ANTI-PROLIFERATIVE AND APOPTOTIC EFFECT OF CANNABINOIDS ON HUMAN
PANCREATIC DUCTAL ADENOCARCINOMA XENOGRAFT IN BALB/C NUDE MICE MODEL

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ผลของสารสกัดกัญชาในการยับยั้งการเจริญและกระตุ้นการตายแบบอะพอพโทซิสของเซลล์มะเร็งท่อตับอ่อนของมนุษย์ที่ปลูกถ่ายในหนูพร่องภูมิคุ้มกันชนิด BALB/c

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Mr. Quang Trung Le
Field of Study	Veterinary Science and technology
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ควาง ตรุง เล : ผลของสารสกัดกัญชาในการยับยั้งการเจริญและกระตุ้นการตายแบบอะพอพโทซิสของเซลล์มะเร็งท่อตับอ่อนของมนุษย์ที่ปลูกถ่ายในหนูพร่องภูมิคุ้มกันชนิด BALB/c. (ANTI-PROLIFERATIVE AND APOPTOTIC EFFECT OF CANNABINOIDS ON HUMAN PANCREATIC DUCTAL ADENOCARCINOMA XENOGRAFT IN BALB/C NUDE MICE MODEL) อ.ที่ปรึกษาหลัก : เกษม รัตนภิญโญพิทักษ์, อ.ที่ปรึกษาร่วม : อนุเทพ รังสีพิพัฒน์

มะเร็งท่อตับอ่อนในคนเป็นมะเร็งที่มีความร้ายแรงอย่างมากและมักทำให้ผู้ป่วยเสียชีวิต สารคาร์นาบินอยที่สกัดจากพืชกัญชา *Cannabis sativa* ถูกนำมาใช้ในการรักษามะเร็งหลายชนิดในคน เนื่องจากมีฤทธิ์ในการต้านมะเร็งอย่างไรก็ดีการศึกษาเกี่ยวกับฤทธิ์ของสารสกัดกัญชาในมะเร็งชนิดท่อตับอ่อนในคนยังมีน้อย ดังนั้นในงานวิจัยนี้จึงมีวัตถุประสงค์ในการศึกษาประสิทธิผลของสารสกัดกัญชาในการยับยั้งการเจริญและการกระตุ้นการเกิดการตายของเซลล์แบบอะพอพโทซิในมะเร็งท่อตับอ่อนของคนที่ปลูกถ่ายในหนูทดลองพร่องภูมิคุ้มกัน ทำการปลูกถ่ายเซลล์เพาะเลี้ยงชนิด Capan-2 บริเวณใต้ผิวหนังของหนูพร่องภูมิคุ้มกันจำนวน 25 ตัว เมื่อก้อนเนื้องอกเจริญจนได้ขนาดประมาณ 200 ลูกบาศก์เซนติเมตร แบ่งหนูออกเป็น 5 กลุ่มการทดลอง ได้แก่ กลุ่มควบคุมลบ (NC) ที่ถูกป้อนด้วยน้ำมันงา ติดต่อกันทุกวันเป็นเวลา 30 วัน กลุ่มควบคุมบวก (PC) จะได้รับยา 5-FU ขนาด 20 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัวเข้าทางช่องท้องสัปดาห์ละ 3 ครั้ง และกลุ่มทดลองที่ป้อนสารสกัดกัญชาคานาบินอยด์ชนิด THC:CBD (1:6) ขนาด 1, 5 และ 10 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัวตามลำดับ ติดต่อกันทุกวันเป็นเวลา 30 วัน จากผลการทดลองพบว่า กลุ่มที่ได้รับสารสกัด THC:CBD (1:6) ก้อนเนื้องอกมีจำนวนเซลล์ที่แบ่งตัว และอัตราส่วนเซลล์ที่แบ่งตัวต่อเซลล์ที่เกิดการตายแบบอะพอพโทซิสลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุมลบ ซึ่งสอดคล้องกับการแสดงออกของยีนและโปรตีนที่เกี่ยวข้องกับการเพิ่มจำนวนของเซลล์ (Ki-67 และ PCNA) ซึ่งพบว่ามีการแสดงออกลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มควบคุมลบ นอกจากนี้ยังพบว่าสารสกัดกัญชา THC:CBD (1:6) เพิ่มการแสดงออกของ BAX อัตราส่วนของ BAX ต่อ BCL-2 และ Caspase-3 แต่ลดการแสดงออกของ BCL-2 ทั้งในระดับยีนและโปรตีน ทั้งนี้ไม่พบการเปลี่ยนแปลงการแสดงออกของ Caspase-8 เมื่อเปรียบเทียบกับกลุ่มควบคุมลบ จากผลการทดลองสรุปได้ว่าสารสกัดกัญชา THC:CBD (1:6) สามารถยับยั้งการเจริญและกระตุ้นการเกิดการตายแบบอะพอพโทซิสในมะเร็งท่อตับอ่อนของคนที่ฝังในหนูทดลอง ทั้งนี้ควรมีการศึกษาเพิ่มเติมเกี่ยวกับประสิทธิภาพของสารสกัดกัญชาชนิดต่าง ๆ รวมทั้ง THC:CBD เพิ่มเติมเพื่อใช้ในการรักษามะเร็งท่อตับอ่อนในคนต่อไปในอนาคต.

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Quang Trung Le : ANTI-PROLIFERATIVE AND APOPTOTIC EFFECT OF CANNABINOIDS ON HUMAN PANCREATIC DUCTAL ADENOCARCINOMA XENOGRAFT IN BALB/C NUDE MICE MODEL. Advisor: Dr. KASEM RATTANAPINYOPITUK Co-advisor: Prof. Dr. ANUDEP RUNGSIPIPAT

Human pancreatic ductal adenocarcinoma (PDAC) is a highly malignant and lethal tumor of the exocrine pancreas. Cannabinoids extracted from the hemp plant *Cannabis sativa* have been suggested as a cancer therapy due to their anti-tumor effects on several human tumors. However, the anti-tumor effect of cannabinoids on human PDAC is still understudied. Therefore, the objective of the current study is to study the inhibition of proliferation and the induction of apoptosis potencies of cannabinoids in PDAC xenograft nude mice model. The human PDAC cell line (Capan-2) was subcutaneously injected into twenty-five nude mice. After tumor reached 200 mm^3 in volume, mice were randomly divided into five groups (5 mice/group), following: negative control (NC) (daily gavaged of sesame oil), positive control (PC) (intraperitoneal administration of 5-fluorouracil at 20 mg/kg body weight (BW), three-time/week), and cannabinoid-treated groups (daily gavaged of THC:CBD (1:6) solution at 1, 5, and 10 mg/kg BW for 30 days, respectively). The findings showed that THC:CBD (1:6) significantly decreased the mitotic cells and mitotic/apoptotic ratio, while the apoptotic cells were dramatically increased compared to the NC group. Parallelly, THC:CBD (1:6) significantly reduced the protein expression level of Ki-67 and PCNA relative to the NC group. Interestingly, THC:CBD (1:6) upregulated BAX, BAX/BCL-2 ratio, and Caspase-3 expression level, meanwhile, downregulated BCL-2 expression level and could not change Caspase-8 expression level relative to the NC group. These findings suggest that cannabinoid solution (THC:CBD at 1:6) could inhibit proliferation and induce apoptosis in human PDAC xenograft models. Cannabinoids, including THC:CBD, should be further studied for use as the potent PDCA therapeutic agent in humans.

Field of Study:	Veterinary Science and technology	Student's Signature ....
Academic Year:	2022	Advisor's Signature ....
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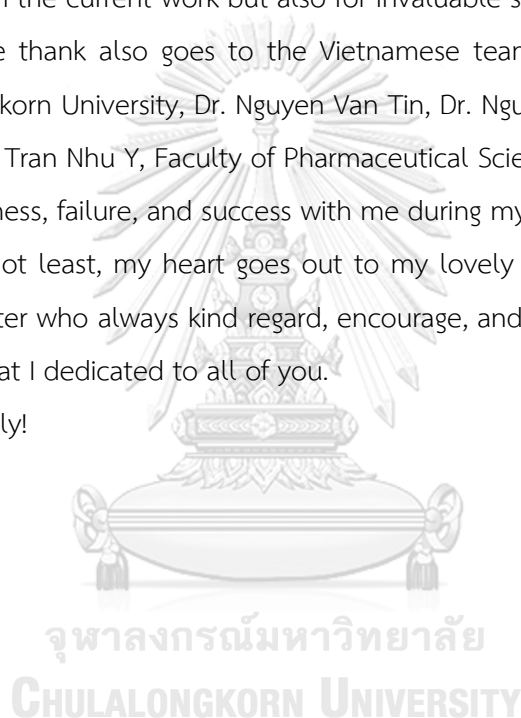
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CHAPTER 1: INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant tumor and the most common neoplasm in the exocrine pancreas, appearing over 90% of all pancreatic malignancies (Wolfgang et al., 2013; Kleeff et al., 2016; Orth et al., 2019). The investigated data from patients associated with pancreatic cancer of the American Cancer Society estimated that high rates of men (79%) and women (80%) patients within the United States would die within the first year of diagnosis and the 5-year relative survival rate of lower than 10% (Siegel et al., 2021). The major causes leading to the ambiguous prognosis of pancreatic cancer patients can be mentioned as highly aggressive growth, early metastases of neoplastic cells to other organs prior to diagnosis, lack of diagnostic biomarkers, and poor response to therapeutic methods (Wolfgang et al., 2013; Kamisawa et al., 2016). Moreover, the anatomical location of the pancreas is also considered a disadvantage, requiring modern equipment and complex methods for early diagnosis. In fact, only a modest proportion of candidate patients (15-20%) are treated with surgical resection during the early stage of the disease (Stathis and Moore, 2010; Kamisawa et al., 2016). At present, although there are significant achievements within the control of pancreatic tumors based on the options including chemotherapy, targeted therapy, immunotherapy, and radiation therapy or optimal combination of individual methods together. It remains a major challenge because of a low response within the late stage of this disease and side effects of therapies (Kleeff et al., 2016; Orth et al., 2019). Therefore, continuously investigating novel, safe, and effective treatments for human pancreatic cancer is still under-investigated.

Herbal medicinal plants and their derivatives have been discovered and used for a long time as potential sources for the treatment of human cancers. Cannabinoid compounds have been reported to an antineoplastic activity since the 1970s (Munson et al., 1975), and until presently it promises therapeutic drugs for various *in vitro* and *in vivo* studies within human cancer cell lines (glioblastoma, leukemia, lung, breast, and pancreas) (Massi et al., 2004; Carracedo et al., 2006a; McKallip et al., 2006; Preet et al., 2008; Ramer et al., 2010; Guo et al., 2018).

Cannabinoids have been shown to inhibit the proliferation and metastasis of tumor cells, correlate with apoptosis, autophagy, and enhance tumor immune surveillance (Sarfaraz et al., 2005; Michalski et al., 2008; Velasco et al., 2012; Kovalchuk and Kovalchuk, 2020). A combination of cannabinoids and radiotherapy offered an outstanding outcome within targeting treatment and improved the quality of life in pancreatic cancer patients (Robson, 2001; Yasmin-Karim et al., 2018). The therapeutic effects of cannabinoids have been performed in laboratory animal models, most commonly xenograft nude mice (Zhao et al., 2013; Guo et al., 2018; Yasmin-Karim et al., 2018). It is demonstrated that cannabinoids extremely reduce the progression of pancreatic tumors by selectively inducing apoptosis and inhibiting of proliferation within tumor cells (Carracedo et al., 2006a).

Even though greater than 60 cannabinoid compounds have been extracted from the hemp plant *Cannabis sativa*, cannabidiol (CBD) and Delta-9-tetrahydrocannabinol (THC) have been widely acknowledged for the study of the therapeutic effects within medicine, and most recently used for the antitumor effects (Guzman, 2003; Velasco et al., 2016). A single effect of CBD and THC or synergistic effect of both CBD and THC were reported on glioma cell line and glioblastoma patients. The synergistic effects of CBD/THC were greater than the single effect of the individual compound (Torres et al., 2011; Nabissi et al., 2016; López-Valero et al., 2018). Furthermore, these results indicated that the antitumor effects were better when CBD and THC were used in combination. In fact, cancer treatment requires further studies on various therapeutic drugs because of the decrease of undesirable medication side effects. Nevertheless, there are a few investigations of cannabinoids within pancreatic cancer animal models. The goal of this study therefore evaluated the anti-proliferative effect and the apoptosis induction in pancreatic cancer xenograft mice treated with cannabinoids.

Objectives of the Study

The study aims to demonstrate the anti-proliferative effect and apoptosis induction associated with the treatment of cannabinoid extracted agent in xenograft human pancreatic tumor cell line (Capan-2) in nude mouse model.

The anti-proliferative and apoptotic-associated protein and the selective gene expressions of the xenograft human pancreatic cancer cell line is evaluated. The objective of the study as follows:

1) Evaluate the apoptotic-related gene expression in xenograft human pancreatic cancer cell line by quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) technique.

2) Evaluate the expression of individual anti-proliferative and apoptotic markers in xenograft human pancreatic cancer cell line by immunohistochemistry (IHC) and Western blot (WB) techniques.

Research questions

Cannabinoids have a potential role in the anti-proliferation and the induction of apoptosis in various cancer cells. Do cannabinoids similarly affect within human PDAC xenograft mouse model?

Hypothesis

Cannabinoids contributed to the anti-proliferative effect and the induction of apoptosis in human PDAC xenograft mouse model.

Advantages of study

The findings of this study expand the fundamental knowledge regarding the potential therapeutic role of cannabinoids for human pancreatic adenocarcinoma among *in vivo* studies within nude mouse models and may contribute to the therapy of PDAC in humans in the future.

CHAPTER 2: LITERATURE REVIEW

2.1. Cannabinoids

Cannabis sativa was early recognized as an ancient herbal medicinal plant and still be valuable for modern scientific medicine. Historically, Chinese physicians were the earliest user of the *cannabis* plant as a medicinal herb for patients over 4,000 BC (Tang and Eisenbrand, 2011; Mechoulam, 2019). These medicinal plants were used for various functions, such as hypnotic, psychotropic drugs, and also recreational purposes (Mechoulam et al., 1970). During the 19th century, *cannabis* became interesting for Western pharmacologists because of its important properties such as analgesic, muscle relaxant, antiemetic, anti-inflammatory and anticonvulsant properties (O'Shaughnessy, 1843). However, the medical use of *Cannabis* plants was illegal because of their characteristics of unwanted psychotropics and addiction (Solomon, 1968). Fortunately, the investigation and identification of *Cannabis's* active components have changed the interest in the uses of cannabinoids. Currently, cannabinoid compounds have been widely accepted in medicine, especially cancer treatments because of their anti-tumor properties (Guzman, 2003; Velasco et al., 2016).

Presently, the three main species of *Cannabis* have been well known as *Cannabis sativa, Cannabis indica,* and *Cannabis ruderalis* (Amar, 2006). It is widely acknowledged that *Cannabis* contains more than 60 active compounds categorized as plant-derived cannabinoids. The most active ingredient of this plant is tetrahydrocannabinol (THC), first elucidated in the 1960s (Gaoni and Mechoulam, 1964). From the starting point, other numerous cannabinoid compounds were continuously discovered such as Delta-8-THC (d8-THC), cannabinol (CBN), cannabicyclol (CBL), cannabichromene (CBC), cannabigerol (CBG) and cannabidiol (CBD) (Devane et al., 1992). Moreover, synthetic cannabinoids including dronabinol and their analogs which are nabilone and rimonabant, were also found (Le Boisselier et al., 2017). The third class, endocannabinoids represent the group comprised of anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG)

are synthesized and involved in various physiological processes of the human body (Huang et al., 2016).

The responses of cannabinoids have been elicited via the transmembrane receptors, highlighted by two identified receptors namely cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). The couple of CB1 and CB2 G-proteins have shared 44% protein identity and exhibit different expression patterns and pharmacological properties (Mackie and Stella, 2006).

2.2. Antitumor effect of cannabinoids

The antitumor effect of cannabinoids showed in four main processes including 1) induction of apoptosis, 2) inhibition of tumor cell proliferation, 3) inhibition of angiogenesis, invasion and metastasis and 4) induction of antitumor immunity (Zhu et al., 1998; Guzman, 2003).

2.2.1. Cannabinoids inhibit tumor cell proliferation

The cell cycle (Fig. 1) has been described as a highly regulated phase of the cell reproductive process or cell division. The cell cycle includes four main phases: G1 (first gap), S (synthesis), G2 (second gap), and M (mitotic). The G1 phase is known as the preparation period for the daughter cells. New components of organelles and proteins are synthesized in this phase. The S phase is mainly related to the replication of DNA, while in the G2 phase, cells continue growth and special proteins are synthesized for preparing mitosis. In the M phase, the synthesized organelles and DNA are divided equally into two daughter cells and these daughter cells are completely detached at the end of this phase (Nurse et al., 1998; Israels and Israels, 2000). Parallelly, there are three checkpoints at the G1, G2, and M phases to control the cell cycle system. Generally, the cells may not run into the next period if it does not pass the previous cell cycle checkpoint. In the G1 checkpoint, the nutrient or growth factor status of the cells is checked. If the cell does not available at that point, it may exit the cell cycle, rest at the G0 phase and turn back to the G1 phase when it is available (Israels and Israels, 2000; Kastan and Bartek, 2004). The checkpoint at the G2 phase is related to the replication of DNA. Protein p53 is responsible for stopping cells that carry the DNA lesions from the previous phase (Hengartner, 2000; Israels and Israels, 2000; Kastan and Bartek, 2004). The last

checkpoint at the M phase checks the chromosome spindle assembly in two new daughter cells (Israels and Israels, 2000; Kastan and Bartek, 2004).
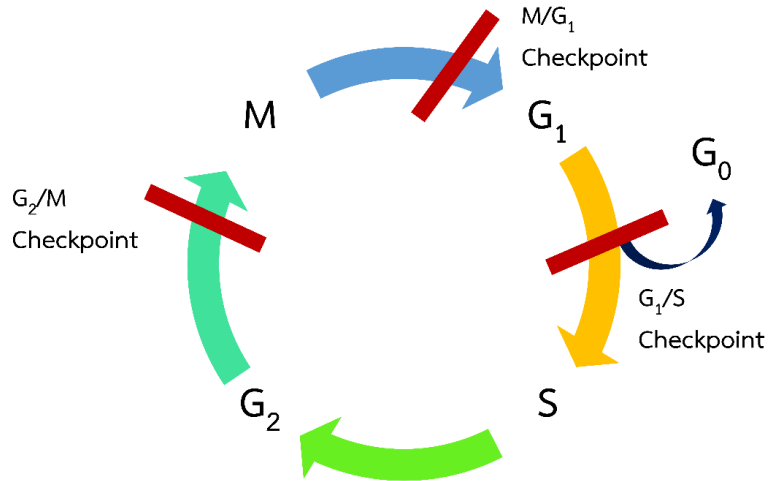
Figure 1: The phase and checkpoints of the cell cycle (Modified from Israels and Israels, 2000; Kastan and Bartek, 2004).

The proliferation of the cells plays a fundamental role that leads to neoplastic development (Smith et al., 2020). Cannabinoids inhibit the proliferation of tumor cells via modulating cell cycle checkpoints. The previous publication demonstrated that the activation of cannabinoid receptors blocked the progression of the cell cycle within melanoma cell lines B16 through the inhibition of Akt and hypophosphorylation (Blázquez et al., 2006). One previous study showed that the G1-S phase of the breast carcinoma cell cycle was blocked by the activation of the CB1 receptors (De Petrocellis et al., 1998). In contrast, the latter study indicated that THC-induced cell cycle arrested at the G2-M phase and led to the reduction of cell proliferation within human breast cancer cells via the activation of CB2 receptors (Caffarel et al., 2006). On the other hand, the results of WIN55,212-2 administration on pancreatic β cells demonstrated the decrease of BCL-2 and cyclin D2 expression, resulting from the cell cycle arrested within the G0-G1 phase (Kim et al., 2016). In a recent report, it is shown that the cell cycle was blocked during the period of the G0-G1 phase under the treatment of CBD for gastric cancer cells (Zhang et al., 2019). Moreover, individuals administered THC or CBD produced cell cycle arrest at only the G0-G1 phase while combined THC and CBD induced cell cycle arrest at the period of the G0-G1 and G2-M phase on human glioblastoma (Marcu et al., 2010). Therefore,

the modulating cell cycle checkpoints by cannabinoids may lead to the inhibition of tumor cell proliferation in various types of cancers.

2.2.2. Apoptosis induced by cannabinoids

"Apoptosis" is translated from Greek with the original meaning "Falling off". In the 1970s, apoptosis was first described as the distinct concept of a suicide program of cells. At present, apoptosis has been widely accepted as one of the most important forms of programmed cell death. It is a complicated process activated by the intrinsic or extrinsic pathway at multiple steps (Fig. 2) (Hengartner, 2000; Duque-Parra, 2005; Roufayel, 2016). The intrinsic pathway or also widely called the mitochondrial or stress pathway is mainly related to the activation of the proteins in the mitochondrial outer membrane by intracellular stress. Intracellular signals stimulate the regulation of the BCL-2 protein family, mainly related to the pro-apoptotic protein BAX and anti-apoptotic protein BCL-2 in the mitochondrial outer membrane. The imbalance in the regulation of protein BAX and BCL-2 leads to the release of cytochrome C from the mitochondrial outer membrane to the cytoplasm, provoking apoptotic protease-activating factor 1 (Apaf-1) to trigger pro-apoptotic protein Caspase-9, and then promote the activation of pro-apoptotic protein Caspase-3, consequence apoptosis is activated in the affected cell via the intrinsic pathway (Hengartner, 2000; Adams and Cory, 2007; Roufayel, 2016). On the other hand, the extrinsic pathway or additionally acknowledged as the death receptor pathway is principally related to the activation of the tumor necrosis factor (TNF) family and Fas receptor on the cell surface. The role of pro-apoptotic proteins Caspase-8 and Caspase-3 is highlighted in this pathway. Protein Caspase-8 is activated through messenger which is the result of an interaction between death ligands and death receptors on the cell surface. Following protein Caspase-3 is processed and activated through the signals of protein Caspase-8, resulting in the activation of apoptosis via the extrinsic pathway (Hengartner, 2000; Kumar, 2007; Roufayel, 2016).
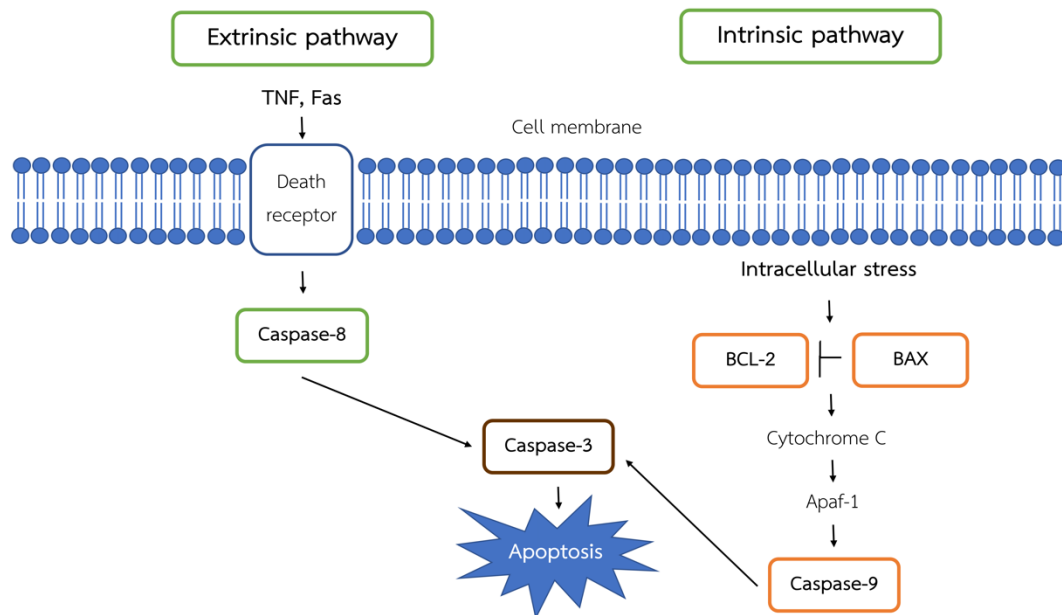
Figure 2: The intrinsic and extrinsic apoptosis pathway. The intrinsic pathway is activated via intracellular stress, which regulates the BCL-2 protein family by promoting protein BAX, inhibiting protein BCL-2. The BAX/BCL-2 ratio imbalance leads to the release of cytochrome C, forming apoptosome via activation of Apaf-1, followed by activation of protein Caspase-9 and -3 that stimulate apoptosis. The extrinsic pathway initially begins from the stimulation of the death receptors on the cell membrane. The activation of protein Caspase-8 is mainly related to the activation of death ligands (TNF, Fas) via interaction with death receptors. Once activated, protein Caspase-8 stimulates the activation of protein Caspase-3, consequently activating apoptosis (Modified from Hengartner, 2000; Adams and Cory, 2007).

Like other antineoplastic drugs, the antitumor effects of cannabinoids are induced via activating apoptosis program and are closely involved in the intrinsic pathway. THC is one of the most popular cannabinoid compounds that was demonstrated in the activated apoptosis-related intrinsic pathway through the endoplasmic reticulum stress (Carracedo et al., 2006a). A previous investigation demonstrated that cannabinoids were associated with the upregulation of BAX levels (Lukhele and Motadi, 2016). Likewise, another additional study showed that CDB promoted the expression of protein BAX and inhibited protein BCL-2 expression, resulting in apoptosis within the human gastric cancer cell line (Zhang et al., 2019). Moreover, previous publications showed that THC related to the regulation of BCL-2

and caspase activity led to triggering apoptosis within immune cell lines (Zhu et al., 1998). Administration of WIN 55,212-2 (CB1) into pancreatic β cells decreased the expression of anti-apoptotic protein BCL-2, and induced cell cycle arrest between the G0 and G1 phase (Kim et al., 2016). The CB2 receptor agonist (JWH133) led to the upregulation of the BCL-2 signaling pathway (Fujii et al., 2014). Furthermore, *in vitro* study of WIN 55,212-2 within glioma cells indicated that contributed to the regulation of the Akt and Erk signaling pathways via Bad protein, a member of the BCL-2 family. Besides, WIN 55,212-2 increased the level of Bad protein which is associated with the leak of cytochrome C from the mitochondria to the cytosol area, thus leading to the caspase activity and finally triggering apoptosis (Ellert-Miklaszewska et al., 2005). Moreover, several studies show that cannabinoids lead to the upregulation of protein Caspase-3, resulting in the activation of apoptosis (Herrera et al., 2006; Marcu et al., 2010; Lukhele and Motadi, 2016; López-Valero et al., 2018; Zhou et al., 2018; Zhang et al., 2019). Contrarily, the effect of cannabinoids on Caspase-8 expression is still under study (Herrera et al., 2006). A previous study showed that THC activated apoptosis in the human leukemia cell line via the intrinsic pathway even though the activation of protein Caspase-8 was found. It can be explained by the activity of protein Caspase-8 at the post-mitochondrial stage (Herrera et al., 2006).

2.2.3. Cannabinoids inhibit the angiogenesis, invasion, and metastasis of tumor cell

Cannabinoids inhibit angiogenesis by blocking the activation of the vascular endothelial growth factor (VEGF) pathway in many cancer cells. The administration of cannabinoids into various skin tumors, gliomas, and thyroid carcinomas showed that cannabinoids contributed to the downregulation of VEGF, resulting in anti-angiogenesis (Casanova et al., 2003; Portella et al., 2003; Blázquez et al., 2006). Additionally, the anti-invasive activity of cannabinoids was firstly reported within prostate cancer cells through CB1 receptor activation by 2-AG (Nithipatikom et al., 2004). Furthermore, the administration of CBD to breast and brain cancer cells showed the downregulation of Id-1 and thus led to the decrease of invasion of the tumor cells (McAllister et al., 2007; Soroceanu et al., 2013). The downregulation of Id-1 was also confirmed to inhibit breast cancer cell invasion through activation of the

CB2 receptor agonist O-1663. (Murase et al., 2014) Besides, the agonist of the CB2 receptor (JWH-133) showed the anti-proliferative and anti-angiogenic effects within lung cancer cells (Vidinsky et al., 2012).

2.2.4. Cannabinoids induced antitumor immunity

The activation of cannabinoid receptors showed a direct effect on immune cells in both *in vitro* and *in vivo* studies. THC affects tumorigenesis by interfering with macrophages and lymphocyte activities on tumor surveillance (Zhu et al., 1998). Besides, *in vivo* studies indicated that using THC at a dose of 10 mg/kg body weight (BW) may affect the total number of T cells, B cells, and macrophages via the decreased cellularity within the thymus and spleen of normal mice (McKallip et al., 2002). On the other hand, the effect of enhancing the immune system of cannabinoids was indicated by several *in vivo* studies. The activated CB1 and CB2 agonist (WIN55,212-2), and activated CB2 agonist (JWH-133) exhibited higher effects on immunocompetent xenograft mice in comparison with immunodeficient xenograft mice in melanoma (Blázquez et al., 2006). Additionally, immunocompetent rats were treated with THC at a high dose of 50 mg/kg BW with 5 times per week during the treatment period of 2 years showed that these rats had longer overall survival time and less numerous types of incidences tumors (Chan et al., 1996).

2.3. Cannabinoid effects on PDAC

PDAC is a mainly popular and aggressive type of pancreatic cancer which highly devastating to patient health (Kleeff et al., 2016; Orth et al., 2019). Previous *in vitro* publications investigated the presence of CB1, CB2, and their agonists within the pancreatic cell lines to understand the antitumor effect of cannabinoids. The previous research indicated that the administration dose-dependent of THC induces a reduction in cell viability via the apoptotic process, specifically via CB2 receptor and ceramide-dependent up-regulation of p8 and ATF-4, and TRB3 stress-related genes (Carracedo et al., 2006b). Meanwhile, there are several studies that focus on the activation of the CB1 receptor. They reported the AM251 agonist receptor-induced apoptosis through the CB1 receptor, which involved MAPK signaling pathways within the MIA PaCa-2 pancreatic cancer cell line (Fogli et al., 2006). Alternatively, the combination of cannabinoids and chemotherapy therapeutics

suggested promising results in *in vitro* study (Yasmin-Karim et al., 2018). On the other hand, *in vivo* studies showed the successful treatment of pancreatic cancer by combining cannabinoids and radiotherapy therapeutic within xenograft mice model (Yasmin-Karim et al., 2018). Furthermore, injection of THC with a dose of 15 mg/kg/day into a xenograft model carrying MIA PaCa-2 pancreatic cancer indicated decreased tumor growth and reduced extension of the tumor cells via the apoptosis process (Carracedo et al., 2006a). KPC mice with combined treatment among CBD and gemcitabine showed three-time longer survival times than the control or gemcitabine group (Ferro et al., 2018).

Although recent studies highlight the therapeutic effect of cannabinoids on pancreatic cancer, there are few studies focused on the proliferation of apoptosis-related genes and proteins. Therefore, the current research focuses on the anti-proliferative and apoptotic-induced effects at various doses of cannabinoids on the PDAC xenograft mice model.

2.4. Conceptual framework

A study conceptual framework was presented in Fig. 3.
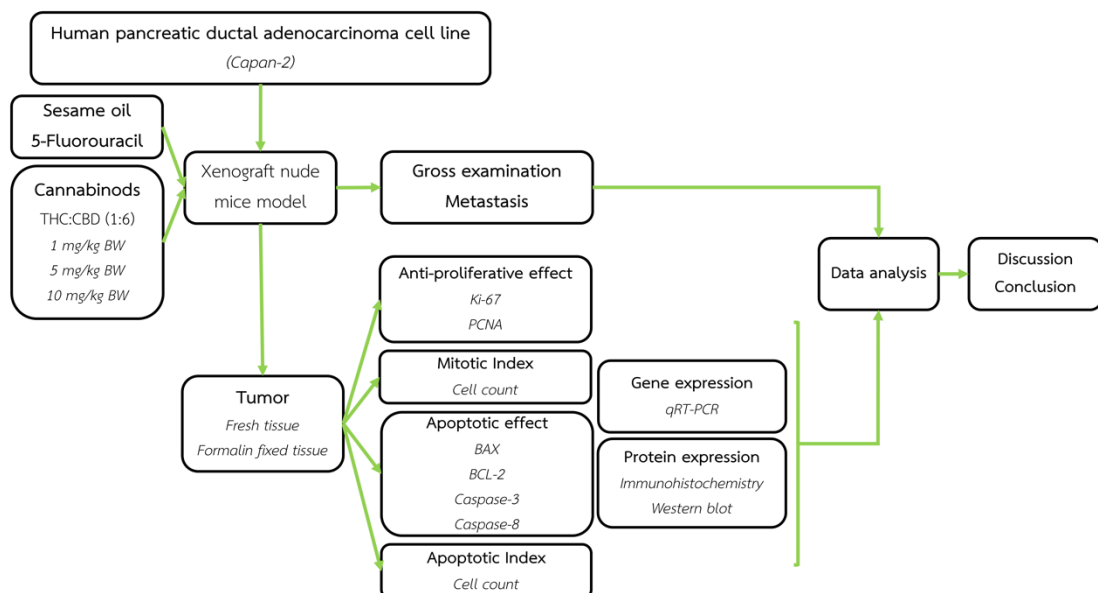
Figure 3: The conceptual framework applied for the present study

CHAPTER 3: MATERIALS AND METHODS

3.1. Animal model

3.1.1. Animal ethics

The tissue samples of the current study were obtained from the previous animal experiment which was approved by the Institutional Animal Care and Use Committee (IACUC) of The National Cancer Institute, Thailand (Approval No.272_2019RB_IN602) and Lerdsin Hospital, Department of Medical Services (Approval No.ACE-F-v03-02). All animal experiment procedures were completely followed according to the approved guidelines.

3.1.2. Human pancreatic ductal adenocarcinoma cell line

Briefly, Capan-2 cell line (HBT-80^{TM}) was purchased from the American Type Culture Collection (ATCC) and maintained growth in other McCoy's 5a cell culture medium (ATCCTM 30-2007, USA) complemented with 10% fetal bovine serum (Cell-culture tested, ATCCTM 30-2007, USA). Cells were cultured at a 37°C incubator containing 5% CO_2 within humidified air. The cell digestion was carried out with trypsin-EDTA solution 0.25%, and then supplemented with 10 ml working phosphate-buffered saline (PBS) pH 7.2 (Gibco, Thermo scientific, USA) prior to the cell line transplantation. The collected cells were stained with 1 mL of 0.4% Trypan blue dye (Gibco, Thermo scientific, USA) to evaluate the availability of the cells by hemocytometer chamber. The individual injection was maintained with 5x10^6 cells within 0.1 mL volume for tumor implantation at the flank of nude mice.

3.1.3. Cannabinoid solution preparation

The THC:CBD (1:6) solution was prepared and obtained from the Government Pharmaceutical Organization (GPO), Thailand. *Cannabis sativa* L. strain was selected for this study and growing within the GPO's greenhouse medical cannabis plantation. The cola of *cannabis* was collected, and then cannabinoids were extracted by using ethanol extraction. Finally, cannabinoids extract was dissolved in pharmaceutical-grade oil.

3.1.4. Xenograft nude mouse model

Twenty-five male immunodeficient mice (nude mice, BALB/cAJcl-nu) at four-week-old were obtained from Nomura Siam International, Co. Ltd. (Bangkok, Thailand). The nude mice were raised at Lerdsin Hospital animal facility within a strictly hygienic conventional laboratory system maintained a temperature of 22°C, relative humidity of 50 to 70% and under a 12-hour light/dark cycle. All mice were acclimatized a week prior to starting an experiment. A xenograft model of human PDAC was then subcutaneously injected with $5x10^6$ Capan-2 cells at the right flank region under aseptic conditions. The xenograft tumors were measured once every 3 days. Tumor volume was calculated according to the described formula: Volume (mm^3) = (Length x Width^2) with the length and width measured by an vernier caliper (Song et al., 2017). The treatments were started when tumor volume developed around 200 mm^3. Five mice were randomly arranged into a treatment group according to the completely randomized design. Two controls and three experimental treatments were following:

Group 1 - Negative control: Mice were gavaged with sesame oil for 30 days.

Group 2 - Positive control: Mice were intraperitoneally injected with 5-fluorouracil (5-FU) with a dose of 20 mg/kg BW, 3 times a week.

Group 3 - Experimental group: Mice were gavaged with THC:CBD (1:6) solution at a dose of 1 mg/kg BW/day treated for 30 days.

Group 4 - Experimental group: Mice were gavaged with THC:CBD (1:6) solution at a dose of 5 mg/kg BW/day treated for 30 days.

Group 5 - Experimental group: Mice were gavaged with THC:CBD (1:6) solution at a dose of 10 mg/kg BW/day treated for 30 days.

The cannabinoid solution with a ratio of THC:CBD (1:6) was used in this study according to the formula of the GPO, Thailand. The tumor volume was measured at the initial (day 0) and the end of the experiment (day 30). Briefly, the experimental scheme was presented in Fig. 4.
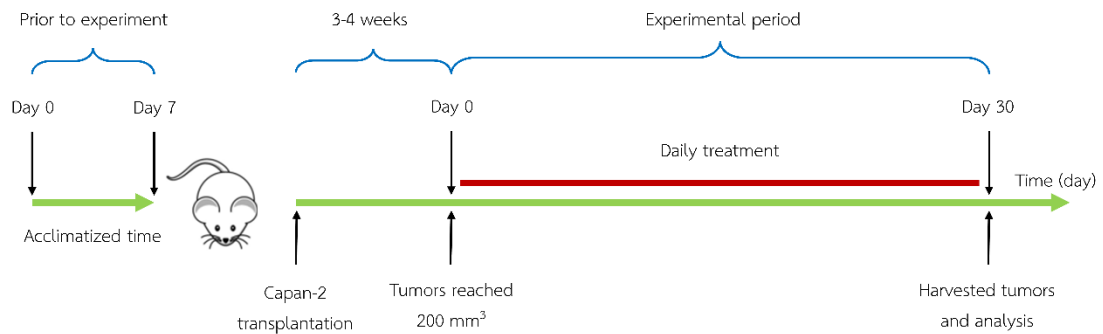
Figure 4: The experimental design of the *in vivo* experiment

3.2. Gross examination

Gross examination was performed subsequent to euthanasia and necropsy. Macroscopic lesions were observed as well as the metastasis of tumors within the various organs.

3.3. Sample collection

The samples of twenty-five xenograft nude mice models including tumors and internal organs (lung, heart, liver, pancreas, spleen, and kidney) were collected and fixed with 10% neutral buffered formalin for 24 hours. In all collected samples, the routine tissue processing was performed (paraffin embedding and hematoxylin and eosin (H&E) stain). The fresh tumors were also kept at -80°C prior to being used.

3.4. Histopathological study

3.4.1. Xenograft tumor examination

The morphological evaluation and subtype classification of PDAC were accomplished according to the previous publication of the World Health Organization (WHO) (Bosman et al., 2010).

These classifications consist of a six-subtype classification including:

1) Adenosquamous carcinoma

2) Colloid carcinoma

3) Hepatoid carcinoma

4) Medullary carcinoma

5) Signet ring cell carcinoma

6) Undifferentiated carcinoma, and undifferentiated carcinoma with osteoclast-like giant cells

In addition, the capsule of tumor mass, morphology of PDAC cells, vascularization, and stroma were evaluated within individual specimens under a light microscope at the high-power field (HPF) (40X, area equals 2.37 mm^2). The number of apoptotic cells and mitotic cells in tumor sections was counted as described in the previous study (Meuten et al., 2016). For the cell count, a total of ten areas were randomly captured at the HPF (40X, area equals 2.37 mm^2) per section. Microscopically, mitotic cells characterized by a set of morphological features which was differentiated in each phase of the cell cycle. Briefly, in prophase, hyperchromatic chromosomes, the nucleoli shrank and disappeared, and the nuclear membrane was irregular or ragged and hairy-like projections. In metaphase, the nuclear membrane disappeared, and the chromosomes were arranged at the metaphase plate. In anaphase, two daughter chromosomes detached and moved to the two poles of the cell, intact cell membrane, and absent nuclear membrane and nucleolus. In telophase, two daughter nuclei form, nucleoli reappeared, less condensed chromosomes, and divided the cytoplasm into two new daughter cells (Ibrahim et al., 2022). Meanwhile, the apoptotic cell was characterized by a blebbing cell membrane, nucleus and cytoplasm reduction in the volume (cell shrinkage), nuclear fragments, condensed organelles and cytosol, and moderately eosinophilic cytoplasm in the morphology of the cell (Häcker, 2000). Apoptotic and mitotic cell count were accomplished by manually counting the section from an individual mouse within each group. The average number of apoptotic and mitotic cells per section was followed by the formula of the number of expressed cells per HPF (cells/HPF). The histopathological evaluation was performed by double-blind study.

3.4.2. Metastasis examination

The internal organ sections stained with H&E were microscopically observed for the metastasis of PDAC cells.

All histological examination results were evaluated as the same as the condition of the xenograft tumor examination. A double-blind study was applied to the evaluation of gross metastasis examination.

3.5. Gene expression study

The gene expression of apoptotic-related genes (*BAX*, *BCL-2*, *Caspase-3*, and *Caspase-8*) within xenograft tumors were determined using the qRT-PCR technique.

3.5.1. RNA extraction

The innuPREP RNA Mini Kit 2.0 (Analytik Jena AG, Jena, Germany) was selected to extract the total RNA from fresh xenograft tumors following the guideline of the manufacturers. Briefly, a maximum of 20 mg of fresh tumors were manually homogenized at room temperature (RT) (20-25°C) and was lysed within 450 µL of Lysis Solution RL. Following, 400 µL of 70% ethanol was supplemented, well mixed, and then transferred into the Spin Filter R column. Continuously, 500 µL of Washing Solution HS and 700 µL of Washing Solution LS were added, well centrifuge at 11,000 rpm for 1 minute between each step. The Spin Filter R column was placed into a new 1.5 mL Elution tube, add on a volume of 50 µL RNAse-free water, and centrifuged at 11,000 rpm for a minute. The aqueous phase of the final mixture was collected and kept at -80°C until used for further process. The spectrophotometer (NanoDrop) (Thermo Fisher Scientific Inc., Wilmington, USA) was used to confirm the concentration of total RNA within the sample over the range from 50 ng to 2 µg.

The synthesis of cDNA was transfected by using the qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, United Kingdom). The synthesis of the cDNA process followed the guideline of the manufacturers. The quality of synthesized products was checked by spectrophotometry (NanoDrop) (Thermo Fisher Scientific Inc., Wilmington, USA) to be ensured the level of cDNA was above 50 ng per 20 µL reaction. The collected products were stored at -20°C until used for PCR amplification.

3.5.2. Quantitative reverse transcription real-time PCR (qRT-PCR)

The expression genes within this study were amplified using the gene-specific primer pairs (Table 1) as focusing the previous publications for *BAX*, *BCL-2*, *Caspase-3,* and *Caspase-8* genes. qRT-PCR was performed on a Rotor-Gene Q thermocycler (Qiagen, Hilden, German). Briefly, 20 ng of cDNA were well mixed in 10 µL of 2x qPCRBIO SyGreen Mix (PCR Biosystems, London, United Kingdom), 400 nM of individual primer, and adjusted into a total of 20 µL final volume, according to the

guideline of the manufacturers. The thermal cycle of the qRT-PCR reaction consisted of polymerase activation at 95°C for 2 minutes, following 40 cycles of denaturation at 95°C for 5 seconds, and extension for 30 seconds at 60°C. The individual sample was carried out in duplicate per each target gene. The qRT-PCR determined the cycle threshold (Ct) value for individual target genes. The relative quantification among target genes was accomplished through the earlier $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The *β-actin* gene was chosen as an internal control following the prior descriptions (Rubie et al., 2005; Iyer et al., 2017). The expression level of the individual target genes among experimental groups was averaged and displayed as a normalized ratio. The *BAX/BCL-2* ratio was calculated by dividing the normalized ratio of *BAX* and *BCL-2* gene expression.

Table 1: The sequences of qRT-PCR primer pairs using in the study

Gene	Sequence (5'-3')	Size (bp)	Reference
β-actin	F: AGCGAGCATCCCCCAAAGTT	285	Garcia-Ortiz et al. (2017)
	R: GGGCACGAAGGCTCATCATT		
BAX	F: TGGCAGCTGACATGTTTTCTGAC	195	Karaliotas et al. (2015)
	R: TCACCCAACCACCCTGGTCTT		
BCL-2	F: CAGGATAACGGAGGCTGGGATG	134	Karaliotas et al. (2015)
	R: AGAAATCAAACAGAGGCCGCA		
Caspase-3	F: GCGGTTGTAGAAGAGTTTCGTG	101	Johnson et al. (2016)
	R: CTCACGGCCTGGGATTTCAA		
Caspase-8	F: AGAGTCTGTGCCCAAATCAAC	78	Borhani et al. (2014)
	R: GCTGCTTCTCTCTTTGCTGAA		

3.6. Protein expression study

The expression of the protein within tumors of xenograft nude mice was investigated regarding IHC and WB techniques of the proliferative (Ki-67 and PCNA) and the apoptotic markers (BAX, BCL-2, Caspase-3, and Caspase-8).

3.6.1. Immunohistochemistry

The paraffin-embedded tissues were cut into a section of 4 μm-thick and placed in the positive charged glass slides, air-dried at RT. The specimens were deparaffinized with xylenes, rehydrated with a gradual decrease within alcohol

concentration (100%, 95%, 80%, and 70% of alcohol), and then washed within running water and distilled water. The slides were pretreated for antigen retrieval, conditions following the antibody (Table 2). Endogenous peroxidase activity was quenched within a chamber containing 3% of H_2O_2 for 10 minutes at RT. For blocking non-specific antibodies, all specimens were blocked with 1% bovine serum albumin for 30 minutes at 37°C, subsequent to stained with primary antibody at 4°C, and incubated overnight. The used primary antibody to dilute within PBS following the guideline of manufacture (Table 2). Subsequently, the secondary antibody was following applied, using a biotinylated goat anti-mouse/anti-rabbit antibody (Envision, Dako, Glostrup, Denmark) for 1 hour at RT. A freshly prepared 3, 3′-diaminobenzidine (DAB) solution (Dako, Glostrup, Denmark) was added to visualize the colored reaction. Finally, the slides were immersed within distilled water, counterstained with Meyer's hematoxylin stain, dehydrated within graded alcohol, and permanently mounted. The lymph node of a mouse was recruited as a positive control, following the same procedure as shown in the experimental specimens. The negative control was experimental specimens, following the same procedure, except for incubating with a primary antibody.

All specimens of immunohistochemical labeling for the individual antibody in each group were evaluated with an HPF (40X, area equals 2.37 mm^2). For each tumor section, a total of ten areas were randomly selected and captured by the digital imaging system. In a group of proliferative markers (Ki-67 and PCNA), the positive cells per all nucleated cells within a minimum of 500 cells observation (numbers of positive cells/in 500 cells) were evaluated. The data were calculated and displayed as a percentage (%) of positive cells. On the other hand, the percentage of positive cells per 1 mm^2 (%IHC positive area/mm^2) within a group of related-apoptotic markers (BAX and BCL-2) were calculated by the image analysis program (NIS-Elements Analysis D, Nikon, Japan). The BAX/BCL-2 ratio was calculated by dividing the normalized ratio of BAX and BCL-2 protein expression. Caspase-3 was analyzed via the number of positive cells per HPF (cells/HPF) according to the prior study (Sakarin et al., 2022).

Table 2: The immunohistochemical primary antibody, working dilutions, antigen retrieval, and positive staining pattern

Antibody	Source	Clone/Cat. Num.	Dilution	Antigen retrieval	Staining pattern
Ki-67	Thermo Fisher Scientific, Massachusetts, USA	8D5	1:100	Tris-EDTA (pH 9.0) Autoclave, 121°C, 20 min	Nucleus
PCNA	Dako, Hamburg, Germany	PC10	1:100	Citrate (pH 6.0) Microwave, 10 min	Nucleus
BAX	Sigma-Aldrich, Missouri, USA	1F5-1B7	1:100	Citrate (pH 6.0) Microwave, 10 min	Cytoplasm
BCL-2	Dako, Hamburg, Germany	124	1:100	Citrate (pH 6.0) Autoclave, 121°C, 20 min	Cytoplasm
Caspase-3	Abcam, Cambridge, UK	Ab4051	1:100	Citrate (pH 6.0) Autoclave, 121°C, 20 min	Cytoplasm, Nucleus
Caspase-8	Santa Cruz Biotechnology, Oregon, USA	8CSP03	1:100	Citrate (pH 6.0) Autoclave, 121°C, 20 min	Cytoplasm

3.6.2. Western blot

Western blot analysis was conducted with the extracted protein from the xenograft tumors following the guideline of Minute™ Total Protein Extraction Kit for Animal Cultured Cells and Tissues (Invent Biotechnologies, Massachusetts, USA) and stored at -20°C until used for the next step. The lysed protein was subjected to using 10% SDS-PAGE polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Santa Cruz Biotechnology, Texas, USA). Thereafter, the membrane was blocked with 5% skim milk for 1 hour at RT, and was incubated overnight with primary antibody (Table 3) at 4°C. Following, anti-mouse IgGk BP-HRP (Cat. Num. sc-516102, Santa Cruz Biotechnology, Oregon, USA) or anti-rabbit IgG-HRP (Cat. Num. sc-2357, Santa Cruz Biotechnology, Oregon, USA) at 1:5,000 dilution was developed as secondary antibody for 1 hour at RT. For visualization, the LumiFlash™ Prime Chemiluminescent Substrate (Energenesis Biomedical, Taipei, Taiwan) and ChemiDoc and ChemiDoc MP Imaging Systems (Bio-Rad, California, USA) were chosen to detect and scan the results, respectively. The individual sample was run with three replications. The labeling intensity of samples was evaluated via the signal strength, using ImageJ software (Ferreira and Rasband, 2012). The strong or weak signals in the visualized results demonstrated the level of presented protein in the samples.

Table 3: The primary antibodies used for WB

Antibody	Source	Clone/Cat. Num.	Dilution
β-actin	Santa Cruz Biotechnology, Oregon, USA	C4	1:500
Ki-67	Thermo Fisher Scientific, Massachusetts, USA	8D5	1:500
PCNA	Dako, Hamburg, Germany	PC10	1:500
BAX	Sigma-Aldrich, Missouri, USA	1F5-1B7	1:500
BCL-2	Dako, Hamburg, Germany	124	1:500
Caspase-3	Abcam, Cambridge, UK	Ab4051	1:500
Caspase-8	Santa Cruz Biotechnology, Oregon, USA	8CSP03	1:500

3.7. Statistical analysis

The one-way analysis of variance (ANOVA) and post hoc test were considered to test the difference in the quantitative variables (apoptotic index, mitotic index, Ki-67, PCNA, and Caspase-3 protein expression) as well as semi-quantitative variables

(BAX and BCL-2 protein expression) among groups. The qRT-PCR and WB results were evaluated by a nonparametric test to compare the differences between the experimental groups, using the Kruskal-Wallis test. All statistical analyses were performed by the SAS software version 9 for Windows (SAS Institute, Inc., USA). The values were presented as mean ± standard deviation (SD). P values below 0.05 were considered the criterion for statistically significant.

CHAPTER 4: RESULTS

4.1. Gross examination

A total of 25 xenograft nude mice were subcutaneously injected with Capan-2 cell line (5×10^6 cells) at the right flank region. Subsequently, the transplanted tumor was weighed and measured tumor size once every 3 days. The experiment was started during the period of the average volume of xenograft tumors reached 200 mm^3. There were five groups (5 mice/group) of treatment included negative control (NC) group, positive control (PC) group, and THC:CBD at a dose of 1 mg/kg BW, 5 mg/kg BW, and 10 mg/kg BW. At the end of the experiment, all mice were euthanized and were grossly examined. Grossly, the tumors were presented subcutaneously at the transplanted areas of all xenograft nude mice. In the xenograft tumors, ulcerated and necrosis were found in several nude mice of each experimental group (Fig. 5). In other internal organs, there was no significant change in gross examination except mild to moderate pulmonary congestion. The metastasis of tumor cells in both intrathoracic and intraabdominal organs was not macroscopically observed.

| a. THC:CBD 1 mg/kg BW | b. THC:CBD 5 mg/kg BW | c. THC:CBD 10 mg/kg BW |

| d. NC | e. PC |
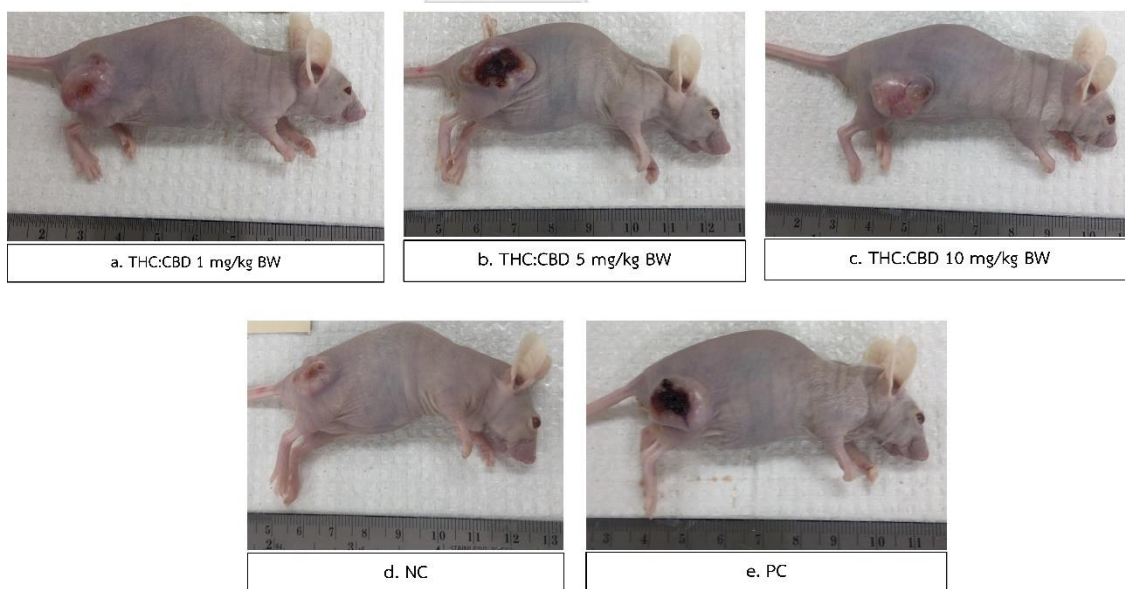
Figure 5: The macroscopic images of the representative mice injected with Capan-2 cell line on the 30 days of the treatment with THC:CBD at a dose of 1 mg/kg BW (a), 5 mg/kg BW (b), 10 mg/kg BW (c), NC group (d), and PC group (e). The transplanted tumors were found subcutaneously at the right flank region with ulceration and necrosis.

4.2. Histopathological study

4.2.1. Xenograft tumor examination

4.2.1.1. Tumor volume

The average tumor volume (mm^3) was calculated on day 0 (initial day) and day 30 (final day) of the experiment. There was no significant difference ($p > 0.05$) between the average volume of the xenograft tumors over the course of treatment (Fig. 6a, b). The percentage of tumor volume change increased in all groups. In comparison to the NC group, there was a decrease in tumor volume change (%) within the PC group (283.96 ± 37.52%) and the treatment groups consisting of 1 and 5 mg/kg BW (276.93 ± 36.41%, 269.32 ± 51.08%, respectively), whereas the greater growth of the tumors was found in the treatment group at a dose of 10 mg/kg BW (499.94 ± 20.51%). However, there was no statistical difference among groups ($p > 0.05$) (Fig. 6c).
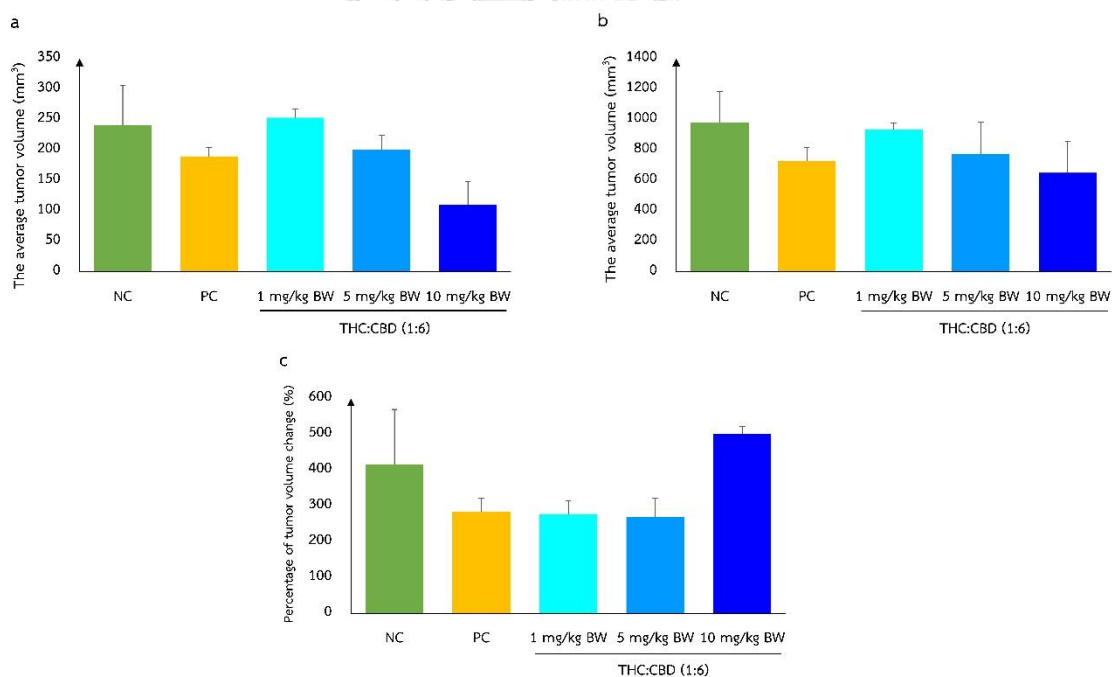
Figure 6: The average tumor volume (mm^3) on day 0 (a) and day 30 (b) over the course of treatment, and percentage of tumor volume change (%) (c) among the NC group, PC group, and treatment groups (THC:CBD at a dose of 1 mg/kg BW, 5 mg/kg BW, and 10 mg/kg BW). The values were presented as mean ± SD. The statistical analysis was calculated by the one-way ANOVA and post hoc test.

4.2.1.2. Histopathological morphology of the xenograft tumors

To evaluate the histopathological morphology of the transplanted tumors on the xenograft nude mice, the H&E-stained section of an individual xenograft tumor was examined and evaluated under a light microscope (40X). As can be seen in Fig. 7, the xenograft tumors were arranged into the glandular structure with fibrovascular stroma surrounding. Neoplastic cells were round to oval, lobulated with the intralesional necrotic area, and surrounded by clearly defined borders. The necrotic areas were frequently represented on the edge of xenograft tumors in the treatment and PC groups, while occasionally found in the center of the xenograft tumor in the NC group. To evaluate the distribution of the necrotic area among groups, the percentage of necrotic tissue was computed by the image analysis program (NIS-Elements Analysis D, Nikon, Japan). The results indicated that there was no statistically change ($p > 0.05$) in the necrotic area between the treatment groups at a dose of 1 mg/kg BW (40.64 ± 3.69%), 5 mg/kg BW (34.88 ± 6.77%), 10 mg/kg BW (27.31 ± 8.84%), the PC group (32.88 ± 2.94%), and the NC group (24.79 ± 4.09%) (Fig. 8).

Among the histopathological examined, the transplanted tumors displayed similar characteristics to the original neoplasm which was pancreatic ductal adenocarcinoma. In subtype classification, all the examined tumors mirrored the characteristic of adenosquamous carcinoma according to the WHO classification. Microscopically, the morphology of the xenograft tumors was characterized by the presence of lobular, tubular, tubulopapillary, and solid patterns (Fig. 9). The tumor patterns were revealed with round lobulated and well-formed glands with lumen lined by the epithelium cells. The tubulopapillary pattern was described as well-differentiated glands, dilated, and larger than the tubular pattern with the protrusion of tumors and fibrovascular stalks in the tubular lumen. In addition, the cribriform pattern was commonly detected in all examined tumors. The tumor cells were tall coluboidal to columnar in shape with moderately basophilic cytoplasm and prominent nucleoli. The nuclei of tumor cells were represented as mild polymorphism, hyperchromasia, chromatin clumping, and numerous mitotic activity

(Fig. 9). Besides, the vascularization examination of the xenograft tissue among all experimental mice demonstrated no proliferation of the blood vessels via H&E stained. Moreover, this study also performed IHC staining of blood vessels in the xenograft tumors via a specific antibody (CD31/PECAM-1). The labeling results of the CD31/PECAM-1 antibody showed no proliferation of the blood vessels in the xenograft tumors among groups (Supplementary data Fig. 1).
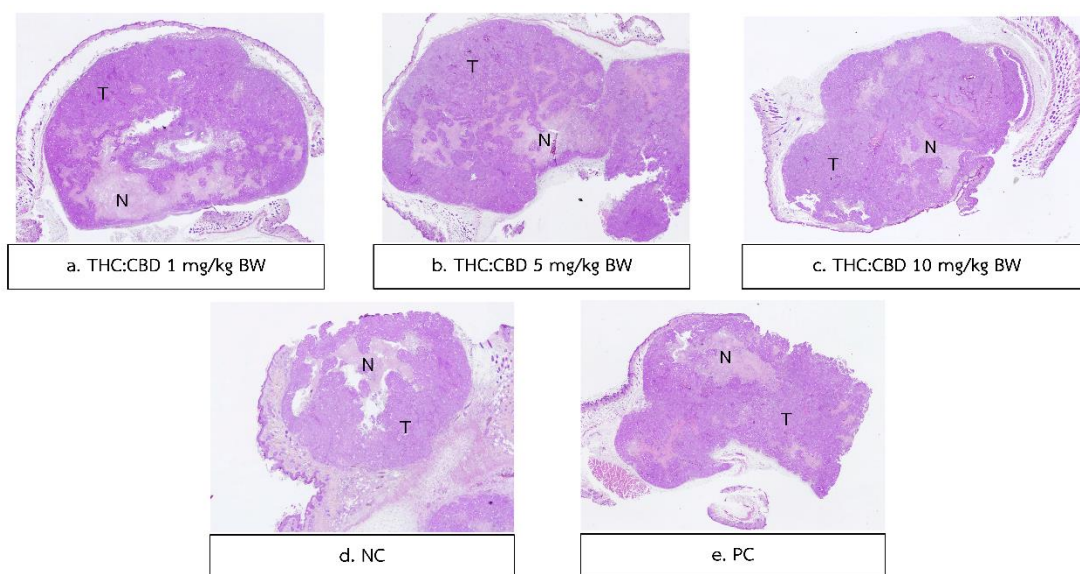
Figure 7: The macroscopic images of the xenograft tumors in the treatment groups with THC:CBD at a dose of 1 mg/kg BW (a), 5 mg/kg BW (b), 10 mg/kg BW (c), NC group (d), and PC group (e). The subcutaneous tumors were presented with tumor area (T) and necrotic area (N) (H&E stained).
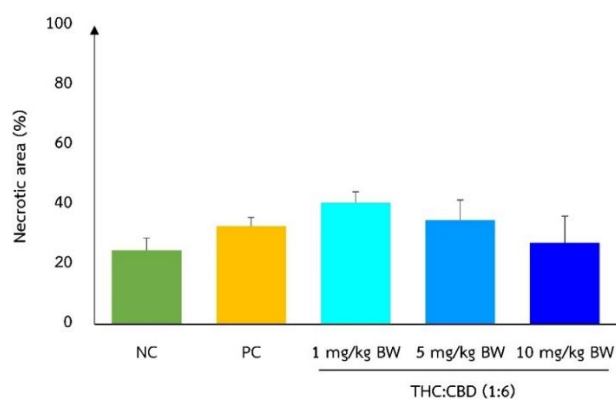
Figure 8: The percentage of necrotic area (%) in the xenograft tumors among the NC group, PC group, and treatment groups (THC:CBD at a dose of 1 mg/kg BW, 5 mg/kg BW, and 10 mg/kg BW). The values were shown as mean ± SD. The statistical analysis was calculated by the one-way ANOVA and post hoc test.
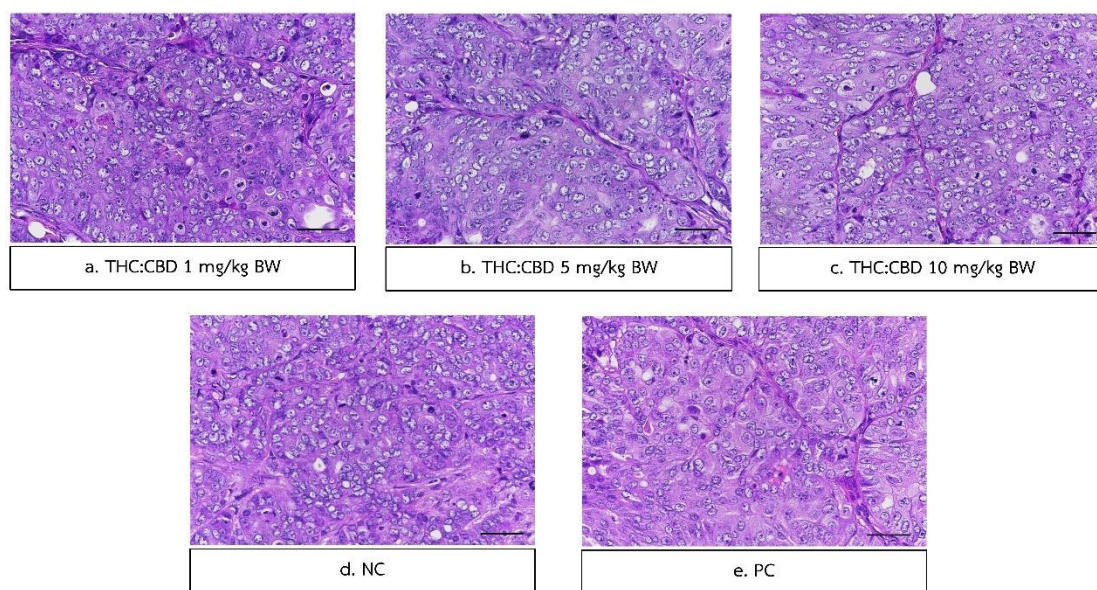
Figure 9: Histopathological morphology of the xenograft tumors among the treatment groups with THC:CBD at a dose of 1 mg/kg BW (a), 5 mg/kg BW (b), 10 mg/kg BW (c), NC group (d), and PC group (e) (H&E stained, 40X, scale bar = 50 μm).

4.2.1.3. Mitotic and apoptotic cell count (cells/HPF)

Ten areas of the H&E-stained section in each xenograft tumor were randomly captured to count the number of mitotic and apoptotic cells under light microscopy at high power magnification (40X). In the area of the predominated number of apoptotic cells, the number of mitotic cells was rare (Fig. 10). As shown in Fig. 11a, the mitotic cell count (cells/HPF) was highest in the NC group (10.50 ± 1.81) compared with those in the treatment group at a dose of 1 mg/kg BW (7.08 ± 1.25), 5 mg/kg BW (6.85 ± 0.88), 10 mg/kg BW (6.40 ± 0.85), and the PC group (6.98 ± 0.39). The difference in the mitotic cell count between experimental groups was significant ($p < 0.05$). In contrast, the apoptotic cell count (cells/HPF) (Fig. 11b) was significant increased ($p < 0.01$) for the treatment group at a dose of 1 mg/kg BW, 5 mg/kg BW, 10 mg/kg BW (14.73 ± 1.34, 15.33 ± 1.73, 15.95 ± 1.01, respectively), and the PC group (13.90 ± 1.93) in comparison with the NC group (7.58 ± 0.95). When comparing the mitotic/apoptotic (M/A) ratio (Fig. 11c), there was statistical difference ($p < 0.01$) between the NC group (1.40 ± 0.29) and the PC group (0.51 ± 0.07), the treatment group at a dose of 1 mg/kg BW, 5 mg/kg BW, 10 mg/kg BW (0.48 ± 0.08, 0.45 ± 0.09, 0.40 ± 0.07, respectively).
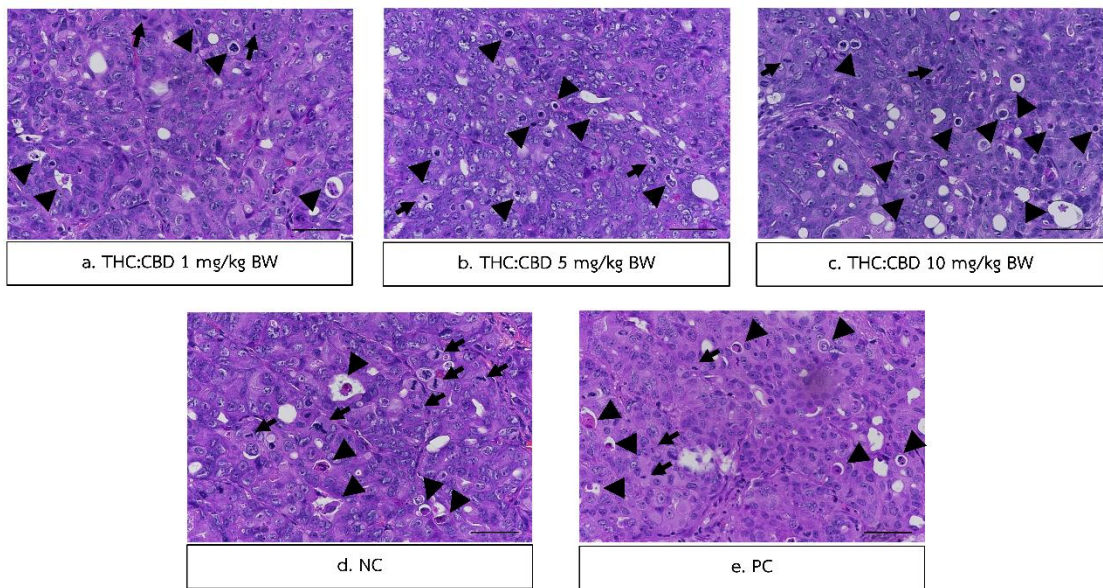
Figure 10: Histopathological morphology of the tumors in xenograft nude mice. The tumors performed scattered mitotic cells (arrow) and apoptotic cells (arrowhead) in the treatment groups with THC:CBD at a dose of 1 mg/kg BW (a), 5 mg/kg BW (b), 10 mg/kg BW (c), NC group (d), and PC group (e) (H&E stained, 40X, scale bar = 50 μm).
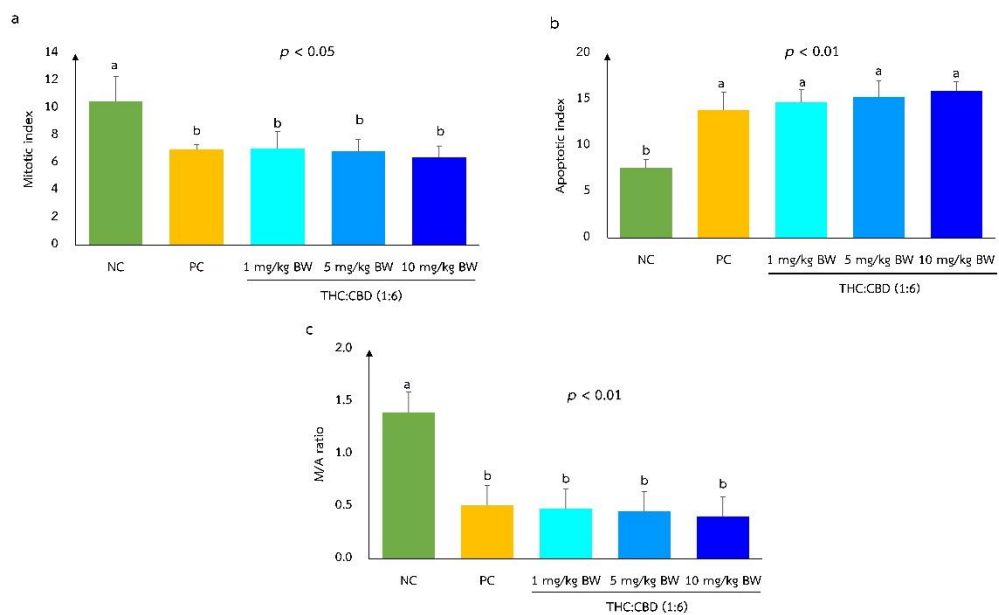
Figure 11: The mitotic (a) and apoptotic cell count (cells/HPF) (b) among the NC group, PC group, and treatment groups (THC:CBD at a dose of 1 mg/kg BW, 5 mg/kg BW, and 10 mg/kg BW). The mitotic/apoptotic (M/A) ratio (c) was calculated via dividing the mitotic index by the apoptotic index. The values were presented as mean ± SD. The statistical difference was calculated by the one-way ANOVA and post hoc test. [a,b] Mean with different letters were significant differences among groups ($p < 0.05$).

4.2.2. Metastasis examination

The metastatic spread of the human PDAC cell line transplanted into the xenograft mice was examined by evaluation in the H&E-stained images of both intrathoracic organs (lung, heart) and intraabdominal organs (liver, pancreas, spleen, kidneys). As shown in Fig. 12, there was no significant change in the selected organs, except mild pulmonary congestion. The evidence of inflammation and necrosis in the representative organs was not discovered. These findings clearly suggested that there was no metastatic spread of the human PDAC cell line to the examined organs.
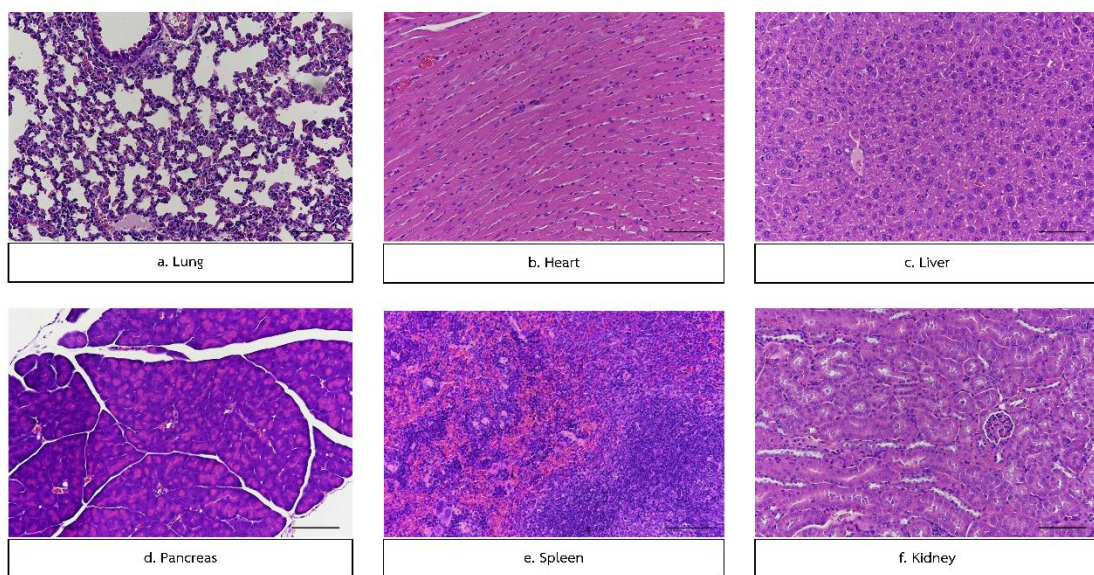
Figure 12: The histological images of the lung (a), heart (b), liver (c), pancreas (d), spleen (e), and kidney (f) from the xenograft nude mice injected with Capan-2 cell line (H&E stained, 40X, scale bar = 50 μm).

4.3. Gene expression study

To investigate the effect of cannabinoids on the human PDAC xenograft tumors through the mechanism of tumor cell death, the mRNA expression of apoptotic-related genes including *BAX, BCL-2, Caspase-3,* and *Caspase-8* was performed. The results were shown in Fig. 13.
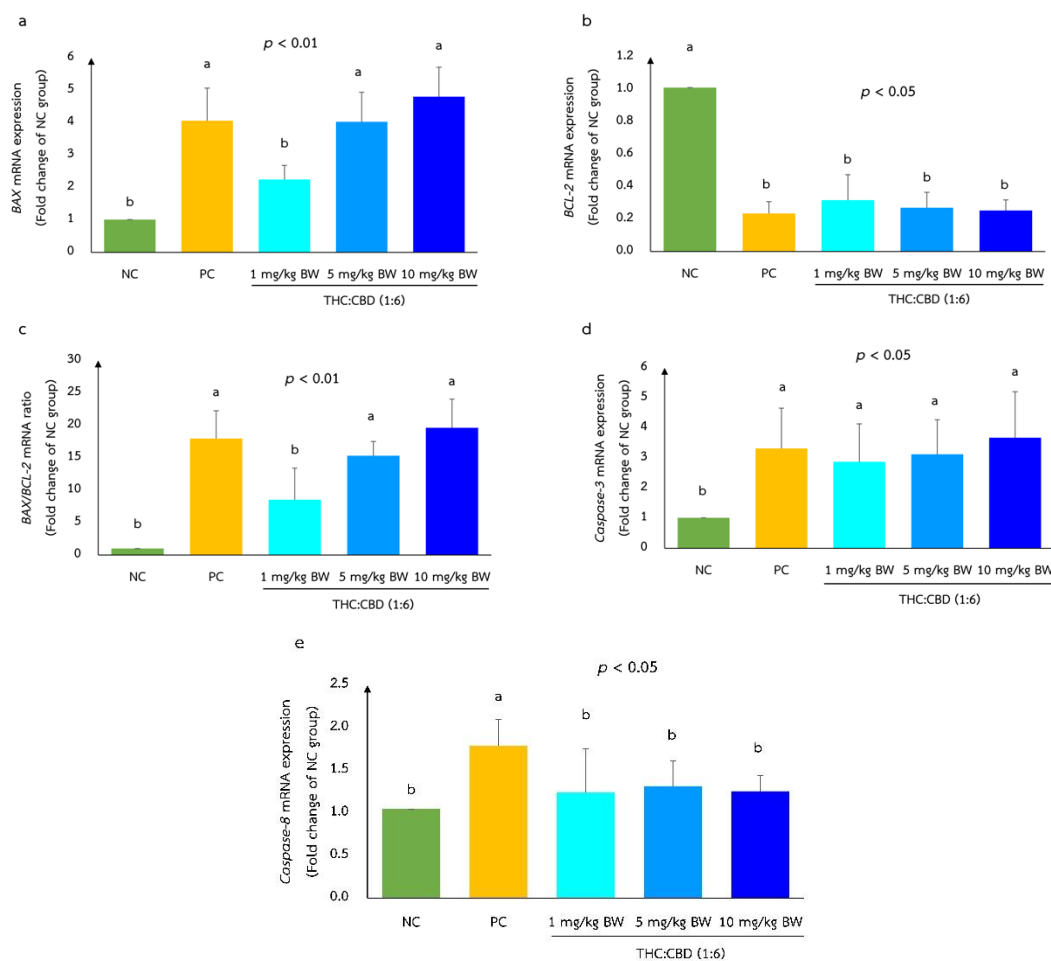
Figure 13: The mRNA expression of apoptotic-related genes: *BAX* (a), *BCL-2* (b), BAX/BCL-2 ratio (c), *Caspase-3* (d), and *Caspase-8* (e) among the NC group, PC group, and treatment groups (THC:CBD at a dose of 1 mg/kg BW, 5 mg/kg BW, and 10 mg/kg BW). The values were shown as mean ± SD. The statistical difference was calculated by the Kruskal-Wallis test. [a,b] Mean with different letters were significant differences among groups ($p < 0.05$).

As demonstrated in the results, the expression of the pro-apoptotic *BAX* gene was significantly higher in the PC and cannabinoid-treated groups at a dose of 5 mg/kg BW and 10 mg/kg BW, whereas the expression of the anti-apoptotic *BCL-2* gene was significantly lower in the PC and cannabinoid-treated groups compared to the NC group ($p < 0.05$) (Fig. 13a, b). Likewise, the *BAX/BCL-2* mRNA expression ratio significantly rises ($p < 0.05$) in the PC and cannabinoid-treated groups at a dose of 5 mg/kg BW and 10 mg/kg BW relative to the NC group (Fig. 13c). However, the mRNA expression of *BAX* gene and *BAX/BCL-2* mRNA ratio in cannabinoid-treated group at a dose of 1 mg/kg BW was not dramatically different ($p > 0.05$) compared to the NC

group (Fig. 13a, c). The mRNA expression of the *Caspase-3* gene increased significantly ($p < 0.05$) in the PC and cannabinoid-treated groups relative to the NC group (Fig. 13d). Moreover, the mRNA expression of the *Caspase-8* gene was not statistically different ($p > 0.05$) among cannabinoid-treated groups and the NC group (Fig. 13e).

4.4. Protein expression study

4.4.1. Immunohistochemistry

Correlation with the aim to investigate the effect of cannabinoids on the human PDAC xenograft tumors via the mechanism of tumor cell death, IHC was carried out to detect the expression of proliferative markers (Ki-67, PCNA), and apoptotic-related markers (BAX, BCL-2, Caspase-3, and Caspase-8) (Table 4).

As presented in Table 4 and Fig. 14a, there was a significant reduction ($p < 0.01$) in the number of Ki-67 positive cells among all cannabinoid-treated groups and the PC group when compared with the NC group (Table 4, Fig. 14a). Similarly, the number of PCNA-positive labeling cells significantly decreased ($p < 0.01$) in cannabinoid-treated groups and the PC group relative to the NC group (Table 4, Fig. 14b). On the other hand, the percentage of BAX-positive cells was dramatically increased ($p < 0.01$) in cannabinoid-treated groups (5 mg/kg BW and 10 mg/kg BW) and the PC group compared to the NC group (Table 4, Fig. 14c). However, the expression of BAX protein was not different ($p > 0.05$) between cannabinoid-treated group at a dose of 1 mg/kg BW and the NC group (Table 4). Contrary to BAX, the percentage of BCL-2 positive cells in the NC group was higher than those in the treatment groups (1 mg/kg BW, 5 mg/kg BW, and 10 mg/kg BW) and PC group ($p < 0.01$) (Table 4, Fig. 14d). The BAX/BCL-2 ratio statistically raised ($p < 0.01$) between the treatment groups (5 mg/kg BW and 10 mg/kg BW) and the PC group in comparison with the NC group, meanwhile, there was no significant difference ($p > 0.05$) between cannabinoid-treated group at a dose of 1 mg/kg BW and the NC group (Table 4). The intranuclear labeling of Caspase-3 was presented in all experimental groups. The number of Caspase-3 positive cells (cells/HPF) significantly decreased ($p < 0.01$) in the NC groups compared to the treatment groups (1 mg/kg BW, 5 mg/kg

BW, and 10 mg/kg BW) or the PC group (Table 4, Fig. 14e). Meanwhile, there was no positive label of Caspase-8 in all groups (Table 4, Fig. 14f).
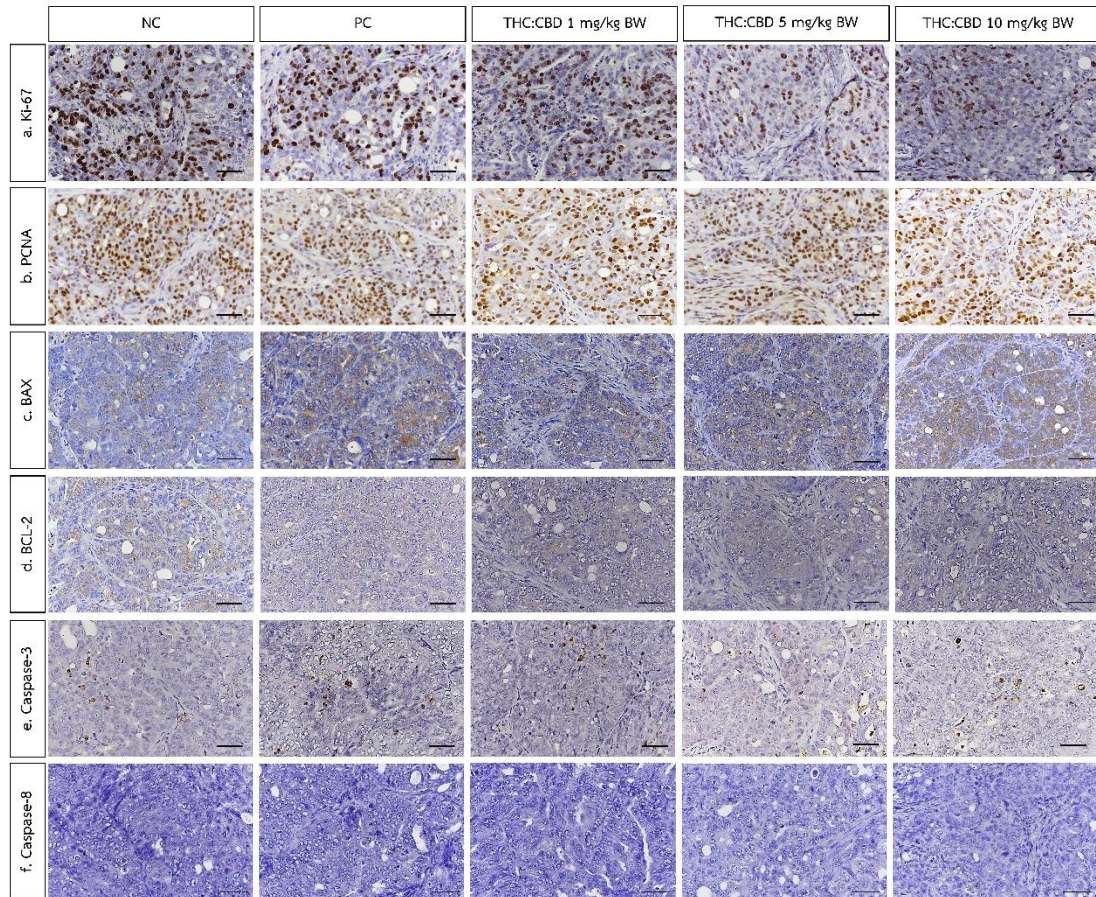
Figure 14: The immunoexpression of Ki-67 (a), PCNA (b), BAX (c), BCL-2 (d), Caspase-3 (e), and Caspase-8 (f) among the NC group, PC group, and the treatment groups with THC:CBD at a dose of 1 mg/kg BW, 5 mg/kg BW, and 10 mg/kg BW. The expression of the target proteins was found in all groups except Caspase-8. The intranuclear labeling of Ki-67, PCNA, and Caspase-3; intracytoplasmic labeling of BAX, BCL-2 in the xenograft tumors were displayed as brown color (DAB labeled, Mayer's hematoxylin counterstained, 40X, scale bar = 50 μm).

Table 4: The distribution of six target protein expressions among experimental groups via IHC evaluation

Parameter	NC	PC	THC:CBD 1 mg/kg BW	THC:CBD 5 mg/kg BW	THC:CBD 10 mg/kg BW
Ki-67 (%)	42.44 ± 8.64[a]	22.13 ± 3.22[b]	28.69 ± 9.25[b]	20.66 ± 8.21[b]	19.17 ± 6.23[b]
PCNA (%)	40.28 ± 5.97[b]	21.70 ± 2.79[b]	25.15 ± 10.66[b]	20.17 ± 5.07[b]	18.50 ± 3.96[b]
BAX (%/mm^2)	20.51 ± 6.62[b]	35.04 ± 5.93[a]	27.31 ± 1.70[b]	34.46 ± 3.77[a]	40.20 ± 7.89[a]
BCL-2 (%/mm^2)	10.97 ± 0.63[a]	5.74 ± 1.34[b]	6.41 ± 0.92[b]	5.86 ± 0.74[b]	5.09 ± 0.76[b]
BAX/BCL-2 ratio	1.88 ± 0.62[b]	6.50 ± 2.35[a]	4.35 ± 0.81[a]	5.98 ± 1.17[a]	8.17 ± 2.48[a]
Caspase-3 (cells/HPF)	9.14 ± 1.83[b]	14.26 ± 2.19[a]	13.86 ± 1.72[a]	15.61 ± 1.73[a]	17.75 ± 2.16[a]
Caspase-8	-	-	-	-	-

The values were shown as mean ± SD; The statistical difference was appraised by the one-way ANOVA and post hoc test

[a,b] Mean with different letters in the same row were significant difference among groups ($p < 0.01$)

4.4.2. Western blot

Although, the expression of proliferative markers (Ki-67 and PCNA) and apoptotic-related markers (BAX, BCL-2, and Caspase-3) were detected via qRT-PCR and IHC, however, WB was conducted to confirm the qRT-PCR and IHC results. The WB results were presented in Fig. 15.

The WB results showed that the relative protein expression levels of Ki-67 were significantly dropped ($p < 0.05$) in cannabinoid-treated groups (5 mg/kg BW and 10 mg/kg BW) and the PC group compared to the NC group. However, there was not significantly different ($p > 0.05$) in cannabinoid-treated group at a dose of 1 mg/kg BW and the NC group (Fig. 15b). Meanwhile, the relative protein expression levels of PCNA were dramatically decreased ($p < 0.05$) in cannabinoid-treated groups compared to the NC group (Fig. 15c).

The relative expression levels of pro-apoptotic protein BAX increased significantly ($p < 0.05$) in cannabinoid-treated groups (5 mg/kg BW and 10 mg/kg BW) and the PC group relative to the NC group (Fig. 15d), while the relative expression levels of anti-apoptotic protein BCL-2 were dramatically decreased ($p < 0.05$) in cannabinoid-treated groups and the PC group compared to the NC group (Fig. 15e). Similarly, the relative protein expression levels of BAX/BCL-2 ratio were statistically different ($p < 0.05$) in cannabinoid-treated groups (5 mg/kg BW and 10 mg/kg BW) and the PC group relative to the NC group (Fig. 15f). Contradictorily, the relative expression levels of protein BAX and BAX/BCL-2 ratio in the treated group of cannabinoids at a dose of 1 mg/kg BW were not significantly different ($p > 0.05$) compared to the NC group (Fig. 15d, f).

The Caspase-3 protein expression levels were statistically significant ($p < 0.05$) in cannabinoid-treated groups and the PC group in comparison with the NC group (Fig. 15g). Besides, the Caspase-8 protein expression levels were dramatically increased ($p < 0.01$) in the PC group when compared with the NC group, meanwhile, there was not statistically significant ($p > 0.05$) in cannabinoid-treated groups and the NC group (Fig. 15h).
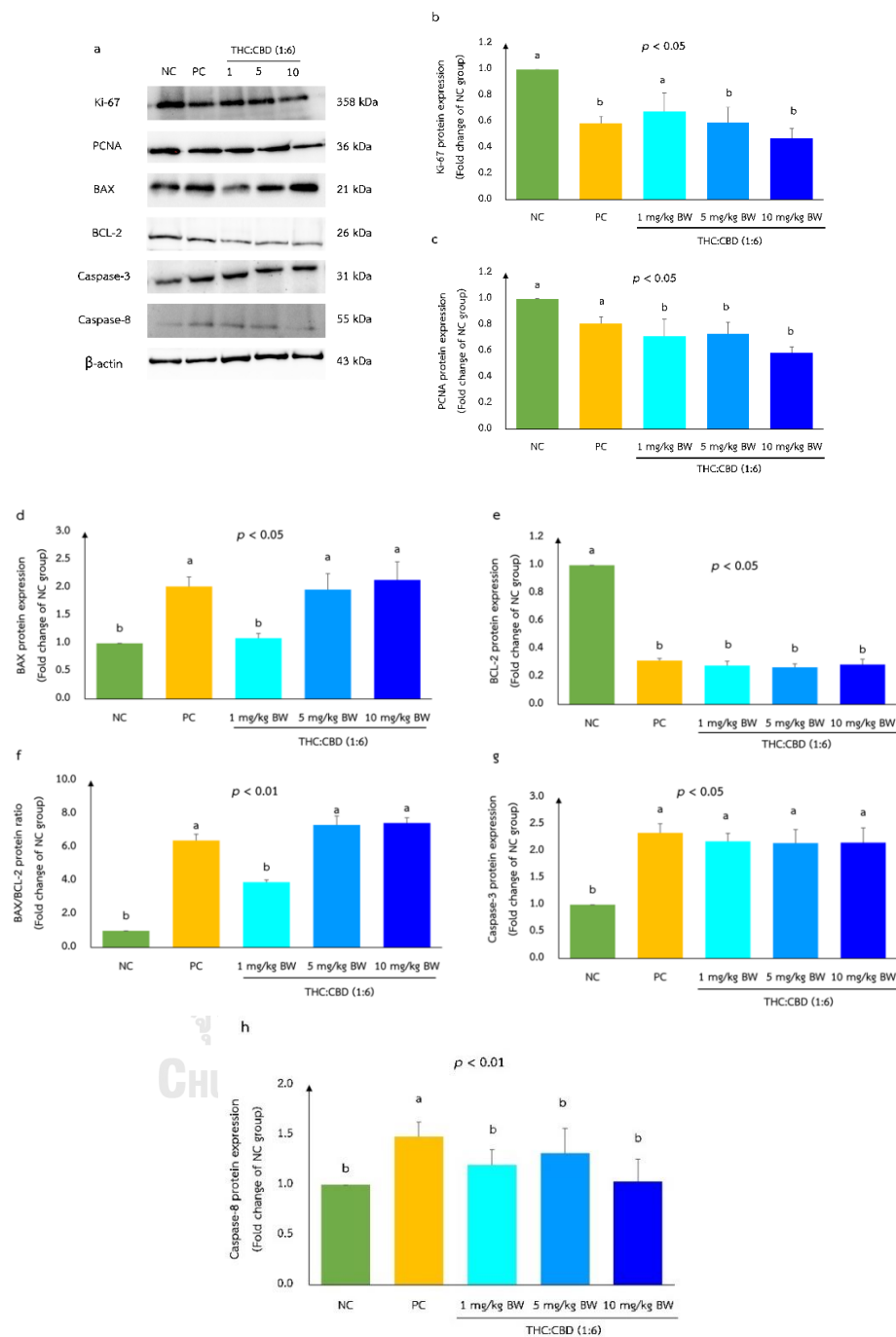
Figure 15: The representative WB results (a) and relative protein expression levels of Ki-67 (b), PCNA (c), BAX (d), BCL-2 (e), BAX/BCL-2 ratio (f), Caspase-3 (g), and Caspase-8 (h) among the NC group, PC group, and treatment groups (THC:CBD at a dose of 1 mg/kg BW, 5 mg/kg BW, and 10 mg/kg BW). The values were shown as mean ± SD. The statistical difference was calculated by the Kruskal-Wallis test. [a,b] Mean with different letters were significant differences among groups ($p < 0.05$).

CHAPTER 5: DISCUSSION

PDAC is the most frequent and lethal neoplasm of human exocrine pancreas (Wolfgang et al., 2013; Kleeff et al., 2016; Orth et al., 2019). Even though several significant methods were prior developed. At present, the suitable therapeutic methods for PDAC are understudied but there is still a major challenge for scientists worldwide. One of the most concerns in the treatment of human PDAC is a poor response at the late stage, resistance exhibited, and the side effects of the therapeutic agents (Kleeff et al., 2016; Orth et al., 2019). Thus, developing novel strategies for the treatment of PDAC in humans are necessarily needed. Herbal medicinal plants have been inclined noticed due to their effects in the treatment of human cancers. Of these, *cannabis* and its compounds have remarkably risen as a potential therapy for the treatment of several human tumors (Massi et al., 2004; Carracedo et al., 2006a; McKallip et al., 2006; Preet et al., 2008; Ramer et al., 2010; Guo et al., 2018). The antitumor effect was reported in four main ways, including 1) inhibition of tumor cell proliferation, 2) induction of apoptosis, 3) inhibition of angiogenesis, invasion and metastasis, and 4) induction of antitumor immunity (Zhu et al., 1998; Guzman, 2003). Besides, the early studies noted that THC and CBD are one of the most active components within the *Cannabis* plants (Guzman, 2003; Velasco et al., 2016). Furthermore, the combination between THC and CBD exerted an increase in antitumor effects compared with the single use of individual components (Torres et al., 2011; Nabissi et al., 2016). In the current study, the combination of THC:CBD with a ratio of 1:6 was investigated the antitumor effects within a human PDAC cell line (Capan-2) derived xenograft nude mice model. The findings of this study illustrated that cannabinoids (THC:CBD) at a ratio of 1:6 could inhibit proliferation and induce apoptosis in human PDAC xenograft nude mice models.

Over the years, laboratory animal models (especially immunodeficient mice) have been considered as the gold standard for testing the preclinical efficacy or evaluating the development of new anticancer drugs in humans (Lu et al., 2017; Yoshida, 2020; Golan et al., 2021; Luo et al., 2022). For PDAC, several previous studies

indicated that BALB/c nude mouse has been a suitable model for exploring the mechanisms of antitumor drugs, including cannabinoids (Li et al., 2011; Jonckheere et al., 2012; Zhao et al., 2013; Guo et al., 2018; Yang et al., 2020). The BALB/c immunocompromised mice lacked T-cell lymphocytes that could be useful for transplanting and proliferating human cell lines into the subcutaneous region (Morton and Houghton, 2007; Okada et al., 2019). In this work, the BALB/c strain mice lacked T-cell lymphocytes which were selected to receive the Capan-2 cell line at the right flank region. The transplanted tumors were well formed in the injected region and increased the volume over the experiment time with no statistical difference in all groups. Besides, there was no metastasis into other internal organs of the xenograft nude mice. The classification of the xenograft tumors showed the same characteristic as the original pancreatic ductal adenocarcinoma in humans based on the guideline of WHO (Bosman et al., 2010). This finding may confirm that BALB/c nude mouse was a suitable model for transplanting the human Capan-2 cell line.

In recent years, THC and CBD have been popularly accepted as the most active ingredient of cannabinoids derived from *Cannabis* plants (Guzman, 2003; Velasco et al., 2016). Various *in vitro* and *in vivo* studies showed a higher effect on the combined use of THC:CBD (1:1) in comparison with the single use of each ingredient agent (Torres et al., 2011; Nabissi et al., 2016). In fact, oral administration of THC:CBD at a 1:1 ratio and Temozolomide on xenograft nude mice model was demonstrated as a potential therapy for human glioblastoma multiforme (López-Valero et al., 2018). Likewise, the daily oral administration of THC:CBD (1:1) on human PDAC-derived knockout mice models was investigated (Yang et al., 2020). However, there are concerns about the differences in transplanted regions, doses, and testing time among prior studies. In the current study, THC:CBD (1:6) at a dose of 1 mg/kg BW, 5 mg/kg BW, and 10 mg/kg BW was daily gavaged into the BALB/c xenograft nude mice models to evaluate the effect of cannabinoids on human PDAC. Although this is not the first report of testing cannabinoids on BALB/c nude mice, the present study first revealed the effect of cannabinoids at a wide range of testing doses and a long time of experiment compared with the previous findings (López-Valero et al., 2018; Yang et al., 2020). Throughout the study, the examination of xenograft tumors displayed

that the increase of THC:CBD dose did not affect the percentage of tumor volume change among groups. Additionally, the percentage of necrotic area was also not significantly different in each group. Meanwhile, the histopathological examination showed that the increase of THC:CBD dose led to the risen of apoptotic cells while the reduction of mitotic cells compared to the NC group. The M/A analysis indicated that higher doses of cannabinoids fell down the M/A ratio among groups. Taken together, increasing a dose of cannabinoids could promote the antitumor effect but did not associate with the tumor volume change. Furthermore, it seems that the appearance of necrotic areas in the xenograft tumors was related to nutrient deprivation and hypoxia (Lee et al., 2018) more than the effect of cannabinoids. Our results additionally imply that daily oral administration at a ratio of 1:6 should be selected for THC:CBD uses, at least on the human PDAC.

Antimetabolite 5-FU was selected as the positive control in the present work. 5-FU has been used worldwide in the treatment of numerous human cancers over the decades (Longley et al., 2003; Wigmore et al., 2010). Metabolically, 5-FU works as a chemotherapeutic agent that inhibits the enzyme thymidylate synthase, accordingly blocking thymidine formation and inhibiting RNA and DNA replication and repair in the sensitive cells. DNA damage leads to the activation of the tumor suppressor p53, consequently the trigger of apoptosis activity in the cells (Longley et al., 2003; Wigmore et al., 2010). Clinically, 5-FU has been well-known as a chemotherapeutic drug for several human cancers, especially human pancreatic cancer (Longley et al., 2003; Wilkowski et al., 2006; Wang et al., 2014). Besides, 5-FU has been combined with other chemotherapeutic agents to increase the therapeutic effects and decrease the side effects in the patients (Longley et al., 2003). In addition, laboratory animal studies have utilized 5-FU as a positive control on the development of new drugs (Miyake et al., 2012; de la Cueva et al., 2013).

The antitumor effect of cannabinoids has been partly reflected via inhibiting tumor cell proliferation. It is shown that cannabinoids modulated cell cycle checkpoints in the tumor cells via CB1 and CB2 receptors, consequently blocking tumor cell proliferation (Zhu et al., 1998; Guzman, 2003). In fact, the prior investigations showed that THC-induced cell cycle arrest at the G2-M phase in

human breast cancer cells (Caffarel et al., 2006). In addition, CBD blocked the cell cycle during the period of the G0-G1 phase in gastric cancer cells (Zhang et al., 2019). Moreover, the separate use of THC or CBD-induced cell cycle arrest at only the G0-G1 phase while combined THC and CBD-induced cell cycle arrest at the period of the G0-G1 and G2-M phase in human glioblastoma (Marcu et al., 2010). The heterogeneous effect of THC and CBD on induced cell cycle arrest in various tumor cells promotes the further investigation of proliferation markers for detailed anti-tumor effects of cannabinoids. For decades, nuclear antigen Ki-67 and PCNA (proliferating cell nuclear antigen) were recruited as the most popular proliferation markers for numerous human cancer studies worldwide (Iatropoulos and Williams, 1996; Gil and Vagnarelli, 2018; Li et al., 2019; Zhao et al., 2020). It is noteworthy that the combination of Ki-67 and PCNA improved the sensitivity and specificity in the diagnosis of proliferated cells (Bantis et al., 2004; Zhong et al., 2008). In fact, Ki-67 and PCNA were widely used in clinical diagnosis and laboratory animal studies of human cancers, especially within the xenograft mice model (Bantis et al., 2004; Zhong et al., 2008; Li et al., 2019; Zhao et al., 2020). Consistent with previous studies, the present study performed both Ki-67 and PCNA markers to detect the proliferated cells in the human PDAC in xenograft nude mice models.

The nuclear antigen Ki-67 has been commonly documented as a classical marker for cell proliferation and upregulated expression in all active phases, except G0 phase (Pezzilli et al., 2016; Gil and Vagnarelli, 2018). Clinically, the Ki-67 score has been utilized for grading types of tumors and contributing to prognostic (Gil and Vagnarelli, 2018). In the laboratory, Ki-67 is useful for evaluating the effect of chemotherapy drugs on human cancer cell lines (Li et al., 2019; Zhao et al., 2020). However, there are no studies that apply the Ki-67 marker for evaluating the antitumor effect of cannabinoids in the human PDAC xenograft model. Of note, a prior study on human glioblastoma cells has demonstrated that only THC dramatically reduced the number of Ki-67 positive cells, meanwhile, CBD was not showing any changes and the effect was abolished when combining THC and CBD (Kolbe et al., 2021). In our study, it was observed that the percentage of Ki-67 positive cells were significantly decreased in all cannabinoid-treated groups

(THC:CBD, 1:6) relative to the NC group. However, cannabinoids at the lowest dose (1 mg/kg BW) could not affect the relative expression level of protein Ki-67 compared to the NC group in human PDAC tumor cells by WB. Indeed, the combination of THC and CBD reduced the expression of Ki-67 in human glioblastoma cells (Kolbe et al., 2021). Based on our results, the interaction between cannabinoids and the expression of Ki-67 should address in further studies.

Regarding our work, the positive labeling of PCNA cells via IHC and the relative level of protein PCNA expression via WB dramatically dropped in the treatment groups compared to the NC group. PCNA has been popularly accepted as the most used cell proliferation marker in previous studies (Iatropoulos and Williams, 1996; Ye et al., 2020). It is a nuclear protein that plays an essential role in the synthesis, resynthesis of DNA, and regulation of the cell cycle. PCNA has upregulated expression in the late stage of the G1, got peak at S, G2, and declined at M phases (Iatropoulos and Williams, 1996; Kelman, 1997; Tehseen et al., 2019; Ye et al., 2020). Clinically, PCNA has been applied to the diagnosis and prognosis of several human tumors (Jurikova et al., 2016; Ye et al., 2020). For PDAC, a prior *in vivo* study demonstrated that PCNA was a vital biomarker to confirm the proliferation of tumor cells and was used to investigate the effect of tumor therapeutic drugs on xenograft animal models. Similarly, the current study suggests that PCNA could be applied as a proliferation marker for assessing the antitumor impact of cannabinoids in the human PDAC xenograft model. These results, coupled with Ki-67 results, imply that THC:CBD (1:6) could inhibit PDAC cell proliferation in xenograft nude mice models.

On the other hand, cannabinoids have been related to the part of the apoptosis induction within various tumors, especially PDAC (Guzman, 2003; Carracedo et al., 2006b; Kim et al., 2016). Apoptosis is a complex physiological process that occurs as the homeostatic mechanism to ensure the balance of cell populations in the tissue of eukaryotes. There are two principal pathways to activate apoptosis that stimulate the intrinsic or extrinsic pathway. In both pathways, it is noted that several involved proteins including BAX, BCL-2, Caspase-3, and Caspase-8 (Hengartner, 2000; Igney and Krammer, 2002; Kumar, 2007). Most prior investigations described that cannabinoids induce apoptotic via the activation of the intrinsic pathway (Carracedo

et al., 2006a) while stimulation of the extrinsic pathway is still understudied (Herrera et al., 2006). This current study investigated the effect of cannabinoids in both intrinsic and extrinsic pathways through the level of mRNA and protein expression of BAX, BCL-2, Caspase-3, and Caspase-8 in human PDAC xenograft tumors. The findings of this study indicated that cannabinoids induced apoptosis by upregulation of BAX and Caspase-3, downregulation of BCL-2, and may not be involved with the activation of Caspase-8. These findings were similar to the results of the previous studies that apoptosis induced by cannabinoids was strictly related to the intrinsic pathway (Carracedo et al., 2006a).

The upregulation of pro-apoptotic protein BAX is associated with the permeabilization of the mitochondrial outer membrane, permitting the release of cytochrome C, thus promoting mitochondrial apoptotic via an intrinsic pathway (Adams and Cory, 2007; Kumar, 2007; Roufayel, 2016). A previous investigation displayed that CBD stimulated the expression of protein BAX and inhibited protein BCL-2 expression, resulting in apoptosis within the human gastric cancer cell line (Zhang et al., 2019). In this study, mRNA expression of *BAX* was increased markedly in cannabinoid-treated groups at a dose of 5 mg/kg BW and 10 mg/kg BW while low expressed in the NC group. Similarly, the expression of protein BAX in cannabinoid-treated groups at a dose of 5 mg/kg BW and 10 mg/kg BW was dramatically higher than those in the NC group. Our results were in agreement with the earlier study that cannabinoids were associated with the upregulation of BAX levels (Lukhele and Motadi, 2016; Zhang et al., 2019). Contrastingly, the statistically low mRNA and protein BAX expression in the NC group may suggest that a small percentage of BAX molecular occurs in an unphosphorylated state under normal conditions (Adams and Cory, 2007). Besides, the current study showed that the lowest dose of cannabinoids (1 mg/kg BW) could not affect the expression of BAX. Altogether, it implies that apoptosis of human PDAC xenograft tumor was involved with the upregulation of BAX protein compared to the NC group.

The anti-apoptotic protein BCL-2 is believed to inhibit the activity of protein BAX, blocking the connection of cytochrome C and caspase proteins, thus preventing downstream activation of apoptosis via an intrinsic pathway (Adams and Cory, 2007;

Kumar, 2007; Roufayel, 2016). Clinically, a previous study conducted on PDAC demonstrated that the upregulation of BCL-2 level was inversely correlated with the overall survival time of the patients (Contis et al., 2018). It is implied that silencing of BCL-2 may stimulate apoptosis in cancer cells (Rückert et al., 2010; Contis et al., 2018). According to this study, the levels of mRNA expression, percentage of protein expression (%), and relative protein BCL-2 expression significantly dropped in the treatment groups compared to the NC group. The obtained results displayed that the downregulation of BCL-2 levels is associated with the activation of apoptosis in human PDAC xenograft tumors. Likewise, recent studies reported that cannabinoids downregulated BCL-2 levels in mouse pancreatic β cell lines and human gastric cancer cell lines (Kim et al., 2016; Zhang et al., 2019).

Remarkably, BAX/BCL-2 ratio has been widely accepted as a protein expression pattern to estimate the eventual outcome of apoptosis expression in cancer patients (Del Poeta et al., 2003; Kulsoom et al., 2018; Krstic et al., 2022). An imbalance of protein BAX and BCL-2 expression may associate with the activation of the apoptosis process in cancer cells (Adams and Cory, 2007; Kumar, 2007; Zhang et al., 2019). However, individual use of BAX and BCL-2 expression patterns could be associated with unclear evidence of apoptosis expression (Kulsoom et al., 2018). Therefore, the current study additionally utilized this ratio to assess the apoptosis expression in the human PDAC-derived xenograft nude mice model. As presented in our results, the BAX/BCL-2 ratio was significantly different between the cannabinoid-treated groups at a dose of 5 mg/kg BW, 10 mg/kg BW, and the NC group. Considering individual expression of BAX and BCL-2 protein showed the upregulation of protein BAX and downregulation of protein BCL-2 in the treated groups compared to the NC group. The consequence was an increased BAX/BCL-2 ratio in cannabinoid-treated groups. The increase in BAX/BCL-2 ratio reflected the critical confirmation of tumor cell death (Del Poeta et al., 2003; Kulsoom et al., 2018; Krstic et al., 2022). Similarly, a previous study demonstrated that CBD stimulated apoptosis via downregulation of protein BCL-2 and upregulation of protein BAX in gastric cancer cell lines (Zhang et al., 2019). Thus, this result confirms that cannabinoids could promote apoptosis in

the human PDAC xenograft model with the upregulated BAX, downregulated BCL-2, and upregulated BAX/BCL-2 ratio.

Pro-apoptotic protein Caspase-3 has been well known as one of the most important proteins in the apoptosis process, which cleavages the substrates of cells. Both intrinsic and extrinsic apoptosis pathways have converged to Caspase-3. Activation of the Caspase-3 protein has directly related to the response of apoptosis in cancer cells (Porter and Jänicke, 1999; Hengartner, 2000; Kumar, 2007; Wong, 2011). This study found that the mRNA expression level of *Caspase-3* was significantly increased in the treatment groups with cannabinoids. Likewise, the expression of protein Caspase-3 was dramatically high in the treatment groups in comparison to the NC group. The level of protein Caspase-3 raised in cannabinoid groups, consequently inducing apoptosis in the human PDAC xenograft model. Consistent with previous studies, cannabinoids upregulated protein Caspase-3, resulting in activated apoptosis (Herrera et al., 2006; Marcu et al., 2010; Lukhele and Motadi, 2016; López-Valero et al., 2018; Zhou et al., 2018; Zhang et al., 2019). For human pancreatic cancer, protein Caspase-3 was activated by cannabinoids in i*n vitro* study, which ultimately resulted in apoptosis (Carracedo et al., 2006a). These studies suggested that activation of protein Caspase-3 could lead to induce apoptosis in cancer cells. Similarly, the obtained results of this study once again confirm that cannabinoids stimulate apoptosis in the human PDAC xenograft model by upregulation of protein Caspase-3.

Contrarily, the current results presented that cannabinoids could not relate to the mRNA expression of the *Caspase-8* gene. Similarly, the relative protein expression of Caspase-8 in xenograft tumors was not significantly different among cannabinoid-treated groups and the NC group. Meanwhile, the expression of protein Caspase-8 was not detected via IHC in all groups. Caspase-8 – a pro-apoptotic protein triggered by outside stimuli through death receptors in the cell membrane. The Caspase-8 expression has related to the extrinsic apoptotic pathway (Hengartner, 2000; Igney and Krammer, 2002; Kumar, 2007). On the other hand, cannabinoids specifically respond with cells via CB1 and CB2 receptors (Mackie and Stella, 2006). Indeed, the earlier investigation showed that the activation of the CB2 receptor was

involved with the activation of the intrinsic apoptosis pathway (Herrera et al., 2006). Likewise, the current finding implies that cannabinoids induced apoptosis in the human PDAC xenograft tumors which may not be associated with the activation of Caspase-8. Taken together with the findings of BAX, BCL-2, and Caspase-3, these data suggest that apoptosis induced via the treatment of cannabinoids in human PDAC xenograft tumors was rather involved with the intrinsic apoptosis pathway.

The present study encountered unavoidable limitations. First, we developed this study from the prior project, all xenograft tumors were inherited from the previous animal experiment. However, we strongly confirm that the xenograft tumor examination, metastasis examination, as well as gene and protein expression study were performed, and these results were interpreted and revised by the author. Furthermore, the present work was carried out to address the limitations of the prior study and was separated from the initial project. Accordingly, it is believed that effectuating this study is necessarily required. Second, the fluorescence assays to demonstrate the DNA fragmentation in apoptotic cells were not performed in the current study. Several studies mentioned the fluorescence assays to detect fragmented DNA strands such as a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay or Hoechst dye (Kraiphet et al., 2018; López-Valero et al., 2018; Jeong et al., 2019). These assays could help to increase the reliability of the study by clearly indicating cell death via fluorescence signals. Nevertheless, the immunoexpression of apoptotic-related proteins in both intrinsic and extrinsic pathways (BAX, BCL-2, Caspase-3, and Caspase-8) has been reflected as a powerful tool and has generally been applied to evaluate program cell death (Wong, 2011; Hasui et al., 2012; Zhao et al., 2013; Shi and Stack, 2015). Besides, the current study recruited WB which is widely mentioned as a gold standard for assessing protein expressions (Murphy and Lamb, 2013; Vallejo-Illarramendi et al., 2013). Therefore, the combination of immunoexpression and WB results are probably sufficient to evaluate apoptosis in human PDAC xenograft tumor cells. Third, the activation of protein p53 was not examined in this study. Protein p53 plays a central role in promoting apoptosis via the upregulation of protein BAX and the downregulation of protein BCL-2 within the intrinsic pathway (Hengartner, 2000;

Adams and Cory, 2007; Wong, 2011). Hence, the current study only focused on the expression of the BCL-2 family members such as BAX and BCL-2. Four, the role of the Apaf-1 protein was excluded in the present study. The Apaf-1 protein is considered as a factor that activates protein Caspase-3 when the appeared cytochrome C combined with dATP in the cytoplasm (Hengartner, 2000; Adams and Cory, 2007; Roufayel, 2016). However, a previous study indicated that the Apaf-1 factor was not necessarily required in the activation of apoptosis within gene-deleted mice. The absence of the Apaf-1 gene is only associated with the rate of the apoptotic process in the gene-deleted mice model (Ekert et al., 2004). For further studies, the limitations of this research should be noticed and addressed.

In summary, this study revealed that cannabinoids (THC:CBD) (1:6) could inhibit the proliferation and induce apoptosis in human PDAC xenograft nude mice models. Furthermore, the study additionally illustrates that the anti-apoptotic effect of cannabinoids on the human PDAC xenograft nude mice model could be associated with the intrinsic pathway. With obtained results, the current study contributes new insights to the fundamental knowledge regarding the potential therapeutic role of cannabinoids for human PDAC cell lines derived xenograft nude mouse model. This study may additionally contribute to accommodating more therapeutic targets for human-occurring pancreatic adenocarcinoma in the future.

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SUPPLEMENTATION

Immunohistochemistry of CD31 (PECAM-1)

The paraffin-embedded tissues were cut into a section of 4 μm-thick and placed in the positive charged glass slides, air-dried at RT. The specimens were deparaffinized with xylenes, rehydrated with alcohol, and then washed with running water and distilled water. The slides were pretreated for antigen retrieval, using Citrate Buffer (pH 6.0) for 20 minutes in autoclave at 121°C. Endogenous peroxidase activity was quenched within a chamber containing 3% of H_2O_2 for 10 minutes at RT. For blocking non-specific antibodies, all specimens were blocked with 1% bovine serum albumin for 30 minutes at 37°C. Then, the primary antibody (anti-CD31/PECAM-1, Cat. Num. sc-376764, Santa Cruz Biotechnology, Oregon, USA, at dilution 1:500) was applied and incubated overnight at 4°C. Subsequently, the secondary antibody was following incubated, using a biotinylated goat anti-mouse/anti-rabbit antibody (Envision, Dako, Glostrup, Denmark) for 1 hour at RT. A freshly prepared 3, 3′-diaminobenzidine (DAB) solution (Dako, Glostrup, Denmark) was added to visualize the colored reaction. Finally, the slides were immersed in distilled water, counterstained with Meyer's hematoxylin stain, dehydrated within graded alcohol, and permanently mounted. The stained specimens of CD31 (PECAM-1) were captured by the digital imaging system with an HPF (40X, area equals 2.37 mm^2).
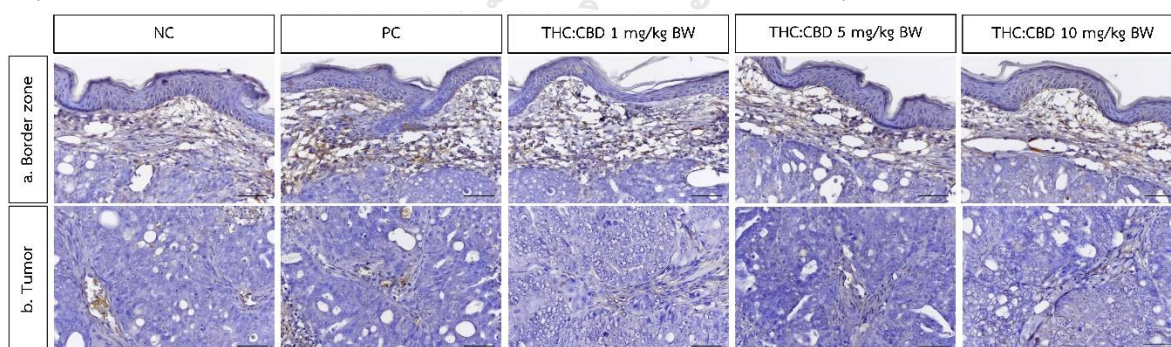

Figure 1: The immunoexpression of CD31 (PECAM-1) among the NC group, PC group, and the treatment groups with THC:CBD at a dose of 1 mg/kg BW, 5 mg/kg BW, and 10 mg/kg BW. The CD31 (PECAM-1) positive labeling in the blood vessels of the border zone (a) and inside of the xenograft tumors (b) (DAB labeled, Mayer's hematoxylin counterstained, 40X, scale bar = 50 μm).

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	2. Trung Quang Le, Latticha Pluemhathaikij, Katriya Chankow, Araya Radtanakatikanon, Anudep Rungsipipat, Kasem Rattanapinyopituk (2022) Case report: BCL-2 and CD31 immunoexpression related to clinical and histopathological evaluation of renal dysplasia in a Welsh Corgi Puppy. Front. Vet. Sci. 9:995765. doi: 10.3389/fvets.2022.995765