# MECHANISMS OF APOPTOSIS INDUCTION BY *ACACIA CONCINNA* EXTRACT IN COLON CANCER CELLS

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# กลไกการกระตุ้นการตายแบบอะพอพโทซิสโดยสารสกัดจากส้มป่อยในเซลล์มะเร็งลำไส้ใหญ่

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# MECHANISMS OF APOPTOSIS INDUCTION BY *ACACIA CONCINNA* EXTRACT IN COLON CANCER CELLS

PORNNAPA SITTHISUK

A Dissertation Submitted in Partial Fulfillment of the Requirements

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MECHANISMS OF APOPTOSIS INDUCTION BY *ACACIA CONCINNA* EXTRACT IN COLON CANCER CELLS

BY

PORNNAPA SITTHISUK

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DOCTOR OF PHILOSOPHY IN MOLECULAR BIOLOGY AT SRINAKHARINWIROT UNIVERSITY

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Colorectal cancer (CRC) ranks as one of the most common cancers and poses a major public health concern worldwide. *Acacia concinna* (AC) extract exhibits many pharmacological properties, including antioxidant, anti-tyrosinase, antimicrobial, and anticancer. However, the anticancer effect of AC extract on colon cancer has not been studied. This study aimed to investigate the effect of AC extract on apoptosis induction and the associated signal transduction pathways in HCT116 colon cancer cells. The effects of AC extract on cell cytotoxicity were determined using MTT assay, while nuclear morphological changes were observed through Hoechst 33342 staining. Mitochondrial membrane potential ($\Delta\Psi$m) was examined using JC-1 staining, and ROS level was measured with DCFH-DA staining. Cell cycle analysis was conducted using flow cytometer. Protein expression was analyzed via western blot. The results demonstrated that AC extract reduced cell viability in HCT116 cells, and increased nuclear condensation and apoptotic bodies in the AC-treated cells. Additionally, AC extract caused a loss of mitochondrial membrane potential, a well-known feature of apoptosis. Western blot analysis revealed a decrease in the expression of anti-apoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1), and increased levels of pro-apoptotic proteins (Bak, Bax, cleaved caspase 7, and cleaved PARP). Furthermore, apoptosis induced by AC extract occurred through the endoplasmic reticulum (ER) stress pathway, as ROS accumulation activated ER stress, leading to increased expression levels of GRP 78, p-eIF2$\alpha$, p-IRE$\alpha$, and CHOP. Additionally, AC extract also stimulated the MAPK pathways while inhibiting the PI3K/Akt and Wnt/$\beta$-catenin pathways. These findings indicate that AC extract suppresses colon cancer cell proliferation and triggers apoptosis through ER stress, along with modulation of the MAPK, PI3K/Akt, and Wnt/$\beta$-catenin pathways in HCT116 cells.

Keyword : Acacia concinna, apoptosis, colon cancer

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# CHAPTER 1
# INTRODUCTION

Cancer is a condition marked by the uncontrolled growth of abnormal cells within an organ or tissue of the body. These cells can invade surrounding tissues and spread to distant parts of the body via the blood and lymphatic systems, a process that significantly contributes to cancer-related mortality [1, 2]. In this study, we are interested in colorectal cancer (CRC), commonly referred to as colon cancer, which poses a significant public health issue in several countries, including Thailand. In Thailand, colon cancer ranks as the third most common cancer among men and the fourth among women, with colorectal cancer rates rising in numerous countries [3]. Moreover, cancer treatment, especially chemotherapy shows side effects such as diarrhea and nausea but is not successful for inhibiting cancer cells [4-6]. Therefore, searching for new candidate agents for cancer treatment is necessary.

Natural phytochemicals, produced by plants through primary or secondary metabolism, provide health benefits and prevent diseases, including cancer. Phytochemicals are generally safe and low toxic, which is an alternative approach to avoid the side effects of synthetic medicines [7, 8]. *Acacia concinna*, known as Sompoi, belongs to the Fabaceae family. *A. concinna* is a traditional medicinal plant that grows in Southern Asia and is used as a shampoo, an anti-dermatophyte, and has antimicrobial properties for skin disorders [9, 10]. A previous study suggested that *A. concinna* extracts present many pharmacological properties such as antioxidant, anti-tyrosinase activity [9], and anticancer activity [11]. However, there have been no reports on the anticancer effects of *A. concinna* extract on colon cancer.

Therefore, in this study, we aim to investigate the mechanisms of apoptosis induction by *A. concinna* extract in HCT116 colon cancer cells. This study highlights the bioactivity of *A. concinna* on colon cancer cells, which may lead to its development as a natural anticancer drug or its use in combination with other cancer drug in the future.

**Objectives**

1. To study the cytotoxic effect of *A. concinna* extract against human colorectal HCT116 cancer cell with *KRAS* gene mutation.

2. To study mechanisms of apoptosis induction associated with signaling pathways in HCT116 cell.

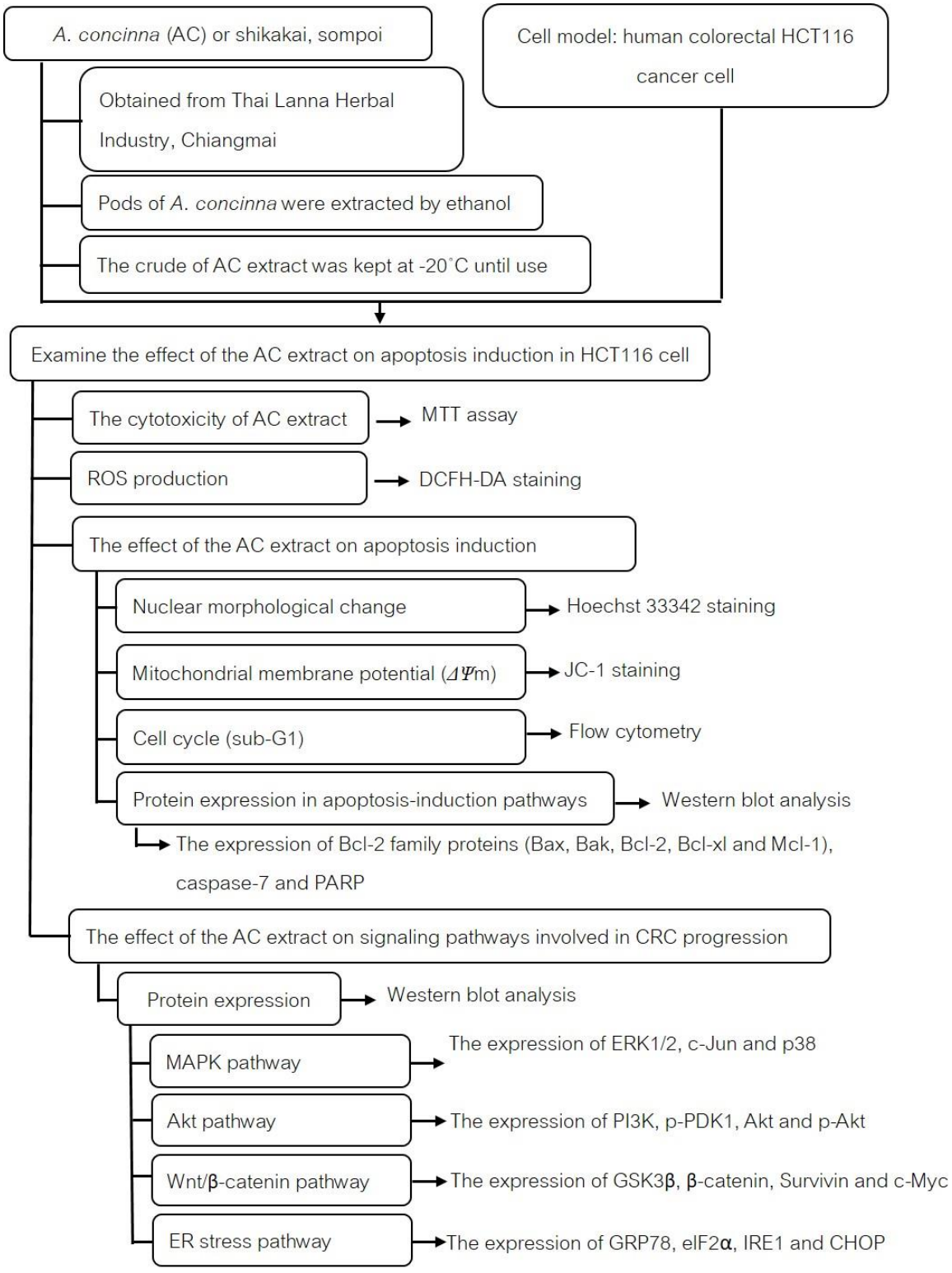


Figure 1 Conceptual framework.

# CHAPTER 2
# LITERATURE REVIEW

## Human large intestine

The intestines are parts of the gastrointestinal tract and digestive system of the human body, including the small intestine, large intestine, and rectum, continuous from the stomach to the anus. The most absorption of nutrients and water (up to 90%) has been at the small intestine [12]. The large intestine, also known as the large bowel or colon, is the final segment of the human digestive system. It receives nearly fully digested food, absorbs water, nutrients, and electrolytes, and ultimately passes waste (stool or feces) to the anus. It is about 1.5 meters long and can be divided into 3 main parts, including the cecum, colon, and rectum [12, 13].

The first part of large intestine is cecum, a pouch that connects last part of small intestine (ileum) to colon that receives undigested food material from small intestine and then continues absorption of water and salts. The cecum has an appendix, a thin tubular that sits in lower right abdomen and contains lymphatic cells. The function of appendix is unsure, but some experts thought it is the site for accumulation of good bacteria [14]. The colon, the longest section of the large intestine, is divided into four parts: the ascending colon, transverse colon, descending colon, and sigmoid colon [15]. The ascending colon is located on right side of abdomen continue from cecum, courses up until meets right lobe of liver, forming the hepatic flexure before continuing as the transverse colon. The transverse colon extends across the abdomen, connecting the ascending colon at the hepatic flexure to the descending colon at the splenic flexure, which is situated on the left side of the abdomen. It absorbs remaining water and other nutrients passed through many parts of colon ultimately into a solid form or stool, stored of stool that will eventually move into the next part of the colon [13, 14, 16]. The last part of colon is sigmoid colon, S-shaped part continuation from descending colon, and connects to rectum. Functions of sigmoid colon is storage site for fecal until it can be moved out of the body by moving into the rectum [12, 16, 17]. Rectum is the final part of large intestine continuation of sigmoid colon and connects to anus. When the feces enter the rectum and become full, that generates

an urge to defecate because the pressure in the rectum is increased, leading to opening of internal anal sphincter and outer anal sphincter, which continue to defecate through the anus [12, 14, 18, 19] (Figure 2).

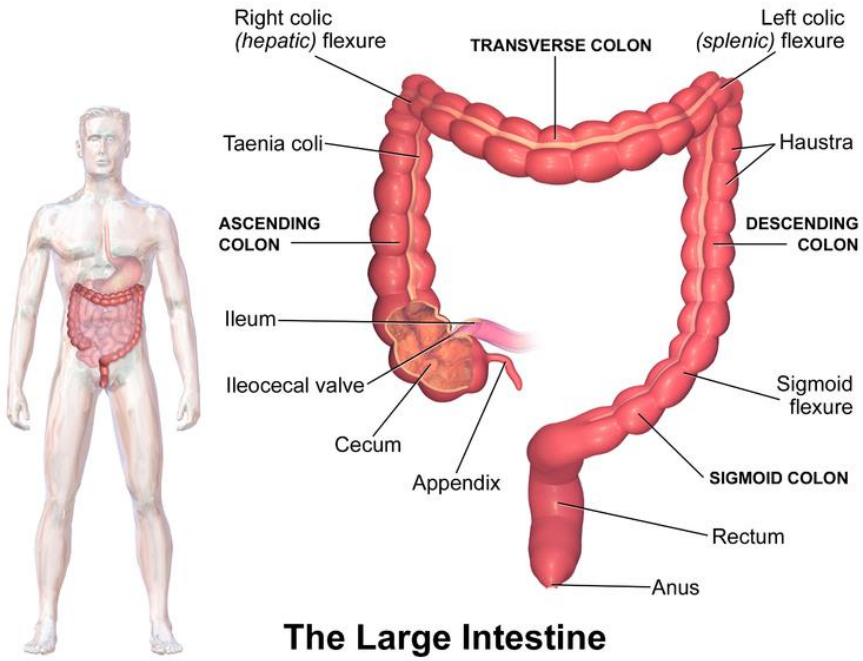


Figure 2 Anatomy of large intestine/colon.

Source: Jones DA. The colon [Internet]. 2018 [cited 2021 Oct 3]. Available from: https://teachmeanatomy.info/abdomen/gi-tract/colon/

The large intestine has no digestive enzymes; chemical digestion occurs in the small intestine. Therefore, chemical digestion occurs exclusively by bacteria in the lumen of the colon. The colon wall consists of four layers: the mucosa (or mucous membrane), submucosa, muscularis (or muscularis propria), and adventitia (or serosa) [20]. The mucosa of the colon is comprised of simple columnar epithelium, which includes enterocytes (absorptive cells) and goblet cells, which are essential to maintain intestinal homeostasis and protect the intestine. The major cell type of the colon includes goblet cells, enteroendocrine cells, and enterocyte cells, which originate from cell differentiation

of intestinal stem cells in the base of the crypt [20, 21]. The thick mucosa has deep crypts, which increase the absorption surface of tissue but are absent of villi. Ordinarily, surface epithelial cells are continued loss into the lumen and renewed by cell proliferation occurring at the crypt base [22]. The goblet cells produce and secretion of mucin, which help the movement of feces and act as a physical barrier to protect the intestine from harmful agents or substances of commensal bacteria. The main function of enterocytes is absorbing water from colon lumen. In addition, the goblet cells produce and secrete mucin, which helps the movement of feces and acts as a physical barrier to protect the intestine from harmful agents or substances of commensal bacteria. The main function of enterocytes is absorbing water from the colon lumen. In addition, the large intestine has several immune cells, such as macrophages, dendritic cells, and B cells, which have an important role in defense against pathogens and protect from diseases [21, 23, 24].

## Cancer

Cells are the smallest structural units and functions of life, including the human body. The adult human is consisting of approximately 1015 cells, many of which are required to grow, divide (proceed through the cell cycle), and differentiate into a specialized cell to develop or replace from cell sloughing, cell injury, or cell death [20, 25, 26]. In multicellular organisms, maintaining homeostasis requires a careful balance between generating new cells through cell proliferation and losing cells through cell death [27]. The processes of cell proliferation and cell death (apoptosis) are tightly regulated by signal transduction pathways [28-30]. Signal transduction pathways, also known as cell signaling, entail the binding of signaling molecules, known as ligands, to receptors located on the cell surface or inside the cell, subsequently triggering events within the cell, resulting in a cellular response that alters the behavior of the cell [31-34]. Disruption of normal regulation of cell proliferation and apoptosis can lead to human diseases, particularly cancer [28, 34, 35].

Cancer cells are cells that abnormally grow and divide uncontrollably to form a benign or malignant tumor; only malignant tumors are referred to as cancers, which cause the accumulation of multiple mutations [36]. Cancer cells can change, which enables them

to survive and reproduce (proliferation and replicative immortality). The characteristics of cancer, as explained by Weinberg and Hanahan, constitute a principle of framework to understand tumor pathogenesis [37, 38]. These major hallmarks consist of originally six biological capabilities, including sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Figure 3). Genome instability is the underlying cause of these traits [39]. Therefore, the knowledge of cancer hallmarks is expected to be an important target for the development of anticancer therapies [40].

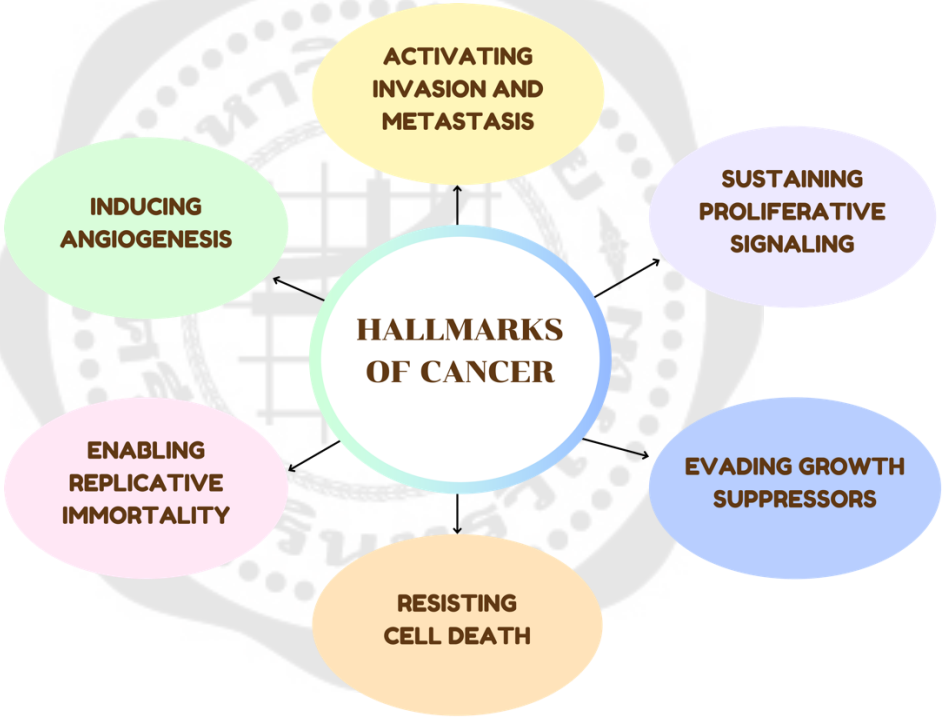


Figure 3 The hallmarks of cancer.

Modified from Hanahan D, Weinberg RA. (2000).

Source: Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.

**Colorectal cancer (CRC)**

Colorectal cancer (CRC), commonly referred to as colon cancer, is a term that encompasses both colon and rectal cancer. It is a malignant tumor development of normal epithelial cells in the inner wall of the colon or rectum to abnormal cells. CRC caused by multistep process mutations of genetics can be either somatic (acquired) and/or germLine (inherited) that accumulate over time leading to a rack of control between cell proliferation and cell death [41, 42]. The abnormal cells heightened replication; these hyper-proliferative cells lead to a benign tumor, survival-immortality advantage, invasion, metastasis, and other cancer hallmarks [42].

**Epidemiology of colorectal cancer (CRC)**

CRC is the most prevalent cancer among both males and females, representing a significant public health issue in numerous countries worldwide. According to GLOBOCAN 2018 data, CRC is the second most common cause of mortality, accounting for an estimated 881,000 deaths, and ranks fourth in terms of new diagnoses, with around 1.8 million new cases reported globally. The incidence of CRC is higher in men than in women. Moreover, the rates of CRC have been rising in developing countries, for example, Southern Europe, Northern Europe, New Zealand, Australia, and Eastern Asia, including Thailand [43].

According to the National Cancer Institute's 2018 report, CRC is the most common cancer among men in Thailand, accounting for 19.7% of new cases, and the third most common cancer among women, representing 11.1% of new diagnoses. The age-standardized rates were 16.2/100,000 men and 11.2/100,000 women, and new cases of cancer diagnosis by staging in patients are 4%-9% for stage I, 17%-22% for stage II, 31%-37% for stage III, and 32%-40% for stage IV. Additionally, the incidence of CRC has been steadily increasing in both sexes in Thailand [3, 44].

**Colorectal cancer (CRC) development**

The histological progression of CRC typically starts from hyper-proliferative of normal intestine epithelium in the inner lining (mucosa) as polyps (benign tumor), which might appear anywhere in the colon and rectum. Most CRC are found in the rectum or sigmoid colon [45, 46]. Polyps are abnormal tissue growths that protrude from the mucosa

of the colon [45]. Size of polyp correlates with CRC development; the larger a polyp, the more likely it is to become cancer. Most polyps that have low-grade dysplasia (mildly abnormal) are harmLess and do not spread to other parts of the body [46, 47]. However, if these polyps are left untreated, they can develop into colorectal cancers due to the accumulation of genetic mutations, epigenetic changes, and local inflammatory alterations [48]. The increases in cellular DNA damage affect the progression of polyps from early to intermediate, then to advanced polyps through a dysplasia stage from low to high-grade (severe) dysplasia. High-grade (severe) dysplasia means that severely abnormal cells look more like cancer that can develop into an adenoma and eventually become a carcinoma or cancer, which increases the degree of risk for progression of CRC [45, 47]. Cancer cells can spread from the colon or rectum to other parts of the body through the bloodstream or to nearby lymph nodes, such as the liver, lung, bone, and brain. This spread is called metastasis, also referred to as advanced colorectal cancer. Therefore, the early screening of polyps and removal of these polyps will reduce the incidence of CRC [42]. However, not all polyps will develop into cancer dependent on polyp type. Types of colon and rectal polyps can be classified as neoplastic and non-neoplastic and grow in two different shapes, including flat (sessile) and with a long stalk that looks like a mushroom (pedunculated) [47, 49]. Non-neoplastics include hyperplastic polyps, hamartomas, benign lymphoid polyps, and inflammatory polyps that have been regarded as harmLess and have no malignant potential. Neoplastic polyps are adenomatous polyps or adenomas that are caused by abnormalities of glandular epithelium and have malignant potential. The main kinds of polyps that may develop into cancer include adenomas and sessile serrated polyps (SSPs) [47]. Most colorectal cancers frequently occur from adenomas polyp [50], which can classify villous components into three types, including tubular, tubulovillous, and villous adenomas [49, 51, 52]. Adenoma polyp have mutation occurs according to the process of adenoma-carcinoma sequence pathway that usually obtain dysplastic changes in a slow and long period about 10 to 15 years before developing invasive carcinoma [42, 49] (Figure 4). CRCs can also develop via a serrated

pathway of serrated polyps, which represent about 10% to 30% of all CRCs [53-55]. Therefore, polyp histology is important for determining malignant potential.

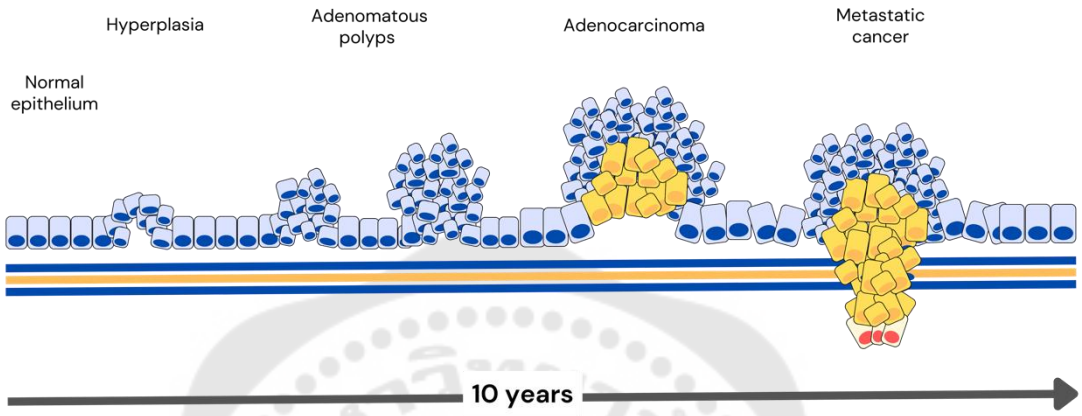


Figure 4 The process of colorectal cancer development.

Modified from https://www.biovendor.com/colorectal-cancer-more-people-screened-more-lives-saved

Source: Colorectal cancer - more people screened, more lives saved [Internet]. 2019 [cited 2021 Oct 1]. Available from: https://www.biovendor.com/colorectal-cancer-more-people-screened-more-lives-saved

**Histologic types of colorectal cancer (CRC)**

According to WHO, the classification of histologic types in CRC are adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, spindle cell carcinoma, and undifferentiated carcinoma [56].

More than 90% of all cases of CRC are adenocarcinomas, and others are rare types of CRC [29, 46]. Adeno is a meaning of granular cells originating from epithelial cells of colorectal mucosa that occur in line the wall of the colon or rectum, whose function is to produce the mucus in the large intestine [57]. CRC adenocarcinoma can be subdivided into subtypes: cribriform comedo-type adenocarcinoma, medullary carcinoma,

micropapillary carcinoma, mucinous adenocarcinoma, serrated adenocarcinoma, and signet-ring cell carcinoma [56]. Mucinous (colloid) adenocarcinoma (MA) accounts for 10-15% of all CRCs and presents in the right colon. MA is characterized by abundant mucinous components that presence of more than 50% of the tumor volume [58]. Micropapillary adenocarcinoma (MPA) is an aggressive tumor with a tendency toward metastasis that presents about 9%-19% of CRC. This variant is more commonly found in the rectum and right colon [46, 59]. Serrated adenocarcinoma (SA) represents 5.8% to 12% of all CRCs and up to 17% of proximal CRCs. Structural and histochemical characteristics of SA resemblance to hyperplastic polyps with glandular serration. The majority of serrated adenocarcinoma (SA) cases are found in the right colon (47% to 57%) or the rectum (15% to 29%) [46]. Cribriform comedo-type adenocarcinoma is a rare histological subtype that can be characterized by large cribriform glands with central necrotic changes, similar to those seen in breast adenocarcinomas [60]. Medullary carcinoma is a rare type, contributing less than 1% of CRC, characterized by a solid growth pattern with poorly glandular differentiation [61, 62] and signet-ring cell carcinoma is also a rare type. Signet-ring cell carcinoma is defined by showing more than 50% of tumor cells with prominent intracytoplasmic mucin and displaced nuclei, representing about 0.7 to 1% of all CRCs [46].

The rare type of CRC, squamous cell carcinoma (SCC), typically affects the esophagus or anal canal. However, when occurring in the colon, it has been reported in the rectosigmoid region, accounting for less than 0.5% of CRC [63]. Adenosquamous cell carcinoma (Ad-SCC) accounts for 0.025% and 0.1% of all CRC. Ad-SCC is characterized by comprising of both adenocarcinoma and a squamous cell carcinoma component, metastasis more than adenocarcinoma, and difficulty to preoperative diagnosis [64, 65]. Spindle cell carcinoma, also called sarcomatoid carcinoma, is an extremely rare tumor that has histologic, cytologic, and molecular properties similar to those of both epithelial and mesenchymal tumors[66]. Spindle cell proliferations are usually present as polyps, uncommonly found in the sigmoid colon, rectum, and anus [67]. Undifferentiated carcinoma

is a term to describe a malignant epithelial neoplasm that is very immature and primitive, caused by not differentiate to form tubules, and is very rare [68].

**Molecular characteristics of colorectal cancer (CRC)**

Most cases of CRC are sporadic, about 70-85%, arise from accumulating a long time of multiple genetic or epigenetic mutations [69], not related to family history or genetic predisposition [70]. Typically, sporadic cancer is due to age and several environments [71]. Cancer involves mutations in two main classes of genes: proto-oncogenes and tumor suppressor genes. Proto-oncogenes, normally promote normal cell growth, when this gene is mutated, it becomes into oncogenes that promote cancer cell growth, such as *RAS* genes comprising three RAS isoforms, including *KRAS*, *HRAS*, and *NRAS*. *KRAS* is the most frequently in CRC, about 50%; KRAS mutations affect many downstream effector pathways. Adenomatous polyposis coli (*APC*) gene is a tumor suppressor gene that highly mutated about 80% of CRC. Additionally, 34% of proximal colon tumors and 45% of distal colorectal tumors in CRCs have *TP53* gene mutations [72]. In addition, epigenetic factors such as DNA methylation can inactivate suppressor genes, therefore promoting cancer, which occurs in about 25% of CRC and involves the p53 tumor suppressor pathway [50, 73].

The histological changes of normal colon to colorectal cancer development result from multistep process mutations of genetics [42, 49]. According to Fearon and Vogelstein, the adenoma-carcinoma sequence is a classical multistep model of colorectal cancer carcinogenesis that describes an accumulation of genetic mutations resulting in the transformation of a benign adenoma into a malignant carcinoma [74]. The three major genetic mechanisms associated with carcinogenesis of CRC are chromosomal instability (CIN), microsatellite instability (MSI or MIN), and the CpG island methylator phenotype (CIMP) [75, 76]. The classical adenoma-carcinoma sequence pathway is primarily linked to chromosomal instability (CIN) and APC and KRAS mutations [77]. In addition, CRCs can also develop via a serrated pathway, which is characterized by BRAF mutations and epigenomic instability (CIMP-high) [46].

CIN pathway is the most common type of genetic instability in sporadic colorectal cancers and is observed in about 65%–70% [75]. CIN refers to alterations in chromosome number and structure, such as the gain or loss of entire chromosomes or segments, caused by aneuploidy, deletions, insertions, amplifications, or loss of heterozygosity (LOH) that results in gene copy number variations (CNVs) [22, 71, 75].

Microsatellite instability (MSI) is the condition of genetic hypermutability caused by the mutations in DNA mismatch repair (MMR) genes such as MLH1, MSH2, MSH6, and PMS2. The loss of MMR function leads to the inability to correct errors during DNA replication, allowing mutations to persist and accumulate, resulting in a hypermutation phenotype [22, 69, 76], and leading to sporadic CRCs. MSI is observed about 10-15% in sporadic CRCs and associates with hereditary CRC syndrome (Lynch syndrome) that is caused by germLine mutations in DNA MMR genes [69, 78]. In addition, inactivation of MMR genes also involves the CpG island methylator phenotype (CIMP) through aberrant methylation (hypermethylation) of promoter CpG of the MutL homolog 1 (*MLH1*) gene [47, 69]. Both CIN and MSI pathways describe the accumulation of genetic abnormalities in specific genes that can result in the loss of tumor suppressor gene function and/or the rise of oncogene function, thereby contributing to CRC carcinogenesis [79].

In addition, epigenetic instability is involved in the pathogenesis of CRC. CIMP is the most well-characterized epigenetic alterations linked to CRC carcinogenesis [79]. CIMP occurs through an epigenetic instability pathway involving the hypermethylation of DNA at promoter CpG islands of several tumor suppressor genes or other tumor-related genes [71, 80], resulting in silence the expression of these genes (*BRAF* and *MLH1*). Consequently, CRC with CIMP is characterized by the epigenetic inactivation of tumor suppressor genes without the presence of mutations [79].

As proposed in the adenoma-carcinoma sequence model, the first step of CRC development includes mutations in the Adenomatous Polyposis Coli (APC) and/or loss of chromosome 5q that includes the APC gene classified as a tumor suppressor gene that regulates the WNT signaling pathway, which is crucial for controlling the proliferation

of intestinal epithelial cells [22, 71, 75, 81]. These events lead to hyper-proliferation of normal colorectal epithelium and to the formation of polyps or adenoma polyps [71, 82], followed by oncogenic *RAS* gene mutations (usually *KRAS* gene) occurring in small adenomas [71, 83]. *KRAS* gene encodes a GTP-binding protein that, when mutated, affects the downstream RAS-RAF-MEK-ERK pathway [81]. These pathways have the role in the cell growth, differentiation, motility, and survival that make an adenoma larger and more severely dysplastic [47, 84] and eventually, p53 mutations (loss of chromosome 18q and deletion of chromosome 17p), which encode a tumor suppressor gene regulated of transcription and apoptosis, and accumulation of these changes leading to promote carcinogenesis [47, 81, 85] (Figure 5).

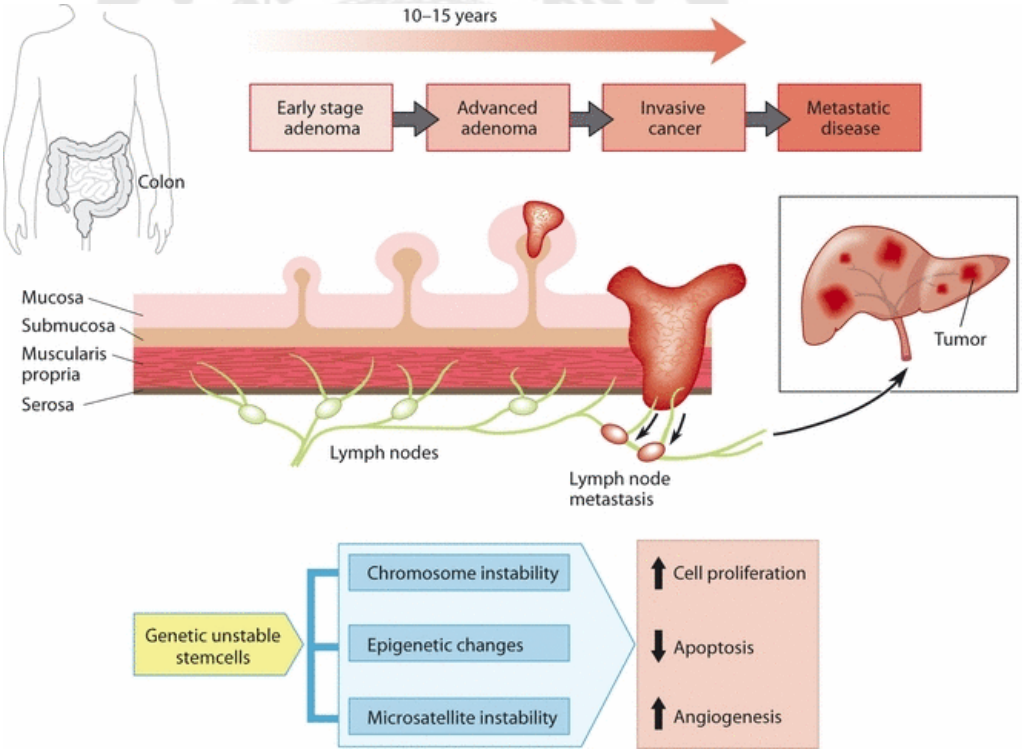


Figure 5 Colorectal adenoma-carcinoma sequence model.

Source: Bretthauer M. Colorectal cancer screening. J. Intern. Med. 2011;270(2):87-98.

**Stage of colorectal cancer (CRC)**

Stage of a cancer describes the growing size or spreading in the body of cancer, which can help determining the severity of disease, selecting the most effective treatment approach, and predicting survival outcomes for patients with CRC [86]. Stage of CRC according to the basis of the American Joint Committee on Cancer (AJCC), the TNM system is the most often used for classification staging of cancer [87]. The TNM system is based on three meanings, including T describes the extent (size) of the tumor, N talks about the cancer having spread to nearby lymph nodes, and M describes the spread (metastasis) to distant parts of the body. The categories of T, N, and M can assign a letter or a number. Higher numbers mean the cancer is more advanced [79, 86, 88]. In the TNM classification, Overall AJCC stage classified of invasive CRC from stage I to IV. The Dukes staging system is a classification system for CRC depend on the extent of local invasion through mucosa and bowel wall. The most commonly is stage from A through D. The Stage classification of CRC in the TNM system has corresponds Dukes staging. Pathologic stage indicates the progression of CRC and important with cancer prognosis. Therefore, if the diagnosis is delayed result to more advanced stage associated with survival rate for CRC patients [50, 89-91].

The development of CRC begins from hyperproliferation of an abnormal cell to the formation of a benign polyp or adenoma and grows only in the innermost lining (mucosa) of the colon or rectum (stage 0 or carcinoma in situ) or into the second layer of tissue (submucosa) (stage 1, T1N0M0, Dukes A). Cancer has larger and grown extending into the muscular layer (muscularis propria), not spreading to nearby lymph nodes or to other sites (stage I, T2N0M0, Dukes B1). Cancer continuous growth through the muscularis propria into the serosa, not spread to nearby lymph nodes or to distant sites (stage II, T3N0M0, Dukes B2). Cancer has invaded past the wall of the colon or rectum, has spread to nearby lymph nodes, but hasn't spread to distant sites (stage III, TxN1M0, Dukes C). Finally, cancer has spread to other parts of the body (metastatic), such as the liver. (stage IV, TxNxM1, Dukes D). The letter X means the information couldn't be assessed [92] (Figure 6).

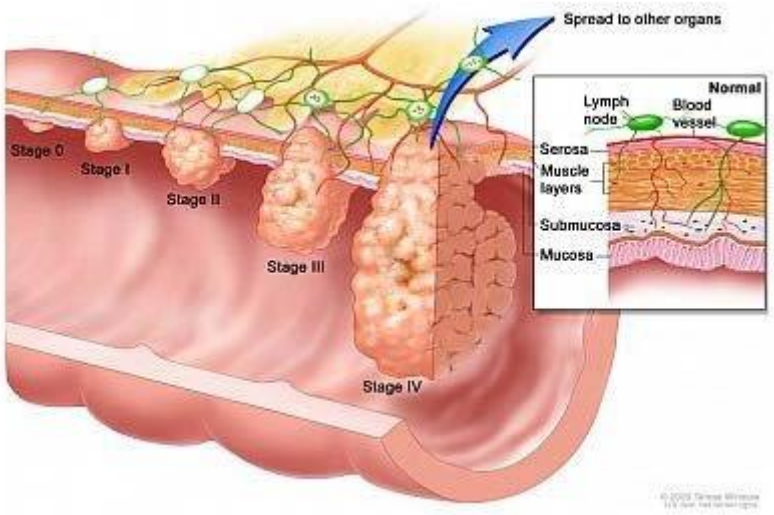


Figure 6 Stages of colorectal cancer (CRC).

Source: Colorectal cancer alliance. Stage of diagnosis [Internet]. 2019 [cited 2021 Oct 1]. Available from: https://www.ccalliance.org/colorectal-cancer-information/stage-of-diagnosis

**Grades of colorectal cancer (CRC)**

Grades of cancer describe the characteristics of the cancer cells compared to normal cells based on differentiation of tumor cells under a microscope. Cancer grading is important for the prognosis of cancer patients and can be helpful in planning patient management, treatment, and prediction of response to treatment [93, 94]. The WHO criteria the CRC classified into three or four grades depend on the percentage of gland formation, including:

**Grade 1** cells were well differentiated; gland formation was more than 95%. Cancer cells that are like normal cells.

**Grade 2** cells are moderately differentiated, accounting for between 50% and 95% of gland formation. Cancer cells are growing more rapidly than normal cells, as opposed to normal cells.

**Grade 3** cells poorly differentiated; gland formation was lower than 50%. Cancer cells that have aberrant characteristics and can spread or develop more quickly.

**Grade 4** cells undifferentiated are rare cases.

The WHO classification at its latest version recommended the use of a two-tiered system, with G1 and G2 of CRCs as low-grade and G3 and undifferentiated as high-grade [93-95].

**Signs and symptoms of colorectal cancer (CRC)**

In the early stages of colorectal cancer, many people experience no symptoms until it is advanced. Signs and symptoms of CRC depend on the cancer's size and location of the tumor. The most common signs and symptoms are a change in bowel habits, blood in the stool, a change in bowel movements, involuntary weight loss, anorexia, muscle weakness, and a feeling of poor health. Additionally, colonic lumen obstruction from large tumors is more likely to cause nausea, abdominal distention, constipation, and pain in the abdomen [50].

**Risk factors of colorectal cancer (CRC)**

Risk factors play a crucial role in the development of diseases. The risk factors for CRC are associated with both environmental and genetic factors [96]. Most cases of CRC are linked to older age and lifestyle choices. Risk factors that may increase the development of CRC include age, gender, diet, smoking, alcohol, and genetics, which account for a small number of cases [97].

**General**

**Age**

CRC may occur at any age. However, older adults over 50 account for the majority of CRC cases, which are mostly related to environmental factors. The incidence of CRC in young people is 2% of all cancer cases. In contrast, in older people, it is about 6% of cases [98]. In Thailand, the age group of 60 to 75 years old had the highest incidence of colorectal cancer (CRC), accounting for around 50% of all cases [3]. The older population is growing faster than all other age groups worldwide, whichncan predict that the number of older patients with CRC will increase.

### Gender

Colorectal cancer occurs more frequently in men than in women, with men having approximately a 1.5-fold higher risk of developing the disease [43]. In Thailand, the National Cancer Institute reported an incidence of 16.2 cases per 100,000 men and 11.2 cases per 100,000 women [3], which may be influenced by lifestyle factors such as smoking and drinking alcohol. Moderate to heavy alcohol intake is more strongly associated with men, likely due to hormone-related differences in alcohol metabolism. Additionally, men tend to consume larger amounts and may underreport their alcohol consumption, both of which are associated with an increased risk of CRC [43].

### History of inflammatory bowel disease (IBD)

The hallmark of inflammatory bowel disease (IBD) is persistent colon inflammation. Chronic inflammation can be a major cause of cancer, resulting in the release of pro-inflammatory cytokines that increase tumor growth and excess blood flow and may induce DNA mutations in cells via oxidative stress as well as additional carcinogenic risk factors. The mortality of colorectal cancer linked with IBD is responsible for approximately 2% per year, and individuals with long-term IBD are twice as likely to get CRC [43, 99].

### Lifestyle

#### Diet

Diet plays an induction or protection role in the development of colorectal cancer, especially diet from high-temperature cooking, curing, grilling, and smoking due to the burning of fat in meat becoming carcinogens. Polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs) are two forms of carcinogens that can be produced by grilling meat. The International Agency for Research on Cancer (IARC) designated red meat as potentially carcinogenic and processed meat as carcinogenic in 2015. These designations may have an increased impact on the risk factor for CRC. On the other hand, fruit and vegetables have shown a protective effect against CRC [43]. The risk of colorectal cancer and adenomatous polyps can decrease linked to drinking five glasses of water a day [100].

### Smoking

Smoking is the primary cause of cancer-related deaths, particularly associated with lung cancer. Additionally, studies have shown that the relative risk of developing colorectal cancer due to smoking is 1.18 [43]. Each puff of cigarette contains over 60 well-established carcinogens. These carcinogens, which are found in cigarette smoke, belong to various chemical classes and play a significant role in the development of human cancers. Metals, aldehydes, volatile organic chemicals, aromatic amines, N-nitrosamines, and polycyclic aromatic hydrocarbons (PAHs) are important classes [101]. In 2009, the International Agency for Research on Cancer (IARC) stated that CRC is caused by smoking [43]. A recent meta-analysis of 188 original studies revealed that, in comparison to non-smokers, the pooled relative risk (RR) for colorectal cancer (CRC) was 1.14 (95% CI 1.10–1.18) for current smokers and 1.17 (95% CI 1.15–1.20) for past smokers. The risk of CRC increased linearly with both the intensity and duration of smoking [102].

### Alcohol

Alcohol refers to ethanol or ethyl alcohol, a chemical compound present in alcoholic beverages like beer, wine, and distilled spirits. The consumption of alcohol may elevate the risk of cancer due to the metabolism of ethanol into acetaldehyde, a toxic substance and probable human carcinogen. This process can trigger cancer-promoting pathways, including the formation of DNA adducts and oxidative stress [103]. Individuals who consume 2 to 3 alcoholic drinks per day face a 20% increased risk of developing colorectal cancer, while those who drink more than three drinks daily have a 40% higher risk [43]. According to a meta-analysis of research published between 1966 and 2013, subjects who consume 56.5 g of alcohol daily have an overall relative risk (RR) of 1.21 (95% CI, 1.01-1.46) [103].

### Genetics

Hereditary factors contribute to 7-10% of all colorectal cancer cases. Lynch syndrome, commonly known as hereditary nonpolyposis colorectal cancer (HNPCC), is the most common inherited syndrome, which accounts for approximately 2-4% of all cases. The second most common hereditary condition is familial adenomatous polyposis (FAP), which represents less than 1% of all cases [43]. A two- to three-fold

increased risk of disease is associated with having two or more first-degree relatives (parents, siblings, etc.), and these individuals make up around 20% of all cases [104].

**Screening of colorectal cancer (CRC)**

The beginning of CRC is polyp and can prevent cancer development by removing this polyp before becoming cancerous. Thus, early detection is necessary. This information led to recommendations for screening in people that have a risk factor, such as people at an age higher than 50 years and people having a higher risk due to family history or other factors [89]. The main screen tests that are used for CRC include testing of blood in the stool by chemical testing. The fecal occult blood test (FOBT) is one of the most used methods to test for CRC screening. Occult blood in the stool may indicate CRC or polyps in the colon or rectum. There are several types of FOBT, including Guaiac fecal occult blood test (gFOBT), a traditional FOBT using a Guaiac-based test. Guaiac is oxidized by hydrogen peroxide that is present in blood in the stool [50, 105]. Immunochemical fecal occult blood test (iFOBT, or FIT) is a new generation of FOBT that measures the globin moiety of hemoglobin, a protein in red blood cells of only intact hemoglobin [105]. Sigmoidoscopy is the method to visualize the sigmoid colon, which is the lower part of the large intestine. If the polyp is found in this method, it is recommended to use a colonoscopy, which is a highly specific test that visualizes inside the entire colon and rectum [89]. Colonoscopy uses a colonoscope as a long, flexible tube. The tip of the tube has a camera and a source of light [106]. The colonoscope is inserted into the anus and then moved slowly into the rectum and through the colon, which control is visualized during the insertion of the scope [107]. During colonoscopy, if found, the polyp can be removed of all polyps before becoming cancer. Therefore, early detection and removal of polyps can reduce the risk and incidence of CRC [108-110].

**Standard treatments of colorectal cancer (CRC)**

The types of treatment for CRC that depend on type of cancer, state, age, and condition of patient [111].The common conventional methods for CRC treatment include radiation therapy, surgery, and chemotherapy, or may include some combination of each type of treatment.

Radiation therapy is most often using X-rays, an energy source to kill cancer cells. It may be used to reduce a large cancer before surgery or relieve symptoms, such as pain. Radiotherapy is usually given aiming to reduce local and regional recurrence [57, 89].

Surgery is the most common treatment for an early stage of CRC, removing the polyp or area with cancer to inhibit the cancer's development or spread to other sites of the body [57].

Chemotherapy is a drug treatment that uses chemicals to kill cancer cells that rapidly divide. The gold standard in adjuvant therapy for advanced CRC was the use fluorouracil (5-FU) or given in conjunction with leucovorin (LV) or new drugs such as oxaliplatin, irinotecan, and capecitabine (Xeloda™) that the oral FU pro-drug was utilized in combination for chemotherapy as well [42, 112]. 5-FU is an antimetabolite drug with a structural formula like uracil, acting during the S phase of the cell cycle. Once inside the cell, 5-FU is converted into several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). These metabolites inhibit thymidylate synthase (TS), a crucial enzyme involved in DNA synthesis, incorporation of its metabolites into RNA and DNA, and induce single and double strand breaks, leading to DNA fragmentation [4, 113, 114]. The event results in DNA damage, and when damage is beyond repair, the accumulation of damage leads to induced G-S phase arrest and finally inducing apoptosis via a p53-dependent pathway in cancer cells [113]. p53 is a tumor suppressor gene that can act to eliminate damaged cells beyond repair by promoting apoptosis through up-regulation of the pro-apoptotic gene and down-regulation of the anti-apoptotic gene [114]. Chemotherapy may be used before or after surgery if the cancer is larger or has spread to the lymph nodes; usually given of chemotherapy after surgery for CRC [57]. However, normally chemotherapy shows side effects in normal cells such as cells in the mouth, stomach, and bowel, hair follicles, and blood cells that rapidly divide [5]. The symptoms such as hair loss, nausea and vomiting, diarrhea, and low blood counts can occur after treatment with chemotherapy [5, 6].

## Apoptosis

Apoptosis, or programmed cell death, is the process for killing cells in multicellular organisms. In 1842, apoptosis was described as a principle for the first time by German scientist Carl Vogt. In 1972, apoptosis (pronounced "a-po-toe-sis") was originally used by Kerr, Wyllie, and Currie to refer to a morphologically special characteristic of cell death in a now-classic study by observing many different researchers during normal development and in response to various toxins. Apoptosis is a word derived from ancient Greek that translates to the "dropping off" or "falling off" of leaves from a tree, indicating the natural role of cell suicide [115]. Apoptosis occurs normally in the process of development of living organisms, both during embryonic development and in adult organisms. In adults, apoptosis occurs in cells to remove damaged cells beyond repair, infected, or potentially neoplastic cells and as an important homeostatic role to maintain cell populations in the body [116, 117]. Cells undergoing apoptosis display morphological change, including cell shrinkage, chromatin condensation, membrane blabbing, nuclear fragmentation, and chromosomal DNA fragmentation, and produce apoptotic bodies that phagocytic cells are able to digest and eliminate before the contents of the cell leak out and harm nearby cells. Apoptosis is clearly distinct from necrosis or accidental cell death, which leads to cell swelling and cell lysis and causes inflammation [116, 118]. Apoptosis occurs at the single cells or small clusters of cells, while necrosis often occurs in a group of cells or in tissue at a particular locus. The process of apoptotic were highly regulated by specific genes and biochemical pathways. The two primary apoptotic pathways are the extrinsic, also known as the death receptor pathway, and the intrinsic, or mitochondrial pathway. The intrinsic pathway is initiated by internal signals, including DNA damage, ischemia, and oxidative stress, while the extrinsic pathway is activated when a death ligand, such as TNF-α, binds to a death receptor like TNFR1. Apoptosis can contribute to a variety of diseases, such as ischemic heart disease, Alzheimer's, Parkinson's, and rheumatoid arthritis, including many types of cancer [116]. Dysregulation of apoptosis results in the accumulation of abnormal cells and finally leads to tumorigenesis induction [119]. Therefore, apoptosis induction is one of the most targeted strategies for cancer therapy.

**Biochemical and morphological changes of apoptosis**

The process of apoptosis can be characterized into biochemically and morphologically distinct phases. The distinct phases can be divided into three key phases, including initiation or induction, effector, and execution phase. First, the apoptotic machinery is activated by inducing apoptosis in response to pro-apoptotic stimuli. Next, the effector phase constitutes the activation of intermediaries that associate in the degradation phase, and the execution phase of apoptosis involves the cleave vital cellular proteins leading to the morphological changes that are the hallmarks of apoptosis [120].

Apoptosis is defined by a series of morphologic features observable through both light and electron microscopy. In cells in the early stages of apoptosis, the process is marked by cell and nuclear shrinkage, along with chromatin condensation (pyknosis) within the nucleus. Cell shrinkage: the cells are decreasing in size. After shrinking, plasma membrane blebbing, and folding around different organelles, the nucleus progressively condenses and breaks up (nuclear fragmentation). The blebs continue to form; cellular organelles and nuclear fragments are packed into the blebs (apoptotic bodies), which are separated from surrounding tissue [120, 121]. Another main biochemical change feature of apoptosis cells is phosphatidylserine (PS) flip-flops from the inner to the exterior of the plasma membrane that are recognized by neighboring cells [122]. Therefore, macrophages and several other neighboring cells, such as parenchymal cells or neoplastic cells, quickly phagocytose apoptotic bodies, which are then broken down inside phagolysosomes [121]. Removal of apoptotic bodies by phagocytes does not stimulate inflammatory and local tissue damage because cellular components of dying cells remained inside the membrane, preventing release into contact with surrounding healthy cells. The quickly phagocytosed, thus likely preventing secondary necrosis, and the phagocytes that ingest apoptotic cells have been demonstrated to release anti-inflammatory cytokines [123-126] (Figure 7).

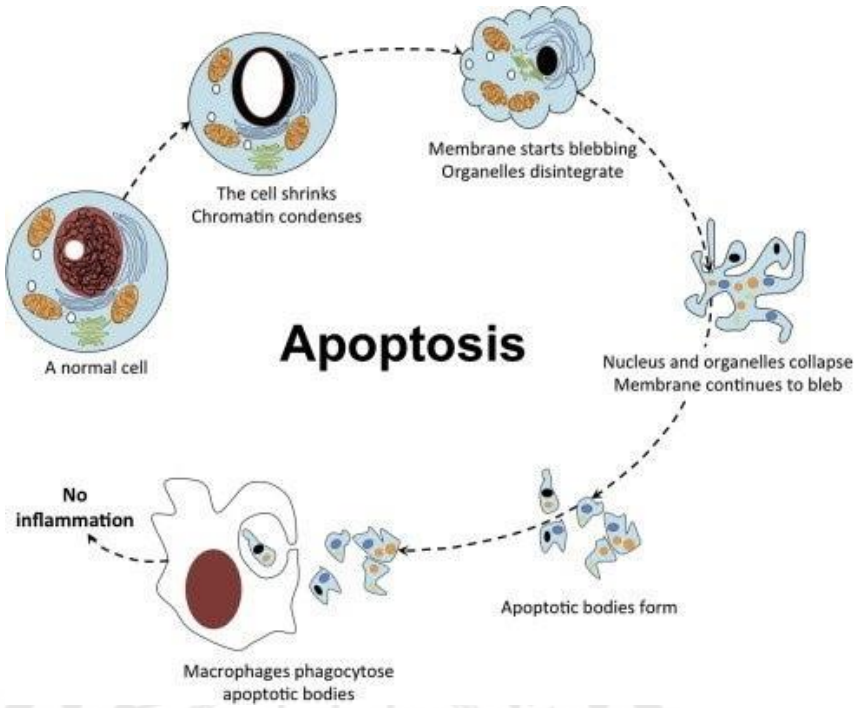


Figure 7 The morphological change in cells during apoptosis.

Source: Sushmita D. Apoptosis: morphological and biochemical changes during apoptosis, intrinsic, extrinsic, and common pathway [Internet]. 2020 [cited 2021 Oct 2]. Available from: https://www.thesciencenotes.com/apoptosis-morphological-and-biochemical-changes-during-apoptosis-intrinsic-extrinsic-and-common-pathway/

**Mediators of apoptosis**

The progression of apoptosis is systematically regulated by a series of signaling cascades involving specific events, such as signal molecules, receptors, enzymes, and gene-regulating proteins [127].

**Bcl-2 family proteins**

Bcl-2 (B-cell lymphoma 2), encoded in humans by the BCL2 proto-oncogene as a family of proteins, is recognized as a major regulator of apoptosis, especially of the intrinsic or mitochondrial apoptotic response [128]. The Bcl-2 (B-cell lymphoma-2) gene was identified at the t(14;18) chromosomal translocation site in B-cell follicular lymphomas [129]. This translocation results in activation of Bcl-2 gene expression

in human tumors [130]. This translocation results in activation of Bcl-2 gene expression in human tumors [128, 131]. The members of the Bcl-2 family are classified into three groups depending on the primary function of the protein and Bcl-2 homology (BH) domains, which include BH1, BH2, BH3, and BH4, and are composed of α-helices segments [128, 132]. All Bcl-2 family proteins contain a BH3 domain function as necessary death-promoting domains [133]. The Bcl-2 family is divided into three groups: (1) anti-apoptotic multidomain members, (2) pro-apoptotic multidomain members, and (3) pro-apoptotic BH3-only members. Anti-apoptotic multidomain members, such as Bcl-2 (B-cell lymphoma 2), B-cell lymphoma-extra-large (Bcl-xl), and Myeloid-cell leukemia 1 (Mcl-1), share sequence homology across all four BH domains (BH1-4) [128]. The main function of anti-apoptotic proteins was to inhibit cell death by restrain pro-apoptotic activity [134]. Pro-apoptotic multidomain members, including Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak), possess four BH domains and play a crucial role as key effectors of apoptosis that act downstream of both the BH3-only and anti-apoptosis members to promote permeabilization of the mitochondrial outer membrane (MOMP) [135, 136]. Pro-apoptotic BH3-only members such as BH3 interacting domain death agonist (Bid), Bcl-2 interacting mediator of cell death (Bim), and Bcl-2 associated agonist of cell death (BAD) containing the sole BH3 domain are initiators of apoptosis by activating Bax and Bak and inhibiting anti-apoptotic proteins In addition, at the C-terminus end, many Bcl-2 proteins also contain a hydrophobic transmembrane anchoring (TM) domain, which is a targeting signal for the mitochondrial outer membrane (MOM) and therefore allowing them to localize to intracellular membranes [128]. The important role of the Bcl-2 family protein is to regulate MOMP, which is well-characterized as releasing activator proteins in activating caspases [131, 137] (Figure 8).

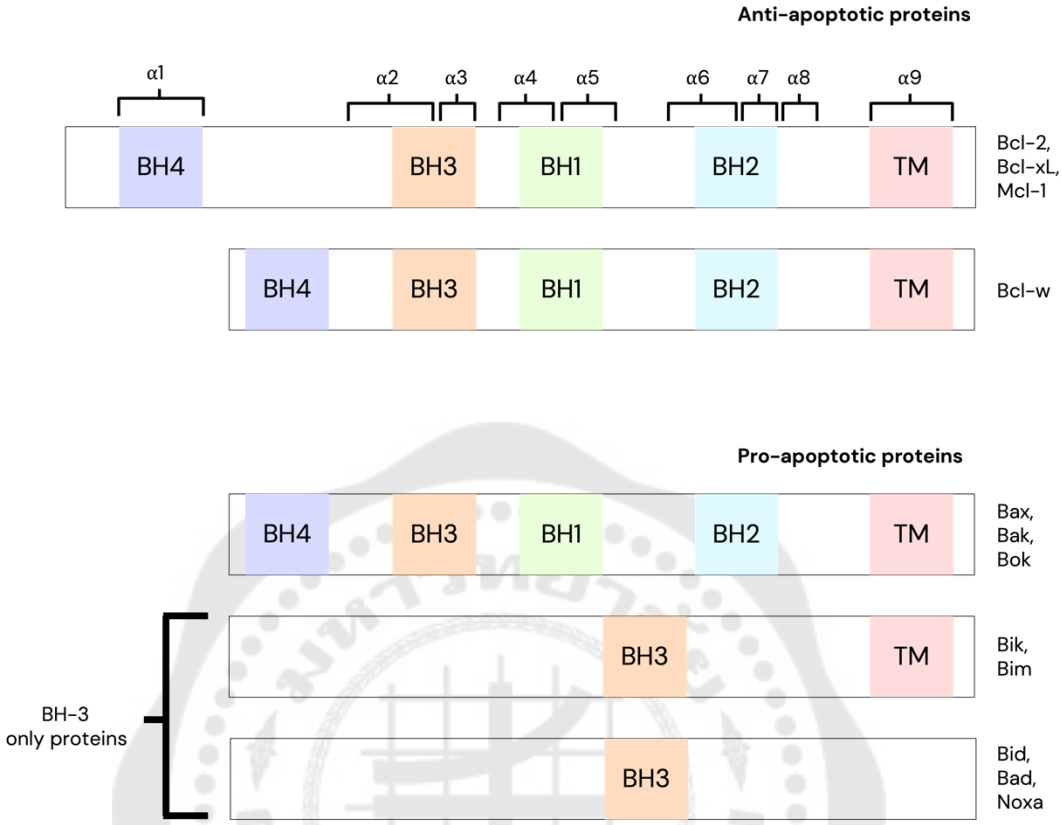


Figure 8 Classification structural of Bcl-2 family proteins.

Modified from Anantram A, Degani M. (2019).

Source: Anantram A, Degani M. Targeting cancer's Achilles' heel: role of Bcl-2 inhibitors in cellular senescence and apoptosis. Future Med. Chem. 2019;11(17):2287-312.

**Caspases in apoptosis**

Caspases (cysteine-dependent aspartate-specific proteases) are a family of cysteine endoproteases encoded by various caspase or CASP genes. They utilize a cysteine residue as the catalytic nucleophile and are characterized by their ability to specifically cleave substrates at aspartic acid residues in target proteins [138]. Caspases that play key roles in programmed cell death or apoptosis. Caspases are zymogens, also called proenzymes. Their typical structure contains three parts: an N-terminal pro-domain,

followed by a large subunit (20 kDa, p20), and a small subunit (10 kDa, p10), which is a catalytic domain. In some caspases, the pro-domain at their N-terminus contains homotypic domains, such as the caspase-recruitment domain (CARD) or the death effector domain (DED) [139, 140].

There are at least 14 distinct caspases in mammals that can be categorized into two functional groups: apoptosis caspases and inflammatory caspases. The members of apoptosis caspases can be divided into two, including initiator caspases, depending on the extended N-terminal prodomain that has an important role for its function. Initiator caspases have long prodomains (>90 amino acids) and include caspase-2, -8, -9, and -10, and executioner caspases, which have short prodomains (20-30 amino acids), include caspase-3, -6, and -7 [141, 142]. There are at least 14 distinct caspases in mammals that can be categorized into two functional groups: apoptosis caspases and inflammatory caspases. The members of apoptosis caspases can be divided into two, including initiator caspases, depending on the extended N-terminal prodomain that has an important role for its function. Initiator caspases have long prodomains (>90 amino acids) and include caspase-2, -8, -9, and -10, and executioner caspases, which have short prodomains (20-30 amino acids), include caspase-3, -6, and -7 [140]. The activation of initiator caspase pro-enzymes occurs by cleavage of a procaspase at the specific caspase cleavage sequences (Aps-X), which causes release of the prodomain and results in the combination of two large and two small subunits by hydrophobic interactions and finally to the formation into the active caspase. Consequently, active initiator caspases cleavage executioner caspases for their activation [139, 140] (Figure 9). Active executioner caspases can cleave of various target proteins that associate with hallmarks of apoptosis, and the active site comprises four surface loops, L1 through L4. The sequence specificity of caspase substrates is determined by these four active-site loops [143, 144]. The most common substrate for apoptosis is poly (ADP ribose) polymerase (PARP). The cleavage of PARP is related to DNA fragmentation, a major recognized hallmark of apoptosis [145, 146].

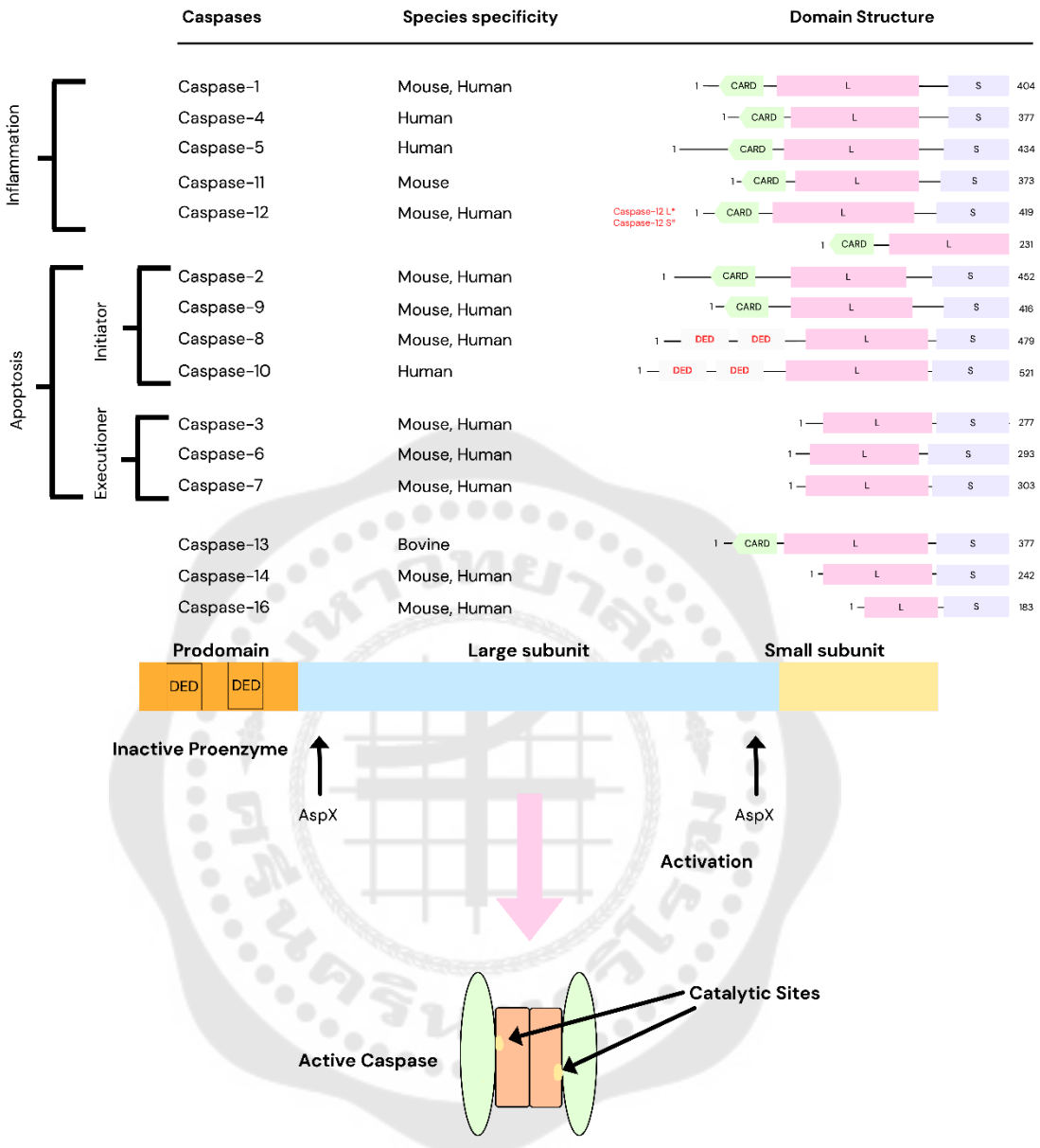


Figure 9 Overview of mammalian caspase domain structure and functional group.

Modified from Hui H, et al. (2004) and Shalini S et al. (2015).

Source: Hui H, Dotta F, Di Mario U, Perfetti R. Role of caspases in the regulation of apoptotic pancreatic islet beta-cells death. J Cell Physiol. 2004;200(2):177-200.

Shalini S, Dorstyn L, Dawar S, Kumar S. Old, new and emerging functions of caspases. Cell Death Differ. 2015;22(4):526-39.

**Poly (ADP-ribose) polymerase (PARP)**

PARP is a family of enzymes in the nucleus of cells that have at least 18 members. Numerous cellular functions, such as the control of chromatin structure, transcription, replication, recombination, and DNA repair, are mediated by these proteins. A conserved catalytic domain unites members of the PARP family, which are encoded by distinct genes. These members can catalyze the transfer of ADP-ribose to target proteins by means of poly ADP-ribosylation. Key domains of the PARP structure consist of four main regions: a DNA-binding domain, a caspase-cleaved domain, an auto-modification domain, and a catalytic domain [147, 148]. One of the most common types of DNA damage is DNA single-strand breaks (SSBs), generally repaired by various DNA repair mechanisms, and PARP is one of the proteins that play an essential role in this SSB repair pathway [149]. PARP also has an important role in cell proliferation and cell death. Especially the activity of PARP proteins in DNA repair that response to DNA damage could help to fix the incurred damage and cell survival [150]. The inactivation of this function leads towards induction of the cell death programs, such as apoptosis, that is recognized as an outcome of DNA damage [150, 151]. During apoptosis, PARP protein is cleaved by caspases, causing its inactivation [152, 153]. Therefore, PARP is one of the important proteins in apoptosis, and inhibition of PARP is one of the targets of cancer therapy, which works by preventing cancer cells from repairing and finally leading to cell death (Figure 10).

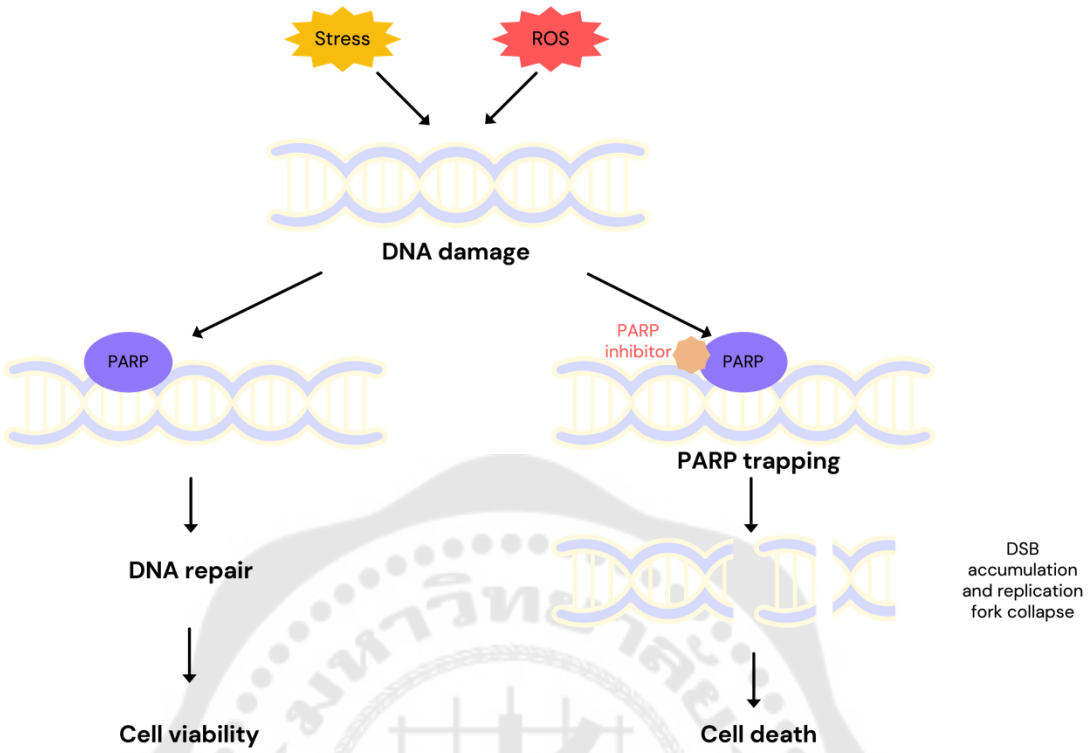


Figure 10 Overview of the function of Poly (ADP-ribose) polymerase (PARP).

Modified from Keung MYT et al. (2019).

Source: Keung MYT, Wu Y, Vadgama JV. PARP Inhibitors as a therapeutic agent for homologous recombination deficiency in breast cancers. J Clin Med. 2019;8(4).

**Apoptosis signaling pathway**

Apoptosis is regulated by high complex mechanisms, involving an energy-dependent cascade of molecular events. There are two major signaling pathways of apoptosis comprise of extrinsic are involve with death receptors, and intrinsic are involve with mitochondrial pathway [121].

**Extrinsic signaling pathway**

The extrinsic pathway is triggered by the interaction between extracellular death ligands and death receptors on the surface of target cells. Transmembrane proteins called death receptors are members of the tumor necrosis factor (TNF) receptor superfamily. They are characterized by shared cysteine-rich extracellular regions called

the "death domain," which is essential for transmitting the death signal into the cell. These receptors are activated by related ligands that belong to the TNF ligand family. The members of TNF family ligands such as tumor necrosis factor-alpha (TNF-α), TNF-related apoptosis-inducing ligand (TRAIL) or Apo 2 ligand and Fatty acid synthetase ligand (FasL), and their corresponding receptors such as TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), TRAIL receptors, Fatty acid synthetase receptor (FasR), and Death receptor (DR). The interaction of ligand to receptor on cell surface, resulting in binding between receptor to adapter proteins in cells such as Fas-associated death domain (FADD) and TNF receptor-associated death domain (TRADD), leads to the formation of a death-inducing signaling complex (DISC), resulting in the autocatalytic activation of procaspase-8. The activation of caspase-8 at the DISC could be regulated by cFLIP. Active caspase-8 activates effector or executioner caspases such as caspase-3 and caspase-7 by proteolytic cleavage of them, then cleaves downstream protein targets that are involved in chromatin condensation and DNA fragmentation, thereby representing the morphological changes of apoptosis, and finally resulting in cell death[154] (Figure 11). In addition, active caspase-8 can activate the intrinsic apoptotic pathway through cleavage of Bid to form tBid that can direct and indirect functions to activate Bax/Bak. This event allows crosstalk between the extrinsic and intrinsic pathways [155, 156].

**Intrinsic signaling pathway**

Intrinsic signaling pathway, or mitochondria-mediated apoptosis, is the signaling that occurs through mitochondria. The intrinsic pathway can be activated from intracellular signals such as growth factors, DNA damage, radiation, free radicals (stress), and toxins and was regulated by the Bcl-2 family. This intracellular process leads to the activation of the pro-apoptotic Bax and Bak in Bcl-2 family proteins. Normally, Bax and Bak activation were prevented by anti-apoptotics in the Bcl-2 family to prevent mitochondria damage. Once Bax/Bak was activated, it dissociated from the anti-apoptotic protein, leading to oligomerization in the mitochondrial outer membrane. This oligomerization forms pores and mediates mitochondrial outer membrane permeabilization (MOMP), resulting in the loss of the mitochondrial transmembrane

potential. All events allow the release of death-promoting proteins such as cytochrome c, which is an essential role for the mitochondrial pathway [121, 157]. Cytochrome c is released from mitochondria to the cytoplasm and forms an apoptosome with a binding to apoptotic peptidase activating factor 1 (Apaf-1) which functions to promote its oligomerization into the apoptosome and can interact with procaspase-9. The apoptosome activates caspase-9, which in turn cleaves and activates caspases-3 and -7. These activated caspases target essential regulatory and structural proteins for proteolysis, ultimately inducing cell death [158] (Figure 11). PARP is one of the important substrates of caspase-3 and -7; cleavage of PARP results in inactive PARP that loses activity of DNA repair associated with cell death [159]. However, activity of caspase-9 can be inhibited directly by X-linked inhibitor of apoptosis (XIAP) and degradation by proteasomes, which are in turn inhibited by mitochondrial protein Smac/DIABLO [158, 160]. Apoptosis occurs through caspase activation, known as caspase-dependent apoptosis pathways.

When apoptosis occurs, cytochrome c is released from mitochondria into the cytosol along with a number of other cell death modulators. These include endonuclease G, which also moves to the nucleus and cleaves nuclear chromatin to produce oligonucleosomal DNA fragments, and apoptosis-inducing factor (AIF), which translocates to the nucleus and causes DNA fragmentation [161-163]. Both AIF and endonuclease G operate within a caspase-independent apoptosis pathway [121].

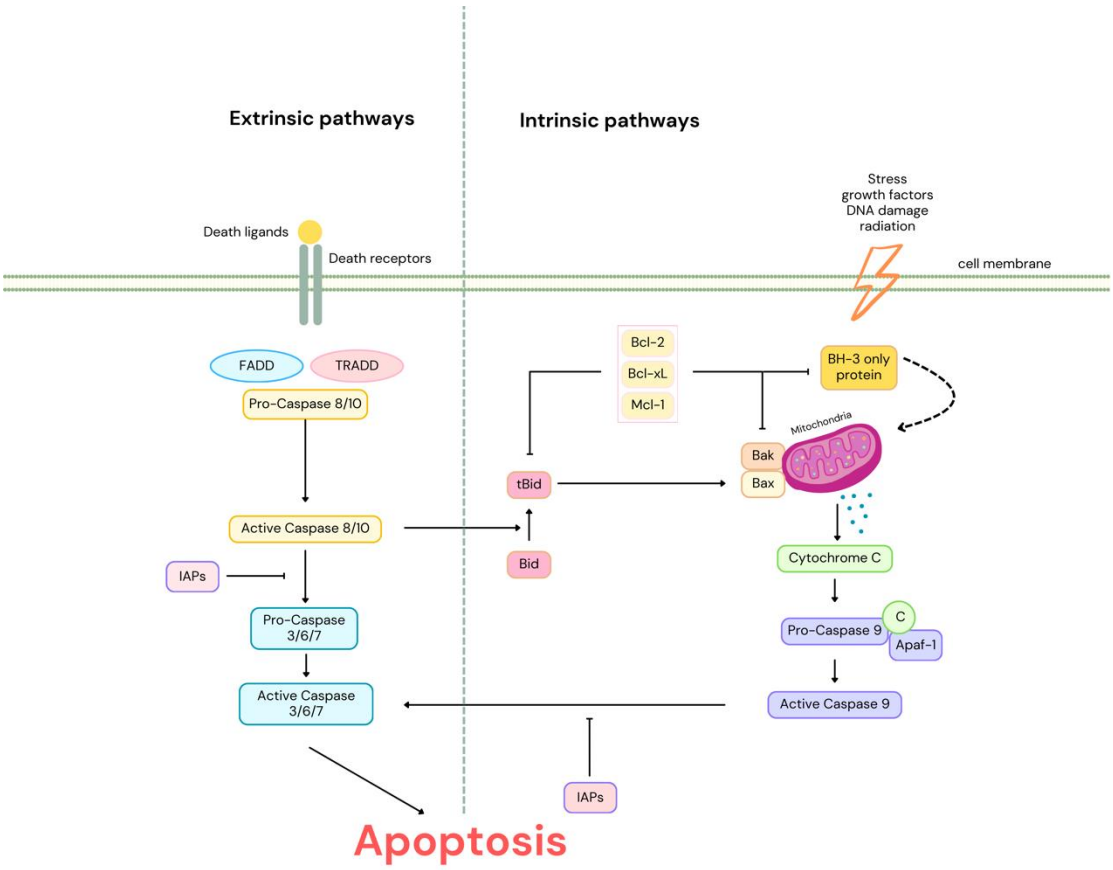


Figure 11 Overview of the intrinsic and extrinsic apoptosis pathways.

Modified from Goldar S et al. (2015).

Source: Goldar S, Khaniani MS, Derakhshan SM, Baradaran B. Molecular mechanisms of apoptosis and roles in cancer development and treatment. Asian Pac J Cancer Prev. 2015;16(6):2129-44.

**Mitogen-activated protein kinase (MAPK) signaling pathway**

MAPK is the family of serine/threonine kinases that regulate a variety of biological processes through signal transduction pathways from the cell membrane to the nucleus, controlling the expression of genes and then cellular responses such as cell proliferation, cell differentiation, and cell death [164, 165]. MAPK can be activated by several extracellular stimuli of environmental including cellular stress, cytokines, and growth factors that bind

to the receptor on the cell surface. In mammalian cells, MAPK signaling pathways activate the downstream in a series of phosphorylations in the MAPK cascade. Each cascade consists of at least three sequences, referred to as a three-tiered MAPK cascade, which includes a MAPK, a MAPK kinase (MAPKK or MAP2K), and a MAPK kinase kinase (MAPKKK or MAP3K) [166, 167]. MAPKKKs are typically activated through phosphorylation and/or interaction with extracellular stimuli, including small GTP-binding proteins (G proteins) from the Ras/Rho family. Once activated, MAPKKK phosphorylates and activates a MAPKK, which subsequently phosphorylates the MAPK, converting it into its active form. They are activated by dual phosphorylation on threonine and tyrosine residues within a conserved Thr-X-Tyr motif found in the kinase activation loop (T-loop) [168]. This phosphorylation event regulates downstream substrates of MAPK, such as transcription factors in the nucleus, leading to cellular function in response to extracellular stimuli [169] (Figure 12).

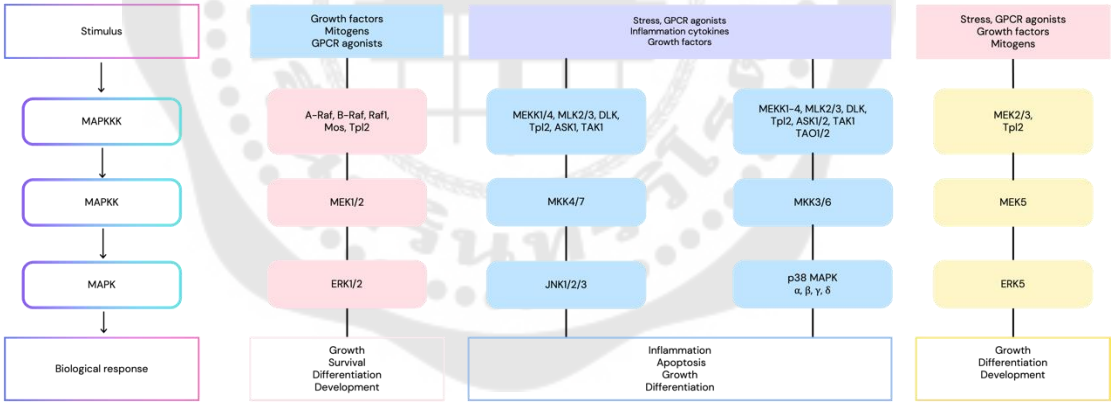


Figure 12 Schematic of MAPK signaling cascades.

Modified from Morrison DK. (2012).

Source: Morrison DK. MAP kinase pathways. Cold Spring Harb Perspect Biol. 2012;4(11).

There are three conventional MAPK signaling cascades include extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK) and p38 mitogen-activated kinases [165].

**Extracellular signal-regulated kinases (ERK1/2) signaling pathway**

The ERK family is a type of serine/threonine protein kinase, generally located in the cytoplasm. The ERK family can be subdivided into ERK 1, 2, 3, 5, and 6, of which ERK1 and ERK2 are two key important members with molecular weights of 44 and 42 kDa, respectively. ERK1/2 cascades are mostly activated by growth factors and play important roles in proliferation, differentiation, and survival [169, 170]. Activation of MEK1/2 leads to activating ERK1 and ERK2 (ERK1/2) by phosphorylation at threonine and tyrosine residues, then activating the target substrate [171]. Key molecules in the ERK pathway include the Raf family (MAPKKK), MAPK/ERK kinase1/2 (MEK1/2) acts as MAPKK, and ERK is MAPK, as well as RAS, an upstream protein of the RAF-MEK-ERK. These key molecules are one of the well-characterized MAPK pathways known as the as the RAS-RAF-MEK-ERK signaling pathway (Figure 13). The ERK1/2 signaling pathway can promote cell survival by activating transcription of pro-survival genes and controlling key molecules in the process of cell death, such as downregulation of pro-apoptotic proteins (Bim and Bad) and upregulation of anti-apoptotic proteins [167, 171]. Dysregulation of this pathway is involved in various diseases, including cancer, by activating mutations of key molecules, especially RAS (mainly KRAS) [170]. In general, ERK activation is involved in cell survival, but previous studies show that the activation of ERK can promote apoptosis. One of the mechanisms in this process is upregulation of p53 protein levels by phosphorylation at Ser15, which depends on the stimuli and cell types [167, 171].

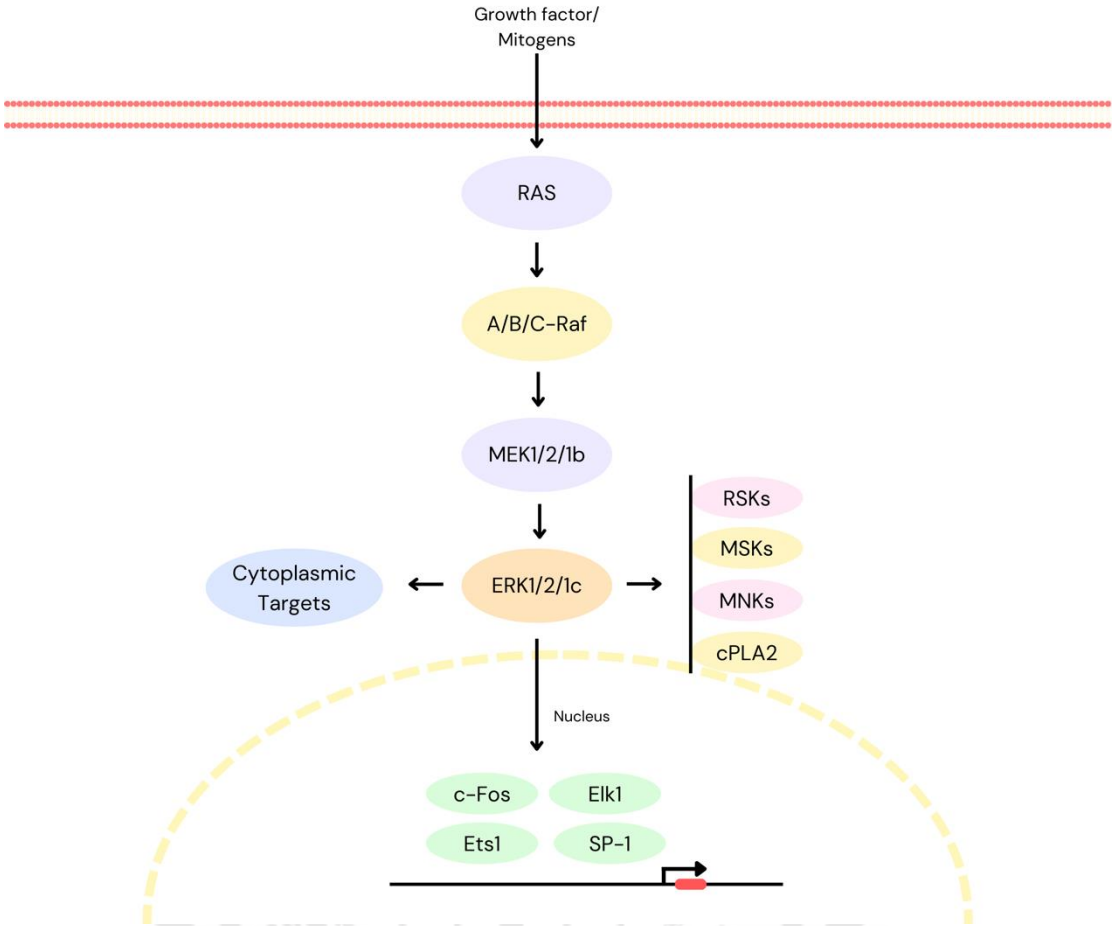


Figure 13 The MAPK/ERK or RAS/RAF/MEK/ERK signaling pathway.

Modified from Guo YJ et al. (2020).

Source: Guo YJ, Pan WW, Liu SB, Shen ZF, Xu Y, Hu LL. ERK/MAPK signalling pathway and tumorigenesis. Exp Ther Med. 2020;19(3):1997-2007.

**c-Jun N-terminal kinases (JNK) signaling pathway**

c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinases (SAPK), is a subfamily of protein kinases that contain a threonyl-prolyl-tyrosyl (TPY) motif. JNK is crucial for numerous cellular functions, including inflammation, cell proliferation, differentiation, metabolism, survival, cell migration, and apoptosis [172]. JNK family proteins, including JNK1 (SAPKγ), JNK2 (SAPKα), and JNK3 (SAPKβ), are encoded by different genes, including Mapk8, Mapk9, and Mapk10 genes, respectively [164, 168, 172].

There are activated by environmental stresses including hypoxia, UV irradiation, oxidative stress, DNA damage, infection, cytokines, and growth factor [169] and signal to the JNK. In response to these stimuli, JNK is activated through phosphorylation of the threonine and tyrosine residues in the JNK module consisting of MAPKKK, including MEKK1 and MEKK4, MLK2 and MLK3, ASK1, TAK1, and Tpl2, that phosphorylate MKK4 and MKK7 is a MAPKK, then activate JNK is a MAPK, and finally the downstream target proteins such as the transcription factor [164, 168, 172] (Figure 14). Transcription factor activator protein 1 (AP-1), which includes members including Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2), is a significant JNK target protein and p53, and the well-characteristic substrate is c-Jun that phosphorylated on Ser-63 and Ser-73 [167, 168]. JNK is implicated in both cell proliferation and apoptosis [173], For example, in cell proliferation, activated c-Jun by JNK1 and JNK2 acts as a positive regulator, resulting in an increase in the activity of AP-1 of cell growth [168] or interacts with NF-κB and JAK/STAT to promote cell survival [172]. Conversely, JNK is recognized for playing a crucial part in apoptosis. JNK can directly phosphorylate pro-apoptotic Bad and Bim to promote the apoptosis or anti-apoptotic Bcl2 to inhibit its activity. JNK can also cause p53 to be phosphorylated and p73 to become activated. As a result, pro-apoptotic proteins like Bax and Bid are up-regulated while anti-apoptotic proteins like Bcl-2, Bcl-xl, and Mcl-1 are down-regulated [167, 172, 174], which is the essential initiating protein in apoptosis induction.

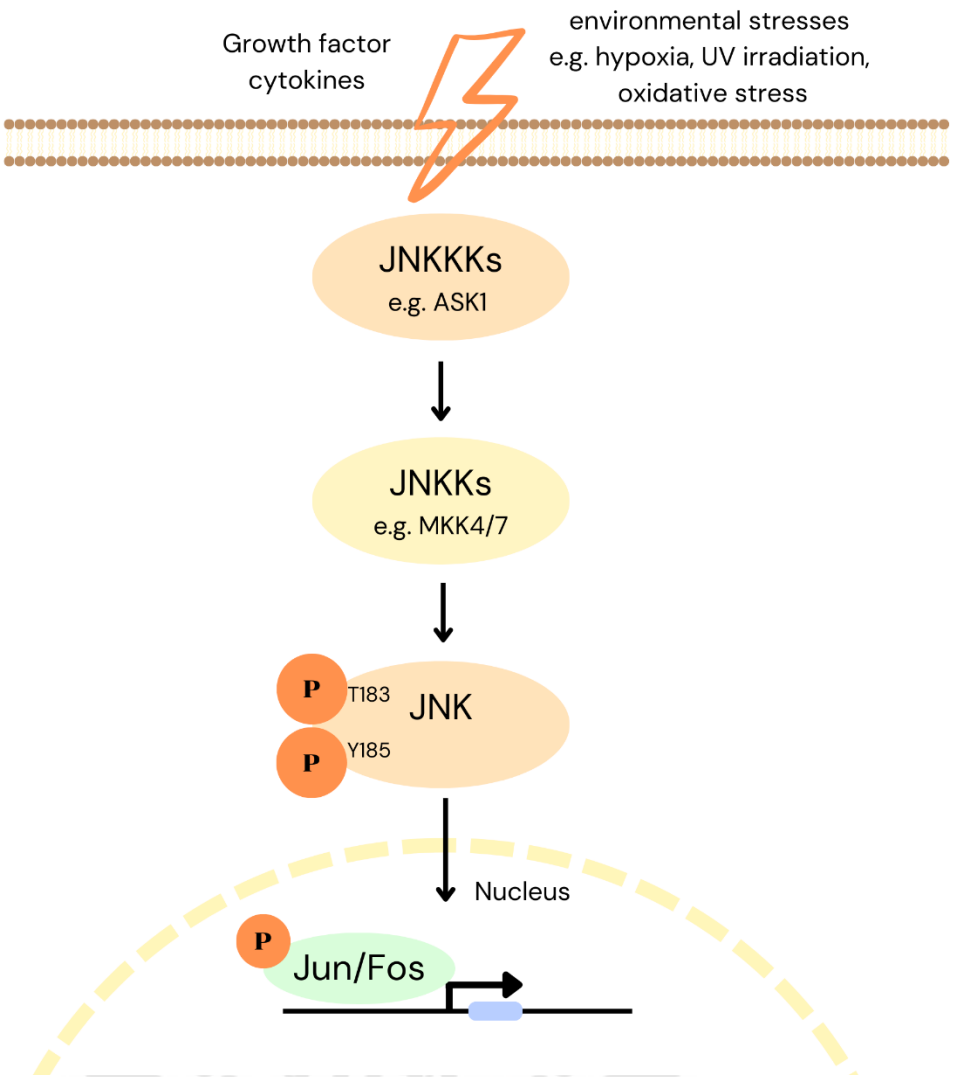


Figure 14 The regulatory system of the c-Jun N-terminal kinase (JNK) signaling pathway.

Modified from Hammouda MB et al. (2020).

Source: Hammouda MB, Ford AE, Liu Y, Zhang JY. The JNK signaling pathway in inflammatory skin disorders and cancer. Cells. 2020;9(4).

**p38-MAPK signaling pathway**

The p38 family are members of the MAPK family of serine/threonine protein kinases and known as stress-activated protein kinases (SAPKs), like a JNK that is associated with cell differentiation, survival, cell cycle regulation, inflammation, and apoptosis. In mammalian cells, there are four isoforms of p38 MAPK, including p38α, p38β, p38γ, and p38δ, of which the most well-characterized is the p38α isoform [164, 169]. Activation of p38 can be caused by environmental stresses including UV irradiation, oxidative stress, hypoxia, proinflammatory cytokines, growth factor, and certain mitogens through upstream molecule signal [168]. The four isoforms of p38 can be activated by the MKK6, which is a MAPKK via phosphorylation in the Thr-Gly-Tyr (TGY) motif at Thr180/Tyr182. Other MKK can phosphorylate some p38 isoforms [166] and the upstream of MAPKK is MAPKKK, which includes MLK2, MLK3, TAK1, ASKs, MEKKs, TAO1, and TAO2, finally leading to activating specifically selected substrates (Figure 15). The important downstream substrates or transcription factors such as p53, CHOP, c-Myc, c-FOS, MK2/3, PRAK, MSK1, and MSK2 [164, 169]. p38 MAPK is involved in several cellular responses, including cell proliferation and survival. p38 controls cell cycle progression and regulates proliferation by activating checkpoint responses. In addition, p38 MAPK can promote cell death by regulating p53 activation phosphorylate at Ser46 instead of Thr81, which encodes as a tumor suppressor. Thus, inducing proliferation arrest triggers apoptosis, and several reports have represented functions of p38 as an antitumorigenic factor [175, 176].

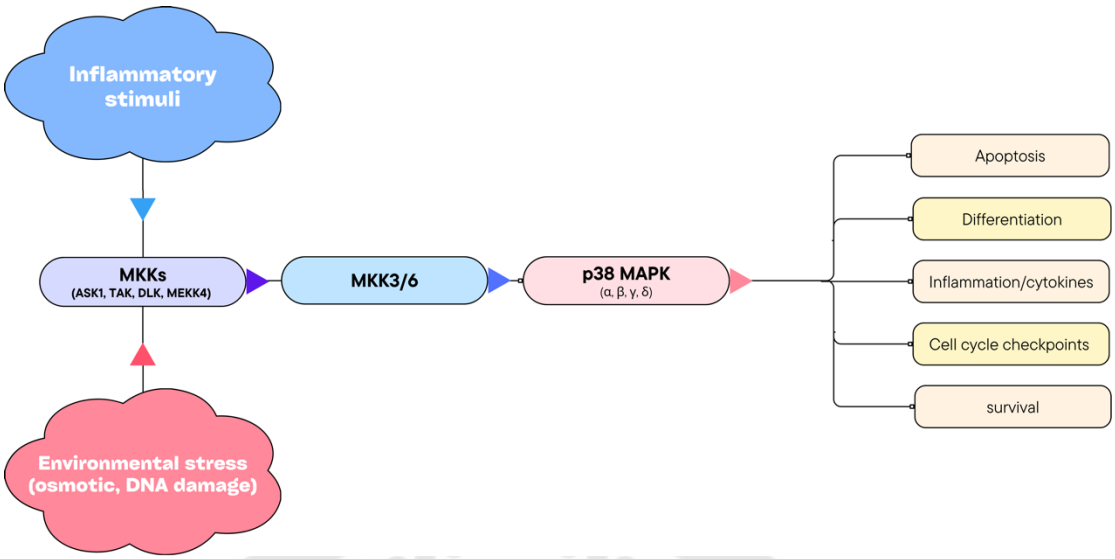


Figure 15 Overview diagram of the p38 MAPK pathway.

Modified from Krementsov DN et al. (2013).

Source: Krementsov DN, Thornton TM, Teuscher C, Rincon M. The emerging role of p38 mitogen-activated protein kinase in multiple sclerosis and its models. Mol Cell Biol. 2013;33(19):3728-34.

**Akt signaling pathway**

Akt serine/threonine kinase, known as protein kinase B (Akt), encoded by the Akt proto-oncogene, is an important kinase that is stimulated by extracellular signals such as several growth factors, nutrients, and hormones to membrane receptors, G protein-coupled receptors (GPCRs), or receptor tyrosine kinases (RTKs). Akt signaling is a crucial pathway involved in regulating various cellular processes such as cell survival, proliferation, cell growth (size), invasion/metastasis, and angiogenesis [177, 178]. The key proteins involved in this pathway are phosphoinositide 3-kinases (PI3Ks) and Akt. Activation of Akt initiates the binding of extracellular signals, such as growth factors, to the receptor on the cell surface. Activation of this receptor complex activates phosphoinositide 3-kinases (PI3Ks) and then phosphorylates phosphatidylinositol 4,5-

bisphosphate PtdIns(4,5)P2, also known simply as PIP2, which converts to the generation of intracellular second messenger phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3), abbreviated PIP3 at the plasma membrane. The process of converting PIP2 to PIP3 was reversed by phosphatase PTEN. PIP3 binds with Akt through the pleckstrin homology (PH) domain and activates PDK1 and PDK2. This leads to phosphorylation at the Thr308 site by PDK and the Ser 473 site by primarily the mammalian target of rapamycin complex 2 (mTORC2) serving as PDK2, which is the active form of Akt, to fully activate its kinase activity. Activated Akt leads to phosphorylation of its downstream targets that involve various cellular processes in various subcellular localizations and nucleus [179-182] (Figure 16). Akt has several downstream targets, including glycogen synthase kinase-3 (GSK-3) and glucose transporter 4 (GLUT4) that involve glucose metabolism and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) that regulate cell proliferation, apoptosis, and survival. One of the major targets is the mammalian target of rapamycin (mTOR), which involves the production of proteins, lipids, and nucleotides in important order to cell growth, division, and survival [183]. However, Akt could be inactivated via dephosphorylation by Akt antagonists (ceramides and PTEN) in certain steps activated by this enzyme [179], which can control the balance of this process in the human body.

The Akt signaling is important in both normal cellular physiology and various disease conditions. Dysregulation of these processes causes the continued activity of this pathway, which results in tumorigenesis [184]. Akt functions through phosphorylation and plays a role in regulating apoptosis. For example, Akt has been shown to inactivate pro-apoptotic factors such as Bad, a member of the Bcl-2 family, by preventing its interaction with Bcl-2 or Bcl-xl, and it also phosphorylates procaspase-9. Additionally, Akt phosphorylates the FOXO transcription factor, which is involved in the expression of pro-apoptotic genes, including the FAS ligand (*FasL*) gene. All these events leading to decreased apoptosis thus promote the survival of cells [179, 181, 185]. Therefore, key protein molecules in the Akt signaling pathway have become an attractive target for cancer therapy.

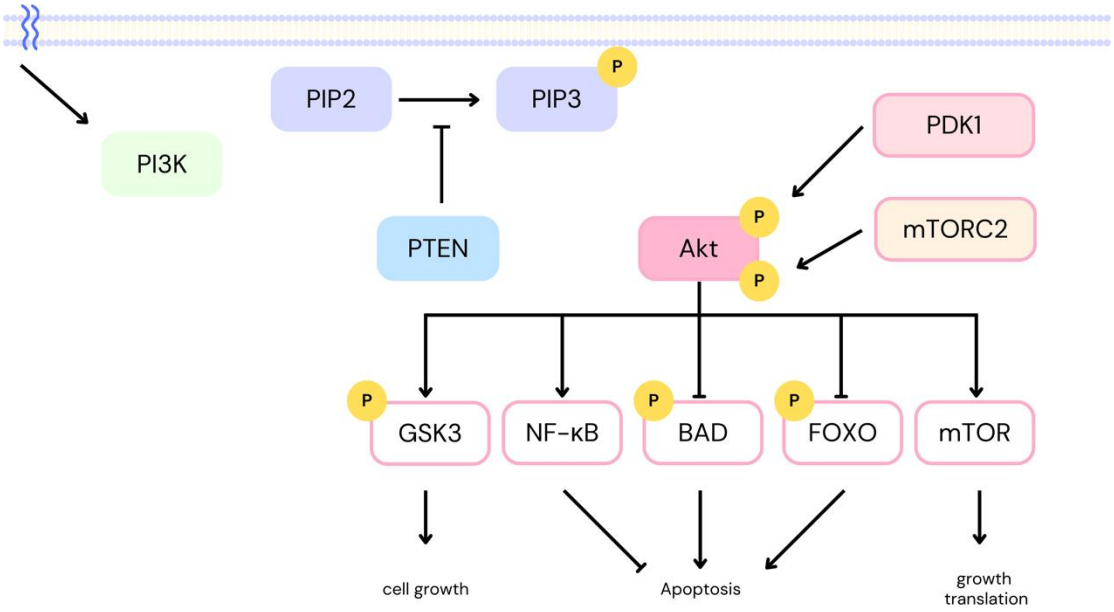


Figure 16 Mechanism of Akt signaling pathway activation.

Modified from Diehl N, Schaal H. (2013).

Source: Diehl N, Schaal H. Make yourself at home: viral hijacking of the PI3K/Akt signaling pathway. Viruses. 2013;5(12):3192-212.

**Wnt/β-catenin signaling pathway**

Wnt/beta-catenin signaling pathway, a signal transduction pathway that participates in a range of biological functions, such as tissue homeostasis, apoptosis, migration, differentiation, and cell proliferation. Wnt proteins are encoded by *Wnt* genes, secreted cysteine-rich glycoproteins that are transported out of the cell [(186)]. Under normal conditions, Wnt-on state: activation by Wnt ligands binding with cell surface receptors involves two distinct receptor families: members of the Frizzled (Fzd) family receptor and co-receptors members of the LDL-receptor-related protein (LRP) family receptor on the cell surface led to stimulate disheveled (DVL), causing the form of destruction complex to the receptor. This destruction complex acts to degrade β-catenin due to the ubiquitination process, which is composed of axis inhibition proteins (AXIN), casein kinase 1 (CK1),

glycogen synthase kinase-3β (GSK-3β), and adenomatous polyposis coli (APC). Subsequently, the phosphorylation and inhibit activity of GSK3β. Normally, β-catenin is phosphorylated by GSK3β, thus still in the form of unphosphorylated β-catenin, leading it to accumulate in the cytosol and finally translocate into the nucleus [186-189]. whereas Wnt ligands are absent or in the Wnt-off state, β-catenin is phosphorylated at serine 45 by CK1, followed by phosphorylation at threonine 41, serine 37, and serine 33 by GSK3 within the destruction complex (181) as a phosphorylated β-catenin. This process is recognized for ubiquitination of β-catenin by ubiquitin ligase E3 and finally degraded by the proteasome result to inhibit translocation into the nucleus of β-catenin (Figure 17). Upon β-catenin translocated into the nucleus, it interacts with the T cell-specific factor (TCF)/lymphoid enhancer-binding factor (LEF) family and coactivators to activate the expression of Wnt target genes such as c-Myc and cyclin D1, which are crucial for cell proliferation, differentiation, and survival [187, 189, 190]. The aberrant Wnt/β-catenin signaling pathway is associated with the tumorigenesis of several cancers [186, 189]. In addition, a previous study found that the interaction between the Wnt/β-catenin and PI3K-Akt pathways fosters tumorigenesis and leads to resistance against cancer treatments [187]. Therefore, inhibiting the Wnt/β-catenin pathway is an intriguing target for cancer treatment.

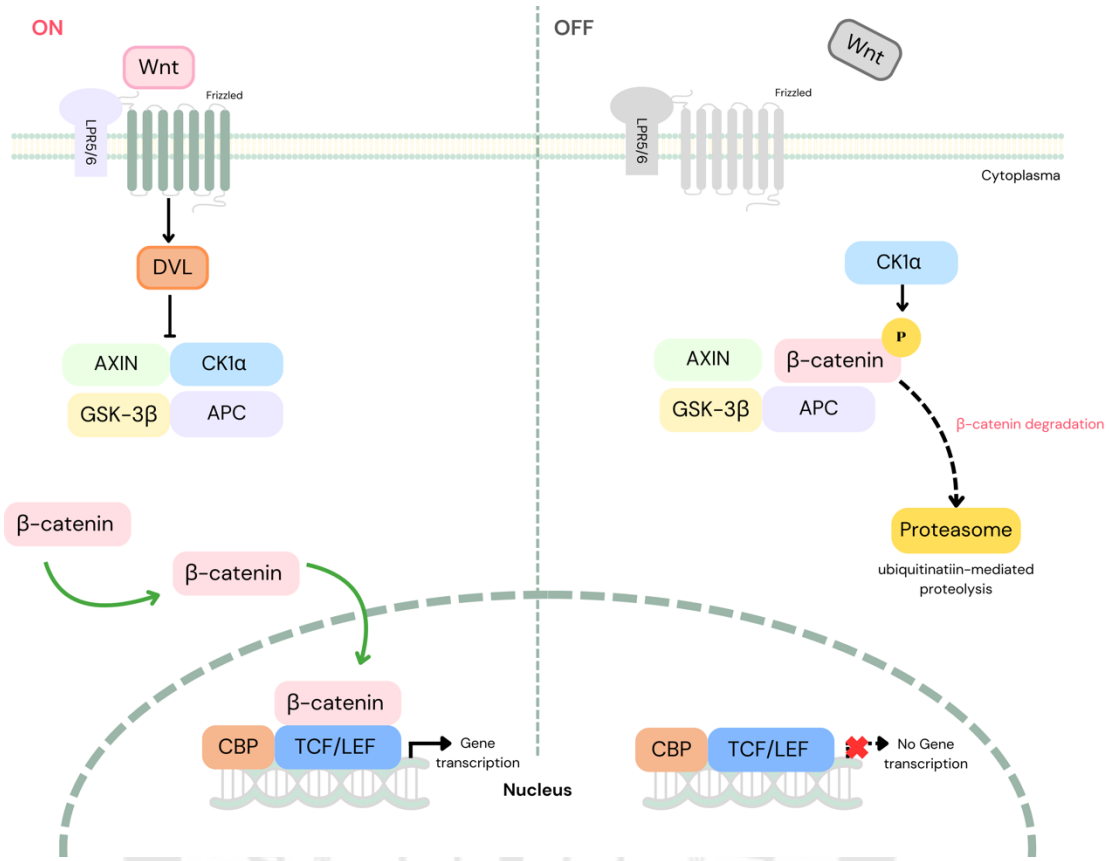


Figure 17 Overview of Wnt/β-catenin signaling pathway.

Modified from Zhang Y, Wang X. (2020).

Source: Zhang Y, Wang X. Targeting the Wnt/beta-catenin signaling pathway in cancer. J Hematol Oncol. 2020;13(1):165.

**Endoplasmic reticulum (ER) stress**

Endoplasmic reticulum (ER) stress refers to the condition in which misfolded and unfolded proteins accumulate in the ER in response to various stimuli, including metabolic stress, oxidative stress, hypoxia, and toxic chemicals. To maintain ER homeostasis, ER stress triggers the unfolded protein response (UPR). The unfolded protein response (UPR) is triggered and controlled by three ER sensors: double-stranded RNA-activated protein kinase R (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). One important regulator of the endoplasmic reticulum (ER)

stress response is glucose-regulated protein 78 (GRP78)/binding immunoglobulin protein (BIP), which binds to and inactivates three ER sensors. During ER stress, GRP78 replaces binding with misfolded and unfolded protein, which separates from the sensors and enables downstream signaling activation that controls transcription and translation to restore ER homeostasis. PERK phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2α) at serine 51, leading to the selective activation of ATF4, which subsequently triggers the transcription of C/EBP-homologous protein (CHOP). CHOP is a well-known transcription factor that, when ER stress persists or worsens, initiates cell death by intensifying oxidative damage. Apoptosis can be induced by CHOP through transcriptional up-regulation of pro-apoptotic proteins (BIM and PUMA) and down-regulation of the anti-apoptotic protein Bcl-2. Additionally, IRE1 also dimerizes and activates in response to ER stress. Activated IRE1 removes an intron from XBP1, converting it into spliced XBP1 (XBP1s). After entering the nucleus, XBP1s promotes gene transcription. During severe ER stress, IRE1α suppresses caspase-2 expression and activates JNK, leading to apoptosis. Meanwhile, activated ATF6 up-regulates CHOP, increasing caspase-3 transcriptional activity (Figure 18) [191, 192].

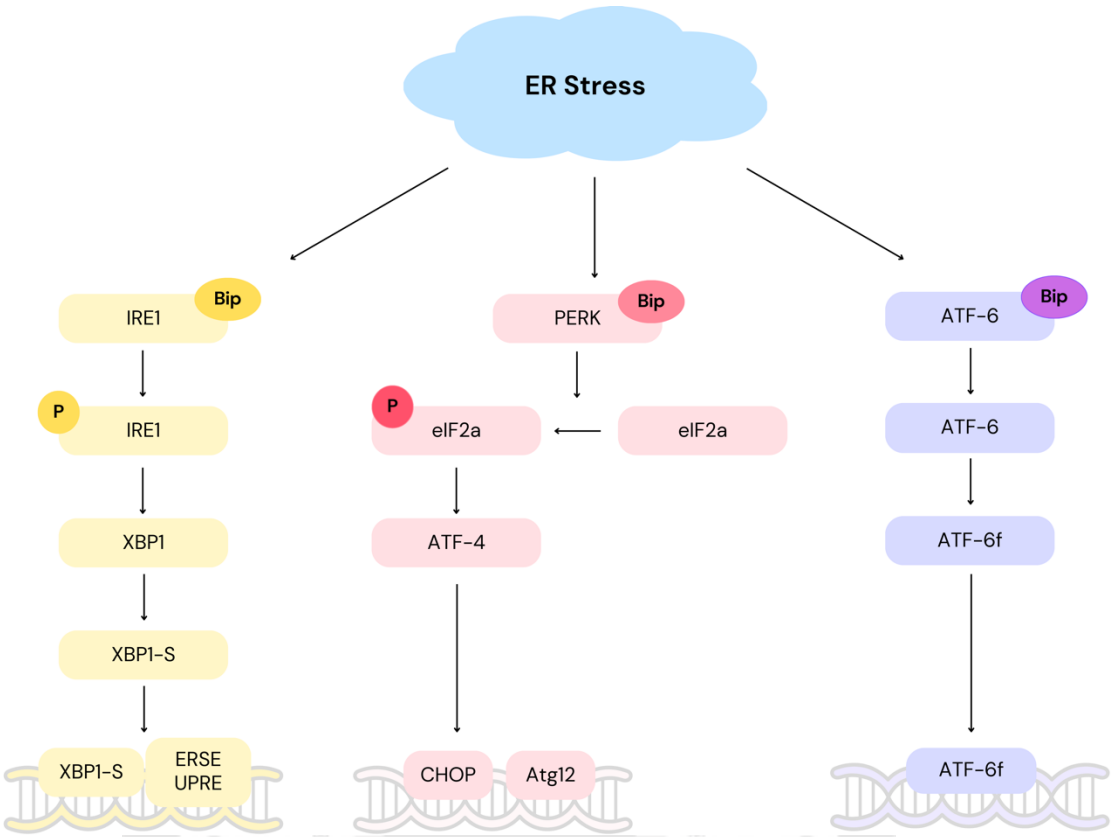


Figure 18 Overview of three UPR classical signaling pathways.

Modified from Fu X et al. (2021).

Source: Fu X, Cui J, Meng X, Jiang P, Zheng Q, Zhao W, et al. Endoplasmic reticulum stress, cell death and tumor: Association between endoplasmic reticulum stress and the apoptosis pathway in tumors (Review). Oncol Rep. 2021;45(3):801-8.

**Acacia concinna**

*Acacia concinna*, known as shikakai or sompoi, belongs to the Fabaceae family. *A. concinna* is a traditional medical plant that is found in southern and southeast Asia, such as India, Myanmar, and Thailand [9]. In Thailand, *A. concinna* is abundant in the north of Thailand. The young leaves and young pods of sompoi are also popular to use in Thai cooking.

*A. concinna* is a shrub plant that stands up to 5 m. The stem and branches are composed with privkly. Leaves are bipinnate with 5-7 pairs of pinnae and shed leaves every year. Bark is a present brown color. Flowers buds are dark red. When blooming (during mid-February-mid-April), flowers are cream or white [193]. Fruit (pod) is dry and present as a long, flat pod. Pods contain 5-12 seeds, flat oval shape, and jet-black seed (Figure 19). Previous studies reported that bark, leaves, and pods are abundant with saponins [194]. Previously, *A. concinna* was used as traditional medicine for treatment of various diseases, such as the roots, which are used for treating fever and intestinal disorder, the stems, and leaves, which are used for laxatives, and the and the stem bark, which is used for expelling phlegm and spoilage. Moreover, pods act as an expectorant, emetic, and purgative [195-197] and are used as a skin tonic and shampoo for reducing dandruff and promoting hair growth [9, 198].

Figure 19 The picture of *Acacia concinna* or Sompoi.

Source: Sitthisuk P, Innajak S, Poorahong W, Samosorn S, Dolsophon K, Watanapokasin R. Effect of Acacia concinna Extract on Apoptosis Induction Associated with Endoplasmic Reticulum Stress and Modulated Intracellular Signaling Pathway in Human Colon HCT116 Cancer Cells. Nutrients. 2024;16(21):3764.

As-well known that, plants can produce a chemical compound called “phytochemicals” through primary or secondary metabolism for plant growth or protection from dangerous pathogens and pest plants [199]. Some phytochemicals show bioactive activities associated with many diseases, including cancer. In this study, focus is on pod extracts of *A. concinna* that show high levels of saponins, about 20.8% [200]. Previous studies demonstrated that pod extract of *A. concinna* contain flavonoids, alkaloids, phenolics, tannin [9] and monoterpenoids [201].

Wuthi-udomLert and Vallisuta (2011) studied the phytochemicals of the *A. concinna* pod ethanolic extract, aqueous lyophilized, and the soxhlet ethanolic and chloroform. The result showed that the extracts presented alkaloids, saponins, tannins, flavonoids, and cardiac glycosides as major compounds but not found antraquinone and cyanotic glycosides [195]. As well as Poomanee et al. (2015) reported *A. concinna* pods extracted by 95% ethanol, 50% ethanol in water, and distilled water found alkaloids, flavonoids, saponins, and phenolics as main compounds. Furthermore, the hydroethanolic extract was dried by spray drier processes (HES) and showed the highest total phenolic (GAE value of the extract at 100.506±0.060 mg/g) and flavonoid content (RE value of the extract at 33.562±0.030 mg/g) [9]. Moreover, Tezuka et al. (2000) reported kinmoonosides A, B, and C are three major saponins found in methanolic pod extract [11].

Previous study showed that the hydroethanolic extracts dried by spray drier processes (HES) of *A. concinna* pods showed the highest scavenging DPPH and ABTS radical activity with $IC_{50}$ values of 0.8280±0.0131 mg/mL and 0.1418±0.0012 mg/mL, respectively. Furthermore, it demonstrated the strongest action against lipid peroxidation, with an $IC_{50}$ of 1.8751±0.0307 mg/mL. In addition, HES showed the greatest antityrosinase activity with an IC50 of 1.1013±0.0130 mg/mL. The results demonstrated that the *A. concinna* pod extracts showed great antioxidant activity and antityrosinase activity [9].

The previous studies suggested that *A. concinna* extracts present many pharmacological properties, such as antioxidant, anti-tyrosinase activities [9], antimicrobial activity [200] and anticancer activity [11]. The evaluation of antimicrobial activity of *A. concinna* pods extract showed activity against fungal including dermatophytes, *Candida*

*albicans*, *Cryptococcus neoformans* and *Penicillium marneffei* and opportunistic pathogens. The inhibitory effects report as the inhibition zone diameters at 18.38, 18.34, 16.67, and 14.06 mm of lyophilized, chloroform, ethanolic (Soxhlet), and ethanolic (macerated) extracts, respectively. The result was compared with ketoconazole at 31.49 mm [(195)]. The antibacterial screening of both aqueous and ethanolic extracts from *A. concinna* pods demonstrated activity against both Gram-positive and Gram-negative bacteria. The aqueous extract showed the strongest inhibition against *Bacillus subtilis*, *Staphylococcus aureus*, *Proteus vulgaris*, *E. coli* and *Micrococcus luteus*, in that order. Similarly, the ethanolic extract exhibited the highest activity against *Staphylococcus aureus*, *Micrococcus luteus*, *E. coli*, *Proteus vulgaris*, and *Bacillus subtilis*, respectively [(202)]. In addition, Todkar (2010) reported that the extracted benzene, methanol, and aqueous of *A. concinna* fresh pods showed maximum inhibition effects in positive and negative gram of bacteria [(10)].

Immunological adjuvant activities are often used to stimulate the immune system's responses to protect diseases. Kukhetpitakwong et al. (2006) indicated that the methanolic fraction (MAC) from *A. concinna* pod extract, at a dose of 40 µg/mL, could stimulate both T and B cells. Furthermore, MAC was effective in activating Th1 and Th2 cells by enhancing the levels of IgG2a, IgG2b, and IgG1. These findings suggest that MAC could be used to stimulate T helper cells (Th1 and Th2) in patients with impaired T cell function [(201)]. Additionally, *A. concinna* pod extract demonstrated significant anti-obesogenic effects by inhibiting pancreatic lipase, promoting lipolysis, and reducing lipid accumulation [(203)].

Furthermore, Tezuka et al. (2000) reported that three saponins extracted from *A. concinna* pods, including kinmoonosides A, B, and C, were obtained using $CHCl_3$, EtOAc, MeOH, MeOH-$H_2O$ (1:1), and $H_2O$. In human HT-1080 fibrosarcoma cells, these extracts demonstrated strong cytotoxicity, while in colon 26-L5 carcinoma cells, they demonstrated modest cytotoxicity, with the effective doses ($ED_{50}$) being >100 µg/mL for $CHCl_3$ and EtOAc extracts, 14.0 µg/mL for the MeOH extract, 21.9 µg/mL for the MeOH-$H_2O$ (1:1) extract, and 56.2 µg/mL for the $H_2O$ extract. This indicates that the MeOH extract

has the strongest activity [11]. Additionally, the hydroethanolic extract, dried using a spray dryer (HES), demonstrated cytotoxic effects on peripheral blood mononuclear cells (PBMCs) comparable to ascorbic acid, with $IC_{20}$ values of 280.85±16.53 µg/mL and 295.94±17.58 µg/mL, respectively. These results suggest that *A. concinna* pod extracts are safe for human use [9].

The pods of *A. concinna* extracts have been presented with many phytochemicals and pharmacological properties, but the research on this plant is very limited, especially anticancer activity. Previous research reported cytotoxicity effects on cancer cells, demonstrating that the extract has the potential to inhibit cancer. However, it has not been studied yet the mechanism and associated signaling pathways. Therefore, the effect of *A. concinna* extracts against cancer cells is interesting.

# CHAPTER 3
# MATERIALS AND METHODS

## Materials and Chemicals

| Name | Company |
|---|---|
| Fetal bovine serum (FBS) | HiMedia |
| Penicillin-streptomycin | HiMedia |
| RPMI 1640 Medium | HiMedia |
| Trypsin-EDTA | HiMedia |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich |
| MTT | Sigma-Aldrich |
| Phenyl methyl-sulfonyl fluoride (PMSF) | Sigma-Aldrich |
| JC-1 | Wako |
| Hoechst 33342 | Thermo Fisher |
| Dichlorodihydrofluorescein diacetate (DCFH-DA) | Merck Millipore |
| Guava cell cycle ® reagent | Merck Millipore |
| Sodium dodecyl sulfate (SDS) | Vivantis |
| Complete mini protease inhibitor cocktail | Roche |
| Bio-Rad Bradford Protein Assays kits | Bio-Rad |
| Bovine serum albumin (BSA) | HiMedia |
| Immobilon™ Western Chemiluminescent HRP Substrate (ECL) | Merck Millipore |
| Tetramethylethylenediamine (TEMED) | HiMedia |
| Tris | HiMedia |
| Ethylenediaminetetraacetic acid (EDTA) | HiMedia |
| Sodium Chloride (NaCl) | HiMedia |
| Triton X-100 | Panreac |
| Ammonium persulfate (APS) | HiMedia |

| | |
|---|---|
| Glycerol | Vivantis |
| 2-mercaptoethanal | ACROS |
| Glycine | HiMedia |
| Tween 20 (Polysorbate 20) | Vivantis |
| Methanol | VWR |
| Acrylamide/Bis-acrylamide solution 40% | HiMedia |
| Polyvinylidene fluoride (PVDF) membrane | Merck Millipore |

| **Equipment and Software** | **Company** |
|---|---|
| Multiskan SkyHigh Microplate Spectrophotometer | Thermo Fisher |
| Fluorescence microscope | DP73+IX71 Olympus |
| Guava EasyCyteTM flow cytometer | Merck Millipore |
| Mini Trans-Blot Cell® | Bio-Rad |
| AllianceQ9 imaging system | Uvitec |
| GuavaSoftTM software | Merck Millipore |
| GraphPad Prism 3.03 | GraphPad Software, Inc. |
| ImageJ software | ImageJ, 1.50i, NIH |
| IBM SPSS 20.0 | SPSS Software, Inc. |

**Cell culture**

Human colon cancer cell line HCT116 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown as a monolayer in Roswell Park Memorial Institute (RPMI) 1640 Medium containing penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% fetal bovine serum (FBS), cultured in a $CO_2$ incubator at 37°C under 5% carbon dioxide ($CO_2$) and saturated humidity (95%). Cells were sub-cultured every 2-3 days by using 0.25% trypsin EDTA.

**Plant extraction**

*A. concinna* pod were purchased from Thai Lanna Herbal Industry, Chiang Mai, Thailand. The specimens were dried and grounded into powder. Then, the plant powder was extracted by maceration technique in 95% ethanol for 3 days. Then, the plant extract was filtered through filter cloth and Whatman® Qualitative filter paper, evaporated to remove ethanol by using rotary vacuum evaporator, and dried by vacuum desiccator for 24 h. The *A. concinna* (AC) extract was kept at -20˚C until use in the experiment.

**Cell viability assay**

3-(4,5,-Dimethylthiazole-2-yl)-2,5 diphenyltetrazoliumbromide (MTT) assay is a colorimetric assay for measuring cell metabolic activity in living cells. This method is based on the ability of mitochondrial enzymes to reduce the MTT (yellow color) to generate insoluble formazan crystals (purple color), which can be dissolved by DMSO solution and appear as the purple color solution. The absorbance of this purple color can be measured at 570 nm by a spectrophotometer [204].

The cytotoxicity of AC extract was evaluated by MTT assay. Briefly, cells were seeded in 96-well culture plates and incubated overnight. After that, the cells were exposed to 0, 25, 50, 100, 200, 300, 400, and 500 µg/mL of AC extract, whereas 0.25% DMSO was administered to the control group for 24 h. After treatment, MTT solution (0.25 mg/mL) was added to each well and further incubated at 37°C in the dark. After incubation, MTT solution was removed and replaced with DMSO to dissolve MTT formazan crystals and shake for 10 min, respectively. The absorbance was measured at 570 nm by using a spectrophotometer. GraphPad Prism 3.03 was used to compute the cell viability percentage. The results were presented as the half-maximal inhibitory concentration ($IC_{50}$).

**Detection of nuclear morphological change by Hoechst 33342 staining**

Chromatin condensation is a hallmark of apoptosis. Briefly, during apoptosis, chromatin undergoes a phase transition from a heterogeneous state, genetically active network, to an increase in nuclear chromatin condensation that is fragmented and

produced into apoptotic bodies [120, 121]. Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]) -2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a fluorescence dye that is specific to interacting with DNA. The apoptosis cells were stained with the blue-brightness color of Hoechst33342 dye. Thus, the Hoechst33342 staining is widely used for the determination of apoptosis cells [205, 206]. To determine chromatin condensation, Hoechst33342 staining was carried out. Briefly, cells were seeded in 24-well culture plates and incubated overnight. Then, the cells were exposed to AC extract at various concentrations, whereas 0.25% DMSO was administered to the control group for 24 h. The cells were then incubated with Hoechst 33342 at 37°C for 30 mins. The images were visualized by using a fluorescence microscope.

**Cell cycle analysis by flow cytometry**

Cell cycle is the process that occurs in cell division. Cell cycle is composed of mitotic phase (M-phase), which divides the cytoplasm into two daughter cells. Whereas in interphase, cells will prepare DNA and environment for DNA synthesis before entering the M-phase. The interphase contains 3 sub-phases, including G1, S, and G2/M phases. In the G1 phase, cells prepare enzymes and an environment for DNA synthesis, while the DNA content in this phase remains diploid (2N). S phase (phase of DNA replication); cells start to synthesize DNA, resulting in tetraploid (4N). Finally. G2/M phase; after DNA replication, cells represent 4N of DNA content. During apoptosis, the DNA is degraded by cellular endonucleases into smaller fragments. Therefore, the DNA content of apoptotic cells is less than the diploid DNA content of healthy G1 cells, known as the sub-G1 phase. Thus, cell cycle analysis by flow cytometry is popular for monitoring apoptosis events in cells through counting of DNA content, called a sub-G1 assay. Cells were stained with propidium iodide (PI), used as a DNA stain. The fluorescence intensity refers to DNA content in each phase of the cell cycle [207, 208].

To investigate the cell cycle progression of cells, Cells were seeded, and different doses of AC extract were added to the cells, and 0.25% DMSO was administered to the control group. Cells were harvested, and ice-cold 70% ethanol was used for fixing. After that, 70% ethanol was removed, and cells were stained with Guava Cell Cycle® reagent.

Then, DNA content was determined by using the Guava EasyCyteTM flow cytometer and GuavaSoftTM software.

**Detection of loss of mitochondrial membrane potential (*ΔΨ*m) by JC-1 staining**

A change of *ΔΨ*m is an indication for the initiation of cells undergoing apoptosis, which can be monitored by using JC-1 fluorescence staining. 5,5',6,6'tetra-chloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1) dye is a lipophilic cationic dye that is selectively transported to the mitochondria and is popularly used to investigate the membrane potential of mitochondria. Normally, the high membrane potential is present in healthy cells, which causes the accumulation of JC-1 dye in J-aggregate form and emission of red fluorescence after staining cells with JC-1 dye. In contrast, in unhealthy cells, cells lose *ΔΨ*m and lose membrane permeability; thus, after stained cells, JC-1 remains in the monomeric form and emits green fluorescence [209].

To investigate the effect of AC extract on the loss of mitochondrial potential, we stained cells with JC-1 dye. Cells were seeded and incubated overnight. Next, different doses of AC extract were added to the cells, and 0.25% DMSO was administered to the control group. Following that, JC-1 was used to stain the cells and further incubated at room temperature for 10 min. The images were observed under a fluorescence microscope.

**Reactive oxygen species (ROS) production measurement**

It is well-established that reactive oxygen species (ROS) can have both positive and negative effects on cancer cells. Elevated ROS levels can inhibit cancer progression by causing oxidative stress, which triggers apoptosis [210]. The level of ROS can be measured by using by DCFH-DA staining. DCFH-DA is a nonpolar molecule that easily penetrates cells, where intracellular esterase converts it into DCFH, a non-permeable form that becomes trapped within the cells. ROS then oxidize DCFH into the highly fluorescent compound 2',7'-dichlorofluorescein (DCF). The level of free radicals present in the cells is indicated by the fluorescence that DCF emits [211].

To examine the impact of AC extract on ROS production, cells were stained using DCFH-DA dye. After seeding and incubating the cells overnight, varying concentrations of AC extract were applied, while 0.25% DMSO was used for the control group. Subsequently, the cells were stained with 20 μM DCFH-DA dye, incubated further, and washed three times with PBS. Fluorescent images were captured under a fluorescence microscope.

**Western blot analysis**

Western blot analysis, also known as protein immunoblotting, is a technique for determining the expression of proteins in tissue or cell samples. The principle of this method is based on the binding between the antibodies against the ligand (target protein). This technique consists of many steps, including SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred or blotted onto membranes such as cellulose or polyvinylidene fluoride (PVDF) membranes. After that, the membranes are probed with primary antibodies specific to the target protein and are detected by using a secondary antibody that will conjugate with horseradish peroxidase (HRP). The immunoreactivity protein bands were detected by using enhanced chemiluminescence's reagent (ECL) substrate system, which produces a chemiluminescence signal under a CCD camera gel documentary machine [212].

**Sample preparation**

Cells were seeded in 6-well culture plates and incubated overnight. Then, the cells were exposed to various concentrations, and 0.25% DMSO was administered to the control group. The cells were collected and lysed using RIPA buffer, which contains 10 mM PMSF and a complete mini protease inhibitor cocktail. The buffer contains 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl, and 0.5% Triton X-100. Then the protein lysates were transferred into a new tube, and the total cellular protein was determined.

**Protein determination**

The total protein concentration was determined by using Bio-Rad Bradford Protein Assays kits. Bradford reagent was diluted with distilled water in a ratio of 1:4 before use. Bovine serum albumin (BSA) was used as standard proteins with concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL. The protein samples and standard protein were mixed with 200 µl of Bradford solution. Then, protein concentration was measured at 595 nm by a spectrophotometer. Then, the protein samples were added with tracking dye (60 mM Tris-HCl; pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanal, and 0.1% bromophenol blue) and heated at 100 ˚C for 5 min in a heat box. The protein sample was kept in the refrigerator until use.

**SDS-PAGE**

Protein mixtures can be separated analytically using SDS-PAGE based on the molecular weight of negative charge proteins. The SDS solution was used to denature the disulfide bond (S-H) of proteins into linear chains and produce negatively charged proteins. Thus, after loading protein samples onto a polyacrylamide gel, all proteins were separated depending on size and migrated to a positively charged electrode. The SDS-PAGE system uses two parts of different polyacrylamide content, including stacking gel and separating gel.

**Preparation of polyacrylamide gel (PAG)**

Polyacrylamide gels are prepared by mixing acrylamide, bisacrylamide, SDS, and Tris-HCl at different pHs. The ammonium persulfate (APS) is the polymerizing agent and catalyzed by TEMED (N,N,N',N'-tetramethylenediamine), leading to the polymerization of acrylamide to form a cross-linked polymer network. The stacking gel and separating gel were prepared according to the table below.

**Separating gel**

The higher acrylamide concentration is suitable for the separation of small proteins. Whereas the lower acrylamide concentration is suitable for the separation of large proteins. Proteins of small size travel more quickly than those of large size through the gel because of the sieving effect of the pores size. Separating gel was prepared according to the table below.

Table 1 Separating gel preparation (0.375 M Tris, pH 8.8) for 1 gel

| Stock solution | 10 % | |
|---|---|---|
| $dH_2O$ | 2.42 | mL |
| 1.5 M Tris-HCl, pH 8.8 | 1.25 | mL |
| 10% SDS | 50 | µl |
| 40% acrylamide | 1.25 | mL |
| 10% ammonium persulfate (APS) | 25 | µl |
| TEMED | 2.5 | µl |
| **Total Volume** | **5** | **mL** |

**Stacking gel**

Stacking gel has a low concentration and lower pH, which has a large pore size; thus, the proteins start migrating from the same level on the base line between stacking and separating gel. In this section, proteins migrate according to their molecular weight. Briefly, stacking gel was created in accordance with the table below once the separating gel had polymerized. After loading the gel, insert a comb and allow the acrylamide to polymerize.

Table 2 Stacking gel preparation (4% gel, 0.125 M Tris, pH 6.8) for 1 gel

| Stock solution | 4 % | |
|---|---|---|
| $dH_2O$ | 1.52 | mL |
| 1.5 M Tris-HCl, pH 8.8 | 0.25 | mL |
| 10% SDS | 20 | µl |
| 40% acrylamide | 0.196 | mL |
| 10% ammonium persulfate (APS) | 10 | µl |
| TEMED | 2 | µl |
| **Total Volume** | **2** | **mL** |

**Electrophoresis and transfer**

Equal amounts of proteins were resolved on 8-12% polyacrylamide gels. Gel electrophoresis was performed under conditions of 100 V for 90 minutes in a running buffer containing 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% SDS. Following this, the proteins were transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane for 1 hour and 30 minutes in transfer buffer (0.025 M Tris-HCl, 0.192 M glycine, and 20% methanol) using a Mini Trans-Blot Cell®.

**Immunobloting**

After the proteins were transferred to the membrane, the membranes were blocked with 5% bovine serum albumin (BSA) or non-fat milk for 1 hour at room temperature. They were then washed three times with TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 10 minutes each. Following this, membranes were incubated with primary antibodies, including primary monoclonal rabbit antibodies against Bcl-2, Bcl-xl, Mcl-1 Bax, Bak, PARP/c-PARP, caspase-7/c-casepase-7, ERK, p-ERK, p38, p-p38, c-Jun, p-c-Jun, PI3K, Akt, p-Akt (Ser473), p-Akt (Thr308), p-PDK1, p-GSK-3β, GSK-3β, β-catenin, c-Myc, surviving, GRP78, p-eIF2α(Ser51), eIF2α, IRE1α (Cell Signaling Technology, Beverly, MA.) and p-IRE1α (Ser724) (InvitrogenTM, Thermo Fisher Scientific Inc., Waltham, MA, USA). The primary monoclonal mouse antibodies against CHOP (Cell Signaling Technology, Beverly, MA.) in a dilution of 1:1000 at 4 ºC, overnight. In addition, primary monoclonal mouse β-actin (Merck KGaA, Missouri, U.S.) was used as an internal control. After incubation, the membranes were washed three times with TBST for 10 minutes each. They were then incubated with secondary antibodies, either anti-mouse IgG-HRP or anti-rabbit IgG-HRP (Cell Signaling Technology, Beverly, MA.) for 1 h at room temperature. After TBST washing, immunoreactivity protein bands were developed by using an ECL reagent and detected by the AllianceQ9 advanced chemiluminescence imaging system (Uvitec, UK). The protein band intensity was assessed using ImageJ software.

**Statistical analysis**

The results were represented as the mean ± standard deviation (SD). All data was taken in triplicate from three independent experiments. Statistical significance differences between groups were analyzed by using one-way analysis variance (ANOVA) and Tukey's post hoc test. The statistical significance was considered at $p < 0.05$ and $p < 0.01$ using the SPSS statistical software package (version 20.0)

# CHAPTER 4
# RESULTS

**Effect of AC extract on cell viability**

First, we investigate the effect of AC extract on cell viability. MTT assay was used to assessed based on the ability of mitochondria enzymes in living cells to reduce the MTT (yellow color) to generate insoluble formazan crystals (purple color) [204]. HCT116 cells were treated with various concentrations of AC extract at 25, 50, 100, 200, 300, 400, and 500 µg/mL for 24 h. While the control group was treated with 0.25% DMSO. As shown in Figure 20, AC extract showed growth inhibitory effects on HCT116 cells line in a dose dependent manner, with an $IC_{50}$ value of 166.0 ± 0.44 µg/mL. These results suggest that AC extract induced cells proliferation inhibition in HCT116 cells. Based on this result, concentrations of AC extract at 50, 100, 200, and 250 µg/mL was selected for the subsequent AC treatment experiments.

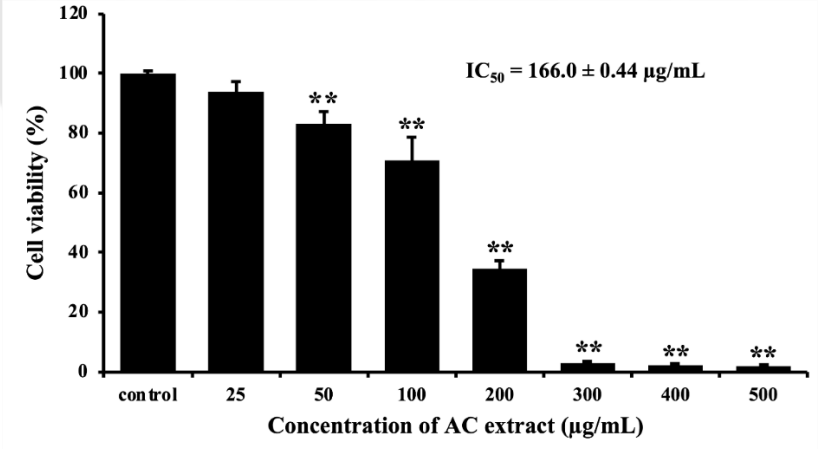


Figure 20 AC extract inhibit cell viability in HCT116 cells.

HCT116 cells were treated with several concentrations of AC extract for 24 h. The cell viability effects were examined by using MTT assay. The percentage of cell viability was showed as mean values ± standard deviation (SD) of three independent experiments in triplicate. ** $p < 0.01$ representing significant differences from the control.

**Effect of AC extract on nuclear morphology changes**

Apoptosis is the popular target for cancer treatment strategies. Therefore, we investigate the effect of AC extract on apoptosis induction. Nuclear condensation and fragmentation are markers of apoptotic cell which can be measured by Hoechst 33342 staining and was visualized under fluorescence microscope [120, 121, 205, 206]. As shown in Figure 21, AC-treated cells are smaller, brighter nuclei than control group, indicating the nuclear condensed, and could observe apoptotic bodies in a dose dependent manner. These results suggest that AC extract showed cells proliferation inhibitory effects on HCT116 cells via induced apoptosis.

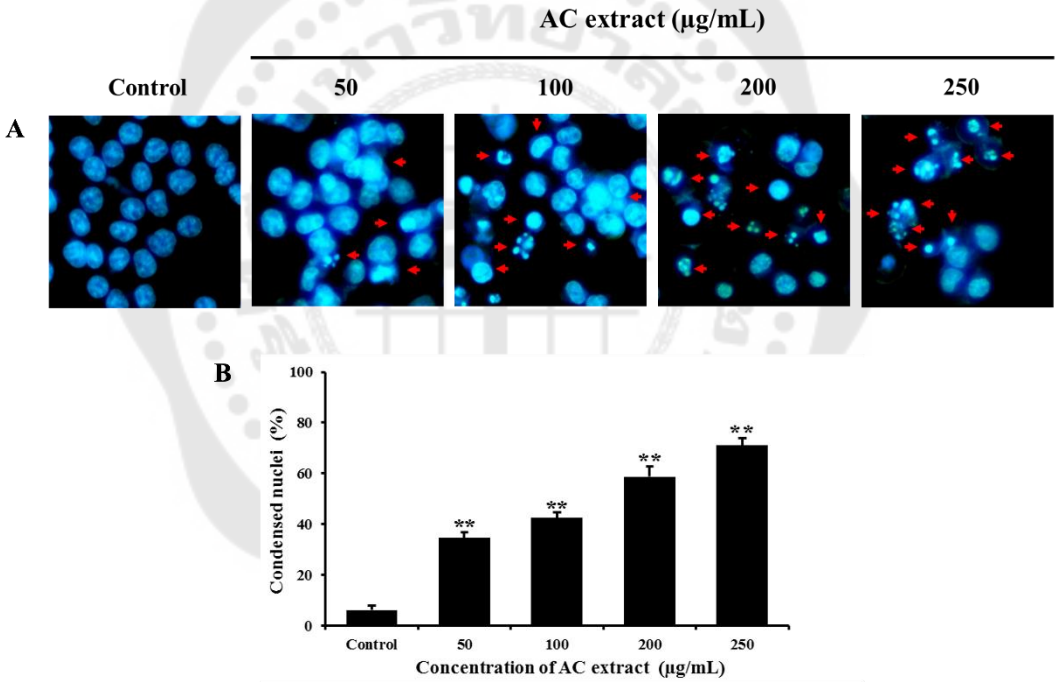


Figure 21 AC extract induce nuclear morphological change.

Cells were treated with AC extract at 0, 50, 100, 200 and 250 µg/mL for 24 h, stained with Hoechst33342 dye and visualized under fluorescence microscope. (A) The image of cells after stained with Hoechst 33342 dye. The arrows present nuclear condensation and apoptotic bodies. (B) The histogram represents the percent of nuclear condensed cells compared to the control cells. Each data bar was presented as the mean ± SD. ** $p < 0.01$ representing significant differences from the control.

### Effect of AC extract on cell cycle distribution

To confirm that AC extract could induced apoptosis. We examined the changes in the sub-G1 population of the cell cycle by flow cytometry analysis. The results found that AC extract increased the sub-G1 population, indicating genomic DNA fragmentation which is one of the markers of apoptosis. The percentages of sub-G1 population increased from 0.18% observed in the control cells to the percentages of 1.23, 2.15, 3.06, and 3.57% for concentrations of 50, 100, 200, and 250 µg/mL, respectively (Figure 22A,B). While the percentages of G1, S, and G2/M populations show no significant changes compared to the control group (Figure 22C). These findings confirm that AC extract induced apoptosis in HCT116 cells.

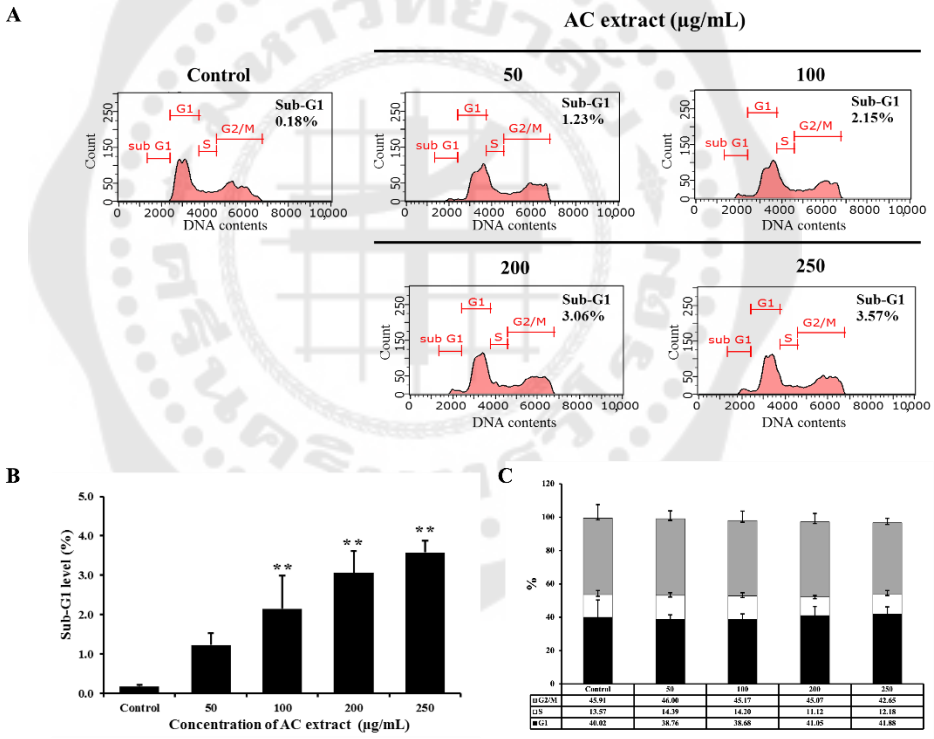


Figure 22 Effects of AC extracts on cell cycle distribution in HCT116 cells.

Histograms showing the cell populations at different stages of the cell cycle (sub-G1, G1, S, and G2/M phases), stained with Guava Cell Cycle® reagent and analyzed through flow cytometry (A). The percentages of the sub-G1 (B) G1, S, and G2/M

populations (C). Data are presented as mean ± SD, with ** $p < 0.01$ representing significant differences from the control.

**Effect of AC extract on mitochondrial membrane potential (*ΔΨ*m)**

The loss of *ΔΨ*m causes mitochondrial fission, which appears to be associated with the progression of apoptosis was examined by JC-1 staining and was visualized under fluorescence microscope. In healthy cells, represented red-fluorescence of J-aggregate form of JC-1 dye. In contrast, in unhealthy cells, represented green-fluorescence of monomeric form of JC-1 dye [209]. Our result showed that AC-treated cells significantly decrease of red fluorescence signal, indicating cells that loss of *ΔΨ*m in a dose dependent manner compare with control group as shown in Figure 23. These results suggest that AC extract induced apoptosis in HCT116 cells associated with mitochondrial damage.

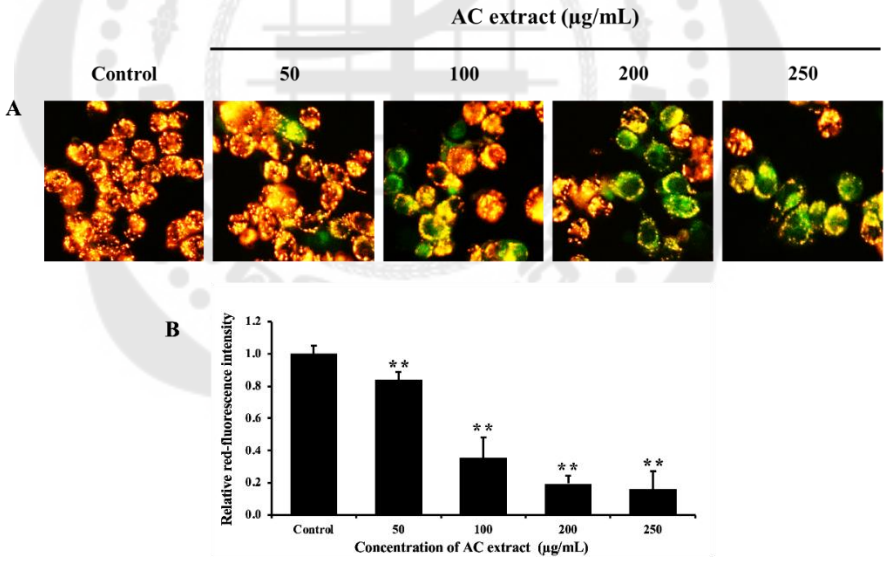


Figure 23 Effects of AC extract on the loss of mitochondrial membrane potential (*ΔΨ*m).

Cells were treated with AC extract for 9 h and stained with JC-1 dye. Green fluorescence signal indicates loss of *ΔΨ*m. (A) The image of cells after stained with JC-1 dye. (B) The histogram represents the relative red-fluorescence intensity compared to the control cells. Each data bar was presented as the mean ± SD. ** $p < 0.01$ representing significant differences from the control.

**Effect of AC extract on reactive oxygen species (ROS) production**

It well knows that the loss of $\Delta\Psi$m has been associated with intracellular ROS [213]. Thus, the intracellular levels of ROS in AC-treated cells were measured by DCFH-DA staining and visualized under fluorescence microscope. Oxidation of DCFH by ROS converts the molecule to DCF, which emits green fluorescence. Therefore, the amount of green fluorescence directly correlates with the amount of ROS [211]. Our result found that AC extract increase of green fluorescence signal, indicating increased ROS level in a dose dependent manner compare with control group in HCT116 cells as shown in Figure 24. These results indicate that AC extract mediated cancer cell apoptosis may be due to increased ROS production in HCT116 cells.

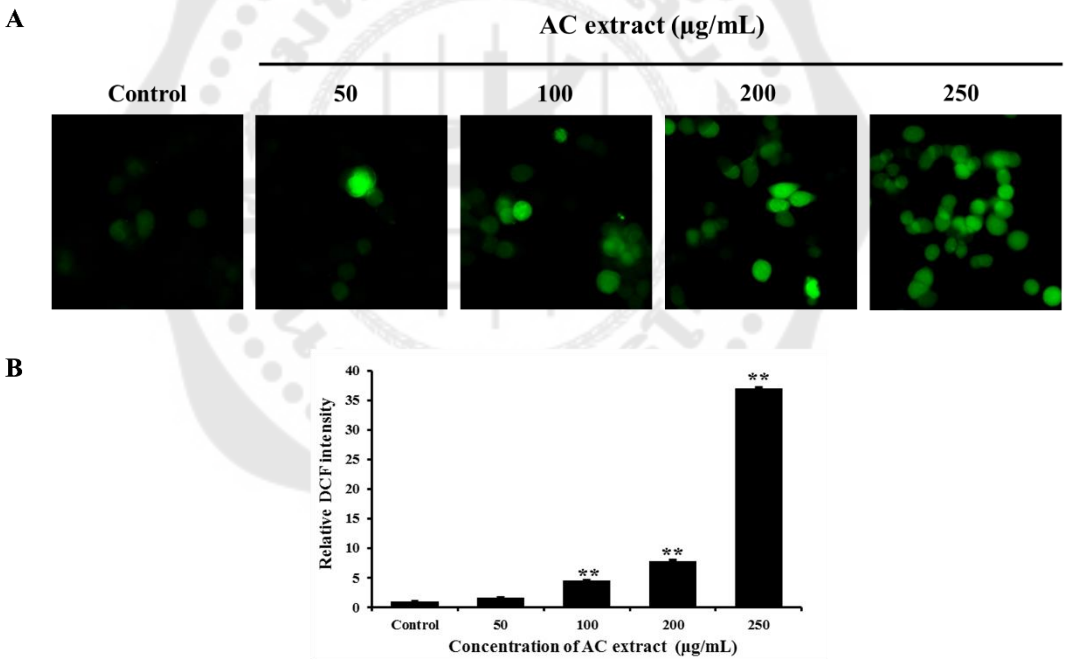


Figure 24 Effects of AC extract on ROS production.

Cells were treated with AC extract for 2 h and stained with DCFH-DA dye. Green fluorescence signal indicates the enhancement of ROS in cells. (A) The image of cells after stained with DCFH-DA. (B) The histogram represents the relative DCF-fluorescence intensity compared to the control cells. Each data bar was presented as the mean ± SD. ** $p < 0.01$ representing significant differences from the control.

**Effect of AC extract on apoptotic-related protein expression**

To clarify the molecular mechanism underlying apoptosis induced by AC extract, the expression of apoptosis-related proteins was analyzed using western blot. Caspase family of proteins plays a role in apoptosis signaling pathway. The activation of initiator caspases produces a cascade reaction, activating several other executioner caspases and its substrate leading to apoptosis [121, 159]. As shown in Figure 25, AC extract increased the level of cleaved caspase 7, an active form of caspase led to inactivated poly-ADP-ribose polymerase (PARP), a DNA repair protein by increased the level of cleaved-PARP. In addition, we also found that AC extract increased the level of the pro-apoptotic protein, Bax and Bak, and decreased the level of the anti-apoptotic protein, Bcl-2, Bcl-xL, and Mcl-1. Therefore, these results indicated that the mechanism of apoptosis induced in HCT116 by AC extract involve in activation of the caspase-dependent mitochondrial apoptosis pathway.

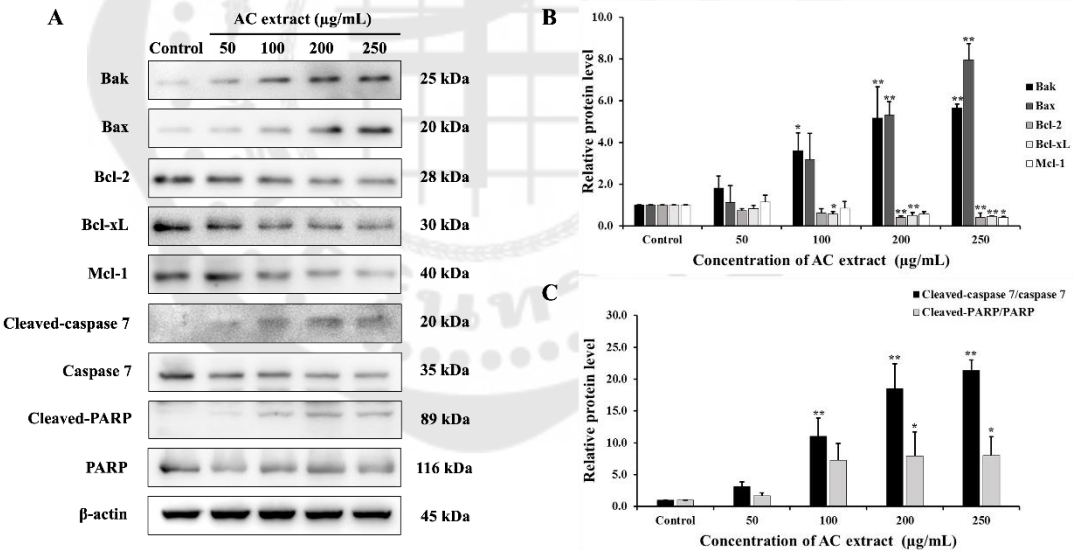


Figure 25 Effects of AC extracts on apoptosis-related proteins level in HCT116 cells.

Representative western blot results showed protein expression of Bax, Bak, Bcl-2, Bcl-xl, Mcl-1, cleaved caspase-7, caspase-7, cleaved PARP and PARP (A). The cells were treated with various concentrations of AC extracts for 24 h and detected by western blot analysis. β-actin was used as an internal control. The relative band intensity compared to the control group was analyzed using Image J software (B, C). Each data

bar was presented as the mean ± SD (n=3). * $p < 0.05$ and ** $p < 0.01$ representing significant differences from the control.

**Effect of AC extract on endoplasmic reticulum stress (ER stress)**

In present study, our results found that AC extract increased ROS production and induce apoptosis in HCT116 cells. The buildup of misfolded or unfolded proteins due to ROS-induced oxidative stress can initiate endoplasmic reticulum (ER) stress, and leads to activation of apoptosis [191, 192]. Thus, we investigate whether AC extract induce apoptosis in colon cancer cells associated with ROS-mediated ER stress pathway. Western blot result revealed that Tunicamycin (TM), an ER stress inducer increased the expression of GRP78, p-eIF2α, p-IRE1, and CHOP. Similarly with AC extract also found to increase the expression of these proteins (Figure 26). These results demonstrated that ability of AC extract on induced apoptosis may involve the accumulation of ROS which activated the endoplasmic reticulum (ER) stress.

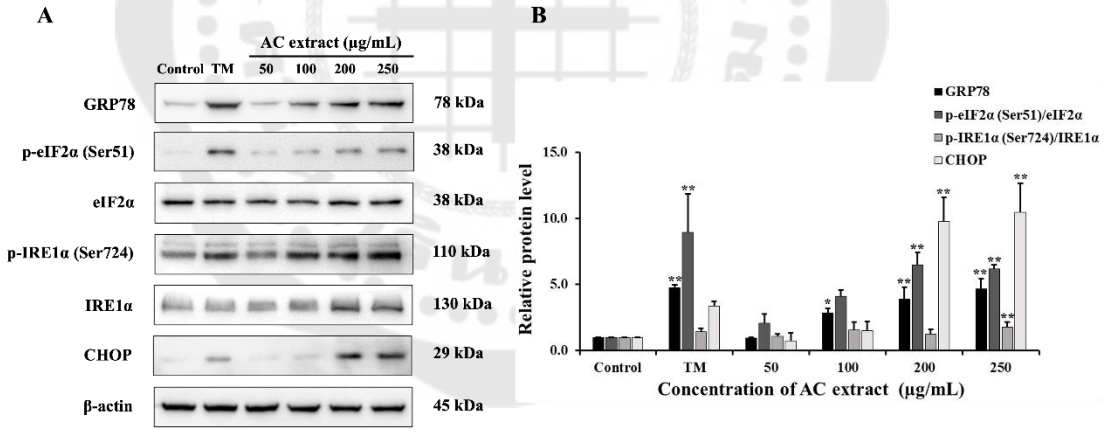


Figure 26 Effects of AC extracts on ER stress-associated proteins level in HCT116 cells.

Representative western blot results showed protein expression of GRP78, p-eIF2α, eIF2α, p-IRE1, IRE1 and CHOP (A). The cells were treated with various concentrations of AC extracts for 18 h and detected by western blot analysis. β-actin was used as an internal control. The relative band intensity compared to the control group was analyzed using Image J software (B). Each data bar was presented as the mean ± SD (n=3). * $p < 0.05$ and ** $p < 0.01$ representing significant differences from the control.

**Effect of AC extract on MAPKs signaling pathway**

MAPKs signaling pathway regulate a variety of biological processes such as cell proliferation, cell differentiation, and cell death [164, 165]. Therefore, we examined the effect of AC extract on the MAPKs signaling pathway in HCT116 cells. The cells were treated with AC extract at the concentration of 50, 100, 200, and 250 µg/mL for 24 h and we examined the levels of the total and phosphorylated form of MAPK protein by Western blotting. The results found that AC extracts increased the levels of phosphorylated ERK1/2 (p-ERK1/2), phosphorylated p38 (p-p38) and phosphorylated c-Jun (p-c-Jun) in HCT116 cells (Figure 27). These results indicated that AC extracts induce apoptosis may be mediated via MAPKs signaling pathway.

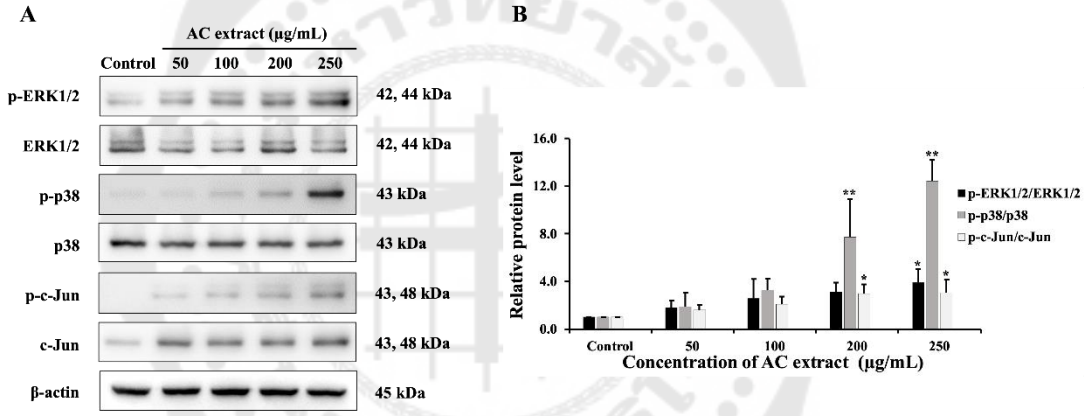


Figure 27 Effects of AC extracts on MAPK signaling pathway in HCT116 cells.

Representative western blot results showed protein expression of phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-c-Jun and c-Jun in HCT116 cells (A). The cells were treated with various concentrations of AC extracts for 24 h and detected by western blot analysis. β-actin was used as an internal control. The relative band intensity compared to the control group was analyzed using Image J software (B). Each data bar was presented as the mean ± SD (n=3). * $p < 0.05$ and ** $p < 0.01$ representing significant differences from the control.

**Effect of AC extract on PI3K/Akt signaling pathway**

PI3K/Akt signaling pathway involved in cell survival, growth, and proliferation by inhibition of apoptosis, which is the commonly activated signaling pathway in human cancers [179, 181, 184, 185]. To explore the potential role of PI3K/Akt pathway in AC-induced apoptosis in HCT116 cells, the protein levels of this pathway were examined. The cells were treated with AC extract at the concentration of 50, 100, 200, and 250 µg/mL for 24 h and examined by western blot analysis. As shown in Figure 28, treatment of HCT116 cells with AC extract significant decreased the levels of PI3K, p-PDK1, p-Akt (Ser473) and p-Akt (Thr308) compared to control group in HCT116 cells. Therefore, AC extract treatment may impair PI3K/AKT signaling pathway, contributing to increased HCT116 cell apoptosis ratio.

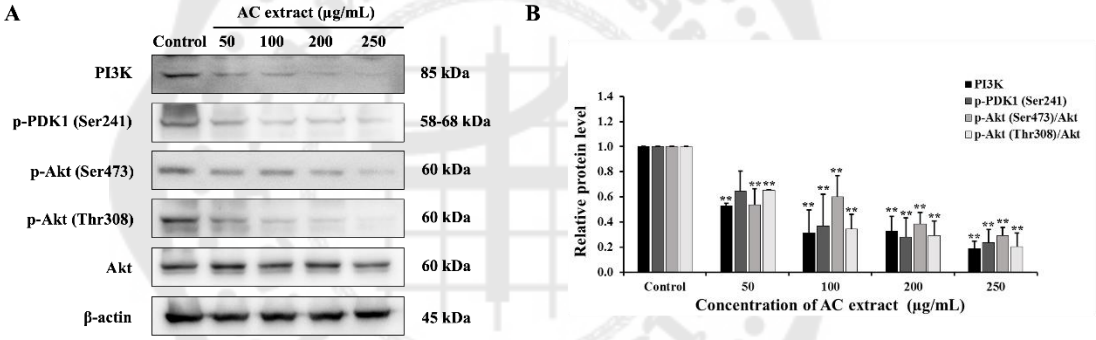


Figure 28 Effects of AC extracts on PI3K/Akt signaling pathway in HCT116 cells.

Representative western blot results showed protein expression of PI3K, p-PDK1, p-Akt (Ser473), p-Akt (Thr308) and Akt in HCT116 cells (A). The cells were treated with various concentrations of AC extracts for 24 h and detected by western blot analysis. β-actin was used as an internal control. The relative band intensity compared to the control group was analyzed using Image J software (B). Each data bar was presented as the mean ± SD (n=3). ** $p < 0.01$ representing significant differences from the control.

**Effect of AC extract on Wnt/β-catenin signaling pathway**

To further explore the effects of AC extract on the molecular mechanisms regarding the cell's proliferation and apoptosis in HCT116 cells, we examined the expression of associated proteins in Wnt/β-catenin signaling pathway plays a central role in the carcinogenesis and maintenance of CRC [186, 189]. The cells were treated with AC extract at the concentration of 50, 100, 200, and 250 μg/mL for 24 h and examined by western blot analysis. According to the Figure 29, we found that AC extract significant decreased the level of phospho-GSK-3β, β-catenin and its downstream target genes, including c-Myc, and survivin in HCT116 cells. These results indicated that AC extracts could inhibit cancer cell proliferation and induce apoptosis via inhibiting Wnt/β-catenin signaling pathway.

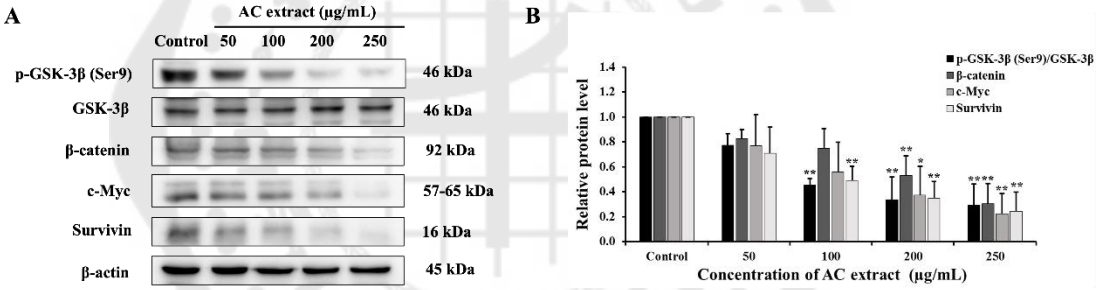


Figure 29 Effects of AC extracts on Wnt/β-catenin signaling pathway in HCT116 cells.

Representative western blot results showed protein expression of phospho-GSK-3β, GSK-3β, β-catenin, c-Myc, and survivin in HCT116 cells (A). The cells were treated with various concentrations of AC extracts for 24 h and detected by western blot analysis. β-actin was used as an internal control. The relative band intensity compared to the control group was analyzed using Image J software (B). Each data bar was presented as the mean ± SD (n=3). * $p < 0.05$ and ** $p < 0.01$ representing significant differences from the control.

# CHAPTER 5
# DISCUSSION AND CONCLUSION

The exploration of plant extracts as potential therapeutic agents against colon cancer has gained significant attention in recent years. This interest is driven by the need for safer, cost-effective, and less toxic alternatives to conventional chemotherapies. Several studies have demonstrated potent anticancer activity of various medicinal plants against colon cancer cells [8, 214]. A previous study has reported that AC extract (spray-dried hydroethanolic extract) showed lower cytotoxicity against human peripheral blood mononuclear cells (PBMCs) than ascorbic acid, with $IC_{20}$ values of 280.85±16.53 µg/mL and 295.94±17.58 µg/mL, respectively. These results indicated the safety profile of AC extract [9]. *A. concinna* extract has been reported to contain several bioactive compounds, including saponins, flavonoids, and monoterpenoids [9, 200, 201]. It's well known that these bioactive compounds have been shown to suppress the growth and proliferation of colon cancer cells [215]. It was discovered that kinmoonosides A, B, and C were present in the methanolic extract obtained from the pod. Four monoterpenoids and five prosapogenins were discovered in the crude saponin fraction of A. concinna after it was hydrolyzed alkalinely, whereas an acacic lactone was discovered after an alkaline hydrolysate was hydrolyzed acidically. The kinmoonosides A-C exhibited significant cytotoxic effects against human HT-1080 fibrosarcoma cells. These saponins demonstrated greater cytotoxicity compared to 5-fluorouracil and showed only weak cytotoxicity against colon 26-L5 carcinoma cells [11]. Another previous study also discovered that the methanolic extract of the *A. concinna* plant contained flavonoids and phenols and exhibited cytotoxic effects on MCF-7 human breast cancer cells [216]. Therefore, the anti-cancer effect of the AC extract may be due to the bioactive compounds presented, such as saponins and flavonoids. Consistent with our results, we found that AC extract decreased cell viability in HCT116 cells, a KRAS mutant colon cell line and showed lower cytotoxicity against normal cells (human keratinocyte cell line; HaCaT) [198]. For which treatment of KRAS mutant colon cancer remains a challenge.

In this investigation, we demonstrated that AC extract has potent anti-proliferation activity in HCT116 cells.

The impact of apoptosis plays a crucial role in the survival of malignant cells. Typically, cancer cells promote faster growth and extended survival compared to normal cells by inhibiting apoptosis. Additionally, defects in apoptosis signaling contribute to tumor cells' resistance to drugs [217]. Therefore, apoptosis is the main goal of cancer treatment. Many cancer drugs have an apoptosis induction potential [218, 219]. Thus, we investigate the effect of AC extract on apoptosis induction. Apoptosis induction is often achieved through the activation of caspase enzymes and the regulation of pro- and anti-apoptotic proteins. These events lead to specific morphological change, including chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies [120, 121]. These changes found in AC-treated cells have been shown to increase nuclear condensed cells, apoptotic bodies, and increased sub-G1 population, as examined by flow cytometry, indicating AC extract induces apoptosis in HCT116 cells. Moreover, AC extract induced loss of $\Delta\Psi$m in HCT116 cells leads to disrupt mitochondrial function. The decrease in $\Delta\Psi$m leads to a loss of membrane permeability, resulting in the release of apoptotic effectors, which activate various caspases and cleavage of downstream death effector protein. These events involve Bcl-2 family proteins [131, 137, 220]. In this study, AC extract increased expression of pro-apoptosis proteins and increased anti-apoptotic protein, resulting in caspase-7 activation, PARP inactivation, and ultimately inducing apoptosis in HCT116 cells. Our findings are related to a previous study indicating that morin (3,5,7,2',4'-pentahydroxyflavone), a flavonoid derived from the Moraceae family, triggers apoptosis via both the extrinsic and intrinsic pathways by upregulating the Fas receptor, activating caspases-8, -9, and -3, and promoting PARP cleavage. Additionally, morin regulates the Bcl-2 family proteins by activating Bid and induced the loss of $\Delta\Psi$m with Bax protein activation and cytochrome c release, while inhibiting the anti-apoptotic proteins Bcl-2 and cIAP-1 in HCT-116 cells [221]. Moreover, Haridas V et al. (2001) indicated triterpenoid saponins from *Acacia victoriae* (Bentham) induce apoptosis via

disruption of mitochondrial function, leading to cytochrome c release, activation of caspase 3, and total cleavage of PARP in the Jurkat human T cell line [222].

Reactive oxygen species (ROS) play a dual role in cancer biology, acting as both signaling molecules for tumor progression and mediators of cell death [223]. This excessive ROS production can overwhelm the ER, leading to ER stress [224]. In prolonged or severe ER stress, the unfolded protein response (UPR) shifts from a protective response to an apoptotic signal, leading to cell death [225]. AC extract has been shown to modulate these pathways by increasing ROS levels, which further activate eIF-2$\alpha$ and IRE1. This leads to the upregulation of pro-apoptotic molecules such as CHOP (C/EBP homologous protein), which plays a central role in ER stress-induced apoptosis by promoting mitochondrial dysfunction and caspase activation. ROS generation not only triggers ER stress but also disrupts $\Delta\Psi$m, finally leading to apoptosis. Additionally, the induction of apoptosis through ROS-mediated ER stress has been linked to the Bcl-2 family proteins. Pro-apoptotic proteins like Bax and Bak are activated in response to ER stress, while anti-apoptotic proteins like Bcl-2 and Bcl-xL are downregulated [226-228]. Interestingly, AC extract has been shown to modulate these proteins, further promoting mitochondrial dysfunction and apoptosis. Similarly, quercetin, a bioactive flavonoid, promotes apoptosis in cervical cancer HeLa cells by activating the tumor ER stress pathway. This flavonoid elevates the expression levels of caspase-3, GRP78, and CHOP, as well as the key receptor proteins, including IRE1, p-PERK, and c-ATF6 [229]. Saikosaponin A is a triterpene saponin induces apoptosis and ER stress via calcium and ROS generation in gastric cancer cells [230]. These results suggest that AC extract has shown potential in inducing apoptosis in colon cancer cells through ROS-mediated ER stress.

AC extracts also showed the anticancer effects involved several pathways that are essential for cancer cell survival, proliferation, and apoptosis, including MAPKs, PI3K/Akt, and the Wnt/$\beta$-catenin signaling pathway. MAPK is downstream of KRAS and consists of three main pathways, including ERK1/2, c-Jun, and p38 [165]. Our result found that AC extract upregulated phosphorylated ERK (p-ERK) as well as phosphorylated p38 (p-p38) and phosphorylated c-Jun (p-c-Jun) in HCT116 cells, whereas Mcl-1 could be

phosphorylated at Ser121 and Thr163 by both p-JNK and p-p38 [231]. Furthermore, JNK deactivates Bcl-2 [232] and activates Bim. Similarly, p-p38 activated Bim and inactivated Bcl-2 to cause apoptosis [233, 234]. While activation of ERK1/2 has also been reported to lead to the induction of apoptosis. Correspondingly, quercetin has been shown to exert anti-cancer effects on A375SM human melanoma cells through the activation of MAPK signaling. Quercetin increased the expression of Bax, cleaved PARP, p-JNK, p-p38, and p-ERK1/2, and decreased Bcl-2 in a concentration-dependent manner. Moreover, immunohistochemistry staining revealed that quercetin treatment also elevated p-JNK and p-p38 levels in quercetin-treated mice [235].

Moreover, our results show that AC extract inhibited PI3K/Akt pathway in accordance with Lim W et al. (2017) reported that quercetin, a prominent dietary flavonol, induced cell death in two choriocarcinoma cell lines, JAR and JEG3, by increasing the proportion of cells in the sub-G1 phase of the cell cycle. Additionally, quercetin modulated the PI3K signaling pathway by inhibiting the phosphorylation of AKT, P70S6K, and S6 proteins [236]. Hyperactivation of the PI3K/Akt pathway promotes cancer cell proliferation, survival, and resistance to apoptosis [237]. Thus, suppression of the PI3K/Akt signaling might be necessary for cell proliferation inhibition via indirectly contributing to the induction of apoptosis due to Akt showing the role to inactivating caspases [222]. Previous studies demonstrated that apoptosis was increased and cell viability was reduced when Akt was inhibited by three non-structurally related inhibitors (GSK690693, AKT inhibitor VIII, and AKT inhibitor IV) [238]. Our study indicated that AC extract may induce apoptosis through the inhibition of the PI3K/Akt pathway.

Furthermore, one molecule regulated by the Akt pathway is GSK-3β, a crucial component of the Wnt/β-catenin pathway. The Wnt/β-catenin pathway is involved in regulating cell proliferation, differentiation, and apoptosis [183, 186-189]. GSK-3β is phosphorylated by Akt at Ser9, which leads to its inactivation. Normally, GSK-3β functions as a kinase that targets β-catenin for degradation. Consequently, Akt prevents β-catenin degradation, resulting in increased nuclear β-catenin and enhanced transcription of Wnt target genes [239-241]. Activation of the Wnt/β-catenin signaling pathway leads to a reduced

Bax/Bcl-2 expression ratio, and inhibition of cytochrome c release [242]. Furthermore, studies have shown that GSK-3β is required for the survival and proliferation of human colorectal cancer cells that depend on mutant K-ras [243]. Therefore, inhibiting this pathway presents a potential therapeutic target for K-ras mutant cancers. Chen T et al. (2019) reported that Paris saponin H (Ps H) able to suppresses cell viability, migration, invasion, and promotes apoptosis in human hepatocellular carcinoma (HCC) cell lines (PLC/PRF/5 and Huh7 cells) through downregulation of β-catenin, a hallmark of Wnt/β-catenin signaling [244]. Moreover, Wang W et al. (2023) reported that quercetin inhibited the growth and migration of human GBM T98G cells, triggered apoptosis, and caused cell cycle arrest in the S-phase. Quercetin increased the Bax/Bcl-2 ratio and elevated the levels of cleaved- caspase 9 and caspase 3. The compound likely caused T98G cells to undergo apoptosis by down-regulating the expression of the essential DNA repair enzyme MGMT through the combined suppression of the Akt/NF-κB and Wnt3a/β-Catenin signaling pathways [245]. Interestingly, our results indicate that AC extract downregulate of p-GSK3β (Ser9), thereby enhancing β-catenin degradation as evidenced by decreased β-catenin and its downstream target genes, including c-Myc, Survivin, and Mcl-1 expression. These results suggest that AC extract induces apoptosis associated with inhibiting the PI3K/Akt and Wnt/β-Catenin signaling pathways.

HCT116 cells harbor a mutation in codon 13 of the KRAS proto-oncogene, which activates downstream signaling pathways, including the RAF/MEK/ERK (MAPK pathway) and the PI3K/Akt pathway. This event leads to the overactivation of both Akt and MAPK signaling, driving cell proliferation and survival in cancer cells [246]. Essentially, the mutation results in continuous signaling through Akt and MAPK, leading to their sustained overexpression. Moreover, KRAS-mutant cells are known to be resistant to several cancer therapies that target upstream receptors, such as the epidermal growth factor receptor (EGFR) pathway. Since KRAS mutations keep RAS in a permanently active state, its signaling remains independent of EGFR activity [246, 247]. Therefore, targeting downstream signaling pathways offers a potential therapeutic strategy. For instance, Encorafenib (Braftovi) for the treatment of BRAF V600E-mutated metastatic colorectal cancer (mCRC)

in combination with cetuximab (Erbitux). Encorafenib is a BRAF inhibitor that blocks the mutant BRAF V600E protein, preventing excessive activation of the MAPK (RAF/MEK/ERK) pathway, which drives tumor growth in BRAF-mutated CRC [248, 249, 250]. However, KRAS mutations also activate PI3K/Akt, promoting survival and therapy resistance. Therefore, combination with inhibitor of this pathway can help to prevent resistance. Currently, several PI3K/Akt pathway inhibitors have been evaluated in clinical trials; however, they have not received FDA approval for colorectal cancer treatment. For instance, Ipatasertib, a novel selective small-molecule ATP-competitive Akt inhibitor, has been utilized in colon cancer therapy. However, long-term use or high doses of Ipatasertib may lead to severe side effects, which restricts its widespread application in clinical practice [251]. Although ERK activation typically promotes cell proliferation, survival, and drug resistance, there are cases where apoptosis occurs despite persistently high ERK levels after treatment. This paradoxical effect is due to the dual role of ERK signaling, where its prolonged activation above a certain threshold triggers pro-apoptotic pathways [167, 171, 252]. For instance, studies have shown that ERK hyperactivation can induce cell death in KRAS-mutant cancers, suggesting that maintaining ERK activity within a specific range is crucial for tumor cell survival [252].

Interestingly, the results of this study suggest that AC extract has the potential to induce apoptosis by regulating downstream signaling pathways of KRAS, including MAPK, PI3K/Akt signaling. In addition, AC extract can also regulate apoptotic cell death through other signaling pathways, such as the inhibition of Wnt/β-catenin signaling. This indicates that AC extract possesses diverse and comprehensive therapeutic potential, making it an effective candidate for colon cancer treatment. Therefore, AC extract may serve as a potential alternative agent for colon cancer treatment.

Additionally, according to the Herbal Medicine List based on traditional knowledge, as announced by the National Drug Committee (Edition 5), the leaves and pods of Som Poi are used in combination with other herbs in the formulation of "Ya Tai Dee Klua Farang", which is indicated for relieving constipation [253]. In general, foods that help stimulate excretion are often beneficial for intestinal health, such as reducing

constipation, supporting digestive function, and minimizing toxin accumulation that can lead to chronic inflammation, a known risk factor for cancer [254, 255]. This may contribute to a lower risk of colorectal cancer (CRC) in the long term. The findings from this study suggest that plant extracts also exhibit anticancer properties. Therefore, further development of these extracts as dietary supplements could aid in cancer prevention or be used in combination with existing treatments to reduce side effects and enhance therapeutic efficacy. This presents a promising avenue for future research and development in cancer therapy.

In conclusion, our results demonstrated that AC extract has the potential to inhibit cell proliferation, and the mechanism of apoptosis induction in HCT116 might involve activation of the caspase-dependent apoptosis pathways associated with ER stress and modulation of several pathways, including MAPK, PI3K/Akt, and Wnt/β-catenin signaling pathways. Further investigation is necessary to examine the interaction between the signaling pathways triggered by AC extract in order to gain a more comprehensive understanding of its inhibitory effects on HCT116 cells.

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# APPENDIX

*nutrients*

MDPI

*Article*

# Effect of *Acacia concinna* Extract on Apoptosis Induction Associated with Endoplasmic Reticulum Stress and Modulated Intracellular Signaling Pathway in Human Colon HCT116 Cancer Cells

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**Abstract:** Background: Colorectal cancer (CRC) stands as one of the most prevalent cancer types and among the most frequent causes of cancer-related death globally. *Acacia concinna* (AC) is a medicinal and edible plant that exhibits a multitude of biological properties, including anticancer properties. This study aimed to investigate the impact of the AC extract on apoptosis induction and the underlying mechanisms associated with this effect in KRAS-mutated human colon HCT116 cells. Methods: The effect of AC extract on cell cytotoxicity was evaluated using MTT assay. Nuclear morphological changes were visualized with Hoechst 33342 staining, while mitochondrial membrane potential (MMP) was assessed via JC-1 staining. Flow cytometry was employed for cell cycle analysis, and intracellular ROS levels were determined using DCFH-DA staining. Results: The results showed that HCT116 cells exposed to AC extract showed reduced cell growth and prompted apoptosis, as indicated by an increase in chromatin condensation, apoptotic bodies, the sub-G1 apoptotic cell population, and disrupted MMP. Expression levels of apoptosis mediator proteins determined by Western blot analysis showed an increase in pro-apoptotic proteins (Bak and Bax) while decreasing anti-apoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1), leading to caspase-7 activation and PARP inactivation. AC extract was also found to enhance intracellular reactive oxygen species (ROS) levels and stimulate endoplasmic reticulum (ER) stress. Furthermore, AC extract increases the phosphorylation of ERK1/2, p38, and c-Jun while downregulating PI3K, Akt, β-catenin, and their downstream target proteins. Conclusions: These results demonstrate that AC extract could inhibit cancer cell growth via ROS-induced ER stress associated with apoptosis and regulate the MAPK, PI3K/Akt, and Wnt/β-catenin signaling pathways in HCT116 cells. Therefore, AC extract may be a novel candidate for natural anticancer resources for colon cancer treatment.

**Keywords:** *Acacia concinna*; apoptosis; colon cancer; KRAS mutant

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## 1. Introduction

Colorectal cancer (CRC) is a malignancy that develops in the colon or rectum, typically arising due to a multi-step process during which cells acquire a series of mutations [1]. Activating mutations in the oncogene Kirsten rat sarcoma (KRAS) are common, and present in up to 40% of CRC patients. These mutations are associated with enhanced cell survival and proliferation as well as reduced apoptosis, and often contribute to resistance against both conventional and targeted chemotherapies [2,3]. CRC is the second most common

cause of cancer-related deaths worldwide and the third most diagnosed type of cancer [4]. According to GLOBOCAN 2020, approximately 1.15 million new cases of colon cancer and 0.58 million deaths were estimated worldwide. The incidences and mortality rates of CRC are constantly increasing due to lifestyle and other factors, with predictions of new cases rising to 1.92 million by 2040 [5]. CRC is primarily treated by surgical removal in the early stages; however, many cases are diagnosed at later stages, leading to chemotherapy treatment in advanced cases. The common chemotherapy drugs used to treat CRC, often in combination, include 5-fluorouracil (5-FU), oxaliplatin, and irinotecan. Nevertheless, the efficacy of chemotherapy drugs is limited due to their side effects on normal cells and the emergence of drug resistance in cancer cells [6]. Thus, the search for new anticancer agents is necessary for effective cancer treatment.

Currently, plant-derived phytochemicals offering health benefits and disease prevention, including cancer, have been extensively investigated for their potential as anticancer drugs [7]. *Acacia concinna* is a traditional medicinal plant belonging to the Fabaceae family and is widely found in Asian countries, including India, Sri Lanka, Myanmar, and Thailand. It has various common names, such as Soap-Pod and Shikakai, and is well-known as "Sompoi" in Thai [8,9]. *A. concinna* is used as a shampoo and in traditional medicine for its anti-dermatophyte and antimicrobial properties in treating skin disorders [10,11]. The plant contains various chemical components, predominantly saponins, flavonoids, and monoterpenoids [9,12], and exhibits numerous pharmacological properties, including antioxidant, anti-tyrosinase activity [10], and anticancer activity [13,14]. However, the mechanisms underlying the anticancer effects of *A. concinna* extract on colon cancer remain unknown.

Apoptosis is a type of programmed cell death occurring during normal processes such as embryonic development, aging, and the removal of damaged cells, thereby maintaining cellular homeostasis [15]. Morphological characteristics of apoptosis include cell shrinkage, chromatin condensation, membrane blebbing, nuclear fragmentation, and the creation of apoptotic bodies, which are phagocytosed by neighboring cells or phagocytes. This process does not trigger any inflammatory reactions and is not harmful to the host [16,17]. The morphological changes occur consecutively to apoptotic signaling events in response to stimuli such as DNA damage and ER stress. During apoptosis, the permeability of mitochondria was disrupted due to the action of the pro-apoptotic Bcl-2 family protein and the consequent release of cytochrome c, leading to caspase-3/7 activation, which in turn cleave several key enzymes, such as poly (ADP-ribose) polymerase (PARP), resulting in irreversible apoptosis cell death [18,19]. Thus, apoptosis serves as a major mechanism of cancer therapy and has become a famous target in various treatment strategies. Additionally, many anticancer drugs exert their anticancer effects by inducing apoptosis [20,21].

Various signaling pathways affect apoptosis. The mitogen-activated protein kinase (MAPK) pathways include three main sub-families: extracellular regulated kinase (ERK), p38 kinase (p38), and Jun N-terminal kinase (JNK), which regulate a variety of biological functions, including cell growth, differentiation, and death. According to previous research, ERK1/2, p38, and JNK are important modulators of both pro-apoptotic and anti-apoptotic protein activities [22]. Meanwhile, the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB, Akt) pathway is involved in cell survival by inhibiting apoptosis via suppressing the expression of pro-apoptotic signals such as FOXO and Bad [23]. In addition, activated Akt has the capability to stimulate the Wnt/β-catenin pathway [24]. Meanwhile, activation of the Wnt/β-catenin pathway results in the suppression of apoptosis by inhibiting caspase family activity, releasing cytochrome c, and decreasing the Bax/Bcl-2 expression ratio [25].

Therefore, in this study, we investigate the effects of *A. concinna* extract on cytotoxicity and apoptosis induction in the KRAS-mutated human colon HCT116 cell line and the underlying molecular mechanism by checking various cellular signaling pathways like ER stress, MAPK, PI3K/Akt, and Wnt/β-catenin. This study presented the potential anti-colon cancer activity of *A. concinna* that may be developed as an anticancer drug or combined with other drugs for colon cancer therapies in the future.

## 2. Materials and Methods

### *2.1. Materials*

The materials for maintenance of cells; RPMI 1640 medium (Roswell Park Memorial Institute), penicillin-streptomycin, trypsin-EDTA, and FBS (fetal bovine serum) were acquired from HiMedia (HiMedia, Laboratories, Mumbai, India). MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Sigma-Aldrich (St. Louis, MO, USA), while Hoechst 33342 dye [2′-(4-Ethoxyphenyl)-6-(4-methyl-1-piperazinyl)-1H,3′H-2,5′-bibenzimidazole] was obtained from Thermo Fisher Scientific (Invitrogen™, Thermo Fisher Scientific Inc., Waltham, MA, USA). JC-1 dye (5,5′,6,6′-Tetrachloro-1,1′,3,3-tetraethylbenzimidazolylcarbocyanine iodide) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). DCFH-DA dye (dichlorodihydrofluorescein diacetate), Guava Cell Cycle® reagent, Immobilon™ Western Chemiluminescent HRP Substrate, and an anti-β-actin antibody were purchased from Merck Millipore (Merck Millipore Corp., Darmstadt, Germany). The antibodies used in Western blot analysis including anti-mouse/-rabbit primary antibody and HRP-conjugated secondary antibodies, were purchased from Cell Signaling (Cell Signaling Technology, Danvers, MA, USA) and Thermo Fisher Scientific, Inc. (Invitrogen™, Waltham, MA, USA).

### *2.2. Plant Extraction*

*A. concinna* pods were purchased from the Thai Lanna Herbal Industry, Chiang Mai province, Thailand, in September 2018. The pods were dried and ground into powder. Then, the powder (50 g) was extracted by maceration in 95% ethanol (500 mL) for 3 days in the dark at room temperature. After that, the extract was filtered through filter cloth and Whatman® Qualitative Filter Paper No. 4, followed by evaporation to remove ethanol by using a rotary vacuum evaporator. The ethanol extract of *A. concinna* (AC) was dried using a vacuum desiccator to give the extract 11.36 g (22.72%). The AC extract was then stored at −20 °C in the dark until used in the experiment. The AC extract was dissolved in DMSO.

### *2.3. NMR and MS Analysis*

The main components of the AC ethanol extract were characterized by liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS), proton nuclear magnetic resonance ($^{1}$H NMR), carbon nuclear magnetic resonance ($^{13}$C NMR), gradient correlation spectroscopy (gCOSY), gradient heteronuclear single quantum correlation (gHSQC), gradient heteronuclear multiple bond correlation (gHMBC), and diffusion ordered spectroscopy (DOSY). LC-ESI-MS analyses were run on a Thermo Scientific Dionex UltiMate 3000 Ultra-High Performance Liquid Chromatography system equipped with an electrospray ionization (ESI) source, an on-line degasser, a quaternary pump, a thermostatted column compartment, and an autosampler. Mass spectrometric detection was performed by a Bruker micrOTOF II. Separation was achieved on a C18 column 2.1 × 150 mm, 3 µm (Thermo Scientific, Waltham, MA, USA). The mobile phases consisting of solvent A (0.1% formic acid in water) and solvent B (acetonitrile) were used for the gradient elution. The $^{1}$H and $^{13}$C NMR spectra were recorded on a 500 MHz Bruker Avance NMR spectrometer, in DMSO-*d6* as solvent, and referenced to the solvent peak at 2.50 and 39.5 ppm, respectively.

### *2.4. Cell Culture and Maintenance*

The human colon cancer cell line, HCT116 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 Medium containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL). The cells were maintained in a $CO_2$ incubator at 37 °C under 5% carbon dioxide ($CO_2$) and saturated humidity (95%). Sub-culturing of the cells was performed every 2–3 days.

### *2.5. MTT Assay*

The cytotoxicity of AC extract was evaluated using the MTT assay. Cells were seeded in 96-well culture plates and incubated overnight. Following incubation, the cells were then treated with AC extract at 0, 25, 50, 100, 200, 300, 400, and 500 μg/mL, while the control cells received 0.25% DMSO for 24 h. Subsequently, the supernatant was removed, and MTT solution (0.5 mg/mL) was added. The culture plates were then incubated for 2 h at 37 °C. Following incubation, DMSO was applied to solubilize the formazan crystals. The absorbance was then measured at 570 nm using a microplate reader (Multiskan Sky Microplate Spectrophotometer, Waltham, MA, USA). Cell viability percentages (%) were calculated in comparison to the control group, and the $IC_{50}$ values were performed in GraphPad Prism 9 Software (GraphPad Prism Software, Inc., San Diego, CA, USA).

### *2.6. Nuclear Morphological Changes Detection*

Hoechst 33342 staining was performed to assess chromatin condensation. Briefly, cells were seeded and treated with AC extract at 0, 50, 100, 200, and 250 μg/mL, whereas the control cells received 0.25% DMSO for 24 h. The cells were then incubated for 30 min after being stained with Hoechst 33342 fluorescent dye. The images were visualized by using a fluorescence microscope (DP73+IX71 Olympus, Tokyo, Japan).

### *2.7. Measurement of Mitochondrial Membrane Potential (MMP)*

JC-1 staining was utilized to evaluate the effect of AC extract on MMP. Cells were seeded and treated with AC extract at 0, 50, 100, 200, and 250 μg/mL, and the control group received 0.25% DMSO for 9 h, then the cells were incubated with JC-1 fluorescence dye at room temperature for 10 min. The images were examined under a fluorescence microscope (DP73+IX71 Olympus, Tokyo, Japan).

### *2.8. Cell Cycle Analysis*

Flow cytometry was utilized to assess the impact of AC extract on cell cycle distribution. In brief, HCT116 cells were seeded and treated with AC extract at 0, 50, 100, 200, and 250 μg/mL. The control group received 0.25% DMSO for 12 h. After collecting the cells, 70% cold ethanol was used to fix the cells, and they were subsequently stained with Guava Cell Cycle® reagent (Merck Millipore Corporation, Merck KGaA, Darmstadt, Germany). The stained cells were then assessed for DNA content using the Guava EasyCyte™ flow cytometer and GuavaSoft™ software version 3.2 (Merck Millipore Corporation, Merck KGaA, Darmstadt, Germany).

### *2.9. Intracellular ROS Measurement*

HCT116 cells were seeded and treated with AC extract at 0, 50, 100, 200, and 250 μg/mL, and the control group received 0.25% DMSO. They were then incubated with 20 μM DCFH-DA fluorescence dye at 37 °C for 30 min. After incubation, stained cells were examined using a fluorescence microscope (DP73+IX71, Olympus, Tokyo, Japan).

### *2.10. Western Blot Analysis*

Protein expression was evaluated by Western blot analysis. The cells were collected, and RIPA lysis buffer (250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% Triton X-100, protease inhibitor cocktail, and 10 mM PMSF) was utilized for extracting the total protein from the cells. Following SDS-PAGE separation, the protein mixture was transferred to PVDF (polyvinylidene fluoride) membranes. The membranes were subsequently blocked with blocking buffer for 1 h, incubated with primary antibodies targeting the specific proteins of interest at 4 °C overnight, and incubated with HRP-conjugated secondary antibodies for 1 h, respectively. The immunoreactivity protein bands were exposed by using chemiluminescent HRP substrate (ECL) and visualized using a gel documentary machine (AllianceQ9 advanced, Cambridge, UK). ImageJ software version 1.53e was used

to quantify the intensity of the protein bands, and the results were presented as the ratio relative to the intensity of β-actin.

### *2.11. Statistical Analysis*

The data were expressed as the mean ± standard deviation (SD). Statistically significant differences between groups were evaluated using one-way analysis variance (ANOVA), and Tukey's post hoc test was then employed. Statistical analysis was performed using SPSS statistical software package version 20.0 (IBM Crop., Albany, NY, USA). Statistical significance was defined as $p < 0.05$ and 0.01.

## 3. Results

### *3.1. NMR and MS Analysis of AC Extract*

The LC-ESI-MS spectrum of the AC ethanol extract indicated the presence of flavonoids in comparison with the previous report on Acacia pod extracts [26]. The main peaks showed $[M + Na]^+$ ion with $m/z$ 325.2 and $[M + H]^+$ ion with $m/z$ 319.2 corresponding to pentahydroxyflavone and hexahydroxyflavone, respectively. In order to obtain more data on chemical compositions in the AC ethanol extract, 1D and 2D NMR spectroscopic analyses were performed. Even though the signals in the $^1$H NMR spectrum (Figure 1A) overlapped, some information was discernible. The presence of angular methyl and methylene protons, sugar protons, and olefinic protons signals in the range of δ 0.7–2.4, 3.0–5.0, and 5.5–6.5, respectively, could be observed. The $^{13}$C NMR (Figure 1B) indicated anomeric carbons, olefinic carbons, and carbonyl carbons signals in the region of 93–112, 121–145, and 162–215, respectively. Comparing the NMR data with the previous report [13] suggested that acacic acid-type saponins were in the extract. The HMBC experimental data indicated that the methylene proton signal observed at δ 2.17 (H-22) correlated with the carbonyl carbon signal at δ 174.5 (C-28). Moreover, the methine proton signal at δ 5.25 (H-21), olefinic proton signal at δ 6.70 (H-3′), and methylene proton signal at δ 4.12 (H-9′) correlated with the carbonyl carbon signal at δ 166.1 (C-1′). This clearly indicates that the extract contained acacic acid-type saponins (Figure 1C).

### *3.2. AC Extract Reduces Cell Viability in HCT116 Cells*

First, we determined whether AC extract can inhibit the proliferation of HCT116 cells by using the MTT assay. We found that AC extract significantly reduced cell viability on HCT116 cells with an $IC_{50}$ value of 166.0 ± 0.44 μg/mL (Figure 2). In addition, we tested the effect of AC extract in normal cells (HaCat cells), and the $IC_{50}$ value was 371.7 ± 1.477 μg/mL (Figure S3). This result suggested that AC extract could inhibit the proliferation of HCT116 cells.

### *3.3. AC Extract Induces Apoptosis in HCT116 Cells*

Most anticancer drugs have properties that induce apoptosis [20]; thus, we next determine whether the proliferation inhibitory effect is associated with apoptosis induction. The nuclear morphological changes and MMP detection, which are major characteristics of apoptosis, were carried out using Hoechst 33342 and JC-1 staining, respectively. As shown in Figure 3A, after treatment for 24 h, the AC-treated cells have smaller, brighter nuclei than the control group, indicating nuclear and cytoplasmic condensation in a dose-dependent manner. Cell shrinkage as well as apoptotic bodies were also observed. Additionally, AC extract could induce the loss of MMP in the HCT116 cells demonstrated by the reduced red fluorescence of JC-1 dye (Figure 3B). This result revealed that AC treatment led to mitochondrial dysfunction. Taken together, our findings demonstrate that AC extract inhibited the proliferation of HCT116 cells through apoptosis induction.

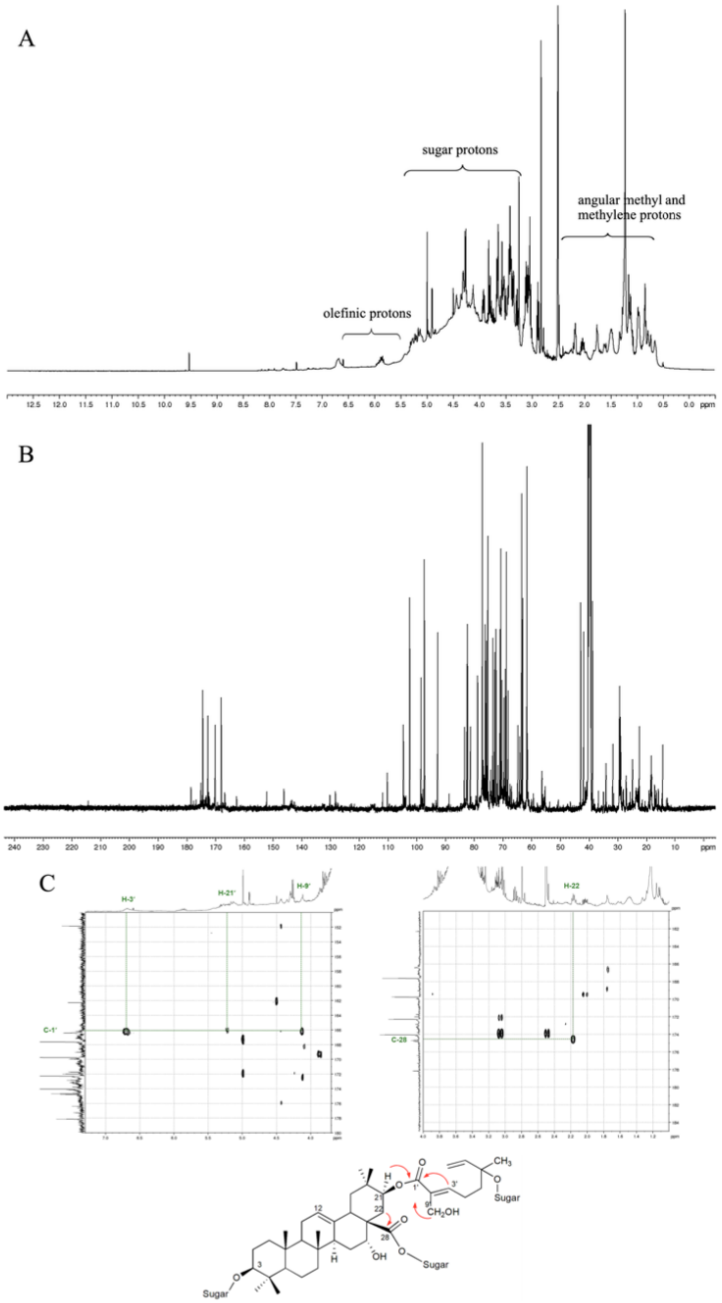


**Figure 1.** NMR spectra of the AC ethanol extract in DMSO-*d6*: (**A**) $^{1}$H NMR spectrum; (**B**) $^{13}$C NMR spectrum; (**C**) HMBC correlations.

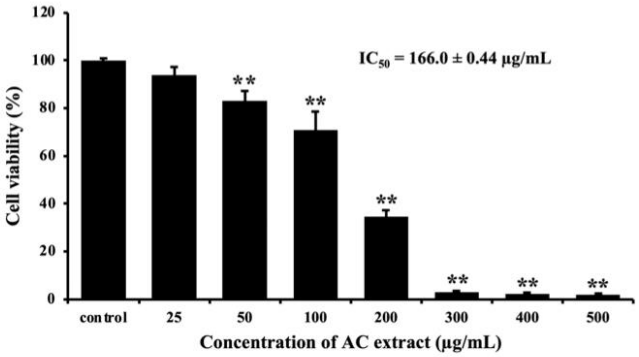


**Figure 2.** AC extract inhibited cell proliferation in HCT116 cells. The cells were treated with different concentrations of AC extract for 24 h and examined using the MTT assay. The cell viability is presented as a percentage compared to the control cells. ** $p < 0.01$, indicating significant differences compared to the control.

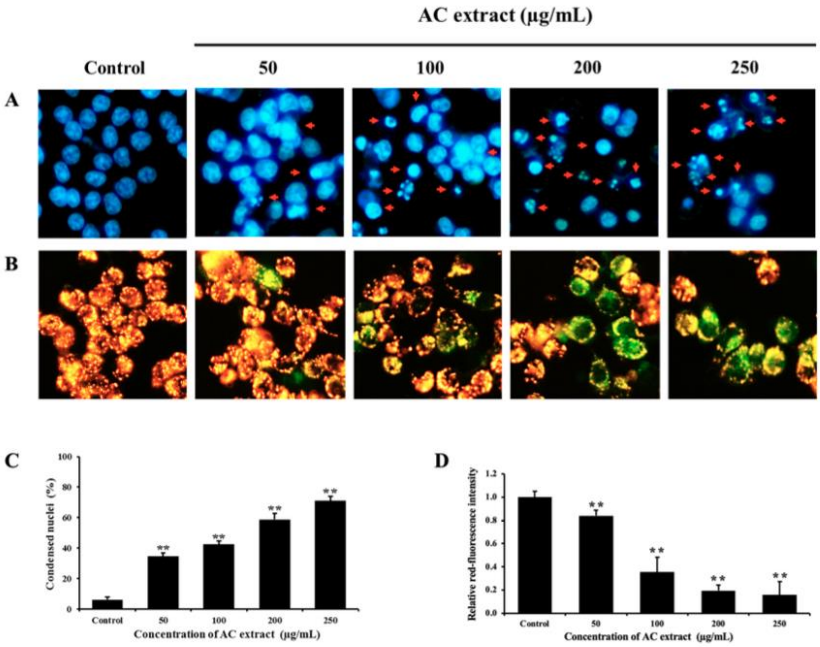


**Figure 3.** Effect of AC extract on the induction of apoptosis in HCT116 cells. (**A**) Nuclear morphological changes were assessed by staining cells with Hoechst 33342 and observed by fluorescence microscopy (20×). The red arrows indicated nuclear condensation and apoptotic bodies. (**B**) The loss of MMP in the cells was evaluated using JC-1 staining and observed by fluorescence microscopy (20×). The green fluorescence indicated the loss of MMP. (**C**) The histogram represented the percent of nuclear-condensed cells relative to the control cells. (**D**) The histogram represented the relative intensity of red fluorescence compared to the control cells. ** $p < 0.01$, indicating significant differences compared to the control.

### 3.4. AC Extract Increased Population of HCT116 Cells in Sub-G1 Phase

The apoptosis-inducing effect of AC extract in HCT116 cells was confirmed by cell cycle analysis. The histograms in Figure 4 show the cell cycle distribution of HCT116 cells, implying that treatment of the cells with AC extract led to an increase sub-G1 population, indicating the presence of apoptotic cells. The percentages of cells significantly increased from 0.18% observed in the control cells to 1.23, 2.15, 3.06, and 3.57% at concentrations of 50, 100, 200, and 250 µg/mL of AC extract, respectively. These findings clearly demonstrated that AC extract induced apoptosis in HCT116 cells.

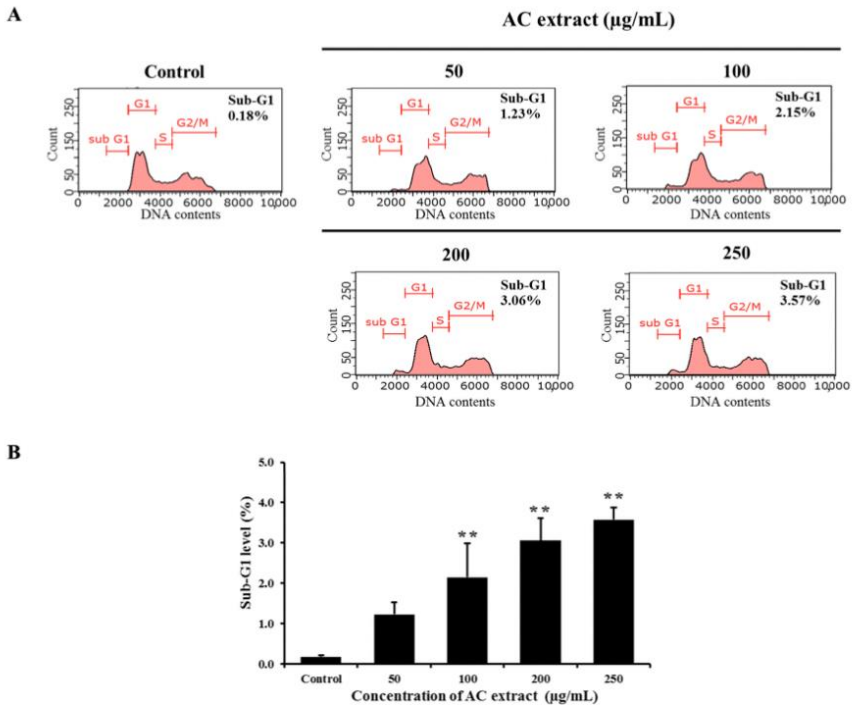


**Figure 4.** Effect of AC extract on cell cycle distribution in HCT116 cells. (**A**) The histograms represent the DNA content analysis performed by flow cytometry. (**B**) The graphical representation compared the relative sub-G1 level to the control cells. ** $p < 0.01$, indicating significant differences compared to the control.

### 3.5. AC Extract Induced Intracellular ROS Production

The accumulation of ROS has been associated with the disruption of MMP [27] considered a trigger for the mitochondrial apoptotic pathway. While our results showed that AC extract caused a loss of MMP, for this reason, the level of intracellular ROS was measured using DCFH-DA staining. As shown in Figure 5, AC extract significantly increased the level of intracellular ROS in HCT116 cells after 2 h of incubation with AC extract as compared to the control cells.

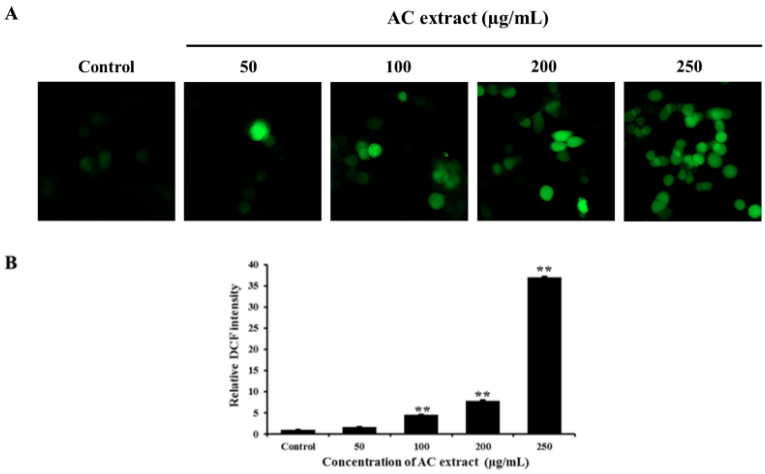


**Figure 5.** Effect of AC extract on intracellular ROS level in HCT116 cells. The cells were treated with AC extract for 2 h, determined by DCFH-DA staining, and observed by a fluorescence microscope (20×). (**A**) DCF fluorescence image in HCT116 cells. The green fluorescence indicated ROS formation in the cells. (**B**) The relative DCF fluorescence intensity compared to the control cells. ** $p < 0.01$, indicating significant differences compared to the control.

### *3.6. Effect of AC Extract on Apoptotic-Related Protein Expression*

To explicate the molecular mechanisms of AC extract-induced apoptosis, the expression of several key apoptotic-related proteins was examined. The treatment with AC extract for 24 h induced a significant decrease in the anti-apoptotic proteins B-cell leukemia/lymphoma 2 (Bcl-2), B-cell lymphoma-extra-large (Bcl-xL), and myeloid leukemia 1 (Mcl-1), while a significant increase in the pro-apoptotic proteins Bcl2-associated X protein (Bax) and Bcl-2-antagonist/killer (Bak) resulted in the activation of the cleaved form of cysteine-dependent aspartate-specific protease (caspase-7). Furthermore, the level of a DNA repair enzyme, cleaved-PARP (inactive form), was also increased in AC-treated HCT116 cells (Figure 6). Therefore, these results indicated that the mechanism of apoptosis induction in HCT116 cells by AC extract was involved in the activation of caspase-dependent apoptosis via the mitochondrial pathways.

### *3.7. Effect of AC Extract on ER Stress*

A previous study indicated that an increase in ROS levels can cause ER stress and lead to cell death [28]. We therefore conducted Western blot analysis to further elucidate the possible apoptotic pathway induced by AC extract associated with the ER stress. As shown in Figure 7, AC extract upregulated the expression of glucose-regulated protein 78 (GRP78), protein kinase RNA-like endoplasmic reticulum kinase (PERK)/eukaryotic initiation factor 2 alpha (eIF2α) pathway, leading to the increased C/EBP homologous protein (CHOP) in HCT116 cells. Moreover, AC extract increased the level of p-IRE1α (inositol-requiring enzyme 1 α). The protein expression patterns observed in AC-treated cells were similar to those in tunicamycin (Tm)-treated cells, a widely recognized inducer of ER stress. These results suggested that AC extract induced ER stress, which may be closely related to apoptosis induction in HCT116 cells.

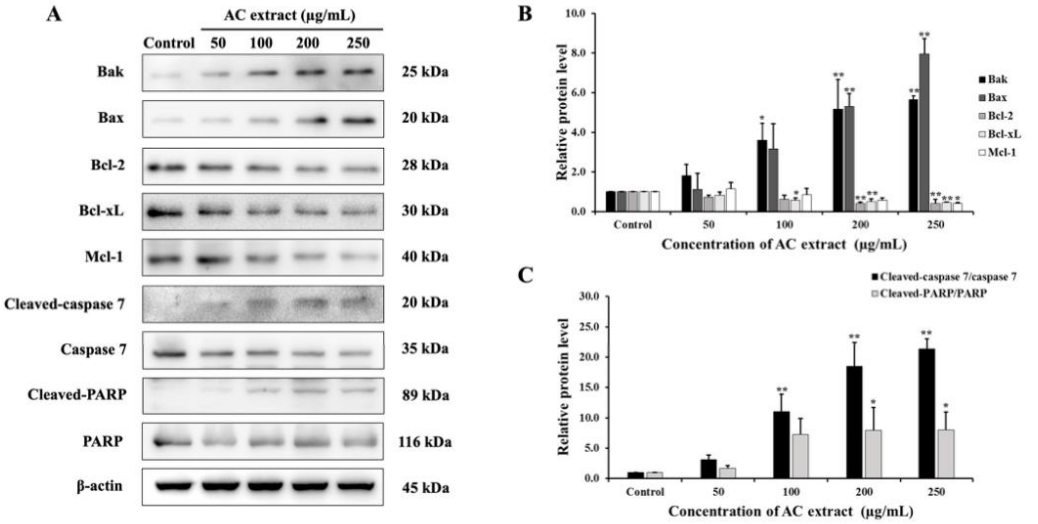


**Figure 6.** Effect of AC extract on apoptosis-related protein expression in HCT116 cells. (**A**) The expression of protein was detected by Western blot analysis. (**B**,**C**) The relative band intensity of apoptosis-related proteins compared to the control group. * $p < 0.05$ and ** $p < 0.01$, indicating significant differences compared to the control.

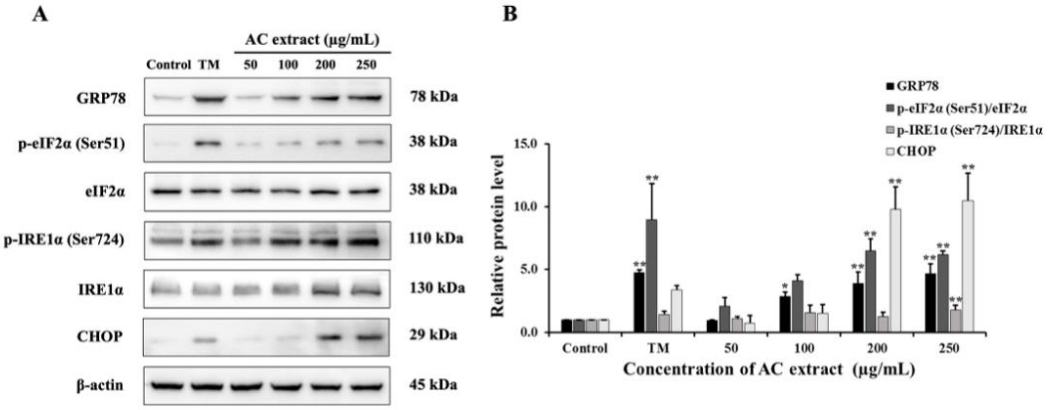


**Figure 7.** Effect of AC extract on ER stress-related proteins in HCT116 cells. The cells were treated with AC extract or Tm (10 μg/mL). (**A**) The protein expression was examined by Western blot analysis. (**B**) The relative band intensity compared to the control group. * $p < 0.05$ and ** $p < 0.01$, indicating significant differences compared to the control.

### *3.8. Effect of AC Extract on MAPK Signaling Pathway*

The MAPK pathway downstream of KRAS plays a major role in cell differentiation, proliferation, and apoptosis [22]. Therefore, we studied the protein levels of this pathway. The results, shown in Figure 8, indicated that AC extract increased the ratio of the phosphorylated form to the total form of ERK1/2, p38, and c-Jun proteins. These results revealed that AC extract may inhibit HCT116 cell growth by regulating the MAPK pathway.

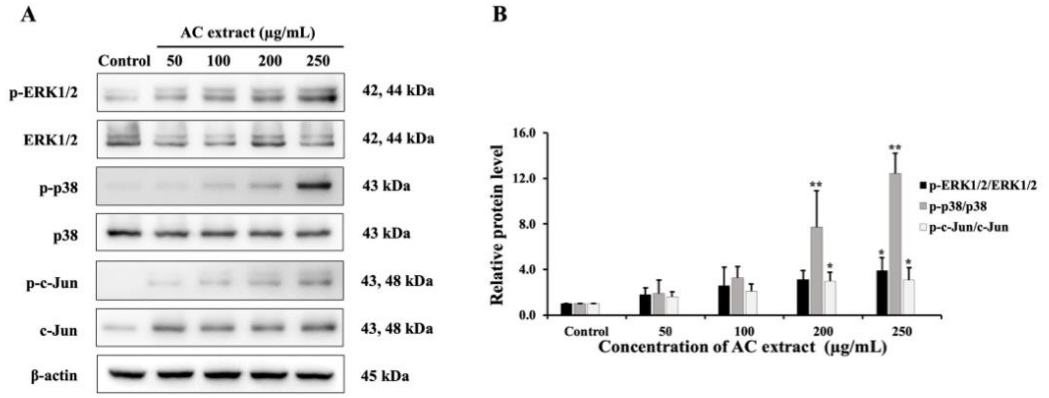


**Figure 8.** Effect of AC extract on MAPK pathway in HCT116 cells. (**A**) The protein expression was detected by Western blot analysis. (**B**) The relative band intensity compared to the control group. * $p < 0.05$ and ** $p < 0.01$, indicating significant differences compared to the control.

### *3.9. Effect of AC Extract on PI3K/Akt and Wnt/β-Catenin Signaling Pathways*

It is well-known that PI3K/Akt serves as a key signaling transduction pathway for regulating cellular survival and inhibiting apoptosis, downstream of KRAS [23]. Thereby, the effect of AC extract on this pathway in HCT116 cells was detected. The results found that AC extract decreased the levels of PI3K, p-PDK1 (3-phosphoinositide-dependent kinase 1), p-Akt (Ser473), and p-Akt (Thr308) (Figure 9A,B). The PI3K/Akt pathway contributes to the triggering of the Wnt/β-catenin pathway [24]; therefore, we next investigated the expression of this pathway. As shown in Figure 9C, AC extract downregulated the expressions of phospho-glycogen synthase kinase-3 beta (p-GSK-3β), the inactive form of GSK-3β, thereby enhancing β-catenin degradation, as evidenced by decreased β-catenin and their downstream target (c-Myc, survivin) expression. Thus, our results suggested that AC extract could induce apoptosis by suppressing the Wnt/β-catenin pathway by restraining the upstream kinase activity of the PI3/Akt pathway.

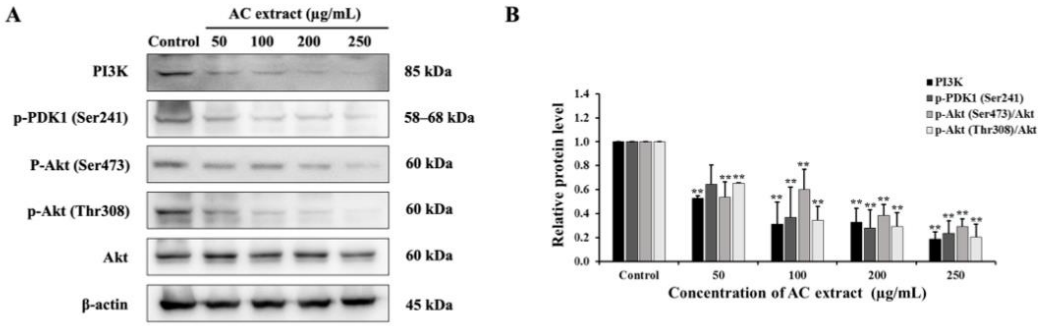


**Figure 9.** *Cont.*

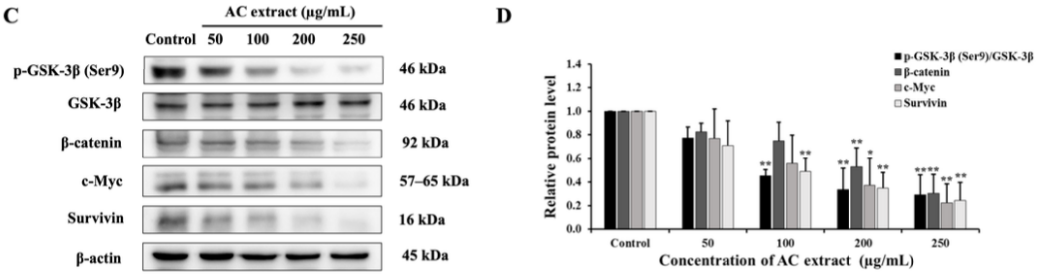


**Figure 9.** Effect of AC extract on PI3K/Akt and Wnt/β-catenin signaling pathway in HCT116 cells. (**A**) The expression of PI3K, p-PDK1, p-Akt (Ser473), p-Akt (Thr308), and Akt. (**B**) The relative band intensity of PI3K/Akt proteins compared to the control group. (**C**) The protein expression of p-GSK-3β, GSK-3β, β-catenin, c-Myc, and survivin. (**D**) The relative band intensity of Wnt/β-catenin proteins compared to the control group. * $p < 0.05$ and ** $p < 0.01$, indicating significant differences compared to the control.

## 4. Discussion

Considerable research suggests the potential impact of bioactive compounds in the prevention and treatment of cancer [29]. Moreover, bioactive compounds from plants or herbs are generally safe and low toxic; this is an alternative approach to avoiding the side effects of synthetic medicines [30]. In this study, we determined whether AC extract has anticancer properties in human colon cancer cells. This study illustrated that AC extract exhibited a significant anti-proliferative effect in HCT116 cells. In a previous study using kinmoonosides A-C, novel saponins isolated from a methanolic extract of *A. concinna* pods also exhibited a significant cytotoxic effect against human HT-1080 fibrosarcoma cells [13], supporting our results and indicating that AC extract has anticancer potential in cancer cells.

Phytochemicals are bioactive compounds derived from plants that offer health benefits, including the prevention of diabetes, obesity, and cancer. These phytochemical compounds contain several groups, such as polyphenols, carotenoids, and saponins [31]. Flavonoids are a large family of polyphenols that exhibit anticancer effects by modulating multiple mechanisms of action such as apoptosis induction, cell cycle arrest, and invasiveness [32]. In this study, the phytochemical compounds of the AC extract detected as the flavonoid (Figure S1) were analyzed by NMR and MS. The main peaks of the MS profile of AC extract corresponded to pentahydroxyflavone, hexahydroxyflavone, and acacic acid-type saponins in comparison with the previous reports of Acacia pods extracts [13,26,33]. A previous study found that the methanolic extract from the *A. concinna* plant was found to contain flavonoids and phenols and showed cytotoxicity on human breast cancer cells, MCF-7 [11]. In addition, the extract containing triterpenoid saponins showed cytotoxicity against HT-1080 fibrosarcoma cells [13]. Therefore, the anticancer effect of the AC extract may be due to the flavonoids and saponins presented. However, further studies are necessary to isolate, purify, and test the active compounds of AC extract to assess their anticancer activity.

The main goals of cancer treatment are the inhibition of cancer cell growth and the elimination of cancer cells while providing low toxicity to normal cells. It is commonly known that most many chemotherapeutic agents can kill cancer cells by activating apoptotic pathways [21]. Therefore, we hypothesized that the inhibitory effect of AC extract on HCT116 cells may involve apoptosis induction. Our study revealed that AC extract selectively induced apoptosis in HCT116 cells indicated by chromatin condensation and loss of MMP as well as increased sub-G1 populations. The loss of MMP is recognized as a key step in the mitochondrial or intrinsic apoptosis pathway. The loss of MMP occurs by activation of pro-apoptotic protein. This event results in the activation of numerous caspases and cleavage of downstream death effector proteins [34]. The Western blot anal-

ysis of AC-treated cells confirmed such a hypothesis. We discovered that AC extract increased the protein expression of cleaved caspase-7 responsible for cleaving downstream substrates, PARP. The activation of effector caspases is well known to be responsible for the hallmarks of apoptosis cells, such as DNA fragmentation [35]. AC-treated cells also increased cleaved-PARP levels, which prevented the cancer cells from repairing damage required for their survival, and finally caused irreversible apoptosis cell death and complete elimination [18,19]. We also confirmed that AC extract regulated Bcl-2 family members, which initiated the apoptosis process in HCT116 cells via the intrinsic pathway. According to previous studies, morin (3,5,7,2′,4′-pentahydroxyflavone) stimulates caspases-8, -9, and -3, causes PARP cleavage, and modulates the Fas receptor and Bcl-2 family members, suggesting that it induces both the extrinsic and intrinsic apoptosis pathways in HCT 116 cells [36].

Several studies have suggested that elevated intracellular levels of ROS can trigger ER stress, potentially leading to mitochondrial dysfunction and ultimately initiating apoptosis [28,37]. Interestingly, AC extract has been found to induce intracellular ROS generation and ER stress by upregulating the expression of GRP78, p-eIF2α, CHOP, and p-IRE1α. Increased levels of GRP78/BiP have been reported to activate PERK by autophosphorylation and homomultimerization. Activated PERK subsequently phosphorylates eIF2α, leading to the activation of ATF4 and its downstream targets [38,39]. Normally, mild ER stress conditions can restore ER homeostasis through the unfolded protein response (UPR). Nevertheless, prolonged ER stress can trigger apoptosis via increased CHOP expression, which is mainly regulated through the PERK/eIF-2α/ATF-6 pathway [40]. The activation of CHOP is regarded as a key event for ER stress-induced apoptosis. Studies have shown that CHOP triggered apoptosis by suppressing anti-apoptosis proteins such as Bcl-2 and Mcl-1 and by enhancing the expression of the pro-apoptosis protein Bim. Subsequently, Bim regulated Bax and Bak, leading to disruption of the permeabilization of the mitochondrial outer membrane [41]. Previous investigation has shown that quercetin, a bioactive flavonoid induced apoptosis in human cervical cancer cells (HeLa) by ER stress induction. The levels of caspase-3, GRP78, p-PERK, c-ATF6, IRE1, and CHOP showed a progressive increase corresponding to the rising concentration of quercetin [42]. Another study showed that quercetin induced cell apoptosis, intracellular ROS production, and ER stress in prostate cancer cells (PC-3) [43]. In addition, a synthetic derivative of quercetin called TEF (5,3′-dihydroxy-3,7,4′-triethoxyflavone) also induced apoptosis and ER stress via the IRE1-α and mito-JNK pathways in HCT-116 cells [44]. Our results were consistent with their observations and demonstrated that AC extract induced ER stress via the GRP78/eIF-2α/CHOP pathway. Additionally, activated IRE1 can activate MAPK pathways including p38 and JNK, which regulate Bcl-2 family proteins [39,45]. Taken together, these results demonstrate that AC extract induced ER stress-mediated apoptosis in HCT116 cells.

KRAS-mutant CRC cancer is linked to decreased survival and increased tumor aggressiveness [46]. Designing drugs that directly target mutant KRAS constitutes a significant challenge. Thus, an alternative strategy is to target downstream pathways such as the MAPK and Akt pathways [2]. Both p38 and JNK respond to both extracellular and intracellular stresses. Activation of p38 promotes cell apoptosis via the regulation of a variety of Bcl-2 family proteins, such as Bax, and the activating caspase family [47]. We reported that the expression of p-p38 and p-c-Jun were increased upon treatment with AC extract. c-Jun is one of the transcription factor AP-1 family members that can be phosphorylated by JNK. The activation of JNK may stimulate apoptosis by upregulating the transcription of pro-apoptotic genes such as the Fas/FasL pathway through c-Jun/AP-1 [48]. Previous studies have indicated that quercetin triggers apoptosis by activating the JNK pathway in KRAS-mutant colorectal cancer cells [2]. It has also been reported that the activation of ERK1/2 is linked to the induction of apoptosis. Kim S et al. 2019 reported the anti-tumor and apoptotic effects are elicited by quercetin through the regulation of MAPK pathways by increasing the levels of Bax, cleaved-PARP, p-JNK, p-p38, and p-ERK1/2, while reducing Bcl-2 in A375SM melanoma cells [49]. In the present study, AC extract increased the

expression of p-ERK1/2 in HCT116 cells. Hence, AC-induced apoptosis in HCT116 cells may be related to MAPK pathway activation.

The PI3K/Akt pathway is widely recognized for regulating cell survival, cell proliferation, and cancer drug resistance. Mutations and activation of protein members within the PI3K/Akt/mTOR pathway are commonly found in cancer [50]. Several studies have indicated that plant extracts can inhibit this pathway, resulting in decreased cell proliferation and increased apoptosis. For instance, quercetin has shown anticancer effects via induced cell death in JAR and JEG3 choriocarcinoma cell lines through PI3K pathways by inhibiting p-Akt, p-P70S6K, and p-S6 proteins [51]. These findings correlated with our research, wherein we observed that AC extract reduced the expression of PI3K/Akt protein in HCT116 cells.

Increased Wnt/β-catenin signaling pathway activity is linked to carcinogenesis in CRC [52]. β-catenin is a key component of this pathway, which is regulated by a destruction complex. GSK3-β is part of the destruction complex that phosphorylates β-catenin in the cytosol leading to its degradation via the ubiquitin-proteasome system. GSK3β activation is regulated by Akt which phosphorylates GSK3β, thereby inactivating it and preventing the formation of the destruction complex [24,52–54]. As reported, we demonstrated that AC extract can inhibit both the PI3K/Akt and the Wnt/β-catenin pathway, which are crucial for tumor initiation and development. [55]. Similarly, quercetin has the potential to enhance apoptosis in human GBM T98G cells by inhibiting the Wnt3a/β-catenin pathway and the Akt/NF-κB signaling pathway [56].

## 5. Conclusions

In conclusion, this study suggested that AC extract can be considered a natural source of anticancer agents for colon cancer treatment. Our findings indicated that AC extract suppressed cell proliferation and apoptosis induction in HCT116 cells through various signaling pathways, including activation of the intrinsic caspase pathway, ROS-induced ER stress, and the MAPK pathway, while inhibiting the PI3K/Akt and Wnt/β-catenin pathways. This is the first report on the mechanism of apoptosis induction of AC extract in HCT116 cells. Therefore, further investigation may be required to explore the relationship between pathways to comprehensively understand the inhibitory effects of AC extract on HCT116 cells.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16213764/s1, Figure S1: Total ion chromatogram by positive mode electrospray ionization mass (A). $^{1}$H-NMR spectrum with expansion of AC extract in DMSO-*d6* (B).; Figure S2: The dried pods of *Acacia concinna* (A) were ground into powder (B) for extraction. The crude extract of *A. concinna* (C) was kept in the refrigerator, in the dark, until used in the experiment.; Figure S3: Effect of AC extract on cell viability in HaCat cells by MTT assay. The cell line was treated with various concentrations of AC extract for 24 h. The results were the mean values ± SD.

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