TÜRKİYE CUMHURİYETİ
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL
BIOENGINEERING DEPARTMENT

INVESTIGATION OF CYTOTOXIC ACTIVITY OF BETA-CARYOPHYLLENE ON PANCREATIC CANCER CELLS

HIBA ANIS

M.Sc.

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THESIS ADVISOR
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ÖZET

BETA-KARYOFİLENİN PANKREAS KANSERİ HÜCRELERİ ÜZERİNDEKİ SİTOTOKSİK AKTİVİTESİNİN ARAŞTIRILMASI

HIBA ANIS
Yüksek Lisans, Biyomühendislik Anabilim Dali
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Beta-Karyofilen (BCP), çeşitli bitkilerden elde edilen ve Gıda ve İlaç Dairesi (FDA) tarafından onaylanan doğal olarak oluşan bir seskiterpendir. Kanabinoid CB2 reseptörleriyle etkileşime girerek anti-inflamatuar ve analjezik etkiler gösterir. Çalışmalar, BCP'nin apoptotik hücre ölümünü indüklemek, anti-inflamatuar etkiler göstermek ve klonojenite, göç ve invazyonu inhibe etmek gibi tümör hücrelerinde birçok biyolojik özelliğe sahip olduğunu göstermiştir. Bu seçici kanser hücrelerini hedefleme yeteneği, BCP'nin kanser tedavisi için umut verici bir terapötik aday olduğunu göstermektedir.

Bu çalışmada, BCP'nin MIAPaCa-2 pankreas kanseri hücreleri üzerindeki etkileri, terapötik performansı artırma potansiyelini değerlendirmek amacıyla kemoterapötik ajan Etoposid ile birlikte incelendi. MIAPaCa-2 hücre hatları için BCP'nin IC50 değerleri 58 µg/mL iken, etoposidin IC50 değeri 10.7 µM idi. BCP ve etoposid kombinasyonu, yalnızca tek başına tedavilere kıyasla MIAPaCa-2 hücrelerinde daha yüksek bir hücre ölümü insidansına yol açtı. BCP ayrıca MIAPaCa-2 hücre hatlarında survivin ve c-Myc'nin ifade seviyelerini baskılarken kaspaz-3'ün ifade seviyesini artırdı ve bu etkiler etoposid ile birlikte kullanıldığında daha da arttı.

BCP, yalnızca MIAPaCa-2 hücrelerinde sitotoksik etkiler göstermekle kalmadı, aynı zamanda etoposidin anti-proliferatif etkilerini de artırarak normal hücreler üzerinde daha az olumsuz etki gösterdi.

Anahtar Kelimeler: Beta- Caryophyllene, Etoposide, Pancreatic Cancer.

ABSTRACT

INVESTIGATION OF CYTOTOXIC ACTIVITY OF BETA-CARYOPHYLLENE ON PANCREATIC CANCER CELLS

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Beta-Caryophyllene (BCP) is an organic sesquiterpene composite derived from multiple plants and authorized by the Food and Drug Administration (FDA). It interacts with cannabinoid receptors CB2, exhibiting analgesic and anti-inflammatory effects. Many researches have demonstrated that BCP exhibits a variety of biological effects in tumor cells, including inducing apoptotic cell death, exerting anti-inflammatory effects, and inhibiting clonogenicity, migration, and invasion. This special approach on cancer cells suggests that BCP is a hopeful therapeutic option for cancer treatment.

Within this study, the influence of BCP on MIAPaCa-2 pancreatic cancer cells were examined in conjunction with the chemotherapeutic agent Etoposide to assess its potential to enhance therapeutic performance. The IC50 values of BCP for MIAPaCa-2 cell lines were 58 µg/mL, while etoposide had an IC50 of 10.7 µM. The combination of etoposide and BCP led to a higher occurrence of cell death in MIAPaCa-2 cells compared to either treatment alone. BCP additionally inhibited the levels of gene expression c-Myc and survivin, concurrently boosting the level of caspase-3 in MIAPaCa-2 cell lines, with these impacts being intensified when combined with etoposide.

BCP demonstrated cytotoxic effects on MIAPaCa-2 cells and augmented the anti-growth effects of etoposide when combined, showing reduced unfavorable effects on normal cells.

Keywords: Beta- Caryophyllene, Etoposide, Pancreatic Cancer.

Dedication

To my professors during this long journey in college,
To my precious family who encouraged me along the way,
To my backbone friend with whom I shared the same path,
To my affectionate husband who believed in me all the time,
To my unborn child who was the hidden hope inside,

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Last but not least, I would like to thank my lifelong friend who has been by my side since the beginning of this journey, my family for their unwavering love and support, and special gratefulness to my husband for his affection and patience, especially during the writing of this thesis, this accomplishment would not have been possible without him.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

BCP	: Beta- Caryophyllene
PC	: Pancreatic Cancer
MIAPaCa-2	: Pancreatic Cancer Cell Lines
DMEM	: Dulbecco's Modified Eagle's Medium
PBS	: Phosphate-Buffered Saline
FBS	: Fetal Bovine Serum
SEM	: Scanning Electron Microscopy
DAPI	: 4',6-diamidino-2-phenylindole, dihydrochloride
IC50	: Half-Maximal Inhibitory Concentration
BSA	: Bovine Serum Albumin
MICs	: Minimum Inhibitory Concentrations

LIST OF SYMBOLS

µL **:** Microliter

mL **:** Milliliter

µM **:** Micromolar

mm **:** Millimetre

1. INTRODUCTION

Cancer is considered to be a pioneer health obstacle. Demographic projections indicate a surge in cancer incidences in the forthcoming decades, with estimates predicting above 20 million fresh instances annually by 2025 (Zugazagoitia et al., 2016). According to the study by (Ferlay et al., 2013) in the European Union only, an approximate of 1.4 million recent cancer cases were reported in males, with a similar number of 1.2 million in females. Furthermore, approximately 555,000 women and 707,000 men were reported to die from this malignancy in the same year.

Pancreatic cancer is known as a lethal and highly aggressive malignancy, and numerous risk factors have been involved in the progression of this malignant disease, consisting of age, obesity, tobacco smoking, heavy alcohol consumption, low physical activity and family history (Kleeff et al., 2016). Due to the fact that it is detected at an advanced level, pancreatic cancer has often metastasized to far organs by the time of diagnosis, presenting a significant challenge in therapy (Hu et al., 2021). Conventional treatment methods usually involve a combination of chemotherapy, surgery, radiotherapy, and palliative care. Yet, new advancements in research have explored other options such as immunotherapy and targeted therapy, offering promising avenues for future treatment modalities which could be integrated with traditional approaches. Furthermore, the treatment strategy for pancreatic cancer depends on the stage of its progression (Zhao & Liu, 2020). therefore, it is crucial to investigate novel treatment approaches aimed at targeting this malignant disease and contributing to its cure.

Plants have been used for medicinal purposes since the earliest days of civilization. Many pharmaceuticals used in industry and commerce derived from secondary metabolites produced within microbial or plant systems. Among the approximately 350,000 known plant species, an estimated 35,000 (with some suggesting up to 70,000) are globally utilized for medicinal purposes. However less than 0.5% of these plants have undergone chemical investigation. Remarkably, abound 100 plant species contribute to 25% of all pharmaceuticals prescribed in developed nations (Shasany, Shukla, & Khanuja, 2007). As per the World Health Organization (WHO), almost 80% of the global population relies on natural compounds as a primary medical

source, while these compounds are also involved in the medical treatment of the remaining 20%. However, despite the extensive use of thousands of traditional natural products, it's assumed that only 5–15% have been scientifically validated (Nassar, Aisha, Al Suede, Majid, & Majid, 2012).

Beta-Caryophyllene (BCP) is an organic sesquiterpene compound derived from multiple plants, including rosemary, red pepper, and various essential oils as well as cannabis. It is a natural compound authorized by the Food and Drug Administration (FDA) that interacts with cannabinoid CB2 receptors, resulting in Pain remedy and anti-inflammatory effects. The cannabinoid receptors CB2 are one type of the endocannabinoid system, which comprises two primary types: type 1 (CB1) and type 2 (CB2) with CB1 being responsible for conventional psychotropic outcomes, and CB2 that is found in the immune system, provides anti-inflammatory effects (Picciolo et al., 2020). Extensive studies have demonstrated that BCP possesses different biological features, including; inducing apoptotic cell death in lung cancer cells (Chung et al., 2019), exerting anti-inflammatory effect on lymphoma and neuroblastoma cells (Sain et al., 2014), inhibiting invasion, migration, and spheroid shaping in colon cancer cells (Saad S. Dahham et al., 2015) and demonstrating anticancer, and cytotoxic effects on the oral cancer KB cell line (Ubiquitous keratin-forming tumour cell line HeLa) (Ramachandhiran, Sankaranarayanan, Murali, Babukumar, & Vinothkumar, 2022), among others.

The goal of this investigation is to explore how BCP's effectiveness in mitigating the malignancy of pancreatic cancer cells could be improved through the use of specific chemotherapeutic agents known for their efficacy in treating pancreatic cancer.
This research aims to augment the existing literature and offer valuable insights for scientists to apply in in vivo studies.

2. THEORETICAL FOUNDATIONS AND LITERATURE REVIEW

2.1 Pancreatic Cancer

Ductal adenocarcinoma stands as the prevalent malignancy of the pancreas, commonly known as pancreatic cancer (PC), posing a significant health challenge (Kleeff et al., 2016). In 2022, 511,000 new cases and 467,000 deaths were recorded attributed to PC. It carries one of the bleakest prognoses, earning it the sixth position among the leading causes of cancer mortality for both genders combined (Figure 2.1.1). It accounts for nearly 5% of all cancer-related deaths worldwide. (Bray et al., 2024).

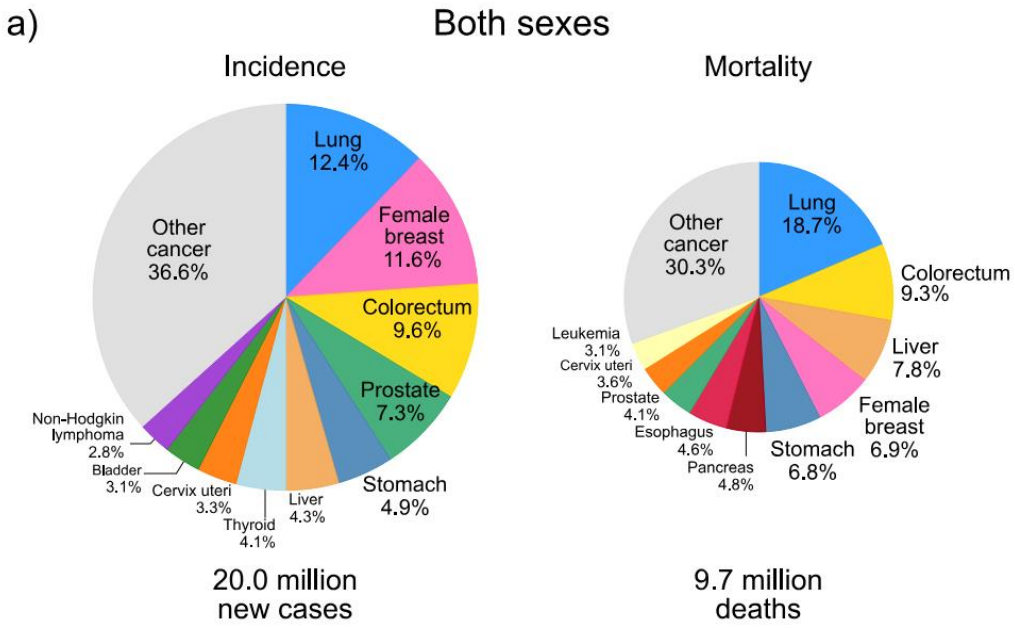


Figure 2. 1: Pie charts shows the breakdown of cases and deaths for the top five cancers in 2022 for both genders. This figure is taken from (Bray et al., 2024)

PC progression and its poor prognosis are driven by numerous genetic alterations. On average, the cancer cells harbor 63 genetic mutations, with the greatest being point mutations. These alterations notably include oncogenic KRAS, present in over 90% of cases, as well as

supressing mutations in some tumor suppressor genes such as SMAD4, TP53, and CDKN2A (Jones et al., 2008).

Furthermore, one of the receptors identified to be present in PC, with elevated expression observed in patients with pancreatic cancer, are the cannabinoid receptors (Falasca & Falasca, 2022)

2.1.1. Risk Factors of Pancreatic Cancer

Risk factors are divided into sections. These are categorized into factors that cannot be changed (non-modifiable) and those that can be influenced (modifiable) (McGuigan et al., 2018), which will be summarized briefly as below

2.1.1.1 Age:

PC mostly affects older adults. It's very uncommon for people under 30 to get diagnosed, and about 90% of new cases occur in people over 55, with most in their 70s and 80s (Midha, Chawla, & Garg, 2016; Wood et al., 2006).

2.1.1.2 Sex:

PC is less common in women compared to men worldwide. In developed nations, this difference is even more noticeable (McGuigan et al., 2018). Research indicates that the estrogen, the feminine hormone, can slow down PC progression, which might be the reason why women when younger, might have a lower chance of getting PC than men, but not necessarily when they're older (Konduri & Schwarz, 2007). A study by Sadr-Azodi et al. found that women who took menopausal hormone therapy (MHT) had a 23% lower risk of the disease in comparison with the ones who didn't take MHT. They also found that the short period of 1-2 years with MHT reduced the risk by 35%, while for over 3 years of MHT it was reduced by 60% (Sadr-Azodi, Konings, & Brusselaers, 2017)

2.1.1.3 Ethnicity:

The occurrence of PC differs across the globe. In the United States of America, African originated Americans experience a bigger frequency compared to Caucasians, whereas Asian

originated Americans and Pacific Islanders have the least occurrence (Midha et al., 2016). The higher rates of PC among African Americans are thought to be connected to more exposure to other risk factors like smoking, drinking alcohol, being overweight, and having diabetes (Silverman et al., 2003)

2.1.1.4 Blood group:

One of the risk factors that play a vital role in the progression of pancreatic cancer is the ABO blood group. A study by Antwi et al. found that people with the blood group O had lower risk of pancreatic cancer compared to other blood group people (Antwi et al., 2018). Wolpin et al. discovered that when contrasted with persons with blood group O, those with blood group A had a 32%, blood group AB had a 51%, and blood group B had a 72% higher risk of progressing pancreatic adenocarcinoma (Wolpin et al., 2009). El Jellas et al. discovered that among cases that were not surgically removed, the occurrence of blood type A and subtype A1 was the highest. Conversely, untreated cases exhibited the lowest occurrence of blood group O. Moreover, O blood grouped patients had a longer survival duration compared to non-O blood grouped ones among unresected cases. (El Jellas et al., 2017).

2.1.1.5 Family and Genetic History:

PC is considered a familial disease in case two or more immediate family members have had this cancer. Patients with family history face a 9-time elevated risk of getting pancreatic cancer in comparison with those that do not have family history, and this risk increases to 32-time higher if three or more close family members have had the disease (Becker, Hernandez, Frucht, & Lucas, 2014). Genetic alterations linked with an elevated risk of PC include CDKN2A (p16), STK11/LKB1, PRSS1/SPINK1/CFTR, BRCA1/2, mismatch repair genes (PMS2/MSH6/MLH1/MSH2), PALB2 and ATM (Ghiorzo, 2014). Another research based on meta-analysis of nine studies found that people with one first-degree family member with PC face an 80% increased likelihood of developing pancreatic adenocarcinoma when contrasted with those with no reported family history (Permuth-Wey & Egan, 2009).

2.1.1.6 Diabetes:

Diabetes is considered to be one of the recognised risk factors to develop PC. The risk of it rises up to 5-10 times for patients with type 1 diabetes for more than 10 years. Those with diabetes for over 20 years face an even higher risk (Bosetti et al., 2014). (B. Z. Huang et al., 2020) found 2,002 new pancreatic cancer cases during nearly 7.5 million person-years of follow-up. Individuals newly detected with diabetes faced nearly 7 times higher risk of pancreatic cancer, when contrasted with those with no diabetes. Between patients who were diagnosed recently with diabetes, the ones who later had PC exhibited quick increases in the levels of HbA1c and glucose in the month preceding the diabetes detection, together with gradual weight loss in preceding years. These shifts were particularly noticeable in specific racial and ethnic groups. Consequently, HbA1c has been suggested as a promising early screening indicator of PC.

2.1.1.7 Smoking:

Data from 12 case-control studies were studied by Bosetti et al. (2012), and summary odds ratios (ORs) were calculated (a measure of how strongly an event is associated with exposure). It was revealed that former smokers faced a 1.2 times higher risk, while current smokers had a 2.2 times higher risk of pancreatic cancer compared to individuals who never smoked. The number of cigarettes smoked had another impact on the risk increase. Also, this risk escalated with the duration of smoking, reaching up to 40 years. There was no observable trend in risk with the age at which smoking began, but the risk decreased with more time passed since quitting smoking, with an OR of 0.98 after 20 years (Bosetti et al., 2012). The Pancreatic Cancer Cohort Consortium discovered similar results, showing that pancreatic cancer likelihood rises with both the time period of smoking and the total of cigarettes smoked (Lynch et al., 2009).

2.1.1.8 Alcohol:

A cohort study with 2,187 pancreatic cancer patients discovered that the susceptibility of the cancer was considerably elevated in people who drank more than 30 grams of alcohol per day (Jeanine M Genkinger et al., 2009).

Other findings showed that alcohol exerts separate influences on the promotion of PDAC, linked with fibrosis formation via a mechanism independent of stellate cells. Moreover, it exacerbates early PDAC development and encourages the induction of M2 macrophages within

the framework of chronic pancreatitis (Xu et al., 2015). Mice expressing an altered K-Ras gene exhibited both advanced and early stages of the most prevalent form of pancreatic cancer in humans. Precise alterations within the K-Ras oncogene might be more prevalent among individuals who consume alcohol and develop pancreatic cancer, potentially acting as catalysts or inhibitors in the development of pancreatic cancer linked to heavy alcohol consumption (Gupta, Wang, Holly, & Bracci, 2010) (Hu et al., 2021).

2.1.1.9 Chronic pancreatitis

Chronic pancreatitis is a disease where the pancreas becomes infected over time, causing scarring and damage to its cells. Chronic pancreatitis can also affect the mitochondria, lysosomal autophagy systems and endoplasmic reticulum in pancreatic cells, potentially causing DNA damage, mutations, and activation of cancer-causing genes. One of the studies revealed that the collective relative likelihood of pancreatic cancer in chronic pancreatitis patients was approximated as13.3.(Yadav & Lowenfels, 2013)

2.1.1.10 Obesity

Several studies have confirmed the relationship between obesity and the higher possibility of pancreatic cancer, leading to exacerbating outcomes in this malignancy. One of the studies revealed that central obesity correlated with elevated mortality rates from pancreatic cancer, regardless of the index of body mass. Moreover, it indicated that being obese, particularly during young adulthood, could substantially raise the chance of dying from pancreatic cancer in later life (J M Genkinger et al., 2015).

2.1.2. Diagnosis

Cancer detection at an early stage is the sole means of identifying small tumors and proceed to potentially curative surgery. PC diagnosis mainly depends on imaging techniques, these methods include Magnetic Resonance Imaging (MRI), Compute Tomography (CT), Positron Emission Tomography (PET) and Endoscopic Ultrasound (EUS), which are used alongside tissue sampling procedures (Zhang, Sanagapalli, & Stoita, 2018).

Multi-Detector Computed Tomography (MDCT) can be considered among the most common and well-validated diagnostic methods for PAC. This approach is essential for detecting the pancreatic tumors, assessing the possibility for surgery, and evaluating their vascular invasion (Singhal, Prabhu, Sethi, & Moorthy, 2017), in addition, it can be beneficial to help recognizing tumors that might be operatable before doing surgery, thereby improving the treatment planning (Zhao & Liu, 2020).

Magnetic Resonance Imaging (MRI) is another detection method for PC patients who show inconclusive results with MDCT or ultrasound. It has the ability to provide detection of early-stage tumors by showing the morphological changes in the pancreatic tissue and ducts. Furthermore, it can show the chemical shift and water molecule movement within the lesion, leading to gain more information about the function and structure of the tumors (Zhang et al., 2018) (Tummala, Junaidi, & Agarwal, 2011).

Positron emission tomography (PET) is a scanning tool that use the tracer 18-fluorodeoxyglucose (FDG) and distinguishes metabolically active proliferative lesions, like cancers, most of which exhibit FDG-avidity, from benign ones which typically do not gather FDG. The effectiveness of PET in the diagnosis and staging assessment of presumed pancreatic cancer is still uncertain, as there is no consensus on whether PET provides additional information more than the ones obtained by contrast-enhanced CT. As a detecting method, PET/CT demonstrates comparable performance to CT only, offering no extra advantage over current primary diagnostic methods for detecting pancreatic cancer. Yet, PET/CT currently may be used as an adjunctive modality in some cases. Moreover, comparative data against MDCT with contrast does not demonstrate any additional information provided by PET or integrated PET/CT. Therefore, further research might be needed to determine the role of PET in detection and staging in pancreatic cancer, especially with patients with indecisive or negative MDCT results (Lee & Lee, 2014) (Tummala et al., 2011) (Zhang et al., 2018).

Endoscopic ultrasound (EUS)/EUS-FNA (Fine Needle Aspiration) are essential tools for diagnosing pancreatic cancer, especially with patients that are suspected to have cancer, but has not been confirmed by standard imaging methods. One feature is highlighted in EUS is offering tissue sampling during the same examination. EUS has emerged as an effective screening tool in patients who were clinically suspected of having PC due to its ability to detect the pancreatic

duct dilation, especially when tumors are smaller than 2 cm or when no definitive mass is observed on a CT scan. Despite all these methods, few aspects of pancreatic cancer screening methods remain undefined, such as the ideal age to initiate screening, its intervals, when to interfere surgically, and the influence of diagnosis on survival rates (Bhutani et al., 2016) (Tummala et al., 2011).

2.1.3. Treatment

Common treatment methods for pancreatic cancer includes chemotherapy, surgery, radiotherapy, and palliative care. However, there have been significant progress in researches related to immunotherapy, microbial therapy research and targeted therapy, which potentially offers promising supplements to the traditional approaches in PC treatment. Moreover, the choice of treatment for PC is heavily influenced by its stage (Zhao & Liu, 2020).

Surgical resection stands as the sole treatment that holds promise for curing pancreatic cancer, and incorporating chemotherapy with the appropriate conditions demonstrating enhanced survival rates. Encouragingly, some studies have indicated additional survival benefits through the application of chemo-radiotherapy in the neo-adjuvant setting. However, additional research is necessary to pinpoint the specific patient groups that stand to gain the most from this approach (McGuigan et al., 2018).

2.1.3.1 Surgery

The evolving role of surgery is reshaping resection rates, leading to improved clinical outcomes for some patients while worsening outcomes for others. Enhanced patient selection is therefore imperative in ensuring optimal results (Buanes, 2017).

Surgical excision of the tumor is the primary curative approach for adenocarcinoma. The typical surgical treatments for operable pancreatic carcinoma include the standard pancreatoduodenectomy (PD) with the pylorus-preserving PD procedure with the Traverso-Longmire method or the Kausch-Whipple. Originally confined to resectable disease (stage I and II), surgical indications have expanded to encompass locally advanced cases (stage III). However, despite these advancements, only a small fraction of patients, approximately 10–20%, are identified with localized, operable condition. Even among those with resectable disease, surgery remains underutilized due to historical concerns about its safety and efficacy.

Nonetheless, recent advancements have significantly improved surgical outcomes, reducing surgery-related mortality to around 3%, and increasing 5-year survival rates to nearly 30% when resection is combined with adjuvant chemotherapy (Kleeff et al., 2016) (Strobel, Neoptolemos, Jäger, & Büchler, 2019).

2.1.3.2 Chemotherapy

Chemotherapy remains a fundamental aspect of the treatment of Pancreatic Ductal Adenocarcinoma (PDAC) throughout every stage. Recent data and approvals from the US Food and Drug Administration (FDA) categorize treatments into three main groups: adjuvant, for use after surgery; unresectable/metastatic, for advanced disease; and neoadjuvant/induction, administered before surgery to shrink tumors or improve surgical outcomes (Roth, Cardin, & Berlin, 2020).

Gemcitabine has been regarded as the primary agent therapy for pancreatic cancer treatment since the 1990s. While numerous agents, either in combination with gemcitabine or as monotherapy, have been assessed, only a few have shown a significant positive effect on longevity in individuals with advanced illness (Burris et al., 1997) (Mohammed, Van Buren, & Fisher, 2014). For patients with advance stages of PC, the recommended treatment is the FOLFIRINOX regimen or can be a combination of gemcitabine and NAB-paclitaxel alongside chemotherapy. If combination chemotherapy isn't suitable for the patient, gemcitabine alone is the main option. Conversely, for cases with locally advanced PC, the advised approach involves a combination of radiation and chemotherapy therapy. Specifically, gemcitabine (Accompanied by or without erlotinib) coupled with radiation therapy is the best suggested treatment for this group of patients (Zhao & Liu, 2020).

Despite concerns regarding its toxicity, FOLFIRINOX has shown promising results in patients with both early and advanced disease, particularly in those with good performance status, indicating potential for improving oncologic outcomes (Mohammed et al., 2014).

Gemcitabine combined with erlotinib presents another regimen that has demonstrated improved overall survival rates and disease-free progression. Nevertheless, owing to their superior

promise for better consequences, FOLFIRINOX and gemcitabine combined with nab-paclitaxel emerge as the favored treatment choices for patients with satisfactory performance status (Moore et al., 2007) (Mohammed et al., 2014).

2.1.3.3 Radiotherapy

The basic principle for radiation therapy is to either damage or demolish cancer cells using radiation therapy, preventing them from multiplying. This treatment modality is primarily utilized in patients diagnosed with locally advanced pancreatic cancer (LAPC) (Zhao & Liu, 2020), where the tumor surrounds the celiac axis or superior mesenteric artery, sometimes with nodal involvement but without distant metastases. By definition, LAPC is deemed untreated and constitutes approximately 25% of cases at the time of detection (F. Wang & Kumar, 2011).

Traditionally, radiation therapy has been employed as a local treatment option in various settings, including adjuvant, neoadjuvant, or definitive treatment, either alone or in combination with systemic therapy. A considerable proportion of patients, ranging from approximately 20% to 80%, have received radiation therapy at some point during their treatment course (F. Wang & Kumar, 2011).

A study conducted revealed that radiation therapy in older patients appears to be well-tolerated and risk-free, especially in regard to prolonged adverse effects, regardless of the applied treatment. Consequently, RT may serve as a viable treatment alternative for pancreatic cancer (PaC), even within an older population (Ciabatti et al., 2019).

2.1.3.4 Targeted Therapy:

A genomic analysis involving 456 cases of pancreatic ductal adenocarcinoma (PDAC) was conducted. Findings revealed 32 consistently altered genes grouped into ten pathways. Expression assessment revealed four distinct subtypes of pancreatic cancer: immunogenic, pancreatic progenitor, squamous and aberrantly differentiated exocrine and endocrine, with each subtype possessing a distinctive molecular pathway, highlighting the heterogeneous nature of the disease (Amanam & Chung, 2018).

One of the most abundantly altered genes in the pancreatic ductal adenocarcinoma is KRAS, with mutations observed in over 90% of these tumors. Consequently, mutant KRAS emerged as an initial candidate target for the treatment of PDAC (Caldas & Kern, 1995).

Other pathways were attested in the treatment of PC, such as angiogenesis (Targeting the VEGFA and VEGFR receptors), tumor stroma (Matrix metallo-proteinases, a family of proteolytic enzymes essential for maintaining tissue homeostasis that showed aberrant expression is implicated in cancer invasion), cancer stem cell, and DNA damage repair (X. Liu, Li, & Wang, 2021).

The US Food and Drug Administration (FDA) has authorized only three targeted therapies for the treatment of pancreatic cancer to date. Erlotinib, which is considered as an inhibitor of the epidermal growth factor receptor (EGFR), is utilized together with gemcitabine hydrochloride, a commonly used chemotherapy drug, for patients with pancreatic ductal adenocarcinoma (PDAC) that is metastatic, locally advanced, or unresectable. Additionally, Everolimus, an inhibitor of the mammalian target of rapamycin (mTOR), and sunitinib, a tyrosine kinase inhibitor (TKI), are approved for use in adults with progressive neuroendocrine tumors that are metastatic, locally advanced, or unresectable (X. Liu et al., 2021).

2.1.3.5 Immunotherapy:

Immunotherapy has the ability to restore immune system function against cancer, which holds the potential to eliminate tumor cells. Although it has experienced great results in various malignancies, it is not yet a traditional therapy for pancreatic cancer. Currently, the only immunotherapeutic authorized by the United States FDA for patients with PC is the anti-PD-1 antibody Pembrolizumab, and only for those who test positive for microsatellite instability (Yoon, Jung, & Moon, 2021). Hence, it is recommended to conduct routine testing for microsatellite instability-high (MSI-H) in PDAC patients who are being considered for checkpoint inhibitor therapy (Sohal et al., 2018), (Yoon et al., 2021).

Currently, various clinical trials are underway to assess its efficacy in pancreatic adenocarcinoma (PAC). These trials examine various approaches, including cancer vaccines, adoptive cell transfer, immune checkpoint inhibitors, and combinations with

chemoradiotherapy or other targeted agents. Unfortunately, most of these experiments did not come with very promising results. It has been found that PAC has low immunogenicity, mainly due to its low tumor mutational burden (TMB). Furthermore, the PAC tissue is known to have a dense stroma with many fibroblasts, that can suppress the immune response (Schizas et al., 2020).

2.1.4. Tumor Microenvironment of Pancreatic Cancer

The tumor microenvironment (TME) of PC is mainly consisting of cancer-related fibroblasts, such as stellate cells, extracellular matrix components, various immune cells, and cytokines released by these cells. These factors helps in enhancing tumor by regulating tumor growth, invasion and metastasis through interactions with the cancer cells (Murakami et al., 2019).

Pancreatic Ductal Adenocarcinoma (PDA) is known to have a rich and diverse stroma, with more components than normal cancer cells. These include both cellular elements (such as fibroblasts, pancreatic stellate cells, immune cells, and myofibroblasts) and acellular elements (extracellular matrix (ECM), blood vessels) (Feig et al., 2012). PC microenvironment can be characterized by dense tissue (Desmoplasia) and extensive immune suppression, both playing crucial roles in PC progression. They play a role in developing metastasis, further worsening the disease's progression. Also, they boost PC cell reproduction and aid in evading immune surveillance through direct suppression of anti-tumor immunity or by inducing the growth of immunosuppressive cells (Neesse, Algül, Tuveson, & Gress, 2015) (Ren et al., 2018)

2.1.4.1 Desmoplasia

Cancer-associated fibroblasts (CAFs) are crucial elements in the desmoplastic reaction within PDA. They contribute in promoting tumor progression by actively interacting with cancer cells using different mechanisms, such as secretion of tumor-promoting factors, remodelling, and extracellular matrix deposition. Consequently, targeting CAFs emerges as a promising strategy against PDA (H. Huang & Brekken, 2020).

Traditionally, CAFs encompass all fibroblasts present within the tumor microenvironment. In the context of PDA, a prevalent model suggests that cancer cells trigger the activation of normal resident tissue fibroblasts, transforming them into CAFs (Apte, Wilson, Lugea, & Pandol, 2013, H. Huang & Brekken, 2020). For instance, the pancreatic stellate cells (PSCs) are considered to be a primary source of CAFs in PDA. PSCs are local cells in pancreatic periacinar, periductal, and perivascular spaces which are dormant cells till they develop into CAFs. This transition into active state shows myofibroblast like appearance, defined by the expression of α-smooth muscle actin (α-SMA), normally involved in pancreas homeostasis and extracellular matrix regulation, due to pancreatic injury or carcinogenesis (Haber et al., 1999, H. Huang & Brekken, 2020).

In addition to PSCs, mesenchymal stem cells (MSCs) have emerged as a well-studied alternative origin of CAFs, which can acquire myofibroblast traits under particular culture environments (exposure to tumor-conditioned media) such as expressing α-SMA and other fibroblast markers (Miyazaki, Oda, Mori, & Kida, 2020). It is likely that CAF populations in PDA are heterogenous, consisting of several subtypes of cells, due to the diverse potential roots of them (H. Huang & Brekken, 2020).

2.1.4.2 Immunosuppression

During the progression of PC, the immune system was found to have a double function. On one hand, it can eliminate cells with mutations before them becoming malignant, thus repressing tumor progression. Alternatively, it can create a suitable environment to immunosuppression and metastasis by enhancing PC development (Gomez Perdiguero & Geissmann, 2014, Ren et al., 2018).

Recent tumor immunology research have focused on cell populations and regulatory immune cells due to their contributions to the expansive immunosuppressive network within tumors (Inman, Francis, & Murray, 2014, Schnurr et al., 2015). These cell types, including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), alternatively activated (M2) tumor-associated macrophages (TAMs), pancreatic stellate cells, fibroblasts, and cellular components of the tumor microvasculature, are involved in establishing the microenvironment crucial for

the ecological and evolutionary processes within pancreatic cancer, even at its early stages (Schnurr et al., 2015, Ren et al., 2018).

The modulation of the immune response in tumors occurs at various levels, encompassing contact-dependent factors like checkpoint ligands expressed by tumor, immune, and stromal cells, soluble secreted factors, and alterations in MHC class I peptide presentation. These factors, either individually or collectively, disrupt the function of T effector cells, contributing to immune evasion within the tumor microenvironment (Schnurr et al., 2015).

2.1.4.3 Genomic characteristics

Recent efforts to classify PDAC have focused on improving patient stratification using genomic criteria. Sequencing studies have identified a few primary driver mutations (in KRAS, TP53, SMAD4 and CDKN2A) that appear in various integrations in most PDAC cases. KRAS is altered in over 90% of PDACs. However, even wild-type KRAS tumors often exhibit somatic genetic mutations that trigger the RAS/mitogen-activated protein kinase pathway. This indicates that even in the absence of KRAS mutations, the RAS pathway continues to be a significant molecular promoter of pancreatic cancer development (Karamitopoulou, 2019, (Caldas & Kern, 1995). A few other alterations (RBM10, KDM6A, and MLL3) happen at a rate of around 10%, while most other identified alterations happen at a frequency of less than 5%. (Raphael et al., 2017).

2.1.4.4 Morphologic Characteristics

A study conducted by (Matsuda et al., 2010) revealed significant findings regarding PDAC cells' behaviour in 3D culture. They observed notable morphological changes, the formation of spheroids, and shifts in cytoskeletal proteins. The investigation involved four distinct cell lines: KLM-1, MIAPaCa-2, PANC-1 and PK-45H. and While KLM-1 and PANC-1 and cells exhibited substantial spheroid formation **(Figure 2.2 B, D)**, MIAPaCa-2 and PK-45H cells did not demonstrate this characteristic in the 3D culture environment **(Figure 2.2 F, H)**. Moreover, the finding indicates that E-cadherin may attribute in facilitating spheroid formation among

PDAC cells. Notably, the levels of E-cadherin mRNA expression were proven to be higher in KLM-1 and PANC-1 cells in comparison with MIAPaCa-2 and PK-45H cells.

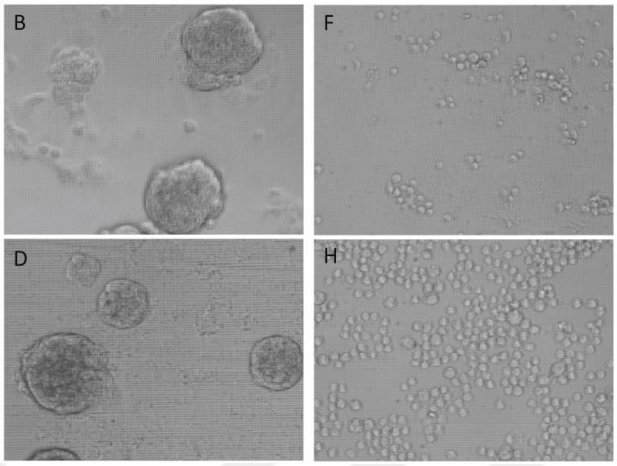


Figure 2. 2: Two-dimensional (2D) and three-dimensional (3D) phase-contrast visuals of pancreatic cancer cells. This figure is taken from (Matsuda et al., 2010).

Another study explained that the implementation of 3D culture effectively highlighted notable morpho functional differences among PDAC cells, discerning between those displaying epithelial or mesenchymal characteristics. Moreover, the persistence of the epithelial phenotype was potentially linked to the inadequacy of the TGF-β pathway. This investigation utilized two distinct cell lines, PK-1 and PANC-1. Within the 3D culture environment, PK-1 cells exhibited elevated E-cadherin and diminished vimentin expression, presenting a rounded morphology surrounded by adipocytes. Conversely, PANC-1 cells displayed heightened vimentin and reduced E-cadherin expression, forming grape-like structures with rough cell surface **(Figure 2.3)**. Additionally, PK-1 cells demonstrated high expression levels of trypsinm, CA19-9, cytokeratin 7, CA19-9, and E-cadherin, unlike PANC-1 cells. Both PK-1 and PANC-1 cells exhibited positivity for transforming growth factor (TGF) β receptor II and phosphorylated smad2/3, while PK-1 cells were negative for Smad4 (Shichi et al., 2019).

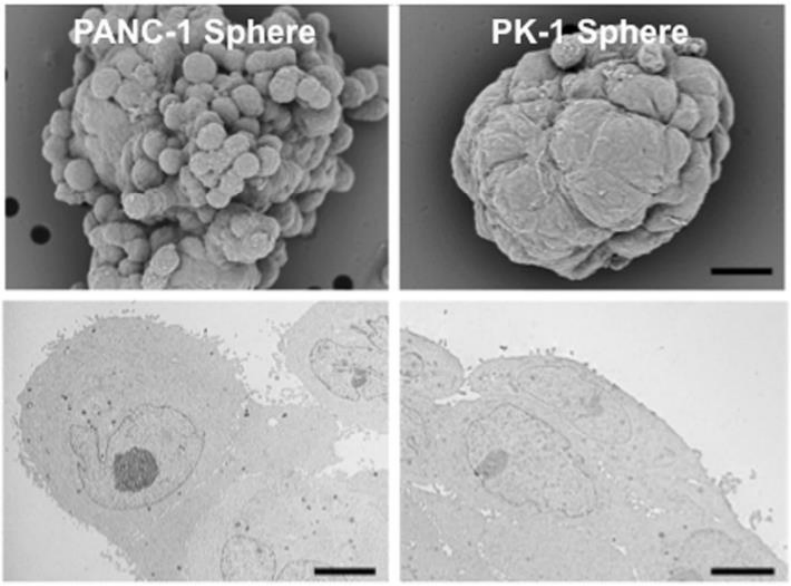


Figure 2. 3 transmission electron microscopy (TEM) and scanning electron microscopy (SEM) and analyses of PK-1 and PANC-1 spheres. This figure is taken from (Shichi et al., 2019).

2.1.5 Treatment of Pancreatic Cancer using Natural Products

Despite the use of traditional treatment methods for pancreatic cancer (PC), including surgery, chemotherapy, and radiotherapy, mortality rates for this lethal cancer remain high compared to other cancer types, possibly due to drug resistance (He, Wang, Zhang, Huang, & Wang, 2022). Consequently, this has prompted research into the potential of natural products as adjuncts to treatment, aiming to enhance efficacy without increasing toxicity.

Kim et al. conducted a study involving 68 natural products, such as Eucalyptus microcorys, Moringa, and Coix seed, which demonstrated anti-pancreatic cancer effects, including apoptosis, anti-metastasis, anti-angiogenesis, and anti-resistance properties. They summarized these effects concerning each anticancer mechanism and highlighted that natural products could serve as promising candidates for treating pancreatic cancer. They proposed that these natural products may enhance the efficacy of conventional anticancer drugs through synergistic effects, leveraging their inherent anticancer properties (Kim et al., 2021).

Another study demonstrated the synergistic effect of Isoflavone and Curcumin on pancreatic cancer proliferation when used together. Z. Wang et al. found that “combining these agents resulted in a significantly greater inhibition of cell growth and induction of apoptosis compared

to using either agent alone. These findings suggest that diets incorporating multiple natural products may be beneficial, potentially preferable over single agents, for both the prevention and treatment of PC" (Z. Wang et al., 2008).

Other study conducted by Dahham et al., suggested that "the essential oils of Agarwood promote cell death and inhibit proliferation in the pancreatic cell line (MIAPaCa-2). One of the major components of these oils is β-Caryophyllene, which demonstrated potent cytotoxic activity against the cancer cells. They found that cell migration and colony formation were inhibited, and MIAPaCa-2 cells exhibited antiproliferative activity, with an IC50 value of 11 ± 2.18 µg/ml" (Saad Sabbar Dahham et al., 2016).

2.2. β-caryophyllene (BCP)

Sesquiterpenoids are a diverse group abundant in plants, made up of secondary compounds and comprising a 15-carbon backbone. They are used in several aspects due to their diverse biological activities, such as antitumor functions, regulating plant growth, acting as antifungal agents, and insect deterrents. Therefore, it gained a lot of attention to learn more about their structure and oxygen content that are involved in their functions (Wu, Shi, & Jia, 2006). In particular, bicyclic sesquiterpenoids had gained attention in the biopharmacological field, often featuring prominently in essential oils, and β-caryophyllene emerges as a notable constituent in volatile oils originated from food and spice plants (Francomano et al., 2019).

(E)-β-caryophyllene [(E)-BCP] is a popular natural compound that is found in the essential oils of different plants and spices. A study performed by (Gertsch et al., 2008) found that "[(E)-BCP] particularly links to the CB2 receptor and serves as a functional CB2 agonist. It interacts with the CP55,940 binding site, which is also known as the THC binding site, on the peripheral hCB2 cannabinoid receptor".

The cannabinoid receptors CB1 and CB2 (CB1R, CB2R) are part of the G protein-coupled receptor (GPCR) family and interact with different types of agonists and antagonists, triggering a wide range of downstream effects (Howlett & Abood, 2017). Upon the binding of (E)-BCP to the CB2 receptor, it inhibits adenylate cyclase, leading to the simulation of intracellular

calcium fluctuations. Additionally, it mildly stimulates the mitogen-activated kinases p38 and Erk1/2 in primary human monocytes (Gertsch et al., 2008).

BCP has gained attention due to its therapeutic traits, including antioxidant and anti-inflammatory effects. In a study conducted by (Gushiken et al., 2022), BCP was chosen to test its efficacy in wound healing and its underlying pharmacological mechanisms. They found "macroscopic wound retraction in subjects treated with BCP. Biochemical analyses demonstrated that the BCP-treated group exhibited antioxidant and anti-inflammatory effects, characterized by elevated levels of IL-10 and GPx, and reduced stages of pro-inflammatory molecules. Immunohistochemical assays further revealed enhanced re-epithelialization following BCP treatment, evidenced by increased immunolabeling of desmoglein-3 and laminin-γ2. Moreover, BCP was found to contribute to the remodelling process by increasing collagen content" (Gushiken et al., 2022).

Furthermore, BCP has exhibited antimicrobial effect on Gram-positive bacteria, including *Streptococcus mutans* (Yoo & Jwa, 2018), and *Staphylococcus aureus* (Saad S. Dahham et al., 2015). Yet, it is noteworthy to mention that BCP demonstrates greater performance in Gram-positive bacteria in contrast to Gram-negative bacteria (Yoo & Jwa, 2018).

2.2.1. Cancer Therapeutic Potential of β-caryophyllene (BCP):

Numerous researches have explored the possible use of cannabinoids in cancer treatment. It is currently hypothesized that the anticancer effects of cannabinoids may primarily stem from three mechanisms: (1) triggering apoptosis, (2) suppression of the cell cycle, and (3) prevention of metastasis and angiogenesis. While the anticancer properties of BCP are not as widely acknowledged as those of conventional cannabinoids, emerging evidence suggests that these natural compounds hold promise as potential complementary treatments for cancer (Fidyt, Fiedorowicz, Strządała, & Szumny, 2016).

During a study performed by (Chung et al., 2019), it was found that BCP demonstrates cytotoxic activity against lung cancer cells by inducing cell cycle cessation. The researchers investigated the cytotoxic effects in two distinct human lung cancer cell lines. Treatment with the essential oils led to apoptotic cell death, characterized by a disruption of the proportion between pro-

apoptotic and anti-apoptotic Bcl-2 proteins, reduction in mitochondrial membrane potential (MMP), and activation of caspase-8, -9, and -3. Interestingly, pre-treatment with a caspase inhibitor significantly mitigated essential oils-induced cell death. Moreover, analysis of essential oils revealed six composites, among which β-caryophyllene showed intense anti-growth effects, emerging as the key active constituent. BCP triggered the G1 cell cycle cessation with the downregulating of cyclin-dependent protein kinase (CDK) -2, -4, and -6, cyclin E, cyclin D1, as well as RB phosphorylation together with the upregulating of p27KIP1 and p21CIP1/WAF1 (Chung et al., 2019).

In another study, it was observed that β-caryophyllene and caryophyllene oxide can trigger cell death in neuroblastoma cells and lymphoma by modulating 15-LOX, an upstream target, Subsequent to up-regulation of pro-apoptotic genes and the down-regulation of anti-apoptotic genes. The researchers treated *Aegle marmelos (Indian Bael)* fractionated extract with Jurkat and human neuroblastoma (IMR-32) cells at various concentrations. Results indicated that a concentration of 50µg/ml of β-caryophyllene and caryophyllene oxide could trigger cell death in the Jurkat cell line. Furthermore, cDNA expression profiling of pro-apoptotic and anti-apoptotic genes was conducted. The study revealed the down-regulation of anti-apoptotic genes such as cmyb, mdm2, bcl-2, and cox2, together with the up-regulation of pro-apoptotic genes including caspase-8, bak1, bax, caspase-9, and ATM in IMR-32 and Jurkat treated cells. These findings shed light on the downstream apoptotic mechanism induced by these compounds (Sain et al., 2014).

On the other hand, it was discovered that BCP demonstrates strong inhibition of colony formation, invasion, and spheroid shaping in colon cancer cells. The impacts of BCP were assessed through a multiple of in vitro antitumor-promoting assays using colon cancer cells. The results indicated that BCP displayed particular antibacterial effect on *S. aureus* and exhibited more prominent anti-fungal activity compared to kanamycin. Furthermore, BCP demonstrated selective anti-growth effects against colorectal cancer cells, with an IC50 of 19 µM. Additionally, the study revealed that BCP triggers cell death through fragmentation pathways and nuclear condensation, including breaking down of mitochondrial membrane potential (Saad S Dahham et al., 2015).

In addition, Ramachandhiran et al. studied the anti-cancer, anti-inflammatory, and cytotoxic effects of BCP in oral cancer KB cells (Ubiquitous keratin-forming tumour cell line HeLa), which are a type of keratin-forming tumor cell line (HeLa). BCP treated cells underwent several changes such as morphological alterations, decreased cell proliferation, apoptosis, losing their metastatic potential through the suppression of NF-κ B via the PI3K/AKT signalling pathway. BCP not only inhibited KB cell growth but also induced apoptosis and disrupted the cell cycle by down-regulating NF-κ B via the PI3K/AKT signalling pathways. These findings highlight the significance of inhibiting NF-κ B and PI3K/AKT as crucial mechanisms by which BCP inhibits cancer cell growth and promotes cell death (Ramachandhiran et al., 2022).

2.3 Etoposide

Etoposide is derived from podophyllotoxin, an organic compound extracted from the rhizomes and roots of plants such as Podophyllum emodi Wallich, found in the Himalayan regions and Podophyllum peltatum Linnaeus, known as May apple or American mandrake (Meresse, Dechaux, Monneret, & Bertounesque, 2012). It was first synthesized in 1966 and accepted as a treatment for testicular cancer in 1983 by the U.S. Food and Drug Administration (Hande, 1998).

2.3.1. Mechanism of action of Etoposide

Podophyllotoxin are known to hinder the cell replication process during metaphase level at mitosis, however, etoposide acts differently. From the beginning, etoposide prohibit the cells from accessing mitosis, by decelerating the cell development in the late S phase, and the early G2 phase. This directs to the cells to arrest in the G2 phase (Meresse et al., 2012).

The connection between topoisomerase II inhibition and the antitumor activity of etoposide was somewhat delayed, coinciding with the gradual elucidation of information about this crucial enzyme. It wasn't until the year 1979 that the term "DNA topoisomerases" was announced (Chen & Liu, 1994). Subsequently, in 1984, several research laboratories showed that mammalian topoisomerase II served as the primary goal for the application of etoposide (Hande, 1998).

DNA topoisomerases are involved in regulating the structural configuration of genetic material by causing short-term breaks in the DNA molecule. The process of unravelling and reassembling the double helix of the DNA, the protein travel along the sequence, and the winding of DNA into more complex structures can result in shape complexities that are fixed by topoisomerases through two transesterification reactions, facilitating topological transformations. Topoisomerases are categorized into two main types depending on their structure and function: type I (TopoI) and type II (TopoII) enzymes. Etoposide functions by poisoning the TopoII cleavage complexes (TopoIIcc) and inhibiting the second step of the reaction, which involves the re-jonining of DNA strands (Montecucco, Zanetta, & Biamonti, 2015).

Topoisomerase II (Topo II) has a pivotal role in controlling DNA topology. With the presence of ATP, Topo II undergoes a structural alteration, facilitating the movement of the unbroken DNA segment through the cleaved helix. Following this, the enzyme repairs the double-stranded DNA break. In vitro studies have demonstrated that etoposide primarily increases Topo II-mediated DNA breakage by supressing the enzyme's ability to religate cleaved nucleic acid molecules. Consequently, either single or double-stranded DNA breaks can be generated, depending on the molar ratio between etoposide and topoisomerase II. In this process, Topo II becomes covalently bonded to the 5′-end of DNA. (Montecucco & Biamonti, 2007).

2.3.2. Etoposide in Cancer:

Etoposide is a medication belongs to the topoisomerase II inhibitors class. It has been used in the treatment of multiple cancers including bladder, stomach, testicular and prostate cancer. The fact of Etoposide being a Topo II inhibitor explains its effectiveness in managing the aforementioned cancers, as well as brain tumors, Hodgkin and non-Hodgkin lymphoma, and other applicable disorders. Since its synthesis, numerous studies have been performed to review the efficacy of Etoposide in treating various types of cancer.

A pioneer study conducted by Bremer et al. involved 165 patients diagnosed with malignant testicular tumors who were administered treatment with etoposide either alone or in combination. Among patients attended to solely with etoposide, a response rate of 24.3% was

seen. On the other hand, when etoposide was combined with ifosfamide, tumor remission was achieved in 58.3% of patients (Bremer et al., 1982).

While in a recent study, 25 patients diagnosed with high-risk stage I and IM non-seminomatous germ cell tumors, along with forty-four individuals with advanced seminoma or non-seminomatous germ cell tumors (NSGCT), underwent treatment with bleomycin, etoposide, and carboplatin (BEC90). Their findings indicated that the BEC90 regimen had minimal impact on fertility and Leydig cell function. However, it was noted that carboplatin-based chemotherapy exhibited lower effectiveness compared to cisplatin-based chemotherapy and is currently not utilized in the treatment of testicular cancer (Pectasides et al., 2004).

Moreover, in a study conducted by (S. Liu & Yamauchi, 2010) the effects of Etoposide on advanced prostate cancer were investigated. Using an in vitro model, they explored the molecular machinery underlying etoposide-induced proliferation inhibition in prostate cancer cells. Their findings revealed that etoposide significantly inhibited androgen-stimulated DNA synthesis and androgen/androgen receptor (AR)-mediated cell growth. Additionally, it down-regulated the expression of AR and prostate-specific antigen (PSA), stopped AR connecting to androgens, and attenuated AR nuclear translocation in prostate cancer cells.

Conversely, (Munk, Memon, Nexo, & Sorensen, 2007) delved into the impact of the epidermal growth factor receptor (EGFR) on the triggering of cell death by etoposide and examined the potential of combining it with gefitinib to prevent cell survival mechanisms in bladder cancer cells. Treatment with VP16 led to the phosphorylation of EGFR and activation of EGFR, which in turn hindered the apoptosis induced by VP16, resulting in a mere 19% apoptosis. However, the study revealed that EGFR activation serves as a cell-survival mechanism. Inhibition of EGFR with gefitinib thwarted VP16-induced EGFR stimulation. The integrated therapy of gefitinib and VP16 yielded 45% dying cells, more than twice the findings achieved with VP16 alone, whereas gefitinib alone did not induce apoptosis in the cells.

A case study conducted by (Kuo et al., 2009) a male patient diagnosed with primary gastric small cell carcinoma underwent chemotherapy combining cisplatin with etoposide. After three months of therapy, a remarkable decline in metastases was observed, and the illness stayed well managed for a period of 10 months. The patient exhibited a response time of 7 months, with a

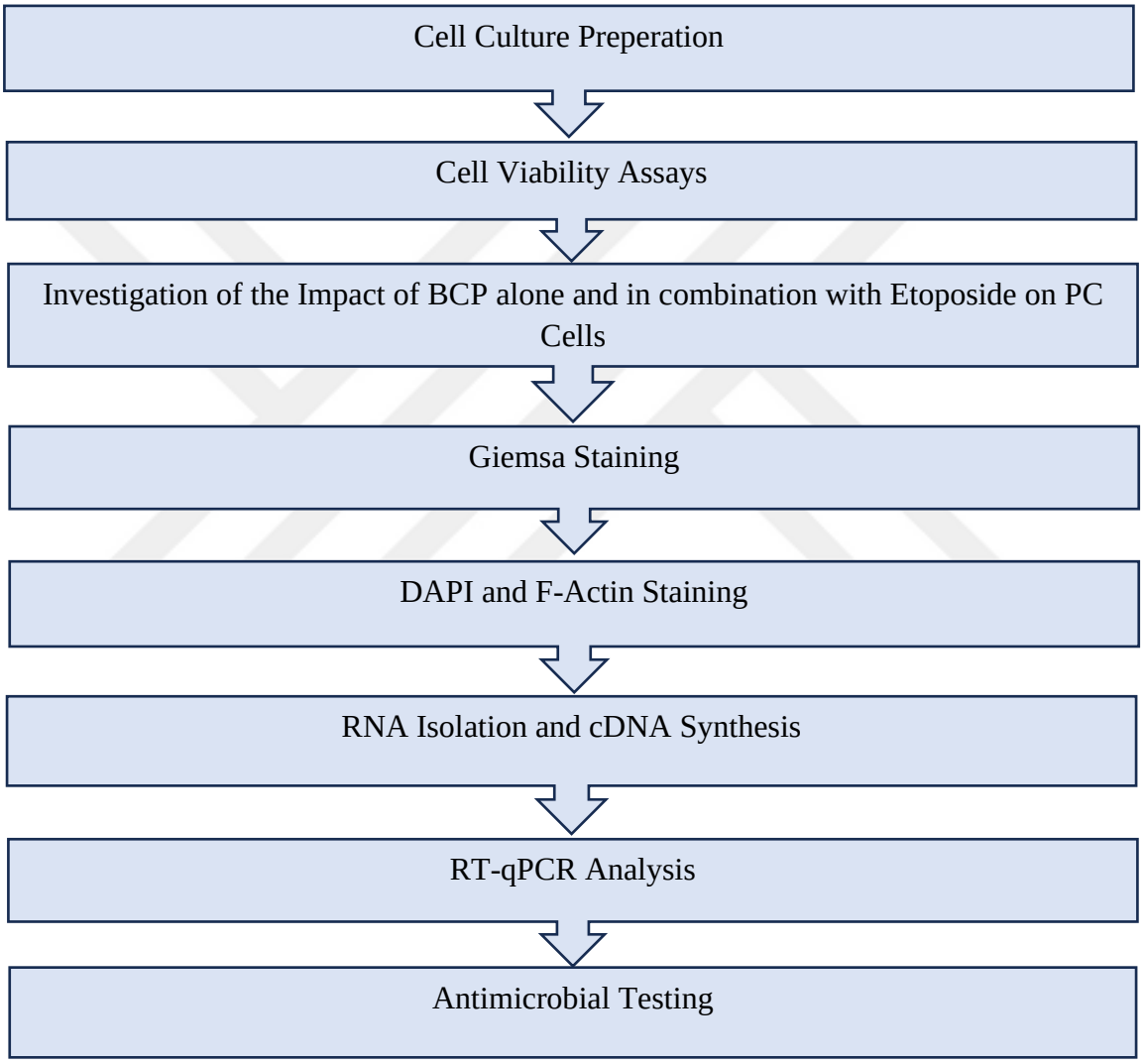
progression-free survival of 10 months. These results are noteworthy considering that most patients diagnosed with this condition typically succumb within one year; however, this particular patient survived for 17 months, as indicated by previous reports.

On the other hand, several studies have explored the use of Etoposide as an adjuvant agent with natural products to enhance its effectiveness, with numerous investigations conducted in this area. One such study by Gokturk et al. used *Juniperus drupacea*, a medicinal herb from the Cupressaceae family, together with Etoposide, on Glioblastoma cell lines. Their observations was as follows "this herb reduced the viability and migration capacity of GBM cells and enhanced the cytotoxic effects of Etoposide" (Göktürk & Özdemir Alkış, 2023). In another study, the effects of Etoposide in combination with *Rosmarinus Officinalis*, commonly used as an essential oil, were investigated on GBM U87 MG cells and Mouse Embryonic Fibroblast (MEF) cells. The study found that "higher concentrations of *Rosmarinus Officinalis* stimulated MEF cell proliferation, while it inhibited the survival of GBM cells. This suggests that Rosmarinus Officinalis may not affect the cytotoxicity of Etoposide on GBM cell cultures; conversely, it promoted proliferation in MEF cell cultures and reduced the impact of Etoposide" (Ozdemir & Gokturk, 2018).

3. MATERIALS AND METHOD

The goal of this study is to see if β-caryophyllene (BCP) can enhance the effectiveness of the chemotherapeutic agent Etoposide against pancreatic cancer cells. The applied methods during this study are shown in Figure 3.1

Figure 3. 1 The Methods Applied in this study.



3.1 Cell Culture Conditions

The PC MIAPaCa-2 (ATCC® CRL-1420 ™) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) enhanced with 10% fetal bovine serum (FBS). The cells were incubated in a humidified incubator (Nüve brand) with a 5% CO_2 concentration at 37°C temperature.

3.2 Cell Viability Assay

In order to assess the cytotoxic effects of BCP on PC cells, the neutral red viability assay was performed. The neutral red uptake assay is a cell viability test used to quantify xenobiotic-induced cytotoxicity in vitro. This assay is based on the capacity of alive cells to take up and connect the neutral red, a weak cationic stain, within their lysosomes. Consequently, cytotoxicity is measured by a concentration-dependent decrease in the uptake of neutral red following exposure to the xenobiotic being studied (Ates, Vanhaecke, Rogiers, & Rodrigues, 2017). The nutrient medium was removed and cells were with PBS. The tested cells were then covered with a neutral red solution (0.033%) in PBS. After that, the cells were incubated for at least 2 hours. Later, the neutral red solution was removed, and a dissolving solution for the neutral red (50% ethanol, 49% pure water, 1% acetic acid) was incorporated. The resulted solution was put on a shaker until the homogenized colour distribution was detected. The absorbance at 540 nm was measured with a microplate reader (Spectrostar Nano).

3.3 Exploring the Impact of BCP Alone and in Combination with Etoposide on PC Cells.

The IC50 value, that is the concentration of BCP required for 50% of the cells to be killed or to cause cell death, was determined.

In order to achieve that, $5x10^3$ MIAPaCa-2 (ATCC® CRL-1420 ™) cells per well were cultured in a 96-well cell plate and kept in a humidified incubator with 5% CO2 at 37°C. Different concentrations of BCP were applied to the cells (ranging as 18, 36, 54 72 and 90 µg/mL) for 24 hours. As mentioned earlier, the cell viability was tested and the IC50 value was found.

To find the collaborative consequence of BCP and Etoposide on MIAPaCa-2 cells, they were subjected to various conditions, including Etoposide, BCP combined with Etoposide, and BCP, for 24 hours. By using the cell viability test, the combined outcome of the specified IC50 value of BCP and Etoposide was evaluated. The IC50 concentration of Etoposide to be applied on

MIAPaCa-2 Cells was determined as 6.298 µg/mL, which was found earlier by (Mohanty, Dilnawaz, Mohanty, & Sahoo, 2010)

3.4 Giemsa Staining

Giemsa staining is one of the well-known histological stains, results in dark blue nuclei and cytoplasm that ranges from blue to pink, depending on the acidity of its contents. By using a 0.22-micron syringe filter, the staining solutions was sterile-filtered. MIAPaCa-2 cells were seeded in six-well plates with four distinct treatments: Control (without treatment), Etoposide, BCP, and BCP/Etoposide combination. Corresponding to their IC50 values; the administration of Etoposide and BCP on MIAPaCa-2 Cells were about 6.298 µg/mL and 58 µg/mL respectively. After 24 hours incubation, the medium was withdrawn, and the cells were rinsed with PBS twice. After that, each well had 1 mL of 60% methanol in PBS, and the plates were incubated for 10 minutes at normal temperature. Later, the solution was removed, the wells were rinsed with pure methanol, treated with 1mL of Giemsa staining solution for each, and kept at room temperature for 20 minutes. Afterwards, the staining solution was withdrawn, and the wells were rinsed with deionized water for three times. The cell shape changing was seen using a LED-inverted microscope (Leica Biosystems).

3.5 DAPI and F-Actin Staining

4',6-diamidino-2-phenylindole (DAPI) and phalloidin dyes were used to assess the vitality and structural components of MIAPaCa-2 cells. DAPI, which binds strongly to adenine-thymine (A-T) rich domains of nuclear DNA through electrostatic interactions, is utilized to visualize nuclear DNA in both living and fixed cells. This binding results in fluorescence due to the inhibition of internal rotation in DAPI's molecular structure (Tarnowski, Spinale, & Nicholson, 1991). Phalloidin, a molecule derived from mushrooms, particularly binds to filamentous actin (F-actin). Thus, fluorescently labelled phalloidin derivatives are frequently used in microscopy across different research domains to observe F-actin (Romani & Auwerx, 2021).

MIAPaCa-2 cells were cultured in 24-well plates with four treatments: Control (without treatment), Etoposide, BCP, and BCP/Etoposide combination. Corresponding to their IC50 values; the administration of Etoposide and BCP on MIAPaCa-2 Cells were about 6.298 µg/mL and 58 µg/mL respectively. After the 24-hour incubation, the cells were visualized. The

medium was withdrawn, and the cells were rinsed with PBS twice. After that, a 4% paraformaldehyde solution was then added to each well and the plates were left for incubation for 20 minutes at room temperature. Later, the cells were rinsed again with PBS twice and treated with PBS containing 0.1% Triton X-100 for 10 minutes to penetrate the cell membranes. Again, the cells were rinsed with PBS twice. On the other hand, the phalloidin-iFluor™488 and DAPI combine was first diluted at a 1:1000 ratio in PBS that contained 1% Bovine Serum Albumin (BSA), then added to the wells, and left for incubation in dark at room temperature for 30 minutes to be able to mark the cellular components accordingly. At the end, the cells were rinsed twice with PBS with BSA and seen with Leica DM IL LED inverted microscope (Leica Biosystems).

3.6 RNA Isolation and cDNA Synthesis

MIAPaCa-2 cells were cultured in 75 cm² cell culture plates. Again, four conditions were done in different plates: Control (without treatment), Etoposide, BCP, and BCP/Etoposide combination. Corresponding to their IC50 values; the administration of Etoposide and BCP on MIAPaCa-2 Cells were about 6.298 µg/mL and 58 µg/mL respectively. After the 24-hour incubation, RNA was isolated using the RNA isolation kit from Thermo Fisher Scientific, Invitrogen™ PureLink™, in order with the method advised by the manufacturer. cDNA synthesis was performed after the RNA being isolated, with the cDNA Synthesis Kit from Thermo Fisher Scientific, RevertAid First Strand, following the suggested method by the manufacturer as well. PCR thermal cycler (Agilent SureCycler 8800) was used for thermal cycling as 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes.

3.7 RT-qPCR Analysis

RT-qPCR test was carried out using a Rotor-Gene Q qPCR device (Qiagen, Hilden, Germany). The factors of this reaction were fixed as:

Initial activation at 95°C for 12 minutes, denaturation at 95°C for 15 seconds, annealing at 55°C for 20 seconds, and elongation at 72°C for 20 seconds. After that, the ΔCt values were attained. The primers used in this study were:

- Survivin forward primer: 5'-TCCACTGCCCCACTGAGAAC-3'
- Survivin reverse primer: 5'-TGGCTCCCAGCCTTCCA-3'

- c-myc forward primer: 5'-TACCCTCTCAACGACAGCAG-3'
- c-myc reverse primer: 5'-TCTTGACATTCTCCTCGGTG-3'
- Caspase 3 forward primer: 5'-TGACTGGAAAGCCGAAACTC-3'
- Caspase 3 reverse primer: 5'-AGCCTCCACCGGTATCTTCT-3'

3.8 Antimicrobial Activity Analyses:

The antimicrobial effect of BCPs on gram-positive (*S. aureus, B. subtilis, B. cereus*) and gram-negative (*E. coli*) and bacteria were determined using the microdilution method in a 96-well microtiter plate. The bacteria in the wells were cultured in Mueller Hinton Broth at a McFarland 0.5 density. BCP solution at concentrations 9, 12, 18, 90 µg/mL was then added to the plates containing the culture medium and microorganisms. The plates were incubated for 24 hours at 37°C. After incubation, the Minimum Inhibitory Concentrations (MIC) value, known as the lowest concentration without visible growth, was observed by inoculating agar plates with Mueller Hinton Agar from the wells without growth, along with controls.

3.9 Statistical Analysis

All assays were performed in triplicate. Statistical analysis was performed using Microsoft Excel 2021. The results were compared using one-way analysis of variance (ANOVA) and t-test. The level of significance was set at $p<0,05$.

4. RESULTS AND DISCUSSION

4.1 IC50 Graph of BCP on MIAPaCa-2 Cells

The cytotoxic impact of BCP was assessed through a neutral red assay, revealing an IC50 value of 58 µg/mL for the MIAPaCa-2 cell line in 24 hours (Figure 4.1). As the concentration of BCP rose, there was a corresponding escalation in the rate of cell death among pancreatic cancer cells. These findings suggest that elevated BCP concentrations resulted in enhanced efficacy in eliminating MIAPaCa-2.

Similar studies on MIAPaCa-2 cell lines was conducted using Agarwood essential oil extracts which one of the major components was identified as β-Caryophyllene (BCP). These oils inhibited the cell growth of MIAPaCa-2 in a dose dependent manner. (Saad Sabbar Dahham et al., 2016). Other studies showed that the IC50 values of BCP increased slightly in PANC-1 cell lines, up to 27 µM, with the lowest value being 19 µM in HCT 116 colon cancer cells (Saad S. Dahham et al., 2015).

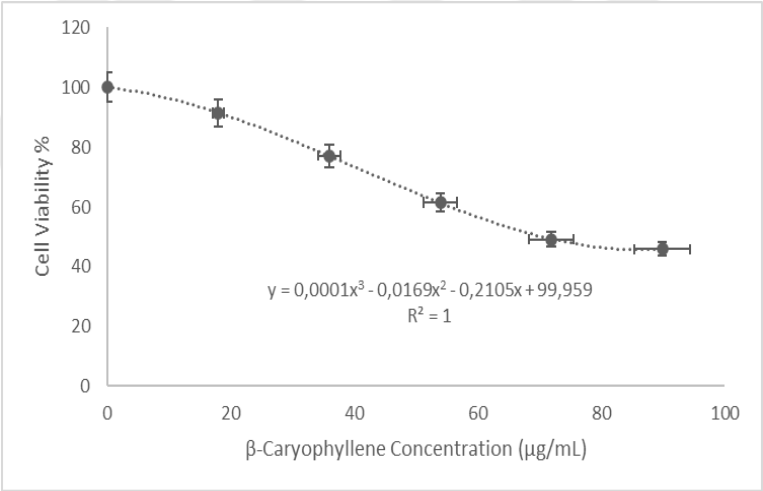


Figure 4. 1 The effect of different BCP concentrations on MIAPaCa-2 cell viability ($p<0.01$)

4.2 The Synergistic Effect of BCP and Etoposide on PC Cell Viability

The combined impact of BCP and etoposide on Pancreatic Cancer MIAPaCa-2 cells was assessed via a neutral red assay. Different concentrations of BCP were applied to the cells (ranging as 18, 36, 54, 72 and 90 ug/mL) for 24 hours. The IC50 concentration of Etoposide to be applied on MIAPaCa-2 Cells was determined as 6.298 µg/mL. These findings suggest that

the cytotoxic effect of etoposide was augmented in the presence of BCP. Moreover, the concurrent administration of BCP and etoposide led to a higher incidence of cell death in MIAPaCa-2 cells compared to using either treatment alone. These results imply that BCP has the potential to enhance the cytotoxicity of etoposide, thereby increasing the rate of cell death in pancreatic cancer MIAPaCa-2 cell lines (Figure 4.2).

Another similar study which supports our findings, showed proliferation inhibition in the same MIAPaCa-2 cell lines using essential oils of Agarwood, which were determined to contain β-Caryophyllene (BCP) as one of their major components. Higher concentrations of these oils decreased cell viability with an IC50 value of 11±2.18 µg/mL. In comparison, 5-fluorouracil, a well-known chemotherapeutic drug used for pancreatic cancer, exhibited significant toxicity toward these cell lines with an IC50 value of 6.5±1.4 µg/mL (Saad Sabbar Dahham et al., 2016)

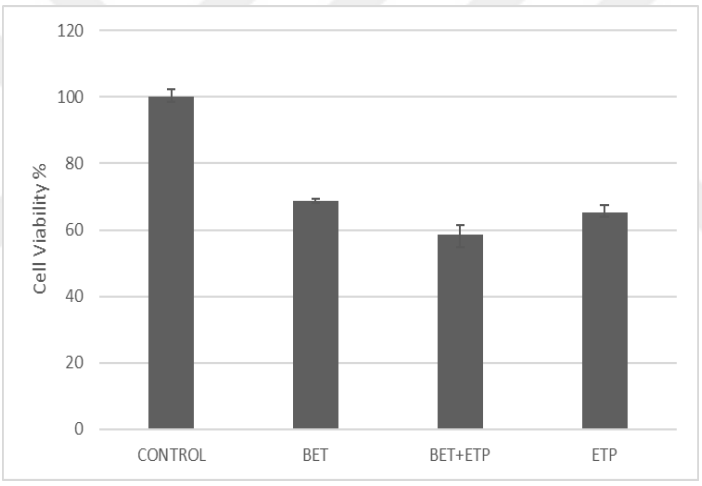


Figure 4. 2 The synergistic effect of β-Caryophyllene with etoposide on MIAPaCa-2 cells ($p<0.01$)

4.3 Giemsa Staining Visuals of Pancreatic Cancer Cells after BCP and Etoposide Application

Giemsa staining was used in order to visualize the MIAPaCa-2 cells during the treatment with BCP and Etoposide. First, the disappearance of the spindle-shape of the cells was observed, with them becoming more individualized and showing signs of weakening. This morphological change indicates and impact on the cytoskeletal organization and cellular adhesion of MIAPaCa-2 cells after being treated with BCP and Etoposide. Furthermore, the cells were

becoming rounder in shape, which is considered to be a remark for apoptosis initiation, and the impact of the cytotoxic effect of BCP and Etoposide on these cells. In addition, the cell counting was observed to be decreasing, meaning that the number of the viable cells was reducing as well. These morphological alterations showed an insight of how the MIAPaCa-2 cells reacted after the treatment with BCP and Etoposide (Figure 4.3).

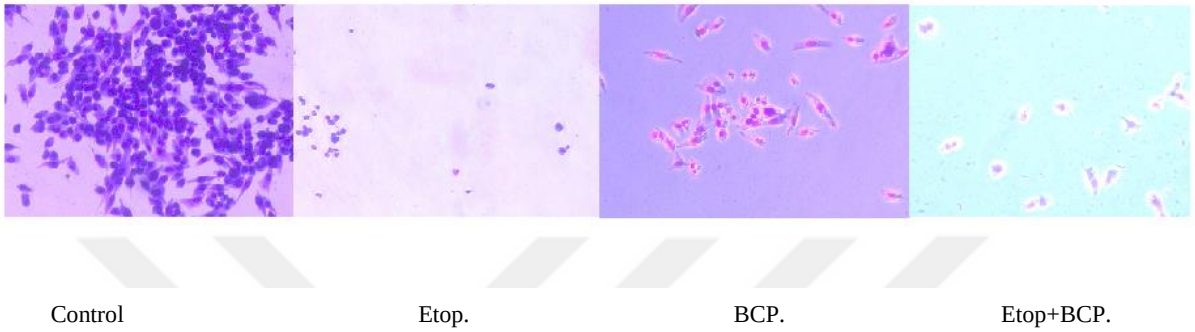


Figure 4. 3 Giemsa staining images of the effect of BCP with Etoposide on MIAPaCa-2 cells.

4.4 DAPI and F-Actin Staining Visuals after BCP and Etoposide Application

F-actin and DAPI staining were utilized to assess the indicates organization of the actin cytoskeleton and the cellular nuclei, respectively. MIAPaCa-2 cells were treated with BCP and etoposide. Corresponding to their IC50 values; the administration of Etoposide and BCP on MIAPaCa-2 Cells were 6.298 µg/mL and 58 µg/mL respectively in 24-well plates and cultured for 24 hours. Following the incubation, the fluorescence intensity was examined using an inverted microscope. Treatment with BCP caused a notable reduction in the nuclei abundance, suggesting a possible decline in cell viability or the initiation of apoptotic pathways. Moreover, there was a pronounced reduction in the F-actin filaments, which constitute the cellular skeletal framework. Additionally, the distinctive spindle-shaped morphology typical of pancreatic cancer cells was no longer apparent after treatment with BCP and etoposide, indicating a disruption in cellular structure and potential alterations in cell behaviours. The robustness of fluorescent nuclei and filaments was altered following administration with Etoposide, BCP, and their integration (Figure 4.4), resulting in the likelihood of some changes in the gene expression linked with the treatment. In this context, fluorescence staining methods have demonstrated that BCP markedly enhances the anti-proliferative impact of etoposide. Given that BCP is a safe

compound commonly found in food products, it holds promise as a natural therapeutic candidate in cancer treatment, particularly in conjunction with potent drugs like etoposide, known for their adverse side effects.

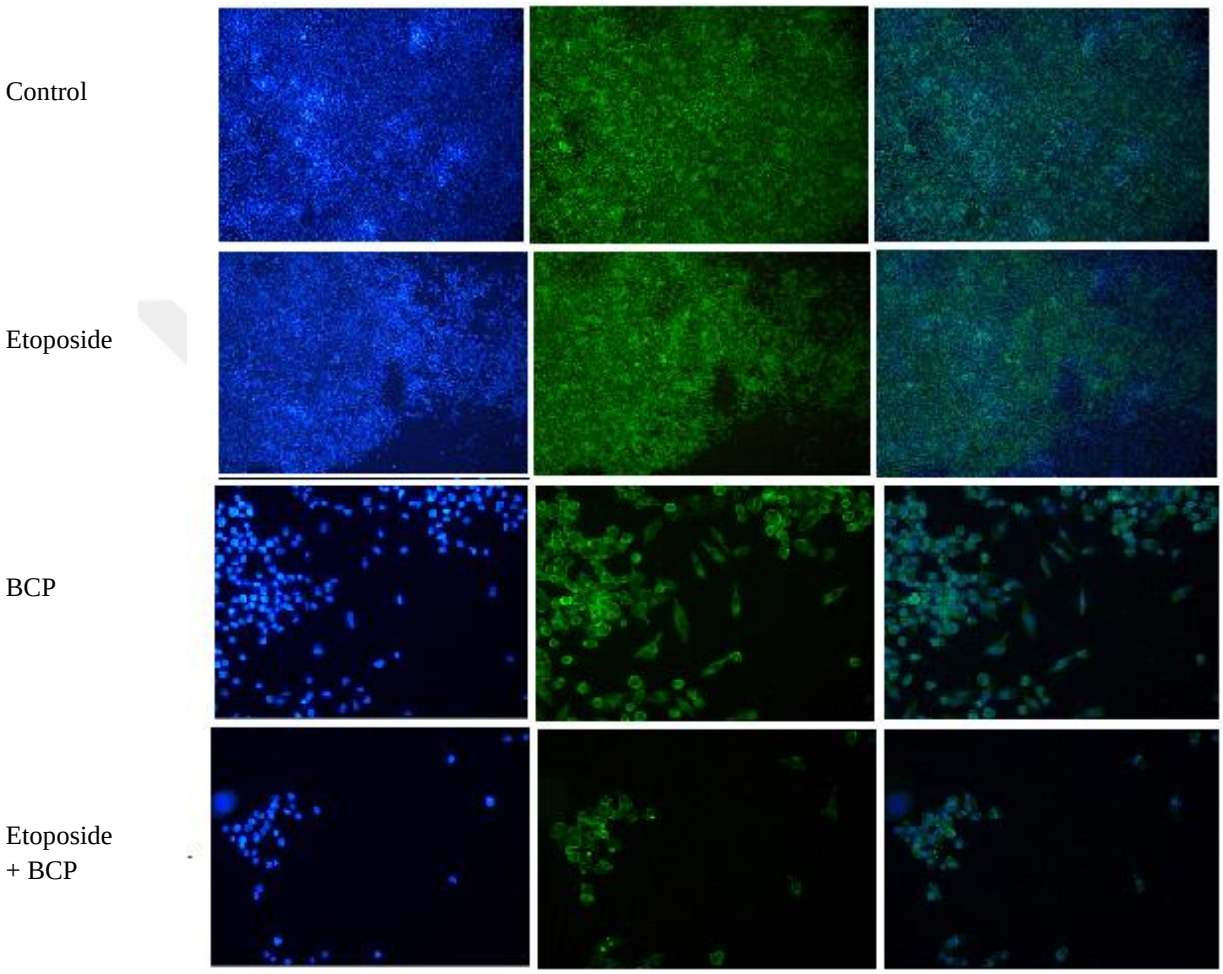


Figure 4. 4 Effect of beta caryophyllene and etoposide on MiaPaCa-2 cell line using DAPI and F-actin staining.

4.5 RT-qPCR Results

RT-qPCR was conducted to check the levels of the expression of Caspase 3, C-Myc, and survivin, in MIAPaCa-2 cells treated with etoposide, BCP, and their combination.

Survivin is a distinctive member of the inhibitors of apoptosis protein (IAP) family, playing a vital role in controlling cell division and preventing cell death. Its anti-apoptotic function is linked to its capacity to inhibit caspases, either directly or indirectly. Considered one of the specific proteins in cancer, survivin is minimally expressed or untraceable in most adult tissues but is significantly overexpressed in numerous tumors. Research has shown that increased survivin levels correlate with poor cancer prognosis, while its downregulation or inactivation can inhibit tumor proliferation. Thus, survivin has gained considerable interest as a promising cancer biomarker and a focal point for anticancer treatments (Zaffaroni & Daidone, 2002) (Guindalini, Mathias Machado, & Garicochea, 2013).

The graph in Figure 4.5 (A) shows a sharp downturn in Survivin mRNA expression when applied etoposide, while BCP alone had little or no effect. However, the combination of both decreased survivin expression. This suggests that the combination treatment effectively aims at the anti-apoptotic pathway, resulting in enhanced cell death and reduced cell viability.

The c-Myc protein, produced by the c-myc proto-oncogene, is a nuclear phosphoprotein with the ability to bind DNA. The uncontrolled expression of C-myc contributes to the formation of experimentally induced tumors and is frequently aberrant in numerous naturally occurring cancers (Kato & Dang, 1992). The substantial contribution of the Myc protein to tumorigenesis likely stems from its ability to promote cell-cycle advancement and impede cellular differentiation. Aberrant expression of myc genes and proteins has been noted in a vast array of human tumors (Herms et al., 2000).

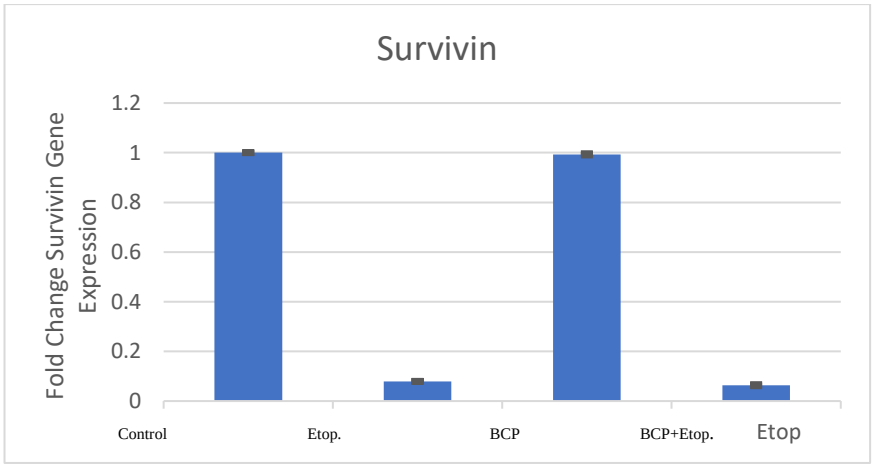
In this study, the combined treatment completely inhibited c-Myc expression in MIAPaCa-2 cells after one day of culture while BCP itself did not have significant impact on the gene expression (Figure 4.5 B), As a main controller of cell growth, the suppression of c-Myc suggests that this therapy might affect the molecular pathways that are associated with cell division and proliferation.

One of the primary genes responsible for mediating apoptosis is caspases, which can be categorized into two main groups: initiator caspases (such as caspases 8, 9, and 10) and downstream effector caspases (such as caspases 2, 3, 6, and 7). Among the effector caspases, caspase 3 is extensively researched. It plays a crucial role in both the death receptor pathway

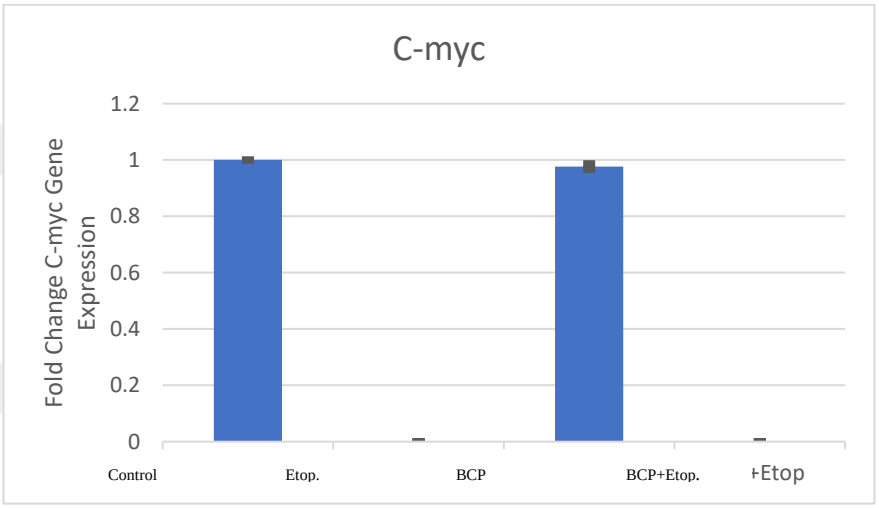
and the mitochondrial pathway. Moreover, several studies have shown that the activation of caspase 3 is crucial in initiating apoptosis in reaction to specific chemotherapeutic medications. Furthermore, it triggers an endonuclease called caspase-activated DNAse, resulting in the distinctive DNA fragmentation seen in apoptosis (O'Donovan et al., 2003).

According to our results, the decrease in survivin and c-Myc expressions, coupled with the increase in Caspase-3 expression, implies that the concurrent administration of BCP and etoposide could trigger cell death and impede the survival and growth of pancreatic cancer cells.

a)



b)



c)

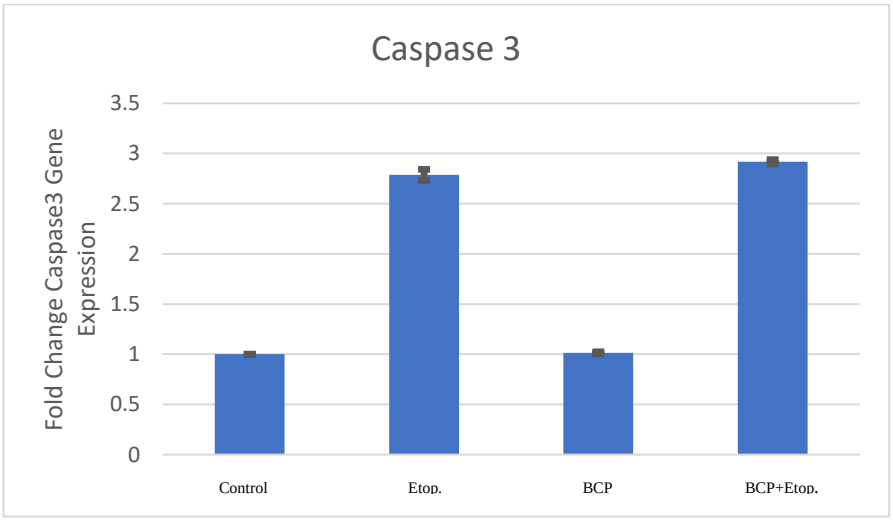


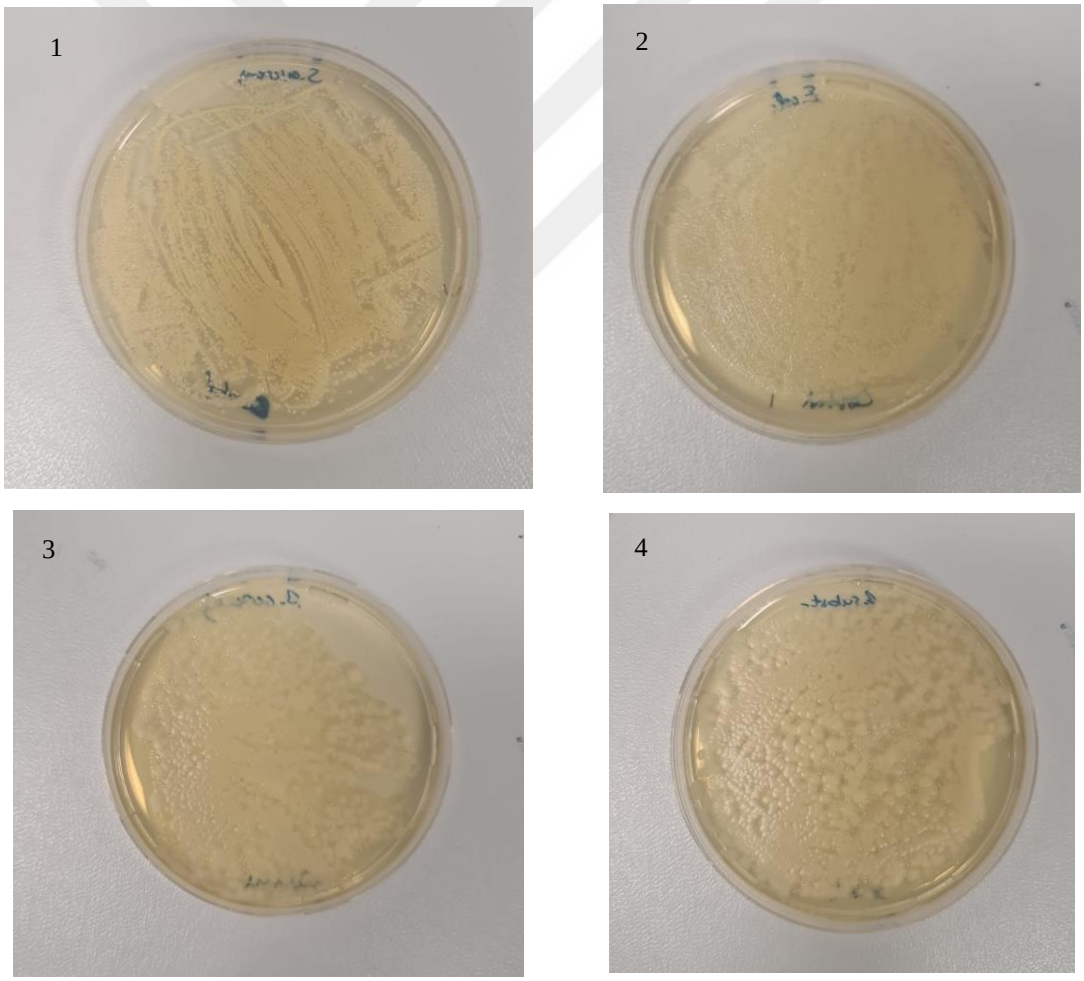
Figure 4. 5 The relative expression of Survivin (a), C-myc (b), Caspase-3 (c) ($p<0.05$)

4.6 Antimicrobial Activity Asay Results:

BCP demonstrated potent antibacterial activity against all examined bacterial strains (*S. aureus, B. subtilis, B. Cereus, E. coli*), which can be attributed to its potent antioxidant activities (Dorman & Deans, 2000). In this study, it was showed that BCP markedly inhibited the growth of all tested bacterial strains, as evidenced by the lack of reproductive activity shown in Figure 4.6. The Minimum Inhibitory Concentrations (MIC) value, was < 9 µg/ml for all strains in this study, where BCP effectively killed the bacterial strains, suggesting that smaller concentrations could be further explored for their antimicrobial efficacy.

One of the antimicrobial studies of BCP had shown similar outcome, with MIC values that ranged from 3 to 14 µM and they tested that BCP exhibited stronger antibacterial activity against Gram-positive bacteria than Gram-negative bacteria (Saad S. Dahham et al., 2015).

A)



B)

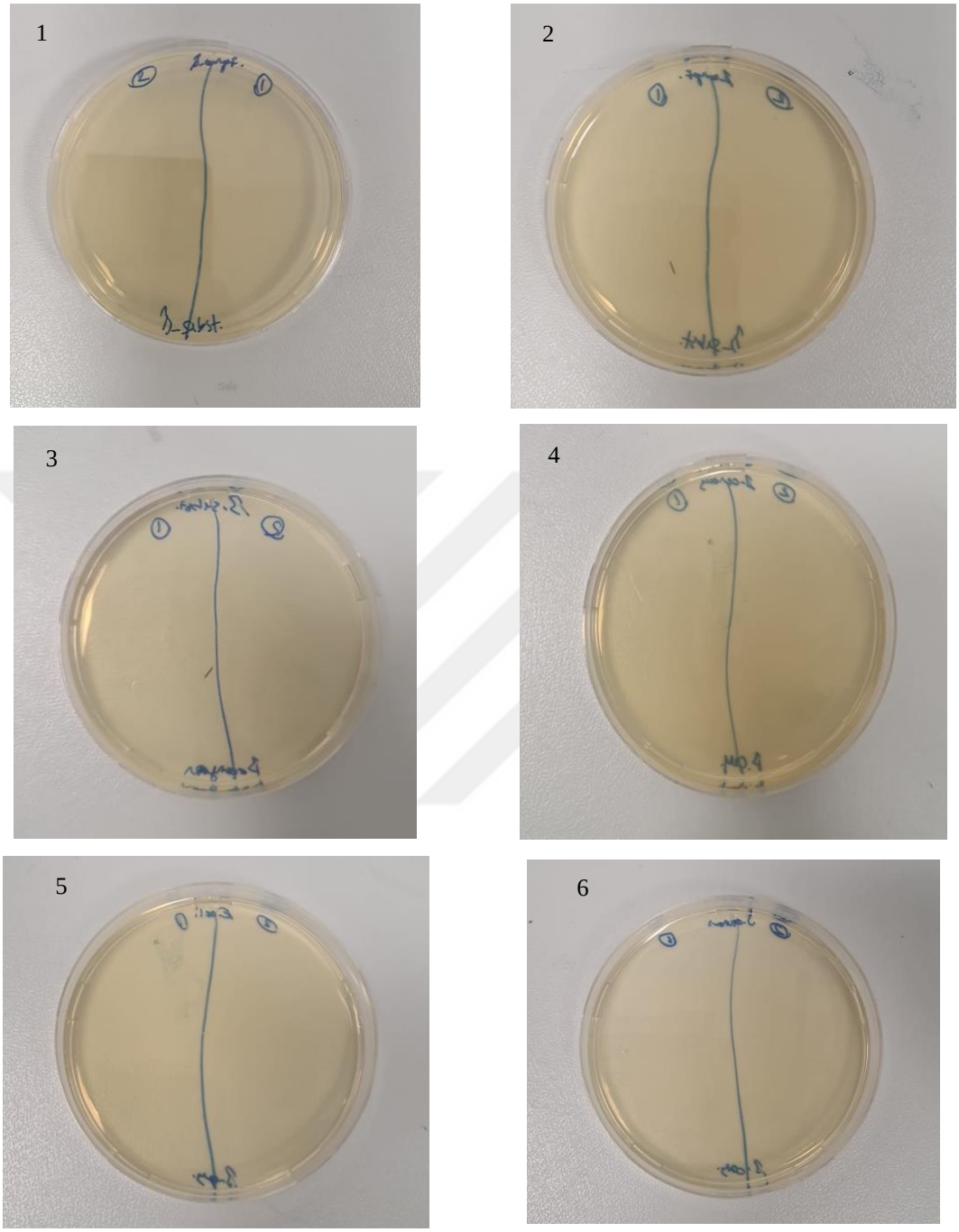


Figure 4. 6 Antimicrobial effect of BCP on E. Coli, S. aureus, B. subtilis, B. Cereus
(A) Control plates (A.1. *S. aureus* A.2. *E. coli* A.3. *B. Cereus* A.4. *B. subtilis*)
(B) Treated with BCP (B.1 and B.2 *B. Cereus,* B.3 and B.4 *B. Subtilis,* B.5. *E. Coli,* B.6. *S. Aureus)*

5. CONCLUSION

The results of this study present strong evidence that BCP is a potential therapeutic enhancer when combined with the chemotherapeutic agent Etoposide. Although several studies have investigated the effects of BCP on different cancer types, such as; inducing apoptotic cell death in lung cancer cells (Chung et al., 2019), exerting anti-inflammatory effect on lymphoma and neuroblastoma cells (Sain et al., 2014), inhibiting invasion, migration, and spheroid shaping in colon cancer cells (Saad S. Dahham et al., 2015) and demonstrating anticancer, and cytotoxic effects on the oral cancer KB cell line (Ubiquitous keratin-forming tumour cell line HeLa) (Ramachandhiran et al., 2022), yet our findings specifically focused on the effect of BCP and its potential to enhance the efficacy of the chemotherapeutic drug Etoposide in treating pancreatic cancer. These results could significantly contribute to existing research, as they are among the first to explore the combination of BCP and Etoposide, potentially inspiring further in vivo studies. The results indicate that BCP has potent selective cytotoxic properties against human pancreatic cancer cells, primarily due to its apoptotic effects. Moreover, in combination, BCP augmented the anti-proliferative impact of Etoposide, leading to reduced adverse effects on normal cells. Detailed further molecular studies are needed to elucidate the mechanisms by which BCP suppresses tumor motility, cell invasion, and tumor aggregation.

In addition, the antimicrobial results showed that BCP demonstrated antibacterial activity against both Gram positive and Gram-negative bacteria. Future research could explore the underlying mechanisms of BCP's antimicrobial action in greater detail, investigate its potential synergistic effects with conventional antibiotics, and assess its efficacy in vivo to further validate its therapeutic potential.

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APPENDIX

Appendix A- ANOVA analyses for dose optimization (IC50 determination) assay

Appendix B- ANOVA analyses for cytotoxicity assays of MIAPaCa-2 cells

Appendix C- t-test analyses for cytotoxicity assay of MIAPaCa-2 cells

Appendix A- ANOVA analyses for dose optimization (IC50 determination) assay

Table A.1. ANOVA analyses for dose optimization (IC50 determination) assay of MIAPaCa-2 ($p<0,001$).

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0 µg/mL	3	2,977	0,992333	0,001016
18 µg/mL	3	2,721	0,907	0,000247
36 µg/mL	3	2,295	0,765	8,1E-05
54 µg/mL	3	1,829	0,609667	0,000177
72 µg/mL	3	1,464	0,488	7,3E-05
90 µg/mL	3	1,366	0,455333	5,03E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,736346	5	0,147269	537,1519	1,13E-13	3,105875
Within Groups	0,00329	12	0,000274			
Total	0,739636	17				

Appendix B- ANOVA analyses for cytotoxicity assays of MIAPaCa-2 cells

Table B.1. ANOVA analyses for cytotoxicity assay of one day cultured MIAPaCa-2 cells ($p<0,001$)

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	2,031	0,677	0,000271
BCP	3	1,044	0,348	0,000016
BCP+Etoposide	3	0,849	0,283	1,3E-05
Etoposide	3	0,921	0,307	0,000013

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,305144	3	0,101715	1299,869	4,32E-11	4,066181
Within Groups	0,000626	8	7,83E-05			
Total	0,30577	11				

Table B.2. ANOVA analyses for cytotoxicity assay of two day cultured MIAPaCa-2 cells ($p<0,001$)

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	1,68	0,56	5,2E-05
BCP	3	0,859	0,286333	0,000114
BCP+Etoposide	3	0,548	0,182667	1,73E-05
Etoposide	3	0,618	0,206	5,7E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,270251	3	0,090084	1497,235	2,46E-11	4,066181
Within Groups	0,000481	8	6,02E-05			
Total	0,270732	11				

Appendix C- t-test analyses for cytotoxicity assay of MIAPaCa-2 cells

Table C.1. t-test analyses for cytotoxicity assay of 1-day cultured MIAPaCa-2 cells (p<0,001)

t-Test: Paired Two Sample for Means

	Control	*BCP*
Mean	0,677	0,348
Variance	0,000271	0,000016
Observations	3	3
Pearson Correlation	0,030373	
Hypothesized Mean Difference	0	
df	2	
t Stat	33,87375	
P(T<=t) one-tail	0,000435	
t Critical one-tail	2,919986	
P(T<=t) two-tail	0,00087	
t Critical two-tail	4,302653	

t-Test: Paired Two Sample for Means

	Control	*BCP+Etoposide*
Mean	0,677	0,283
Variance	0,000271	1,3E-05
Observations	3	3
Pearson Correlation	0,210598	
Hypothesized Mean Difference	0	
df	2	
t Stat	42,40401	
P(T<=t) one-tail	0,000278	
t Critical one-tail	2,919986	
P(T<=t) two-tail	0,000556	
t Critical two-tail	4,302653	

t-Test: Paired Two Sample for Means

	Control	*Etoposide*
Mean	0,677	0,307
Variance	0,000271	0,000013
Observations	3	3
Pearson Correlation	-0,96875	
Hypothesized Mean Difference	0	
df	2	
t Stat	32,08307	
P(T<=t) one-tail	0,000485	
t Critical one-tail	2,919986	
P(T<=t) two-tail	0,00097	
t Critical two-tail	4,302653	

Table C.2. t-test analyses for cytotoxicity assay of 2-days cultured MIAPaCa-2 cells (p<0,001)

t-Test: Paired Two Sample for Means

	Control	*BCP*
Mean	0,56	0,286333
Variance	5,2E-05	0,000114
Observations	3	3
Pearson Correlation	-0,09078	
Hypothesized Mean Difference	0	
df	2	
t Stat	35,29755	
P(T<=t) one-tail	0,000401	
t Critical one-tail	2,919986	
P(T<=t) two-tail	0,000802	
t Critical two-tail	4,302653	

t-Test: Paired Two Sample for Means

	Control	*BCP+Etoposide*
Mean	0,56	0,182667
Variance	5,2E-05	1,73E-05
Observations	3	3
Pearson Correlation	2,82E-17	
Hypothesized Mean Difference	0	
df	2	
t Stat	78,49008	
P(T<=t) one-tail	8,11E-05	
t Critical one-tail	2,919986	
P(T<=t) two-tail	0,000162	
t Critical two-tail	4,302653	

t-Test: Paired Two Sample for Means

	Control	*Etoposide*
Mean	0,56	0,206
Variance	5,2E-05	5,7E-05
Observations	3	3
Pearson Correlation	0,936766	
Hypothesized Mean Difference	0	
df	2	
t Stat	231,7474	
P(T<=t) one-tail	9,31E-06	
t Critical one-tail	2,919986	
P(T<=t) two-tail	1,86E-05	
t Critical two-tail	4,302653	