ANTICANCER EFFECT OF PEANUT HAIRY ROOT CULTURE CRUDE EXTRACT ON HUMAN CHOLANGIOCARCINOMA CELL LINES

DONRUETAI THALAPPHET

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Biochemistry)
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By DONRUETAI THALAPPHET

has been approved by the Graduate School as partial fulfillment of the requirements

for the Master of Science in Biochemistry of Naresuan University

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Title ANTICANCER EFFECT OF PEANUT HAIRY ROOT CULTURE CRUDE EXTRACT ON HUMAN CHOLANGIOCARCINOMA CELL LINES

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ABSTRACT

Cholangiocarcinoma (CCA) is a major health problem of Thailand especially North and Northeast regions. Most of the cases of CCA are diagnosed at an incurable advanced or metastatic stage. The major limitations of current anti-CCA therapy are recurrence, chemoresistance and toxic effects resulting in poor prognosis and high mortality rate. Therefore, the discovery of novel anti-CCA agents remain an urgent challenge. Stilbenoid is a natural stilbene-base compound that possesses anti-oxidant, anti-inflammatory, cardioprotective, and anticancer properties. It has been recently reported that peanut hairy root culture crude extracts (PCE) are rich in 3 majors stilbene derivative compounds including trans-arachidin-1, -3 and resveratrol. Since, resveratrol has been extensively studied for anticancer activity. Interestingly, knowledge about anticancer effect of trans-arachidin-1 and -3 are little. In this study, we aimed to investigate the anticancer activity of PCE on CCA cells. Anti-proliferative effect of PCE in 4 CCA cell lines with different cancer potential, namely KKU-213, KKU-214, KKU-055, and KKU-213L5 were investigated by using MTT assay. The results showed that PCE significantly inhibited cell proliferation of those a CCA cells by dose and time dependent manners compared with the vehicle control. The strongest effect was detected in KKU-213L5, a highly metastatic cell line, with inhibitory concentration 50% (IC50) values of 947.64 ± 11.49, 403.65 ± 14.83 and 226.9 ± 16.31 µg/ml at 24, 48 and 72 h treatment, respectively. Then, effect of PCE on apoptotic induction was investigated in KKU-213L5 by using Annexin-V/7AAD

staining, Hoechst33342 staining and Western blot. The results revealed that PCE treatment at high doses significantly induced CCA cell apoptosis characterized by increasing early and late apoptotic populations and DNA condensation. Moreover, PCE increased the expression of cleaved Poly (ADP-ribose) polymerase (PARP) but not cleaved-caspases 3 and 9 suggested that PCE may induce apoptosis in KKU-213L5 cells through caspase-independent pathway. Furthermore, anti-metastatic effects of PCE in KKU-213L5 were determined on cell migration and invasion abilities by scratch wound healing and transwell invasion assay, respectively. The results demonstrated that low doses (non-cytotoxic concentration) of PCE effectively reduced cell migration and invasion abilities in KKU-213L5. In addition, the gelatin zymography showed that the activities of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in PCE treated KKU-213L5 cells were not suppressed when compare to untreated KKU-213L5 cells. These results suggest that peanut hairy root culture extract can potentially inhibit CCA cell growth and metastasis suggests the therapeutic potential on anti-cancer candidates.

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CHAPTER I

INTRODUCTION

Rational of this study

Cholangiocarcinoma (CCA), also known as bile duct cancer. It has significant increase in incidence rates over the last several decades. The incidence of CCA has been reported worldwide. There are high incidences of CCA especially in northeast of Thailand. It is caused by liver fluke (*Opisthorchis viverrini*: OV) infection (Young ND *et al*., 2010). CCA is a highly lethal cancer that occurs in the biliary tract. It is characterized by early invasion, poor prognosis, and resistance to current chemotherapies. To date, an effective therapeutic strategy for this advance stage and deadly disease is lacking. The current treatments are surgical resection, systemic chemotherapy and targeted radiation therapy. Many anticancer drugs, such as 5-fluorouracil and gemcitabine have been frequently used for the treatment of CCA. Despite all of the recent advancements in CCA diagnosis and treatment, the current therapies are unable to completely eliminate CCA cells. Controlling cancer growth and the development of chemo-preventive agents are the major goals in current basic research in oncology. The purpose of this study was to identify a novel treatment method for CCA.

In addition, apoptosis is a therapeutic goal of cancer therapy because its targeting is more advantageous than other cell death mechanisms. Previous studies on anti-cancer activity of stilbene compound have indicated that resveratrol and its derivatives inhibit cell viability by induce apoptosis and inhibit cancer metastasis such as call migration and invasion abilities in many cancer cells. Stilbenoids exist widely as natural phytoalexins produced by plants such as grapes, berries, and peanuts. Trans-resveratrol and its derivatives, trans-arachidin-1 (Ara-1), trans-arachidin-3 (Ara-3), are stilbenoids that have been isolated from peanut hairy root culture. Hairy root culture is a biotechnology to harvest the products in the form of secondary metabolites that are significantly important for their biological activities and

pharmacological properties. It was recently reported that peanut hairy root culture crude extracts (PCE) are rich in stilbene derivative compounds like trans-resveratrol, trans-arachidin-1 and -3, and have a high antioxidant activity (A. Limmongkon *et al*., 2019). Among these peanut stilbenoids, the biological functions of trans-resveratrol have been studied extensively. Much existing evidence indicates that trans-resveratrol has therapeutic advantages for cancer prevention and therapy, improving insulin sensitivity, controlling blood glycemia and lipid levels, suppressing inflammation diseases, and aging (Chen *et al*., 2018) and it was found to be a chemopreventive phytochemical for cardiovascular disease and cancer. It also exhibits potent bioactivities as an antioxidant, anticancer, and anti-inflammatory agent and it's can reduce the metastasis of prostate and breast cancer cells that through the inhibition of cell migration and invasion. The anticancer activity of trans-arachidin-1 isolated from germinated peanut kernels was demonstrated in HL-60 cells through the caspase-dependent and caspase-independent pathways (Huang CP *et al*., 2010). Moreover, trans-arachidin-1 and trans-arachidin-3 possess anti-inflammatory, anti-proliferative properties and have bioavailability better than resveratrol (Chang *et al*. 2006 and Djoko *et al*. 2007). These data indicated that, trans-arachidin-1 and trans-arachidin-3 might represent a novel class of compounds with significantly improved biological activity that might be exploited for future development as efficacious pharmaceutical drugs. However, very few studies have investigated about the anticancer and/or anti-metastasis activity of trans-arachidin-1 and trans-arachidin-3. In our preliminary data, we have investigated the effect of PCE on human cancer cell lines. The results show that PCE can inhibit cancer cell line especially in KKU-214 cholangiocarcinoma (CCA) cells. Therefore, PCE has potential to be anti-CCA agents.

In the current study, trans-resveratrol, trans-arachidin-1, trans-arachidin-3 and other stibenoids were isolated from peanut hairy root culture, and their anticancer activities were assessed in cholangiocarcinoma cells. The potency of PCE as an anticancer agent and its efficacy-associated contribution to the induction of cell apoptosis and inhibition of cancer metastasis, were further determined

Hypothesis

1. PCE exhibited potent anti-proliferative activity in CCA cell lines.

2. The high doses of PCE treatment significantly induced cell apoptosis.

3. The low doses of PCE were effectively suppressed CCA cell metastasis via inhibiting of cell migration, invasion and MMPs activities.

Objectives of the study

1. To investigate the effect of PCE on cell proliferation in different type of CCA cells

2. To analyze the effect of PCE on cell apoptotic induction in CCA cell

3. To investigate the effect of PCE on the metastatic phenotypes of CCA cell

Scope of study

To prove the hypothesis and objectives proposed in the study, the experiments were divided into three parts. For part one, the effect of PCE on cell proliferation in four CCA cells with the different severity of the disease was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and calculated IC50 (50% inhibiting concentration) of PCE in CCA cell lines. In part two, the CCA cell that show the strongest anti-proliferative effect of PCE were selected to analyze the induction of cell apoptosis. Three methods including of Annexin-V/7-AAD staining, Hoechst 33342 staining and the protein expression of apoptotic molecules such as cleaved-caspase-9, cleaved-caspase-3 and cleaved-PARP were investigated by flow cytometry, fluorescent staining and western blot, respectively. The last part aimed to evaluate the effect of PCE on metastatic phenotype. The non-cytotoxic concentration of PCE was determine by MTT assay. Then, the effect of PCE on cell migration and invasion abilities were analyzed by wound healing assay and trans-well invasion assay. In addition, the Matrix metalloproteases (MMPs) activities were analyzed by using gelatin zymography.

Expected outcomes

1. Obtain the potentially anti-cancer effect of peanut hairy root culture crude extract by anti-growth, anti-metastasis and apoptosis induction in CCA cell lines

2. Understand the basic molecular mechanism of PCE to inhibit CCA cells

3. Provide the basic information to further explore how to develop PCE as an anticancer drug

Experimental designs

Objective I. To investigate the effect of PCE on the cell proliferation in different type of CCA cells

Objective II. To analyze the effect of PCE on cell apoptosis in CCA cells

Objective III. To investigate the effect of PCE on the metastatic phenotype of CCA cell

Part I : Cell proliferation

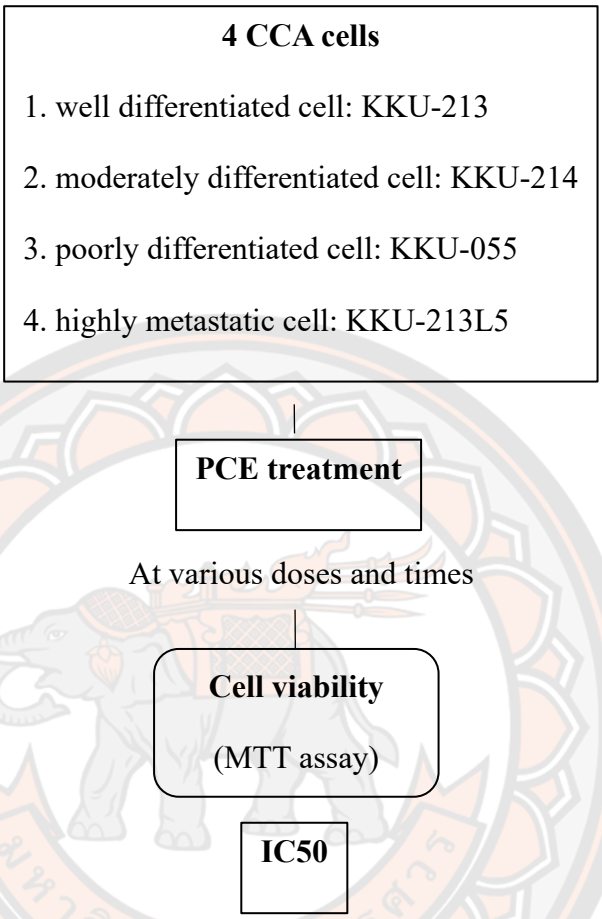


Figure 1 Schematic diagram shown the scope of part one in this study.

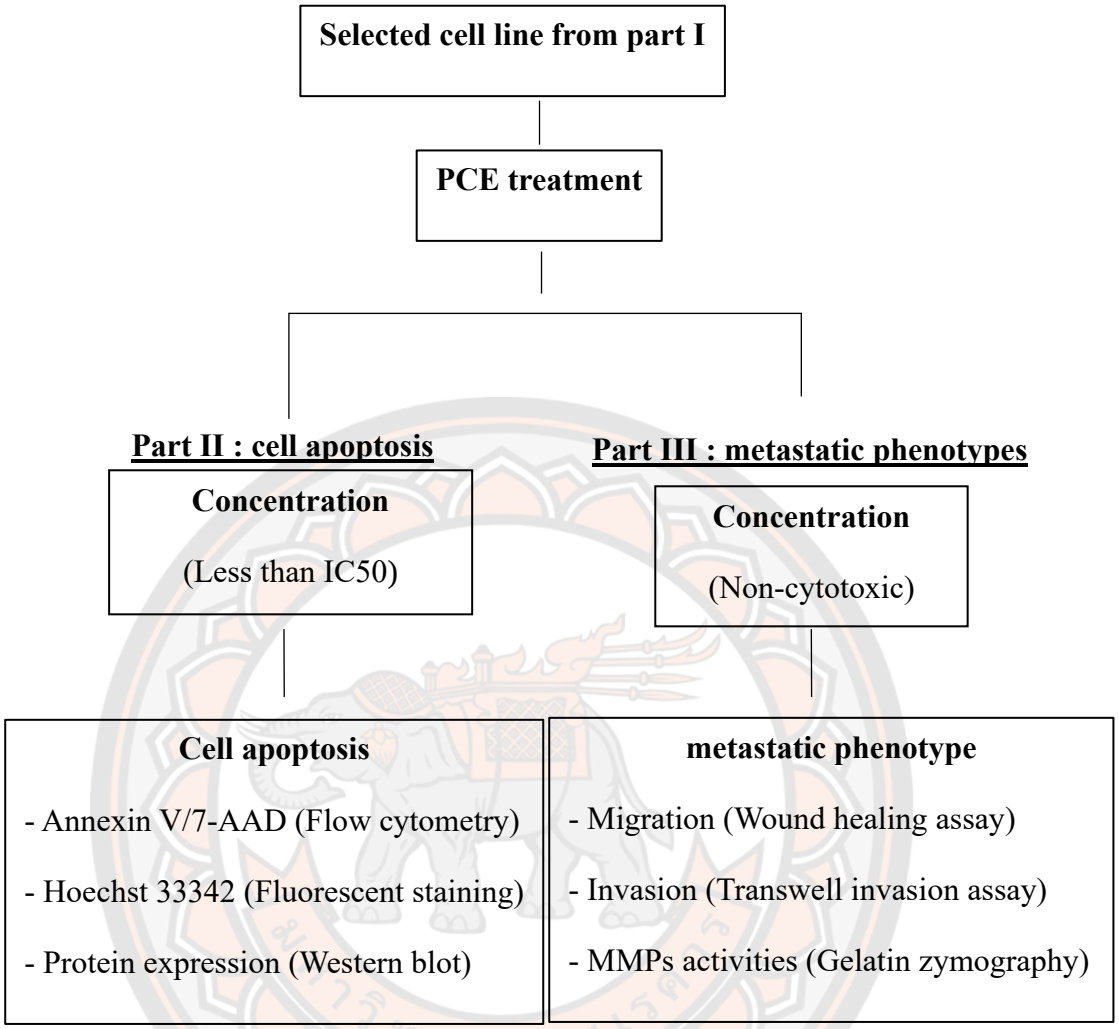


Figure 1 Continue.

CHAPTER II

LITERATURE REVIEWS

Cholangiocarcinoma (CCA)

1. Definition and cause of CCA

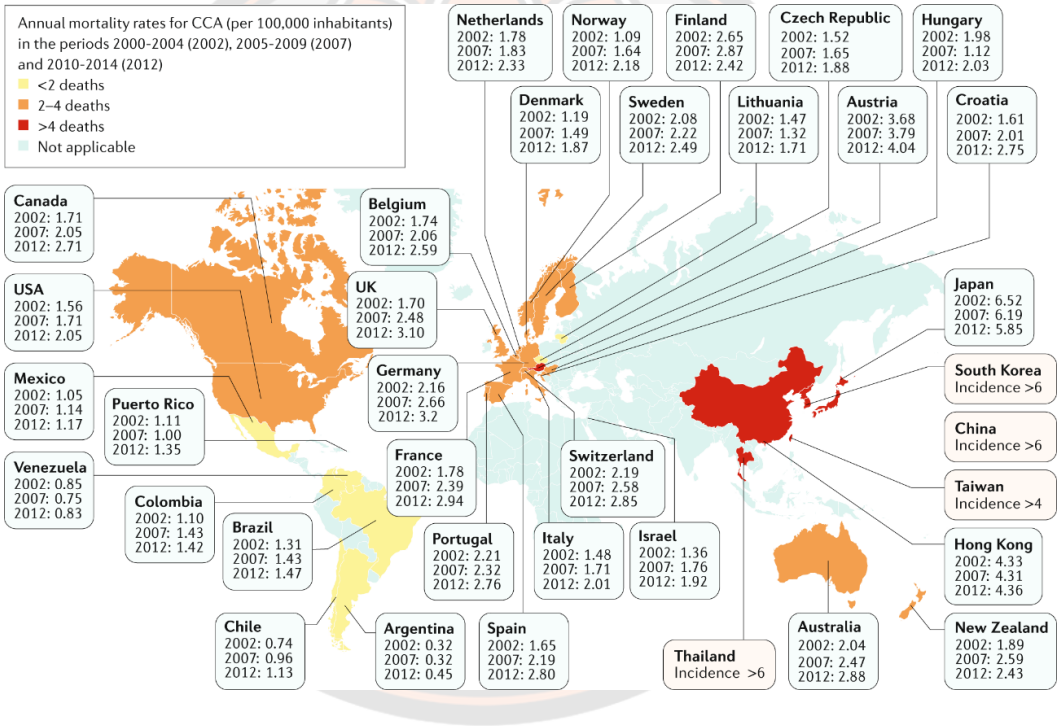


Figure 2 Mortality rate of CCA in worldwide**.**

Source: Wedro B, Shiel Jr WC. Bile Duct Cancer: Cholangiocarcinoma

Cholangiocarcinoma (CCA) is one of the major liver cancers in Asia. Thailand has reported a high incidence of cholangiocarcinoma, especially in the Khon Kaen Province, and is more prevalent in men than in women with an incidence of 84.6 *vs.* 36.8 per 100,000 persons per year, respectively (Fried B *et al*., 2011) (Figure 2). CCA is a cancer at the lining of the bile duct wall, which includes the internal and external

bile ducts. The cancerous tumors that occur in the bile duct within the liver are called “Intrahepatic CCA”, cancerous tumors in the bile duct outside the liver are called “Extrahepatic CCA”. (Klatskin G *et al*., 1965) (Figure 3). CCA is usually asymptomatic during the early stages. The symptom of CCA has been reported that patients were jaundice due to biliary tract obstruction. Moreover, the advanced stage of this disease has other symptoms include asthenia, abdominal pain, malaise, nausea, anorexia, and weight loss (Alvaro, D. *et al*.,2011). There are many risk factors that cause bile duct cancer, such as liver fluke infection, liver stones or chronic inflammation of the bile duct. In recent years, new cases and the mortality rate of CCA have increased, especially in America and Asia (Saha *et al*., 2016 and Yanala *et al*., 2019), where the 5-year survival rate only 15%. Most of patients are diagnosed at the advanced stage of disease as it is highly aggressive by non-specific symptoms. It was found bile duct cancer in patients are caused by liver fluke infection, *Opisthorchis viverrini* (*O. viverrini*) (Haswell-Elkins *et al*., 1992) due to their behavior of eating raw fish that certain cause cancer from the larvae of the liver fluke (*Metacercaria* of *Opisthorchis viverrini*) growing in bile ducts. Cholangiocarcinoma occurs when cells in the bile ducts develop changes (mutations) in their DNA. DNA mutations cause changes in the instructions. One result is that cells may begin to grow out of control and eventually form a tumor or a mass of cancerous cells.

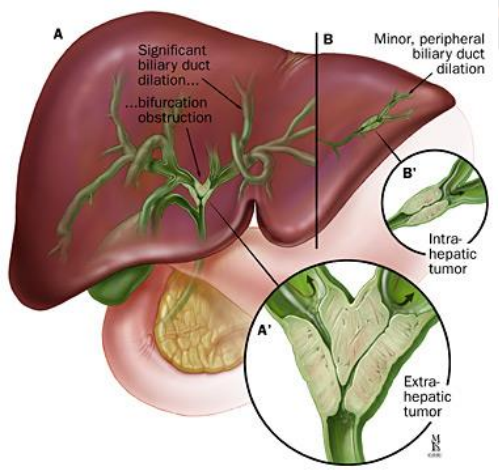


Figure 3 The position of the CCA.

Source: Wedro B, Shiel Jr WC. Bile Duct Cancer: Cholangiocarcinoma

Cholangiocarcinoma can be developed by several stages of cancer, ranging from a more normal early stage (hyperplasia) to metaplasia and an abnormal growth (dysplasia), resulting in cancer in the end. CCA, a process similar to the process of developing colon cancer and believed that chronic inflammation of the bile duct, gallbladder (Sirica A. *et al*., 2005) blockage, and bile duct blockage may contribute to the conversion of normal bile duct cells to cancerous (Holzinger F *et al*., 1999). The differentiation stage is strongly associated with tumor behavior, and generally an immature tumor is more aggressive than the more differentiated counterpart (Annika Jögi, *et al.*, 2012). Well-differentiated cancer cells look more like normal cells and tend to grow and spread more slowly than moderate and poorly differentiated or undifferentiated cancer cells (Annika Jögi *et al*., 2012). Differentiation is used in tumor grading systems, which are different for each stage of cancer as shown the cancer cell differentiation in figure 4.

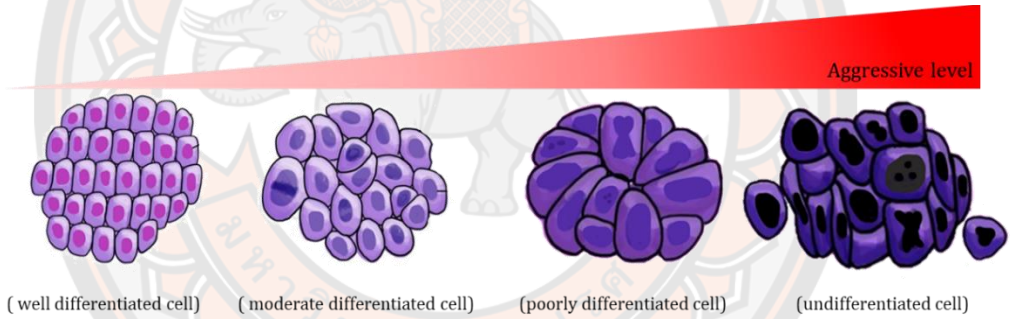


Figure 4 Cancer cell differentiation**.**

Previously, CCA represented a diverse group of epithelial cancers united by late diagnosis and poor outcomes. A diagnosis may be confirmed by molecular genetic testing, which detects gene mutations in patients (Anna P. Sokolenko *et al*., 2018). CCAs are generally asymptomatic in early stages and are usually diagnosed when the disease has already metastasized, by combining nonspecific biomarkers in serum and/or biopsy samples, as well as imaging methods (Rizvi *et al.*, 2018). Late diagnosis compromises the effective therapeutic options, which are based on surgical resection, whereas chemotherapies are virtually palliative given the marked chemoresistance of this cancer. Therefore, there is an urgent need for novel

therapeutic strategies or potential effective anticancer agents that can improve the clinical outcomes of CCA.

2. CCA Therapy

There are currently 3 treatments: surgery is the main treatment of bile duct cancer. Best performance if the disease can be detected at an early stage, it can be treated with surgical methods, but because of bile duct cancer is often diagnosed at the stage of cancer development to the advance stage or metastasis. Therefore, surgical treatment is not always possible (Khan *et al*., 2002). Chemotherapy and radiotherapy used in cases where the cancer cannot be removed. Therefore, the treatment aims to control cancer growth or enlargement and relieve symptoms resulting from cancer and use in postoperative treatment to control cancer in the area repeatedly (Anderson *et al*., 2004). Cancer treatment has side effects from treatment, is surgery may cause loss of organs, blood loss, wound infection and risking anesthesia. Treatment by radiotherapy has side effects on the skin and joints various organs that have been irradiated (National Institutes of Health, 2013). For patients presenting with unresectable or metastatic CCA, systemic chemotherapy remains the mainstay palliative treatment modality. The use of chemotherapy, which provides chemotherapy to cancer patients, is giving drugs to destroy or stop the growth of cancer cells (Sarah Elizabeth Harrington *et al*., 2018). However, because chemotherapy is not specific to cancer cells. Therefore, it may show the side effect with normal cells and other organ functions such as nausea, vomiting, mouth inflammation, loss of appetite, low immunity, diarrhea, hair loss, which these symptoms are more or less dependent on the type of medication body strength and the patient's mental readiness, etc (Koedoot CG *et al.*, 2004).

Treatment options for CCA are limited and associated with high mortality rates of tumor recurrence and short survival. There are several options used for CCA treatment, including of surgical resection, which is the only potentially curative approach currently available (Yezaz Ahmed Ghouri *et al*., 2015). Chemotherapy has been used to control disease and to improve survival and quality of life in patients with unresectable, recurrent, or metastatic CCA (C. Verslype *et al*., 2008). Chemotherapy is a treatment for control the disease to make the tumor smaller or not

grow up and not spread to other organs, relieve the symptoms for metastatic cancer patients to improve the quality of life for patients. The role of adjuvant chemotherapy is not clearly defined in CCA, but for patients with local recurrence after resection of CCA, chemotherapy has been recommended (Mansour, J *et al*., 2015). The most promising approaches involve the use of single-agent such as gemcitabine (GEM). Combination regimens of GEM with agents, such as 5- FU, docetaxel, oxaliplatin, cisplatin (Dingle, BH *et al*., 2005). In addition, in advanced CCA patients that are unsuitable for curative resection, radiotherapy alone or in combination with other chemotherapy (Ghafoori, A. P *et al*., 2011). Chemotherapy drugs attack cells that are dividing quickly, which is why they work against cancer cells. But other cells in the body, such as those in the bone marrow (where new blood cells are made), the lining of the mouth and intestines, and the hair follicles, also divide quickly. These cells can be affected by chemotherapy drugs, which can lead to side effects. The side effects of chemotherapy depend on the type and dose of drugs given. Therefore, it has to divide the methods or chemotherapy drugs that are suitable for treatment.

Apoptotic cell death

One way of treating cancer is to gain control or possibly terminate the uncontrolled growth of cancer cells. Using the cell's own mechanism for death is a highly effective method. Cell death is critical for diverse physiological processes and dysregulation of cell death mechanisms is implicated in various pathological such as cancer disease. Morphologically, cell death can be classified into three different forms: apoptosis, autophagy, and necrosis (Yun Chen *et al*.,2020) as shown in figure 5. Apoptosis and cell death associated to autophagy that can be distinguished by certain biochemical events. However, necrosis is characterized by the absence of caspase activation, cytochrome c release and DNA fragmentation. A particular difficulty in defining necrosis is that in the absence of phagocytosis apoptotic cells become secondary necrotic cells with many morphological features of primary necrosis (Dmitri V Krysko *et al*.,2008). Additionally, targeting apoptosis is the most successful treatment. Targeting apoptosis is also effective for all types of cancer, as apoptosis evasion is a hallmark of cancer. There are many anticancer drugs that target

various stages in both the intrinsic and extrinsic apoptosis pathways (Liu Y, Zhu X, 2017).

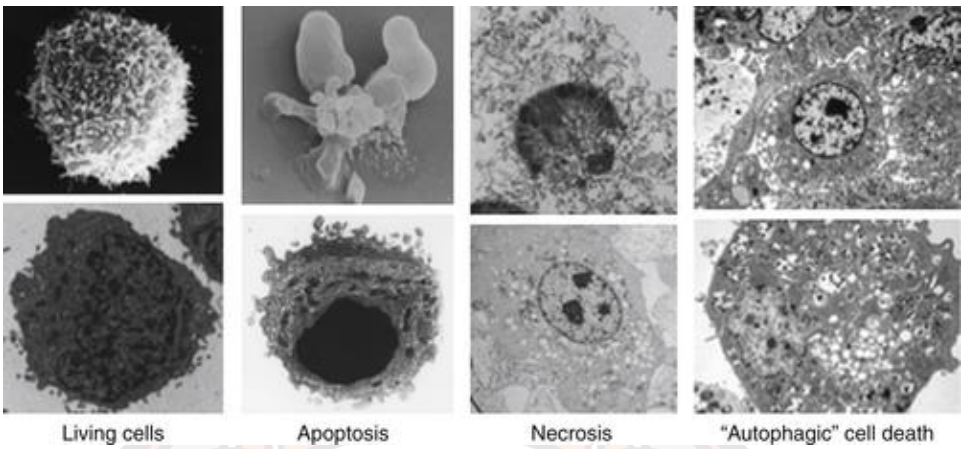


Figure 5 Types of cell death showing features of each under electron micrographs.

Source: *J Clin Invest*. 2008;118:79-88

The term apoptosis to describe cell death was introduced by Kerr and colleagues in 1972. Apoptosis is a programmed cell death, which is a death pattern used to eliminate abnormal cells in the body and plays an important role in the development and balance of cells (Kerr *et al*., 1972; Paweletz, 2001 and Kerr, 2002). Therefore, the abnormalities of controlling apoptosis can cause many types of diseases or pathogens, including induction defects, death of apoptosis of the cells that may cause cancer and autoimmune diseases (Formigli *et al*., 2000; Sperandio *et al*., 2000 and Debnath *et al*., 2005). As soon as apoptosis is signaled, changes start to the cell. These changes include activation of caspases which cleave cellular components required for normal cellular function such cytoskeletal and nuclear proteins. As a result of caspase activity, apoptotic cells begin to shrink and undergo plasma membrane changes that signal the macrophage response (Hassan M *et al*., 2014). With the contraction of organelles (membrane blebbing) (chromatin condensation) to become more condense DNA and nuclear fragmentation. Finally, the cells are formed into apoptotic bodies as shown in Figure 6,7 (Saraste *et al*., 2000 and Ewies *et al*., 2003).

There are several techniques available to study apoptosis cell death, including annexin-V staining, the TUNEL assay, caspase detection, and measurement of mitochondrial membrane potential (Michelle M. Martinez *et al*., 2010).

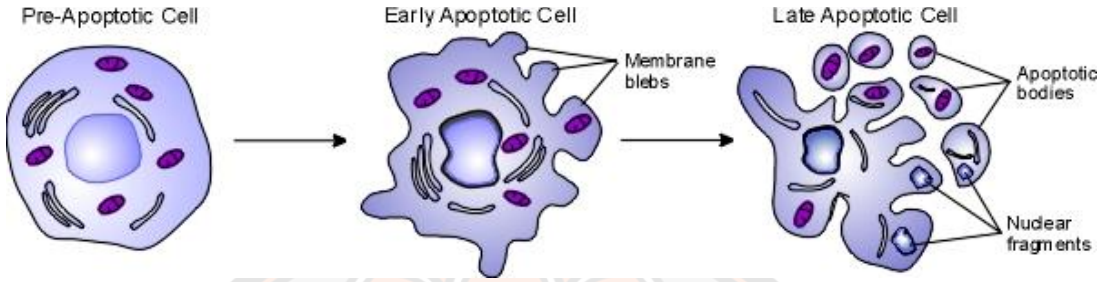


Figure 6 Cytology of apoptosis**.**

Source: https://sphweb.bumc.bu.edu/otlt/MPHModules/PH/Aging/mobile_pages/Aging2.html

Apoptosis is known as a physiological process of cell deletion and is also a process of programmed cell death, resulting in morphological change and DNA fragmentation. It is stimulated by external or internal events of cells, one of which is the extrinsic pathway mediated by the death receptor. The death receptors include Fas receptors, tumor necrosis factor (TNF) receptors, and TNF-related apoptosis-inducing ligand (TRAIL) receptors (Hirsch, 1997; Zeiss, 2003). As a surface receptor, for example, TNF receptor-1 (TNF-R1), it will interact with TNF to induce the recruitment of adaptor proteins such as Fas-associated protein with death domain (FADD) and Tumor necrosis factor receptor type 1-associated death domain protein (TRADD), which recruits a series of downstream factors, including Caspase-8, which is a critical mediator of the extrinsic pathway, resulting eventually in cell apoptosis. The other pathway is the intrinsic pathway or mitochondrial pathway regulated by the BCL-2 family of proteins (Zaman S, 2014). Various apoptotic stimuli result in the upregulation of BH3-only proteins, which then activate both BAX and BAK (Lomonosova E, Chinnadurai G, 2008). Once activated, BAX and BAK oligomerize, which leads to mitochondrial outer membrane permeabilization (MOMP). The

permeabilization allows the release of cytochrome c proteins to cytoplasm. Absorption and merging with the protein Apaf-1 causes an apoptosome which activates the enzyme caspase-9 and caspase-8, which stimulates the enzyme activity caspases-3, -6, and -7 which will result in apoptosis death sis as well (Denault JB *et al*., 2002) as shown in figure 7.

Cancer cells evade apoptosis through a variety of mechanisms. For instance, in almost half of all human cancers, the antiapoptotic BCL-2 expression is elevated and the proapoptotic proteins are suppress (Yip KW, Reed JC, 2008). Two common strategies for therapeutic targeting are stimulation of proapoptotic molecules and inhibition of antiapoptotic molecules (Claire M. Pfeffer *et al.*, 2018). The ability to target and activate an apoptotic pathway would provide a more universal cancer therapy. Particularly promising compounds to trigger apoptosis are many plant-derived compounds that are additionally nontoxic to healthy cells (Fridlender M *et al*., 2015).

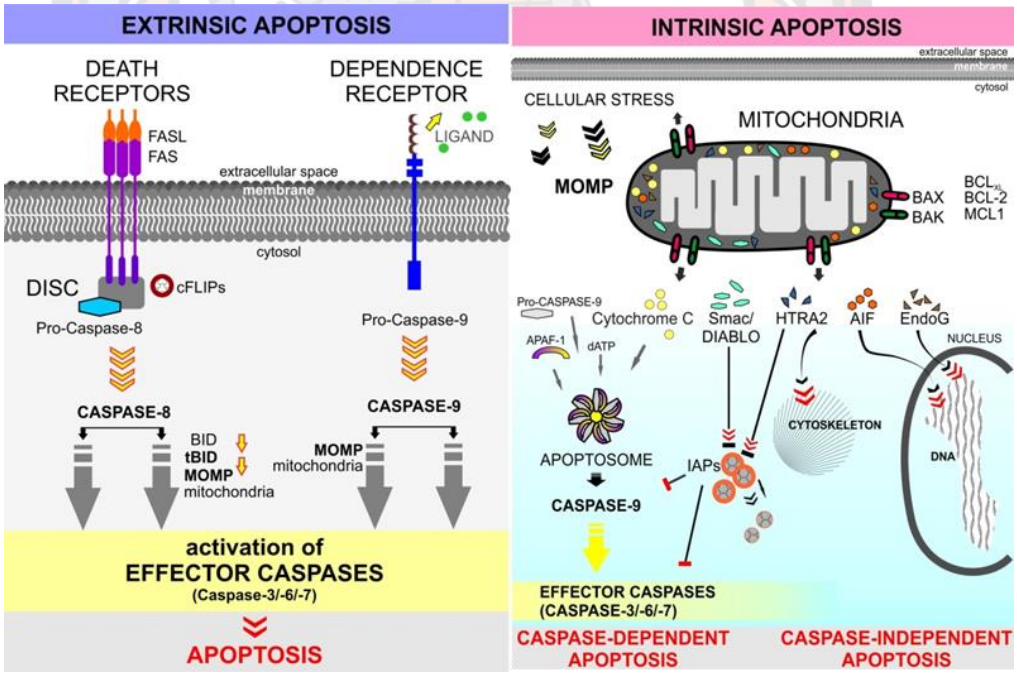


Figure 7 Apoptotic pathway.

Source: https://biologydictionary.net/apoptosis/

Cancer metastasis

The main reason that cancer is so serious its ability to spread in the body. Cancer cells can spread locally by moving into nearby normal tissue (Thomas N. Seyfried *et al*., 2013). Cancer can also spread regionally, to nearby lymph nodes, tissues, or organs. It's can spread to distant parts of the body. When this happens, it is called metastatic cancer. Cancer cells spread through the body in a series of steps. These steps include : growing into, or invading, nearby normal tissue, moving through the walls of nearby lymph nodes or blood vessels, traveling through the lymphatic system and bloodstream to other parts of the body, stopping in small blood vessels at a distant location, invading the blood vessel walls, and moving into the surrounding tissue, growing in this tissue until a tiny tumor forms, causing new blood vessels to grow, which creates a blood supply that allows the tumor to continue growing (Tarin D, 2011; Welch DR *et al*.,2006 and Bacac M *et al*., 2008) as shown in figure 8. As advanced stage of cancer grows, it can cause symptoms. These symptoms can almost always be managed with treatment, even when the cancer itself no longer responds to treatment (Seyfried TN *et al*., 2012).

The pattern of metastasis in CCA are starts with lymphatic spread to the regional lymph nodes, Then, spread via the hematogenous route to other organs, including the liver, lung, peritoneum and, occasionally, to the bone and brain (Chindaprasirt J *et al*., 1995).

The metastatic cascade paradigm illustrates how cancer cells must invade through the extracellular matrix (ECM) to intravasate into the surrounding blood supply and extravasate into the tissue of the metastatic site (S Valastyan, 2011). The ECM is a non-cellular meshwork of crosslinked macromolecules including collagens, proteoglycans, and glycoproteins, that form a dynamic, supramolecular, scaffold (Christian Frantz *et al*., 2010). Tumor cell invasion is initiated through the breakdown of the interactions (i.e., cell-cell and cell-ECM) at the primary tumor site, allowing cells to invade into the adjacent tissue (Tracey A. *et al*., 2013). The ability to process the ECM is a key tool used by cancer cells to negotiate these steps. The release and activation of ECM-sequestered growth factors [e.g., transforming growth factor (TGF)-β, fibroblast growth factors (FGFs)] and invasive enzyme such as MMPs and

cathepsin may also play a part in this malignant process (Thiery JP *et al*., 2002). Matrix metalloproteinases (MMPs), a family of 23 secreted and membrane-bound proteinases, have long been associated with intravasation and extravasation because of their ability to process the majority of ECM components. There relationship between MMP expression and the invasive activity of various cancers has been well documented. MMP-2 and MMP-9 are examples of how MMP family members can contribute to disease progression by processing a vast repertoire of ECM substrates (Lynch CC *et al.*, 2010).

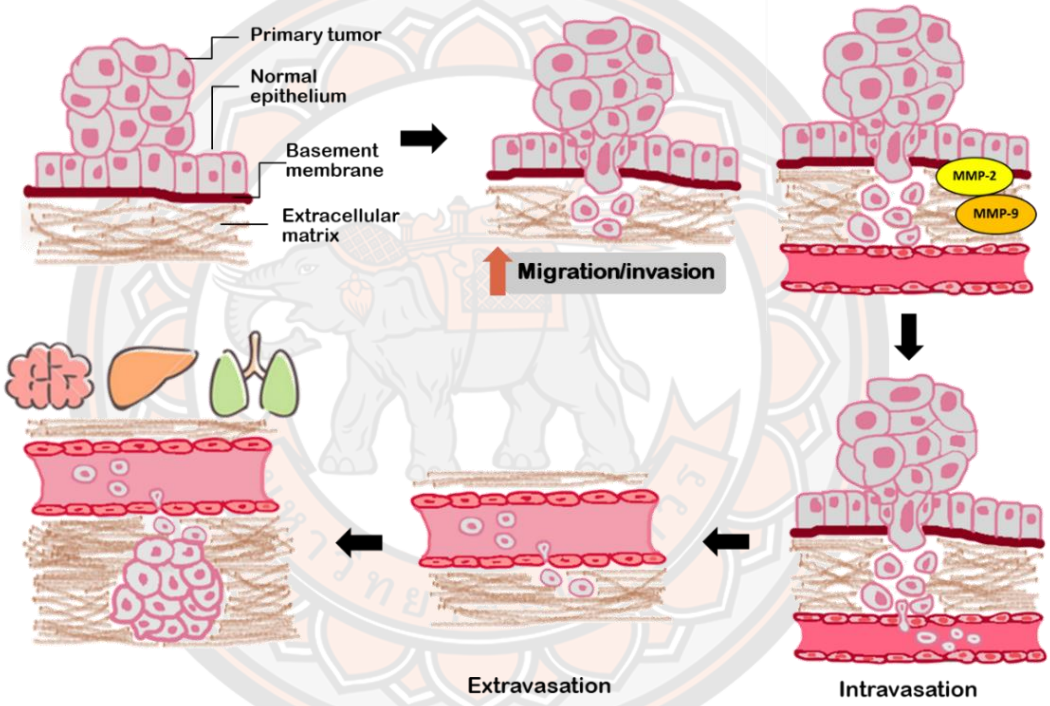


Figure 8 Steps of metastasis in cancer.

Matrix metalloproteinase in cancer metastasis

Extracellular proteolysis mediates tissue homeostasis. In cancer, altered proteolysis leads to unregulated tumor growth, tissue remodeling, inflammation, tissue invasion, and metastasis. The matrix metalloproteinases (MMPs) represent the most prominent family of proteinases associated with tumorigenesis. MMPs are a family of zinc-dependent endopeptidases first described almost half a century ago (Gross and Lapiere, 1962). They play a crucial role in various physiological processes including tissue remodeling and organ development (Page-McCaw *et al*., 2007), in the regulation of inflammatory processes (Parks *et al*., 2004), and in diseases such as cancer (Egeblad and Werb, 2002). There are 23 MMPs expressed in humans are categorized by their architectural features. The discovery that inhibition of MMPs suppresses the invasive potential of tumors in animal studies was swiftly implemented into clinical trials. Yet, these failed to increase survival rate of the patients (Kai Kessenbrock *et al*., 2010 and Coussens *et al*., 2002). Recent observations also suggest that MMPs play a role in cancer cell survival. The function of MMPs and their substrate as shown in table 1 (Niina Reunanen *et al*., 2013).

Table 1 Human MMPs, their expression profile, and substrates. Modified from Niina Reunanen *et al.,* 2013**.**

Enzyme	Expression or function	Substrates
Collagenases		
Collagenase-1 (MMP-1)	Development, tissue repair, malignant tumors	Col I, II, III, VII, VIII, X, aggrecan, MBP
Collagenase-2 (MMP-8)	Leukocytes, cartilage	Col I, II, III, aggrecan,, serpins, α2M
Collagenase-3 (MMP-13)	Bone development,	Col I, II, III, IV, IX, X, XIV, gelatin,
Stromelysins		
Stromelysin-1 (MMP-3)	Keratinocytes and fibroblasts	Col IV, V, VII, IX, X, XIV, fibronectin, elastin, gelatin, laminin, aggrecan,
Stromelysin-2 (MMP-10)	Keratinocytes and fibroblasts	Col IV, V, IX, X, XIV, fibronectin, elastin, gelatin, laminin, aggrecan, nidogen
Stromelysin-like MMPs		
Stromelysin-3 (MMP-11)	Invasive human carcinomas	α1PI

Macrophage elastase (MMP-12)	Constant expression in macrophages	Col IV, gelatin, fibronectin, laminin, MBP, elastin, vitronectin, nidogen, α1PI,
Matrilysins		
Matrilysin-1 (MMP-7)	Constant expression in ductal epithelial cells of exocrine glands	Col IV, elastin, fibronectin, laminin, nidogen, tenascin, osteonectin, MBP, decorin, versican, α1PI
Matrilysin-2 (MMP-26)	Uterus, placenta, reproductive processes	Col IV, gelatin, fibronectin, fibrin, fibrinogen, type I gelatin, α1PI, β-casein,
Gelatinases		
Gelatinase A (MMP-2)	Degradation of BM and of fibrillar collagens	Col I, IV, V, VII, X, gelatin, fibronectin, tenascin, fibrillin, osteonectin, MBP
Gelatinase B (MMP-9)	collagenases, invasion of malignant tumors	Col IV, V, VII, XI, XIV, XVII, gelatin, elastin, fibrillin, osteonectin, fibronectin,
Membrane-type MMPs		
MT1-MMP (MMP-14)	Skeletal development, angiogenesis, trophoblast invasion, tumors	Col I, II, III, gelatin, fibronectin, laminin, vitronectin, aggrecan, tenascin, nidogen,
MT2-MMP (MMP-15)	Liver, brain, placenta, heart	fibronectin, laminin, aggrecan, tenascin, nidogen, perlecan
MT3-MMP (MMP-16)	Brain, heart, placenta, carcinomas	Col III, gelatin, casein, fibronectin, laminin, aggrecan, vitronectin
MT4-MMP (MMP-17)	Brain, leucocytes, colon, ovary, testis, breast carcinoma	gelatin, TNF-a precursor, fibrin,
MMP-19-like MMPs		
MMP-19	Capillary endothelial cells, inflamed synovium	Col IV, gelatin, laminin, nidogen, tenascin, fibronectin, aggrecan, COMP
MMP-28	Testis, lung, keratinocytes	ND
Other MMPs		
Enamelysin (MMP-20)	Tooth development	Amelogenin, aggrecan, COMP
MMP-23	Reproductive processes	Synthetic MMP substrates

Bioactive compounds

Bioactive compounds are substances from natural organisms that are effective to human beings, animals and plants. Good bioactive substances must be specific substances having specific effects such as killing the cancer cells, and the substances do not have a negative effect on the body or have little side effects. For example, bioactive compounds such as Polyphenolic compounds, Carotenoids, Tocopherols, Phytosterols, Organosulfur compounds (Carbonell-Capella *et al*., 2014; Porrini & Riso, 2008) (Figure 9). Many bioactive substances have different chemical structures and functions (Kris-Etherton, P. M. *et al*. 2002). Among these, phenolic compounds were also found to be active ingredients biologically with antioxidant activity (Burn, J. *et al*. 2000), helps prevent cancer and the effect of lowering cholesterol levels. The research has been shown that phenolic compounds (Figure 9) also have properties that have antioxidant activity (Ganguly A. *et al*.,2007) anti-inflammatory (Athar M. *et al*, 2007) and anti-cancer effects by reducing cancer cell proliferation (Jow, G. *et al*., 2004) and inducing cancer cells apoptosis. (Ganguly A. *et al*., 2007)

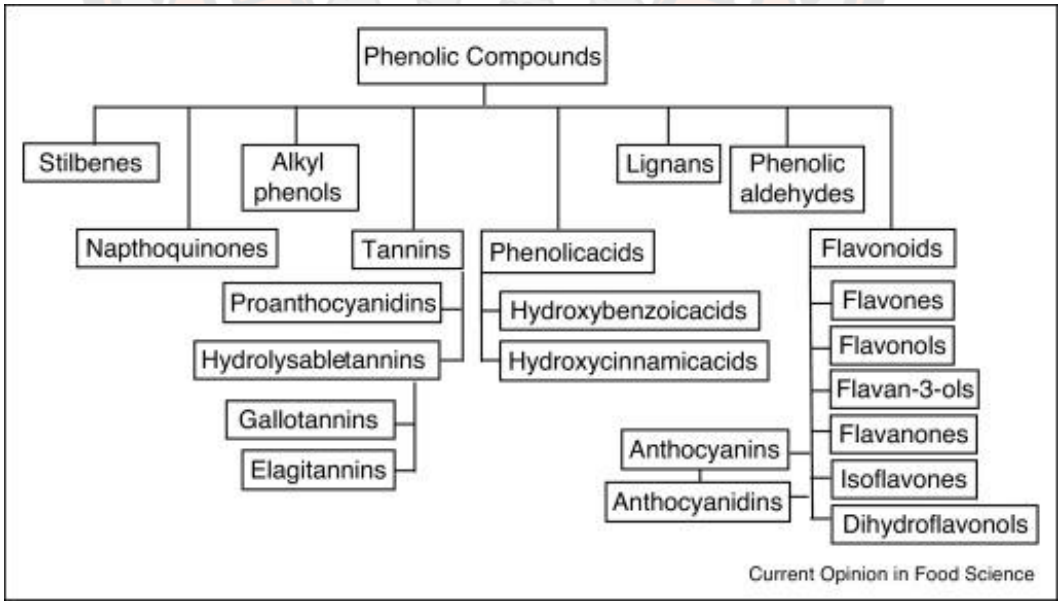


Figure 9 Phenolic compounds**.**

Source: https://www.sciencedirect.com/science/article/pii/S2214799317300425

Peanut

Peanuts (peanut or groundnut) may be called ground nuts, digging nuts, or yeast beans. It is a biennial plant that is a legume crop (Leguminosae) as well as soybeans and green beans. The scientific name *Arachis hypogaea L*. is dried beans (legume) which has high oil organized in the oil crop sector which is economically important. General characteristics of peanuts are peanut seeds in the pod which is underground caused by complete flowers, sex after pollination. The petals will shrink and fall, but the stem of the ovary expanding longitudinally along the vertical line, called the needle of the needle, thrust into the soil and then develop into a pod.

1. Kalasin 2 peanut

"Tiger patterned beans" or "Peanuts of Kalasin 2" cultivated for a long time in the north of Thailand (Royal Thai Government and The World Bank, 2012). There are many varieties of queen beans, royal peas, jumbo nuts, distinctive characteristics. When the seeds are removed from the pods, there will be a seed coat of beans similar to the tiger pattern, as shown in Figure 10. The taste is quite sweet and crisp, softer than peanuts including of bioactive substances such as trans-resveratrol, trans-arachidin, piceatannol etc (Victor S Sobolev *et al*., 2006).

Figure 10 Kalasin 2 peanut**.**

Source: http://www.thaigrowth.com/product/

In addition, the previous study on the amounts of antioxidants or antioxidants in peanuts, Kalasin 2 found that various parts peanut trees have a high level of antioxidant activity in the seed and root membranes, respectively (Apinun Limmongkon *et al*., 2018). In addition, these results showed that the increasing of the root part led to increase the amounts of phenolic compounds and antioxidants as shown in Figure 11.

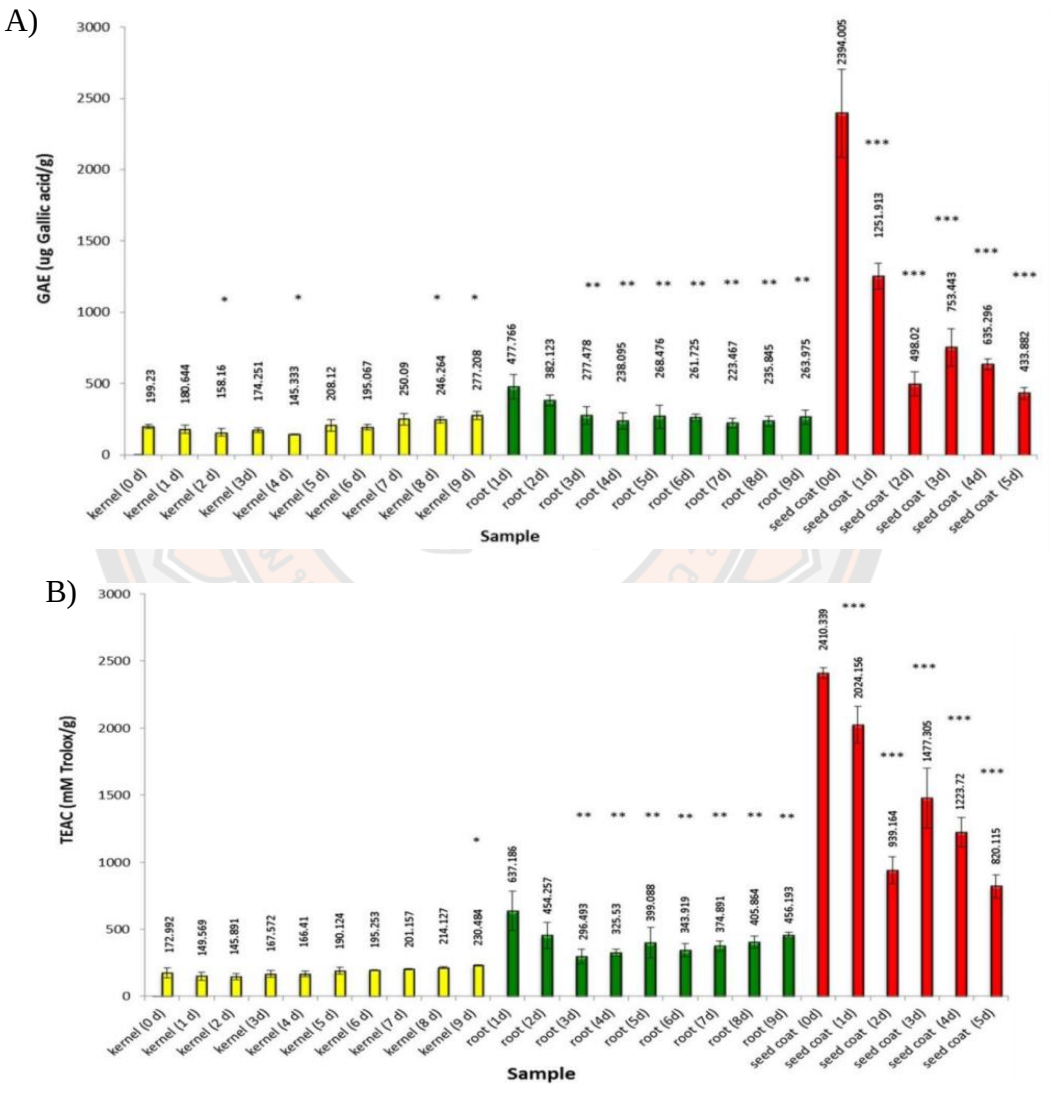


Figure 11 The amounts of phenolic compounds and antioxidant activity of various parts from the growing peanut**.** (a) total phenolic compound, (b) antioxidant activity.

Source: A. Limmongkon *et al*., 2019

Peanut hairy root culture crude extract (PCE)

The hairy root culture system is the great biotechnology that can be induction for plant secondary metabolite production. Hairy root cultures were transformed by *Agrobacterium rhizogenes* grow rapidly on phytohormone-free medium. Hairy roots are useful to produce biologically active molecules because large amounts can be produced with highly cost effectiveness (Mallikarjuna *et al*., 2016). Resveratrol production from hairy root cultures has been several studied in several plants. Hairy root cultures of peanut have been established to produce of resveratrol and its derivatives (Pilaisangsuree *et al*., 2018). The most promising strategy to enhance the production of trans-resveratrol, piceatannol, trans-arachidin-1, and trans-arachidin-3 is co-treatment with methyl jasmonate and cyclodextrin, which promotes a high resveratrol yield in peanut hairy root cultures (Yang *et al*., 2015).

Previous study have been conducted on the induction of peanut glutinous cultivation to produce an increased amount of antioxidant (T. Soomboon *et al*., 2019). The two different types of elicitors, paraquat (PQ) followed by methyljasmonate (MeJA) combined with cyclodextrin (CD) and extracts from the peanuts hairy root that elicited by methyljasmonate combined with cyclodextrin and followed by paraquat for 24 hours. The hairy root culture medium from each elicitor treatment was extracted with ethyl acetate. The ethyl acetate extracted fractions from culture medium extracts were separately evaporated to dryness. The crude extracts were dissolved in EtOH and determine the phenolic component by High-performance liquid chromatograph (HPLC). The results showed that the extract obtained was a substance in the group of 3 types of stilbene derivatives, namely trans-resveratrol, trans-arachidin-1, trans-arachidin-3, where the extracts elicited by PQ + MeJA_CD had a higher resveratrol content than the extracts elicited with MeJa_CD + PQ as shown in Figure 12-13.

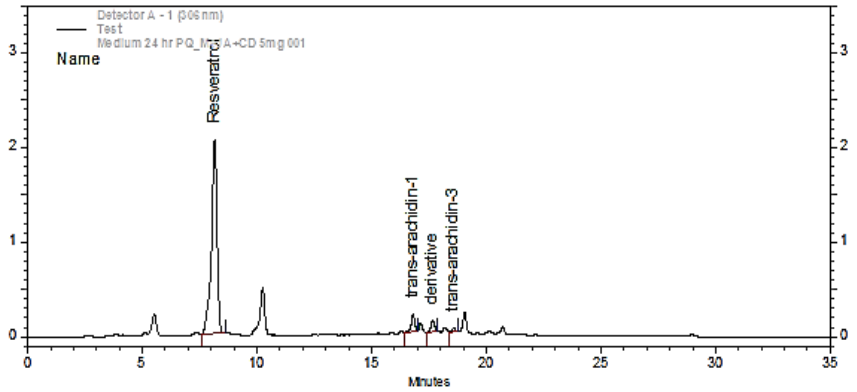


Figure 12 The amount of substances obtained from the activation of peanut hairy root with PQ + MeJA_CD at 24 h**.**

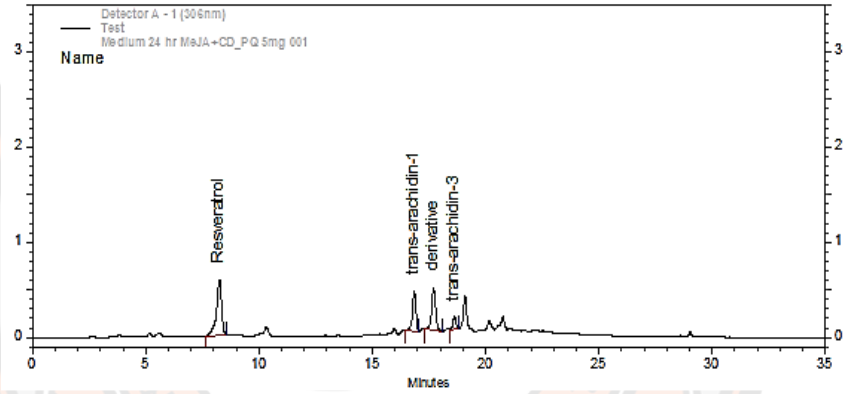


Figure 13 The amount of substances obtained from the activation of peanut hairy root with MeJA_CD + PQ at 24 h.

Stilbene derivatives

Stilbenoids are a group of phenolic compounds with different structures (Figure 15). The acrylonitrile stilbene monomer (trans-1,2-diphenylethylene) consists of two phenyl rings linked by ethylene. Stilbenes can be in the form of a cis- or trans-isomer, but in the form of trans-isomer is a more general and stable structure. Stilbenes are derived from the synthesis of plants through the phenylpropanoid biochemical synthesis pathway (Figure 14) begins with changing phenylalanine that is a cinnamate based on enzyme activity phenylalanine ammonia lyase (Allwood EG *et al*., 1999) such as trans-resveratrol, piceatannol, trans-arachidin-1, trans-arachidin-3 and oxyresvertarol (Figure 15).

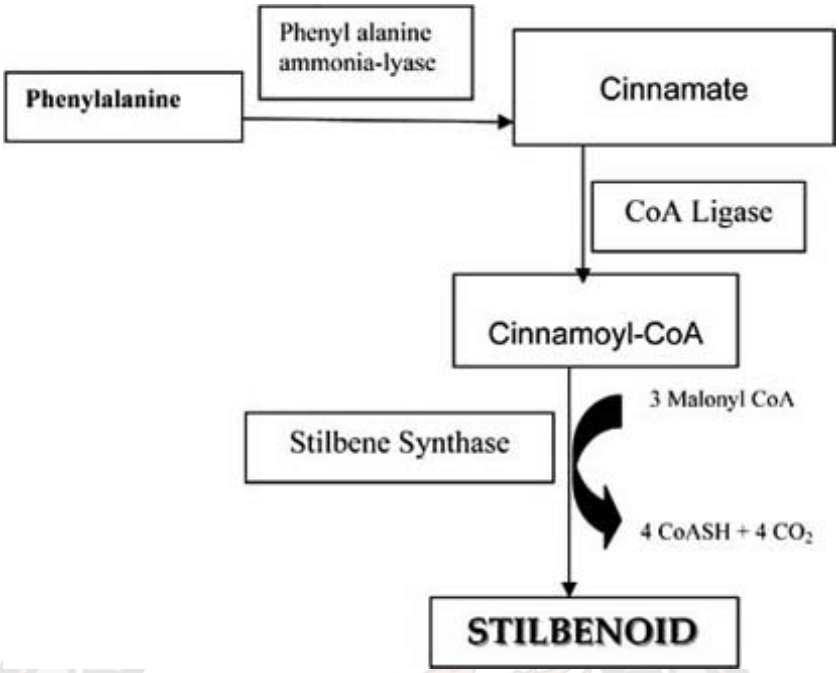


Figure 14 Phenylpropanoid synthesis pathway of stilbenoids**.**

They are produced by plants to protect themselves against stressing situations such as excessive ultraviolet (UV) irradiation, heat exposition, insect attacks, and fungus or bacterial infections. Since the original research by Jang *et al*. where a resveratrol was shown as a potent chemopreventive agent (M. Jang *et al*., 1997). The reason for this extensive study in the number of biological activities stilbene carries such as antioxidative (Gulc E. 2010), antitumoral (Khan *et al*., 2016), antiviral (Yiu *et al*., 2010), anti-inflammatory (Tili *et al*., 2010) and life span extension (Khan *et al*., 2019). Therefore, these compounds have awakened the interest of the scientific community involved in anticancer drug development.

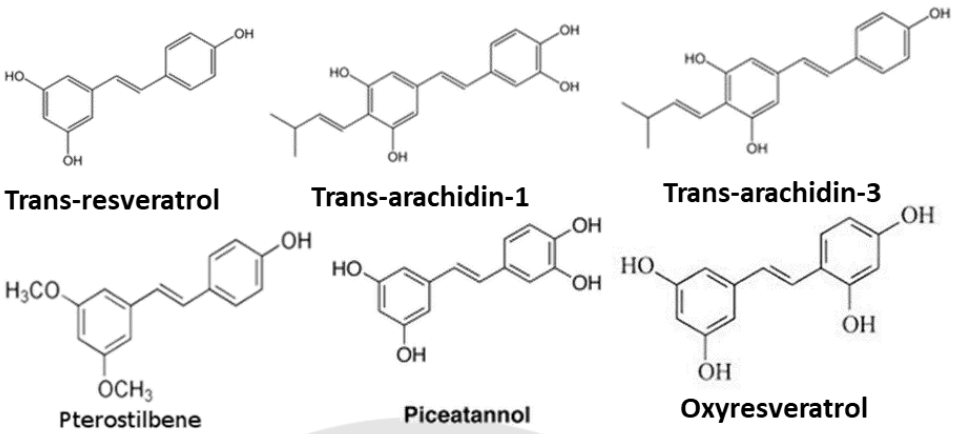


Figure 15 The structure of the Stilbene group**.**

Source: Tava Shelan Nagapan *et al.*, 2018

Stilbenoids act as anticancer

11.1 stilbenoids induce cell death

The stilbene compounds have received a lot of attention due to their potentially biological properties. Resveratrol has antioxidant, anti-inflammatory, and anticancer properties. There are several data shows that resveratrol acts as an anticancer agent such as colorectal cancer (Aggarwal BB *et al*., 2004; Bishayee A *et al*., 2009 and Bishayee A *et al.*, 2010). Consumption of resveratrol reduces cancer cell proliferation. The results of a previous study suggested that daily consumed doses of resveratrol at 0.5 or 1.0 g could reduce cancer cell proliferation in colorectal cancer patients by 5% (Patel *et al*., 2010). In addition to resveratrol and its derivative, the stilbenoid trans-arachidin-1 demonstrated anticancer activity by inducing caspase-independent programmed cell death in human leukemia HL-60 cells with a four-fold lower the IC50 than resveratrol (Huang *et al*., 2010). Molecular modelling study have indicated that the prenylated products of trans-arachidin-1 and trans-arachidin-3 improve that can binding affinity to cannabinoid receptors (CBR) (LK Brents *et al*., 2012). This observation demonstrates that CBR antagonists produce a variety of therapeutic

effects, such as anti-obesity, anti-carcinogenic, and anti-inflammatory activities (Brents *et al*., 2012).

Resveratrol (trans-3,5,40-trihydroxystilbene) is found in grapes, red wines and various plants (Ngo KS and Brown GD, 1998). Previously, resveratrol was able to inhibit growth and induce cancer cells into the apoptotic death process in the cholangiocarcinoma KKU-100 and KKU-M156 by does and time dependent manner as shown in Figure 16 (C. Hahnvajanawong, *et al*., 2011)

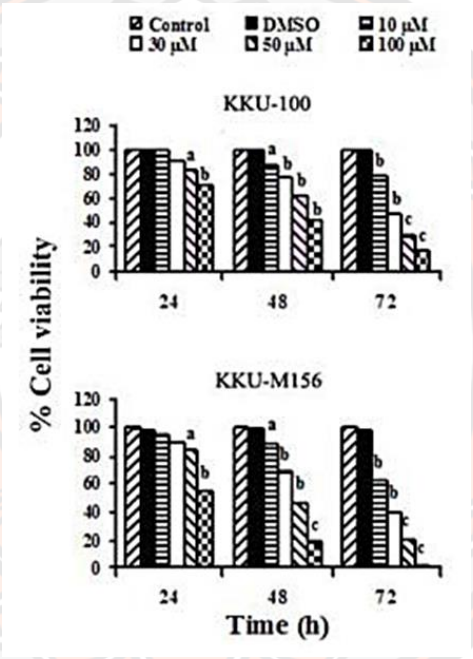


Figure 16 Experimental results on Resveratrol and cholangiocarcinoma cell line KKU-100 and KKU-M156.

Source: C. Hahnvajanawong, *et al*.,2011

The study of resveratrol in breast cancer cells by Genes R Venkatadri *et al*., 2016 showed that resveratrol treatment in breast cancer cells can also increase mortality as shown in figure 17.

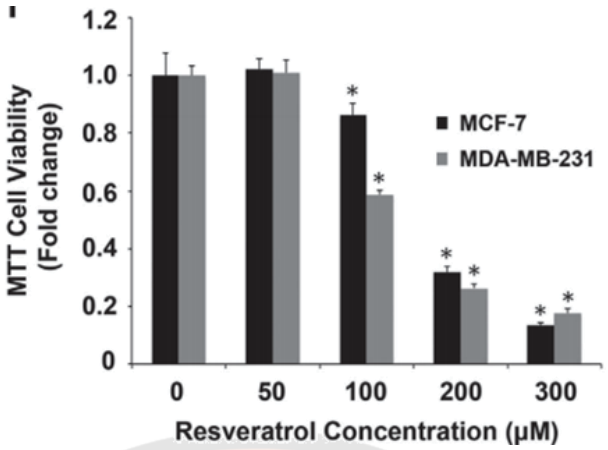


Figure 17 Death rate of breast cancer cells when receiving resveratrol at various concentrations

Source: R Venkatadri *et al*., 2016

The study was further investigated whether resveratrol causes apoptosis in breast cancer cells. Using Hoechst 33343 staining, they observed increased numbers of apoptotic cells in the resveratrol treated cells for 18 h compared with the control which characterized by DNA condensation and fragmentation. These results suggested resveratrol exhibit a significant increase of apoptosis as shown in figure 18.

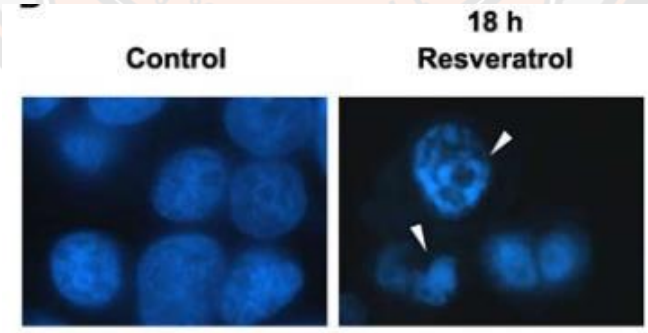


Figure 18 Resveratrol induced apoptosis by DNA condensation and fragmentation**.**

Source: R Venkatadri *et al*., 2016

Numerous studies of resveratrol-induced apoptosis were also demonstrated in many other cells, such as colon cancer. Leukemia Oral cancer (Andrew K. Joe *et al*., 2002) Ovarian cancer (Anthony W. *et al*., 2004; Daniele Vegara *et al*., 2012) and lung cancer (Lijie Ma *et al*., 2015), (Michinori *et al*., 2017) etc.

In addition, resveratrol also causes apoptosis of the cell death with caspase-8-dependent pathway (receptor mediated type I) or caspase-9 dependent pathway (mitochondrial; type II). It can reduce the risk of heart disease by up to 40%. At the same time, resveratrol also has antioxidant properties by inhibiting the coagulation of blood well, helping to regulate fat metabolism (Jang *et al*., 1997) reduce tissue inflammation, reduce the occurrence of tumors of cancer (Ahmad *et al*., 2000).

According to a research study (N. Kaewduangdee, 2004), resveratrol was used to test the toxicity on inhibition of growth and kill bile duct cancer cells, the KKU-100 by SRB assay method. Resveratrol can inhibit the growth of bile duct cancer KKU-100.

Recently, several reviews have summarized the effects of resveratrol treatment on many cancer types. Numerous *in vitro* studies have shown that resveratrol has multiple anticancer effects. However, the data from animals and humans are limited due it's low solubility, rapid metabolism, poor bioavailability, low bioactive absorption and low targeting efficacy (Brents LK *et al*., 2012). Some compounds structurally related to resveratrol are present in plants and also show a wide spectrum of biological activities such as pterostilbene, picatannol and trans-arachidins.

The multiple benefits of pterostilbene in treatment and prevention of human diseases have been attributed to its antioxidant, anti-inflammatory, and anticarcinogenic properties leading to inhibition of malignant cells (A. M. Rimando, *et al*., 2002 and C. M. Remsberg *et al*., 2008). In recently, pterostilbene inhibited the growth of xenotransplanted triple-negative MDA-MB-468 tumors consisting of a population of in vivo-selected and thus highly tumorigenic cells resembling cancer stem cells (Chiou YS *et al*., 2008). In addition, this data suggest that pterostilbene might be potentially useful not only for prevention and treatment, but also the reduction of recurrence of human breast cancer, especially triple-negative breast cancer (Rei Wakimoto *et al*., 2017).

Piceatannol is a phenolic compound (stilbenoid) and a hydroxylated analogue of resveratrol (M. Boccellino *et al*., 2019). Piceatannol possesses potent antioxidant activity and has chemopreventive and anticancer properties against different cancers through induction of both mitochondrial-dependent and independent pathway of apoptosis by elevating the levels of pro-apoptotic proteins, lowering the levels of anti-apoptotic proteins (T.C. Hsieh *et al*., 2012); and regulation of the Akt/mTOR, NF-κB, and JAK-STAT3 pathway. It also demonstrates potent anti-metastatic effects via suppression of MMPs, Wnt signalling pathways and PI3K/Akt/ mTOR, epithelial to mesenchymal transition (R.G. Jayasooriya *et al*., 2013).

Trans-arachidin-1 and trans-arachidin-3 have antioxidant activity and anti-inflammatory effect (Chang J *et al*., 2006), similar to resveratrol, including anti-cancer effects in colon cancer cells Leukemia (Huang C *et al*., 2010) and lung cancer and also found that inhibition of CB 1 and CB 2 (Brents LK *et al*., 2012) is a receptor for the brain area that is overexpressed in many cancers such as liver cancer, lung cancer and breast cancer. However, these two substances are very few studies on trans-arachidin-1 and trans-arachidin-3. The anticancer activity of trans-arachidin-1 isolated from germinated peanut kernels was demonstrated in HL-60 cells through the caspase-dependent and caspase-independent pathways (Huang CP *et al.*,2010). Moreover, trans-arachidin-1 and trans-arachidin-3 possess anti-inflammatory, anti-proliferative properties and have bioavailability better than resveratrol (Chang *et al*. 2006 and Djoko *et al*. 2007) indicate that, trans-arachidin-1 and trans-arachidin-3 might represent a novel class of compounds with significantly improved biological activity that might be exploited for future development as efficacious pharmaceutical drugs.

The knowledge of epigenetic mechanisms regulating gene expression has allowed significant advances understanding of cancer biology. Cancer can caused by the accumulation of genetic and epigenetic alterations, which induce alterations in the expression of oncogenes and tumor suppressor genes (Sharma *et al*., 2010; McCleary-Wheeler *et al*., 2013 and Aggarwal *et al*., 2015). Recent reports indicate that dietary supplements and natural compounds may recover the normal epigenetic marks which are altered during carcinogenesis. The phytochemicals most studied in cancer are epigallocatechin-gallate (EGCG), quercetin and resveratrol. In particular, these compounds blocked the development and progression of tumors by targeting key signaling transducers resulting in the restoration of tumor suppressor genes, and inhibition of oncogenes expression (Greenlee, 2012; Guo *et al*., 2015; Zhou *et al*., 2016). These effects are mediated, in part, by the modulation of epigenetic machinery which included the regulation of DNMTs and HDACs activities (Figure 19) (Shukla et al., 2014; Thakur *et al*., 2014; Li *et al*., 2015; Deb and Gupta, 2015; Shankar *et al*., 2016 and Sundaram *et al*., 2017).

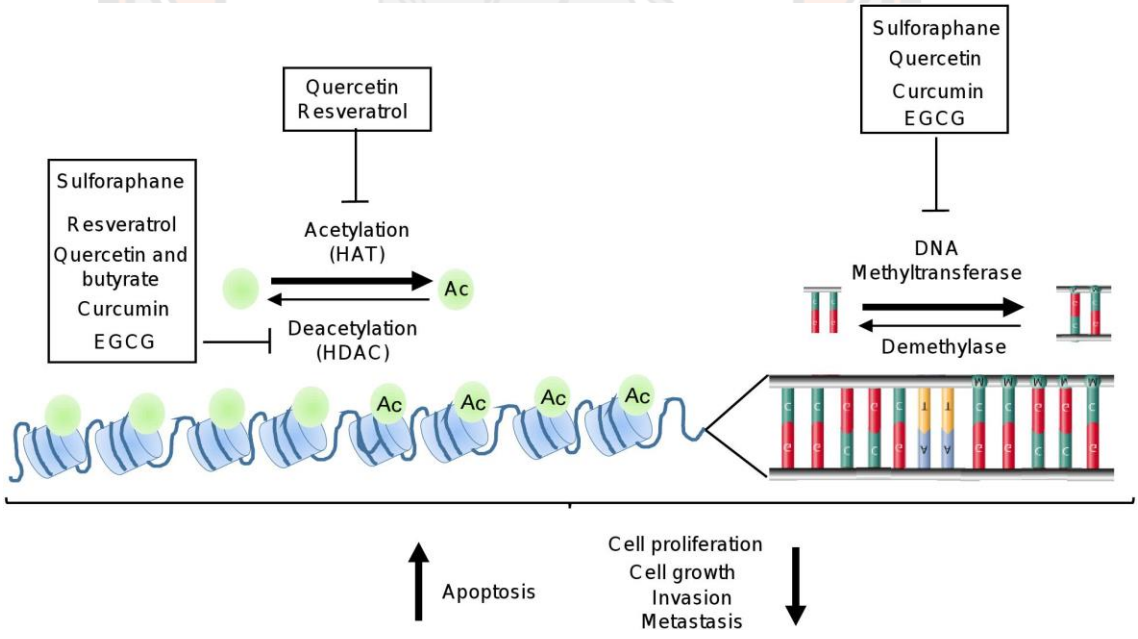


Figure 19 Epigenetic machinery as targets of polyphenols

Source: Ángeles Carlos-Reyes *et al*., 2019

11.2 Stilbenoid on Cancer Treatment and Prevention

The prevention or treatment the cancer cell at specific stages of carcinogenesis such as initiation, promotion and progression can be summarized as shown in figure 20. Stilbenes block metabolic activation of pro-carcinogens by inhibiting specific isoforms of cytochrome P450 (CYP) enzymes and thus prevented the initiation of carcinogenesis in cultured human tumor cells (Chang, T.K *et al*., 2000 and Chun, Y.J. *et al*.,2009). Resveratrol target on carcinogenesis by inhibiting the initiation of tumor which consists of the DNA mutation of a normal cell, which is an abnormal and fast change (Jeong-Hyeon Ko *et al.,* 2017). The initiated cell is capable to autonomous growth. The anti-initiation activity of resveratrol is linked to the suppression of the metabolic activation of carcinogens (Signorelli, P and Ghidoni, R, 2005).

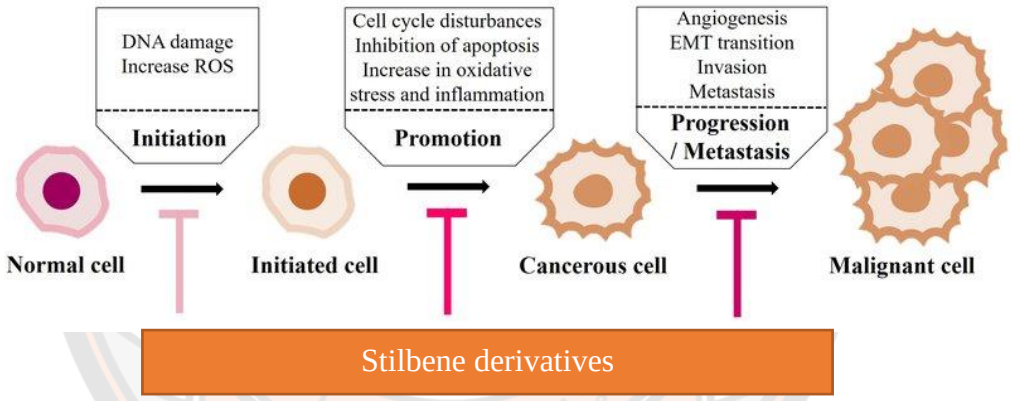


Figure 20 Anticancer activity of stilbene derivatives on carcinogenesis**.**

Resveratrol can prevent the progression of cancer by inhibiting actions of transcription and growth factors, such as p53, FoxO, and ATF3, which are involved in the initiation and promotion of cancer in cell culture studies (Whitlock, N.C., Baek, S.J, 2012). Furthermore, resveratrol and pterostilbene prevent proliferation and induce apoptosis in various cancer types including breast, prostate, pancreatic, liver and colorectal cancer (Carter, L.G *et al*., 2014 and McCormack, D.; McFadden, D, 2012). Mechanisms that have been concerned in the anticancer properties of resveratrol and pterostilbene include induction of apoptosis, inhibition of proliferation, cell cycle arrest and inhibition of angiogenesis (Kosuru, R *et al.*, 2016 and Pavan, A.R. *et al*., 2016).

11.3 The effect of the stilbene derivatives on the inhibition cancer cell metastasis

Stilbenes are known to possess a wide range of biological activities including antioxidative, anti-inflammatory, antifungal, antibacterial, antiviral, and antitumor activities. Previous studies shown that the effect of resveratrol on the inhibition of cancer cell proliferation showed that it could inhibit the spread of many cancer cells such as liver cancer (Chao-Bin Yen *et al*., 2013), bladder cancer Pigment cancer (J Chen *et al*., 2013), prostate cancer, colon cancer and ovarian cancer (Aline Pavan *et al*., 2016) etc.

In addition, there are other types of stilbene agents that have the effect of inhibiting the proliferation of cancer cells. For example, oligostilbenes are oligomers of the natural molecule resveratrol and have been reported to exhibit a broad variety of biological activities, including antioxidant, antitumor, anti-inflammatory and antimalarial activities (He S *et al*., 2009 and Aggarwal BB *et al*., 2004). The resent study has focused on the effects of oligostilbenes on the migration and invasion of MDA-MB-231 breast cancer cells in order to determine their effects on the metastasis of tumor cells. At a lower dose that did not affect the cancer cell growth, all oligostilbenes inhibited the migration and invasion capability of MDA-MB-231 cells in *vitro* (Ying g *et al*., 2017). Trans-4,4′-dihydroxystilbene (DHS) inhibits the spread of lung cancer cells (B. Saha *et al*., 2018) and inhibits distribution of breast cancer cells (C. Maccario *et al*., 2012). The effect of stilbene derivatives on various cancer are summarized in the table 2.

Table 2 Summary of anticancer effect from stilbenoids on cancer

Stilbenoid	Model	Anticancer effect	References
Resveratrol	Breast cancer	Downregulation of PI3K/Akt/mTOR Reduced beta catenin and cyclin D1 Cell cycle arrest Upregulation of p53 and Bax/ apoptosis	Khan A *et al*., 2014 Chatterjee M *et al*., 2011 Back JH *et al*., 2012 Boily G *et al*.,2009
	Colorectrol cancer	Inhibition of invasion/metastasis Activation of ER-stress and apoptosis Cell cycle arrest	Karimi DF *et al*., 2016 Squd SM *et al*., 2014 Yuan SX *et al*., 2016
	Hepatocellular cancer	Inhibition of angiogenesis/metastasis Inhibition of invasion Increase apoptosis	Li Y *et al*., 2013 Weng CJ *et al*., 2010 Du Q *et al*., 2013 Guo L *et al*., 2016
	Gastric cancer	Induction of apoptosis Inhibition of migration/invasion	Shin KO *et al*., 2012 Aquilano K *et al*., 2009 Gao Q *et al*., 2015 Feng M *et al*., 2016
	Lung cancer	Inhibition of invasion/metastasis Induction of apoptosis Inhibition of STAT3 and invasion Cell cycle arrest at S-phase	Ma L *et al*., 2015 Wang H *et al*., 2013 Yang YP *et al*., 2012 Wang G *et al*., 2015
Trans-arachidin-1 **Trans-**	Ovarian cancer Leukemia	Reduce cell viability Cytotoxicity Induce cell apoptosis	Huang *et al*., 2010 Amin *et al*., 2009 Ko *et al*., 2013

arachidin-3	Colon cancer	Inhibition of cell viability	Macy R. *et al.*, 2017 Ball *et al*., 2015
	Lung cancer	Inhibition of cell viability	Ko *et al.*, 2013
pterostilbene	Colon cancer	Inhibition of cell viability	Nutakul *et al* ., 2011
	Breast cancer Prostate cancer	Affecting epigenetic pathway	Chen RJ *et al*., 2010 Hsieh MJ *et al*., 2017
	lung cancer Bladder cancer	Induce apoptosis	Li K *et al*., 2013 Kala R *et al*., 2015
	Lung cancer Breast cancer	Anti metastasis against migration and invasion Decrease MMP2 and MMP9 activities	Pan MH *et al*.,2011 Ko HS *et al*., 2014 Ma Z *et al*., 2017 Principe DR *et al*., 2017

12. The effects of the stilbene derivatives from peanut hairy root in various cancers

As mentioned previously (Apinun Limmongkon *et al*., 2019) that the extract from the peanut hairy root culture which are stimulated by two different stimulants, paraquat (PQ) followed by methyljasmonate (MeJA) combined with cyclodextrin (CD). The extracts from peanuts hairy root that elicited by PQ, MeJA combined with CD for 24 h. There are exhibits the strong antioxidative effect and stilbenes are the major bioactive compounds in which different pattern of stilbene compounds (Apinun Limmongkon *et al*., 2019). Therefore, in both extracts may exert the anti-cancer effect. To demonstrate whether the peanut hairy root culture extract has anti-growth effect, we determined the effect of those extracts on cell viability of four types of cancer cells that high incidence in Thailand which are the KKU-214; a Cholangiocarcinoma cell, A549; a lung cancer cell, MCF-7; a breast cancer cell and KB; an oral cancer cell line. The cells were treated with both extracts at

concentrations of 0-800 µg / ml for 24 h. After that, the survival of the cells was measured by using the MTT assay technique. The results showed that the extracts stimulated by PQ + MeJA_CD reduced the viability of cancer cells greater than the extracts stimulated by MeJA_CD + PQ. Interestingly, the anti-growth effect of the extracts was clearly observed in cholangiocarcinoma cell compared with other cancer cells. These data indicated that CCA cell is the most sensitive to inhibit by peanut hairy root culture extracts (Figure 21).

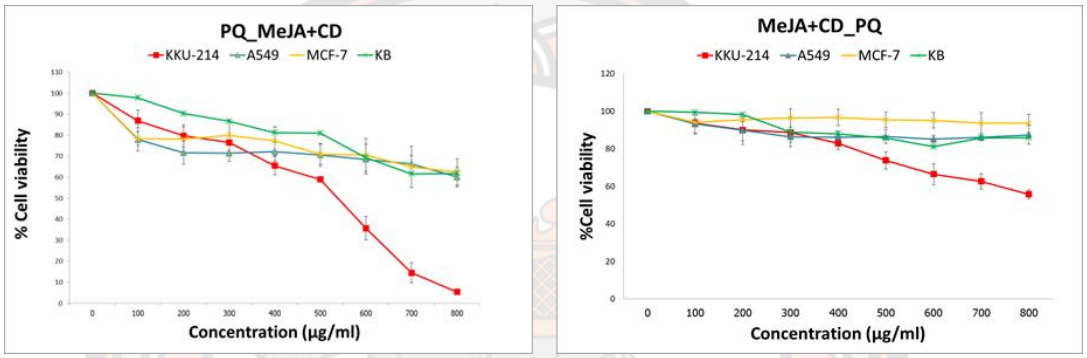


Figure 21 The effect of two extracts from peanut hairy root culture on cell viability of cancer cells (D. Thalapphet, unpublished data).

According to the benefit of trans-arachidin-1 and trans-arachidin-3 that has a greater bioavailability than resveratrol. However, only few studies are working on these compounds. The main reason is that trans-arachidin-1 and trans-arachidin-3 can only found in peanut. Therefore, using the technology of hairy root culture from the peanut can lead to the way of discovery the novel compounds that might be exploited for future development as efficacious pharmaceutical drugs.

CHAPTER III

RESEARCH METHODOLOGY

Materials

1.Biological materials

1.1 Instruments

Table 3 List of instruments

Instrument	Product of
25 cm^3 culture plate	SPL Life Science, Korea
96-well culture plate	SPL Life Science, Korea
12-well culture plate	SPL Life Science, Korea
6-well culture plate	SPL Life Science, Korea
15 ml centrifuge tube	SPL Life Science, Korea
1.5 ml microcentrifuge tube	SPL Life Science, Korea
Autopipette and tips	Corning Inc, USA
CO_2 incubation	Gibco, Grand Island, NY
Laminar flow	Esco Lifesciences
Inverted microscope	LEICA, USA
Hemocytometer	Microbehunter, Austria
Water bath	Julab, Germany
Microplate reader	Labsystems, Finland

1.2 Chemicals and reagents

Chemicals, reagents, commercial kits, and antibodies, are listed in List of chemicals and suppliers

Table 4 List of chemicals and suppliers

Chemicals	Product of
Cell culture	
3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide MTT	Bio basic Canada, Inc, CA)
Crystal violet	Panreac, E.U.
Dimethyl sulfoxide	Sigma-Aldrich.St. Louis, MO
Dulbecco's Modified Eagle Mediun (DMEM)	Gibco, Grand Island, NY
Fetal bovine serum (FBS)	Gibco, Grand Island, NY
Penicilin-Streptomycin	Gibco, Grand Island, NY
Phosphate buffered saline (PBS)	Amresco, USA
Trypan blue	Gibco, Grand Island, NY
Trypsin/EDTA	Gibco, Grand Island, NY
Protein expression (Western blot)	
Acrylamide	Amresco, USA
Ammonium peroxidisulphate	Amresco, USA
Beta-mercaptoethanol	Gibco, GranIsland, NY
Bromophenol blue	Fluka, China
Chemiluminescence (ECL)	Bio-rad, USA
Dithiothreitol	Amresco, USA
Ethylene diamine tetraacetic acid	Ajax finechem, New Zealand
Glycerol	Amresco, USA
Glycine	Amresco, USA
Methanol	Rcilabscan, Thailand
Primary antibody	Cell signalling technology
Secondary antibody	Cell signalling technology
Sodium chloride	Amresco, USA
Non-fat skim milk	Amresco, USA
NP-40	Amresco, USA
Protease inhibitor cocktails	Amresco, USA
Protein ladder	Genedirex, Inc., USA
PVDF Immobilon FL	Millipore, Billerica, MA
Sodium deoxycholate Zealand	Ajax finechem, New
Sodium dodecyl sulfate: SDS	Amresco, USA
Tetramethylethylenediamine	Sigma-Aldrich.St. Louis, MO
Tris-base	Amresco, USA
Tween-20	Amresco, USA
Apoptosis detection	
Annexin V / 7-AAD	EMD Millipore corp
Hoeshct 33342	Sigma-Aldrich.St. Louis, MO
Triton-X 100	Iobachemie
Gelatin Zymogram	
Calcium Chloride	Ajax finechem, New Zealand
Coomassie blue R250	Fluka, China
Gelatin	Amresco, USA
Triton-X 100	Iobachemie
Zinc chloride	Ajax finechem, New Zealand

CCA cell models

KKU-055 (poorly differentiated adenocarcinoma), KKU-214 (moderately to poorly differentiated adenocarcinoma), KKU-213 (well differentiated adenocarcinoma) and KKU-213L5 (highly metastatic cholangiocarcinoma cells) were shown in Figure 22. All CCA cell lines were kindly provided from Prof. Sopit Wongkham, department of Biochemistry, faculty of Medicine, Khon Kaen University. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 100 units/mL of penicillin and 100 g/mL of streptomycin. The cultured cell lines were incubated at 37˚C in a humidified incubator with 5% CO_2 atmosphere. Cells were subcultured twice a week. At 80% confluence, cells were detached from culture flask using trypsin/EDTA (0.25% w/v) and processed according to the particular assay.

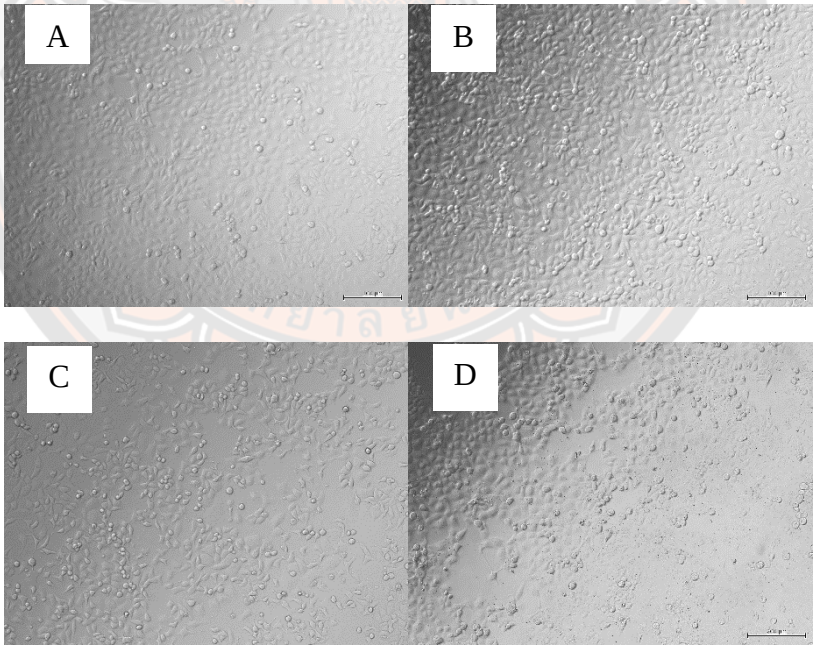


Figure 22 The morphological observation of 4 CCA cell lines. There were KKU-213 (A), KKU-214 (B), KKU-055 (C) and KKU-213L5 (D). Images were captured under a magnification of 10X

Methods

1. Preparation PCE extract

Peanut hairy root crude extract or PCE was kindly obtained from Assoc.Prof. Apinun Limmongkon, department of Biochemistry, faculty of Medical Science, Naresuan University. The extracts were prepared by the cultivation of peanut hairy root and then stimulated with Paraquat, Methyl jasmonate and cyclodextrin for 72 h. The culture media were extract by ethyl acetate. The compounds in the extracts were determined by HPLC. The stilbene group is the most component in the extracts as shown in Figure 23. These stilbene compounds, such as trans-resveratrol, trans-arachidin-1 and trans-arachidin-3, were previously detected in the culture medium and exhibited potent antioxidant activity (Pilaisangsuree *et al*., 2018)

In this study, PCE were dissolved with 100% DMSO, then diluted with cell culture media and filtered with filter paper (Whatman 25 mm, 0.2 um) to get the maximum concentration of 400 µg / ml.

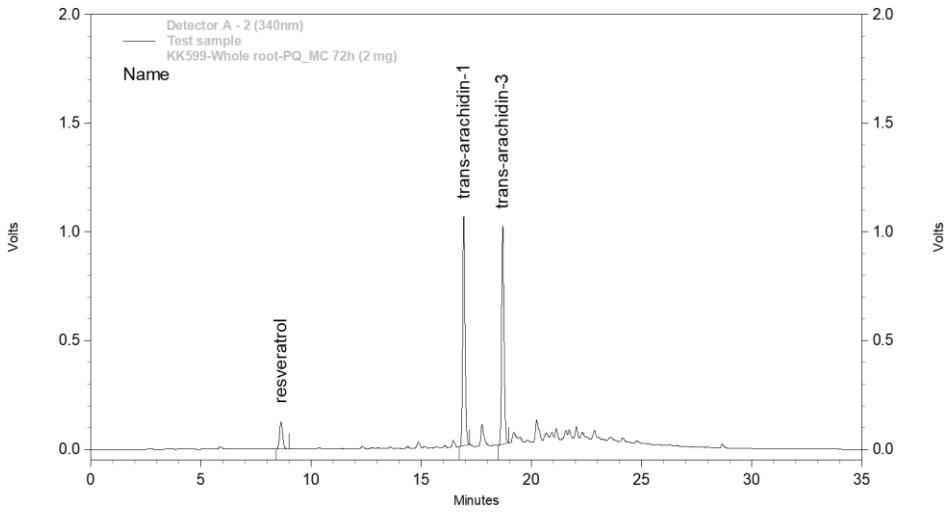


Figure 23 The amount of substance derived from peanut hairy root stimulation with PQ + MeJA_CD at 72 h**.**

Source: Apinun Limmongkon *et al.*,2019

2. Cell proliferation (MTT assay)

Cell proliferation and non-cytotoxicity were determined by MTT assay. The MTT assay was measure the cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. The number of viable cells in each well were estimated by the uptake of the tetrazolium salt, 3-(4,5-dinethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) to purple formazan crystals by the active cells (Vistica, D. T *et al*., 1991). Approximately 15,000 cells per well of each cell line were plated in 96-well plates and incubated for 24 h at 37◦C with 5% CO_2 to allow for the cells to adhere to the bottom. Then, the cells were treated with peanut hairy root extract (PCE) at 0-800 µg/ml for 24, 48 and 72 h. After the treatments, the cells were incubated with 100 µL of 0.5 mg/ml MTT solution for an additional 4 h at 37 ◦C. Then, the MTT solution were aspirated and 200 µL of 100% DMSO were added to each well to dissolve the formazan crystals. Finally, the absorbance was read at 540 nm using the microplate reader. The cell proliferation was calculated as a percentage relative to the vehicle control treated by DMSO. The IC50 values were calculated by using Graph Pad Prism software.

3. Apoptotic Assay

3.1 Annexin V / 7-AAD staining

To analyze apoptosis of KKU-213L5 cells, an Annexin/7-AAD Apoptosis Detection Kit was used. In living cells, phosphatidylserine is transported to the inside of the lipid bilayer. An early event in apoptosis is the flipping of phosphatidylserine of the plasma membrane from the inside surface to the outside surface. Annexin-V binds specifically to phosphatidylserine and labelled Annexin V can be used to detect an early apoptosis cell. Propidium iodide (PI) or 7-aminoactinomycin D (7-AAD) is a fluorescent nuclear and chromosome counterstain as shown in Figure 24. Since propidium iodide and 7-AAD are not permeant to live cells, it is also commonly used to detect late apoptotic cells in a population.

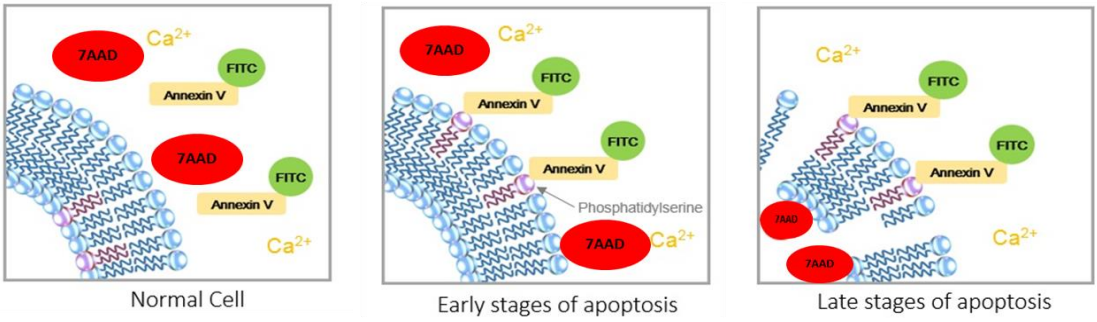


Figure 24 Principles of Annexin V/7-AAD binding.

Source: Maya Zimmermann *et al.*,2011

Apoptosis was detected using Annexin/7-AAD apoptosis detection kit according to the protocol enclosed in the kit. Apoptosis of CCA cells induced by PCE were quantitatively determined by flow cytometry while using the Annexin V/7-AAD kit. Approximately 250,000 cells were seeded in each well of 6-well plates and left for 24 h at 37 ◦C with 5% CO_2 for adherence and recovery. The cells were then treated with the PCE at concentration from IC50 value. After that, the cells were harvested by brief trypsinization and washed with PBS. The cells were stained with Annexin V/7-AAD for 10 min and incubated at room temperature in the dark. The cells were then measured the early apoptosis and late apoptosis by flow cytometry using Muse cell analyzer**.** The AnnexinV-FITC uses annexin V conjugated with fluorescein isothiocyante (FITC) to label phosphatidylserine sites on the membrane surface of early apoptotic cells. The kit includes 7-AAD to label the cellular DNA in late apoptotic and necrotic cells where the cell membrane has been totally compromised. This combination allows the differentiation among early apoptotic cells (annexin V positive, PI negative), late apoptotic cells (annexin V positive, PI positive), and viable cells (annexin V negative, PI negative).

3.2 Hoechst 33342 staining

A cell that is undergoing apoptosis demonstrates nuclear condensation and DNA fragmentation, which can be detected by staining with Hoechst 33342 and fluorescence microscopy. At first, $2x10^4$ cells were plated in the 4-well chamber slides and incubated for 24 h. Then, PCE at different concentration were treated into the cells for 48 h. After that, cells were wash with ice-cold 1X PBS. The cells were fixed with 4% (v/v) paraformaldehyde at room temperature for 30 min. Then, wash cells with 1X PBS and permeabilize them with 1% triton X-100 at room temperature for 30 min. Hoechst 33342 dye were added and incubated for 10–15 min at room temperature in the dark. After that, the cells were mount with coverslip. Finally, staining cells were captured using fluorescence microscopy. The result of apoptotic cells were count for the cell with DNA condensation and DNA fragmentation. Then, apoptotic cell calculated as a percentage relative to the vehicle control treated by 0.1% (v/v) DMSO. (Figure 25).

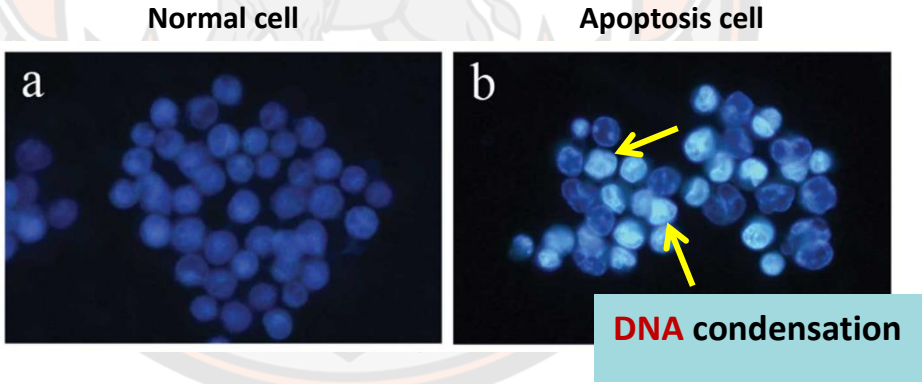


Figure 25 Example result of Hoechst 33342 staining**.**

Source: M. Emília Juan *et al*.,2010

3.3 Protein expression (Western blot)

3.3.1 Cell lysate and protein determination

Cells were seeded $3x10^5$ cells/well in 6-well plates and incubated for 24 h. KKU-213L5 cell were treated with various concentrations of the PCE for 48 h. The cells were washed with ice-cold 1x phosphate buffered saline (PBS) and lysed using RIPA lysis buffer (0.1% (w/v) sodium dodecyl sulfate (SDS), 0.5% (w/v) sodium deoxycholate, 50 mM Tris, 1% (v/v) Tween 20 and protease cocktail inhibitor). The cells were harvested by cell scraper into microcentrifuge tubes and incubated on ice for 30 min. Then, the lysed cells were centrifuge at 12,000 rpm for 10 min at 4°C. The supernatants were collected into new microcentrifuge tubes and determine the total protein concentration using a spectrophotometer by Bradford assay.

3.3.2 SDS-PAGE and western blot analysis

To denature, use a loading buffer with the anionic detergent sodium dodecyl sulfate (SDS), and boil the mixture (Protein and loading buffer with beta-mercaptoethanol) at 95°C for 5 min. Proteins were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein (20 µg) was loaded into an individual well and separated with 12% separating gel at a constant current of 120 V at 1.30 h. Until the tracking dye front reached the bottom of the gel, then proteins in electrophoresed polyacrylamide gel were transferred onto a polyvinylidene difluoride (PVDF) membranes using transfer buffer for 90 mins. After electro-transferring, the membrane was blocked with 5% (w/v) non-fat skim milk at room temperature for 1 h and then washed 3 times with washing buffer [1X TBST (Tris-Buffered Saline, 0.1% TWEEN® 20 Detergent]. The membrane was incubated with 1:1000 primary antibody or 1: 1,000 anti-β-actin antibody or apoptosis marker (rabbit anti-caspase 3 antibody 1:1000, rabbit anti-cleaved caspase-3 antibody 1:1000, mouse anti-Caspase 9 antibody 1:1000, mouse anti- cleaved caspase-9 antibody 1:1000, rabbit anti-PARP antibody 1:1000, rabbit anti- cleaved PARP antibody 1:1000) for overnight at 4°C and washed 3 times with washing buffer. Incubate the membrane in

goat anti-rabbit conjugated to HRP (secondary antibody) for 1 h. The immunoreactive bands were detected by Chemiluminescence ECL Prime Western Blotting Detection System. The membranes were captured with Image Quant TM 4000 image analyzer and analyze band density by ImageJ.

4. Wound healing assay

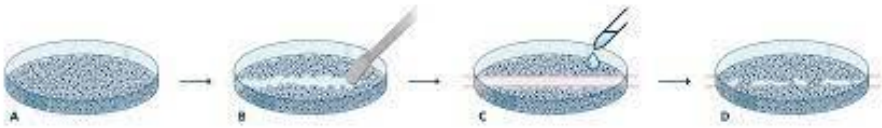


Figure 26 The step of wound healing assay.

Source: https://www.reactionbiology.com/services/cell-based-assays/migration-assay-scratch-assay-and-oris-assay

The wound healing assay is one of the earliest developed methods to study cell migration *in vitro* (Todaro, G. J *et al*., 1965). This method is based on observation of cell migration into a "wound" that is created on a cell monolayer. A wound healing assay is a laboratory technique used to study cell migration and cell–cell interaction (Figure 26). KKU-213L5 cells were placed in 6-well plates at density 300,000 cell/wells in DMEM medium supplement with 10% (v/v) FBS and incubated for 24 h at 37◦C with 5% CO_2. A linear scratch was subsequently made through the confluent cell monolayer by using a sterile 10 µl pipette tip. Then, PCE was treated in CCA cell at 0, 25, 50, 100 µg/ml. The scratch healing area of cells was detected every 6 h for 24 h. Then, the results were photographed at a constant magnification (10X) and measured the relative migration area of wound closure.

5. Transwell invasion assay

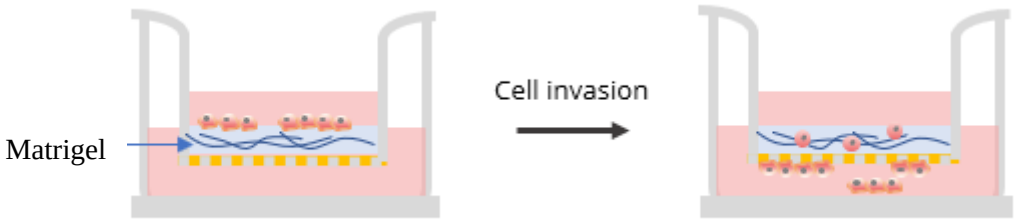


Figure 27 The principle of Transwell invasion assay.

Source : https://www.thewellbio.com/vitrogel-hydrogel-system-for-invasion-assay/

The cancer cell invasive ability with or without indicated treatment was examined by membrane transwell culture system. The transwell invasion assay are provides thorough analysis of the ability of cells to sense a particular chemo-attractant and invade through a layer of extracellular matrix or a layer of endothelial cells on top of the transwell membrane to mimic the process of ECM invasion and extravasation (Kramer N *et al.*, 2013) as shown in figure 27. Before the measurement of invasion ability, the plates were pre-coated with 0.4 mg/ml Matrigel and KKU-213L5 cell were treated with various concentrations of the PCE for 24 h and trypsinization for harvest cell, The cells were resuspended in the serum-free medium. Then, 200 µl of cell were placed into upper chamber at density 20,000 cells/well and 500 µl of DMEM medium supplement with 10% (w/v) FBS were added into the lower chamber. After incubation at 37°C for 16 h, 4% (v/v) paraformaldehyde and 0.5 % (v/v) crystal violet were used for fixation and staining of the invading cells. The number of cells were evaluated in five random areas by a microscope (100X).

6. Preparation of conditioned media for gelatin zymography assay

A $2x10^5$ cells/well were cultured in fetal bovine serum (FBS) in a 60 mM dish (2 mL/dish). At 70–80% confluency, FBS media were removed and wash cells twice with PBS. Cells were continue treated cells with PCE in FBS-free media for 24 h. Then, the conditioned media were collected and centrifuged or filter to eliminate dead cells at 4000 rpm for 2 h 30 min and concentrate conditioned media 10X.

7. Gelatin zymography

Type IV collagenases/gelatinases matrix metalloproteinases MMP-2 and MMP-9 (Massova I *et al*., 1998) that have consistently been shown to be associated with cancer progression. Gelatin Zymography is used to detect gelatinase activity, specially MMP-2 and -9. MMP-2 (gelatinase A, 72 kD) and MMP-9 (gelatinase B, 92 kD) can be detected on gelatin zymograms as two-three white bands (pro and active forms) after staining with Coomassie Blue staining.

The separating gel were prepared by 7.5% (v/v) acrylamide gel containing 0.1% gelatin. Then, the samples were load to each well typically 20 µl protein per well is suitable. The gel was run at 200 V for 1 h until good band separation is achieved. Then, the gel were washed 2 times 30 min with renaturing buffer (2.5% v/v Triton-X 100) to removes SDS from the gel at room temperature. Next, the gel was replaced with fresh developing buffer (0.5 mM Tris pH 7.5, 10nM $CaCl_2$, 1mM $ZnCl_2$, 1% (v/v) Triton-X 100) and incubate for 16-18 h at 37°C. Then, the gel was staining with Coomassie blue at room temperature for 30 min. After that incubate the gel with de-staining solution until bands can clearly be seen.

8. Statistical Analysis

Quantitative data was expressed as means ± SD. Statistical significance was determined by the Student’s t-test and One way-ANOVA with Dunnett's multiple comparisons test and Sidak's multiple comparisons test. Data were analyzed using GraphPad Software (San Diego, CA, USA). A p-value less than 0.05 were considered statistically significance. (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$)

CHAPTER IV

RESULTS

The effect of PCE on cell viability in 4 CCA cells

MTT assay was utilized to determine the effect of the various PCE on CCA cell viability over a series of concentrations ranging from 200 μg/mL to 800 μg/mL at 24 h, 48 h, and 72 h time points. The morphology of 4 CCA cells after treated with PCE show that PCE induced morphology change in all CCA cell lines. As a vehicle control, 0.2% DMSO was used in this experiment and showed non-cytotoxicity towards the tested cell lines. KKU-213L5 cells were more inhibited by the PCE compared to other CCA cells. In addition, the data were supported by morphological observations of the extract-treated cells, showing a change in cell morphology characterized by cell shrinkage and cell wall blabbing at the high concentration. Also, there was a clear reduction of the cell populations treated with the higher concentrations of extract compared to untreated cells, typically indicating cytotoxicity in the cells by the PCE (Figure 28A, 29A, 30A and 31A). Generally, when treated with PCE, all CCA cells showed a reduction in percentage cell viability by dose- and time-dependent manners. The reduction in cell viability showed statistical significance. Based on the IC50 values (Table 5), KKU-213L5 cells were more sensitive to PCE treatment than other CCA cells.

PCE had a relatively higher cytotoxic potency in KKU-213L5 than other CCA cell lines, with IC50 at 24, 48 and 72 h about 947.64 ± 11.49 ,403.65 ± 14.83 and 226.9 ± 16.31 μg/ml, respectively. The IC50 values of each cell were shown in table 5. Therefore, the most potent cytotoxic sensitivity, KKU-213L5 cell was selected for further analyze the effect of PCE on cell apoptosis and their related mechanism.

Table 5 IC50 of PCE on 4 CCA cells at different incubation time.

Times (h)	IC50 (µg/ml)			
	KKU-213	**KKU-214**	**KKU-M055**	**KKU-213L5**
24	>800	>800	>800	>800
48	449.44 ± 29.38	643.17 ± 24.22	498.51 ± 25.74	403.65 ± 14.83
72	354.09 ± 5.68	558.2 ± 32.56	273.95 ± 9.97	226.9 ± 16.31

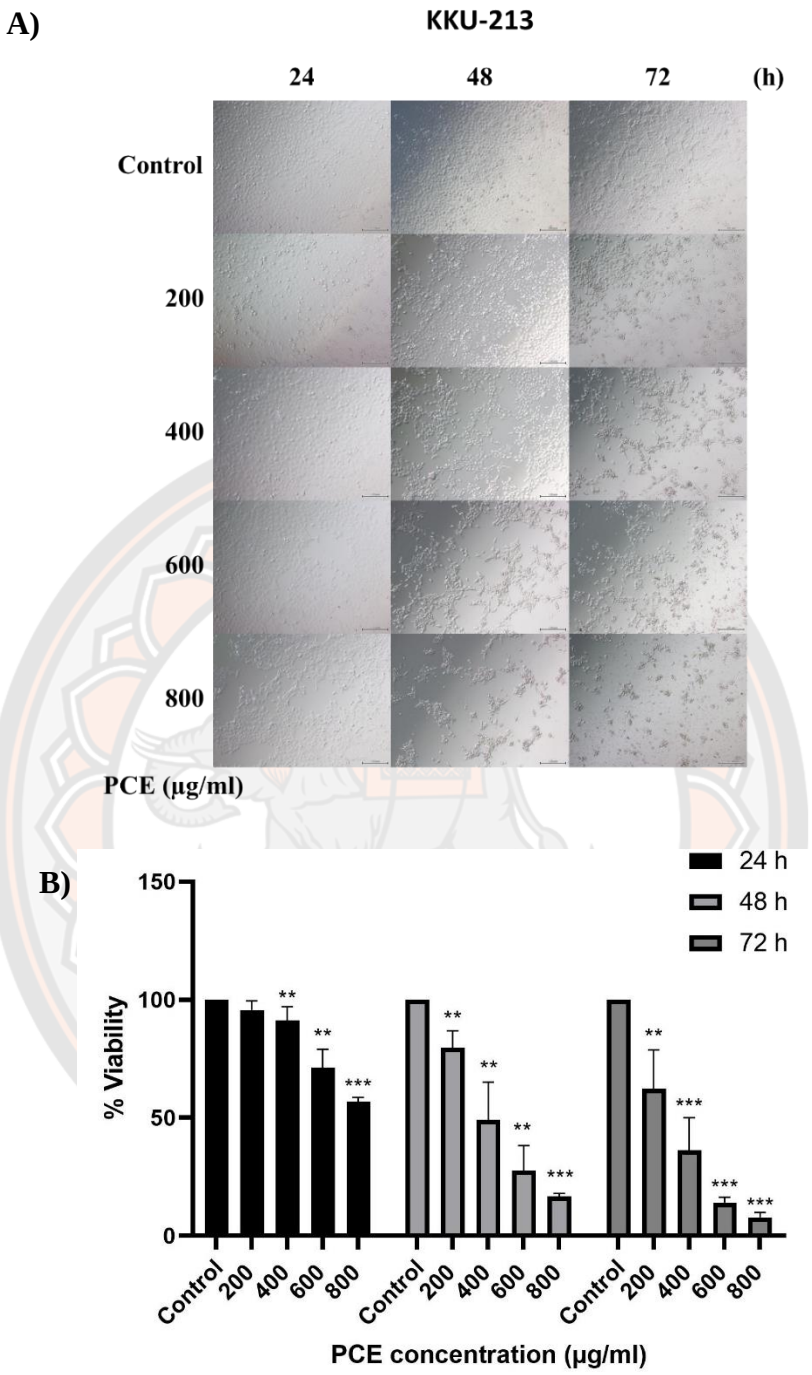


Figure 28 Effect of PCE on KKU-213 cell lines. (A) The cell morphology of KKU-213 cell lines after treatment with different concentration of PCE for 24, 48 and 72 h. (B) The relative cell proliferation compared between the untreated group and treated with PCE group. The results are shown as mean ± S.D. from three independent experiments [**p < 0.01 and ***p < 0.001 vs control (DMSO)].

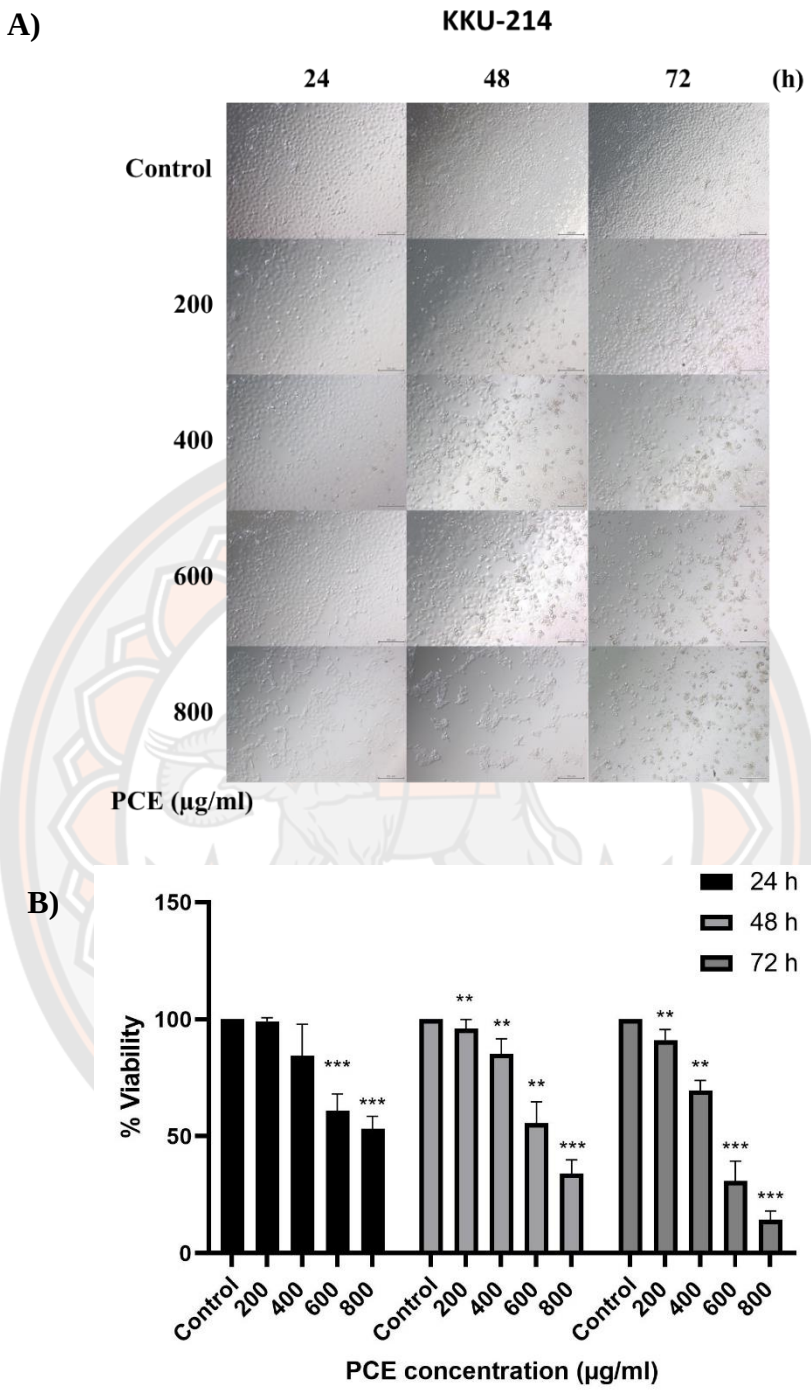


Figure 29 Effect of PCE on KKU-214 cell lines. (A) The cell morphology of KKU-214 cell lines after treatment with different concentration of PCE for 24, 48 and 72 h. (B) The relative cell proliferation compared between the untreated group and treated with PCE group. The results are shown as mean ± S.D. from three independent experiments [**p < 0.01 and ***p < 0.001 vs control (DMSO)].

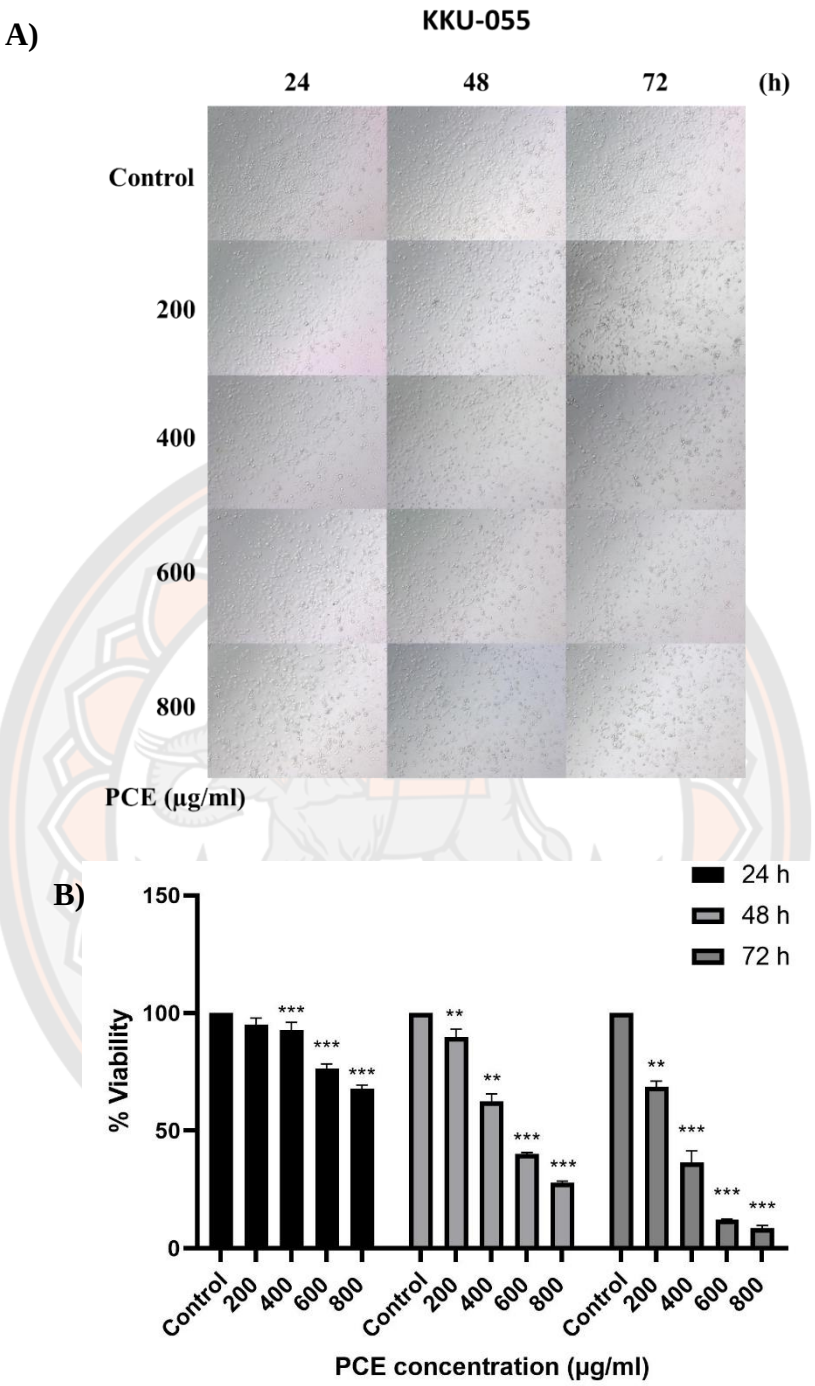


Figure 30 Effect of PCE on KKU-055 cell lines. (A) The cell morphology of KKU-055 cell lines after treatment with different concentration of PCE for 24, 48 and 72 h. (B) The relative cell proliferation compared between the untreated group and treated with PCE group. The results are shown as mean ± S.D. from three independent experiments [**p < 0.01 and ***p < 0.001 vs control (DMSO)].

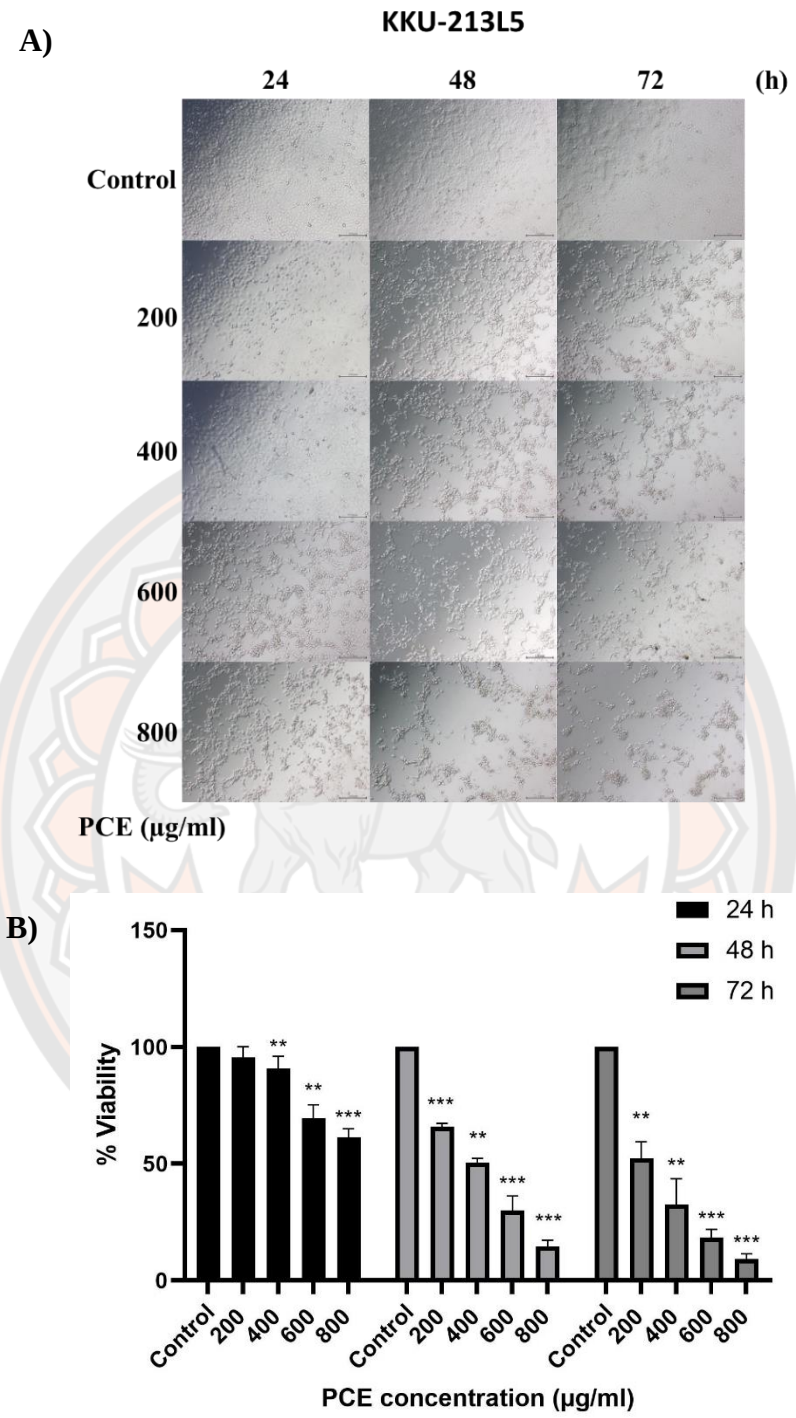


Figure 31 Effect of PCE on KKU-213L5 cell lines. (A) The cell morphology of KKU-213L5 cell lines after treatment with different concentration of PCE for 24, 48 and 72 h. (B) The relative cell proliferation compared between the untreated group and treated with PCE group. The results are shown as mean ± S.D. from three independent experiments [**$p < 0.01$ and ***$p < 0.001$ vs control (DMSO)].

Effect of PCE on apoptotic induction in KKU-213L5 cell line by Annexin V/ 7-AAD staining

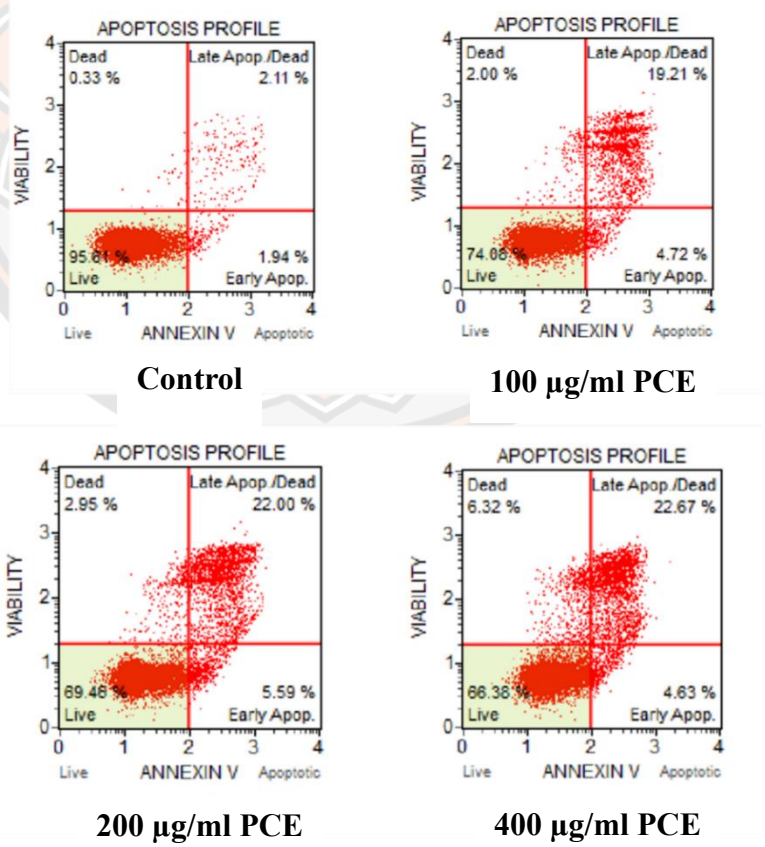
Resveratrol is a well-known stilbene derivative with apoptosis cell death effects, there is no evidence about the effects of PCE on human cholangiocarcinoma cell apoptosis. We aimed to determine if the PCE induced KKU-213L5 death through apoptosis. To confirm that induction of apoptosis underlies the cytotoxic effect of the PCE, apoptosis was evaluated in KKU-213L5 by using Annexin V/7-AAD staining and flow cytometry. KKU213L5 were treated with PCE at the concentration less than IC50 from control, 100, 200 and 400 µg/ml for 48 h. Consistently, changes in cell morphology were observed under an inverted microscope as shown in figure 32A. PCE induced cell death by reduce the cell density.

In figure 32B and C, the percentages of cells apoptosis analyzed by flow cytometry following Annexin V and 7-AAD staining could be classified into four categories. The populations of cells residing in the Annexin V+/7-AAD- and the Annexin V+/7-AAD+ quadrants were determined as early and late apoptotic cells, respectively. The Annexin V−/PI - and the Annexin V−/PI+ quadrants were determined as living cells and necrotic death cells, respectively. The results showed that PCE significantly induced cell apoptosis in KKU-213L5 cell by increasing of early (5.023 ± 0.452 at 400 µg/ml) and late apoptosis (24.923 ± 3.581 at 400 µg/ml) as a dose-dependent manner. These results suggested that PCE reduced cell viability of KKU-213L5 was due to the induction of apoptosis.

A)


Control
100 µg/ml PCE
200 µg/ml PCE
400 µg/ml PCE

B)


APOPTOSIS PROFILE
Dead
0.33 %
Late Apop./Dead
2.11 %
95.61 %
Live
1.94 %
Early Apop.
VIABILITY
ANNEXIN V
Live
Apoptotic
Control
APOPTOSIS PROFILE
Dead
2.00 %
Late Apop./Dead
19.21 %
74.08 %
Live
4.72 %
Early Apop.
100 µg/ml PCE
APOPTOSIS PROFILE
Dead
2.95 %
Late Apop./Dead
22.00 %
69.48 %
Live
5.59 %
Early Apop.
200 µg/ml PCE
APOPTOSIS PROFILE
Dead
6.32 %
Late Apop./Dead
22.67 %
66.38 %
Live
4.63 %
Early Apop.
400 µg/ml PCE

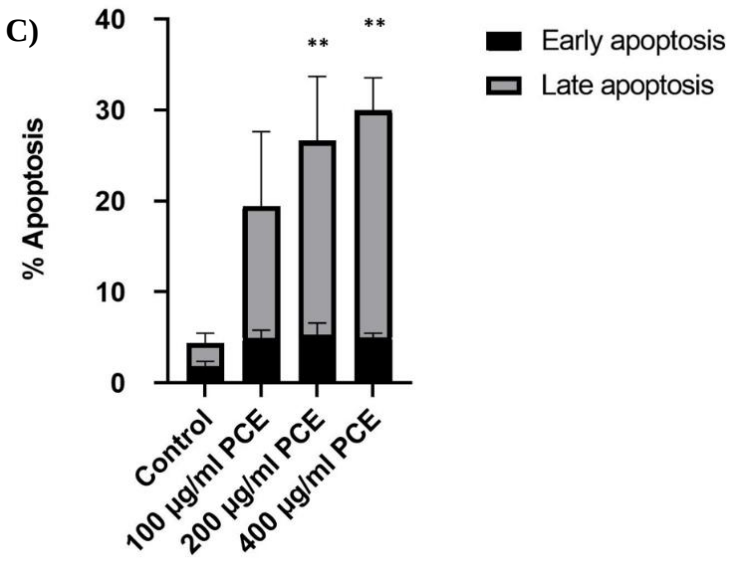
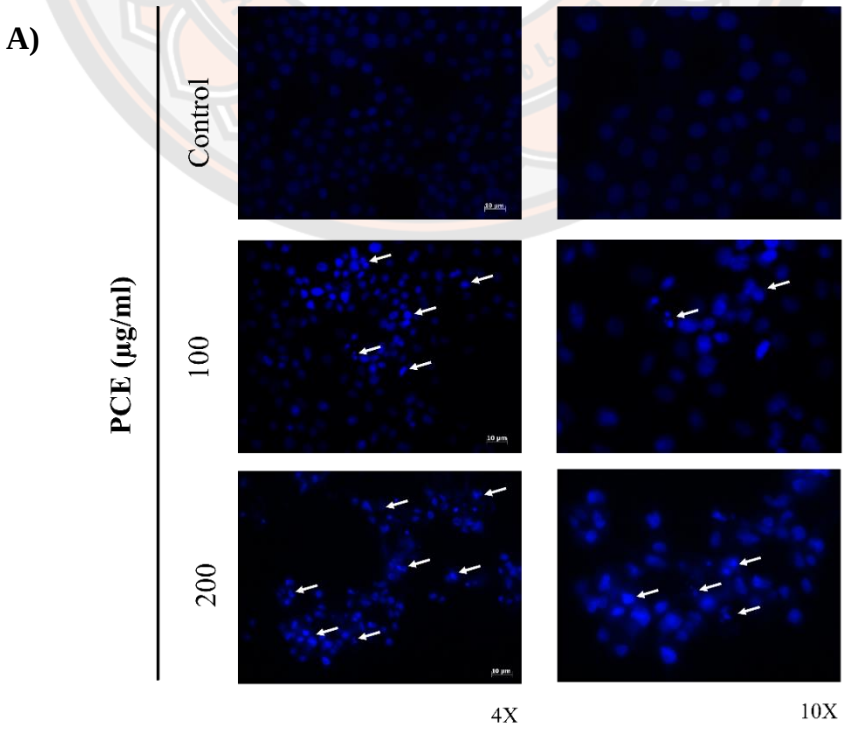


Figure 32 The effect of PCE on KKU-213L5 cell apoptosis in different concentration for 48 h. (A) The KKU-213L5 morphology after treatment with PCE for 48h. (B) Apoptotic cells were stained with Annexin V/7-AAD solution and analyzed by flow cytometry. (C) The relative cell apoptosis compared between the untreated group and treated with PCE group. [**$p < 0.01$ vs control (DMSO)].

Effect of PCE on apoptotic induction in KKU-213L5 cell line by Hoechst 33342 staining

To ensure that PCE can potentially apoptosis induction in KKU213L5 cells, another apoptotic assay should be further determined. A cell that is undergoing apoptosis demonstrates nuclear condensation and DNA fragmentation, which can be detected by staining with Hoechst 33342. In this study, KKU213L5 were treated with 0, 100 and 200 µg/ml of PCE for 48 h and further performed the Hoechst 33342 staining. From the fluorescent microscope showed that the nuclei are generally fragmented and stained more intensely because of condensation of the DNA by PCE dose-dependent manner.

The number of apoptotic cells which characterized by DNA condensation and DNA fragmentation (white arrows) of KKU-213L5 cell was increased with the dose of PCE (Control = 1.81 ± 0.88, 100 µg/ml = 20.94 ± 0.33 and 200 µg/ml = 28.81 ± 0.95). The representative images of Hoechst 33342 staining were shown in figure 33A. These results suggesting that PCE significantly induced cell apoptosis by increasing the number of DNA condensation and DNA fragmentation (Figure 33B).



B)

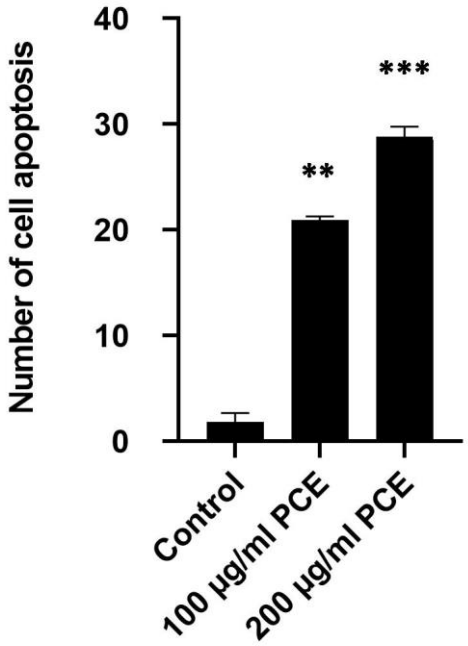
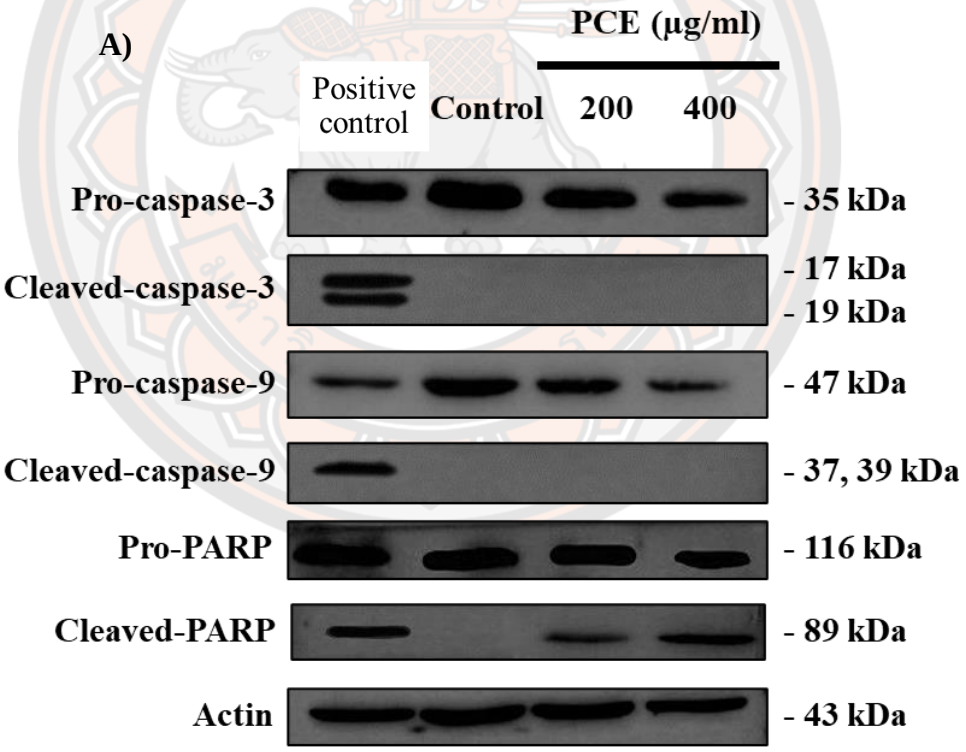


Figure 33 Cell apoptosis observed using Hoechst 33342 staining. (A) The morphology of KKU213L5 cells were treated with PCE for 48 h. Apoptotic cells exhibited DNA condensation and fragmentation typical of apoptosis. Photographs were taken under a fluorescence microscope (10X, 20X). (B) Cells that accumulated dye due to apoptosis were counted [**$p < 0.01$ and ***$p < 0.001$ vs control (DMSO)].

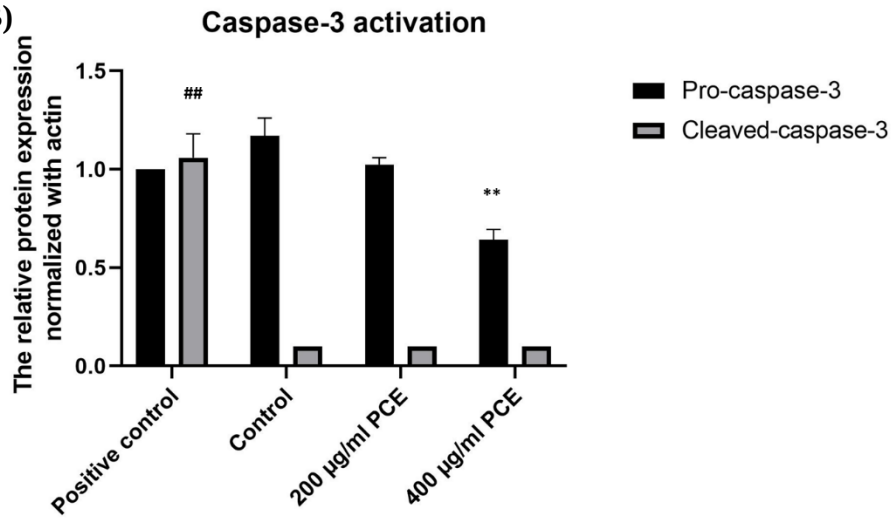
Effect of PCE on protein expression of apoptotic molecules

Next, we investigated the mechanism of PCE-induced apoptosis in human KKU-213L5 on caspase-3, caspase-9 and poly ADP-ribose polymerase (PARP) activation. Recent evidence indicates that caspases play the important roles to denaturing the cellular infrastructure during apoptosis, and that many chemotherapeutic agents activate caspases to kill cancer cells. To identify the mechanisms underlying PCE-induced apoptosis in KKU-213L5 cells, activation of caspases-9, and -3 and cleavage of PARP were detected by western blot. KKU-213L5 cells were treated with 0, 200 and 400 µg/ml PCE for 24 h and subjected to perform western blot. In this experiment, the hydrogen peroxide treated HCT116 was used as a positive control for caspases and PARP activation. We observed that after PCE treatment for 48 h, the majority of cells detached the bottom of the culture wells, floated in the medium and exhibited apoptosis-like morphological characteristics such

as cell shrinkage and membrane blebbing. All the attached and floating cells were collected to measure the protein expression. The western blot results in figure 29 showed that PCE treatment in KKU-213L5 cells extensively increased the expression of cleavage PARP as compared with the control untreated cells (Fig.34) similarly with the positive control cell that show the prominent signal of all cleavage forms. In contrast to PARP activation, PCE treatment didn't increase the expression of cleavage caspase-3 and cleavage caspase-9 even at the high concentration of PCE. Interestingly, PCE decreased the expression of pro-caspase-3 and pro-caspase 9 as a dose dependent manner indicated that PCE down-regulated caspase-3 and caspase-9. Therefore, PCE induced apoptosis by activation of PARP but not activation of caspase-3 and -9 suggesting that PCE induced apoptosis via caspases-independent pathway.



B)


Caspase-3 activation
The relative protein expression normalized with actin
1.5
1.0
0.5
0.0
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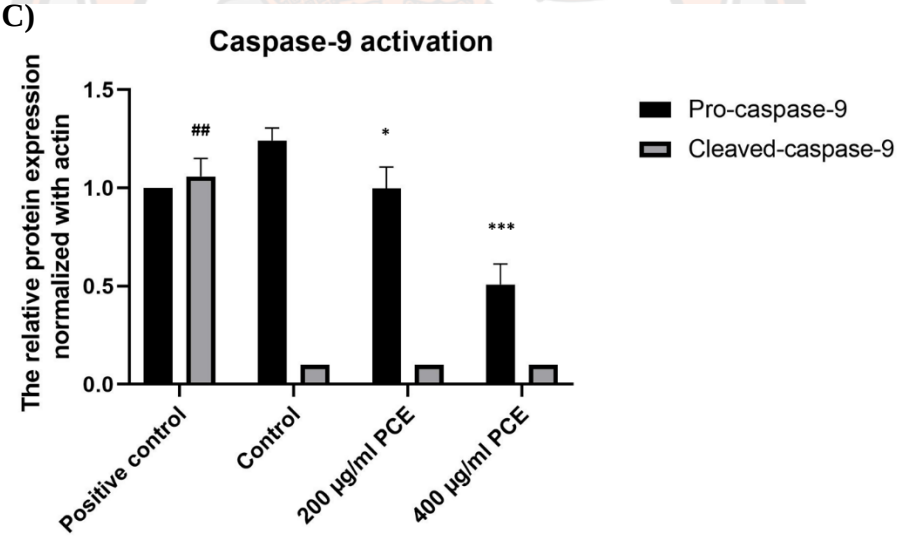
Pro-caspase-3
Cleaved-caspase-3
Positive control
Control
200 µg/ml PCE
400 µg/ml PCE

C)


Caspase-9 activation
The relative protein expression normalized with actin
1.5
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Pro-caspase-9
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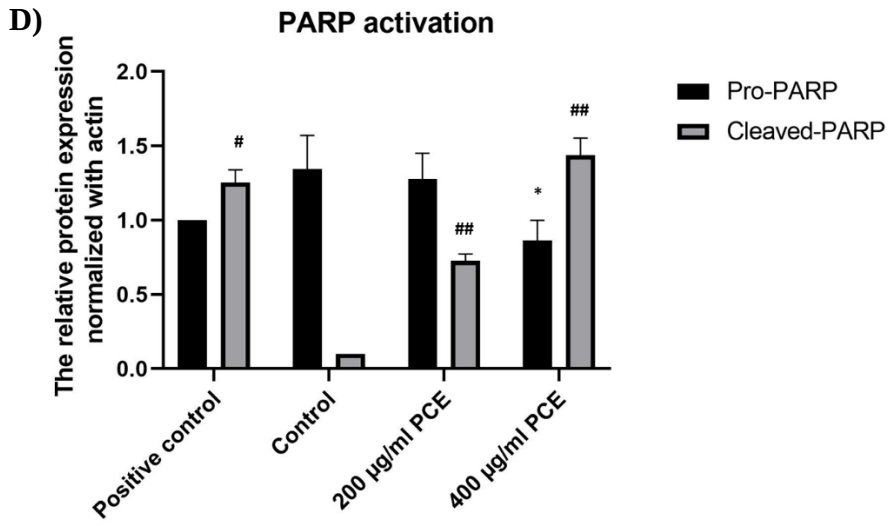


Figure 34 Western blot analysis of apoptosis-related proteins in KKU-213L5 cells**.** (A) KKU-213L5 were treated with 0, 200 and 400 µg/ml of PCE for 24 h. Hydrogen peroxide (500 µM) treated HCT-116 cell was used as a positive control (PC). (B, C and D) Quantitative protein expression of caspase-3, caspase-9 and PARP activation. [*p< 0.05, **p < 0.01 and ***p < 0.001 vs Pro-form control (DMSO)] [#p<0.05 and ##p<0.01 vs cleaved-form control (DMSO)].

The effect of PCE on anti-metastasis in KKU213L5 cells

Metastasis is one of the biggest hurdles in cancer management. The inhibition of metastatic phenotypes as an attractive therapeutic strategy, because it can prevent the severity of the diseases. According to the multi-step process, cell migration and cell invasion are the basic capabilities of metastatic cells. To investigate the anti-metastatic effect of PCE on KKU-213L5 which is a highly metastatic cell line, the sub-lethal concentrations of PCE were selected for further analysis. Therefore, the low doses of PCE were determined the cytotoxicity by MTT assay. KKU-213L5 were treated with 0, 25, 50,100 and 200 µg/ml PCE for 48 h. The results show that PCE at concentration of 200 µg/ml significantly decreased cell viability of KKU-213L5. Thus, the non-cytotoxic concentrations of PCE of KKU213L5 cells were 0, 25, 50 and 100 µg/ml (Fig. 35) and could be used for further determining anti-metastatic assay.

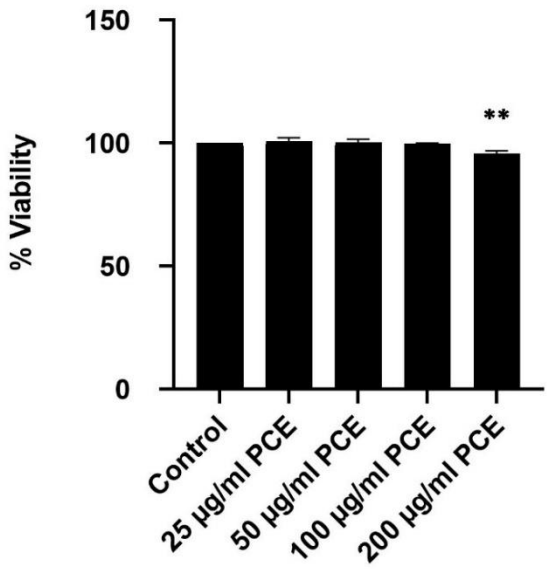


Figure 35 KKU-213L5 cell was treated with non-cytotoxic concentration at 24 h. The relative cell proliferation compared between the untreated group and treated with PCE group. The results are shown as mean ± S.D. from three independent experiments [**p < 0.01 vs control (DMSO)].

The effect of PCE on cell migration in KKU213L5 cells

The ability of PCE to inhibit KKU-213L5 cancer cell migration was subsequently determined by wound healing assay (Figure 36). At sub-lethal concentrations of 0, 25, 50, 100 µg/ml PCE, KKU-213L5 were treated for 0, 6, 12, 18 and 24 h. Then, the cells were created the gap wound and monitored the wound closer every 6 h until 18 h that can represent the cell migration ability. The results demonstrated that the non-treated control group showed the complete closing of the wound gap while the 25 µg/ml PCE-treated cells were still remained the wound gap (Figure 37A). Moreover, increasing the PCE concentration to 50 and 100 µg/ml dramatically suppressed the migration abilities of the cells when compared with the control cells. The wound distance in figure 36B showed that PCE significantly inhibit cell migration in KKU-213L5 cells by dose- and time-dependent manners. From these results suggested that the anti-metastatic effect of PCE was not due to reduced cell proliferation, as we did not find its cytotoxicity at the concentrations where the cell migration assays were carried out (Figure 36).

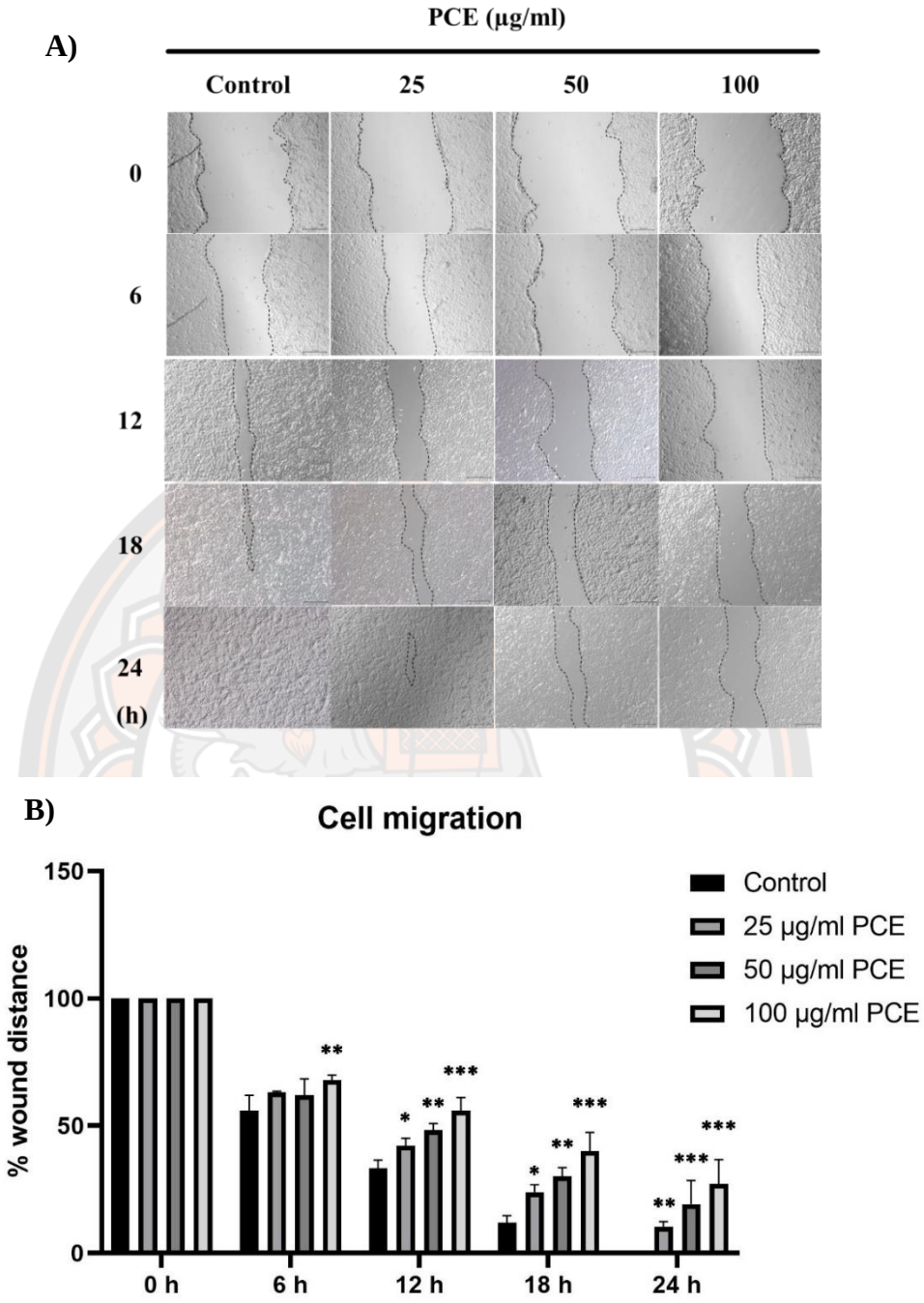
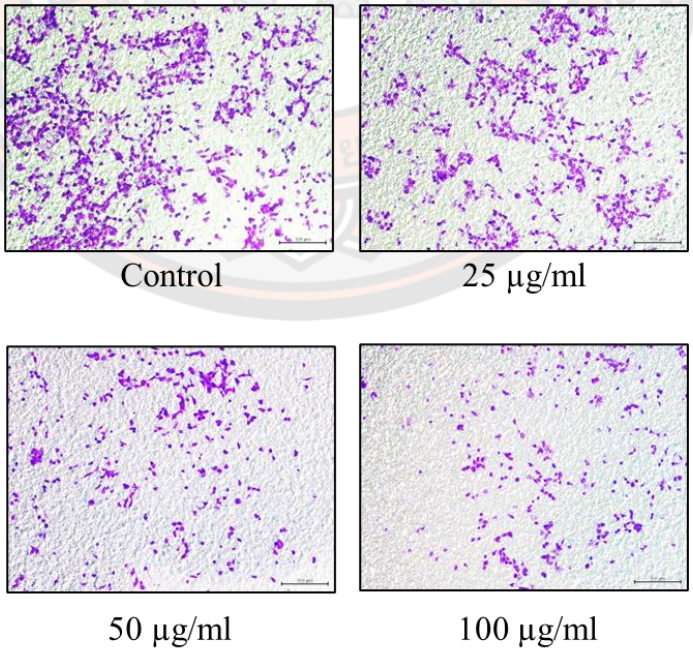


Figure 36 The inhibition of KKU-213L5 cell migration treated with PCE in different dose and time. (A) The effects of PCE on migration potential of KKU-213L5 cells were examined using wound healing assay. Quantitative analysis of the scratch sizes in the wound healing assay [*p< 0.05, **p < 0.01 and ***p < 0.001 vs control (DMSO)].

The effect of PCE on cell invasion in KKU213L5 cells

To further examine the possible role of PCE in the prevention of cancer cell invasion, we analyzed the invasion induced by using Boyden chamber coated with Matrigel. This assay was used to measure the cell invasion ability in which the cells can invade the Matrigel of the upper chamber through the lower chamber by chemoattractant. KKU-213L5 cells were treated with PCE at non-cytotoxic concentration similar as cell migration assay for 24 h. Then, cells were harvest and placed into the upper chamber with serum free media and incubated for 16 h. The results showed that in the present of PCE, the numbers of invading cells which was observed as the stained cells were diminished compared with the untreated cells (Figure 37). After counting the invading cells as shown in figure 37, PCE significantly decrease the numbers of invading cells as a dose-dependent manner (85.32 ± 2.87 at 25 µg/ml, 47.43 ± 5.56 at 50 µg/ml and 10.84 ± 3.11 at 100 µg/ml) as shown in figure 38B. These results suggested that PCE was the important compound for inhibiting the invasiveness of KKU-213L5 cell.

A)



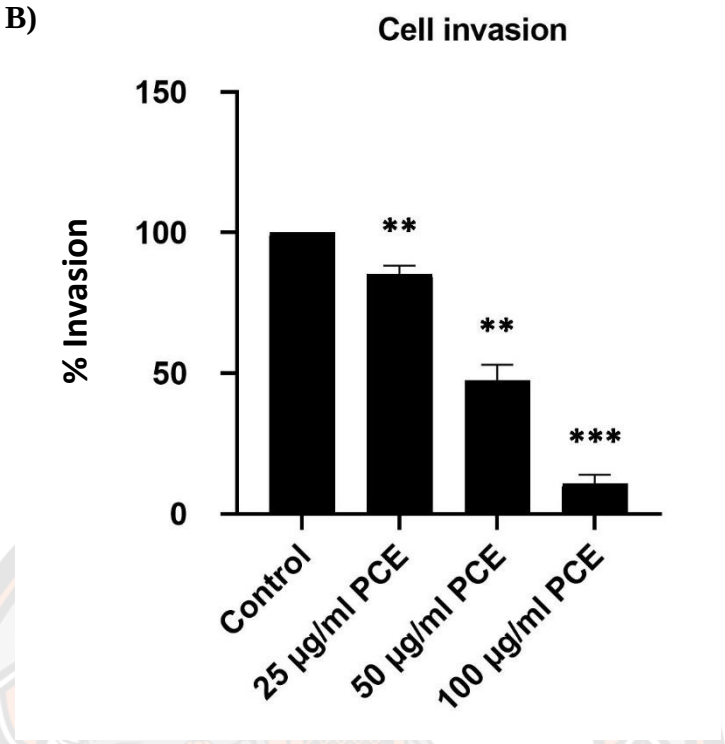


Figure 37 Effect of PCE on cell invasion in KKU213L5 cells. (A) Cell invasion ability was determined by transwell assay after treated with PCE. The relative cell invades compared between the untreated group and treated with PCE group. The results are shown as mean ± S.D. from three independent experiments [**$p < 0.01$ and ***$p < 0.001$ vs control (DMSO)].

The effect of PCE on MMP-2 and MMP-9 activities in KKU213L5 cells

Previously, resveratrol which is an effective chemoprevention agent has been shown to potentially block tumor development by suppress the MMP-2 and MMP-9 activities (Le Corre, L. *et al.*, 2005). In the current study, we examined the possibility whether PCE could suppress tumor cell invasion by modulating the secretion of MMP-2 and MMP-9 activities. To examine the effects of PCE on MMP-2 and MMP-9 activities, the conditioned media of PCE treated KKU-213L5 were collected and determined the MMPs activities by gelatin zymography. In this experiment, KKU-213L5 cells were treated with PCE at various concentrations of 0, 25, 50 and 100 µg/ml for 48 h in serum free media. The conditioned media were concentrated and evaluated by electrophoresis of soluble protein on a gelatin containing polyacrylamide gel. Then, the enzymatic reaction was developed and measured the activities of MMPs from the clear band at their sizes. As the results in figure 38, PCE treatment

didn’t alter the activities of both MMP-2 and MMP-9 which located at 72 and 92 kDa. These results suggested that PCE inhibited cell invasion of KKU-213L5 was not associated with the activation of MMP-2 and MMP-9.

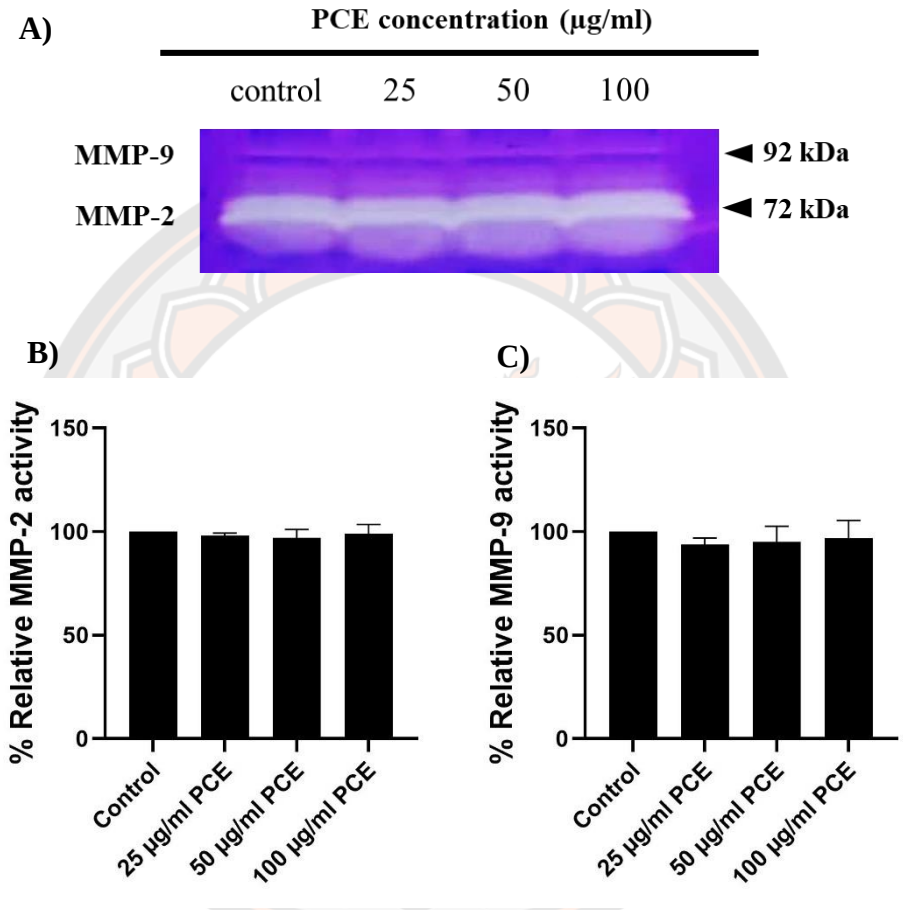


Figure 38 Gelatin zymography analysis of MMP-2 and MMP-9 activities in KKU-213L5 cells. (A) Photographs of the active MMP-2 and active MMP-9 bands, which are representative of three independent experiments, are shown. (B, C) The intensities of the representative band of both MMP-2 & MMP-9 were calculated and plotted. The results are shown as mean ± S.D. from three independent experiments.

CHAPTER V

DISCUSSION

Cancer is one of the biggest challenges that must be handled as a global health issue. In fact, cancer is still the second leading cause of death worldwide and was responsible for 9.6 million deaths in 2018 (Ferlay J *et al.,* 2019). However, advances in the knowledge of cancer, supported by frontier research and advanced technologies for its diagnosis and treatment, the discovery of new therapeutic agents is a hot topic in cancer research. The major limitations of conventional chemotherapy are the recurrence of cancer, drug resistance, and toxic effects on non-targeted tissues (X Wang *et al.*, 2019). Moreover, side effects can restrain the use of anticancer drugs and thus impair a patient's quality of life (Pritchard, J.R *et al*., 2012). Numerous plants and isolated bioactive compounds have gained immense attention due to their ability to target heterogeneous populations of cancer cells and regulate key signaling pathways involved in cancer development at different stages and their wide safety profile (Rates SM. 2001 and Fang J *et al*., 2017).

Stilbene is a naturally occurring phytoalexins, which plants produce in response to stress such as a bacteria attack. Stilbenes presents in natural products with a variety of important biological including antioxidant, anti-inflammatory, anti-bacterial, anti-viral and anti-cancer properties (Chang *et al*., 2006 and Djoko *et al*., 2007). Stilbene derivatives demonstrated promising activity in cancer prevention and cancer chemotherapy by targeting a wide variety of intracellular pathways including of apoptosis pathway (K. Z. Xiao *et al*., 2008 and Y. Zhou *et al*., 2016). Among these, resveratrol is the most extensively studied with multiple anticancer effects in several types of cancer (M. Reinisalo *et al.,* 2015 and A. Csiszar *et al*., 2011). However, few research studies have been reported about trans-arachidin-1 and trans-arachidin-3 due to the substance can only be found in peanuts. Recently, peanut hairy root culture technique has been developed to increase the stilbene production by stimulation with particular elicitors (Limmongkon A *et al*., 2018). Moreover, peanut hairy root culture

extracts (PCE) are rich in stilbene derivative compounds especially trans-arachidin-1, -3 and trans-resveratrol, and exhibit high antioxidant activity. From our preliminary results demonstrated that PCE potentially inhibit viability of bile duct cancer cell or cholangiocarcinoma (CCA) cell better than other cancers including lung cancer, breast cancer, and oral cancer. In the present study, we explored whether PCE has anti-cancer potential for cholangiocarcinoma. Our results demonstrated that PCE exhibit anti-CCA properties with a significant decrease cell growth, induce apoptosis and inhibit metastasis.

The effect of PCE on anti-proliferation in CCA cell lines

Cholangiocarcinoma (CCA) is a major health problem in especially in North and Northeast of Thailand. Currently available anti-CCA drugs are disadvantages such as cancer recurrence or side effects. Therefore, an alternative anti-CCA drugs using a substance derived from natural compounds could be promising for CCA prevention and treatment. CCA is usually diagnosed at the advanced stage where the cancer is difficult to treatment due to cancer aggressiveness and metastasis. Mostly chemotherapeutic drug for CCA could not be cured effectively. To determine whether PCE is potentially overcome this limitation, the effects of PCE was determining the anti-proliferative effect on the CCA cells with different stages including KKU-213; a well differentiated adenocarcinoma, KKU-214; a moderately to poorly differentiated adenocarcinoma, KKU-055; a poorly differentiated adenocarcinoma, and KKU-213L5; a highly metastatic cholangiocarcinoma cell. The results demonstrated that peanut hairy root culture crude extract significantly inhibited all CCA cell lines by dose- and time-dependent manners (Figure 28-31) consistent with previous report demonstrating that the peanut testa extracts could inhibit the growth of several other human cancer cell lines (S. Khaopha *et al.*, 2015). However, the growth inhibition efficiency of peanut hairy root culture crude extracts is varied among cholangiocarcinoma cell lines. Interestingly, in comparison of IC50 values, KKU-213L5 is the most potent anti-proliferation with the lowest IC50 compared with the IC50 of KKU-213, KKU-214 and KKU-055 cells (Table 5). As previously mentioned, KKU-213L5 is a type of CCA cell with highly metastatic potential as demonstrated *in*

vitro and *in vivo* (Kwuntida Uthaisar, *et al.*,2016). These data indicated that PCE is the valuable agent to specifically inhibit the highly metastatic cells which is the most difficult to remove. The reason of this phenomenon could be possible that PCE has a specific target molecule that highly expressed in KKU-213L5 cells. Since, KKU-213L5 is an established human CCA cell subline which possesses higher metastatic behaviors, i.e., growth rate, stem cell marker characteristics, migration and invasion abilities, than those of the parental, KKU-213 cell. Therefore, the genes responsive for those behaviors in KKU-213L5 could be overexpressed and might be the specific target molecule of PCE.

It has been reported that trans-arachidin-1 and trans-arachidin-3 from peanut hairy root extract can bind to cannabinoid receptor (CBRs) (Brents L *et al*.,2011) which highly expressed in cancer cell than normal cell (Surang Leelawat *et al*.,2010 and Xu X, Liu Y., 2006). CBRs might represent novel molecular targets responsible for at least some of the anti-cancer activity produced by PCE. In addition, the PCE exhibited the most potent for anti-proliferative effect to high aggressive cell KKU-213L5, it could be a possibility that KKU-213L5 has CBRs expression higher than other CCA cell. In addition, PCE is the crude extract in which the stilbene derivatives are major compound as shown in figure 23. In the extracts might contain other stilbenes or compounds that exhibit the anti-cancer properties or exert the synergistic effect with the know stilbene derivatives.

The mechanism of PCE reduced cell proliferation might be related to cell cycle arrest. It is well known that a number of checkpoints exist within the mammalian cell cycle, which ensure that cell proliferation proceeds normally. The transition from G1 to S-phase is strictly regulated by sequential formation, activation and inactivation of a series of cell cycle regulatory molecules (Li and Brooks, 1999). The progress of the cell cycle in cancer cells is regulated by three protein families: Cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs). However, multiple changes occur in cancer cells, including cyclin amplification, CDK or substrate mutation, as well as inactivation of inhibitors. This results in abnormal CDK activity, mutation of checkpoint, as well as dysregulation of programmed cell death or apoptotic processes, inducing the selective growth advantage of cancer cells (McDonald E *et al*., 2000). In

view of the progression of the cell cycle, cells prepare for S phase during the G1 phase, which is triggered by regulation of cyclin D1, CDK4 and CDK6 (Bockstaele L *et al*., 2009). Recently, resveratrol and pterostilbene induced cancer cells to arrest in the G0/G1 phase and it was associated with a decrease in the protein level of cyclin D1, CDK4 and CDK6. CDK-cyclin complexes (Chatterjee SJ *et al*., 2004 and Dong Wang *et al*.,2019). Since the discovery of p53 in 1979 (Linzer DI *et al*., 1979) numerous studies have been conducted related to its functions in response to stress and its regulatory mechanisms. p53 is a sequence-specific nuclear transcription factor that binds to defined consensus sites within DNA as a tetramer and represses transcription of a set of genes involved in cell growth stimulation, while activating a different set of genes involved in cell cycle control. Many *in vitro* studies have shown that resveratrol induce overexpression of wild-type p53 in several cancer cell lines (Tanigawa S *et al*., 2008 and Lee DH *et al*., 2012). The expression of wild-type p53 is often associated with a G1 or G2/M phase cell cycle arrest together with transcriptional regulation of target genes such as p21, Bax, PUMA and apoptosis induction (Basile V *et al.,* 2009 and Lee DH *et al*., 2009) In addition, there is no investigation revealed about the inhibition of cell cycle molecule on PCE or trans-arachidin-1 and trans-arachidin-3 induced cell cycle arrest. However, further studies are still needed.

Numerous *in vitro* studies have shown that resveratrol has anticancer effects, protecting against both tumor initiation and cancer progression. Unfortunately, resveratrol possesses unfavorable pharmacokinetic properties. Various *in vivo* studies have shown that resveratrol is impaired by a short half-life, rapid metabolism, and low bioavailability (M. S. Gerova *et al*.,2016). It was recently reported that other stilbene such as piceatannol (N. Koolaji *et al*.,2013), pterostilbene (H. S. Lin *et al*.,2009), trans-arachidin-1 and trans-arachidin-3 (Lisa K. Brents *et al*.,2012) has a better bioavailability than trans-resveratrol. Therefore, PCE can be a source to produce anticancer drugs with a good bioavailability.

The effect of PCE induced KKU-213L5 apoptosis by caspase-independent pathway

Various studies have been shown that targeting of apoptotic pathway is a valuable strategy for cancer drug discovery process. (Evan and Vousden 2001; Kim *et al*. 2014; Wang *et al*. 2014; You and Park 2014; Zhang *et al*. 2015; Zhang *et al*. 2014 and Zheng *et al*., 2014). Presently, apoptosis is a therapeutic goal of cancer therapy because its targeting is more advantageous than other cell death mechanism, apoptosis could kill cancer cells without causing damage to normal cells or surrounding tissues (Evan GI *et al*.,2001). Stilbene derivatives have previously shown to possess apoptotic induction in the various cancer cell lines (Carter, L.G *et al*.,2014).

In this study, the results demonstrate that peanut hairy root culture crude extract (PCE) induced apoptosis through apoptosis regulatory proteins. PCE significantly increased phosphatidylserine externalization and apoptosis population by flow cytometry (Figure 32). The results showed that peanut stilbenoid significantly stimulated induction of apoptosis in KKU-213L5 cell by increasing of late apoptosis more than early stage. Late stages of apoptosis are characterized by DNA condensation, DNA fragmentation and loss of cell membrane permeability (S Elmore *et al*.,2007). Our results are consistency with previous report that resveratrol is increased the major population of late apoptotic cells in leukemic cells (Michinori Takashina *et al*.,2017). In addition, our results showed that PCE concentration of 400 µg/ml which is nearly the IC50 value (403.65 ± 14.83 µg/ml) induced apoptotic population about 30 percent and necrotic population about 5 percent suggesting the other 15 percent of dead cells may come from other cell death such as autophagy. Oxyresveratrol (Mohan, N *et al.*, 2011) and resveratrol (Jun Zhang *et al*., 2013) has been shown to activate autophagy cell death in neuroblastoma cells and A549 lung adenocarcinoma cells.

In this study, PCE induced DNA condensation, DNA fragmentation and PARP cleavage, indicated cell apoptosis. There is considerable evidence of apoptosis effects in different natural polyphenolic compounds on cancer cells (Singh *et al*., 2011). Resveratrol and pterostilbene were found to induce the mitochondrial apoptotic molecule Bax (pro-apoptotic protein) and reduce Bcl-2 (anti-apoptotic protein) in

cancer cells (Chakraborty *et al*., 2010 and Gogada *et al*., 2011). Moreover, the other stilbene piceatannol showed concentration-dependent induction of cell death in HL-60 cells due to activation of caspases -3, -8 and -9 (Chowdhury *et al*., 2005). Although there is more evidence of pro-apoptotic effects of some stilbene derivatives on cultured cell lines or cancer cells (Ko *et al*., 2011; Liu *et al*., 2011; Radhakrishnan *et al*., 2011 and Zhou *et al*., 2011). Caspase belongs to the effector group of caspases which are responsible to apoptosis (Fan *et al*., 2005). Caspase activation from their pro-caspase form has been widely described in cells undergoing apoptosis, including cholangiocarcinoma and many cancer cells. In our results, the treatments with PCE down-regulated pro-caspase-9 and pro-caspase-3 in KKU-213L5 cells by does-dependent manner. Surprisingly, we did not observe a significant activation of caspase-9 and -3 from the Western blotting as the undetectable of cleavage forms. (Figure 34). Since, the catalytic activities of caspases exist when the pro-caspases are cleaved into the smaller size. Therefore, the downregulation of pro-caspase-3 and pro-caspase-9 might not represent the cells undergo apoptosis. We speculated that the decreasing of pro-caspase-3 and -9 may resulted from PCE induced protein degradation process, and possibly via the transcription factor regulation which can prove by investigating the mRNA expression level. Nowadays, trans-resveratrol represents such an ideal molecule, due to its capacity to target multiple signaling molecules that collectively promote cancer cell survival and tumor growth. Resveratrol downregulated c-MYC in medulloblastomas in which 73% of tumor tissues expressed this oncogene and its downregulation was accompanied by S phase arrest and induce apoptosis (Zhang P *et al*., 2006). The prototypic c-MYC oncogene encodes a transcription factor (Cole MD, Henriksson M, 2006). Moreover, the transcription factor activator protein-4 (AP-4) could encode a c-MYC-inducible repressor to inhibit p21 expression (Jung P et al., 2008), and presumably played an important role in mediating the proliferative activity of c-MYC (Jung P *et al*., 2009). In addition, AP-4 influenced the sensitivity to apoptosis by regulating the expression of Caspase-9 (Kim MY *et al.,* 2006). In addition, the positive control HCT-116 treated with hydrogen peroxide was clearly shown to induce apoptosis through activation of cleavage caspase-9 and -3 and decrease pro-caspase -9 and -3, including

of Pro-PARP. In the present study, we demonstrated that the mechanism of PCE induced apoptosis in KKU-213L5 may be via caspase-independent pathway.

Although coordinated activation of caspases plays a central role in the execution of most types of apoptosis, there is now accumulating evidence supporting that apoptosis can occur in caspase-independent manner (Shi Y.2002 and Degterev A, 2003). Caspase-independent programmed cell death could result from AIF release from the mitochondria to the nuclei, thereby activating PARP and endonucleases. Previous report has been shown that oxyresveratrol (OXY), a hydroxyl-substituted stilbene, drives a caspase-independent cell death with apoptosis-like features, through the induction of ROS, resulting in translocation of apoptosis inducing factor (AIF) into the nucleus, PARP activation, DNA fragmentation and cell death (Sunilkumar, D *et al*., 2019). Similarly, trans-arachidin-1 from germinating peanut kernels induced apoptosis by caspase-independent pathway through inducing factor (AIF) in HL-60 leukemia cell (Cheng Po *et al.*, 2010). Accumulating evidence demonstrating that stilbenes can induce apoptosis both caspases-dependent and -independent pathways. To prove the actual pathway of PCE-induced apoptosis, determination of AIF activation or treatment with v-VAD FMK, the pan-caspases inhibitor may need to be further analysis.

The effect of PCE on anti-metastasis in CCA cell

In most CCA patients, metastasis has already occurred by the time of diagnosis. Thus, inhibition of metastasis is unlikely to be of therapeutic benefit for CCA patients. The cancer metastasis consists of a series of multi-steps, including cell movement, adhesion, migration, invasion and angiogenesis to form new tumors in other sites of the body. Inhibition of the one or several steps in the process of metastasis can prevent the formation of secondary lesions. Active compounds with anti-metastatic properties such as stilbene derivatives have been identified as new chemotherapeutic agents (Middleton, G *et al*., 2008 and Renouf, D *et al*.,2010). Resveratrol and its analogues could inhibit the metastastic phenotypes of lung, breast, cholangiocarcinoma and liver cancers (Fulda, S *et al*.,2010 and Tang, F. *et al*., 2008). In this study, we explored whether PCE has the anti-metastatic properties on KKU-213L5, a highly metastatic CCA cell. Our study demonstrated that PCE at non-cytotoxic concentration could effectively suppress cell migration and invasion activities as shown in figure 36-37. These results indicated that PCE could be used as an anti-metastatic agent in CCA. The current studies revealed that stilbene derivatives suppressed the viability, migration and invasion of myeloma, breast, liver and gastric cancer (Aqilano K *et al*., 2009; Khan A *et al*., 2014 and Li Y *et al*.,2013). Resveratrol analog-3,5,4′-trimethoxy-trans-stilbene inhibits invasion of human lung adenocarcinoma cells by suppressing the MAPK pathway (Ya-Ting Yang *et al*.,2009). In other stilbenoid, pinosylvin is a natural stilbenoid with a structure similar to that of resveratrol. Pinosylvin was previously highly correlated with the inhibition of cell migration, cell invasion and suppress of cyclooxygenase-2 and MMP-9 expression, and also down-regulate the p-Akt expression in human fibrosarcoma HT1080 cells (E.J. Park *et al*., 2012). Both VEGF and hypoxia-inducible factor-1α (HIF-1α) are over-expressed in several human tumors and their metastases, and are closely linked to a more aggressive tumor phenotype. It has been reported that resveratrol suppressed the expression of VEGF and HIF-1α in human ovarian cancer cells via abrogating the activation of the PI3K/Akt and MAPK signaling pathways (Cao Z *et al*.,2004). Resveratrol caused inhibition of the expression of these molecules, which suggests that it could be part of an efficacious anti-cancer therapy for preventing cancer and its metastasis (Trapp V,2010 and Zhang M *et al*.,2014) (Fig.40).

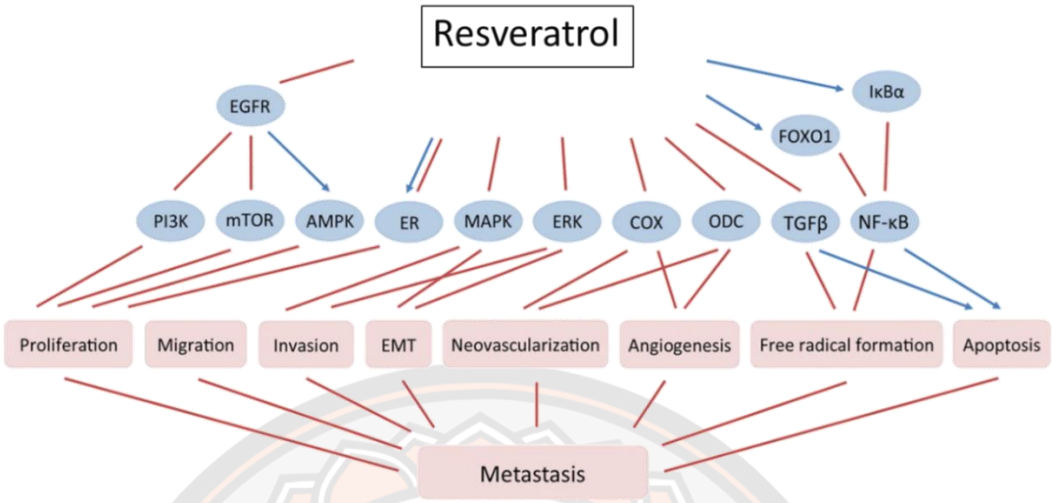


Figure 39 Resveratrol effect on metastasis molecules

Source: Dimiter Avtanski *et al*., 2018

The essential features of the metastatic process include tumor cell invasion and migration. Matrix metalloproteinases (MMPs) degrade mechanical barrier such as extracellular matrix and basement membrane leading to cell movement (Gabelloni P *et al*.,2010). MMPs cleave various matrix proteins such as collagen, elastin, gelatin and casein. It is well documented that the proteolytic activities of MMPs are involved in the metastasis process, including enabling cell migration, and invasion (Kim SY *et al*., 2008). MMP-2 and MMP-9 are often implicated in pathological processes, such as cancer progression including metastasis (Kai Kessenbrock *et al*.,2010). In this study, we further investigate the effect of PCE on MMPs activities using gelatin zymography. Our results demonstrated that PCE treatment couldn't be able to alter the activities of MMP-2 and MMP-9 in cholangiocarcinoma KKU-213L5 cell as shown in figure 38. These results suggesting that MMP-2 and MMP-9 may not be the target of PCE and the mechanism of PCE inhibited cell invasion in KKU-213L5 might be via another matrix degrading enzymes such as cathepsins and urokinase plasminogen activator (uPA) (Yeh CB *et al*., 2017).

Relative to the development of cancer chemopreventive agents, the development of cancer chemotherapeutic agents is more straightforward. For example, non-toxic to normal cells, much higher levels of toxicity can be tolerated under life-threatening conditions, the phases of clinical trials are clear, and therapeutic endpoints are well defined (Hong WK, 1997). PCE could be the best source of drugs and due to our interest in the identification of new anticancer natural products that overcome the limitations of cell toxicity and adverse reactions. As this study, it is difficult to make definitive health claims, because of it has no evidence about PCE on normal cells or about combination with anticancer drugs. In addition, a literature search shows that few of trans-arachidin-1 and trans-arachidin-3 have already been experimentally screened for their anti-cancerous activity, relative molecular mechanisms and found active. As well, to obtain the greatest anticancer effect, it is essential to determine which compound from PCE are work together well in a combined manner. By understanding the exact mechanisms by which compound can destroy tumors, the ultimate combination therapy to treat cancer can be designed.

The effect of partial purify compound from PCE on KKU-213L5 cell

Stilbenoids exist widely as natural phytoalexins produced by plants such as grapes, berries, and peanuts in response to microbial infection. Trans-resveratrol and its derivatives, trans-arachidin-1 and trans-arachidin-3 are stilbenoids that have been isolated by various research groups from peanut kernels following various treatments (Ingham J et al., 1976; Aguamah G et al., 1981 and Sobolev, V et al., 2007). Among these peanut stilbenoids, the biological functions of resveratrol have been studied extensively, and it was found to be a chemopreventive phytochemical for cardiovascular disease and cancer. The bioactivities of trans-arachidin-1 and trans-arachidin-3 have not been extensively studied, with the exception of their reported antioxidant and anti-inflammatory activities (Chang J *et al*., 2006 and Nichanan Eungsuwan *et al*., 2020). From our study suggests that PCE with high amounts of tran-arachidin-1 and trans-arachidin-3 has a potent to be anticancer agents in CCA cells. These results suggested that trans-arachidin-1 and -3 may act as a bioactive compound with anticancer activities. To prove this hypothesis, trans-arachidin-1 and -

3 were partially purified from PCE as shown in figure 41-42 (Apinun Limmongkon, unpublished data). Furthermore, we did comparison the anti-growth effect between the partially purified compounds with PCE on KKU-213L5 cell.

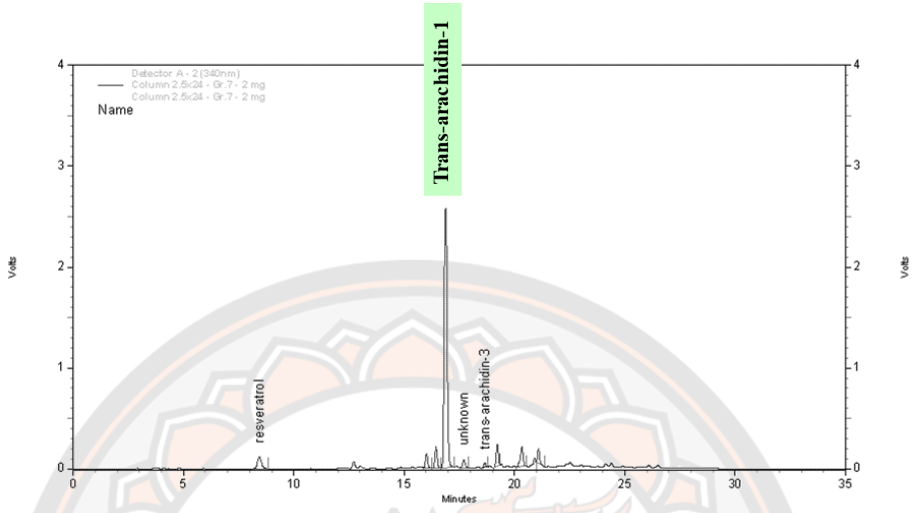


Figure 40 The amount of trans arachidin-1 from peanut hairy root culture crude extract.

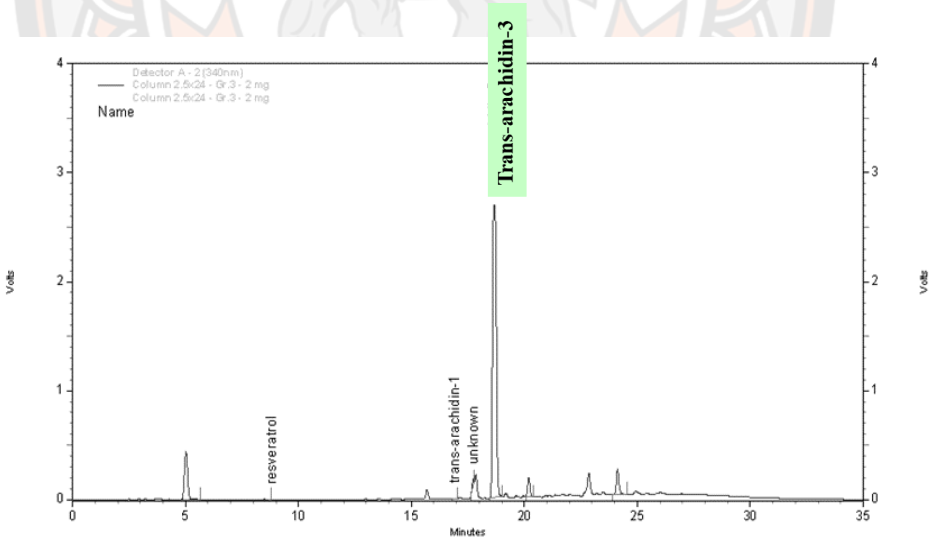
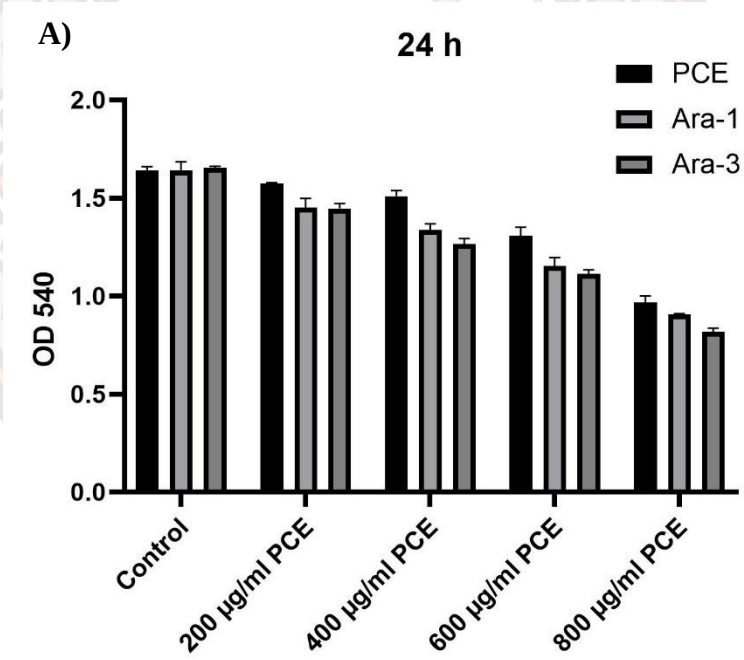


Figure 41 The amount of trans arachidin-3 from peanut hairy root culture crude extract.

The preliminary results demonstrated that over a concentration range of 0 to 800 μg/ml, partial trans-arachidin-1, -3 and PCE (crude extract) showed concentration- and time-dependent growth inhibitory effects on KKU-213L5 cells. PCE clearly exhibited less effect on KKU-213L5 cells than partially purified compounds as shown in figure 43. Therefore, trans-arachidin-1 and trans-arachidin-3 has the most potent cytotoxic compound. The results suggest that, partial compound of both trans-arachidin-1 and -3 are a promising candidate for cancer treatment especially in CCA which plays a significant role in determining its potential therapeutic effect on cancer.



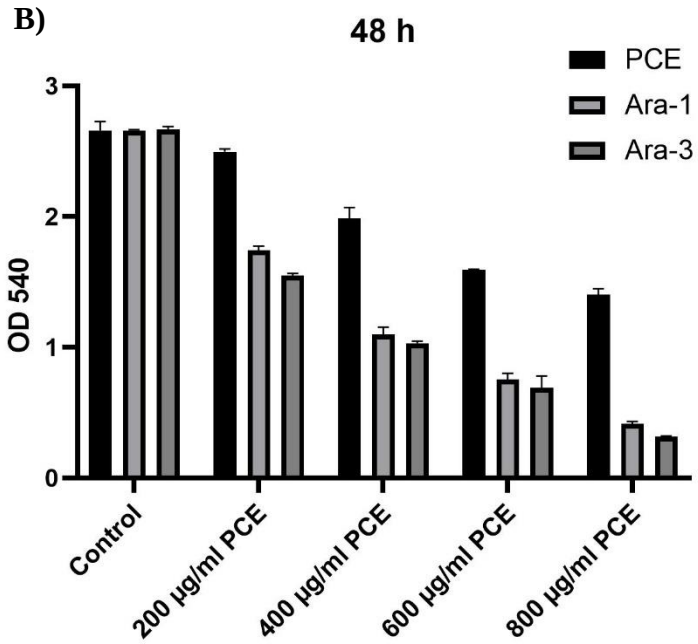


Figure 42 The effect of PCE and partially purified compounds (trans-arachidin-1 and trans-arachidin-3) on KKU-213L5 cells using MTT assay. Cell were treated for 24 h (A) and 48 h (B). The results were obtained from single experiment.

Perspective studies

Our results indicated that PCE with high trans-arachidin-1 and trans-arachidin-3 as a chemotherapeutic agent by the induction of cell apoptosis and inhibition of cancer metastasis in CCA cells. Besides, drug resistance remains as one of the most important disadvantages of chemotherapy. In order to conquer drug resistance or multidrug resistance, the above drawbacks should be minimized by developing more effective drug which can significantly improve the therapeutic efficacy to chemotherapeutic drugs with minimal toxicity (Hu and Zhang, 2009; Egusquiaguirre *et al.,* 2012). In this study, we have investigated the effects of PCE on cholangiocarcinoma cells which has not been investigated the effects of PCE on normal bile duct cell. Therefore, the cytotoxicity on normal cell could be further explored.

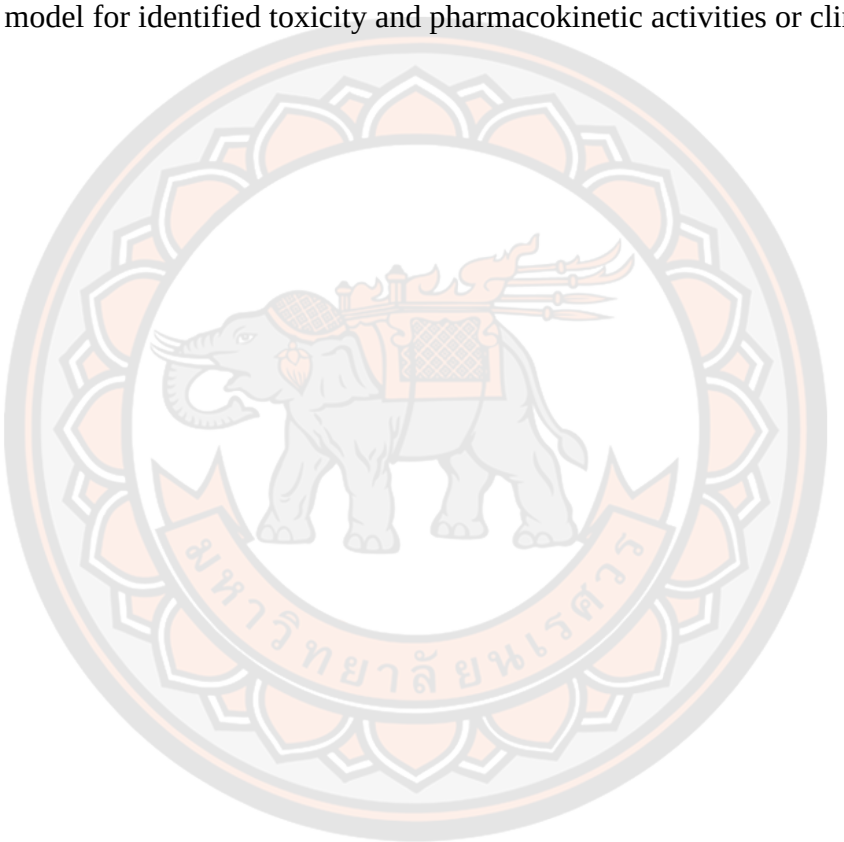
Nowadays, over 60% of the current anticancer drugs were derived in one way or another from natural sources (Newman DJ *et al*.,2010). The essential role played by natural products in the discovery and development of novel anticancer agents from natural product sources have been extensively reviewed (Cragg GM *et al.*,2009) (Grothaus PG *et al*.,2010). One of the most notable discoveries resulting from this program of natural product cancer chemopreventive agent discovery is stilbenoid resveratrol. The poor aqueous solubility and the weak bioavailability of resveratrol are great drawbacks of this natural and no toxic polyphenol which prevent its clinical trials. Numerous resveratrol derivatives have been produced from the powerful technology and synthesized with the aim of modifing physico-chemical features and improving biological activities of the parent polyphenol (Latruffe, N *et al.*,2018) (Barbara De Filippis *et al*., 2017). Recently, stilbene derivatives have been produced by peanut hairy root culture technology to increase the secondary metabolite compounds such as trans-resveratrol, trans-arachidin-1, trans-arachidin-3 and other stilbenoids. This study has been investigated the effect of peanut hairy root culture crude extract (PCE) with high amount of trans-arachidin-1 and trans-arachidin-3 on CCA cell. The result showed that PCE have a potent to be an anti-CCA agents. Synergistic sensitization properties of the crude extract have been highlighted. It is possible that these anticancer activities are from combined the extract and/or only one

stilbene compound. Moreover, Resveratrol plays the role of an adjuvant with pro-apoptotic drugs (CH11 and Trail) and with 5-fluorouracil (5-FU) caspases-3. A cell death marker is activated in chemoresistance HT29 colon tumor cell line by CH11 and trail after resveratrol sensitization (Delmas, D *et al*.,2004). Therefore, the effect from which compound was further investigate by purified the PCE or investigate the PCE with chemotherapy drugs. Interestingly, trans-arachidin-1 and trans-arachidin-3 possess anti-inflammatory, anti-proliferative properties and have bioavailability better than trans-resveratrol (Chang *et al*. 2006) (Djoko *et al*. 2007). Additionally, the previous studies show that trans-arachidin-1 and trans-arachidin-3, peanut hairy root-derived isoprenylated analogs of trans-resveratrol, exhibits slower metabolism/enhanced bioavailability and retain biological activity via cannabinoid receptor (CBR) (Lisa K. Brents *et al*., 2012) which is overexpression in many cancer cells (Anju Preet *et al*., 2011). Recently, Cannabinoid Receptors, CBR1 and CBR2, as novel targets for inhibition of non-small cell lung cancer growth and metastasis.

In this sense, chemotherapy is one of the main alternatives for cancer treatment. using molecules capable of inhibiting proliferative signaling pathways, replicative immortality mechanisms and angiogenesis, besides inducing apoptosis of tumor cells (R.S.Y. Wong *et al*., 2011; L. Zitvogel, *et al.,* 2013; T. Otto, *et al*., 2017). However, the efficacy of conventional chemotherapeutics has been limited by drug resistance mechanisms (G. Housman *et al.*, 2014). Most of the identified chemosensitizer natural compounds are phytochemicals, which are classified as phenolic derivatives, flavonoids, alkaloids, carotenoids, terpenoids, quinones, saponins and steroids depending on their molecular structure (B.S. Vinod *et al.*, 2012; P. Dandawate *et al.*, 2013). In general, these compounds act by increasing the residence time of chemotherapeutics in tumor cells, inducing cell death by up-regulation of pro-apoptotic targets, promoting DNA damaged or regulating the expression of altered and unaltered drug targets. Interestingly, several phenolic derivatives and flavonoids commonly found in medicinal plants were investigated as chemosensitizers, including resveratrol (S. Fulda *et al*., 2004), curcumin (T.C. Hour *et al.*, 2002), naringenin (F.Y. Zhang *et al*., 2009) and myricetin (L. Wang, *et al*., 2014). When associated, PCE which contain trans-resveratrol, trans-arachidin-1 and trans-arachidin-3 may could enhance the cytotoxic effect of anticancer drugs, promoting a synergistic effect even

in cells with acquired resistance. In the future, PCE may acts not only as a chemopreventive or chemotherapeutic agent, but also as a chemosensitizer.

These data indicated that, trans-arachidin-1 and trans-arachidin-3 might represent a novel class of compounds with significantly improved biological activity that might be exploited for future development as efficacious pharmaceutical drugs. However, if PCE can actually be used in the treatment of CCA patients. Further studies are still needed in the future such as combination to chemotherapy drug, animal model for identified toxicity and pharmacokinetic activities or clinical trials.



CONCLUSION

In the present study demonstrated that stilbenoids from peanut hairy root crude extract potentially inhibited all cholangiocarcinoma cell lines with different stages include KKU-213; a well differentiated adenocarcinoma, KKU-214; a moderately to poorly differentiated adenocarcinoma, KKU-055; a poorly differentiated adenocarcinoma, and KKU-213L5; a highly metastatic cholangiocarcinoma cell. Among these cell lines, KKU-213L5 exhibited the strongest anti-proliferative effect with IC50 value of 947.64 ± 11.49, 403.65 ± 14.83 and 226.9 ± 16.31 (µg/ml) at 24, 48 and 72 h PCE treatment, respectively. KKU-213L5 was selected for further analysis. We found that PCE significantly decreased cell growth through induction of apoptosis which characterized by DNA condensation and fragmentation. The protein expression analysis of apoptotic-related molecule revealed that PCE induced apoptosis via activation of PARP but not caspases-3 and -9 suggesting PCE extract-induced apoptosis via the caspase-independent pathway in KKU-213L5. Additionally, PCE treatment at non-cytotoxic concentration effectively suppressed cell migration and invasion abilities in KKU-213L5 which is not associated with MMP-2 and MMP-9 activities. These results suggest that stilbene derivatives from peanut hairy root culture extract potentially inhibit CCA cell growth and metastasis. Thus, we suggest that the extract of peanut hairy root culture may be a potential candidate for developing anti-cancer agents against human cholangiocarcinoma cancer metastasis. (Figure 41).

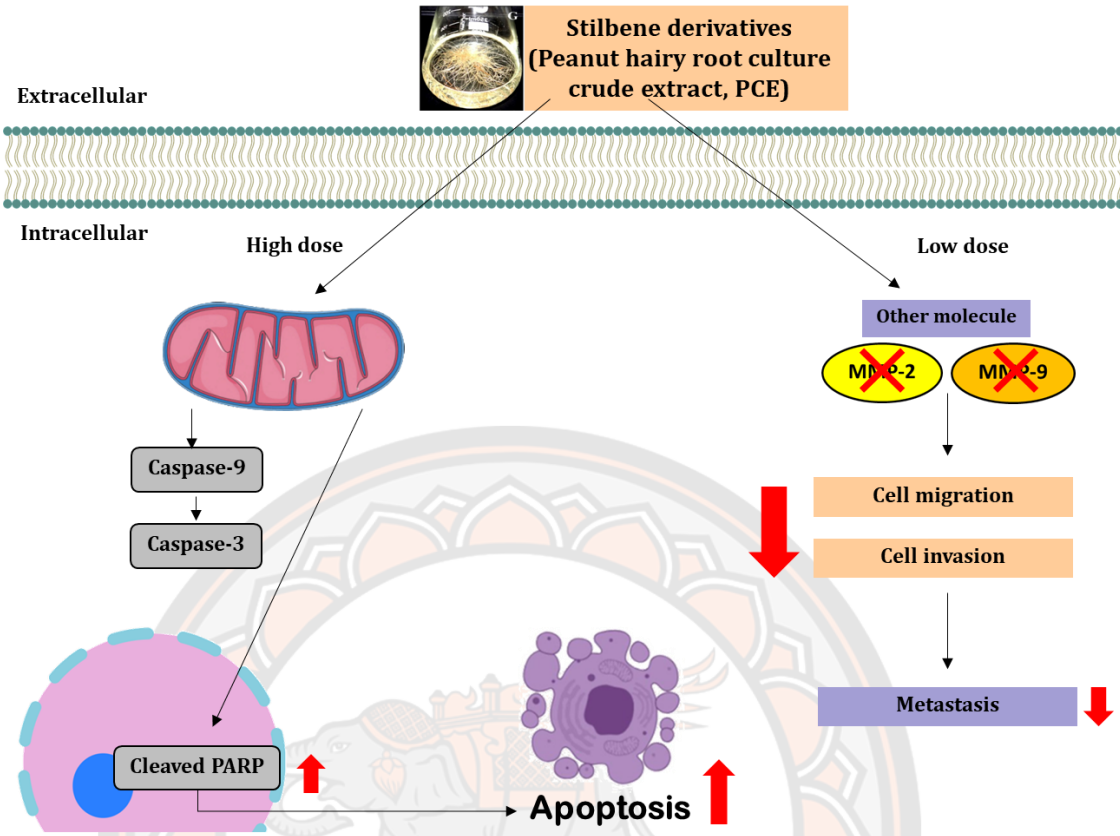


Figure 43 Schematic diagram of anti-cancer effect of PCE on CCA cell

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APPENDIX

Table 1 The effect of PCE on cell viability in KKU-213 cell were analyzed by MTT assay. The results were obtained from three separate experiment.

Conc. (µg/ml)	N1	N2	N3	Mean	S.D.
24 h					
Control	100	100	100	100	0
200	92.7273	94.25484	100	95.66071	3.834762
400	91.97521	85.24841	96.70123	91.30828	5.755463
600	74.51431	62.62799	76.87719	71.33983	7.636619
800	55.79923	58.87372	56.00094	56.8913	1.719787
IC50	892.7877	941.9795	896.015	910.2607	27.51662
48 h					
Control	100	100	100	100	0
200	85.88082	71.88307	81.26339	79.67576	7.132645
400	58.54146	30.72513	58.04968	49.10542	15.9197
600	39.76024	19.92231	23.33643	27.67299	10.60614
800	17.54915	17.27691	15.21003	16.6787	1.279169
IC50	468.3317	415.5987	464.3974	449.4426	29.37562
72 h					
Control	100	100	100	100	0
200	66.78219	43.64074	76.03339	62.15211	16.6853
400	45.20672	20.40097	43.28783	36.2985	13.80106
600	16.65509	12.3817	12.9124	13.98307	2.329208
800	8.408177	9.542888	5.23322	7.728095	2.233875
IC50	359.6538	354.3256	348.3026	354.094	5.679103

Table 2 The effect of PCE on cell viability in KKU-214 cell were analyzed by MTT assay. The results were obtained from three separate experiment.

Conc. (µg/ml)	N1	N2	N3	Mean	S.D.
24 h					
Control	100	100	100	100	0
200	100	100	97.31709	99.1057	1.548979
400	99.58399	79.81324	73.68075	84.35933	13.53679
600	69.1046	57.85727	55.8874	60.94975	7.130651
800	59.29497	50.73268	49.8096	53.27908	5.230314
IC50	848.7195	811.7229	798.9536	819.7987	25.84716
48 h					
Control	100	100	100	100	0
200	100	92.10191	95.80218	95.96803	3.951656
400	92.40733	83.2909	80.05921	85.25248	6.403505
600	66.10424	48.6836	51.66845	55.48543	9.316473
800	40.07203	28.66242	33.88053	34.20499	5.711722
IC50	641.1525	668.3272	620.0214	643.167	24.21583
72 h					
Control	100	100	100	100	0
200	96.17919	87.61237	89.6333	91.14162	4.478153
400	69.02001	74.16981	65.63539	69.6084	4.297528
600	35.59008	36.12737	21.16136	30.9596	8.489779
800	17.75556	14.81067	10.3667	14.31098	3.719688
IC50	552.1601	593.3585	529.0831	558.2006	32.56065

Table 3 The effect of PCE on cell viability in KKU-055 cell were analyzed by MTT assay. The results were obtained from three separate experiment.

Conc. (µg/ml)	N1	N2	N3	Mean	S.D.
24 h					
Control	100	100	100	100	0
200	92.10671	95.51934	97.64916	95.09174	2.795861
400	90.8326	96.6339	90.84539	92.77063	3.345698
600	78.38969	74.51364	76.28841	76.39725	1.940316
800	67.10625	66.84151	69.64284	67.86353	1.546602
IC50	1073.7	1063.464	1114.285	1083.817	26.87859
48 h					
Control	100	100	100	100	0
200	89.99348	92.98836	86.1375	89.70645	3.434436
400	63.20012	65.16531	58.82448	62.39664	3.245879
600	39.8297	40.77623	39.67048	40.09214	0.597768
800	28.05438	27.15223	28.48541	27.89734	0.680322
IC50	503.6004	521.3225	470.5959	498.5062	25.74412
72 h					
Control	100	100	100	100	0
200	66.03558	68.75867	71.02096	68.60507	2.496238
400	30.87046	39.40398	39.43929	36.57124	4.937056
600	12.08728	11.99628	12.58488	12.22281	0.316841
800	7.238514	9.4587	8.888623	8.528612	1.153045
IC50	264.1423	273.6159	284.0838	273.9474	9.974891

Table 4 The effect of PCE on cell viability in KKU-213L5 cell were analyzed by MTT assay. The results were obtained from three separate experiment.

Conc. (µg/ml)	N1	N2	N3	Mean	S.D
24 h					
Control	100	100	100	100	0
200	90.38963	99.26845	97.09284	95.58364	4.627811
400	89.94442	85.98945	96.4293	90.78772	5.270766
600	73.92733	63.07749	71.66837	69.55773	5.724585
800	65.47722	60.0556	58.77011	61.43431	2.642454
IC50	941.64	960.8836	940.3812	947.6349	11.49094
48 h					
Control	100	100	100	100	0
200	67.13472	64.18607	66.1066	65.80913	1.496665
400	52.25976	48.549	50.57442	50.46106	1.857975
600	36.85828	28.5465	24.99484	30.1332	6.088806
800	15.64286	11.25	16.61481	14.50256	2.85841
IC50	418.018	388.392	404.5354	403.6485	14.8329
72 h					
Control	100	100	100	100	0
200	51.72632	45.75488	59.70781	52.39634	7.000556
400	39.2631	19.96489	38.70193	32.64331	10.98342
600	21.68421	14.55474	18.80547	18.34814	3.586672
800	8.757918	11.58634	7.248538	9.197598	2.20207
IC50	208.3053	233.547	238.8312	226.8945	16.31411

Table 5 The effect of PCE on cell apoptosis in KKU-213L5 cell were analyzed by flow cytometry. The results were obtained from three separate experiment.

Conc. (µg/ml)	**Apoptosis population (Early apoptosis)**				
	N1	**N2**	**N3**	**Mean**	**S.D**
Control	1.23	1.94	2.31	1.826667	0.548847
100	4.19	4.72	5.9	4.936667	0.875348
200	3.87	5.59	6.43	5.296667	1.304965
400	4.7	4.83	5.54	5.023333	0.452143

Conc. (µg/ml)	**Apoptosis population (Late apoptosis)**				
	N1	**N2**	**N3**	**Mean**	**S.D**
Control	1.83	2.11	3.8	2.58	1.0657865
100	19.24	5.06	19.21	14.5033	8.1781803
200	13.9	22	28.03	21.31	7.0902256
400	23.04	22.67	29.05	24.92	3.5814668

Table 6 The effect of PCE on cell apoptosis in KKU-213L5 cell were analyzed by Hoechst 33342 staining. The results were obtained from three separate experiment.

Number of DNA condensation and fragmentation					
Conc. (µg/ml)	**N1**	**N2**	**N3**	**Mean**	**S.D**
Control	1.161242	2.81779	1.4567	1.811911	0.883555
100	21.01582	21.23576	20.5702	20.94059	0.339097
200	29.42579	27.7183	29.3068	28.81696	0.953329

Table 7 The effect of PCE on cell migration in KKU-213L5 cell were analyzed by wound healing assay. The results were obtained from three separate experiment.

N1	Concentration (µg/ml)			
Times (h)	**Control**	**25**	**50**	**100**
0	100	100	100	100
6	48.74383	63.66867	66.14896	65.5528159
12	30.82754	45.53225	45.60665	53.5297171
18	10.244789	20.750466	28.243518	34.71321049

N2	Concentration (µg/ml)			
Times (h)	**Control**	**25**	**50**	**100**
0	100	100	100	100
6	60.18762	62.54341	54.623939	68.7074
12	36.9980302	40.67146	50.460375	61.88311
18	15.1366001	26.64885	34.113571	48.4907

N3	Concentration (µg/ml)			
Times (h)	**Control**	**25**	**50**	**100**
0	100	100	100	100
6	58.477729	62.7775	65.1500933	69.346624
12	32.32851	40.23308	49.08695	52.359469
18	10.586424	24.28447	27.888637	36.74602

Table 7 The effect of PCE on cell invasion in KKU-213L5 cell were analyzed by trans-well invasion assay. The results were obtained from three separate experiment.

	Number of invading cells				
Conc. (µg/ml)	**N1**	**N2**	**N3**	**Mean**	**S.D**
control	100	100	100	100	0
25	85.49641	88.10076	82.3549	85.31736	2.877111
50	44.07895	53.86145	44.36291	47.43444	5.56777
100	9.748804	8.418959	14.3429	10.83689	3.108247