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Anti-cancer Evaluation of Lebanese Cannabis Oil Extract on Lung and Breast Cancer Cells

BY

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Anti-cancer Evaluation of Lebanese Cannabis on Lung and Breast Cancer Cells

Jane Abdallah Saliba

ABSTRACT

Cannabis sativa L. is one of the oldest plants used in traditional medicine. Lebanese cannabis is recognized around the world for its quality and recreational use; however, little is known about its medicinal importance. Cannabis is a distinctive plant containing a variety of compounds most notably cannabinoids and terpenoids. Cannabis oil extract (COE) has been shown to possess anti-inflammatory and anticancer activities, which could be attributed to the interaction between terpenes and cannabinoids as "entourage effect". In this study, we look at the anti-tumorigenic effects of Lebanese COE on non-small cell lung cancer cell line (NSCLC) A549 and the triple negative breast cancer cell line (MDA-MB-231). GC-MS analysis of COE revealed CBD dominance (59.1%) followed by THC (20.2%). Cell viability was evaluated using MTS cell proliferation assay and COE demonstrated more selectivity against MDA-MB-231 (IC_{50} =26.7 μg/mL) and A549 (IC_{50} =30.2 μg/mL), compared to rat stem cells (IC_{50} =42.31 μg/mL). COE caused more significant cell migration inhibition in A549 cells than MDA-MB-231 cells. Western blot analysis showed significant increase in MAPK/ERK pathway and the autophagic marker LC3B. Cell cycle analysis by flow cytometry and western blot assay revealed a minor involvement of apoptosis in cell death. In conclusion, the results confirmed that COE has dose/time dependent effect and is effective in inhibiting cancer cell migration and inducing autophagy. These results necessitate further studies to better

understand the complex cell-death mechanisms of COE and provide supportive evidence for its use in cancer treatment.

Keywords: Entourage effect, Breast cancer (MDA-MB-231), Lung cancer (A549), Cannabis, Cannabinoids.

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List of Abbreviations

2-AG	2-arachindonoylglycerol
AEA	anandamide
AMPK	adenosine monophosphate-activated protein kinase
ATG	autophagy related genes
CB	cannabinoid
CBC	cannabichromene
CBD	cannabidiol
CBG	cannabigerol
CBL	cannabicyclol
CBN	cannabinol
COE	cannabis oil extract
COX-2	cyclooxygenase-2
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERK	extracellular-regulated kinase
FAAH	fatty acid amide hydrolase
FDA	Food and Drug Administration
GPR55	G-protein coupled receptor 55
JNK	c-Jun N-terminal kinase
LIR	LC3 interacting region
MAGL	monoacylglycerol lipase

MAPK	mitogen activated protein kinases
MOMP	mitochondrial outer membrane permeabilization
mTOR	mammalian target of rapamycin
PCD	programmed cell death
PE	phosphatidylethanolamine
PI3K	phosphatidylinositol 3 kinase
PPAR	peroxisome proliferator-activated receptors
ROS	reactive oxygen species
SQSTM1	sequestosome 1
THC	Δ^9-tetrahydrocannabinol
TRAIL	Tumor necrosis factor (TNF)-related apoptosis-inducing ligand
TRIB3	tribbles homolog 3
TRPV1	transient receptor potential cation channel subfamily V member 1
UBD	ubiquitin binding domain

Chapter One

General Introduction

1.1. History of Cannabis as a Medicinal Plant

The history of cannabis dates to the ancient world with its medicinal use going back about 5000 years ago. In the 28th century B.C, the benefits of cannabis were praised by the Chinese Emperor Shen Nung, as having healing powers for diseases like rheumatism, gout, and malaria (Bridgeman & Abazia, 2017; Wilkie et al., 2016). Furthermore, cannabis seeds, specifically *C. sativa*, were used by Chinese physicians for their richness in γ-linoleic acid. The seeds were prescribed for eczema and psoriasis topically, and orally for inflammatory diseases (Bonini et al., 2018). From the Christian Era to the 18th century, the medical use of cannabis was concentrated in India; it later spread to the Middle East and Africa. In Arabia, many renowned physicians mentioned cannabis in their medical compendiums, such as Avicena, in the year 1000 A.D (Nahas, 1982; Zuardi, 2006). Besides, evidence dating back to 3000 years ago, on the use of cannabis was found on Assyrian clay tablets and the Egyptian Ebers Papyrus. In ancient Greece and the Roman Empire, the use of cannabis was documented as well. The Roman historian Pliny the Elder reported the use of *C. sativa* roots for pain while a Greek physician, Pedacius Dioscorides, author of The *Materia medica*, included *C. sativa* in his classification of medicinal plants and described its benefits (Bonini et al., 2018; Lozano, 2001). Fast forward to the 19th century, British surgeon W.B.O'Shaughnessy introduced

C. sativa as an anti-inflammatory, anti-convulsant, analgesic, and anti-emetic, to Western medicine (Bonini et al., 2018; Wilkie et al., 2016). During this same time, Jacques-Joseph Moreau, a French psychiatrist, found that cannabis decreased headaches, helped with insomnia, and improved appetite. Many of these findings contributed to the introduction of cannabis to the US Pharmacopeia in 1850 (Wilkie et al., 2016). In the 20th century, a lot of controversies revolved around the use of cannabis mainly due to its psychoactive effects and it was banned in many countries such as the UK and the USA (Bonini et al., 2018). Nowadays, cannabis research is advancing, and evidence of its therapeutic advantages is changing the way it is consumed, used, and perceived by both patients and health care providers.

Figure 1.1. (A) Miniature from the late 12th century currently found in the British Library's collection of Ourscamp monastery, (B) Emperor Chen Nung illustration containing the word "Cannabis sativa" in Chinese, (C) Seshat the Egyptian goddess of architecture, writing, wisdom, and knowledge depicted with what is thought to be a cannabis leaf over her head (Bonini et al., 2018). Modified from Bonini et al., 2018.

1.2. Cannabis in Lebanon

Cannabis cultivation existed in Lebanon since Roman times. A cannabis leaf is found engraved on the ruins of an ancient Roman temple in Baalbek. For decades, the plant was grown unlawfully in the Beqaa valley. In April 2020, the Lebanese Parliament passed a bill legalizing the cultivation of cannabis for medical and industrial use. This decision made Lebanon the first Arab country to legalize cannabis growing and it is thought to be an incentive to boost the economy (Lebanese Official Gazette; issue 23; 2020).

1.3. The Cannabis Plant

Cannabis, an aromatic annual herbal plant of the Cannabaceae family, has been known since antiquity and studied for its therapeutic benefits. There are three main types of *Cannabis* plants*: C. sativa*, *C. indica*, and *C. ruderalis*. All three species differ in terms of height and composition (Bonini et al., 2018; Hartsel et al., 2016). The major strains cultivated in Lebanon are *C. sativa* L.ssp. sativa, *C. sativa* L. ssp. indica (Lam.), and a dominant hybrid strain. Phytochemical analysis of the cannabis plant demonstrated the abundance of terpenes, phenolic compounds, and cannabinoids (McDonald and Gough, 1984).

1.3.1. Biology of the plant

C. sativa is mostly dioecious with stems that could reach around 5 m in length and green, palmate leaves composed of five to seven leaflets. The male and female plants have small differences in their morphologies and are thus identifiable. Furthermore, the

cannabis plant is rich in trichomes, small glandular bulges covering the leaves, stems, and bracts of the plant, and containing secondary metabolites such as phytocannabinoids and terpenoids. Phytocannabinoids are involved in the plant's defense mechanisms while terpenoids are responsible mostly for the plant's smell. Cannabis continuously produces new leaves, branches, and nodes, in the pre-flowering phase. Both the plant appearance and composition vary depending on the environmental conditions and geographical region (Bonini et al., 2018; Farag & Kayser, 2017).

Figure 1.2. Trichomes in *Cannabis sativa* L. (Bonini et al., 2018). Modified from Bonini et al., 2018.

1.3.2. Chemical aspect

1.3.2.1. Phytocannabinoids

Phytocannabinoid composition is influenced by extreme environmental conditions (ie, humidity, radiation, temperature, parasites, and soil nutrients). To date, more than 100 different phytocannabinoids have been identified and share a similar chemical structure composed of a lipid structure with alkylresorcinol and monoterpene moieties. Phytocannabinoids are mostly found in the resin secreted by the trichome of the female plants. Cannabinoids are synthesized and stored as acids (with a carboxyl group), and later decarboxylated into the known neutral forms (Bonini et al., 2018). Among the herbal cannabinoids, Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most abundant and most studied. Other cannabinoids are present to a lesser extent; however, they are recently gaining more attention such as cannabinol (CBN), a degradation derivative of THC, cannabichromene (CBC), and cannabigerol (CBG). THC is the primary psychoactive component of the cannabis plant and to a lesser extent CBN. CBD, CBG, and CBC are devoid of psychoactive potential (Javid et al., 2016). Cannabicyclol (CBL) occurs during the storage of *C. sativa* in the presence of light, however, its biological properties are still unknown (Bonini et al., 2018).

1.3.2.2. Terpenoids

Terpenoids are chemical compounds mainly responsible for the fragrance of the cannabis plant, they also act as defenses against many predators along with phytocannabinoid acids with which they have a synergistic mechano-chemical protective approach. Over 200 terpenoids have been identified and they comprise up to 10% of the trichome contents in leaves and flowers. Among those compounds, pinene, limonene and

myrcene are the most abundant. Terpenoids exhibit many pharmacological properties such as anti-inflammatory, analgesic, anti-nociceptive, anti-platelets, and sedative (Bonini et al., 2018; Tomko et al., 2020).

1.4. The Endocannabinoid System

The endocannabinoid system is comprised of the cannabinoid receptors CB1 and CB2, both being G protein-coupled receptors (GPCRs), N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), the main endocannabinoids intrinsic ligands, and specialized enzymatic machinery (fatty acid amide hydrolase FAAH, monoacylglycerol lipase MAGL). AEA and 2-AG are derived from the non-oxidative metabolism of membrane phospholipids. CB1 and CB2 are the most known targets of endocannabinoids, however, other receptors may play a role. As an example, AEA may activate the potential vanilloid receptor type 1 (TRPV1); also, G protein-coupled receptor 55 (GPR55), and the peroxisome proliferator-activated receptor (PPAR) (Massi et al., 2013; Afrin et al., 2020). Three main categories of cannabinoid receptor ligands exist and are known as phytocannabinoids, endogenous cannabinoids, and synthetic cannabinoids. Their effects are mediated by the activation of either CB1 or CB2 receptors or both. CB1 receptors are found mainly in the brain and nervous system, whereas CB2 receptors are expressed predominantly by immune and hematopoietic cells. The psychoactive effects of THC are due to its interaction with the CB1 receptors, whereas its immune-modulatory activities are mostly related to its interaction with the CB2 receptors. In contrast, CBD has a low affinity to both CB1 and CB2 (Seltzer et al., 2020). CB1 and CB2 receptors have been found to be expressed in many cancer types and are nowadays considered to be

responsible for most of the cannabinoids' physiological effects (Shrivastava et al., 2011). The anti-cancer mechanisms of cannabinoids are yet to be fully uncovered, however, most studies suggest that THC's anti-cancer effects are mostly CB receptor-dependent while the non-THC cannabinoids, such as CBD, are mostly CB receptor-independent. Nonetheless, significant crosstalk exists between GPCRs, mainly CB1 and CB2, and non-GPCR signaling pathways in inducing anti-cancer mechanisms (Afrin et al., 2020).

1.5. The "Entourage Effect"

The assemblages of the phytocannabinoids and terpenoids are substantial for cannabis therapies since whole cannabis extracts have been found to be more active than single, purified phytocannabinoids (Russo, 2019). The use of the crude extract of cannabis has been shown to relieve chronic pain in humans and animals, as well as enhance cytotoxic activity against cancer cell. This phenomenon has been termed "the entourage effect", as it originates from the synergistic interactions between different cannabis compounds, specifically cannabinoids, and terpenoids (Gallily et al., 2018). These phytochemicals are known for their medicinal properties, including anti-inflammatory and anticancer activities (Namdar et al., 2019). In a published study, Blasco-Benito et al. (2018) confirmed the benefit of using whole cannabis extract, in comparison to pure cannabinoids, on breast adenocarcinoma cell lines. The suggested synergistic effect between the different cannabis compounds makes it evident and crucial to study the anti-cancer effects of the crude oil. The combined use of THC and CBD showed superior benefits compared to pure compounds. The psychotropic effects of THC were reduced, and the anticancer activity of THC was enhanced in the presence of CBD. This

combination helped in limiting the doses of THC needed to prevent tumor growth and, hence, improve the tolerance to cannabis-based medications (Blasco-Benito et al., 2018; Tomko et al., 2020; Baram et al., 2019; Milian et al., 2020). It also induced apoptosis and reduced cell viability and migration in human glioblastoma (Milian et al., 2020).

1.6. Cancer

1.6.1. Lung cancer

Lung cancer is the most diagnosed cancer and is the leading cause of cancer death worldwide. Non-Small-Cell Lung Cancer (NSCLC), which constitutes around 80-85% of all lung cancer types, can be further divided into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma, with adenocarcinoma being the most prevalent (Hoenerhoff et al., 2009). Most NSCLC patients develop resistance to chemotherapeutic agents and endothelial growth factor receptor (EGFR) inhibitors. This is possibly due to EGFR overexpression and abnormal signal transduction. EGFR is known to modulate tumor cell proliferation and subsequently lead to tumor growth (Milian et al., 2020). The expression of CB1 and CB2 receptors has been reported in NSCLC patients and the NSCLC cell lines A549 as well as the TRPV1 receptors (Ramer et al., 2012; Laezza et al., 2020). According to Preet et al. (2011), CB1 was found in 24% of samples examined while CB2 in 55% of the samples. These findings allow for cannabis-based medicine to be a potential candidate in treating this type of cancer.

1.6.2. Breast cancer

Breast cancer is the most frequently diagnosed and the second leading cause of death in women worldwide. The most aggressive type is Triple-Negative Breast Cancer

(TNBC), which is negative for the Estrogen Receptor (ER), Human Epidermal Growth Factor Two (HER2), and Progesterone Receptor (PR), making nonspecific and cytotoxic chemotherapy the most common treatment option (Sultan et al., 2018). The cannabinoid receptors CB1 and CB2 were detected in many breast cancer cell lines including MDA-MB-231. In breast cancer, the tumor aggressiveness is related to the high expression of CB2 while CB1 expression is moderate. Furthermore, overexpression of GPR55 receptors was also reported (Kisková et al., 2019; Chakravarti & Ganju, 2014; Velasco et al., 2016; Laezza et al., 2020).

1.7. Anti-cancer Mechanisms

In general, a variety of proposed anti-cancer mechanisms of action are displayed, but not limited to induction of apoptosis, autophagic cell death, cell cycle arrest, inhibition of migration, invasion, and metastasis (Ramer et al., 2012; Seltzer et al., 2020).

1.7.1. Apoptosis

Apoptosis is a type I programmed cell death (PCD). PCD is a critical process that ensures tissue homeostasis and development by protecting the body against damaged cells which can become carcinogenic (Shrivastava et al., 2011). Apoptosis is usually divided into intrinsic and extrinsic pathways. Caspases are proteases that play a crucial role in apoptosis. They are classified as initiator caspases such as caspase-8 and -9 and executioner caspases such as caspase-3, -6, and -7. The extrinsic pathway is essentially driven by caspase 8 while the intrinsic pathway is characterized by the mitochondrial release of Cytochrome c and mediated by caspase 9. Initially, the initiator pro-caspases are enzymatically cleaved to active caspases which will subsequently cleave the

executioner pro-caspases in turn to active caspases and thus promoting apoptosis. Both the extrinsic and intrinsic pathways join to activate specifically caspase 3 and subsequently 7. The Bcl2 family of proteins usually mediates the cell's decision to undergo mitochondrial outer membrane permeabilization (MOMP) and resulting in apoptosis. The Bcl-2 family is divided into pro-apoptotic and anti-apoptotic proteins with BAX being pro-apoptotic and Bcl-2 being anti-apoptotic (Tompkins & Thorburn, 2019).

1.7.1.1. MAPK/ERK and Apoptosis

The mitogen-activated protein kinase (MAPK) signaling pathway is implicated in the regulation of a variety of biological processes including apoptosis (Yue & López, 2020). MAPKs are divided into three subcategories: Jun N-terminal kinase (JNK), p38, and extracellular-regulated kinase (ERK). MAPK/ERK is a chain of signaling protein kinases considered to be downstream of different transmembrane receptors such as EGFR and unfold diverse regulatory mechanisms implicated in cellular processes such as apoptosis and proliferation. The MAPK/ERK pathway is known as the Ras-Raf-MEK-ERK pathway. The extracellular ligand binds to the designated membrane receptor and triggers a cascade. It is initiated first by the conversion of GDP to GTP by Ras. GTP will then activate the Raf kinase which will phosphorylate and activate the MEK1 and MEK2 tyrosine/threonine kinases (or MAPKK). Subsequently, MAPK/ERK1/2 will be activated and ERK 1/2 will dissociate and thus regulate multiple transcription factors such as c-Jun, c-Myc, NF-kB, and AP-1 (Harvey, 2019; Lee et al., 2021).

1.7.2. Autophagy

More recently, autophagy was considered as a type II PCD, where cellular components are engulfed and destroyed inside newly synthesized, double membrane-

enclosed vesicles called "autophagosomes" (Seltzer et al., 2020; Shrivastava et al., 2011; Ramer et al., 2012). Autophagy is usually involved in the cell's recycling system, where damaged cellular material especially proteins and organelles, are degraded in lysosomes and parts of them are reused, to maintain cellular homeostasis (Tompkins & Thorburn, 2019). The process of autophagy is divided into two pathways: microautophagy and macroautophagy, which are later divided into canonical and non-canonical forms. The differences between those pathways are in the transport of the degradation material, their types, and regulation. It is in macroautophagy that we find the formation of autophagosomes. The autophagy-related genes (ATG) and their affiliated pathways are implicated in autophagy. At the cellular level, pro-LC3 is cleaved by Atg4 protease, resulting in the formation of LC3-I which is conjugated by Atg7, Atg3, and Atg12-Atg5-Atg16L multimers to phosphatidylethanolamine (PE) moiety for the generation of LC3-II form. This will add a lipophilic character to LC3-II enabling its insertion into the autophagosome membranes and its degradation when the autophagosomes fuse with lysosomes. LC3B-II is especially important since it is the only well-defined protein throughout the entire process of autophagy (Tanida & Kominami, 2008). In contrast, p62 is considered a scaffold protein as well as an autophagy receptor. The numerous binding domains of p62 such as ubiquitin-binding domain (UBD) and LC3-interacting region (LIR), enable it to recognize the cargoes that need degradation and subsequently interact with the autophagosomes to deliver the material that will be lysed by lysosomes. Moreover, p62 also known as sequestosome 1 (SQSTM1) can interact with other effector proteins like beclin-1. Beclin-1 is a critical autophagy effector that has vital roles in the crosstalk with the apoptosis pathway (Kang et al., 2011). In addition, a potential modulator

between apoptosis and autophagy is p62. It was found to be linked to apoptosis through the activation of caspase 8 in the extrinsic pathway (Islam et al., 2018; Su et al., 2013).

1.7.2.1. Possible signaling pathways in autophagy

Phosphatidylinositol 3-kinase (PI3K)/AKT and the mammalian target of rapamycin (mTOR) are two distinct pathways that are highly interconnected to the point where they are regarded as a single pathway. They are crucial for cell growth and survival in both physiological and disease states (Harvey, 2019). Among the multiple signaling pathways that can activate autophagy when subjected to cannabinoid treatment, is the PI3K/AKT/mTOR. Inhibition of this pathway leads to the induction of autophagy and has an anti-tumorigenic action. Another potential pathway for autophagy regulation is the adenosine monophosphate-activated protein kinase (AMPK). Under stressful situations, AMPK promotes autophagy by inhibiting mTOR (Calvaruso et al., 2012; Lee et al., 2021). Also, an interesting pathway is through EGFR and MAPK/ERK signaling. In EGFR mutated tumors, there is an increased level of MAPK/ERK signaling pathway proteins leading to autophagy induction. With autophagy being upstream of apoptosis, effective cell death is attained as it was shown to cause cancer cell death in apoptotic deficient cancers (Sooro et al., 2018). Although the regulation of autophagy through the MAPK pathway is indirect, ERK has shown to be implicated in the induction of autophagy in response to certain anti-tumor/cytotoxic agents. ERK also appears to be implicated in mitochondrial autophagy known as mitophagy and can protect cells from apoptosis (Sridharan et.al, 2011).

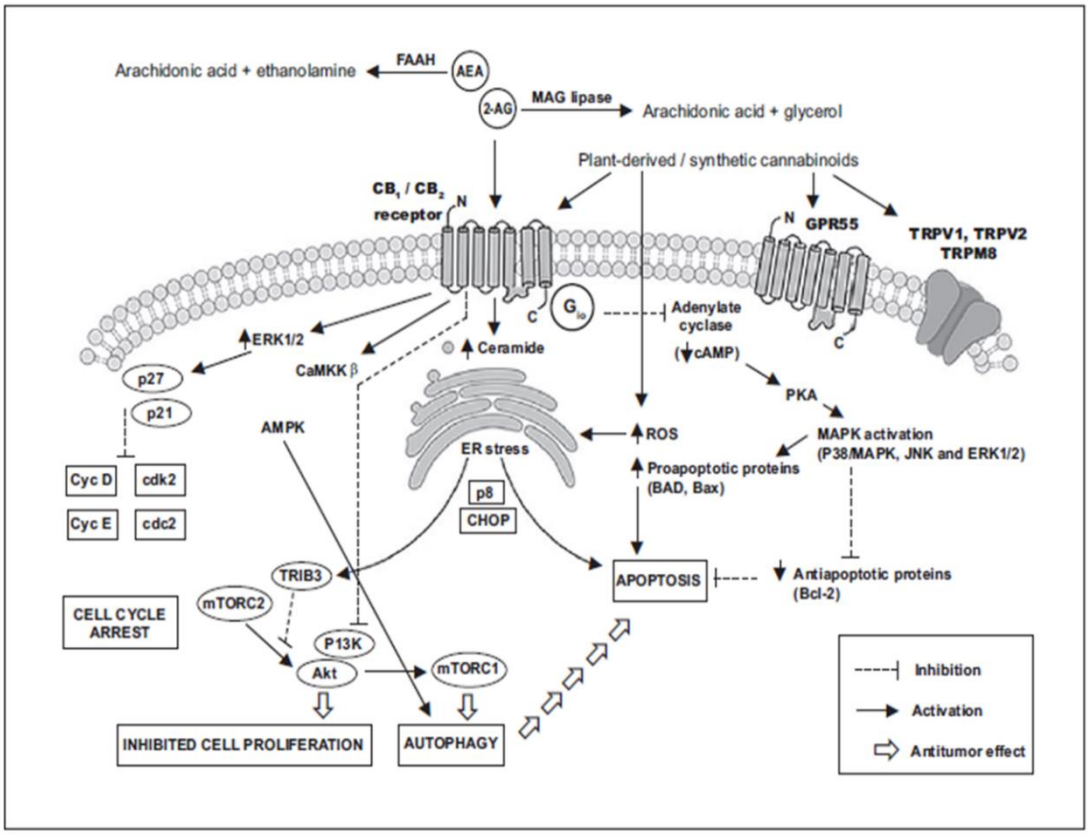


Figure 1.3. Schematic representation of the main signaling pathways downstream of CB receptor activated by cannabinoids (Daris et al., 2019).

1.7.3. The interplay between autophagy and apoptosis

Apoptosis and autophagy have a controversial relationship where they may coexist, cooperate, or antagonize each other to balance survival versus death signaling. Several injuries (i.e, calcium imbalances, oxidative stress) will cause stress to the endoplasmic reticulum (ER) and might activate either autophagy, or apoptosis, or both. This complex interplay makes it difficult to distinguish between the two mechanisms because some stimuli may activate both mechanisms and thus the pathways undertaken are purely dependent on the cell type (Calvaruso et al., 2012; Kang et al., 2011). In a study done on A549 cells treated with resveratrol, cell death occurred in the absence of apoptosis

and due to an increase in autophagic flux (Jung et al., 2020). The inhibition of apoptosis by autophagy might be due to multiple mechanisms one of which is mitophagy. Mitophagy is the selective breakdown of the mitochondria by autophagy. A damaged mitochondrion is usually prone to activate apoptosis, however, its removal by mitophagy might increase the apoptotic induction threshold. Furthermore, autophagy can also decrease the abundance of pro-apoptotic proteins in the cytosol. In colon cancer cells, autophagy facilitated the selective removal of active caspase 8 rendering the cells resistant to TRAIL unless autophagy is inhibited (Mariño et al., 2014). The exact mechanism of apoptosis inhibition is not clear; however, it may be attributed to unregulated autophagy as an anti-injury mechanism to clear apoptotic cells. Beclin-1 is thought to function as an anti-apoptotic protein in numerous settings including chemotherapy, TRAIL, immunotherapy, and irradiation (Kang et al., 2011).

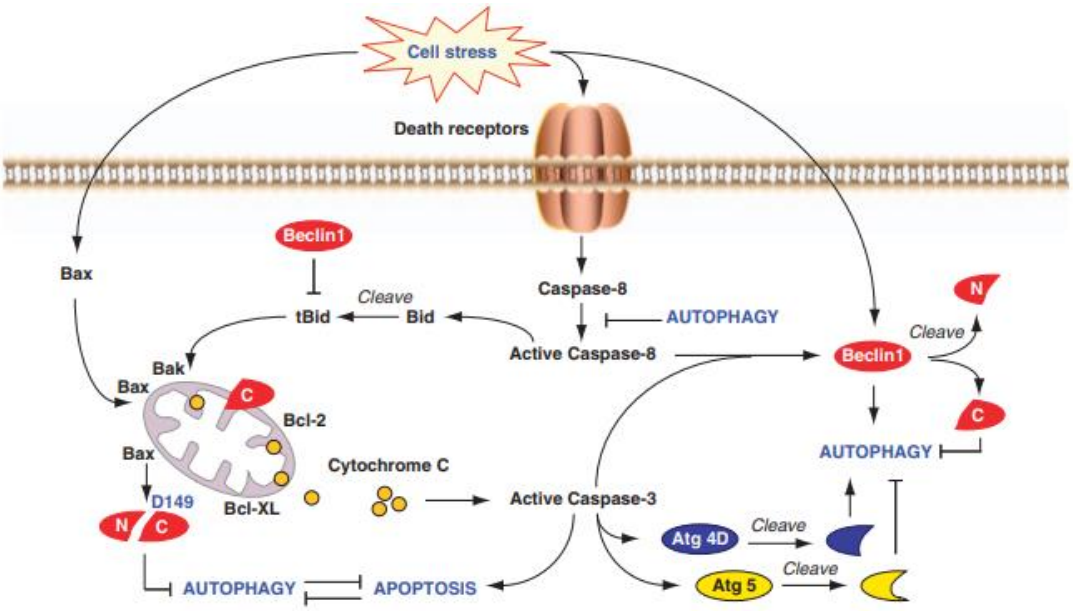


Figure 1.4. Autophagy and Apoptosis crosstalk (Kang, et al., 2011).

1.8. Anti-cancer Mechanisms of Cannabinoids

Extensive in-vitro and in-vivo research on cannabis and its cannabinoids found that the administration of cannabinoids, such as THC, and CBD, had a selective anti-cancer activity in a broad range of cancer cell lines (Afrin et al., 2020). The use of cannabis oil containing the mixture of cannabinoids and terpenoids is considered beneficial and works by blocking cell cycle progression, cell growth and induce cancer cell apoptosis and autophagy (Kisková et al., 2019). The cancer cells follow different pathways that lead to cell death, most of which are cell line specific. Several studies have shown the contribution of COX-2 and PPAR-γ to CBD's tumor-regressive and proapoptotic action in A549 cells (Chakravarti & Ganju, 2014; Ramer et al., 2012). According to Ramer et al, (2013), PPAR-γ activation plays a crucial role in the induction of apoptosis in different tumor cells such as NSCLC cells, highlighting one of the important CB receptors-independent pathways. Such findings further emphasize that the molecular targets of CBD are partly dependent on the cancer cell type present and the receptors expressed (Afrin et al., 2020; Laezza et al., 2020). In MDA-MB 231 cell lines, cell type-dependent mechanisms of action were also demonstrated. CBD was shown to induce oxidative stress leading to apoptosis by activation of CB2 and TRPV1 receptors (Javid et al., 2016). Furthermore, both CBD and THC were found to induce apoptosis and autophagy in breast cancer cells (Seltzer et al., 2020; Salazar et al., 2009). Moreover, activation of the CB receptors triggers the induction of the MAPK/ERK pathway, leading to an increase in ROS which subsequently leads to cell cycle arrest and thus apoptosis (Afrin et al., 2020).

In many cultured cancer cells, cannabinoids induced autophagy, and any attempts to inhibit this mechanism resulted subsequently in preventing cannabinoids antitumoral mechanisms. The anticancer mechanism of cannabinoids relies largely on the capacity of these molecules to induce autophagy-mediated apoptotic cancer cell death with autophagy possibly being the general mechanism leading to cancer cell death. This proves that autophagy is upstream of apoptosis in the mechanism of cannabinoid-induced cell death. Additional mechanisms (mostly cell-specific) may collaborate with autophagy to further promote cancer cell death. One way THC can induce autophagy in a CB receptor-dependent manner is by causing ER stress which will up-regulate tribbles homolog 3 (TRIB3), known to inhibit the AKT/mTORC1 axis, thus promote autophagy (Das et.al., 2019; Velasco et al., 2016; Śledziński et al., 2018; Afrin et al., 2020). THC did not show any cytotoxic effects on normal cells and it could be considered to preferentially target cancer (Afrin et al., 2020; Fluda, 2017). In MDA–MB 231 cell line, CBD induced ER stress, inhibition of AKT/mTOR pathway, and thus the up-regulation of autophagy-mediated cell death (Calvaruso et al., 2012; Dariš et al., 2019; Shrivastava et al., 2011). In addition, cannabinoids deprived of psychoactive properties displayed anti-cancer activity mostly independently from CB receptors. It has been noted that the action of CBD on other receptors (TRPV1, GPR55), had a more significant role (Śledziński et al., 2018). The anti-cancer activity of cannabinoids was proven decades ago, however, the underlying signaling pathways of cancer cell death have remained uncertain and necessitate more studies (Lee et al., 2021).

1.9. Effect of Cannabinoids on Cell Migration

Cancer cell migration and invasion are characteristic elements that will lead to metastasis and thus reduce patient survival. Cancer patient survival is mostly determined by tumor spread, and invasion of cancer cells is usually mediated by tumor cell motility, thus inhibiting migration will increase patient survival by decreasing metastasis. Angiogenesis is also known to be linked to cancer metastasis. The restricted efficacy of chemotherapy is demonstrated in many cancer types and there is a need to develop new complementary drugs that would be able to target migration, invasion, and metastasis (Chakravarti & Ganju, 2014). CB receptor agonists regulate crucial signaling pathways inhibiting cancer cell migration and invasion. The major pathways include MAPK/ERK, PI3K, and ceramide (Milian et al., 2020). Usually, the activation of ERK promotes cell growth, however, according to McAllister et al, (2011), the activation of ERK through the MAPK inhibits the growth of cancer cells depending on the duration of the stimulus. The consistent upregulation of ERK will lead to inhibition of cell growth. EGFR is also an important player in cancer cell migration. The over-activity of EGFR is a characteristic of NSCLC and when stimulated by cannabinoids such as THC, EGFR is known to activate the MAPK/ERK pathway (Gao et al., 2005; Milian et al., 2020). Other crucial receptors, independent of CB receptors play a role in the cannabinoid effects on cancer cell migration such as TRPV1 and GPR55, and eventually leading to the phosphorylation of ERK (Afrin et al., 2020). Also, studies confirmed that cannabis oil extract diminishes angiogenesis and tumor metastasis in animal breast cancer models (Kisková et al., 2019).

1.10. Rationale, Aim, and Objectives

Cannabis is a reputable plant known for its history of both medicinal and recreational use. In recent years, it has been gaining increasing acceptance among physicians and the public, with many countries legalizing its use. Although cannabinoids have a favorable safety profile and have proven to be effective, studies are still needed, especially in oncology, to enable their approved clinical use (Wilkie et al., 2016; Bridgeman & Abazia, 2017). According to the United Nations' National Report on the Drug Situation in Lebanon (2017), Lebanese cannabis is known to be of high quality due to the geographical location of the country, providing a favorable environment for cannabis cultivation. With Lebanon being one of the largest global suppliers of cannabis resins mostly for recreational use, little is known of its medicinal benefit. Studies on Lebanese cannabis are very scarce (Bercht et al., 1974; McDonald and Gough, 1984; Ohlsson et al., 1971; Valle et al., 1968), which gives an incentive to investigate the plant and learn more about its potential therapeutic values. Furthermore, people specifically in the Bekaa valley, use the plant for several diseases including cancer. In January 2021, a research study was conducted depicting the phytocannabinoid content of the Lebanese cannabis plant and it was shown to be rich in CBD, a molecule highly appreciated for its therapeutic benefits especially in the cancer field (Shebaby et al., 2021). The different phytocannabinoid percentages in the extract, elucidate the "entourage effect" and contribute to the anti-cancer activity, making the Lebanese cannabis plant an attractive target for investigation. With cancer being one of the most serious medical problems and responsible for millions of deaths worldwide, the need for new therapies is rising. Cannabis is well known for its palliative effects in oncology, however, nowadays, it is studied for its anti-cancer activity as cannabinoids are usually well-tolerated and do not

have the common toxic effects of conventional chemotherapies (Javid et al., 2016; Tomko et al., 2020).

The present study aims to investigate the anti-cancer activity of Lebanese *Cannabis* oil extract (COE) in-vitro against human lung adenocarcinoma (A549) and TNBC (MDA-MB-231) and highlight the different anti-cancer mechanisms present.

1.10.1. Specific objectives

The specific objectives of this study are as follows:

- To extract the crude oil from the Lebanese cannabis flower sample using ethanol which is the traditional extraction method used in Lebanon.
- To identify the major compounds of the COE by GC/MS method.
- To assess the *in vitro* cytotoxic effects of the COE on MDA-MB 231 and A549 cell lines using the MTS cell proliferation assay.
- To evaluate the *in vitro* effect of COE on MDA-MB 231 and A549 cancer cell migration using "wound healing" assay.
- To conduct western blot analysis to elucidate the possible anti-cancer mechanisms of COE on MDA-MB 231 and A549 cell lines, including MAPK/ERK, apoptotic and autophagic markers.
- Flow cytometry assessment of cell death using annexin/7-AAD staining to evaluate the apoptotic involvement in the anti-cancer mechanisms of cell death of A549 cells.

Chapter Two

Materials and Methods

2.1. Chemicals and Reagents

Primary Rabbit antibodies (LC3B, p62, β-Actin, BAX, Bcl-2, Cytochrome c, ERK, and pERK), as well as horseradish peroxidase (HRP)-coupled secondary antibodies were purchased from Abcam (Cambridge, MA, USA). Primary Rabbit antibodies (cleaved Caspase-3) was purchased from Cell Signaling Technology (Danvers, MA, USA). Fetal bovine serum (FBS), penicillin-streptomycin, 2X Laemmli buffer solution, acrylamide (30%), blotting pads (9x10.5 cm), PVDF membranes, Precision Plus Protein ladder, Bio-Rad Protein Assay, and enhanced chemiluminescence (ECL) substrate kit were purchased from Bio-Rad (Hercules, CA, USA). Dimethyl sulfoxide (DMSO), glycine, Tris-base, Tris-HCl, NaOH, NaCl, ammonium persulphate, 2-mercaptoethanol, 2-propanol, methanol, Tween 20, trypan blue, sodium dodecyl sulfate (SDS), well plates, Dulbecco's phosphate-buffered saline (PBS), (with $MgCl_2$ and $CaCl_2$ and without $MgCl_2$ and $CaCl_2$), bovine serum albumin (BSA), TEMED, Trypsin EDTA with phenol red 1X, RPMI 1640 culture medium (with 20 mM HEPES/ L-glutamine/ without sodium bicarbonate/ liquid, sterile-filtered), Dulbecco's modified Eagle's medium (DMEM), (with 4.5 g/L glucose/ L-glutamine/ sodium bicarbonate/ sodium pyruvate/ liquid, sterile-filtered), Dulbecco's modified Eagle's medium/Nutrient mixture F-12 Ham (with 15 mM HEPES/ sodium bicarbonate/ L-glutamine/ phenol red/ liquid, sterile-filtered) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Guava® Nexin Reagent mix-and-read assay kit was acquired from Luminex (Austin, TX, USA). Etoposide Mylan 20 mg/mL was provided

from Benta Pharma Industries (Dbayeh, Lebanon). Phenazine metho sulphate (PMS) and cell proliferation assay MTS reagent were acquired from Acros Organics Fisher Scientific (Geel, Belgium). The GCMS solvents: dichloromethane (DCM), ethyl acetate, and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.1. Cell lines

The two human epithelial cell lines A549 from a 58-year-old male lung carcinoma, and MDA-MB-231 from a 51-year-old female breast adenocarcinoma were purchased from ATCC (Gaithersburg, MD, USA).

2.1.2. Solutions and buffers

The following solutions and buffers were prepared:

- Buffer for separating gel (500 mL): 90.75 g Tris-base, 500 mL distilled H_2O, pH=8.8
- Buffer for stacking gel (200 mL): 12 g Tris-base, 200 mL distilled H_2O, pH=6.8
- Ammonium persulphate (APS 10%): 10g ammonium persulphate, 100 mL distilled H_2O
- Sodium dodecyl sulfate (SDS 10%): 10g sodium dodecyl sulfate, 100 mL distilled H_2O
- Separating gel (10%), for 2 gel: 6 mL distilled H_2O, 4.95 mL acrylamide (30%), 3.75 mL buffer (pH=8.8), 150 µL SDS (10%), 150 µL APS (10%), 7.5 µL TEMED
- Stacking gel (5%), for 2 gel: 2.8 mL distilled H_2O, 850 µL acrylamide (30%), 1.25 mL buffer (pH=6.8), 50 µL SDS (10%), 50 µL APS (10%), 5 µL TEMED
- Running buffer 5X (1 L): 15 g Tris-base, 75 g glycine, 25 mL SDS (20%), pH=8.3

- Tris Base Saline TBS 10 X (1 L): 24.2 g Tris-base, 80 g NaCl, 25 mL SDS (20%), pH=7.6
- Washing buffer/TBST (400 mL): 360 mL distilled H_2O, 40 mL TBS (10X), 0.1% Tween 20
- Transfer buffer 5X (500 mL): 7.6 g Tris-base, 37.5 g glycine, pH=8.5
- Transfer buffer 1X (100 mL): 20 mL Transfer buffer (5X), 60 mL distilled H_2O, 20 mL methanol
- Blocking buffer (5%): 5g bovine serum albumin (BSA) in 100 mL TBST

2.2. Plant Collection and Oil Extraction

The dried samples of Lebanese cannabis were obtained through the Drug Enforcement Office in Zahle, Beqaa Governorate. The samples were originally cultivated in Yammoune, Beqaa valley (Yammoune:34◦07′46.4″ North, 36◦01′40.8″ East; altitude, 1375 ± 10 m) in October 2019. On campus, the plant samples were stored in a secured facility. A 10g, air-dried, cannabis flower sample was extracted with ethanol for 48 h. The extract was filtered and concentrated at 45◦C under reduced pressure to yield 1.17 g of cannabis oil extract (COE).

2.3. Gas Chromatography and Mass Spectroscopy (GC-MS) Analysis

For compounds identification, GC-MS analysis was performed using a Shimadzu GCMS-QP2020NX. The GC-MS contained an AOC-20i/s liquid autosampler, and an HP5MS Restek separation column was used, (0.25mmX30m, 0.25 µm film thickness).

Helium was the carrier gas of choice. Three solvents (DCM, Ethyl acetate, and methanol), were used to wash the injection needle before and after each run. The injection volume was 1 µl with a split ratio equal to 6.6. The column flow was 1.3 $ml.min^{-1}$ while the injector temperature was set at 280°C during the whole experiment. The total run time was 100 minutes, with the oven temperature set first at 60°C, then heating it to 240°C with a ramp of 3°$C.min^{-1}$. The temperature was held for 5 minutes then it was increased to 280°C with a ramp of 5°$C.min^{-1}$ and kept for 15 minutes. Finally, the temperature was increased to 290°C with a ramp of 15°$C.min^{-1}$ and it was maintained for 10 minutes. The ion source temperature was 220°C and the transfer line temperature was 280°C. A 2.10^2 V detector voltage and a 70-eV electron impact ASSP scan mode were used. The scan range was from 41.00 to 500.00 m/z. All samples were evaluated without dilution.

2.4. Cell Survival Assay

2.4.1. Stem cells extraction

Stem cell extraction was performed using rat bone marrow. Briefly, 12 weeks old rats were sacrificed with chloroform anesthesia. The femur and tibia bones were aseptically recovered, the bone marrows were flushed out with Dulbecco's Modified Eagle's Medium DMEM (10% FBS and 1% Penicillin/Streptomycin) and the collected cells were allowed to adhere in a humidified chamber at 37 °C and 5% CO2. After 5 days, the culture medium was replenished, and the cells were grown for 2-3 weeks before plating. Rat stem cells were extracted from the bone marrow.

2.4.2. Plating and treatment

Stem cells were seeded at $5x10^4$ cells/mL in a 96 well plate. Each well contained 100 µL of DMEM medium and cells were allowed to adhere overnight (37 °C; 5% CO2). The stem cells were later treated with the following concentrations of 5, 10, 20,40, and 80 µg/mL of cannabis oil extract (COE). A549 and MDA-MB-231 cells were seeded at $5x10^4$ cells/mL and 10^5 cells/mL respectively in 96-well plates. A549 in complete RPMI medium (10% FBS and 1% Penicillin/Streptomycin), and MDA-MB-231 in complete DMEM-high glucose medium (10% FBS and 1% Penicillin/Streptomycin) and were allowed to adhere overnight in a humidified chamber (37 °C; 5% CO2). In one experiment, both cell lines were treated with etoposide as follows: 2.75, 5.5, 11, 22, 44, 88, 176 µg/mL. In another experiment, the cells were treated with the following concentrations of 5, 10, 20,40, and 80 µg/mL of cannabis oil extract (COE).

2.4.3. MTS cell proliferation assay

Cells were incubated for 72 h then the media was removed, and an MTS cell proliferation assay was performed to measure cell viability. A volume of 2.4 mL of MTS reagent was transferred to a test tube along with 120 µL of PMS and 9.5 mL Dulbecco's modified Eagle's medium/Nutrient mixture F-12 Ham media. In each well, 100 µL of the MTS/PMS/DMEM F-12 Hamm mixture was added, and the plate was incubated in the dark for 1h. The absorbance was measured at 492 nm using a Multiskan FC microplate ELISA reader (Thermo Fisher Scientific, Rockford, IL, USA).

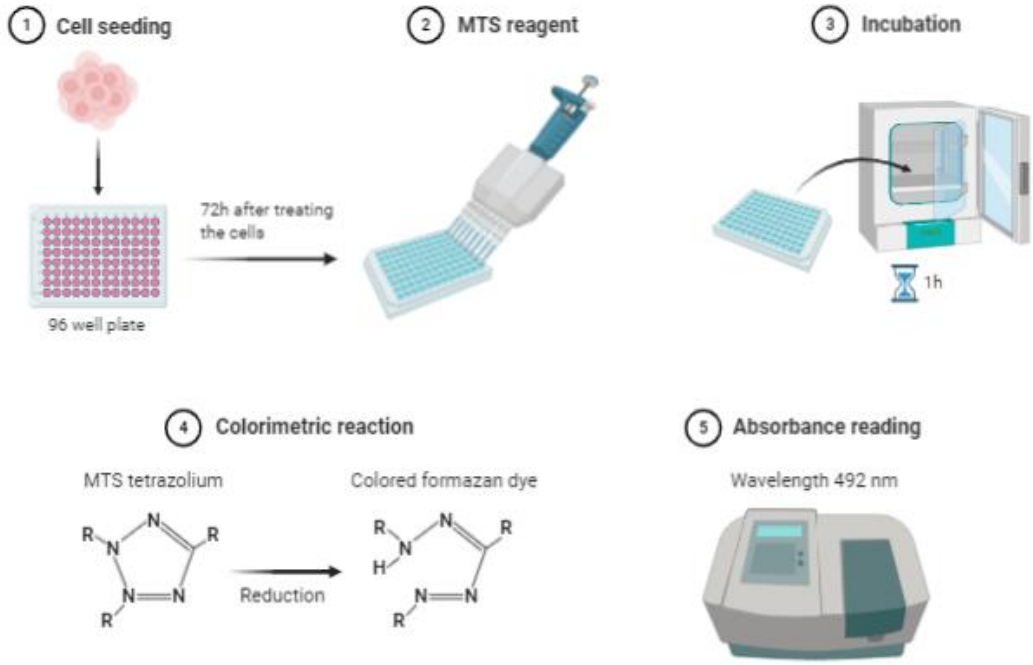


Figure 2.1. MTS cell proliferation assay demonstration

2.5. Wound Healing Assay

A549 and MDA-MB-231 cell lines were seeded at $2.5x10^5$ cells/mL and $3x10^5$ cells/mL respectively in 12 well plates and grown to confluence for 24h. Cells were then washed and starved overnight with serum-free media (RPMI for A549 and DMEM for MDA-MB-231). Afterward, cells were wounded using a sterile pipette tip washed with PBS, and media was replenished. Culture plates were then treated with increasing concentrations of COE (7.5, 15.1, and 30.2 µg/mL for A549 and 6.7, 13.4, and 26.75 µg/mL for MDA-MB-231) for 24h. These concentrations represent the $IC_{50}/4$, $IC_{50}/2$, and IC_{50} of each cell line determined from the cytotoxicity assay. A control group was present, and each condition was performed in triplicates. Phase-contrast images of the wounded area were taken at 0 h and 24h after wounding. Around 8 pictures per well were taken at

each time point. The area of the wound was outlined and measured in pixels using Axiovision systems. The average area of the wound at 24h was subtracted from the area at 0 h and % wound closure was calculated using Microsoft excel.

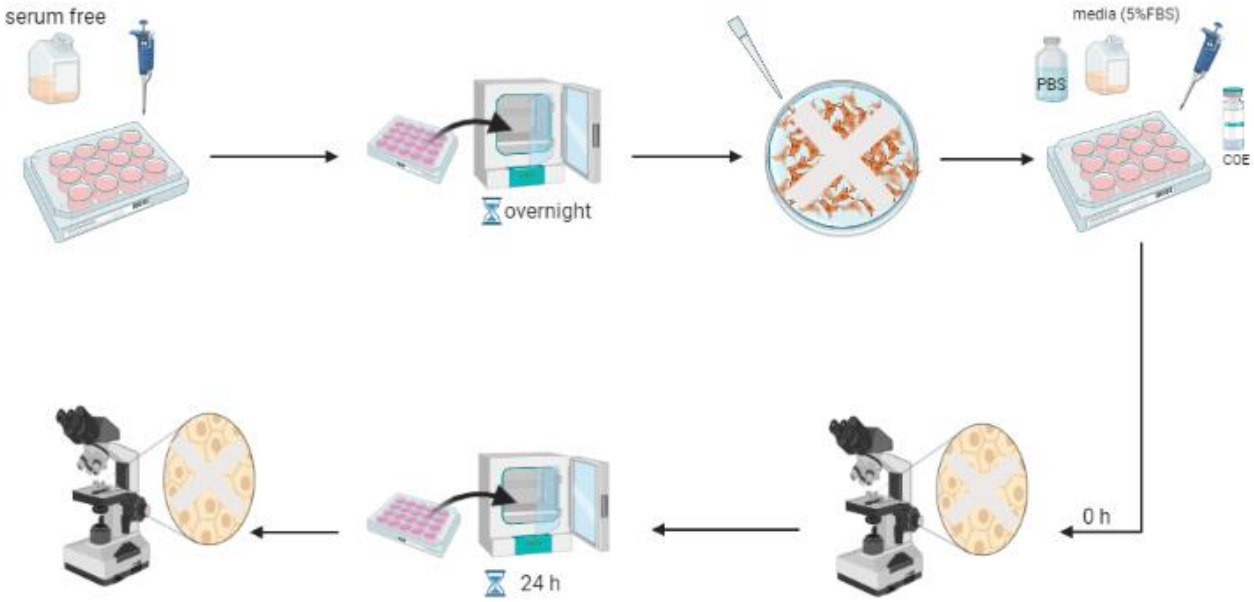


Figure 2.2. Wound healing assay demonstration

2.6. Cell Lysate Preparation

A549 and MDA-MB-231 cancer cell lines were seeded in 3 separate 6-well plates Cells were then treated with increasing concentrations of the COE (20 and 40 µg/mL) for 24 and 48h. The adherent and non-adherent cells were collected on ice and washed twice with cold PBS and lysed with 100µL 2X Laemmli buffer to which 2-mercaptoethanol was added. The samples were incubated on ice and vortexed every 10 minutes for 30 minutes, then centrifuged at 13000 rpm for 10 minutes at 4°C. The collected cell lysate was then heated at 100 °C for 10 min.

2.7. Western Blot Analysis

Cell lysate samples of equal protein concentrations were subjected to 10% SDS-PAGE. (5% stacking gel, 10% separating gel and 1X running buffer: 0.3% Tris Base, 1.4% glycine, 20% SDS, pH = 8.3) at 90 V for 30 min and then at 120 V for 2 h. Separated proteins were then transferred onto the PVDF membranes using a Semi-dry electro blotter (PEQLAB, Erlangen, Germany) and 1X transfer buffer (25mM Tris base, 0.2 M glycine, 20% methanol, pH 8.5) at 10V for 30 min. The membranes were blocked with blocking buffer (1X TBS, 0.1% Tween-20, 5% BSA) for 1 h, and then probed with primary antibodies against LC3B, p62, β-Actin, BAX, Bcl-2, Cytochrome c, cleaved Caspase-3, ERK, and pERK at 4 ºC overnight. Later, the antibodies were washed away with TBST for 30 minutes and the membranes were treated with rabbit HRP-coupled secondary antibodies for 1 hour and 30 minutes and washed with TBST afterward. Detection of proteins was performed using the chemiluminescence ECL kit. and blot images were obtained using the ChemiDoc imaging 3.8 instrument (Bio-Rad, Hercules, CA, USA) and analyzed with ImageLab™ Software (BioRad, Hercules, CA, USA).

2.8. Flow Cytometry

A549 cells ($5x10^4$ cells/mL for 48 h incubation time) were seeded in 6-well plates and treated with different concentrations of COE (20 and 40µg/mL) as well as Etoposide (positive control) at a concentration of 71.68 µg/mL (double IC_{50}) for 48 h. The non-adherent cells were collected, and adherent cells were washed with PBS and trypsinized with 1 mL/well of Trypsin 1X for 2 minutes. Subsequently, the cells were collected and centrifuged at 1500 rpm for 5 minutes at 4 °C. The pellet was resuspended in 500 µL PBS

and cells were counted under the inverted microscope (Nikon Eclipse TE300), by mixing 50 µL of cells with 50 µL of Trypan blue and placing 10 µL of the mixture on a hemocytometer. A final concentration of 500 cells/µL was obtained and the required volume was transferred into eppendorf tubes labeled for each condition and centrifuged at 1500 rpm for 5 minutes at 4 °C. The pellet was then resuspended in 100 µL Guava Nexin Reagent, containing Annexin V-Phycoeryhtrin (Annexin V-PE) and 7-AAD (7-amino-actinomycin D). The samples were incubated in the dark for 10 minutes. and then analyzed using the Guava® easyCyte 8HT Benchtop Flow Cytometer (Millipore, Luminex, USA). Annexin V/7-AAD data was measured on FL1-H versus FL2-H scatter plot and analyzed with FlowJo™ software (BD Biosciences, NJ, USA).

Chapter Three

Results

3.1. Chemical Composition of COE

The results summarized in Table 3.1 represent the GC-MS analysis of the COE. The major compounds identified consisted of cannabinoids (85.15%), with cannabidiol (CBD) having the highest percentage (59.1%), followed by tetrahydrocannabinol (THC) (20.2%). The remaining cannabinoids with lower percentages comprised cannabinol (CBN) (3.63%), and cannabichromene (CBC) (2.22%). Monoterpenes consisted in total of 4.12% represented by β-Myrecene (1.94%), α-Pinene (1.01%), and D-Limonene (0.51%). Another group of phytochemicals identified in the COE was the sesquiterpenes (5.89%) which included β-Caryophyllene (1.78%), α-Bergamotene (1.74%), Caryophyllene oxide (1.54%), and α-Humelene (0.53%) (Shebaby et al., 2021).

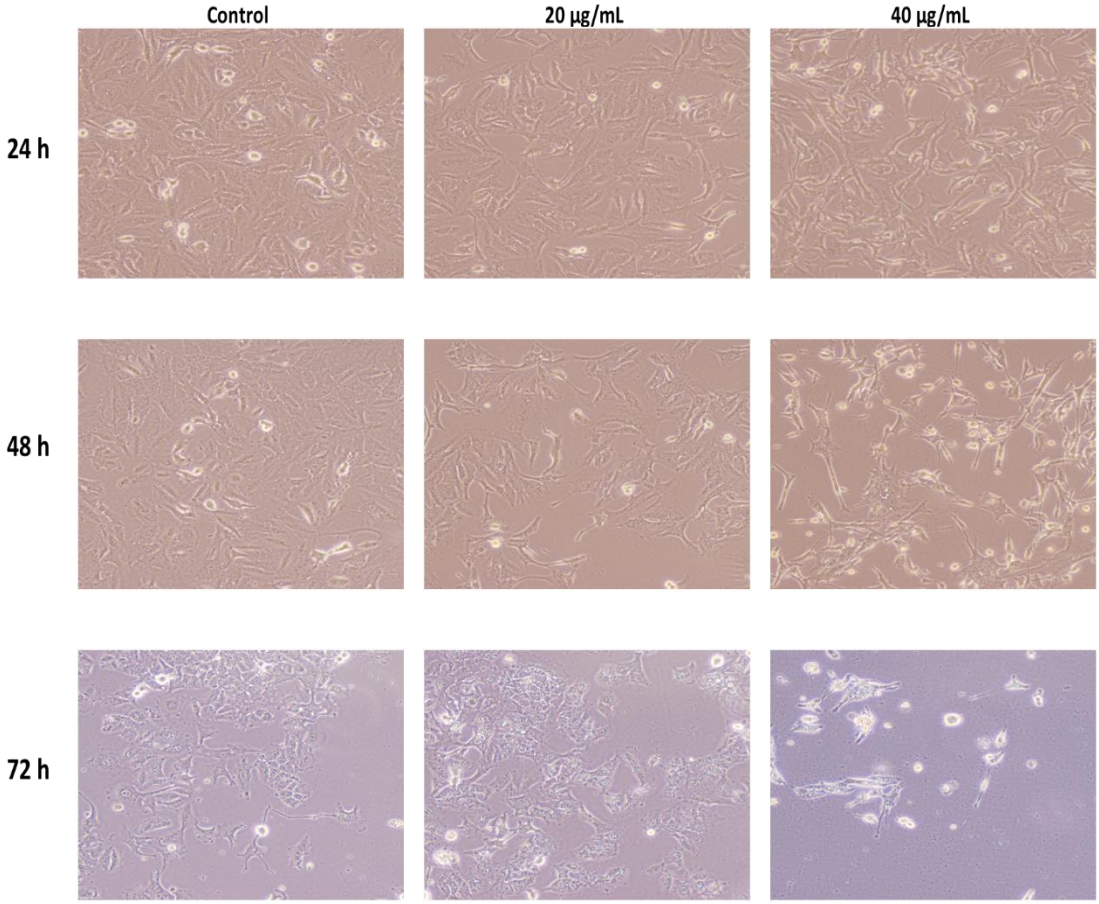
Table 3.1. Main identified constituents (>0.18%) of Lebanese COE.

Retention time (min)	Constituents	Percentage
5.83	α-Pinene	1.01
7.23	Camphene	0.50
7.72	β-Myrecene	1.94
9.02	D-Limonene	0.49

11.9	β-Ocymene	0.18
26.2	β-Caryophyllene	1.78
26.8	α-Bergamotene	1.74
27.7	α-Humelene	0.35
34.1	Humulene epoxide II	0.38
36.0	Caryophyllene oxide	1.64
47.3	Hexadecanoic acid	1.16
52.1	Phytol	1.41
52.7	Linoelaidic acid	0.56
52.9	α-Linolenic acid	0.95
61.3	CBD	59.1
61.6	CBC	2.22
64.2	Δ^9-THC	20.2
66.2	CBN	3.63
Total identified		99.24

3.2. The Effects of COE on Cancer Cell Survival

The cytotoxic effects of COE are evident under microscopic observations. The morphological changes (cell rounding, formations of cell protrusion, and autophagosomes), of both A549 and MDA-MB 231 cell lines are seen at 20 µg/mL of COE and are more prominent at 40 µg/mL (Figure 3.1 and Figure 3.2). As observed, the effects of COE increase with time also and are mostly evident after 72h were practically most of the cells are dead.



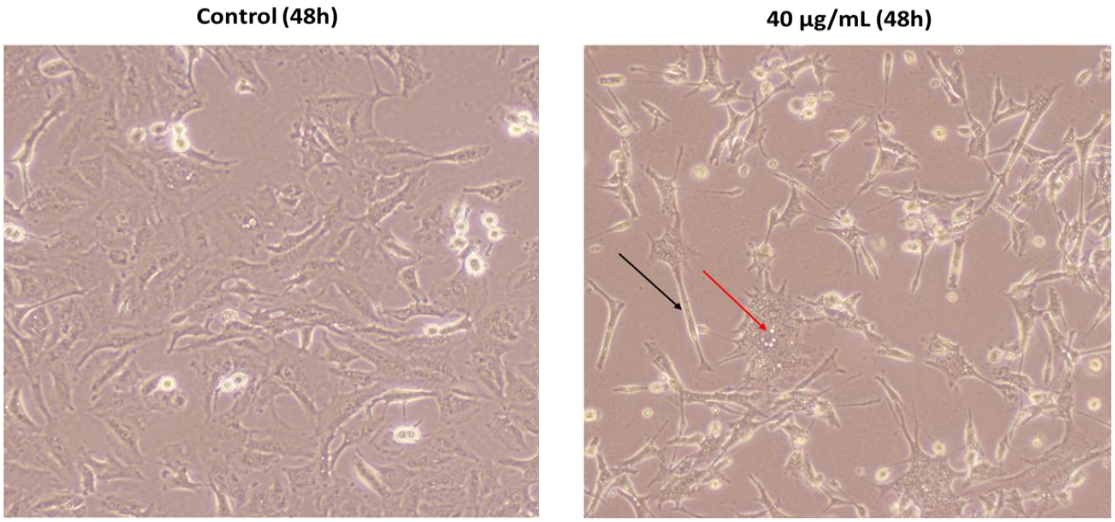
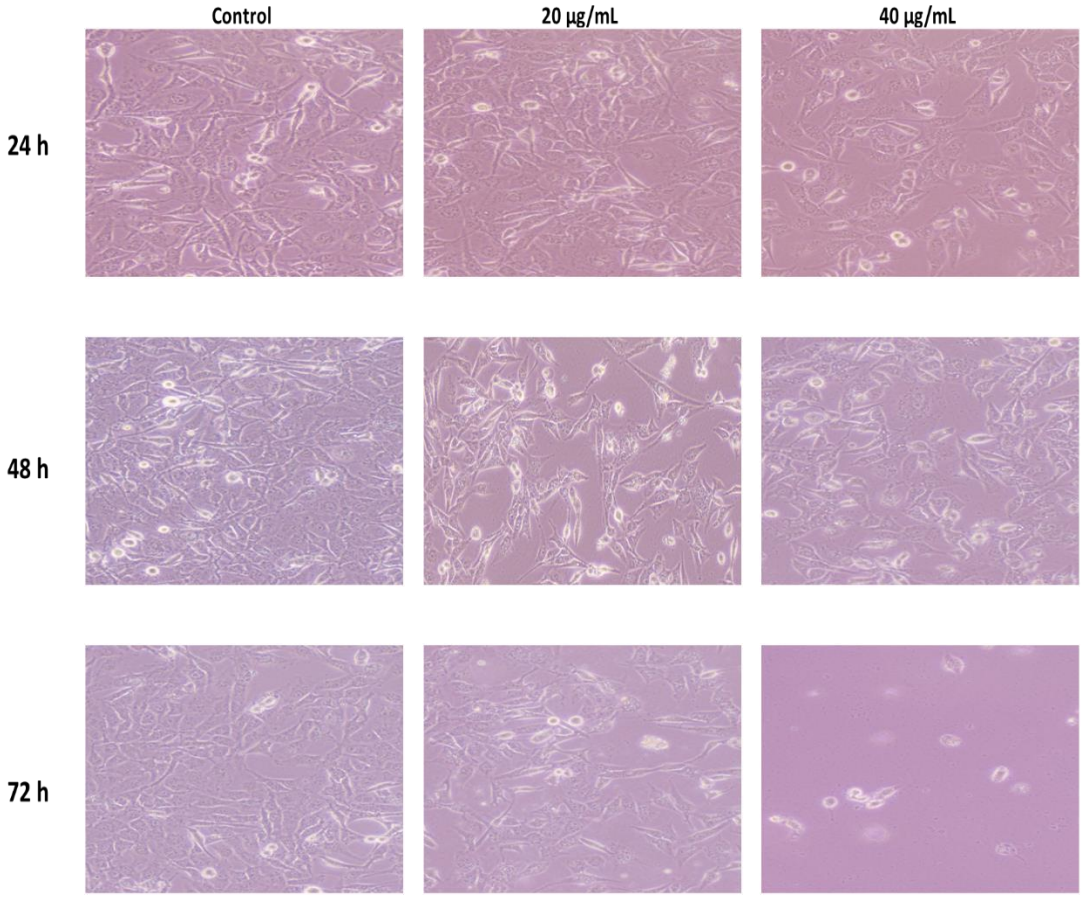


Figure 3.1. Morphological changes of A549 cancer cell lines exposed to various concentrations of COE for 24h, 48h and 72h. The morphological changes at 48h include formations of cell protrusion (→), and autophagosomes (→).



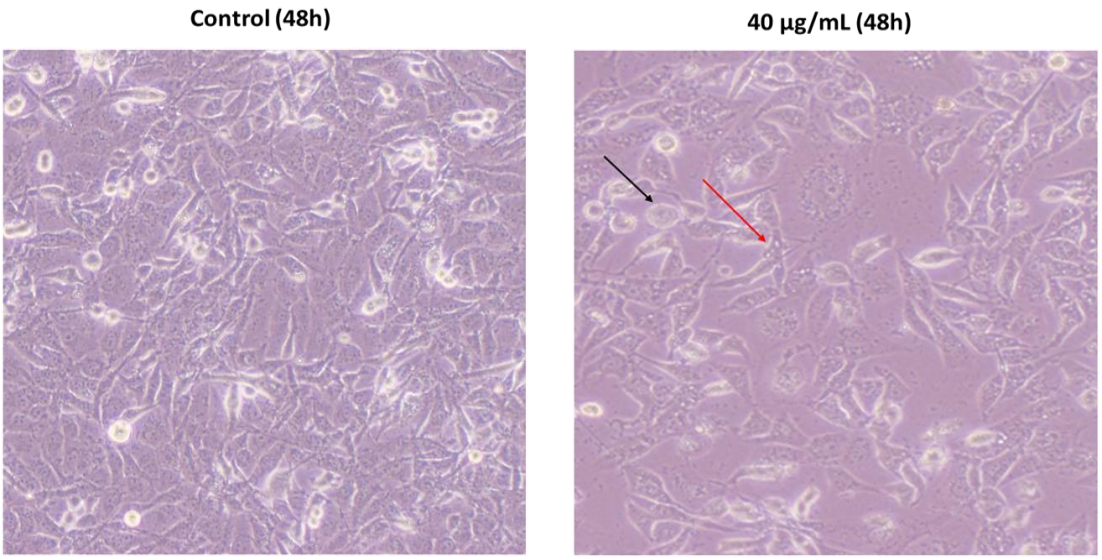
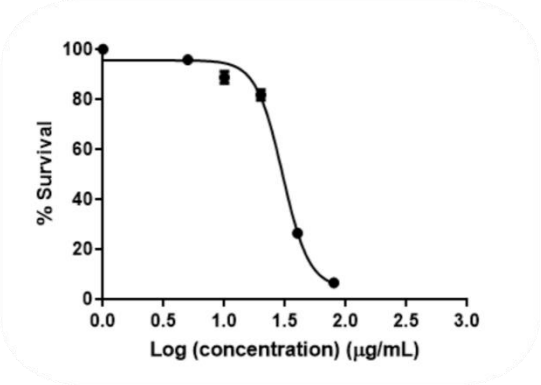
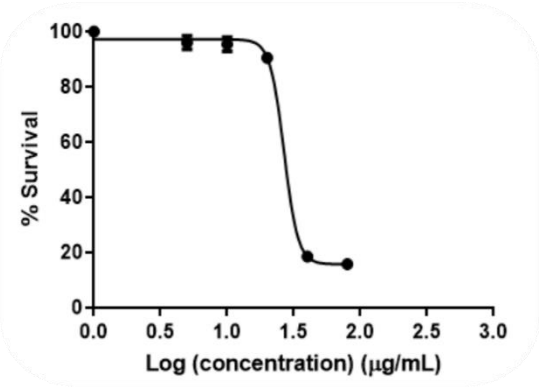


Figure 3.2. Morphological changes of MDA-MB 231 cancer cell lines exposed to various concentrations of COE for 24h, 48h and 72h. The morphological changes at 48h include cell rounding (→), and autophagosomes (→).

The cytotoxic effect of the COE was studied on A549 and MDA-MB 231 cancer cells for 72 hours using the MTS cell proliferation assay. Etoposide (20 mg/mL) was used as a positive control. The cytotoxic effects of COE were also examined on rat stem cells to highlight the selectivity of the extract at killing cancer cells (Figure 3.4). Multiple concentrations (5, 10, 20, 40, 80 µg/mL) of COE were used for treating A549 and MDA-MB 231 cells (Figure 3.3). Results showed a dose-dependent inhibitory effect on cell proliferation after 72h of incubation for both cell lines. The IC_{50} values of COE were 30.2 µg/mL and 26.7 µg/mL for A549 and MDA-MB 231 cell lines respectively (Table 3.2). The same concentrations of COE were also used on rat stem cells and the IC_{50} value was 42.31 µg/mL (Table 3.2). Furthermore, different concentrations of etoposide (2.75, 5.5, 11, 22, 44, 88, 176 µg/mL) were applied on both cancer cell lines (Figure 3.5). The IC_{50} values were 35.84 µg/mL and 10.87 µg/mL for A549 and MDA-MB 231 respectively (Table 3.2).

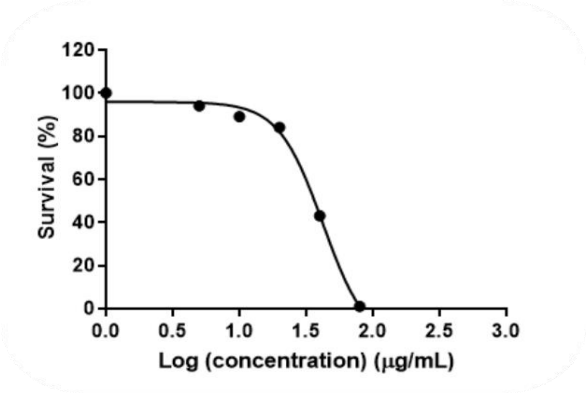


A549



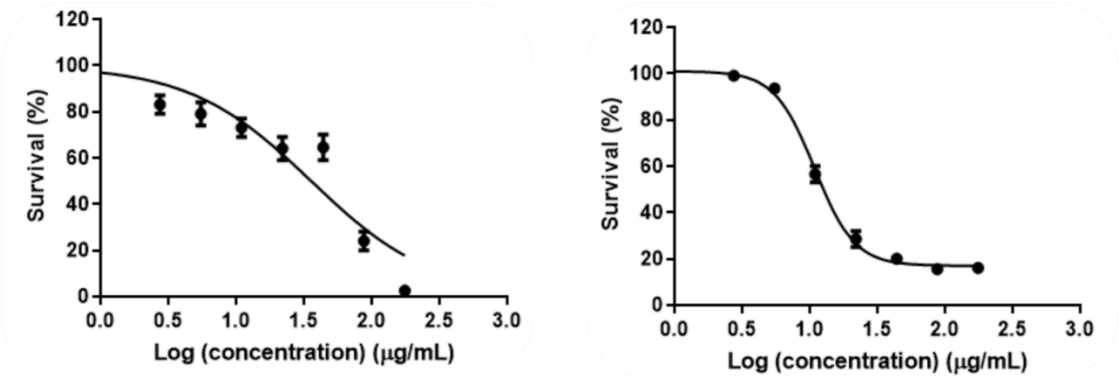
MDA-MB 231

Figure 3.3. Cytotoxic effect of COE on A549, MDA-MB 231. Cells were treated with COE concentrations ranging from 5 µg/mL to 80 µg/mL using serial dilutions for 72h. Data are expressed as % survival of cells relative to the control. Data points represent mean ± SEM.



Rat Stem Cells

Figure 3.4. Cytotoxic effect of COE on rat bone marrow stem cells. Cells were treated with COE concentrations ranging from 5 µg/mL to 80 µg/mL using serial dilutions for 72h. Data are expressed as % survival of cells relative to the control.



A549 MDA-MB 231

Figure 3.5. Cytotoxic effect of Etoposide on A549, MDA-MB 231. Cells were treated with etoposide concentrations ranging from 2.75 µg/mL to 176 µg/mL using serial dilutions for 72h. Data are expressed as % survival of cells relative to the control Data points represent mean ± SEM.

Table 3.2. IC50 values (µg/mL) of COE treatment on A549, MDA-MB 231 The IC50 values were also computed for the positive control etoposide.

IC_{50}	COE	Etoposide
A549	30.2 ± 0.33	35.84 ±7.98
MDA-MB 231	26.75 ± 0.75	10.87 ± 0.67

3.3. Effects of COE on Cell Migration

To determine the effect of COE on the migration of cancer cells A549 and MDA-MB 231, a "wound-healing" assay was executed over 24 h. The IC_{50} values of COE used on each cell line were determined in the cytotoxicity analysis (Table 3.2). For the A549 cell line, the % wound closure was 33% in the control group, 17.1%, 10.1%, and 9.7% respectively in the $IC_{50}/4$, $IC_{50}/2$, and IC_{50} groups (Table 3.3). A substantial decrease in cancer cell migration is observed especially in the $IC_{50}/2$ and IC_{50} groups (Figure 3.6). For the MDA-MB 231 cell line, the % wound closure was 54.2% in the control group, 49.2%,

44%, and 24.9% respectively in the $IC_{50}/4$, $IC_{50}/2$, and IC_{50} groups (Table 3.4). A decrease in cancer cell migration is observed particularly in the IC_{50} group (Figure 3.7). The results show a clear difference in wound closure between the two cell lines, with the COE having a more significant effect on A549 cell migration.

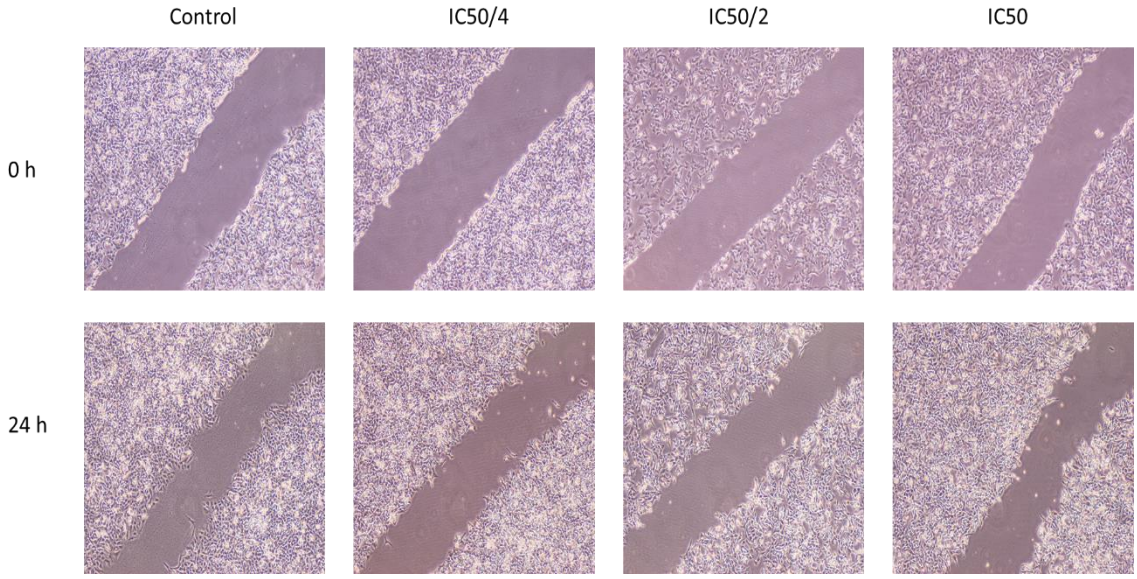


Figure 3.6. Microscopic images of A549 cells after inducing a wound at 0h and 24h. Wound closure was observed at different concentrations and compared to a control group.

Table 3.3. Wound closure (%) in A549 cells at different concentrations.

	Control	**IC50/4**	**IC50/2**	**IC50**
% Wound closure	33.0	17.1	10.1	9.7

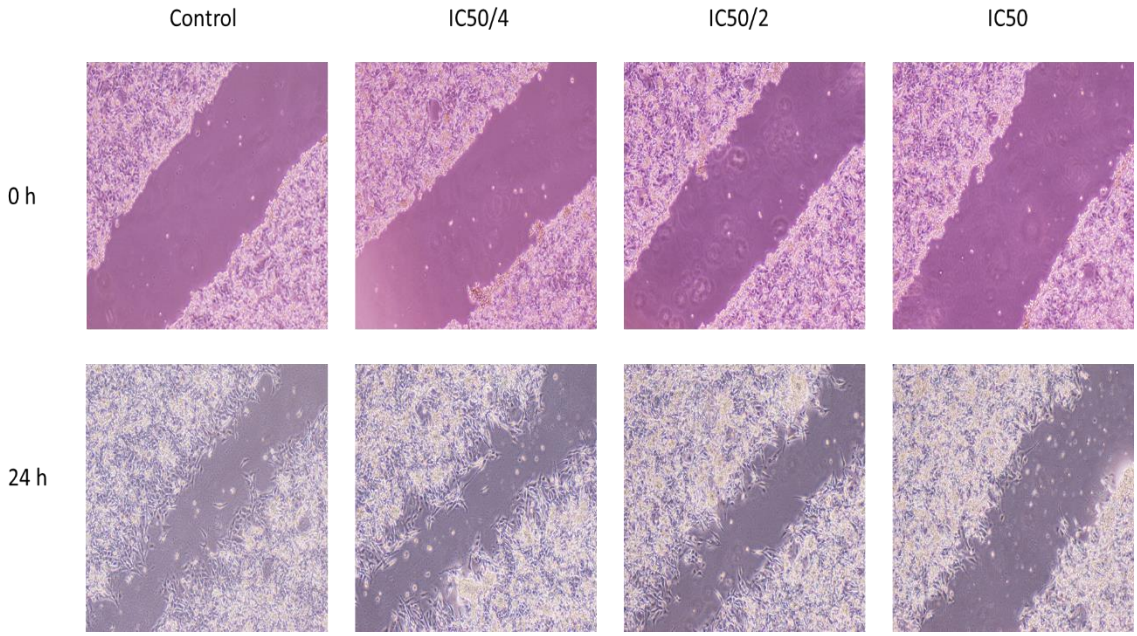


Figure 3.7. Microscopic images of MDA-MB 231 cells after inducing a wound at 0h and 24h. Wound closure was observed at different concentrations and compared to a control group.

Table 3.4. Wound closure (%) in MDA-MB 231 cells at different concentrations.

	Control	**IC50/4**	**IC50/2**	**IC50**
% Wound closure	54.2	49.2	44.0	24.9

3.4. Western Blot Analysis

The effects of COE on the expression of apoptotic, autophagy and MAPK/ERK protein markers in A549 and MDA-MB 231 cells were assessed at 24 and 48h. Cells were treated with 20 µg/mL and 40 µg/mL of COE, respectively, and the western blot results were analyzed using ImageLab software and normalized accordingly.

Treatment of MDA-MB 231 cells for 24h with 20 µg/mL of COE did not show any significant variation in the expression levels of the apoptotic proteins including cleaved caspase-3 (Figure 3.9, a), Bax (Figure 3.11, a), Bcl-2 (Figure 3.10, a) and cytochrome c (Figure 3.12, a) compared to the control. However, treatment of cells with 40 µg/mL of COE for 48h caused a slight increase in the expression levels of Bax (Figure 3.11, a) and cytochrome c (Figure 3.13, a) and a considerable increase in the cleaved caspase-3 level (Figure 3.9, a). The ratio of Bax/Bcl-2 showed to be increasing after 48h particularly at 40 µg/mL where it increased by 3-folds, indicating apoptotic cell death (Figure 3.8, a).

As for the A549 cell line, 40 µg/mL treatment showed an insignificant increase in levels of cleaved caspase-3 after 48h (Figure 3.9, b) and an increase in levels of cytochrome c at 24h followed by a decrease at 48h (Figure 3.12, b). The anti-apoptotic Bcl-2 protein levels displayed a significant decrease at both time points (Figure 3.10, b), while Bax protein levels showed a minor increase after 48h (Figure 3.11, b). The ratio of Bax/Bcl-2 showed to be significantly increasing at 24h, and it decreased markedly after 48h at 40 µg/mL (Figure 3.8, b).

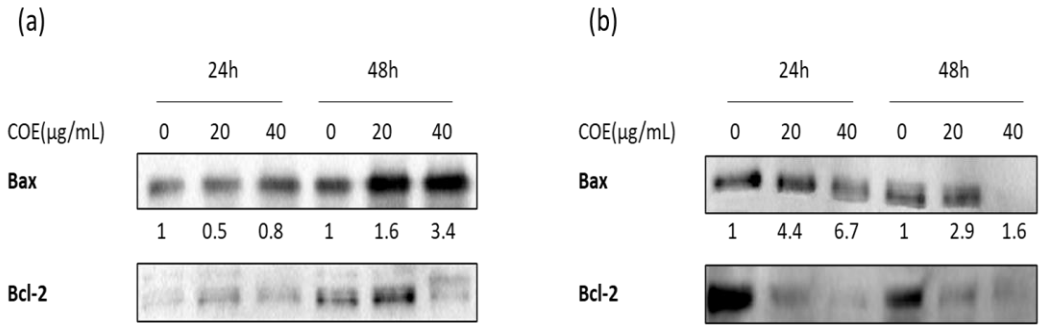


Figure 3.8. Western blot analysis of Bax/Bcl-2 in MDA-MB-231 and A549 cells. (a) MDA-MB 231 cells and (b) A549 cells were treated with 20 and 40 µg/ml of COE for 24 and 48h. The densitometer intensity of each band was determined relative to the bands of Bcl-2 and is shown under the immunoblot as a fold change compared with the control.

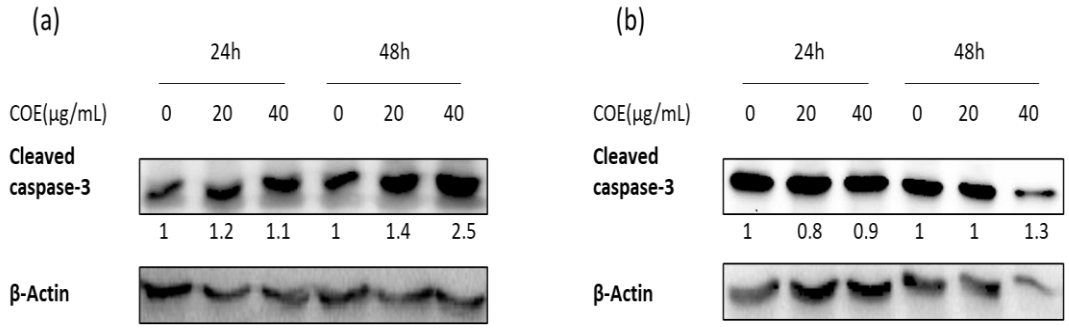


Figure 3.9. Western blot analysis of cleaved caspase in MDA-MB-231 and A549 cells. (a) MDA-MB 231 cells and (b) A549 cells were treated with 20 and 40 μg/ml of COE for 24 and 48h. The densitometer intensity of each band was determined relative to the bands of β-actin and is shown under the immunoblot as a fold change compared with the control.

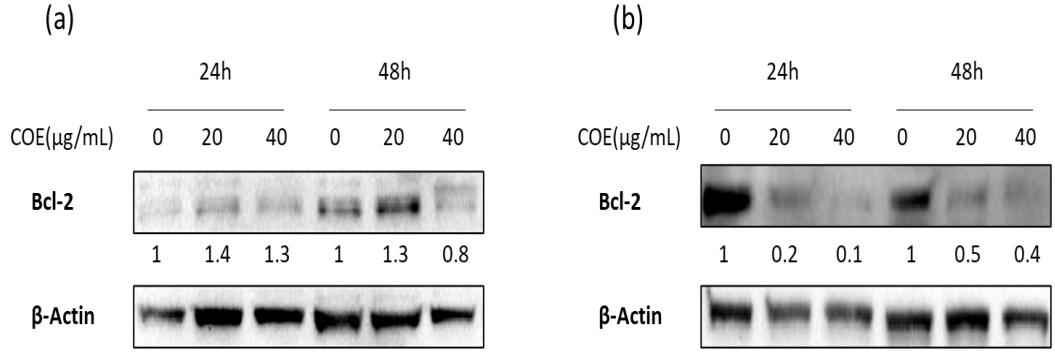


Figure 3.10. Western blot analysis of anti-apoptotic protein Bcl-2 in MDA-MB-231 and A549 cells. (a) MDA-MB 231 cells and (b) A549 cells were treated with 20 and 40 μg/ml of COE for 24 and 48h. The densitometer intensity of each band was determined relative to the bands of β-actin and is shown under the immunoblot as a fold change compared with the control.

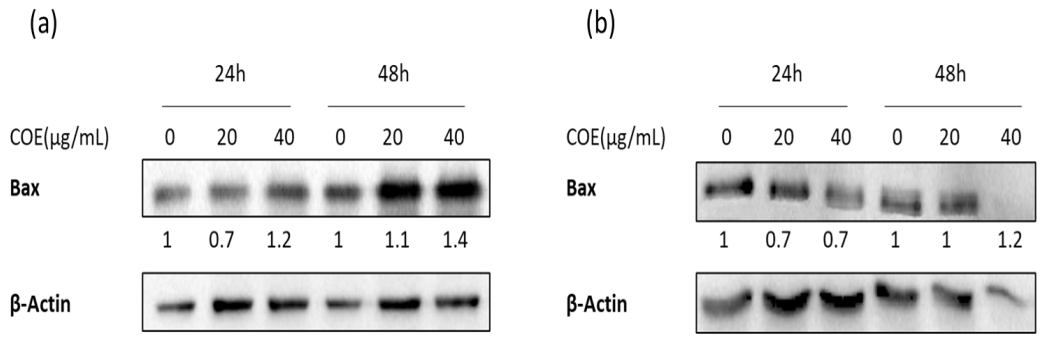


Figure 3.11. Western blot analysis of pro-apoptotic protein Bax in MDA-MB-231 and A549 cells. (a) MDA-MB 231 cells and (b) A549 cells were treated with 20 and 40 µg/ml of COE for 24 and 48h. The densitometer intensity of each band was determined relative to the bands of β-actin and is shown under the immunoblot as a fold change compared with the control.

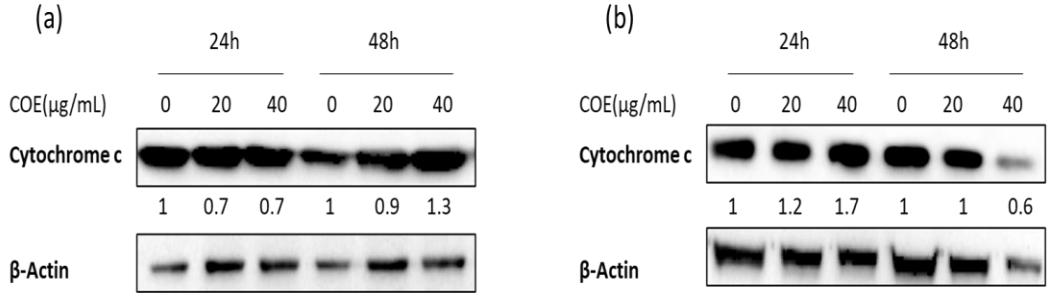


Figure 3.12. Western blot analysis of cytochrome c in MDA-MB-231 and A549 cells. (a) MDA-MB 231 cells and (b) A549 cells were treated with 20 and 40 µg/ml of COE for 24 and 48h. The densitometer intensity of each band was determined relative to the bands of β-actin and is shown under the immunoblot as a fold change compared with the control.

For the autophagy markers, the MDA-MB 231 cell line displayed a slight increase in LC3B and a greater increase in p62 to β-Actin expression after 20 and 40 µg/ml of COE treatment at both time points (Figure 3.13, a), while the A549 cell line exhibited a major increase in the LC3B protein especially after 48 h at 40 µg/mL (Figure 3.13, b). These

results further accentuate the fact that the mechanisms of cell death induce by COE are in fact specific to the cancer cell type.

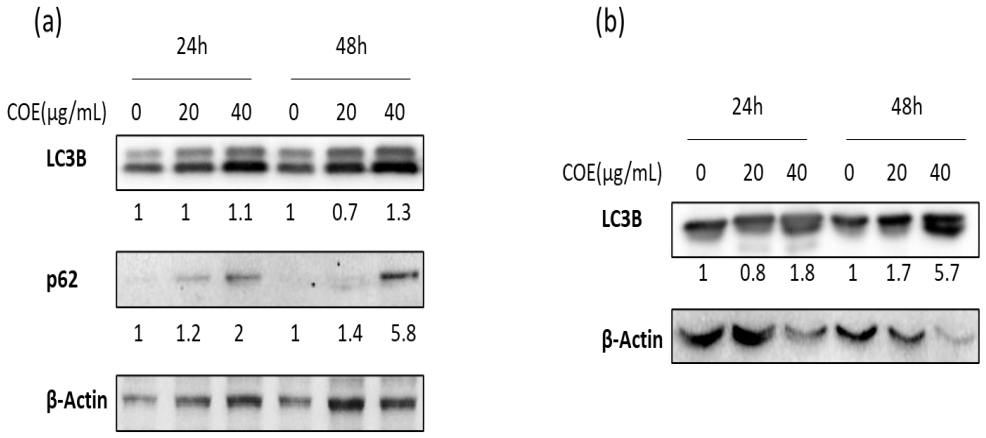


Figure 3.13. Western blot analysis of autophagy markers LC3B and p62 in MDA-MB-231 and A549 cells. (a) MDA-MB 231 cells and (b) A549 cells were treated with 20 and 40 µg/ml of COE for 24 and 48h. The densitometer intensity of each band was determined relative to the bands of β-actin and is shown under the immunoblot as a fold change compared with the control.

The results for MDA-MB 231 cells demonstrated an increase in pERK to ERK expression at 24 h followed by a major increase after 48 h at 40 µg/mL (Figure 3.14, a). In A549 cells, the level of pERK to ERK expression was significantly increased in both the 20 µg/mL and 40 µg/mL treated groups, at 24 and 48h (Figure 3.14, b).

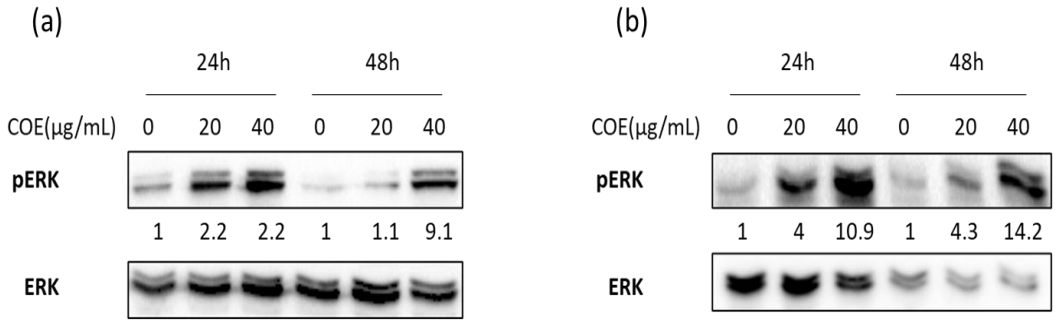


Figure 3.14. Western blot analysis of MAPK/Erk pathway in MDA-MB-231 and A549 cells. (a) MDA-MB 231 cells and (b) A549 cells were treated with 20 and 40 µg/ml of COE for 24 and 48 h. The

densitometer intensity of each band was determined relative to the bands of non-phosphorylated ERK and is shown under the immunoblot as a fold change compared with the control.

In the western blotting analysis, experiments were conducted once and the possible decrease in the β-Actin expression at 48h in the 40 µg/mL group in some of the samples is mostly due to the increase in cancer cell death.

3.5. Flow Cytometry

To further determine whether the cytotoxic effect of COE on A549 cells was correlated with the induction of apoptosis, Annexin V/7-AAD staining method was applied. Etoposide was used as a positive control. The A549 cells were treated with 20 µg/mL and 40 µg/mL of COE, respectively for 48 h. The population of cells in quadrant four (Q4) was negative for both Annexin V and 7-AAD represented the viable cells. Cells in quadrant three (Q3) were positive for Annexin V and negative for 7-AAD represented early apoptosis, while cells in quadrant two (Q2) were Annexin V positive and 7-AAD positive and represented late apoptosis. Cells in quadrant one (Q1) were Annexin V negative and 7-AAD positive and were stated necrotic. Flow cytometry results showed the presence of a dose dependent effect. As shown in Figure 3.15, treatment of A549 cells with 20 µg/ml of COE revealed an increasing trend in both the early and late apoptotic cell population. However, a significant increase in necrotic cell population was observed after treatment with 40µg/ml of COE. In the control group around 97% of cells were healthy as compared to etoposide treated cells which had 87% healthy cells.

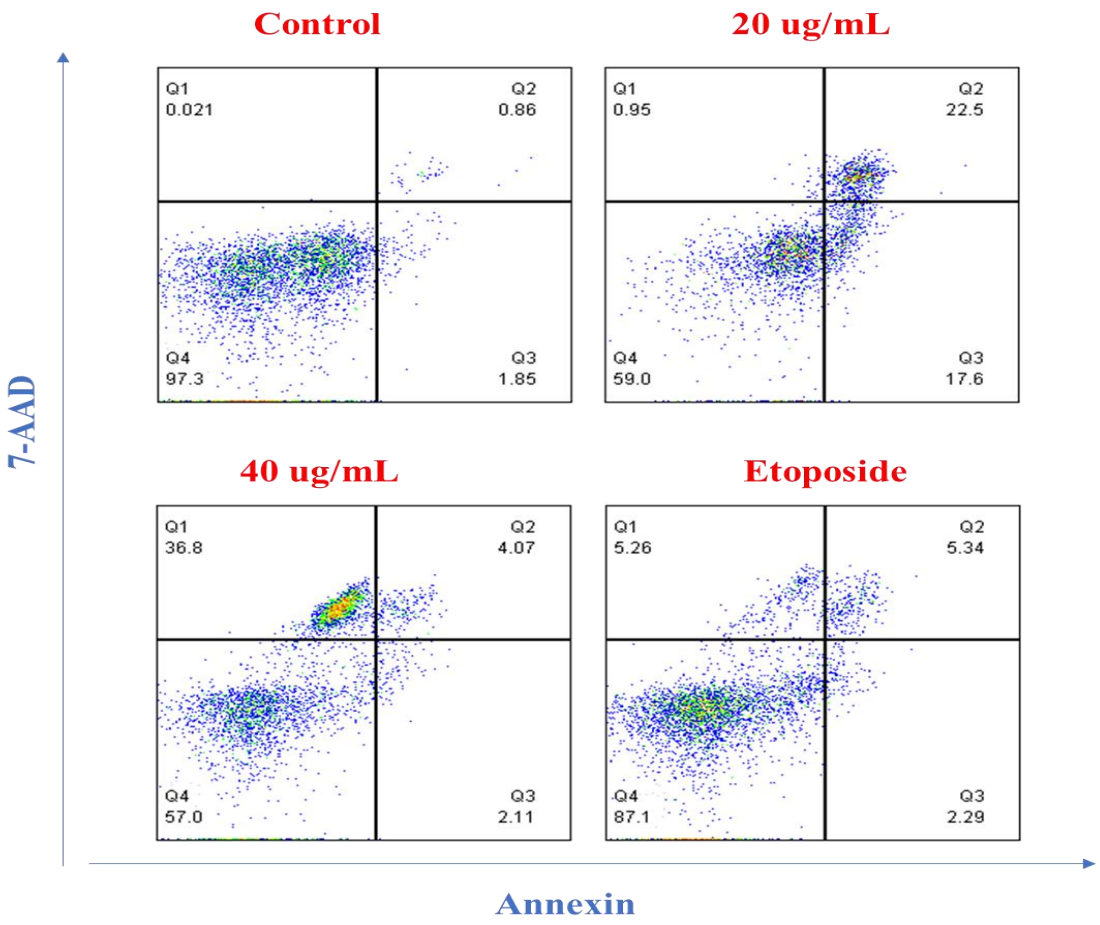


Figure 3.15. Flow cytometric analysis of Annexin V-FITC and 7-ADD quantifying the COE -induced apoptosis in A549 cells. Dot plots of A549 cells treated with 20 or 40 µg/ml of COE for 48 h. Etoposide was used as a positive control.

Chapter Four

Discussion

Cannabis was used in traditional medicine for anxiety, pain, convulsions, and sedation, for almost 5000 years. In recent times, cannabis started to gain a lot of attention especially in the field of oncology (Afrin et al., 2020). For many cancer patients, chemotherapy is inevitable along with its devastating side effects rendering cannabis-based medicine a highly favored candidate in the treatment of cancer. The diverse composition of a COE, particularly terpenes or cannabinoids, has shown synergistic effects with current chemotherapies, allowing for a lesser dosage and thus less adverse effects. In addition, cannabinoids display a relatively safe profile as compared to chemotherapy (Tomko et al., 2020). This synergism between the different COE compounds, creates what is known as the "entourage effect", rendering the plant's extract more potent than isolated pure compounds (Milian et al., 2020; Russo, 2011; Blasco-Benito et al., 2018; Tomko et al., 2020; Baram et al., 2019). In the fight against cancer, researchers are racing to find the best drug to either kill cancer cells or provide an alternative to the present treatment options. Non-small cell lung cancer (NSCLC) and triple negative breast cancer (TNBC) are two of the most life-threatening types of cancer. Cannabis has proven to have anti-tumoral effects such as inhibition of migration, cell cycle arrest, apoptosis and autophagic cell death on cancer cells, including MDA-MB 231 and A549 (Ramer et al., 2012; Seltzer et al., 2020; Kisková et al., 2019; Calvaruso et al., 2012; Daris et al., 2019; Shrivastava et al., 2011).

The medicinal benefits of Lebanese cannabis were overlooked for many years despite it being one of the finest in the world. This study explores the anti-cancer effects and the different anti-cancer mechanisms of Lebanese cannabis extract, *in-vitro*, on NSCLC cells (A549) and TNBC cells (MDA-MB-231).

The GC-MS analysis of the Lebanese cannabis oil extract of *C. sativa L. ssp. indica (Lam.)* (Table 3.1), showed an abundance in phytocannabinoids (85.15%), specifically CBD (59.1%). THC also occupied a significant portion of the COE (20.2%). CBD and THC are mostly highlighted for their anti-cancer effects in a multitude of cancer types including lung, breast, brain, colon, leukemia, and prostate (Shebaby et al., 2021; Afrin et al., 2020; Kis et al., 2019). Cannabichromene (CBC) occupied 2.22% of the total COE while cannabinol (CBN) consisted of 3.63%. Although CBC and CBN are considered minor phytocannabinoids, as they are not very abundant, however they are not devoid of anti-tumoral activity (Shebaby et al., 2021; Tomko et al., 2020). Like CBD, CBC lacks psychoactive activity, and was shown to be a potent inhibitor of cell viability in the MDA-MB 231 cell line while CBN, a degradation product of THC, possessed minimal psychoactive effects and showed antiproliferative effects in aggressive breast cancer (Ligresti et al., 2006; McAllister et al., 2007). Other compounds commonly found in cannabis extracts are the terpenes (4.12%) represented by β-Myrecene, α-Pinene, and D-Limonene and the sesquiterpenes (5.89%) which included β-Caryophyllene, α-Bergamotene, Caryophyllene oxide, and α-Humelene (Shebaby et al., 2021). In a review by Tomko et al. (2020), the anti-tumorigenic effects of these products were illustrated. *In vitro*, myrcene showed cytotoxic effects on cancer cells, pinene reduced cell viability and induced apoptosis, and limonene decreased migration and induced apoptosis and

autophagy; while β-Caryophyllene had cytotoxic effects and induced apoptosis, and humelene expressed cytotoxic effects. According to Vergara et al., (2017), total THC levels varied in commercial cannabis flower samples taken from different U.S. cities and showed around 15% in Denver and Oakland, while having higher levels (19%) from samples taken in Seattle. The diversity of compounds and their different activities comes together to reinforce the idea of the "entourage effect" and shed light on the importance of having all those compounds working together to achieve cancer cell death.

The cytotoxic effects of the Lebanese cannabis were also assessed. Experiments were conducted on A549, and MDA-MB 231 cell lines and results showed a dose-dependent effect (Table 3.2 & Figure 3.3). In a study conducted by Baram et al. (2019), extracts with high CBD content or high THC-type cannabinoids content (>35%) are proven to have dose-dependent effects on A549 cell survival. This further proves the efficacy of the Lebanese COE and reinforces the findings discussed previously. Furthermore, the results of COE on rat stem cells (Figure 3.4), illustrate the possible selectivity between cancer cells and normal cells. According to Solinas et al. (2015), CBD was as potent as THC in selectively inhibiting the proliferation of MDA-MB-231 cells *in vitro* while having a far less potent effect on normal cells. In addition, THC as well as CBD, did not show significant cytotoxic effects on normal cells proving once more the selectivity to cancer cells (Fluda, 2017; Dariš et al., 2019). A review done by Russo (2019), highlighted the synergism between the different plant derivatives (terpenes, cannabinoids, flavonoids), and the ability to have better pharmacological effects when using the whole extract rather than single purified cannabinoid. Nallathambi et al (2018), provided evidence of

synergism between different cannabis compounds to produce cytotoxic effects in colon cancer cells.

To determine whether the Lebanese COE affects cellular migration of A549 and MDA-MB-231 cell lines, a "wound healing" assay was used. Cell migration, after 24h, was markedly decreased in the A549 cell line (Table 3.3 & Figure 3.6), particularly in the $IC_{50}/2$, and IC_{50} groups while the effect on MDA-MB-231 migration was lower (Table 3.4 & Figure 3.7). This shows the selectivity of COE on cancer cells. The two cell lines being studied differ in the expression of certain vital receptors used in the COE anti-cancer mechanisms. In a review by Massi et al. (2013), CBD was the most potent inhibitor of MDA-MB-231 migration by regulating the expression of important genes involved in migration, proliferation, and invasion through the downregulation of Id-1 expression. In fact, the upregulation of phosphorylated ERK (pERK) mediated the effect of CBD on Id-1 expression (McAllister et al., 2011). These findings are conformant with the results as the CBD rich extract used in this study generated an increase in pERK demonstrated in western blotting analysis (Figure 3.14). EGFR is also a key player in cancer cell migration especially A549 since the over-activity of EGFR is a characteristic of NSCLC. It was recently shown that THC inhibited migration of A549 cells induced by EGFR and subcutaneous metastasis in mice with severe immunodeficiency, while CBD had an additive effect on the inhibition of THC-mediated cell migration. This confirms the beneficial use of both cannabinoids together, as CBD enhances the effects of THC and reduces its psychotropic activity (Milian et al., 2020). Also, the role of GPR55 in inhibiting cancer cell migration and metastasis should not be ignored. GPR55 may lead to a rapid intracellular release of Ca^{2+} and phosphorylation of ERK (Afrin et al., 2020). These

findings highlight the diversity of pathways a cell might undertake as a response to the COE treatment, and more studies are needed to be able to untangle these pathways, however, it is obvious that the Lebanese COE affects cancer cell migration.

The western blotting analysis comes to reinforce the idea previously discussed. In Figure 3.14, there is a clear increase in the expression of pERK to ERK mostly at the concentration of 40µg/mL in both cell lines. The MAPK/ERK pathway might be activated through EGFR. Furthermore, MAPK/ERK plays a dual role as it may either inhibit or activate apoptotic pathways depending on the stimulus and cell type (Yue & López, 2020). It is also implicated in the activation of autophagy which is known to be upstream of apoptosis in cannabinoid treated cells (Sooro et al., 2018; Afrin et al., 2020; Velasco et al., 2016; Das et.al., 2019). The presence of autophagy is confirmed by looking at the expression of LC3B which is fairly increased in MDA-MB-231 and markedly elevated in A549 cells (Figure 3.13), confirming the involvement of autophagy in cancer cell death. The results also showed an increase in the expression of p62 in MDA-MB-231 cells. The upregulation of LC3 is the most reliable marker for autophagy while an elevated p62 level is usually correlated to cancer cell resistance to therapy. As an example, the cisplatin-resistant ovarian cells (SKOV3/DDP) displayed high levels of p62. In addition, p62 is thought to play an anti-apoptotic role rendering these cells resistant to drugs (Islam et al., 2018). Further studies are needed to unlock the complete role of these markers, specifically p62, as it has multiple roles to play in both tumor cell death and survival, thus cannot be overlooked when designing cancer treatments. Moreover, apoptotic cell death was analyzed and revealed a 2.5-fold increase of cleaved caspase-3 expression in MDA-MB-231 cells after 48h and a 1.3--fold increase in A549 cells (Figure 3.9). The anti-

apoptotic protein Bcl-2 was decreased in A549 cells compared to MDA-MB-231 cells which showed a decrease in Bcl-2 only after 48h at 40 µg/mL (Figure 3.10), while the pro-apoptotic protein Bax expressed a slight increase in both cell lines (Figure 3.11). An increase in the Bax/Bcl-2 ratio is observed in the MDA-MB-231 cell line after 48h at 40 µg/mL (Figure 3.8, a). A sharper increase in the Bax/Bcl-2 ratio is noticeable in the A549 cell line at 24h, followed by a decrease at 48h (Figure 3.8, b). This comes in accordance with the flow cytometry results (Figure 3.15), which showed that A549 cells underwent early/late apoptosis at 20 µg/mL and went into necrosis at 40 µg/mL. The expression of cytochrome c was also slightly increased in MDA-MB-231 cells after 48h at 40 µg/mL while it increased at 24h in A549 cells (Figure 3.12). Looking at the results, one might consider that MDA-MB-231 cells have a bit more apoptotic activity, mostly through the intrinsic pathway, than A549 cells, however, in both cases apoptosis is thought to have a reduced expression. This might be due to several factors. First, autophagy might inhibit apoptosis by a process known by mitophagy, where the damaged mitochondrion is removed, and apoptosis is hindered. Also, autophagy can actively remove pro-apoptotic proteins from the cell's cytoplasm (Mariño et al., 2014). Second, as seen previously, p62 might also have anti-apoptotic properties along with the MAPK/ERK which in many cases inhibits apoptosis in a dose-dependent and cell specific manner (Yue & López, 2020; Islam et al., 2018).

In the cell death analysis using flow cytometry, A549 cells were assessed after 48h of treatment with COE. The results showed that in the 20 µg/mL COE group, A549 cells underwent early/late apoptosis while at 40 µg/mL cells went into late apoptosis/necrosis (Figure 3.15). These findings are compatible with the western blotting results and further

accentuate the fact that the effect of the COE is dose dependent as well as time dependent. In a study conducted by Baram et al. (2019), A549 cells were treated with different cannabis extracts, for 24h, the doses being 4 or 8 µg/mL and the results showed a dose dependent induction of apoptosis.

Chapter Five

Conclusion

Lebanese cannabis is very well known around the globe for its high quality, where it is mainly used recreationally. The current study is the first to evaluate the anti-tumorigenic activity of Lebanese cannabis oil extract (COE) against lung and breast cancer cells, where it showed significant anti-cancer effects. The effects were time- and dose-dependent, cell specific and based on receptor expression. The results revealed multifactorial mechanisms, including cell migration, apoptosis, and autophagy. Autophagy was highlighted as an important mechanism of cancer cell death and a possible apoptosis regulator. Migration inhibition was clearly observed especially in NSCLC, and possibly related to EGFR overexpression. In addition, there is a strong possibility that MAPK/ERK pathway is involved in the activation of autophagy, inhibition of cancer cell migration and probably apoptosis. Further studies are needed for better understanding the complexity and interplay between these different signaling pathways.

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