when invade other organs. It has been reported that menopausal bleeding and intermenstrual bleeding are symptoms of cervical cancer. Moreover, the symptom of cervical cancer includes persistent lower abdominal/pelvic pain, continual smelly vaginal discharge, discomfort or pain during sex, or blood in urine/stool [20].

Cervical cancer screening

The Papanicolaou (Pap) smear test is a very useful method for collecting sample from the cervix under a microscope for precancerous cervical epithelial lesions. The test is highly specific when combined with cytology, which is the most widely used HPV DNA or HPV mRNA detection. The Pap test is safe, and easy to access. Early diagnosis is essential to reduce treatment burden, morbidity, and mortality [17, 21].

Cervical cancer prevention

The risk factors of cervical cancer are early age of sexual intercourse, multiple sexual partners, and having multiple partners. Reduction of risk factors can reduce HPV infection or treatment to prevent cancer invasion. When women are diagnosed with cervical cancer usually be healed with the effective treatment to reduce symptoms, reduce severe regions, and reduce the mortality from disease.

Cervical cancer stages and treatments

The stages of cervical cancer are referred to the International Federation of Gynecology and Obstetrics (FIGO) staging system in 1994. Stages are determined on size of the tumor and its spread.

Stage 0: the cancer is *in-situ* or intraepithelial carcinoma

Stage I: the cancer is so small, its growth is still less than 7 mm on cervix tissues but not spread. The cancer can be seen with or without a microscope or colposcope. Cervical cancer in stage 1 is generally treated by radiotherapy or surgery.

Stage II: the cancer grows and spreads to the surrounding cervix tissues, but it spreads to the muscles or ligaments. Cervical cancer in stage 2 is generally treated by radiotherapy or chemoradiotherapy.

Stage III: the cancer grows and spreads away from cervix tissues such as the lower third of vagina, spreading to the pelvic wall or block kidneys tubes. Moreover, the cancer spreads to the muscles or ligaments. Cervical cancer in stage 3 is generally treated by chemoradiotherapy.

Stage IV: the cancer grows and spreads away from cervix tissues to nearby organs such as rectum, bladder or spread to other organs including lung. Cervical cancer in stage 4 is generally treated by chemoradiotherapy [6].

Chemotherapy usually results a long list of side effects. The treatment is usable to discriminate between healthy and cancerous cells. High doses of chemotherapy can disrupt immune cells in your body. Moreover, the available chemotherapeutic agents have not been fully effective against the patients with advanced cervical cancer due to less chemosensitivity of cervical cancer cells. The efficient chemotherapeutic agents are apparently needed for patients to alleviate 5-year survival rate. For 40 years, natural products have indicated a very important role as established cancer chemotherapeutic agents.

***Colocasia esculenta* var. *aquatilis* Hassk**

Alternative medicine has been used for a long time for human health to prevent and treatment of various diseases. Recently, the phytocomponents in plants have been shown as a chemotherapeutic agent for treating cancer. Our research, we aim to examine the effect of *Colocasia esculenta* var. *aquatilis* Hassk against cervical HeLa cell line.

Colocasia esculenta var. *aquatilis* Hassk, a tropical plant called elephant ear (EE). The tree height was estimated 1.5 meters and leaf cylindrical shape like elongated heart and length about 7 to 40 centimeters [22]. It is originally distributed in Thailand and Southeast Asia, India, China, southern Japan, northern Australia, and Melanesia [23]. Moreover, it has been known as an invasive weed in southeastern United States (Texas, Mississippi, and Alabama). This tree grows near fresh water or aquatic habitats including marshes, lake, waterway margins, and drainage sloughs [24]. *Colocasia esculenta* var. *aquatilis* Hassk is abundant in Thailand, especially in Northern part, called “Bon”. The evidence demonstrated that a stem of Bon has been used as traditional foods and treatment of some diseases such as reducing fever [25]. But the stem also contain oxalate which is a toxic compound resulting in itchy and allergic reactions. However, this compound is degraded under high temperature [10].

Currently, there are not many scientific reports on *C. esculenta* var. *aquatilis* Hassk activities. Thus, we reviewed the activity of close species *C. esculenta*, which has numerous varieties into sub-species. The various sub-species are divided by size, shape, color, number of tubes, texture, chemical composition of tubers, and degree of rhizome

formation. Many reports showed bioactive compounds of *C. esculenta* extract consist of flavonoids, tannins, alkaloids, saponins, phenols, hexadecanoic acid, and octadecanoic acid [10, 26-29]. These compounds have anti-fungal, anti-bacterial, anti-inflammatory, anti-diabetic, anti-allergic, and anti-cancer effects [22, 29, 30]. Moreover, the *C. esculenta* extract was reported to be used against cancer proliferation and survival. The *C. esculenta* extract inhibits proliferation in rat colon cancer cell line via inducing apoptosis and non-specifically activating lymphocytes [9]. In addition, it has been reported *C. esculenta* extract were against cancer proliferation in various cell lines such as murine EpH4 breast cancer cell, MCF-7 breast cancer cells, MDA-MB-231 breast cancer cells, MCF10A breast cancer cells, lung cancer cells, HepG2 hepatoma cells, U87MG glioblastoma cells, and B16BL6 melanoma cells [10]. In renal carcinoma cell line, the vitexin extracted from *C. esculenta* was able to induce apoptosis via increased caspase-3, caspase-9 and decreased Bcl-2 protein. In addition, the vitexin inhibited survival via Akt pathway [31]. Based on these data, *C. esculenta* inhibited cancer cell growth and induced apoptosis. However, the effect of *C. esculenta* on ER stress associated apoptosis induction in cervical cancer has not been reported.

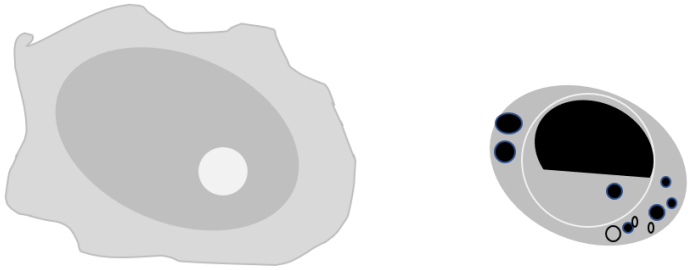
Figure 4 *Colocasia esculenta* var. *aquatilis* Hassk (Elephant ear). [Modified from Sanga Sabhasri Research and Development Department. Bon. The Botanical Organization [Internet]. Available from: http://www.qsbg.org [32]]

Apoptosis

Apoptosis or programmed cell death controls both physiological and biological changes of cells. Apoptosis involves in various processes including immune system, embryonic development, and cell death. Apoptosis imbalance leads to enhance many diseases such as autoimmune disorder, neurodegeneration, ischemic damage, and cancers. Interestingly, apoptosis is an effective cancer cell death induction.

Morphological characteristics of apoptosis

Kerr et al., 1972 described apoptosis definition by morphological characteristic through electron microscope [33], which showed apoptotic information and phagocytosis by phagocytes [34]. Briefly, cells undergoing apoptosis present cell shrinkage and pyknosis. Cell shrinkage is smaller than normal cell by condensation of cytoplasm and organelles are tightly packed while chromatin condensation results pyknosis. Pyknosis or karyopyknosis is cell undergoing apoptosis, followed by self-break up, producing two or more fragments, or known as karyorrhexis. This event occurs under nuclear membrane condensation. After that, the extensive plasma membrane blebbing and leaving of cell fragments into apoptotic bodies. Apoptotic bodies consist of packed organelles with or without nuclear fragments. The apoptotic bodies are rapidly phagocytosed and degraded by the lysosomal enzymes [33, 35, 36]. The phagocytic cells recognized and engulfed apoptotic cells by eat-me signaling such as phosphatidylserine (PS) exposure on surface of membranes in apoptotic cells and changes in surface sugars detected by phagocyte lectins. In a latent PS expose to the inner-membreane, once the apoptosis induction, PS exposed on outer-membrane [37]. Thus, cells undergoing apoptosis are not presenting inflammatory response. However, the fragmented not phagocytosed will undergo degradation resembling necrosis in a process called secondary necrosis [33, 36].



Characteristics	Normal cell	Apoptotic cell
Cell shape	Varies according to function	Rounded and shrunk
Chromatin	Well-defined domains of euchromatin and heterochromatin	Condensed, marginalized, later fragmented
Cell membrane	Intact	Intact, later apoptotic bodies
Nuclear envelope	Intact	Intact
Organelles	Well defined, numerous	Shrunk
Cytoplasm	Contained	Contained

Figure 5 The morphological distinction of apoptosis and necrosis. [Modified from Anne K. Voss, et al. 2020[38]]

Biochemical characteristics of apoptosis

The biological changes in apoptosis induction are regulated by numerous biomolecules such as Bcl-2 family proteins, caspases enzymes, and poly (ADP-ribose) polymerase (PARP) protein. This event results in protein cleavage, protein cross-linking, DNA condensation, DNA breakdown, and phagocytic program leading to the characteristic structural pathology described previously [39].

The Bcl-2 family

The Bcl-2 family proteins containing Bcl-2 homology (BH) domains which are divided into two groups including pro-apoptosis and anti-apoptosis. The pro-apoptosis proteins are divided into two sub groups; pro-apoptosis initiators and pro-apoptosis effectors. Different combination of proteins determines functions of Bcl-2 family on proliferation and cell death [40]. Bcl-2 family protein balance also regulates survival and apoptosis of cancer cells [41].

Pro-apoptosis initiators

The pro-apoptotic Bcl-2 proteins including Bcl-2-like protein 11 (Bim), p53 upregulated modulator of apoptosis (Puma), BH3 Interacting Domain Death Agonist (Bid), Bcl-2 associated agonist of cell death (Bad), Phorbol-12-myristate-13-acetate-induced protein 1 (Noxa), Bcl2 Modifying Factor (Bmf), and Bcl2 Interacting Protein (Hrk). In humans, the Bcl-2 protein family also contains BH-3 only proteins, which initiate apoptosis pathway by directly binding and activation to pro-apoptosis effectors like Bcl-2 Associated X (Bax), Bcl-2 homologous antagonist/killer (Bak), and Bcl-2 related ovarian killer (Bok). In addition, the BH-3 only proteins inhibit anti-apoptotic proteins like B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), Bcl-2-like protein 2 (Bcl-w) [40, 41] by BH-3 domain binds hydrophobic cleft of anti-apoptotic proteins [42]. Bim, Puma, and Bid bind strongly to all anti-apoptotic proteins but Bad inhibits Bcl-2, Bcl-xL, and Bcl-w. Noxa inhibits Mcl-1 [40, 41].

Pro-apoptosis effectors

The pro-apoptotic Bcl-2 protein include Bax, Bak, and Bok which contain three BH domains, BH1, BH2, and BH3 [42]. The pro-apoptotic Bcl-2 proteins induce apoptosis pathway by interacting with outer mitochondrial membrane i.e. activated Bax and Bak formed homo-dimer followed by oligomerization on outer mitochondrial membrane. The changed conformation induces releasing apoptosis-inducing protein such as cytochrome C and mitochondrial outer membrane permeabilization (MOMP). Following this release, the caspase proteins are activated by the enzyme cleaved caspase cascade, resulting in cellular destruction and cell death [41]. We are also interested in the role of some pro-apoptosis effectors like Bax.

Anti-apoptotic protein

The anti-apoptotic proteins such as Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 contain BH1, BH2, BH3, and BH4 domains. The four-domains proteins induce apoptotic cell and maintain cells survival [41, 43]. The BH4 domain of anti-apoptotic protein is bound directly BH3 domain of pro-apoptotic Bcl-2 initiators like Bim for apoptosis induction. While the BH4 domain binds pro-apoptotic Bcl-2 effectors like Bax, preventing MOMP, to maintain cell survival. Anti-apoptotic Bcl-2 protein; Bcl-2, which associates with apoptotic protein as Bax, undergoes conformation change healthy cell to apoptotic cell [40, 43]. Both events are occurred by Bcl-2 conformation change. Thus, we focused particular attention on role of anti-apoptosis initiators such as Bcl-2, Bcl-xL, and Mcl-1.

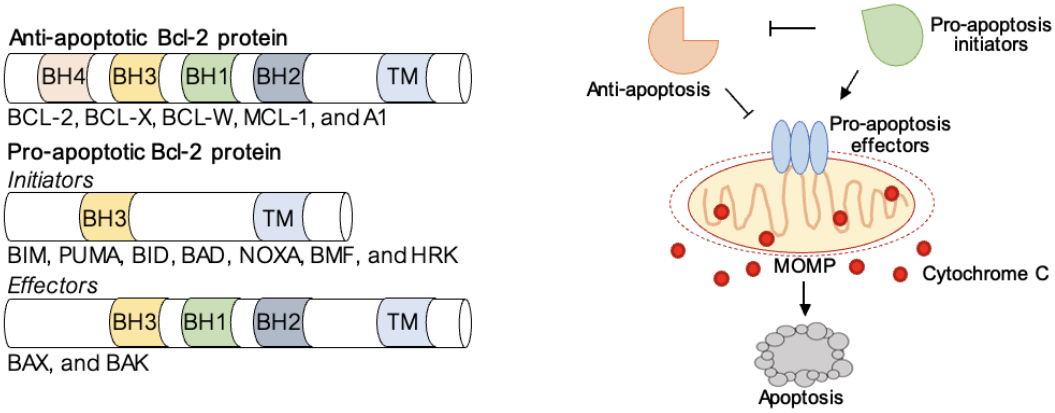


Figure 6 The group of Bcl-2 family proteins and functional Bcl-2 protein induced cell death. [Modified from Clare M. Adams, et al. 2018[43]]

Caspase family

Cysteinyl aspartate-specific protease (Caspase) is synthesized as zymogens as an inactive form. The zymogens comprise of N-terminal domain that has a death effector domain (DED) or a caspase recruitment domain (CARD) and C-terminal domain consists catalytic domains [44, 45]. The catalytic domains of caspases consist of large and small subunits. The active form of caspases was activated by cleavage two heterodimer of enzymes into heterotetramer and the activated caspase then activates other caspases [46]. The caspase is divided into three subfamilies including apoptosis initiator caspase consists of caspase-2, -8, -9, -10, apoptosis executioner consists of caspase-3, -6, and -7, and inflammatory mediator consists of caspase-1, -4, -5, and -12 [47].

In unstressed cells, initiator caspase is a long pro-domain which contains inactive caspase or zymogen. When cells are under stress, initiator caspase is induced by dimerization and induced cell death and inflammation. After that, the interchain of dimer caspase undergoes autocatalytic cleavage from heterodimer to heterotetramer or known as active form. While executor caspase is a short pro-domain containing inactive dimer form. Activated initiator caspases activate effector caspases consist of heterotetramers derived from two large and two small subunits [48].

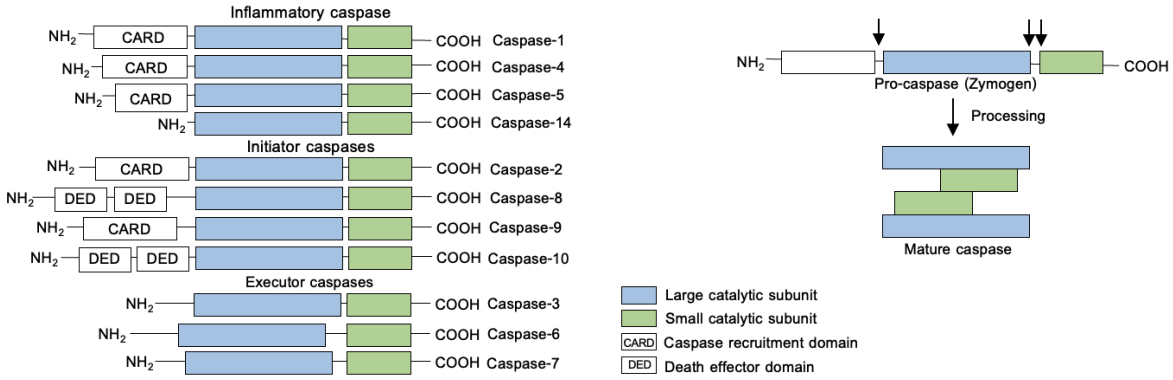


Figure 7 The characteristic and function of caspase. [Modified from Mar Orzáez, et al. 2009[44]]

PARP family

The poly (ADP-ribose) polymerases (PARPs) family involved with DNA repair which is activated by reactive oxygen species (ROS) [49] and radiation [50]. The role of PARPs is to transfer of ADP-ribose to target protein on DNA lesion and enhances Ligase III, DNA polymerase beta, AP endonuclease, and XRCC1 protein activities [50, 51]. It has been shown that PARP plays a key role on base excision repair (BER) [51] and nucleotide excision repair (NER) to prevent DNA mutation. Thus, PARP inhibition accumulated DNA damage and attenuated repairing. Furthermore, PARP is a substrate of casapas-3/-7. Active caspase-3/-7 directly cleave PARP and induce PARP degradation [52-54]. The cleaved PARP consists of two fragments; 89 kDa catalytic fragment and 24 kDa DNA binding fragment. When DNA strand breaks, the 24 kDa PARP fragment will irreversible bind to DNA damage and inhibit DNA repair enzymes [55]. Therefore, PARP is considered as a marker of apoptosis. The detection of cleave-PARP is one of the most used indicator for apoptotic cell.

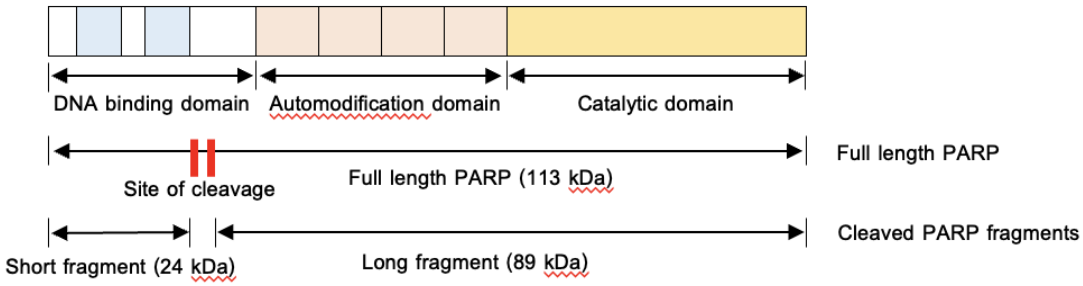


Figure 8 The important domain of PARP and their functions. [Modified from Mi Young Kim, et al. 2005[53]]

Apoptosis pathway

Apoptosis pathways are divided into two pathways consist of extrinsic and intrinsic apoptosis pathway. The extrinsic pathway is stimulated by external signals or death ligand such as Fas Ligand (FasL), tumor necrosis factor alpha (TNF-α), Apo3 ligand (Apo3L), and Apo2 ligand (Apo2L) [56]. Whereas the intrinsic pathway is stimulated by internal signals such as radiation, toxins, hypoxia, hyperthermia, viral infections, free radicals, growth factors, hormones, and other cytokines [35].

Extrinsic pathway

The initiation of extrinsic apoptosis pathway associated with transmembrane death receptor activation of the tumor necrosis factor (TNF) receptor family proteins, containing a cysteine in extracellular domains and 80 amino acids in cytoplasmic domain called death domain [36]. The extrinsic pathway is triggered by binding of ligand and specific receptor such as TNF-α/TNFR1 (DR1), FasL/FasR (DR2), Apo3L/ TRAMP (DR3), Apo2L/ TRAILR1 (DR4) and Apo2L/ TRAILR2 (DR5) [33, 57]. Briefly, the ligand binds to N-terminal region of receptors and induces formation of death-inducing signaling complex (DISC) via the FADD (Fas-associated death domain protein) activation [58]. DISC complex binds to pro-caspase8 leading to autocatalytic activation of pro-caspase-8 to caspase-8 [59] or also called FADD- like IL-1 converting enzyme (FLICE). The enzyme then triggers a cascade of activated caspase by cleaving and thereby activating other [36, 60] including pro-caspases-3, -6, and -7. Activated caspase-3 and -7

actives a variety of cellular substrates, including DNA repair enzymes, structural proteins of cell and nuclear, and endonuclease inhibitors [61]. It has been reported that activated caspase-7 cleave various substrates such as PARP, assist in the degradation and destruction of the cell that resulting in apoptosis induction.

Intrinsic pathway

Intrinsic pathway is stimulated by intracellular signals such as toxins, radiation, free radical, DNA damage, hyperthermia, hypoxia, and lacking growth factors leading to disrupt mitochondrial permeability [33, 59]. This event is controlled by the balance between the pro- and anti-apoptotic proteins. Normally, anti-apoptosis proteins bind to pro-apoptosis proteins preventing apoptosis induction. In apoptosis, Bcl-2 family consists of BCL-2 homology domain 3 (BH3)-only proteins including Bim, Bid, and Bad, directly interact and block the function of Bcl-2 and Bcl-xL leading to enhance pro-apoptotic protein (Bax and Bak) forming oligomers that insert into the outer mitochondrial membrane and create channels, called mitochondrial outer membrane permeabilization (MOMP) leading to the release of intermembrane space protein, cytochrome c to cytoplasm [36, 62]. Then, cytochrome c binds to apoptotic protease activating factor 1 (Apaf-1), ATP and procaspase-9 forming apoptosome [33, 63]. The apoptosome complex induces self-cleavage of pro-caspase-9 into cleaved caspase-9, which in turn cleaves and thereby activates effector caspase including caspase-3, -6, and -7. These effector caspases cleave variety of proteins and in some cases proteolytically activate or inhibit other enzymes. These processes lead to chromatin condensation and cell content pack into apoptotic bodies and which are removed by phagocytosis [38, 64].

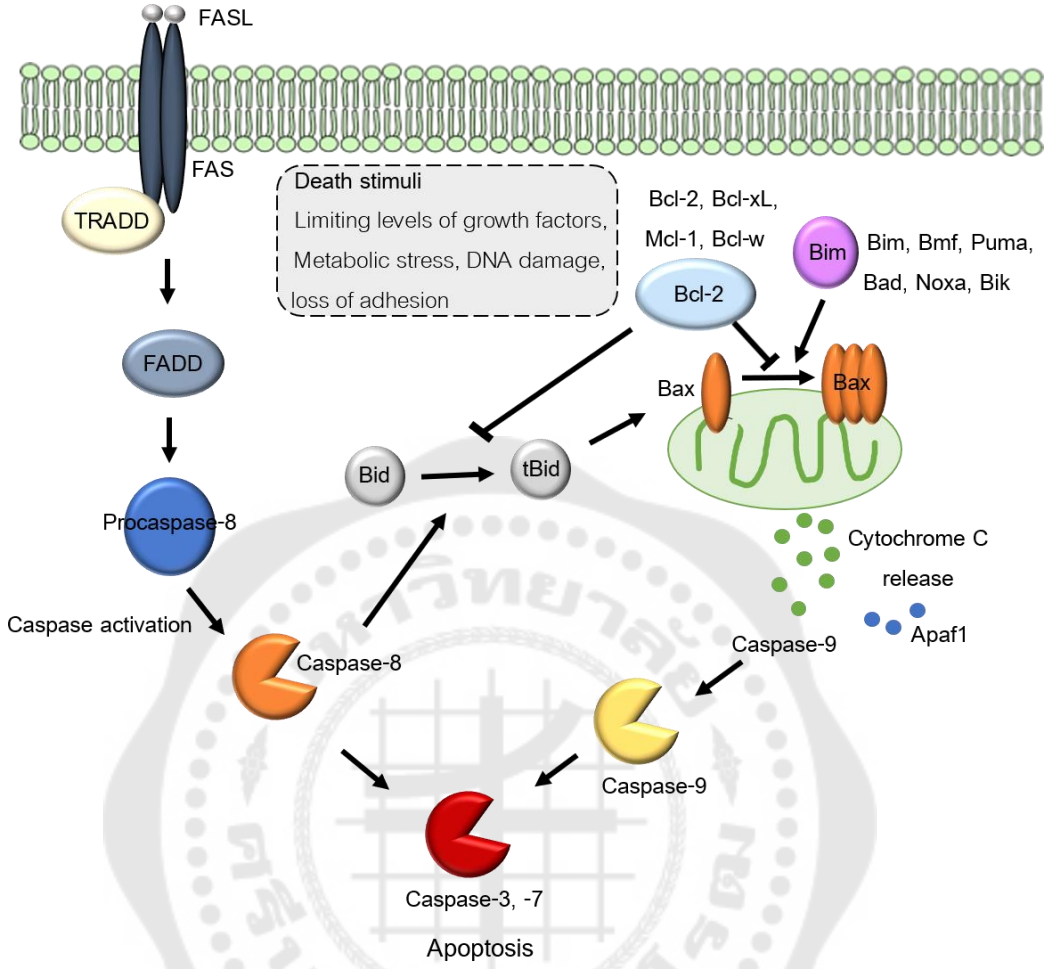


Figure 9 Simplified schematic drawing of extrinsic and intrinsic pathways of apoptosis.

[Modified from Anne K. Voss, et al. 2020[38]]

Endoplasmic reticulum (ER) stress

The endoplasmic reticulum (ER) is a cellular organelle which has multifunction involving in the synthesis, degradation, transport of protein, calcium storage, and lipid metabolism [65]. The ER contains cisternae, tubules and vesicles that implicate with modification of proteins and lipids [66]. The ER is divided into rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) [67]. Both have different function and structure such as the SER lacks ribosomes and is involved in the synthesis and storage of lipids, phospholipids, and steroids. Furthermore, it also actualizes the metabolism of carbohydrates, steroid, and attachment of receptors on cell membrane proteins [68]. In cells of the liver, SER contains cytochrome P450, which catalyzes and detoxifies natural metabolism products of carcinogens and other organic molecules [69]. The RER consist of ribosomes on the flattened membrane, which are involved in the synthesis of secreted, cytosolic and membrane proteins, protein folding, quality control, and dispatch. After transcription, a process of mRNA synthesis in nucleus, is then transferred outsides nucleus to produce a specific amino acid chain or polypeptide wherewith recognized by mRNA-ribosome-signal recognition particle (SRP) complexes that are situated at the ER membrane [8, 70]. These proteins are modified and folded by folding factors and ER chaperones [71]. After polypeptide synthesis, it is transferred from the RER to the Golgi apparatus via membrane-bound vesicles for protein storage and to be further processed or to organelles. The end step of protein synthesis is pinched off from the Golgi complex and released outside to perform its function [72].

The functions of the ER are suppressed by various intracellular and extracellular stimuli such as sugar deprivation, asparagine (N)-linked glycosylated inhibition, oxidative stress, ischemia, hypoxia, virus infection, and calcium ion imbalance, which resulted in the accumulation of unfolded or misfolded proteins in the ER lumen known as endoplasmic reticulum stress (ER stress). However, the stabilizing ER functions is controlled by three major mechanisms [73] known as unfolded protein response (UPR). The UPR is the major preventive mechanism against deleterious and toxic effects of ER stress by (I) inducing transcription of ER chaperone proteins and other components of

polypeptide folding and processing capacity in the ER [70, 73], (II) inducing proteasome-dependent ER-associated degradation (ERAD) to remove proteins from the ER and (III) controling protein translation to extenuate the polypeptide traffic into the ER [73].

The UPR is initiated by three ER transmembrane proteins including protein RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1α) [70]. In normal ER, three ER transmembrane proteins are maintained in inactive state binding to the ER chaperon, called glucose-regulated protein 78 (GRP78) [74]. GRP78 is a heat shock protein of 78 KDa that binds to hydrophobic amino acid sequences exposed to ER stress response [75]. Under ER stress condition, GRP78 will be dissociated from transmembrane and interacts with unfold proteins resulting in UPR-activated ER stress pathway [74], activate ER stress pathway. However, prolong or chronic ER stress led to failed UPR and cells are switched to cell death induction [70, 74].

The unfolded protein response (UPR) signaling pathway

The Inositol-Requiring Transmembrane Kinase/Endoribonuclease 1 (IRE1α) signaling pathway

IRE1 is a transmembrane protein with serine/threonine kinase domain and an endoribonuclease (RNase) domain at C-terminal cytosolic region. There are two homologs of mammalian cells, IRE1α is expressed in many cell types whereas IRE1β is expressed in intestinal epithelial cells [71], and mucosal surfaces [76]. When ER stress occurs, the dissociation of GRP78 from luminal domain activates IRE1α with oligomerization and autophosphorylation of its kinase domain to activate RNase, which splices 26 nucleotide intron of X-box binding protein (XBP1) mRNA to spliced XBP1 (XBP1s). This event produces a translational frameshift and generates a 41 kDa cAMP-response element-binding protein/Activating transcription factor (CREB/ATF), basic leucine zipper (bZiP) containing transcription factor [70, 75-77]. XBP1s transcriptional factor induces the production of target proteins required under ER stress such as ER degradation-enhancing alpha mannosidase- like 1 (EDEM), ER-localized DnaJ 4 (Erdj4), protein disulfide isomerase (PDI), chaperones, and other transcription factors [77]. In addition, IRE1 plays essential roles in the terminal differentiation of B-lymphocytes in plasma cells, placental development and promotes embryonic viability which activate pro-

survival effect during stress conditions [71]. However, long term ER stress, IRE1 induces apoptosis signaling pathway via the c-Jun N-terminal kinase (JNK) function and recruitment of TNF receptor associated factor 2 molecule (TRAF2) activating apoptosis signal-regulating kinase 1/ MAP3K5 (ASK1), leading to activation of MAPKs, JNK, and p38 function [70].

The Activating Transcription Factor-6 (ATF6) signaling pathway

ATF6 is a transmembrane protein with a basic leucine zipper protein (bZIP)-containing transcription factor, which presents two isoform genes including *ATF6*α and *ATF6*β. Upon accumulation of misfolded and unfolded proteins, ATF6 dissociates from GRP78 and translocates from the ER membrane to the Golgi apparatus by vesicular transport. Wherein it is cleaved by site-1 protease (S1P) at luminal domain and site-2 protease (S2P) at transmembrane domain generate N-terminal cytosolic domain of ATF6, known as ATF6 p50. Then, ATF6 p50 contains a bZIP domain, translocates to the nucleus and activates target genes expression [71, 75-78] such as ER chaperones (e.g., *BiP*, *GRP94*, *PDI*), and *XBP1* gene. These genes are translated to proteins and induce ERAD components such as ER degradation enhancing alpha-mannosidase-like protein (EDEM), yeast Der1 like protein (Derlin), mammalian homolog of yeast HMG-CoA reductase degradation 1 protein (Hrd1p), mammalian homolog of Hrd3p (SEL1L), and homocysteine-induced endoplasmic reticulum protein (Herp) [71], and CHOP [77]. Furthermore, ATF6 regulates calcium dependent pro-apoptotic functions through calcineurin 1 (RCAN1), which dephosphorylates Bad and inhibits Bcl-xL function [70].

The double-stranded RNA-dependent protein kinase (PKR)-like eukaryotic initiation (eIF2α) kinase (PERK) signaling pathway

PERK is a transmembrane protein with serine/threonine kinase domain. When ER stress occurs, PERK dissociates from GRP78 then undergoes oligomerization and auto-phosphorylation of its kinase domain to trigger eukaryotic translation initiation factor 2 (eIF2α) phosphorylation at Ser51 region, which attenuates newly synthesized polypeptides into the ER lumen [76, 77]. Moreover, PERK requires for induction of mRNAs translation such as activates transcription factor 4 (ATF4), which increase CHOP level [78], Additionally, this event results in restoration of redox homeostasis, protein folding,

autophagy, and cell survival. Under chronic ER stress, ATF4 induces apoptosis pathway through pro-apoptosis signaling proteins such as growth arrest and DNA damage-inducible protein 34 (GADD34), ER oxidoreductin 1α (Ero1α), and BH3-only proteins like Bim, Puma and Noxa via CHOP upregulation [70]. GADD34 and Ero1α increase ROS production and calcium release was restrained by IPR3. The increase of cytosolic calcium triggers PTP induced apoptosis in the cell. On the other hand, the motivation of Bim, Puma, and Noxa induce cytochrome *C* release from mitochondria via Bax and Bak induction. Furthermore, CHOP and ERO1α decrease the glutathione (GSH) level which leads to ROS formation by re-oxidation of protein disulfide isomerases (PDIs) induces hydrogen peroxide (H_2O_2) reduction in active proteins reconstitution. Moreover, CHOP increase inositol- 1,4,5-trisphosphate receptor (IP3R) contributing to apoptosis [70, 77].

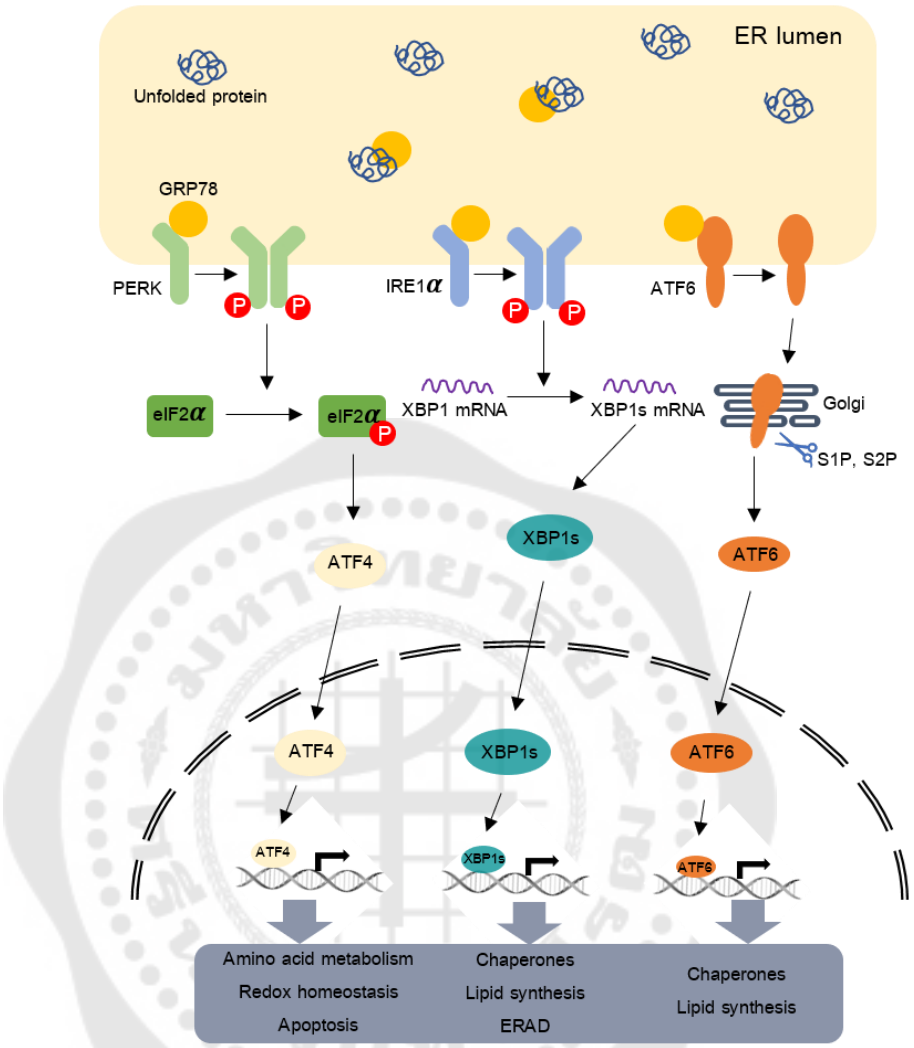


Figure 10 The unfolded protein response (UPR) signaling pathway. [Modified from Scott A Oakes, et al. 2020[78]]

CHAPTER 3
MATERIALS AND METHOD

Chemicals

Dulbecco's Modified Eagle Medium (DMEM) and other supplements; fetal bovine serum (FBS), Penicillin/streptomycin (Pen/Strep), and trypsin were purchased from HIMEDIA (HiMedia Laboratories, India). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB (USB Co., USA). 2'-(4-Ethoxyphenyl)-6-(4-methyl-1-piperazinyl)-1H,3'H-2,5'-bibenzimidazole (Hoechst 33342) was purchased from Thermo Fisher Scientific (Thermo Fisher Scientific Inc., Massachusetts,U.S.). 5,5',6,6'-Tetrachloro-1,1',3,3-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was purchased from Wako (Wako Pure Chemical Industries, Ltd., USA). Bovine serum albumin (BSA) was purchased from VWR (VWR International Ltd., USA). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Merck (Merck KGaA, Missouri, U.S.). Dimethyl sulfoxide (DMSO) was purchased from RCI Labscan (RCI Labscan Co.,Ltd., USA). Antibodies for immunoblotting analysis antibody against CHOP, PERK, GRP78, Caspase-7, c-Caspase-7, PARP, c-PARP, Mcl-1, Bcl-2, Bcl-xL, Bax, anti- rabbit and anti-mouse immunoglobulin G horseradish peroxidase (HRP) conjugated secondary antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology Inc., Danvers MA, USA), and β-actin was purchased from Merck (Merck KGaA, Missouri, U.S.).

Cell culture

The cervical cancer HeLa cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% of fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37˚C in a humidified atmosphere of 5% CO_2. HeLa cells were sub-cultured by 0.25% trypsin/EDTA in every 2-3 days. After that, the total numbers of cells were counted by a hemocytometer counting chamber for each experiment.

Plant used and extraction

The fresh stem elephant ear (EE) was harvested from Chiang Mai in October, 2020 for preparation of crude extract (Fig. 11). Briefly, the fresh EE stem was cut and dry at 50˚C for 5 days. After that, dry sample was ground and soaked in 50% acetone (5% w/v of dry weight). Then, immersed plants were vortexed and primary filtered through a filter paper Whatman No.4, and evaporated. Next step, the acetone-water extract was used for fractionation based on solvent-solvent partition by different solvents with various polarity index which are ethyl acetate and water (ratio of 1:1). After partition, the ethyl acetate part (upper) was collected and evaporated. The crude EE extract was kept at 4˚C until used.

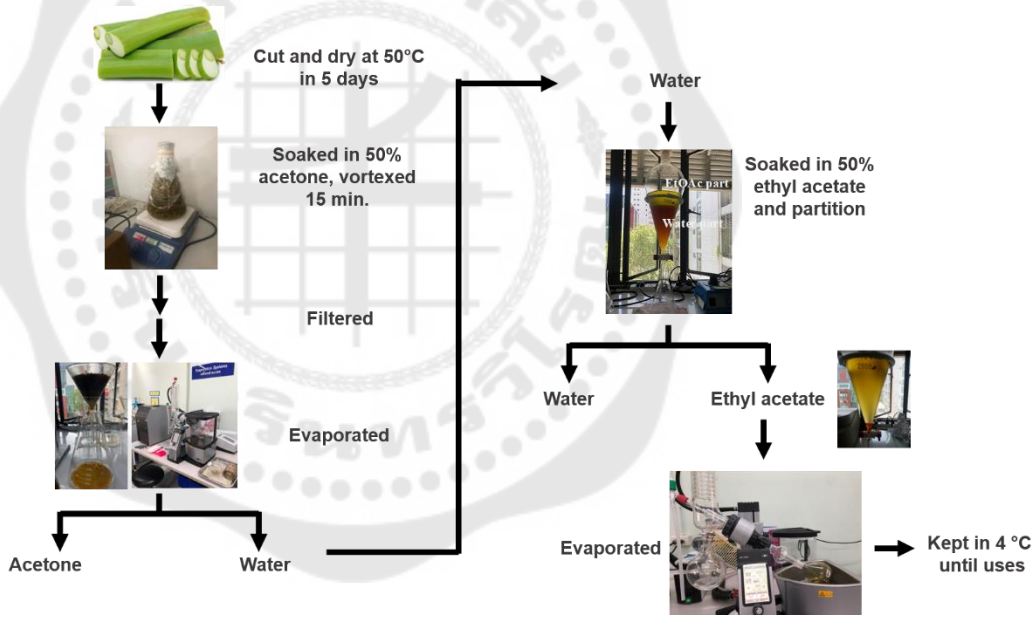


Figure 11 The extraction of fresh stem elephant ear (EE).

Cell viability assay

The cytotoxicity of EE extract was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT assay is a colorimetric assay for measuring cell viability via activity of mitochondrial dehydrogenase. This enzymes the present reduction activity of MTT to (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenyl-formazan (formazan crystals) in viable cells which changes yellow tetrazolium sait of MTT

solution to purple color of formazan crystals (Fig. 12). Thus, to determine cell viability of HeLa cells, MTT assay was carried out. Briefly, the cells were seeded at 7×10^3 cells/well in a 96-well plate and incubated for overnight. After that, the medium was replaced by EE extract at concentration of 25, 50, 100, 200, 300, and 400 µg/ml whereas the control was added with 0.5% DMSO. After 24 h of treatment, medium was taken out and replaced with 0.5 mg/ml of MTT solution, at 37°C for 2-4 h. The formazan crystal was dissolved by addition of DMSO. The purple color of formazan was measured at 570 nm using a microplate reader (Multiskan Sky Microplate Spectrophotometer, USA). The cell viability and half maximal inhibitory concentration (IC_{50}) value were estimated by using the GraphPad Prism 9 software (GraphPad Prism Software, Inc.).

Figure 12 Reduction of MTT to formazan crystals by mitochondrial reductase. [Modified from Enrique Chacon, et al. 1997[79]]

Detection of chromatin condensation

The morphological changes of cells including chromatin condensation and DNA fragmentation are the characteristic of cells undergoing apoptosis which can be detected by fluorescence staining. The Hoechst 33342 is a fluorescence dye that binds to A-T rich sequences in the minor grove of double-stranded DNA (dsDNA). Apoptotic cells stained with Hoechst 33342 dye will appear brightness of fluorescence dye in the nucleus. Briefly, HeLa cells were seeded at 6×10^4 cells/well in a 24-well plate and incubated overnight. Then, cells were treated with EE extract at various concentrations for 24 h. Then, cells were stained with 5 µg/ml of Hoechst 33342 dye and observed under a fluorescence microscope at 20X magnification (Fluorescence microscope, IX71 Olympus, Japan).

Sub-G1 detection

One of the apoptosis hallmarks is DNA fragmentation. The DNA fragmentation or DNA endonucleolytic cleavage is caused by endonuclease activity. This event can be determined by staining cellular DNA with fluorescent dye such as propidium iodide (PI) and observed by a flow cytometer. To determine the effect of EE extract on fractional DNA content, sub-G1 detection assay was conducted by using flow cytometry. HeLa cells were seeded at 10 $\times 10^4$ cells/well in a 12-well plate and incubated overnight at 37ºC in 5% CO_2 atmosphere. The cells were treated with EE extract at various concentrations and 0.5% DMSO as a control for 12 h. Next, the whole cells were kept and fixed with 70% cold ethanol. After fixation, the cells were stained with Guava Cell Cycle® reagent (Merck Millipore Corporation, Merck KGaA, Darmstadt, DE) and detected by the Guava easyCyte™ flow cytometer and GuavaSoft™ software (Merck Millipore Corporation, Merck KGaA, Darmstadt, DE). Data was shown as a percentage of sub-G1 DNA content, indicating as apoptotic cells.

Detection of mitochondrial membrane potential ($\Delta\Psi$m)

Mitochondria plays an important role on an ATP synthesis. The loss of mitochondrial membrane potential ($\Delta\Psi$m) was widely found in cells undergoing apoptosis. This event leads to loss of ATP energy resulting in cell death induction. Therefore, the mitochondrial membrane potential can be used as an indicator of apoptosis cell death. The lipophiliccationic 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye is a fluorescence dye that has properties to react with mitochondrial membrane. In healthy cells, JC-1 dye accumulates and generates in J-aggregates form which emitted red fluorescence signal. Conversely, in unhealthy cells with low mitochondrial membrane potential, the JC-1 dye enters and displays of monomeric form which emitted green fluorescence signal (Fig. 13). To investigate EE extract induced early apoptosis induction, JC-1 dye staining was carried out. Briefly, HeLa cells were seeded at 6×10^4 cells/well in a 24-well plate and incubated for 6 h at 37˚C. The cells were treated with EE extract at various concentrations for 6 h. After that, cells were stained with 5 µg/ml of JC-1 dye and incubated at 37˚C. Then, cells were washed with PBS and observed

under a fluorescence microscope at 20X magnification (Fluorescence microscope, IX71 Olympus, Japan).

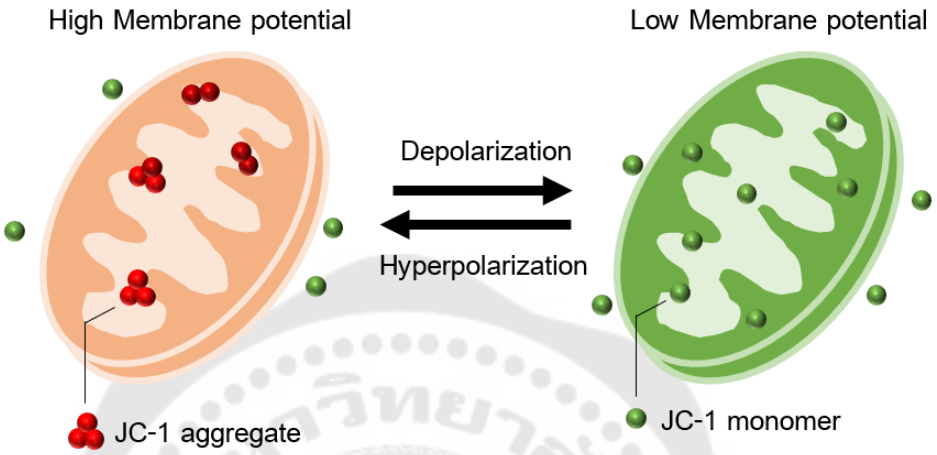


Figure 13 The principle of JC-1 dye staining assay. [Modified from Farzane Sivandzade, et al. 2019[80]]

Analysis of proteins expression by immunoblotting

The immunoblotting, also called "Western blotting", is a technique for detecting protein expression detection in living cells. This method contains many steps including gel electrophoresis, transferring proteins from gel into membranes, incubating with the specific antibodies, and detection, respectively. SDS-PAGE electrophoresis is used to separate proteins (negatively charged) depending on molecular weight of proteins. The protein samples are separated through a slab of porous polyacrylamide gel. After gel electrophoresis, proteins are transferred onto the surface of the positive membrane such as polyvinylidene fluoride (PVDF) membrane. Then, the non-specific binding proteins are blocked by bovine serum albumin (BSA) or non-fat dry milk. After that, the interested proteins on membrane are incubated with the specific antibody that recognizes a specific protein and detected by using chemiluminescent reaction.

Treatment and protein sample preparation

HeLa cells were seeded at 2.8×10^5 cells/well in a 6-well plate and incubated for 24 h. Then, cells were treated with EE extract at various concentrations or 10 µg/ml of

tunicamycin (Tm) and incubated at 37˚C for 24 h. The Tm is a common agent produced by Streptomyces lysosuperificus, which inhibits N-linked glycosylation as a result of interruption of oligosaccharide are added to nascent polypeptides and therefore blocks protein folding in ER. Consequently, Tm was used as a positive control on ER stress induction. After incubation time, cells were collected and lysed with RIPA buffer (RIPA buffer; 50 mM Tris, pH7.5, 5 mM EDTA, 250 mM NaCl, 0.5% TritonX-100, protein inhibitor tablet). Then, cells were centrifuged at 13,000 rpm at 4˚C for 30 min and the protein lysate was collected.

Determination of protein concentration

The concentration of total protein was determined by Bradford's assay using Bio-Rad assay kits (Bio-Rad Laboratories, Inc., USA). BSA at 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL were used as standard protein to measure protein concentration in each sample. Briefly, standard or samples of protein were mixed with 200 µl of Bradford's assay solution, then measured absorbance at 595 nm using the microplate reader (Multiskan Sky Microplate Spectrophotometer, USA). The protein concentration was calculated by calibration curve of standard protein. The total protein was mixed with 5X Dye before incubating on a heat box at 100˚C for 5 min, then the protein samples were used for SDS-PAGE.

Preparation of SDS-PAGE

The polyacrylamide gel for separating protein includes separating gel (lower) and stacking gel (upper) which differ in pH and polyacrylamide content. Proteins were separated depending on molecular weight. Briefly, large size of proteins usually spends longer time for separation than small size at the same polyacrylamide content.

Separating gel

Percentage of polyacrylamide gel has effective range between 8-12%. The total protein is separated by the pore size of a polyacrylamide gel, a higher percentage has smaller pores and suits for small proteins whereas lower percentage of polyacrylamide suits for large proteins. The preparation of separating gel according to the percentage of gels is shown in Table 1.

Table 1 Separating gel preparation

Chemicals	8%	10%	12%
dH_2O	2.67 ml	2.42 ml	2.17 ml
1.5 M Tris-HCl, pH8.8	1.25 ml	1.25 ml	1.25 ml
10% SDS stock	50 µl	50 µl	50 µl
40% Acrylamide/Bis-acrylamide	1 ml	1.25 ml	1.5 ml
10% Ammonium persulfate	25 µl	25 µl	25 µl
TEMED	2.5 µl	2.5 µl	2.5 µl
Total volume	5 ml	5 ml	5 ml

Stacking gel

The stacking gel uses as a baseline to start the protein separating at the same line. The stacking gel contains lower polyacrylamide content than that of perorating (separating) gel as shown in table 2.

Table 2 Stacking gel preparation

Chemicals	Volume
dH_2O	1.52 ml
1.0 M Tris-HCl, pH6.8	0.25 ml
10% SDS stock	20 µl
40%Acrylamide/Bis-acrylamide	0.196 ml
10% Ammonium persulfate	10 µl
TEMED	2 µl
Total volume	2 ml

Preparation of immunoblotting

To detect protein expression, 20 µg of each protein samples were loaded into 8-12% of polyacrylamide gel. The proteins were separated by gel electrophoresis at 100 V for 90 min. Then, proteins were transferred from the gel onto PVDF membrane. After transferred, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline

(TBS; 10 mM Tris, pH7.5, 150 mM NaCl) containing 0.1% Tween-20 (TBST) for 1 h. Then, the membranes were incubated with specific primary antibodies including PERK, GRP78, CHOP, cleaved-PARP, PARP, caspase-7, cleaved-caspase-7, Bax, Bcl-2, Bcl-xL, Mcl-1 (Cell Signaling Technology Inc., Danvers MA, USA), and β-actin (Merck KGaA, Missouri, U.S.), in a dilution of 1:1000 for overnight at 4˚C. After that, the membrane was washed with TBST for 3 times and followed by incubation with the anti-rabbit immunoglobulin G or anti- mouse immunoglobulin G conjugated with horseradish peroxidase (HRP) secondary antibodies (Cell Signaling Technology Inc., Danvers MA, USA) in a dilution of 1:1000 for 1 h at 25°C. After incubation, membranes were washed with TBST for 3 times. The protein expressions were amplified using Immobilon™ Western chemiluminescent Horseradish peroxidase (HRP) substrate solution (Millipore Co., USA) and protein bands were captured by using CCD camera under gel documentary machine (AllianceQ9 advanced chemiluminescence imaging system from Uvitec, UK).

Statistical analysis

Each experiment was played as mean ± standard deviation (SD) obtained from three independent experiments (n=3). IC_{50} value analysis was operated by using GraphPad Prism version 9 (GraphPad Prism Software, Inc.). The treatment groups were assessed by one-way analysis of variance (one-way ANOVA) at P-value of 0.05 ($p<0.05$) for statistically significant considering by using SPSS statistical software version 25 (IBM Crop., NY, USA). Image J densitometer (National Institutes of Health, MD, USA) was used for protein intensity determination.

CHAPTER 4
RESULTS

1. Effect of EE extract on cell viability in HeLa cells.

The cytotoxicity of HeLa cells treated with EE extract was shown in Figure 14. The result demonstrated that after 24 h of treatment, the EE extract significantly increased cell death induction in HeLa cells in a dose-dependent manner. The IC_{50} value was found to be 187.2 ± 0.3368 µg/ml. This result indicate that EE extract induced cytotoxic in cervical HeLa cells. Based on this result, we chose EE extract at concentration of 0, 50, 100, 150, 200, 250, and 300 µg/ml for further experiments.

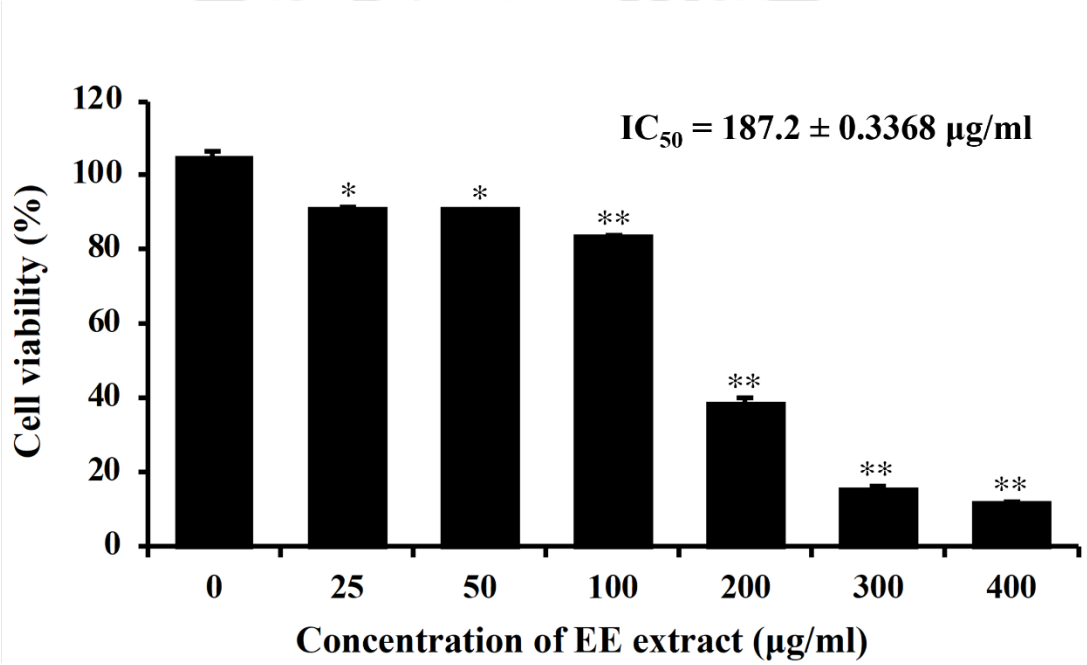


Figure 14 Cytotoxicity of EE extract against HeLa cells. The Cells were treated with EE extract at various concentration for 24 h and estimated by MTT assay. The IC_{50} of EE extract is 187.2 ± 0.3368 µg/ml at 24 h of treatment. The data shows as mean±SD from three experiments. *$p < 0.05$ and **$p < 0.01$ vs. control group.

2. EE extract induced nuclear changes in HeLa cells.

The important characteristics of apoptotic cell are chromatin condensation, nuclear fragmentation and followed by cellular collapse into apoptotic bodies. To determine whether the effect of EE extract on apoptosis induction in HeLa cells, cells were treated with various concentration of EE extract (0, 50, 100, 150, 200, 250, and 300 µg/ml) and incubated for 6 h. The results showed EE extract induced chromatin condensation combined with apoptotic bodies in a dose response manner in HeLa-treated cells (Fig. 15A). Over 50 percentage of chromatin condensation cells were significantly increased after treated cells at 200, 250, and 300 µg/ml of EE extract, compared to the control group ($p<0.01$) (Fig.15B). This event described to EE extract induced cell death through the process of apoptosis in HeLa cells.

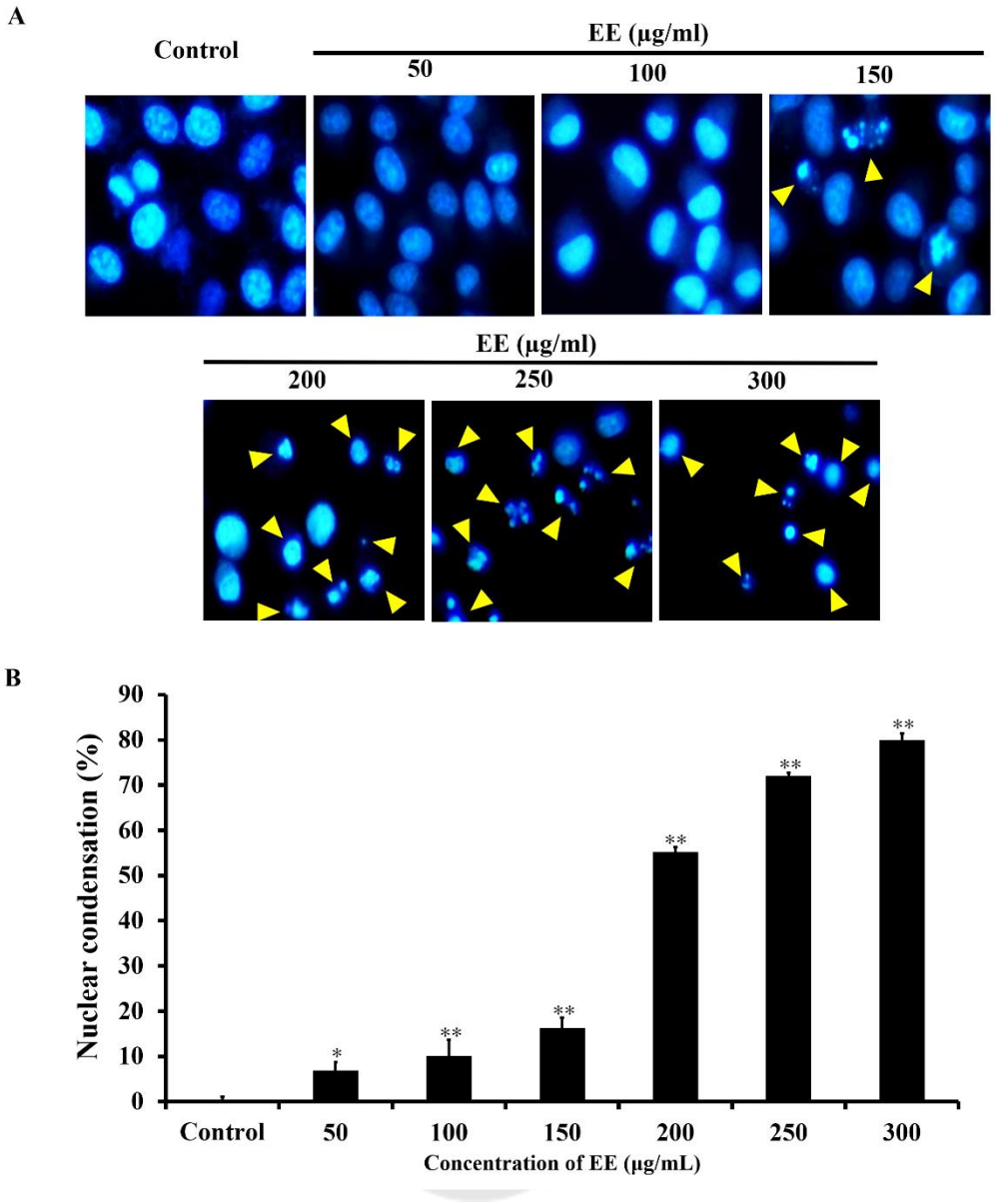


Figure 15 EE extract induced nuclear condensation and apoptotic bodies in HeLa cells. Cells were seed and treated with EE extract at various concentrations for 6 h, then cells were stained with Hoechst 33342 dye and observed under fluorescence microscope (20X). (A) Images shows the nuclear morphological changes. Yellow arrow presents nuclear condensation and apoptotic bodies. (B) The histogram shows percentage of nuclear condensation and apoptotic bodies in cells. The data was mean±SD from three experiments. *$p < 0.05$ and **$p < 0.01$ vs. control group.

3. Effect of EE extract on sub-G1 DNA content in HeLe cells.

Cells undergoing apoptosis present DNA endonucleolytic cleavage which lead to increase DNA fragmentation in cells. Thus, we investigated the effect of EE extract on sub-G1 DNA content in cell cycle distribution which indicating apoptotic cells by using flow cytometry analysis. HeLa cells were treated with 0, 100, 150, 200, 250, and 300 µg/ml of EE extract for 12 h and following stained whole cells with Guava Cell Cycle® reagent. The results showed that non-treated cells presented sub-G1 DNA content 1.17% (control group). While cell treated with EE extract at various concentrations increased sub-G1 DNA content in a dose-dependent manner compared to the control group as shown in Figure 16A. At this point, we found that at concentration of 250 and 300 µg/ml EE extract significantly increased the sub-G1 DNA content a $p<0.01$ (Fig. 16B). These results suggest that EE extract induced apoptotic cell death in HeLa cell.

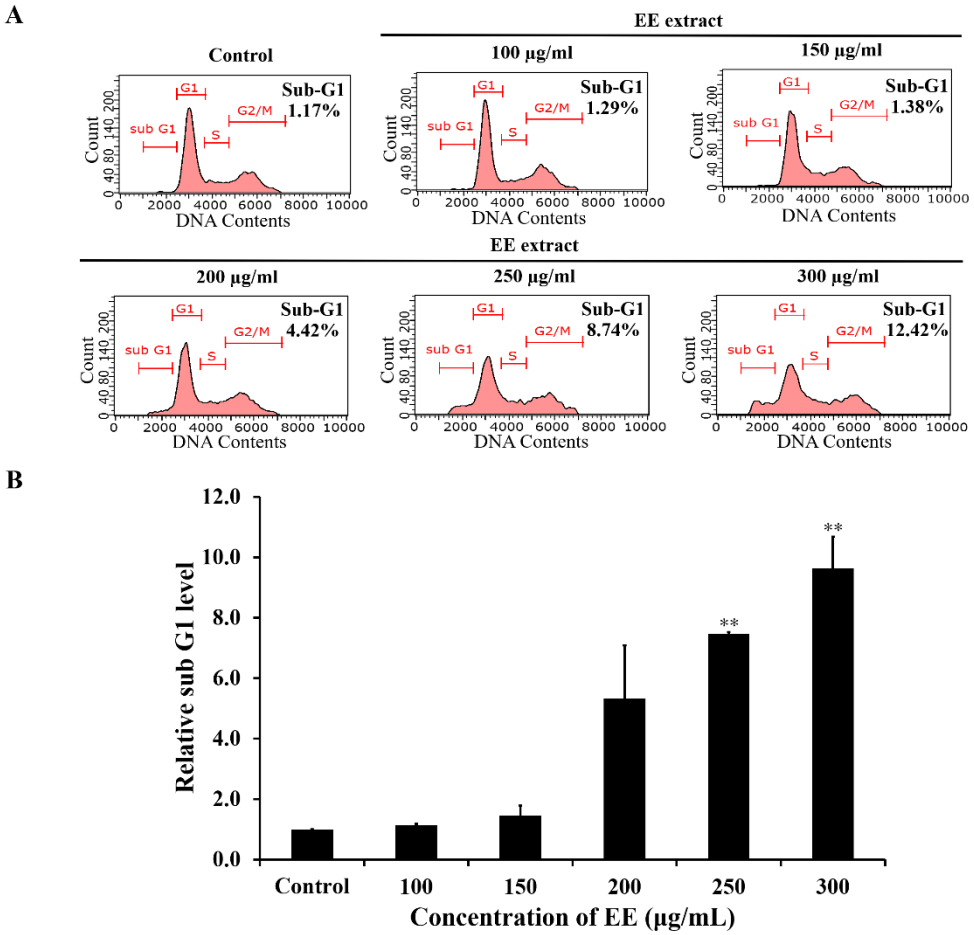


Figure 16 Effect of EE extract on the cell cycle distribution and sub-G1 DNA content in HeLa cells. Cells were treated with EE extract at various concentrations. After 12 h of treatment, cells were stained with Guava Cell Cycle® reagent and cell cycle distribution was analyzed by using flow cytometer. (A) The histogram shows sub-G1 DNA content in HeLa cells. (B) The graph shows the relative of sub-G1 DNA content in EE-treated cells. The data presents as mean±SD of three experiments. $^{**}p < 0.01$.

4. Effect of EE extract on loss of mitochondrial potential in HeLa cells.

The loss of mitochondrial membrane potential is a remarkable characteristic of the intrinsic apoptotic pathway. Therefore, to determine the effect of EE extract on loss of mitochondrial potential in HeLa-treated cells, the JC-1 staining was carried out. Briefly, HeLa cells were treated with various concentration of EE extract (0, 50, 100, 150, 200, 250, and 300 µg/ml) and incubated for 6 h before staining with JC-1 dye. This study showed that the loss of mitochondrial membrane potential occurred in EE-treated cervical cancer cells at high concentration in a dose-dependent manner (Fig. 17A), while the control group showed red fluorescence signal indicating as normal mitochondrial membrane potential or healthy cells. Less than 50 percentage of red intensity significantly decreased in cells treated with 200, 250, and 300 µg/ml of EE extract, respectively compared to the control group (Fig. 17B). The results presented that EE extract decreased mitochondrial membrane potential and induced membrane permeability in treated HeLa cells, presumably caused the intrinsic apoptotic pathway induction.

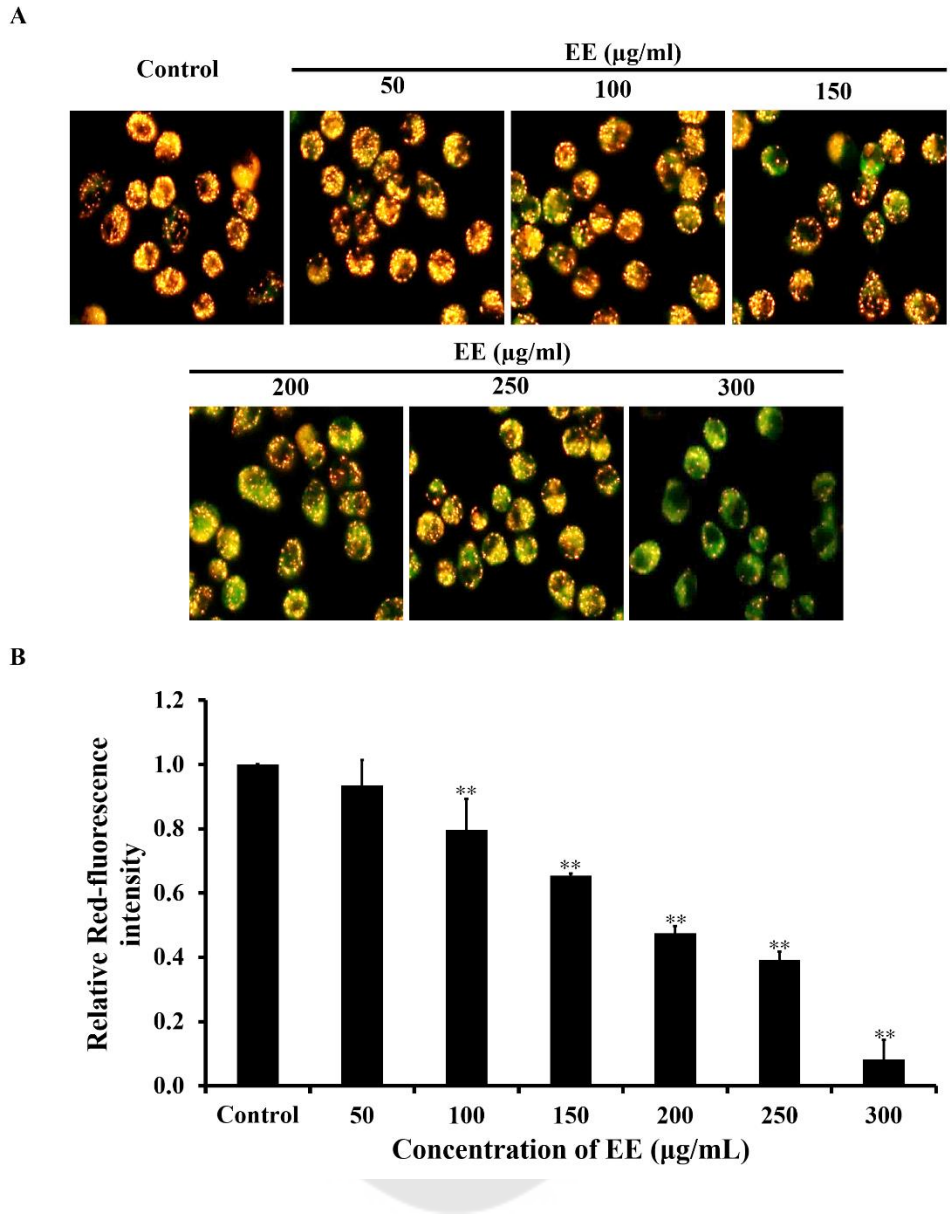


Figure 17 Loss of mitochondrial membrane potential in HeLa cells by EE extract. Cells were seeded and treated with EE extract at various concentrations for 6 h, then cells were stained with JC-1 dye and observed under fluorescence microscope (20X). (A) Images show the changes of mitochondrial potential in cells. The green fluorescence indicates the loss of mitochondrial membrane potential of cells, this event is a characteristic of early apoptosis, and the red fluorescence indicates cells with normal mitochondrial membrane potential. (B) The graph shows the relative red-fluorescence intensity. The data shows as mean±SD from three experiments. $^{**}p < 0.01$ vs. control group.

5. Effect of EE extract on the proteins related to ER stress induction in HeLa cells.

ER stress occurs in cells with an accumulation of un- and misfolded proteins in ER. This event leads to apoptosis induction to eliminate abnormal cells. To study the effect of EE extract on ER stress in HeLa cells, the proteins related to ER stress induction including PERK, GRP78, and CHOP were determined by using Western blotting. The results displayed that the expression of GRP78 and CHOP proteins were enhanced and PERK protein expression was down-upregulation in EE-treated HeLa cells in a dose-dependent manner (Fig. 18A). Interestingly, we found that EE extract predominately increased CHOP level as well as Tm-group, the ER stress inducer (Fig. 18B). This result suggests that EE extract induced ER stress via mainly activating CHOP in HeLa cells.

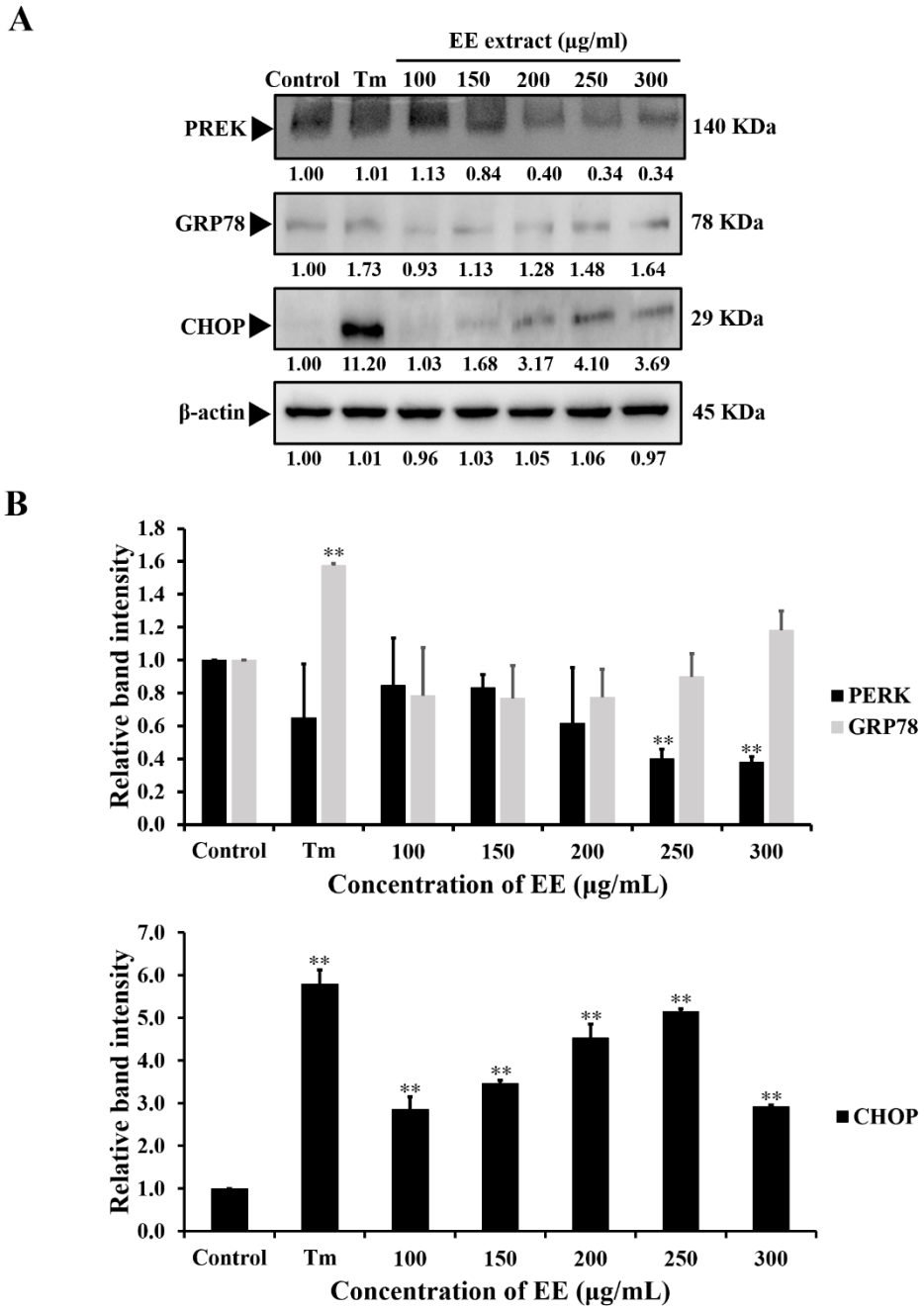


Figure 18 Effect of EE extract on the protein expression associated ER stress. HeLa cells were treated with EE extract at 0, 100, 150, 200, 250, and 300 µg/ml or Tm at 10 µg/ml for 24 h. (A) Protein expressions were estimated by Western blotting. (B) The graph shows the relative protein intensity of PERK, GRP78, and CHOP. β-actin was used as an internal control. Tm; tunicamycin, the ER stress inducer. The data shows as mean±SD of three experiments. $^{**}p < 0.01$.

6. Effect of EE extract on ER stress related to apoptosis induction in HeLa cells.

Above results, we found that EE extract induced CHOP level in HeLa cells. As well-known that CHOP activities are associated with apoptosis induction via inhibition of anti-apoptosis as Bcl-2 and enhanced pro-apoptosis as Bax expression. We hypothesized that EE may enhance ER stress to activate apoptosis in cervical cancer HeLa cells. Thereby, we investigated the expression of proteins involving apoptosis pathway by using Western blotting. Our result demonstrated that EE extract induced the increase of pro-apoptotic protein, Bax and c-caspase 7 which resulting in the inhibition of PARP degradation in HeLa cells. In contrast, EE extract decreased anti-apoptotic proteins such as Bcl-2, Bcl-xL, and Mcl-1 compared to control group (Fig. 19A) in dose-dependent manner in HeLa cell. These results suggest that EE extract enhanced ER stress leading to apoptosis induction in HeLa cells.

In conclusion, our results showed that EE extract induced apoptosis in cervical cancer HeLa cell The increase of CHOP in EE-treated cells, correlate to Tunicamycin-group. Furthermore, we found that the EE extract induced the loss of mitochondrial membrane potential, chromatin condensation and apoptotic bodies as well as increased sub-G1 content in HeLa cells. In addition, EE extract increased pro-apoptotic proteins and caspases resulting in attenuated PARP degradation. These events demonstrated the EE extract induced ER stress related to apoptosis induction in HeLa cells. These data guide that EE extract is a new candidate that may be developed as an anti-cancer agent for cervical cancer treatment in the future.

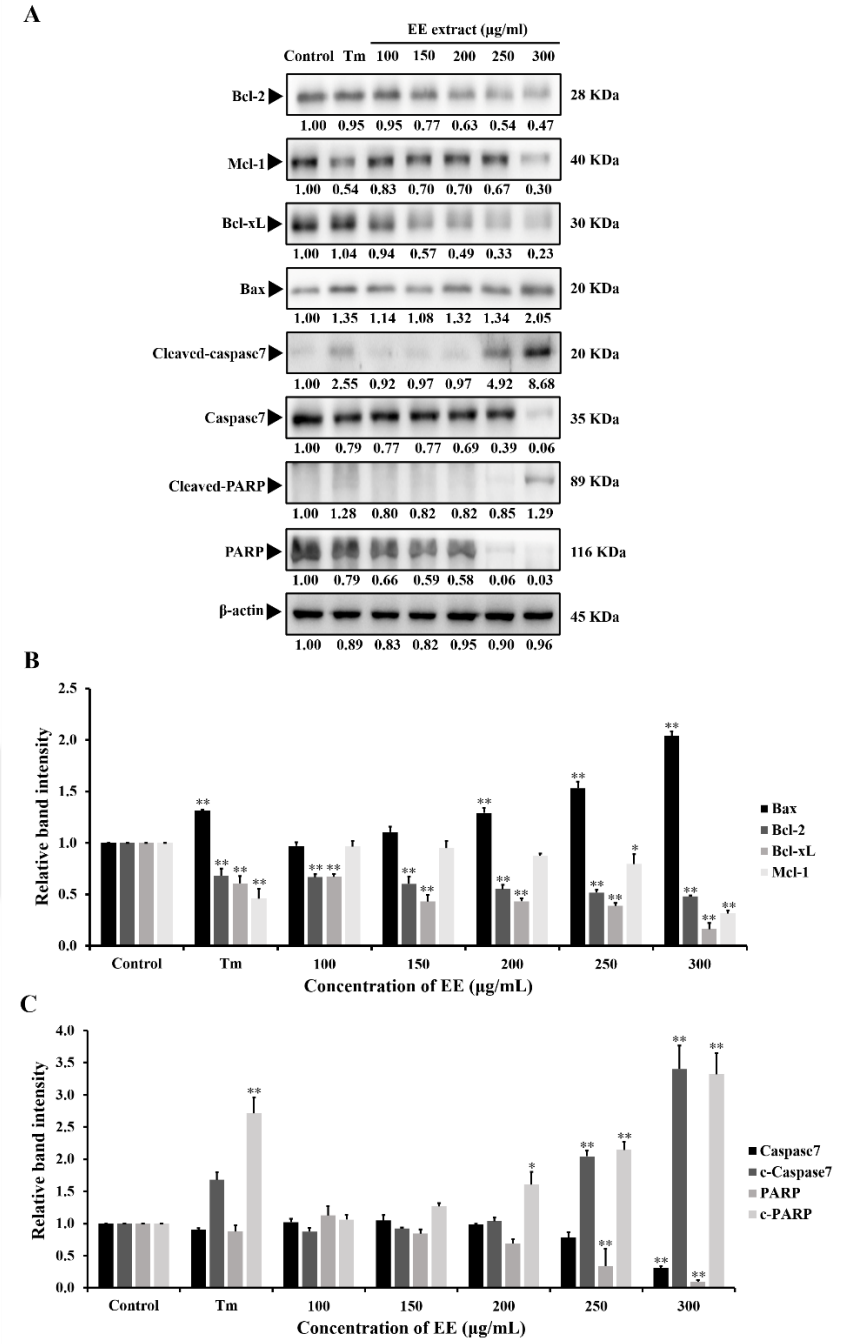


Figure 19 Effect of EE extract on apoptosis induction in HeLa cells. Cells were treated with EE extract at 0, 100, 150, 200, 250, and 300 μg/ml or Tm at 10 μg/ml for 24 h. (A) Protein expressions were estimated by Western blotting. The graph shown relative band intensity of (B) Bax, Bcl-2, Mcl-1, Bcl-xL, (C) Caspase 7, c-Caspase 7, PARP, and c-PARP. β-actin was us as an internal control. Tm: tunicamycin. The data shows as mean±SD of three experiments. *$p < 0.05$ and **$p < 0.01$.

CHAPTER 5
DISCUSSION

At present, many reports showed that cervical cancer remains one of the most common of death in many countries. The incidence and mortality of cervical carcinoma are in the top five of cancers in Thailand. The treatment of cervical cancer is limited due to surgical technique, serious side effects of chemotherapeutic drugs, and chemoresistance leads to less effective cancer treatments and a poor quality of life.

Currently, traditional medicine or natural compound extracts from plants is interesting because it might relieve harmful effect from conventional therapies. In various studies have found that *C. esculenta* had pharmacological treatment including antimicrobial, antihyperglycemic, antihepatotoxic, anti-cancer, antioxidant, anti-inflammatory responses, antibacterial, antifungal, anthelmintic activity, antidiabetic activity, synergistic properties [81], hypolipidemic, anti-melanogenic, estrogenic, neuropharmacological effects and anti-cancer activity [82-88]. However, the effect of *C. esculenta* on cervical cancer cell growth never has been reported. Therefore, in our study is the first report showed the effect of *C. esculenta,* elephant ear (EE) extract on cervical cancer HeLa cell line.

Previous studies presented that the stem and leaf methanolic *C. esculenta* extracts were abundant in flavonoids as major component such as luteolin 7-O-sophoroside, vitexin, schaftoside, orientin, isovitexin, isoorientin, isoschaftoside, gallic acid, cafeic acid, and chlorogenic acid [89-92]. Vitexin caused apoptosis in leukemia cells by inducing oxidative stress mediated cytotoxicity. Vitexin induced apoptosis in human non-small cell lung cancer A549 cells through reduction of Bcl-2/Bax ratio and activation of cleaved-caspase 3 expression [93-95]. Similarly, orientin showed a strongly apoptosis induction through p53 upregulation and Bcl-2 downregulation in esophageal cancer EC-109 cells [96]. In addition, isoschaftoside, schaftoside, isovitexin, and isoorientin suppressed cell growth in human prostate cancer (PC3) and mouse malignant melanoma (B16F10) cell lines [97]. In 2017, Ahmed et al. found that the methanol extract of *C.*

esculenta stem decreased blood glucose levels as well as glibenclamide, a standard antihyperglycemic drug in glucose-challenged mice ([98]). Likewise, the 95% ethanolic extract of *C. esculenta* stem showed high phenolic contents and inhibited oxidative stress [99]. In 2012, Ferreres et al. reported that the aqueous extract of *C. esculenta* contains 41 phenolic compounds, divide into 11 hydroxycinnamic acid derivatives and 30 glycosylated flavonoids, which may serve as therapeutic agents for cancer [92]. In this study, we determined the effects of Elephant ear (EE); *C. esculenta* stem extract on HeLa cells. The results displayed that EE extract decreased cell viability in a dose-dependent manner at 24 h in EE-treated cervical cancer HeLa cell with IC_{50} value at 187.2 $\pm$ 0.3368 µg/ml. According to the previous studies, the *C. esculenta* extract can inhibit cancer cell growth. Park et al. reported that the *C. esculenta* fraction decreased cell viability and migration on Yac-1 cells, mouse lymphoma cell line [100]. While Brown et al. and Kuete et al. showed that *C. esculenta* extract induced anti-cancer in human breast (MCF-7, MDA-MB-231, MDA-MB-435 and T47D) cancer cell line, and rat colon cancer (YYT) cell line [9, 101].

Apoptosis is a form of programmed cell death that played an important role on regulate cell proliferation and cell death. The apoptosis process presents clearly the changes of morphological characteristic in cells which is called hallmarks of apoptosis. The remarks are the flip out of phosphatidylserine on cell membranes, mitochondrial dysfunction, chromatin condensation and fragmentation, and apoptotic bodies [102-104]. Many reports suggested that during apoptosis the various molecules such as AIF (apoptosis inducing factor), activated nuclease, acinus, and PLA2 (phospholipase A2) induce nuclear chromatin condensation and shrinkage. Then, chromatins are packed together with nuclear proteins and nuclear disassembly leading to apoptotic bodies [105]. Our results found that EE extract significantly induced apoptosis in HeLa cells through increased nucleus condensation and apoptotic bodies, estimated by Hoechst 33342 staining after cells were treated with EE for 6 h and compared to the control group (Fig. 15A). Our results are consistent with Choi et al. (2014) which showed caffeic acid induced apoptosis through a remarkable nuclear condensation and nucleolar shrinkage in treated

cells [106]. In addition, in the late apoptosis, the activated nuclease cleaved genomic DNA into smaller fragments, each approximately 180 bp, estimated by sub G1 assay [107, 108]. We observed that the sub G1 DNA content was enhanced in EE-treated cells compared to control group (Fig. 16A). Consistent with our results, caffeic acid and orientin induced apoptosis and arrested cell cycle of human head and neck squamous carcinoma (Detroit 562) cells and colorectal carcinoma HT29 cells in G0/G1 phase [109, 110].

The major function of mitochondria is to produce cellular energy, adenosine triphosphate (ATP) through the oxidative phosphorylation. This event displays proton (H^+) cross over between intermembrane space and mitochondrial inner membrane via ATP synthase resulting to electrochemical gradient or mitochondrial membrane potential in normal cell [111]. In mitochondria-mediated apoptosis, pro-apoptotic proteins such as Bax and Bak are activated and oligomerize at the mitochondrial outer membrane (MOM) resulting in the formation of large pore, called MOM pore. This event leads to the decrease of mitochondrial membrane potential and leak of cytochrome *c* and apoptogenic factors [112]. Then, the cytochrome *c* activates apoptosome dependent-apoptosis cascade [113]. In our studies, we used fluorescence JC-1 dye staining to observe the loss of mitochondrial membrane potential. In healthy cells, JC-1 dye accumulates J-aggregates form which emitted red fluorescence signal. Conversely, in unhealthy cells with low mitochondrial membrane potential, the JC-1 dye enters and displays of monomeric form which emitted green fluorescence signal. Our results demonstrated that EE extract enhanced the loss of mitochondrial membrane potential in EE-treated cells (Fig. 17). These results are compatible with caffeic acid and vitexin treatment promoted low mitochondrial membrane potential and induced apoptosis-associated caspase activation in human fibrosarcoma HT-1080 and human non-small cell lung cancer A549 cells line [94, 114].

Previous studies have reported the relationship between the ER stress and apoptosis. Generally, ER stress is caused by un-/misfolded proteins accumulation in ER lumen, which activates stress response, adaptive signaling induction called as UPR to restore normal ER function [115, 116]. The UPR has specific gene binding for regulation of essential proteins including ER chaperones GRP78, which restore proteins folding in ER

lumen [117, 118]. Prolong ER stress trigger autophagy and apoptosis induction through transmembrane proteins function, including PERK, IRE1α, and ATF6. During ER stress, these transmembrane proteins are activated and formed oligomer and autophosphorylation which initiated downstream molecules induction [118-121]. For example, the active PERK stimulates apoptosis signaling pathway through CHOP expression [122-124]. CHOP is a transcription factor that regulated specific target genes expression such as pro-/anti-apoptotic gene [125, 126]. CHOP suppressed *Bcl-2* genes and enhanced the expression of *Bax* genes resulting to apoptosis [120, 127]. Our studies found that the CHOP and Bax protein levels were increased whereas Bcl-2, Bcl-xL, and Mcl-1 were decreased in EE extract-treated cells. Likewise, Kamiya et al. reported that caffeic acid treatment induced apoptosis mediated by CHOP/Bcl-2 pathway in human breast cancer MCF-7 Cells line [128]. In fact, cells undergoing apoptosis, cytochrome *c* is released and binds to Apaf-1 led to activate caspase-9. Caspase-9 is an initiator caspase which is mainly responsible for induction of execution caspase, caspase-3/-7 [129-132]. Active-caspase-3/7 inhibits PARP function by PARP cleavage into cleaved-PARP, resulting in attenuating DNA repair mechanism and inducing apoptosis pathway [133, 134]. To confirm EE extract induced apoptosis in HeLa cells, we determined the expression of apoptosis proteins. Our results showed that EE extract increased cleavd-caspase-7 and cleaved-PARP in HeLa cells in a dose-dependent manner. This finding displayed that EE extract induced apoptosis in HeLa cells.

In summary, EE extract induced chromatin condensation and apoptotic bodies as well as sub-G1 DNA content in HeLa cells. In addition, EE extract increased the expression of CHOP protein to promote pro-apoptotic proteins, Bax and suppressed anti-apoptotic protein including Bcl-2, Bcl-xL, and Mcl-1. This event disrupted mitochondria function led to increase c-caspase-7 activities and PARP degradation. Based on this data indicated that EE extract induced ER stress associated apoptosis pathway in cervical cancer HeLa cells.

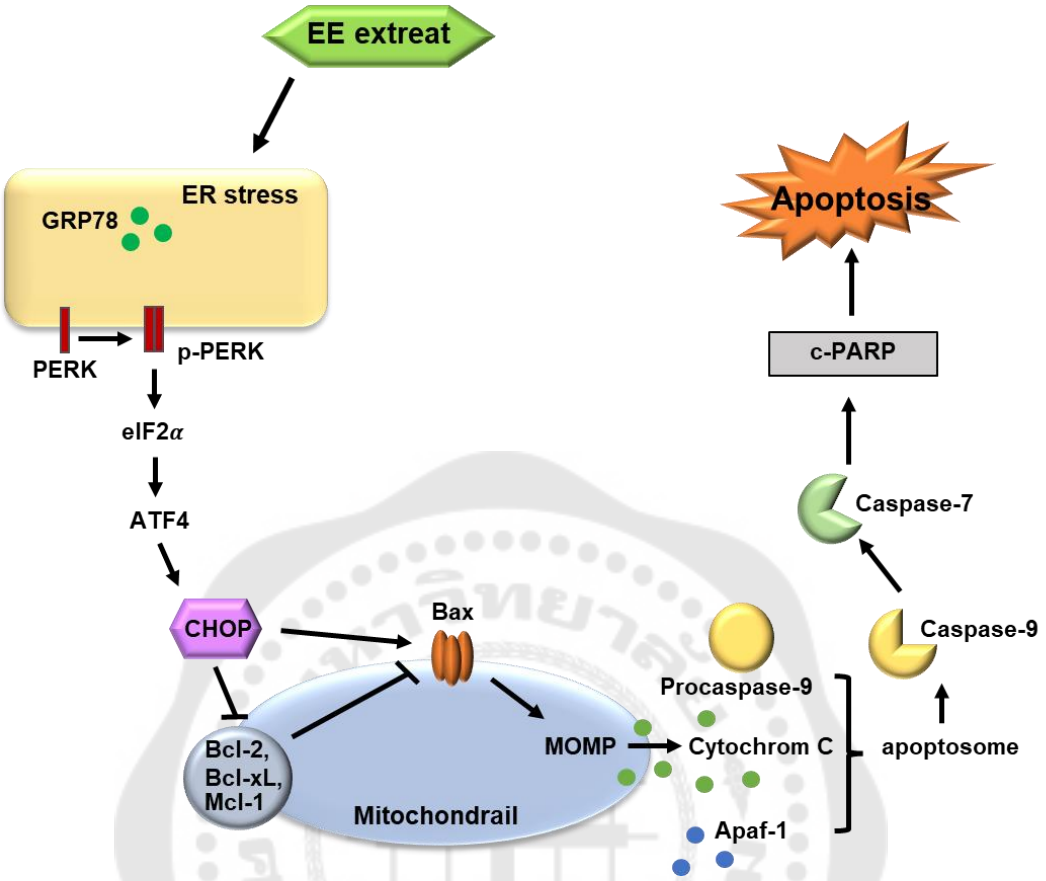


Figure 20 The mechanism of apoptosis induction via ER stress was activated by EE extract.

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Appendix

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