# THE EFFECTS OF *CANNABIS SATIVA* EXTRACTS ON MELANOMA CANCER CELLS IN VITRO

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## DEDICATION

I want to dedicate this to my parents for their endless love and dedicated partnership for success in my life.

## ABSTRACT

Melanoma is the deadliest form of skin cancer, and its mortality continues to rise. Although early diagnosis and development of new treatments have improved melanoma prognosis, many patients develop resistance to current therapies. Research demonstrates that the main active ingredients of the cannabis plant, cannabinoids, have potential anti-cancer effects in different models of cancer. Cannabis plants, however, contain more than 545 secondary metabolites, which have shown to have medicinal potential and are believed to create an entourage effect when used together. The current study explores the anti-proliferative effects of ten cannabis extracts on melanoma cells in vitro. We discovered that studied cannabis extracts are cytotoxic for melanoma cells in a time- and dose-dependent manner and that high THC cannabis extracts exert higher cytotoxicity compared to their corresponding amount of THC alone. This anti-tumor activity may be via regulation of MAPK/ERK and PI3K/AKT pathway.

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

2-AG: 2-arachidonoylglycerol
AEA: Anandamide
AKT: Serine/threonine-specific protein kinase B
AMPK: Adenosine monophosphate-activated protein kinase
BCC: Basal cell carcinoma
PBST: Phosphate-buffered saline with tween
CB: Cannabinoid
CBC: Cannabichromene
CBCA: Cannabichromenic acid
CBD: Cannabidiol
CBDA: Cannabidiolic acid
CBG: Cannabigerol
CBGA: Cannabigerolic acid
CBGA: Cannabigerolic acid
CBN: Cannabinol
CDK: Cyclin-dependent kinase
DAGL: Diacylglycerol lipase
DMSO: Dimethyl sulfoxide anhydrous
ECS: Endocannabinoid system
EMT: Epithelial-to-mesenchymal transition
ER: Endoplasmic reticulum
ERK: Extracellular signal-regulated kinases
FAAH: Fatty acid amide hydrolase
GPR119: G protein-coupled receptors 119
GPR18: G protein-coupled receptors 18
GPR55: G protein-coupled receptors 55
HPLC: High performance liquid chromatography
MAGL: Monoacylglycerol lipase
MAPK: Mitogen-activated protein kinases
MEP: Plastidial methylerythritol phosphate
MEV: Cytosolic mevalonate
MMP-2: Matrix metaloproteinase-2
MMPs: Matrix metalloproteinase
mTOR: Mammalian target of rapamycin
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NAPE: N-Acylphosphatidylethanolamine
NAPE-PLD: NAPE-phospholipase D
NMSC: Non-melanoma skin cancer
p21/p27: Cyclin-dependent kinase inhibitor proteins
PI3K: Phosphatidylinositol 3-kinase
PTEN: Phosphatase and tensin homolog
PVDF: Polyvinylidene difluoride
RB: Retinoblastoma tumor suppressor protein
ROS: Reactive oxygen species
RIPA: Radioimmunoprecipitation assay
SCC: Squamous cell carcinoma
SDS: Sodium Dodecyl Sulfate
TERT: Telomerase reverse-transcriptase
THC: Tetrahydrocannabinol
THCA: Tetrahydrocannabinolic acid
THCV: Tetrahydrocannabivarin
TIMP-1: Matrix metalloproteinases
TP53: Tumor protein p53
TRPA1: Transient receptor potential ankyrin channel 1
TRPV2: Transient receptor potential cation channel 2
VEGF: Vascular endothelial growth factor

# 1. INTRODUCTION

## 1.1 *Cannabis sativa* plant

*Cannabis sativa*, also known as marijuana, is a plant that has many medicinal properties. It belongs to *Cannabacaea* family, and the species *Cannabis sativa* contains many highly heterogeneous varieties. Therefore, the taxonomic classification of this plant has always been disputed. Cannabis has three main species: *C. sativa L.* (known as fibre-type), *ruderalis Janisch,* and *C. indica Lam* (known as drug-type) [1].

### 1.1.1 History of use

For several thousand years cannabis species have been used for a variety of medical purposes including pain and sleep disorders. Cannabis was known as an unapproved therapeutic product besides being hallucinogenic intoxicant and it has the longest recorded history of human use among plants [2]. Cannabis is indigenous to central Asia and India, and its earliest medicinal use is reported in China, India, and Israel [3]. Cannabis agriculture has reached North America in the early 1600s, but as a medicine, it was introduced to western medicine by William B. O'Shaughnessy only in the 19$^{th}$ century [4, 5].

Throughout the latter half of the nineteenth century, the medicinal use of marijuana expanded, and it was commonly used in the treatment of migraine headaches in Britain and America [6]. In the early twentieth century, physicians prescribed cannabis products to their patients in the USA [7]. Nevertheless, the use of this plant was criminalized in Canada under the Opium and Narcotic Drug Act in 1923, followed by the same restriction known as Marijuana tax Act in 1937 in the USA [7, 8]. Those rules placed many obstacles in the way of research into the medicinal properties of cannabis.

Finally, in 1986, a synthetic version of tetrahydrocannabinol (THC) was approved as a licensed drug, dronabinol, for the treatment of chemotherapy-induced nausea and vomiting, and in 1992, it started to be used for the treatment of anorexia associated with AIDS wasting syndrome. Dronabinol set the stage for other cannabis-based drugs to be legally available. Sativex, a whole cannabis plant extract with a THC: CBD ratio of 1:1, for example, is now used for the treatment of central pain associated with Multiple Sclerosis and cancer-related pain, and is a medicinally approved drug in Canada and Europe, but not in the USA [9].

The potential use of cannabis has led to legalization changes in some countries and since October 2018, cannabis recreational use has been federally legalized in Canada [10].

### 1.1.2 Composition of cannabis extracts

Secondary metabolites are extremely diverse chemicals produced by plants and they have a significant role in plants survival in their environment. There are at least 545 identified compounds in cannabis plant's including cannabinoids, terpenoids, flavonoids, and phytosterols and numerous other compounds responsible for each cultivar's unique qualities and medicinal effects [1].

#### 1.1.2.1 Cannabinoids

Cannabinoids are terpenophenolic compounds that are predominantly produced and accumulated in the glandular trichomes of pistillate (female) cannabis plants but can also be produced by male plants [11]. Cannabigerolic acid (CBGA) is the first formed cannabinoid. It converts to tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA) which are then decarboxylated to delta-9tetrahydrocannabinol (Δ9-THC), cannabidiol (CBD), and cannabichromene (CBC),

respectively in the presence of heat or light [12]. Moreover, cannabinoids can undergo oxidation and transform into other cannabinoids, such as, for example conversion of THC to cannabinol (CBN) [12].

Over 100 phytocannabinoids have been identified so far, however, only a few of them are abundant and believed to be active [12]. These compounds are oxygen containing aromatic hydrocarbons, and one of the most well-known of them is delta-9tetrahydrocannabinol (Δ9-THC), a principle psychoactive compound in cannabis, and its isomer (Δ8-THC) [1].

Non-psychotropic phytocannabinoids are CBD which modifies THC diverse effects, CBN that exist particularly in aged samples, cannabigerol (CBG), CBC, Δ9-tetrahydrocannabivarin (THCV), and various other phytocannabinoids [1, 13].

#### 1.1.2.2 Terpenes

120 out of 545 identified chemical compounds in *C. sativa* L. are terpenes, most of them being 10 carbon monoterpenes or 15 carbon sesquiterpenes [14, 15].

Like cannabinoids, terpenoids are also formed in glandular trichomes. They are typically accumulated in cannabis flowers up to 2-5% of dry weight [16]. The general precursor of all terpenes, 5-carbon isoprenoid diphosphate, is produced through two pathways, plastidial methylerythritol phosphate (MEP) and the cytosolic mevalonate (MEV). The first pathway leads to monoterpenes formation and the latter contributes to sesquiterpenes production [11]

Myrcene, (+)-α-pinene, (-)-limonene, (+)-β-pinene, terpinolene, and (E)-β-ocimene are the most frequent monoterpenes in *Cannabis sativa*, and β-caryophyllene and α-humulene are the most frequent sesquiterpenes [11]. Reports have shown that terpenes may

have anti-inflammatory, antioxidant, analgesic, antibiotic, anti-allergic, and anticancer effects [17].

### 1.2. Endocannabinoid system

Endocannabinoid system consists of endocannabinoids, G-protein coupled receptors (CB1, CB2), and the enzymes for synthesis and degradation of endocannabinoids. The most studied endocannabinoids are N-arachidonoylethanolamine (AEA), and 2-Arachidonoylglycerol (2-AG). The ECS plays a crucial role in a variety of processes, including pain, sleep, immune function, metabolism, and generally in maintaining body homeostasis [18]. Most cancer cell types express cannabinoid receptors, demonstrating the importance of studying endocannabinoid system for treating cancer tumors [19].

#### 1.2.1 Endocannabinoids

AEA and 2-AG are the best-known endocannabinoids. However, other arachidonic acid derivatives are also proposed to be endogenous cannabinoids. Endocannabinoids are produced in response to the increment changes in intracellular Ca2+ concentrations, from the plasma membrane of postsynaptic cells, followed by binding to cannabinoid receptors and reducing the release of the neurotransmitters [20, 21].

AEA is formed from the N-Acylphosphatidylethanolamine (NAPE) by a phospholipase D-like enzyme (NAPE-PLD); after being taken up by cells, AEA is degraded to arachidonic acid and ethanolamide by an intracellular fatty acid amide hydrolase (FAAH) enzyme, while 2-AG is formed mainly by hydrolyzation of diacylglycerol (DAG) by diacylglycerol lipase (DAGL), and it is finally hydrolyzed by monoacylglycerol lipase (MAGL) to arachidonic acid and glycerol [22]. Endocannabinoids can also be metabolized through other pathways. 2-AG, for example, can be oxidized by Prostaglandin-

endoperoxide synthase 2 (COX-2) under certain circumstances and produce prostaglandin glycerol esters [23].

### 1.2.2 Cannabinoid receptors

CB1 and CB2 receptors are known as main cannabinoid receptors. They belong to the G Protein-Coupled Receptor (GPCR) superfamily and share 44% amino acid sequence identity [24]. AEA and 2-AG, bind with different affinities to cannabinoid receptors. The expression of CB1 receptor is abundant in central nervous system. It is also present in peripheral nerve terminals and in many extra-neural sites, but at much lower levels [25]. By contrast, CB2 is preferentially expressed in immune and blood cells, but it has also been shown to be expressed in other tissues [26].

There is evidence indicating that endocannabinoids might apply their effects also through non-classical receptors, including the transient receptor potential vanilloid receptor 1 (TRPV1), TRP vanilloid 2 (TRPV2), certain orphan G protein-coupled receptors, GPR55, GPR119 and GPR18, peroxisome proliferator-activated receptors (PPARs), and many others [27-29].

TRP vanilloid receptors are involved in the transduction of stimuli, such as the sensation of temperature, pressure, smell, vision and taste [30]. Beside endocannabinoids, CBD and CBG, but not THC, are known as TRPV1 full agonists, and CBD has even a higher binding affinity for TRPV1 than CB1 and CB2 [12, 27, 31]. Also, CBD and THC are known as activators of TRPV2 [27, 32].

Studies have further reported CBD as an antagonist and 2-AG and AEA as potent activators of GPR55 [33, 34]. It was found that CBD could be an antagonist of CB1 and an inverse agonist of CB2 receptor [12, 35]. THC is known as a full agonist of GPR18

receptors, and TRPV1 and TRPV2 are demonstrated to be involved in THC-, CBD-, and CBN-modulated cell fate [12, 36].

### 1.2.3 Endocannabinoid system of skin

Skin is the largest human organ and has an important role in both innate and adaptive immune system. Components of ECS have been discovered on most of the cutaneous cell types; hence, endocannabinoid system contributes to healthy physiological functions of skin by regulating cutaneous cells growth, differentiation and survival, and its dysregulation could lead to skin disease such as, fibrosis, cutaneous contact allergic dermatitis, and skin cancer [18, 37-39].

CB1 and CB2 were immunodetected in melanocytes, dermal fibroblasts, sweat glands, skin nerve fibres, hair follicles, and vascular endothelium [37, 40-42]. Moreover, the expression and function of CB receptors has been also demonstrated in mast cells, macrophages, and in T and B cells presence in the skin [40, 43].

TRPV1, as another important receptor in ECS signalling, was also to be expressed in numerous cells of human skin including basal and suprabasal keratinocytes, dermal mast cells, Langerhans cells, sebocytes, hair follicle, and sweat glands, but not in melanocytes [44-48]. However, other data show the presence of TRPV1 in primary melanocytes [49].

The expression of AEA and 2-AG has also been confirmed in keratinocytes, melanocytes, and fibroblast [49-51].

### 1.2.4 Endocannabinoids and cancer

Many studies suggest that endocannabinoid receptors and their endogenous ligands are overexpressed in tumor tissue and this upregulation correlates with disease progression

and tumor aggressiveness. However, the biological role of endocannabinoid system is not completely understood yet [52].

In agreement with this, upregulation of CB1 receptor was associated with poor prognosis for pancreatic cancer patients and with disease severity in prostate cancer [53, 54]. High expression of CB2 receptor was observed in variety of cancer types, e.g., melanoma, breast cancer, and in bladder tumors comparing to healthy tissue [55-57]. Moreover, endocannabinoids were found at higher concentrations in colorectal cancer, and 2-AG was found to be upregulated in glioma and in prostate cancer [58-60]. Increased concentration of AEA was reported in hepatocellular cancer, while meningioma tumor cells showed lower levels of AEA compared to healthy tissue [61, 62]. Analysis of plasma samples obtained from 304 patients suffering from several types of melanoma cancer further revealed decreased levels of AEA and increased levels of 2-AG. Also, upregulated amounts of 2-AG in mice paralleled the disease progression [39].

Thus, it can be considered that over-expression of ECS receptors and endocannabinoids may be pro-tumorigenic. In support of this, CB1/2 receptors deficit mice showed a high resistance to UVB-induced skin carcinogenesis. This shows that cannabinoid receptors are required for UV-induced inflammation and skin cancer development [63].

However, anticancerogenic properties of ECS were also observed in many studies. In agreement with this, the endocannabinoid-degrading enzymes were detected to be upregulated in cell lines and human tumors, and AEA was found to have cytotoxic effects on melanoma and breast cancer through the induction of apoptosis and cell cycle arrest, respectively [64, 65].

### 1.3. Cannabis uses in cancer

Nowadays, cannabis is known as a potent therapeutic cancer agent. For the first time, more than 40 years ago, Munson et al. demonstrated that THC could inhibit lung cancer cell growth in vivo [66].

Cannabinoids modulate cell cycle and signalling pathways by mimicking endogenous substances. They have demonstrated antineoplastic effects in preclinical research in a wide variety of cancer cells and some animal models. There is some controversy regarding the cannabinoids effects on tumor cells as published data obtained in lung, brain and genitourinary carcinoma cell lines demonstrated that while high concentrations of cannabinoids exert antiproliferative effects, low concentrations may result in cancer cell proliferation [67]. These effects, however, are very cancer specific, and are dependant on the type of tissues, the affected pathways, and perhaps additional underlying mutations. Also, different cannabinoids may exert very different effects on cancer cells in vitro and be different from those effects in vivo.

Much information is yet to be obtained concerning the benefits and drawbacks of the cannabinoids, but cannabis have already shown to have some properties as a single agent therapy or adjunct drug for cancer [3].

#### 1.3.1 Cannabinoids as a co-therapy

Cannabinoids are known to exert palliative effects in cancer patients by reducing chemotherapy-induced nausea and vomiting, stimulating appetite, and pain relief [1, 3]. The THC capsule, Dronabinol (Marinol; Solvay Pharmaceuticals), and its synthetic analogue, Nabilone (Cesamet; Meda Pharmaceuticals), are prescribed for reducing the nausea and vomiting induced by chemotherapy [68]. Dronabinol is also used for anorexia

in patients with AIDS [68]. Moreover, THC and CBD combination, nabiximols (Sativex; GW Pharmaceuticals), is approved for the treatment of neuropathic pain in adult patients with multiple sclerosis and management of pain in adult patients with advanced cancer [69, 70].

The combined use of cannabinoids with conventional treatment methods results in augmented effects, dose reduction of each drug, and thus reduction of the side effects [71]. For instance, cannabinoids and synthetic cannabinoids are demonstrated to induce synergistic inhibitory effects by enhancing autophagy in pancreatic cancer, glioblastoma multiforme, and colorectal cancer tumor when combined with chemotherapy methods. As an example, combined consumption of THC and temozolomide is a potent antitumor strategy in glioma xenograft model and in temozolomide-resistant tumors [72-74]. A murine model demonstrated that combined with radiation therapy, THC and CBD further enhance the antitumor effects against glioma [75].

Moreover, cannabinoids have a potential role in sensitizing cells to chemotherapy as they can modify the expression of ABC efflux pumps which induce chemotherapy resistance [76]. For instance, combined administration of THC with cytotoxic agents enhance the sensitivity of leukemia cells to chemotherapy [77].

### 1.3.2 Antiproliferative effects of cannabinoids

A plethora of data reports the antiproliferative effects of cannabinoids on cancer cell lines and in some in vivo mouse models. This outcome is achieved by activating different receptors and modulating numerous pathways, but often results in the same fate for the tumor cell. However, antitumor effects of cannabinoids depend on their concentration and type of cancer, among others [71, 78].

Induction of cell cycle arrest, autophagy and apoptosis are identified as mediators of cannabinoids antiproliferative action in melanoma [79, 80].

Despite numerous experimental studies that demonstrate the antiproliferative effects of cannabinoids, there are also few reports indicating tumour promoting effect of cannabinoids in melanoma cancer [67, 81, 82]. As an example, Carpi et al. demonstrated the tumor promoting effects of CB1 receptor and claimed that silencing of CB1 receptor significantly reduced the number of viable melanoma cells as compared to control cells [81]. As another example, cannabinoid receptors type 1 and 2 were directly activated by UV irradiation and absence of those receptors in mice decreased the in UVB-induced skin carcinogenesis [63].

#### 1.3.2.1 Autophagy induction

Autophagy is a degradative process that could serve as a survival or a death pathway depending on the molecular expression profile of the cell type. There are three types of autophagy: microautophagy, macroautophagy (or autophagy), the most frequent form of autophagy, and chaperone-mediated autophagy. Autophagy, at least in solid tumors, could trigger apoptosis and it is believed that autophagy is upstream of apoptosis in cannabinoids-induced cell death [71, 79].

The PI3K/AKT/mTOR signaling pathway and AMPK are responsible for the induction of autophagy. Protein kinase b (AKT) is a prooncogenic protein that mediates cell survival, and its modulation is critical for autophagy and apoptosis induction. AKT could positively regulate mTOR and they both are repressors of autophagy. AMPK activation, however, decreases mTOR and phosphorylates p53 which will result in autophagy induction and cell cycle arrest, respectively [83, 84].

Administration of cannabinoids has been shown to increase ceramide level, induce ER-stress, and activate autophagy via TRB3 inhibition of the AKT/mTOR axis and stimulation of AMPK in glioma, hepatocellular, and breast cancer [85-87].

#### 1.3.2.2 Apoptosis induction

Autophagy mediated apoptosis is one of the main mechanisms of action of cannabinoids in apoptosis induction, however, there are other mechanisms that accelerate the programmed cell death.

Cannabinoids triggered modulation of MAPK pathways (p38 MAPK, JNK, and ERK1/2) or inhibition of PI3K pathway are responsible for enhancing apoptosis [77, 88].

Moreover, ceramide synthesis could promote apoptosis. Ceramide is responsible for cells proliferation and programmed cell death. CB1 and CB2 activation regulates *de novo* synthesis of ceramide and enhance the mitochondrial intrinsic pathway which results in canonical apoptosis [88, 89]. Similarly, cannabinoid receptors activation has shown to induce cell death in prostate, pancreatic, and lymphoma cancer [90].

Reactive oxygen species (ROS) increased production has also been associated with apoptosis induction. CB and TRP vanilloid receptors activation by cannabinoids are responsible for the formation of ceramide and ROS [71]. Ceramide, also, could augment the formation of ROS. These two intracellular mediators induce the ER-stress by increasing the activity of ER-stress mediators like CHOP, P8, TRB-3, and GRP-78 and lead to mitochondrial intrinsic apoptosis [71]. Similarly, activation of CB2 receptor in pancreatic cancer cells causes *de novo* synthesis of ceramide that leads to up-regulation of p8, TRB-3, and ATF-4 and induces apoptosis [91].

There is evidence that while THC induces apoptosis in a CB receptor-dependant manner, CBD exerts its proapoptotic effects by activating TRPV2 receptors and is independent from CB receptors [78].

#### 1.3.2.3 Cell cycle arrest

The other crucial pathway involved in cannabinoids anti-tumor effect is cell cycle arrest. In line with this hypothesis, activation of CB receptors in gastric cancer cells on the one hand, have shown to downregulate p-AKT and activate ERK1/2; on the other hand, have shown to increase ROS production which result in G0/G1 cell cycle arrest [92, 93].

Similarly, CB receptors activation-induced cell cycle arrest at G0/G1 phase of melanoma cells is accompanied by an increase in the expression level of P53 and P27 and key intermediate protein 1 (KIP1) [55]. Conversely, two other studies showed that CB1 receptor silencing precedes G1/S phase cell cycle arrest in two melanoma cell lines and a selective CB1 receptor antagonism (AM251) promotes cell cycle arrest at G2/M in a melanoma cell line [81, 94].

### 1.3.3 Antiangiogenic effects of cannabinoids

Tumor progression is dependent on the adequate supply of oxygen and nutrients. For that purpose, angiogenesis is essential for cancer progression and inhibition of that is known as a way of slowing down the cancer [95, 96].

Cannabinoid administration could mitigate tumor angiogenesis and impair vascularization at microenvironmental sites of malignant tissues. These effects are accompanied by a reduced expression level of vascular endothelial growth factor (VEGF), VEFG receptors, placental growth factor (PlGF), and angiopoietin-2 (Ang-2). Casanova et al. in vivo experiment on skin tumors has demonstrated that CB receptors activation on the

one hand, could alter the vessel morphology, on the other hand, could significantly decrease the blood vessel size thereby impairing the tumor vascularization [7, 41]. Moreover, CBD-treated mice model of malignant gliomas, AEA-treated breast cancer cell line, and THC- or CBD-treated mice xenograft of lung cancer all have shown the inhibition of angiogenesis and pro-angiogenetic factors [97-101].

### 1.3.4 Anti-invasive effects of cannabinoids

Invasion and metastasis of aggressive tumor cells is the determinant and fatal step in cancer progression. Metastasis is represented by detachment of cancer cells from primary tumors, migration, invasion, intravasation, attachment at a distant location, extravasation, and formation the secondary lesions [102].

Evidence indicates that cannabinoids effects on tumor cells invasiveness and metastasis potential are mediated by modulating Matrix metalloproteinase (MMPs) (e.g. matrix metaloproteinase-2 (MMP-2) blockage), downregulation of ld-1 expression, upregulation of tissue inhibitor of matrix metalloproteinases (TIMP)-1, and mitigation the markers of epithelial-to-mesenchymal transition (EMT), among others [103-106].

Likewise, cannabinoid administration could inhibit the migration of breast cancer cells. In melanoma, CB1 and CB2 receptors activation has shown to decrease the metastasis potential. CB2 receptor activation also could prevent melanoma cancer cells transmigration through the Blood-Brain Barrier therefore reducing brain metastases potential [107-109].

### 1.3.5 Terpenes effects on cancer

Most of the cannabis effects are dependent on cannabinoids, but not all medicinal potential is attributed to cannabinoids. Diverse clinical and in vitro studies indicate that terpenes have biomedical properties, such as antioxidant, anticancer, antitumor, and anti-

inflammatory, among others. They often induce the anti-cancer activity by pro-apoptotic actions, but they do not affect the normal cells and tissues [17]. Moreover, terpenes are well tolerated and have very low toxicity without side effects [17].

In agreement with this assumption, limonene has been recognized to inhibit the progression of pancreas, stomach, colon, skin, and liver cancer in animal models. In prostate cancer, co-administration of limonene with conventional drugs could inhibits the tumor growth more effectively through increasing the reactive oxygen species (ROS) generation and the caspase activity [110].

Similarly, there has been increasing body of evidence that other terpenes could inhibit tumor progression. Tomko et al. 2020, have reported that myrcene is cytotoxic against human cervical carcinoma, lung carcinoma, and colon adenocarcinoma [16]. β-Caryophyllene was reported to inhibit the cell cycle progression in G1 phase in lung cancer cells [111]. Other studies revealed that β-elemene and linalool could induce apoptosis in lung and oral cancer cells respectively [112, 113]. Humulene, as one of the core cannabis terpenes, was shown to enhance the antiproliferative effect of the conventional drugs in colon and ovarian cell lines [16].

### 1.3.6 The entourage effect

For the first time, In 1998, Professors Raphael Mechoulam and Shimon Ben-Shabat reported that in the endocannabinoid system, a variety of inactive molecules increase the activity of the endogenous cannabinoids anandamide and 2-arachidonoylglycerol [114]. Then, they used this to demonstrate the reason why the botanical drugs are usually more effective than their isolated components [115]. In cannabis extract also, where all its secondary metabolites come together, a synergistic relationship is formed, and this is called

the “entourage effect”. For instance, limonene induces apoptosis synergistically with CBD and CBG in breast cancer cells, and α-pinene and β-myrcene anti-inflammatory effects are more efficient when combined with CBD [116].

In addition, combinations of CBD with THC are more effective in reducing melanoma and glioblastoma cells proliferation than applying THC or CBD alone [79, 117]. Generally, cannabis secondary compounds modulate the anxiety, psychoses, motor discoordination, and other THC-induced unwanted effects and could boost the THC beneficial effects [4]. They could also reduce the effective dose of THC and enhance the tolerability of cannabis-based medicine [4].

There is evidence indicating that high THC extracts inhibit melanoma and breast cancer cells more efficiently than THC alone [118, 119]. Moreover, a high CBD extract exhibited significantly higher cytotoxicity than pure CBD in a glioma cell line [120]. Therefore, cannabis extract might be more efficacious in cancer treatment comparing to THC or CBD individually.

### 1.4. Melanoma

Skin malignancies are one of the most common types of cancer in young adults. There are three major types of skin cancer: basal cell carcinoma (BCC), squamous cell carcinoma (SCC), which are known as non-melanoma skin cancer (NMSC), and melanoma.

Melanoma is a malignant skin tumor that arises from uncontrolled proliferation of melanocytes. 91.2% of melanomas are cutaneous, however there is an uncommon form of this cancer known as non-cutaneous melanoma that occurs most commonly at eye and mucosa [121].

### 1.4.1 Melanoma epidemiology

Melanoma is the least common but deadliest form of skin cancer [122]. The prevalence of this cancer varies between populations and GLOBOCAN (2020) has determined that the melanoma incidence rate ranges from 0.39 per 100,000 persons in South-Central Asia to 35.8 per 100,000 persons in Australia and New Zealand [123]. It was estimated that in 2021, 8,700 Canadians will be diagnosed with melanoma [124]. Worldwide, 0.7% of all cancer deaths are due to malignant melanoma annually, and the incidences have increased dramatically by more than 250% in the last four decades [125]. It will keep rising at least until 2022 in Norway, Sweden, the UK, and the USA. Although, the melanoma incidences have been decreasing since 2005 in Australia; also, it is anticipated that New Zealand will experience the same trend in the next four years [126].

Skin cancer is a global major public health concern and beside high rate of occurrence and mortality, it is a financial hardship not just for patients, but also for the governments. Considering direct medical costs and indirect costs related to lost productivity, melanoma will account for 75.5% of total economic burden of all skin cancers in Canada by 2031, with over 690 million dollars spent annually [127].

### 1.4.2 Melanoma pathophysiology

Cancer develops from genetic instability. Firstly, initial mutations stimulate cell proliferation, and when followed by mutations in cell cycle controlling genes, DNA repair and apoptosis genes, it could result in cancer [128].

Numerous gene alterations are observed in melanoma cancer, many of them being derived from UV exposure. These mutations are characterized by a high abundance of the cytosine to thymine transitions indicative of UV mutagenesis [129]. However, two

important somatic mutations, BRAF and NRAS, which are present in benign nevi are not C>T transitions [129]. BRAF alterations (presents in 50-60% of melanomas) are mostly found in melanomas that are caused by intermittent sun exposure, whereas NRAS mutations (presents in 15% of melanomas) are present in patients with chronic sun damage of the skin [128, 130].

BRAF (Val600) mutation is known as a typical feature of benign nevus formation. For melanoma progression into intermediate lesion and obtaining invasive potential, however, additional mutations are required: NRAS, telomerase reverse-transcriptase (TERT) promotor, cyclin-dependent kinase-inhibitor 2A (CDKN2A, cell cycle controlling gene), and AT-rich interaction domain (ARID)1A, ARID1B, ARID2 (chromatin-remodelling genes) [129, 131-133].

Metastatic phenotype in melanoma is correlated with mutations in phosphatase-and-tensin homologue (PTEN) and tumor-protein p53 (TP53). The copy-number alterations are also invariably present in invasive melanoma [133].

### 1.4.3 Melanoma treatment

Therapeutic options for melanoma are different and they are used based on the tumor features such as stage, location, and genetic profile. Surgery is a primary treatment followed by adjuvant therapies like chemotherapy and targeted therapy [134, 135].

#### 1.4.3.1 Chemotherapy

Chemotherapy was the first proposed therapy for malignant melanoma. Dacarbazine and Temozolomide are prescribed as approved chemotherapy medication for advanced melanomas. However, chemotherapy resistance occurs because of acquiring resistance to apoptosis in melanoma tumors [134, 136]. Electrochemotherapy is a technique

which uses a combination of bleomycin and cisplatin with high-intensity electric pulse, to stimulate the drug delivery into the tumor cells. There is evidence that this procedure is useful in the treatment of cutaneous and subcutaneous nodules of melanoma [137-139].

#### 1.4.3.2 Immunotherapy

Immunotherapy induces longer response duration compared to other therapies [140]. Immunotherapy approaches include using cytokines like high-dose Interferon (IFN) α-2b, Interleukin-2 (IL-2), and Peginterferon α-2b (Peg-IFN) which is a combination of IFN α-2b with the molecule polyethylene glycol [141-143]. Moreover, targeted antibodies like Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade and Programmed cell death protein 1 (PD-1)/ PD-1 ligand (PD-L1) blockade are used to stimulate T-cell activation in metastatic melanoma [145]. Other immunotherapy approaches are vaccines such as gp100 Peptide [144].

#### 1.4.3.3 Targeted therapy

Mutations in genes responsible for signaling pathways are frequent in melanoma. About 50% of melanoma patients harbor a BRAF mutation. Vemurafenib has shown the overall response rate of 53% in metastatic melanoma patients [145]. Vemurafenib, dabrafenib, and encorafenib, are FDA approved BRAF-mutant inhibitors, and are used in clinical trials in monotherapy and in combination with other therapies [146, 147]. BRAF inhibitors administration improves the overall survival, but almost all melanomas expand resistance mechanisms to these drugs. MEK inhibitors are used to target the downstream of BRAF and overcome the tumor resistance. Other approaches that are used to overcome BRAF inhibitor resistance include AKT or mTOR inhibition, and combined inhibition of PI3K and mTOR [148, 149].

Other targeted therapies are CKIT inhibitor, VEGF inhibitor, PI3K/AKT/mTOR pathway inhibitors, and CDK inhibitors [150-154].

By developing new treatment methods, better outcomes are achieved for advanced melanoma patients, however, the current therapies effectiveness is reduced because of development of numerous resistant mechanisms by tumors and short response duration. To aim to solve this problem, combined application of chemotherapy, immunotherapy, and targeted therapy might be an appropriate strategy [134]. Despite development of new treatments for melanoma cancer, there is an increased demand for more effective therapeutic strategies for this malignancy.

Previous data suggest that cannabinoids are promising therapeutic agents against the progression of various types of cancers including melanoma cancer [79, 80, 107]. Terpenes were also found to have anticancer effects [17]. Considering the entourage effect that will likely occur when whole flower extracts are used, we decided to test some of our cannabis extracts on melanoma cancer cells and study their effect on melanoma and normal melanocyte cells viability.

## 2.HYPOTHESIS

In this study, we hypothesized that cannabis extracts will reduce the viability of melanoma cancer cells, and that the extracts reduce the cell proliferation more efficiently than pure THC or CBD alone.

# 3.MATERIALS AND METHODS

## 3.1 Main reagents

Δ9 -THC (Cat#T4764) and CBD (Cat#C-045) and were purchased from Sigma. 1.0 mg/ml stock solutions were prepared by dissolving cannabinoids in methanol and stored at -20℃. DMSO (Dimethyl sulfoxide anhydrous) was purchased from Thermo Fisher Scientific (Cat#D12345) and was used to make 60 mg/ml cannabis extracts and 1 mM vemurafenib stock solutions.

## 3.2 Cell lines

The human cutaneous melanoma cancer cell line A375 (CRL-1619), the primary normal melanocytes; normal, human, adult (HEMa, PCS-200-013), and the hTERT-immortalized human fibroblast foreskin cell line BJ-5ta (CRL-4001) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). A375 and BJ5ta cells were cultured in DMEM medium supplemented with a final concentration of 10% heat-inactivated Premium Grade Fetal Bovine Serum (Cat# 97068- 085; VWR International LLC, Radnor, USA). HEMa cells were cultured in dermal cell basal medium (PCS-200-030) supplemented with adult melanocyte growth kit (PCS-200-042). Cells were maintained at 37℃ in a humidified atmosphere containing 5% $CO_2$. The cells culture medium was changed to fresh medium every 2 or 3 days and cells were incubating until the confluency reached 80-90% for further experiments.

## 3.3 Treatments

The cannabis plants were grown, flowers were harvested, and extracted with ethanol in a licensed facility at the University of Lethbridge. After evaporation, resin was dissolved in DMSO to the concentration of 60 mg/ml and stored at -20°C. Cultivars number #4, #6,

#8, #10, #14, #20, #45, #130, #131, #132 were chosen for the experiments based on the previous data from the lab – these cultivars were pre-selected based on their capacity to inhibit the growth of breast cancer cells. Different concentrations of the treatments (7 μg/ml, 15 μg/ml, and 30 μg/ml) were prepared by diluting the extracts in the complete media; these concentrations were shown to be effective for the inhibition of the growth of breast cancer cells.

A range of concentrations of pure THC and CBD were obtained by diluting the cannabinoids in the fresh complete media. Vemurafenib (Cat#S1267; Selleckchem, Houston, USA) was used as the positive control as it is an approved FDA drug for the treatment of melanoma cancer harboring BRAF (V600e) mutation [155]. Different concentrations were tested to determine the $IC_{50}$ value of Vemurafenib for A375 melanoma cells (0.05 μM to 1.6 μM) solutions these concentrations were prepared by dissolving Vemurafenib powder in DMSO and then diluting it in the complete media. All the treatment samples were filtered with 0.22 μm filter after being diluted in the growth media, and were kept at 4°C.

**Table1. Summary of *Cannabis sativa* extracts used for the experiment.**

| High THC C*annabis sativa* extracts | High CBD C*annabis sativa* extracts |
|---|---|
| #4 | #6 |
| #8 | #10 |
| #14 | #20 |
| #132 | #45 |
| | #130 |
| | #131 |

### 3.4 Terpene analysis

The analysis of terpenes of extracts #14 and #132 was done on dry flowers with help of 8610C GC coupled with a flame ionization detector (SRI Instruments at Canvas Labs, Vancouver, BC, Canada). Extracts were sent to Canvas Labs at different time points.

### 3.5 High performance liquid chromatography (HPLC)

The levels of cannabinoids (CBD and THC) were analyzed with Agilent Technologies 1200 Series HPLC system. The system had the G1315C DAD, G1316B column compartment, autosampler (G1367D), and the binary pump (G1312B). The Phenomenex Kinetex EVO C18 column with a Phenomenex SecurityGuard ULTRA guard column was used for the separation. The data acquisition, control of the instrument, and integration were performed with help of software, ChemStation LC 3D Rev B.04.02

(Agilent Technologies). For calibrating the standards and analysis of samples, the injection volume of 2 µL was used. The detection of compound peaks was done for 230 nm and 280 nm. On the A side, the mobile phases included 50 mM ammonium formate (Sigma-Aldrich) in HPLC grade water (Fisher Chemical), and 100% methanol on the B side. The flow rate was 0.3 ml/min. Per each cultivar, two samples were analyzed, with two technical repeats for each sample.

### 3.6 Protein extraction and quantification

Cells were incubated until they reach the 80-90% confluency in 10 cm petri dishes and they were washed twice with 2ml of cold PBS. For extracting the total protein, cells were collected using a cold cell scraper and after transferring cell lysate to a 1.5 mL microcentrifuge, cells were resuspended in 120 µl of RIPA lysis buffer with 50 mM Tris-HCl (PH 7.4), 1mM EDTA, 150 mM NaCl, 1% Na-Deoxycholate, 1% Triton X-100, 0.1% SDS (10 µl protease inhibitor cocktail solution [Cat#04693116001] was added per 1 ml of RIPA lysis buffer before use) and incubated on ice for 15 minutes. Samples were bead beat in a Bullet Blender (Next Advance, Averill Park, NY) at speed 2 for 10 seconds and incubated on ice for 5 seconds this step was repeated three times. After this samples were bead beat again at speed 3 for 10 seconds and then incubated on ice for 5 seconds this step was repeated twice. As the last step for extracting the cells protein, the samples were centrifuge for 10 minutes at 13,000 × g at 4℃. The supernatant was carefully aliquoted into new microcentrifuge tubes and kept at -20℃ for further analysis. Protein concentration was determined by Bradford assay. Briefly, 2 µl of cell lysate was diluted with distilled water in a 1:20 ratio and 25 µl of the diluted sample was added to 1.25 ml Bio-Rad assay reagent, and then incubated at room temperature for 5 minutes. Finally, the protein

concentration was determined by a NanoDrop 2000/2000c Spectrophotometer (ThermoFisher Scientific Company, Wilmington, DE).

### 3.7 Electrophoresis and Western blot analysis

An equal concentration of samples (100 μg) was achieved by adding loading buffer and RIPA buffer to samples. samples were then incubated for 10 minutes in 95℃. The Prestained Protein Ladder (Cat#26620; Thermo Scientific, Massachusetts, USA) and samples were then loaded in a 10% (v/v) SDS-PAGE (poly-acrylamide gel electrophoresis) gel and electrophoresis was run for 1 and half hour at the voltage of 90 V to separate the proteins.

The protein bands were transferred to a PVDF o polyvinylidene difluoride membrane (GE Healthcare Biosciences) by running the Western blot for 2 hours at 100 V at 4℃. The membrane was blocked in 5% (w/v) non-fat dry milk in BPST (phosphate-buffered saline with 0.1% (w/v) tween) for 1 hour on a shaker at room temperature. The membranes were incubated with the primary antibodies overnight on a shaker at 4℃.

CB1 receptor expression was determined using the mouse monoclonal CB1 Antibody (2F9) (Cat#sc-293419; Santa Cruz Biotechnology, Texas, United States; 1:500 dilution), and CB2 receptor expression was measured by mouse monoclonal CB2 Antibody (3C7) (Cat#sc-293188; Santa Cruz Biotechnology, Inc., Texas, United States; 1:500 dilution). p-ERK1/2 levels were detected using a rabbit monoclonal phospho-p44/p42 MAPK (ERK 1/2) antibody (Cat# CS-9101, Cell Signaling Technologies, Massachusetts, United States; 1:1,000 dilution). ERK1/2 expression levels were detected using a rabbit monoclonal p44/p42 MAPK (ERK1/2) antibody (Cat#CS-9102, Cell Signaling Technologies, Massachusetts, United States; 1:1,000 dilution). pAKT1/2/3 levels were

detected using a mouse monoclonal p-AKT1/2/3 antibody (Cat#sc-271966, Santa Cruz Biotechnology, Inc., Texas, United States; 1:250 dilution). AKT1 expression levels were detected using a mouse monoclonal AKT1 antibody (Cat#sc-377457, Santa Cruz Biotechnology, Inc., Texas, United States; 1:200 dilution). GAPDH antibody (Cat#sc-47724; Santa Cruz Biotechnology, Inc., Texas, United States; 1:500 dilution) was used as a loading control. The membranes were then washed with 0.1% Tween-20 in PBS (PBS-T) and incubated with Bovine anti-mouse secondary antibody (Cat#sc-2371; Santa Cruz Biotechnology, Inc., Texas, United States; 1:1000 dilution) for 2 hours on a shaker at room temperature. Following by three washes with PBS-T, UltraScence Pico/Femto Ultra Western Substrate (Cat# CCH345- B, FroggaBio, Inc., Ontario, Canada) was added to the membranes and they were visualized using the FluorChem HD2 Imaging System (Cell Biosciences, California, United States). Data were analysed from three repeats of Western blot by ImageJ program.

**3.8 Cell proliferation assay (MTT)**

Cell viability was measured by MTT (3-((4,5)-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium) assay. For that, cells were incubated until they reach the 80-90% confluency in a 10 cm petri dish. A375 and BJ5ta cells were trypsinized by TRYPSIN/EDTA (0.25% Trypsin and 2.21 mM EDTA-4Na; Cat#325-043-EL; WISENT Inc., Quebec, Canada) and HEMa cells were trypsinized by Trypsin-EDTA for Primary Cells (PCS-999-003; ATCC, Virginia, USA) and then neutralized by Trypsin Neutralizing Solution (PCS-999-004; AYCC, Virginia, USA) as suggested in ATCC. After trypsinization and centrifuging, fresh media was added to the cells, and they were plated in 96-well plates with the following density: A375 cells with $2 \times 10^3$ cells/well in 100 µl culture medium, BJ5ta cells with 6 ×

$10^2$ cells/well in 100 µl cultural medium, HEMa cells with $2 \times 10^3$ cells/well in 100 µl cultural medium. Cells were then maintained at 37℃ in a humidified atmosphere containing 5% $CO_2$ for 24 hours and then the media was replaced with fresh media containing the treatments. Treatment media were changed every 24 hours and the concentration of methanol and DMSO in culture media was normalized in all treatments.

After the specified treatment time (0, 1, 2, 3, 4 and 5 days) 10 µl of MTT kit I (#11465007001, Roche, Ontario, Canada) was added and plates were incubated at 37°C in the $CO_2$ incubator for 4 hours. At 4 h after incubation, 100 µl of MTT solution was added to each well and the plate continued to incubate at 37°C overnight. Absorbance was measured at 590 nm using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Results were calculated by comparing the treatments to the control treatment. All treatments were run in triplicate and each test was performed three times.

### 3.9 Statistical analysis

Data were analyzed using ImageJ and GraphPad Prism 8 software. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was done to analyze MTT results, and One-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's multiple comparisons test was done to analyze Western blot. Results were presented as the mean ± SD of data. Differences were considered statistically significant when $p < 0.05$.

# 4. RESULTS

## 4.1 Cannabinoids and terpenes profile of the used *Cannabis sativa* extracts

To confirm whether the extracts are high THC or high CBD, the cannabinoids percentage was analysed by HPLC (Table 2).

**Table 2. HPLC profile of the used *Cannabis sativa* extracts.**

| | Total THC, % | Total CBD, % | Total cannabinoids, % |
|---|---|---|---|
| #4 | 33.6 | 1.72 | 35.78 |
| #6 | 10.3 | 23.4 | 37.1 |
| #8 | 32.5 | 0.33 | 33.37 |
| #10 | 1.2 | 29.6 | 31.74 |
| #14 | 44.3 | 1.1 | 45.63 |
| #20 | 0.99 | 33.75 | 35.13 |
| #45 | 0.44 | 24.92 | 25.63 |
| #130 | 2.43 | 28.43 | 32.02 |
| #131 | 0.84 | 34.9 | 36.68 |
| #132 | 42.82 | 1.2 | 44.86 |

## 4.2 A375, HEMa, and BJ5ta cells express cannabinoid receptors type one and two

A375 melanoma cell line is one of the most frequently used cell lines in melanoma studies with 1663 PubMed citations [156]. BRAF V600 mutation is present in about 50% of melanomas [129], and A375 cell line harbors the BRAFV600E mutation which makes

this cell line a good candidate for melanoma research. The other used cell line, HEMa, derived from a normal melanocyte from adult donor, was used to check if our cannabis extracts have any effects on normal non-cancerous melanocyte cells or not. BJ5ta fibroblast cell line was also used as another normal cell which is present in the skin to be compared with the melanoma cancer cells.

To establish a cell model system that allows us to study the effects of *Cannabis sativa* extracts on cancer and normal cells, we performed Western blot analysis to determine the protein levels of CB1 and CB2 cannabinoid receptors in the cell lines. The results show that all examined cell lines express both CB1 and CB2 receptors which are the main cannabinoid receptors, and this make the chosen cell lines suitable for our research. Western blot results also showed that CB1 receptor expression is significantly higher in A375 cells compared to HEMa cells.

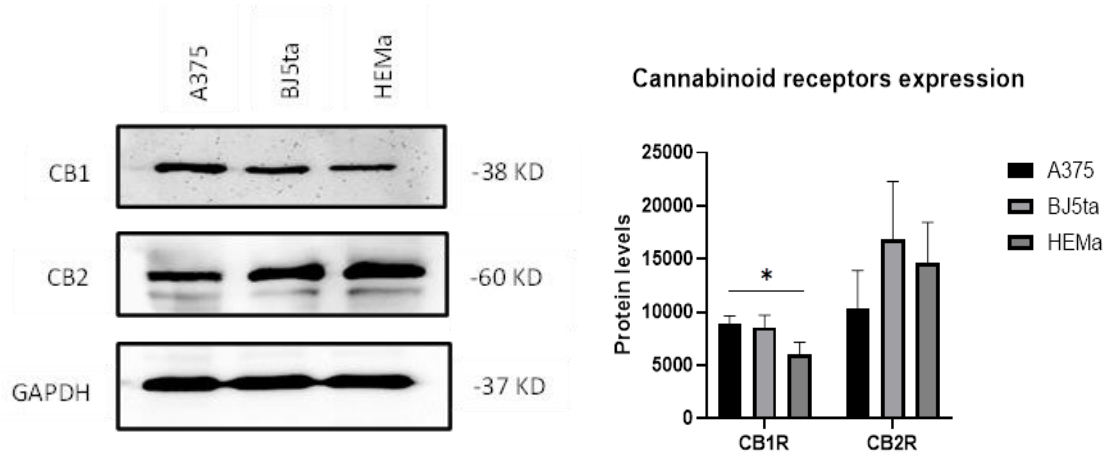
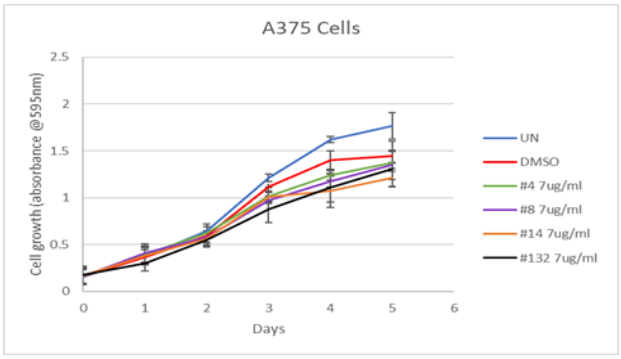


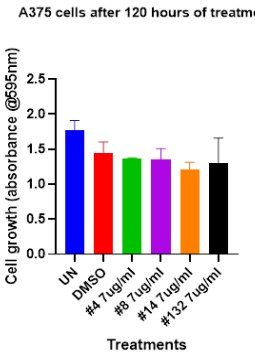

**Figure 1. Quantification of cannabinoid receptors in cancerous (A375) and non-cancerous (BJ5ta, HEMa) cells.** Statistical analysis was performed by ANOVA followed by Tukey's multiple comparisons test. Data are expressed as the mean ± SD (N=3), asterisks indicate significance ($p < 0.05$).

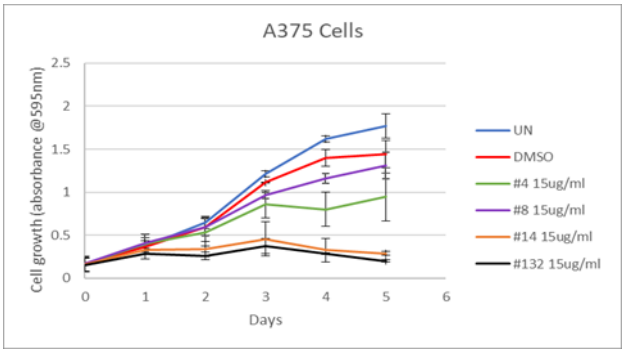
### 4.3 *Cannabis sativa* extracts suppress A375 cells growth in a time and dose dependent manner

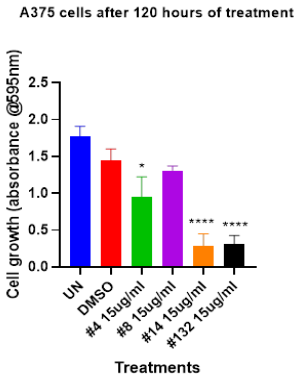
To study the potential antiproliferative effects of *Cannabis sativa* extracts on melanoma cancer cells, ten different cannabis extracts were tested on A375 cells. Melanoma cells were plated in 96-well plates as described in the methodology and they were treated with three different concentrations of the extracts (7 μg/ml, 15 μg/ml, and 30 μg/ml) for five days. The MTT assay or the cell proliferation assay measures the cell metabolic activity which is the indicator of cell viability. Measuring the cell viability of cells after treatment with extracts helps us to study the antiproliferative effect of the extracts on the tested cell lines. Four high THC extracts (extracts number #4, #8, #14, #132) and six high CBD extracts (extracts number #6, #10, #20, #45, #130, #131) were used in this experiment. MTT assay indicated that extracts number #4, #14, #20, and #132 have suppressed cell growth significantly in the concentration of 15 μg/ml, and all the extracts have reduced the cell growth significantly in the highest used concentration (30 μg/ml). Moreover, analyses revealed that there is no significant difference between the effectiveness of high THC extracts and high CBD extracts on A375 cells.

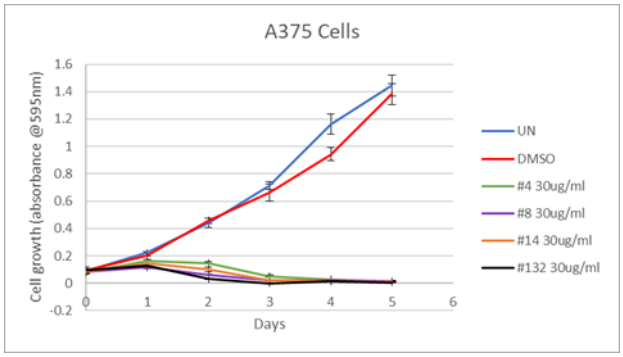
A.

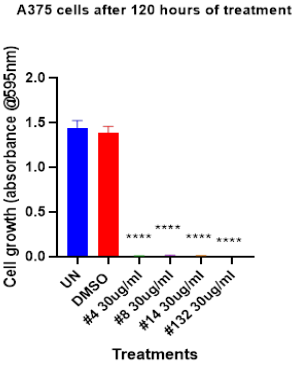

A375 Cells
Cell growth (absorbance @595nm)
Days
UN
DMSO
#4 7ug/ml
#8 7ug/ml
#14 7ug/ml
#132 7ug/ml


A375 cells after 120 hours of treatment
Cell growth (absorbance @595nm)
UN
DMSO
#4 7ug/ml
#8 7ug/ml
#14 7ug/ml
#132 7ug/ml
Treatments

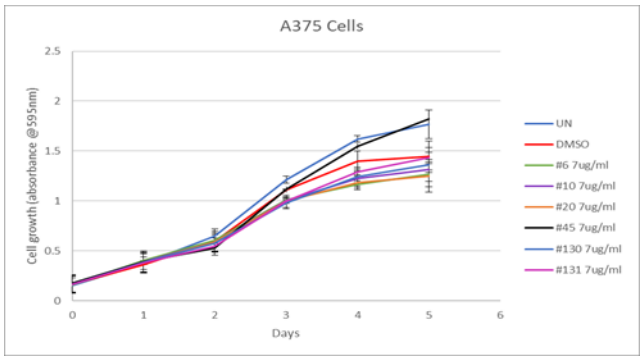

A375 Cells
Cell growth (absorbance @595nm)
Days
UN
DMSO
#4 15ug/ml
#8 15ug/ml
#14 15ug/ml
#132 15ug/ml

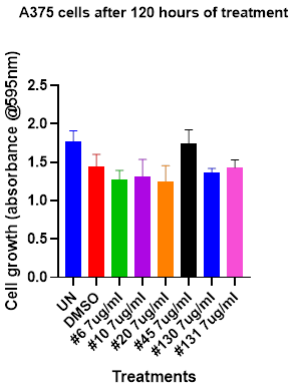

A375 cells after 120 hours of treatment
Cell growth (absorbance @595nm)
UN
DMSO
#4 15ug/ml
#8 15ug/ml
#14 15ug/ml
#132 15ug/ml
Treatments

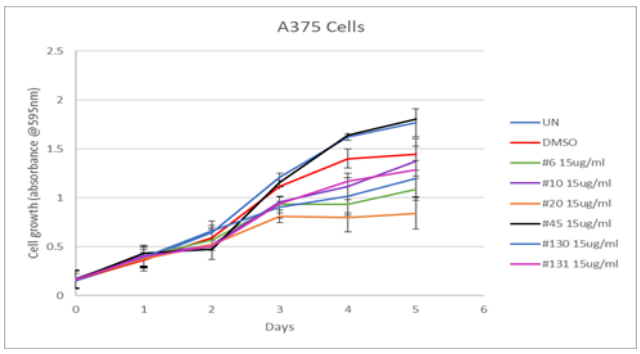

A375 Cells
Cell growth (absorbance @595nm)
Days
UN
DMSO
#4 30ug/ml
#8 30ug/ml
#14 30ug/ml
#132 30ug/ml

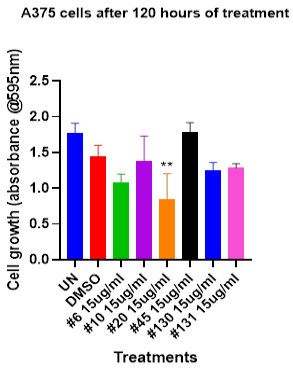

A375 cells after 120 hours of treatment
Cell growth (absorbance @595nm)
UN
DMSO
#4 30ug/ml
#8 30ug/ml
#14 30ug/ml
#132 30ug/ml
Treatments

B.


A375 Cells
Cell growth (absorbance @595nm)
Days
UN
DMSO
#6 7ug/ml
#10 7ug/ml
#20 7ug/ml
#45 7ug/ml
#130 7ug/ml
#131 7ug/ml


A375 cells after 120 hours of treatment
Cell growth (absorbance @595nm)
UN
DMSO
#6 7ug/ml
#10 7ug/ml
#20 7ug/ml
#45 7ug/ml
#130 7ug/ml
#131 7ug/ml
Treatments


A375 Cells
Cell growth (absorbance @595nm)
Days
UN
DMSO
#6 15ug/ml
#10 15ug/ml
#20 15ug/ml
#45 15ug/ml
#130 15ug/ml
#131 15ug/ml


A375 cells after 120 hours of treatment
Cell growth (absorbance @595nm)
**
UN
DMSO
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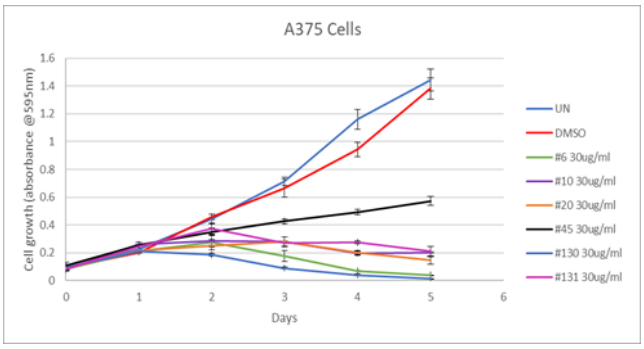
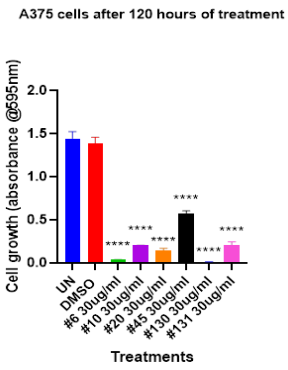
#10 15ug/ml
#20 15ug/ml
#45 15ug/ml
#130 15ug/ml
#131 15ug/ml
Treatments


A375 Cells
Cell growth (absorbance @595nm)
Days
UN
DMSO
#6 30ug/ml
#10 30ug/ml
#20 30ug/ml
#45 30ug/ml
#130 30ug/ml
#131 30ug/ml


A375 cells after 120 hours of treatment
Cell growth (absorbance @595nm)
****
UN
DMSO
#6 30ug/ml
#10 30ug/ml
#20 30ug/ml
#45 30ug/ml
#130 30ug/ml
#131 30ug/ml
Treatments

C.

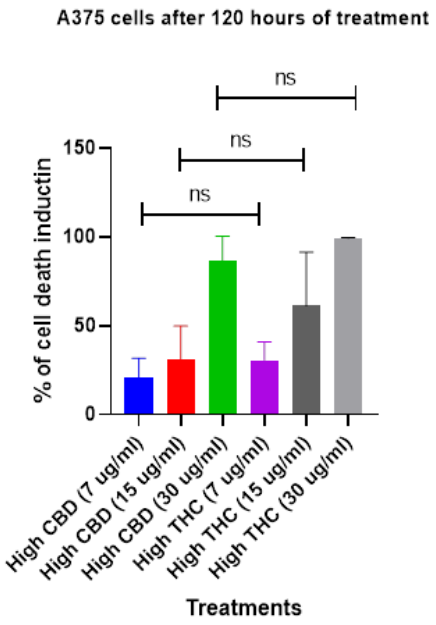
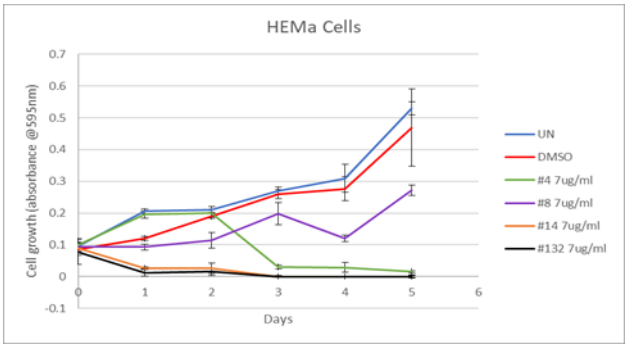


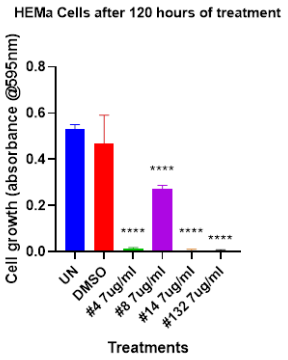

**Figure 2. MTT analysis of the effect of *Cannabis sativa* extracts on human melanoma cancer cells.** (A) Antiproliferative effect of high THC cannabis *extracts* with concentration of 7 µg/ml, 15 µg/ml, and 30 µg/ml on A375 cells after 120 hours of treatment. (B) Antiproliferative effect of high CBD cannabis extracts with concentration of 7 µg/ml, 15 µg/ml, and 30 µg/ml on A375 cells after 120 hours of treatment. (C) Effectiveness of high THC cannabis extracts in comparison to high CBD extracts on A375 cells in the three tested concentrations. Results are expressed as means of calculated cell growth or cell death induction ± standard deviations of each group in triplicate. Significant differences between groups are marked with ns -non-significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

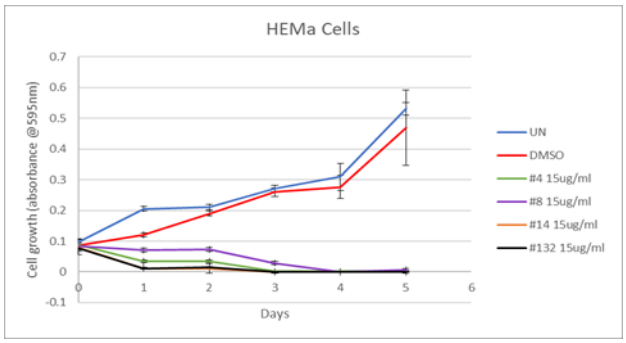
### 4.4 *Cannabis sativa* extracts are cytotoxic for HEMa cells

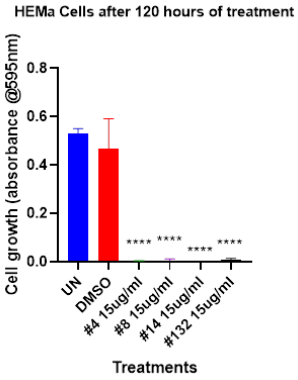
Two different concentrations of the extracts (four high THC and six high CBD) were tested on normal melanocytes (7 µg/ml, 15 µg/ml). The MTT analysis was continued for five days, and data showed that normal melanocytes are sensitive to the used extracts at the lowest tested concentration (7 µg/ml). As HEMa cells showed sensitivity to cannabis extracts, the highest concentration (30 µg/ml) was not tested anymore.

A.



B.

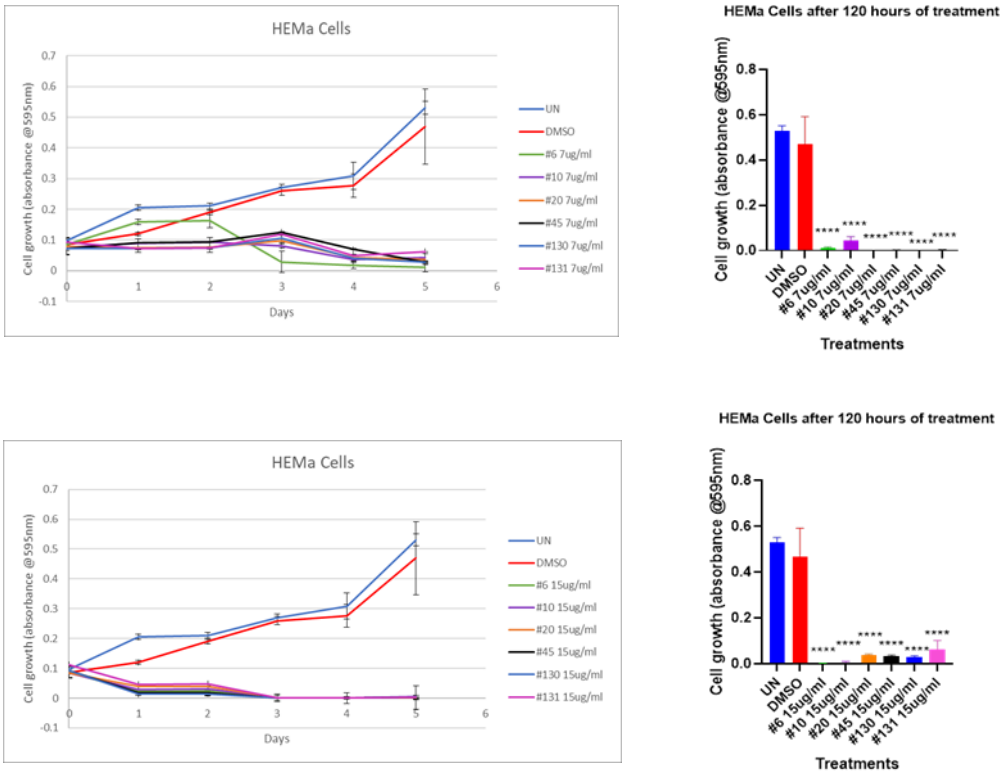
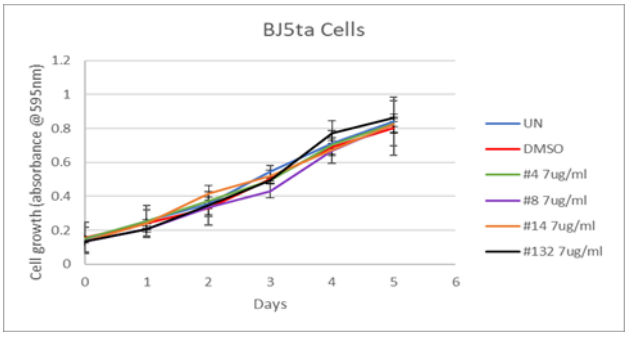


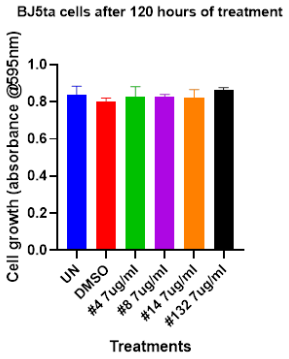

**Figure 3. MTT analysis of the effects of *Cannabis sativa* extracts on Human Epidermal Melanocytes.** (A) High THC *Cannabis sativa* extracts antiproliferative effect on HEMa cells after five days (120 hours) of treatment with two different concentrations of the extracts (7 µg/ml, 15 g/ml). (B) High CBD cannabis extracts antiproliferative effect on HEMa cells after five days (120 hours) of treatment with two different concentrations of the extracts (7 µg/ml, 15 µg/ml). Results are expressed as means of calculated cell growth ± standard deviations of each group in triplicate. Significant differences between groups are marked with: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

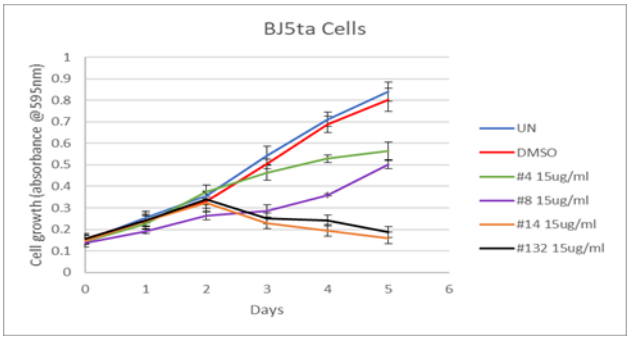
### 4.5 *Cannabis sativa* extracts effect on BJ5ta cell

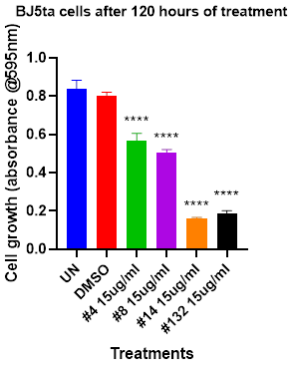
Two different concentration of the extracts (four high THC and six high CBD) were tested on BJ5ta cells (7 µg/ml, 15 µg/ml). Treatment was continued for 5 days (120 hours) and comparing the growth of treated cells to untreated ones showed that BJ5ta cells are not sensitive to any of our extracts at the lowest tested concentration (7 µg/ml; figure 4). However, all the high THC extracts were cytotoxic for normal fibroblasts at 15 µg/ml (figure 4A). Also, all high CBD extracts except #45 have reduced the growth of BJ5ta cells significantly comparing to untreated cells at 15 µg/ml (figure 4B).

A.



B.

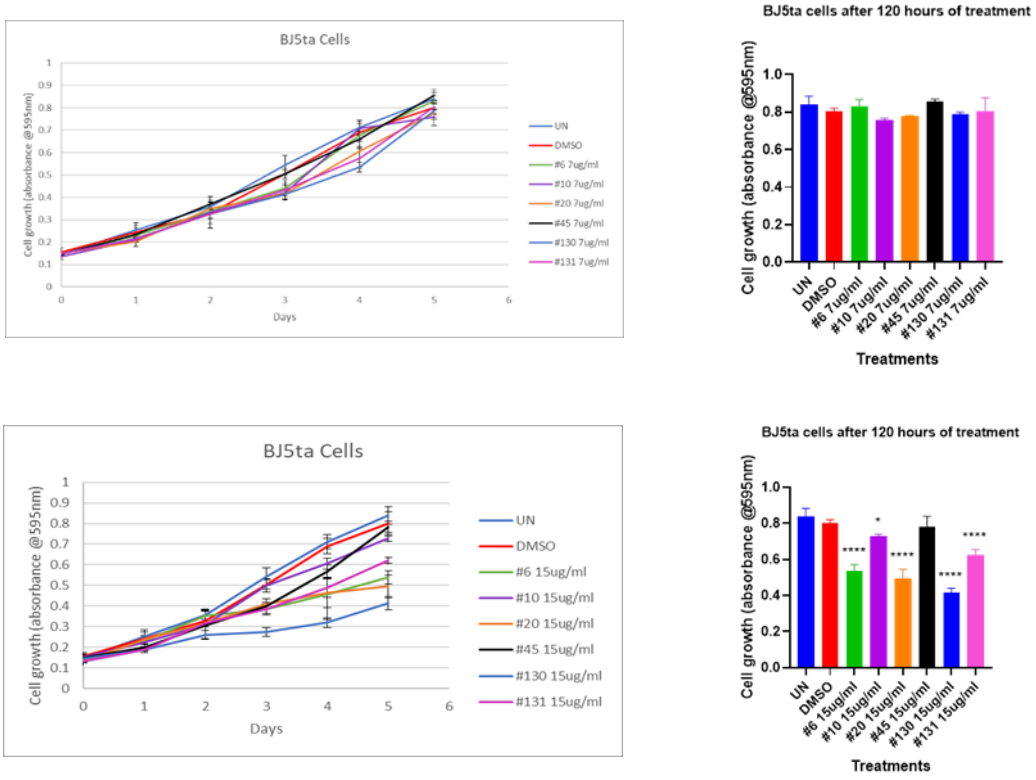


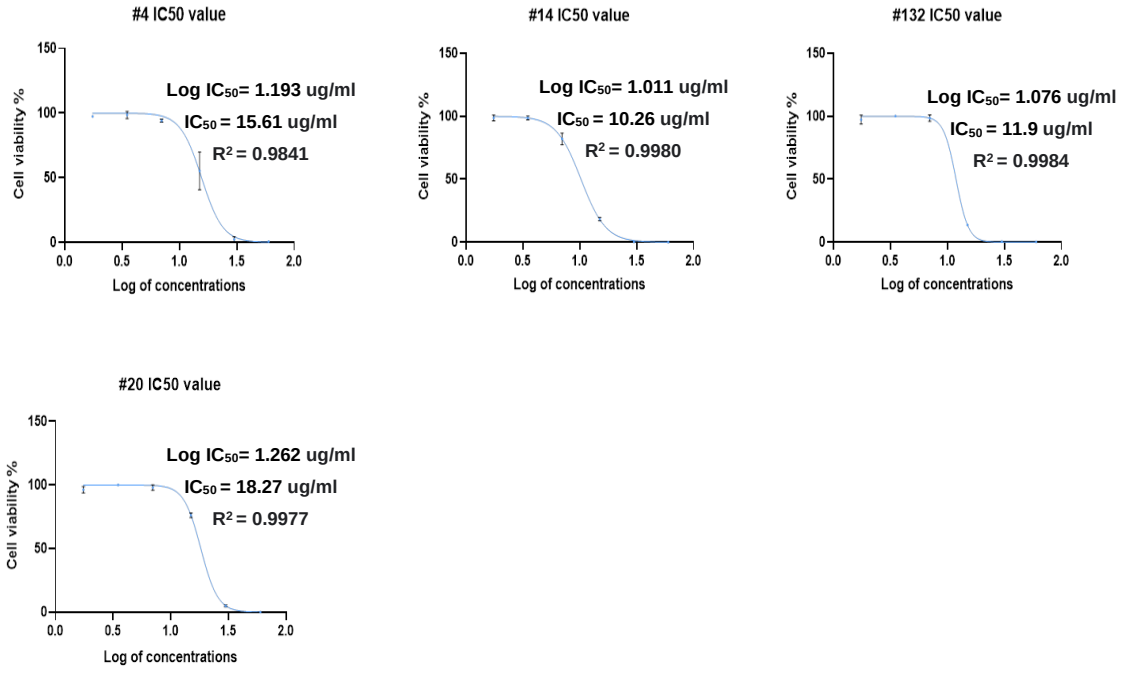

**Figure 4. MTT analysis of the effects of *Cannabis sativa* extracts on foreskin fibroblast cells growth.** (A) High THC *Cannabis sativa* extracts antiproliferative effect on BJ5ta cells after five days (120 hours) of treatment with two different concentrations of the extracts (7 µg/ml, 15 µg/ml). (B) High CBD *Cannabis sativa* extracts antiproliferative effect on BJ5ta cells after five days (120 hours) of treatment with two different concentrations of the extracts (7 µg/ml, 15 µg/ml). Results are expressed as means of calculated cell growth ± standard deviations of each group in triplicate. Significant differences between groups are marked with: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

### 4.6 Extract #14 has lower $IC_{50}$ value for A375 cells than for BJ5ta fibroblast cells

The half-maximal inhibitory concentration or the $IC_{50}$ value of four extracts was tested for A375, HEMa, and BJ5ta cells. Measuring the $IC_{50}$ value of extracts allows us to compare their cytotoxicity for the targeted cell lines. The four chosen extracts (three high

THC, and one high CBD extracts) have effectively decreased the cell growth of melanoma cancer cells in the concentration of 15 ug/ml. Cells were treated with six different concentration of each extract for five days to determine the $IC_{50}$ values. A375 cells were treated with 1.75 ug/ml to 30 ug/ml of each extract and fibroblasts were incubated with 0.875 ug/ml to 15 ug/ml of each extract. The cell viability percentage at day five was used to calculate the half-maximal inhibitory concentration of extracts. Data indicated that high THC extracts #4, #14, and #132 have $IC_{50}$ value of 15.61 ug/ml, 10.26 ug/ml, and 11.9 ug/ml for A375 cells, respectively (Figure 5A). The high CBD extract #20 has the highest $IC_{50}$ value among all for A375 cells (18.27 ug/ml, Figure 5A). The $IC_{50}$ value of extracts #4, #14, #132, and #20 were 13.69 ug/ml, 10.94 ug/ml, 9.95 ug/ml, and 16.23 ug/ml for BJ5ta cells, respectively (Figure 5B). The MTT assay indicated that extract #14 has slightly less $IC_{50}$ for A375 cells than for BJ5ta fibroblasts.

A.



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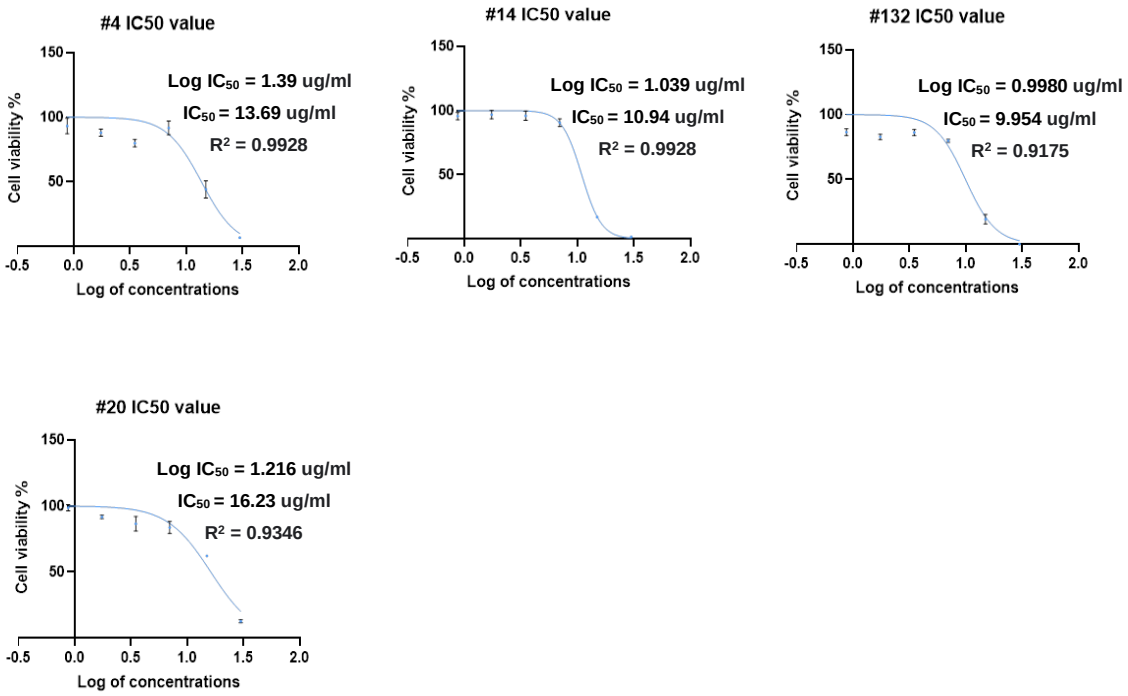



**Figure 5. MTT analyse for the extracts $IC_{50}$ value for A375, BJ5ta and HEMa cells.** **(**A) The $IC_{50}$ value of four *Cannabis sativa* extracts after 5 days (120 hours) of treatment for A375 cells. (B) The $IC_{50}$ value of four *Cannabis sativa* extracts after 5 days (120 hours) of treatment for BJ5ta cells. Statistical analysis was performed by Nonlinear regression (curve fit), Dose – response - Inhibition, Log (inhibitor) vs. normalize response -- Variable slope test. Data is expressed as mean ± standard deviations of each group (N=3).

### 4.7 Vemurafenib has $IC_{50}$ value of 0.073 µM for A375 cells

Vemurafenib is an FDA approved drug for treatment of melanoma cancer harboring BRAF (V600e) mutation [155]. This mutation is the same mutation as the one which A375 cells have, and this makes vemurafenib as a suitable positive control for our experiments. As explained in the methods MTT assay was performed and the $IC_{50}$ value of Vemurafenib for A375 melanoma cells was measured 0.073 µM. This concentration was used in further experiments as the positive control and to be compared with the effectiveness of pure Δ9 - THC, CBD, and cannabis extracts.

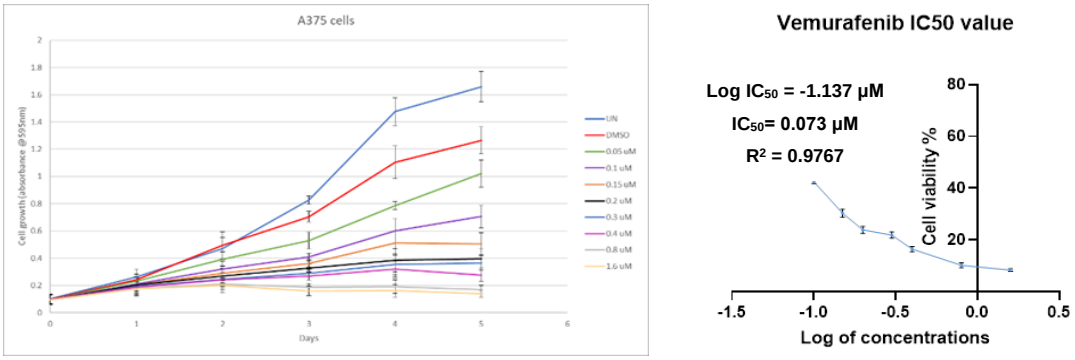



**Figure 6. MTT assay for the Vemurafenib $IC_{50}$ value for A375 melanoma cells.** The $IC_{50}$ value of vemurafenib after 5 days (120 hours) of treatment for A375 cells. Statistical analysis was performed by Nonlinear regression (curve fit), Dose – response - Inhibition, Log (inhibitor) vs. normalize response -- Variable slope test. Data is expressed as mean ± standard deviations of each group (N=3).

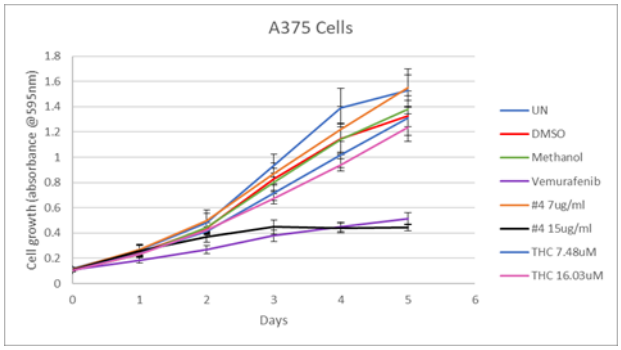
### 4.8 High THC extracts have higher inhibitory effect on A375 cells growth comparing to pure THC

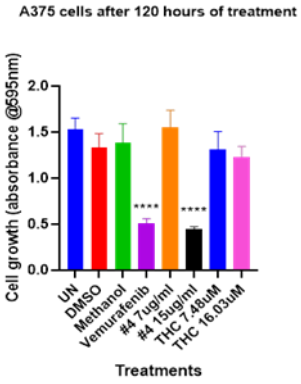
Those *Cannabis sativa* extracts which had significant inhibitory effect on the growth of A375 cells in the lower concentration (15 µg/ml) were chosen to be compared to their corresponding amount of THC or CBD. Only two concentrations of each extract were used (7 µg/ml and 15 µg/ ml). As the highest tested concentration (30 µg/ml) of all tested extracts have suppressed the proliferation of the melanoma cells, we didn't test that concentration here anymore. The corresponding THC and CBD amount of each extract was calculated using the HPLC profile of the extracts (table 3). Finally, the MTT analysis was performed for five days to compare the effectiveness of each chosen extract and its corresponding THC or CBD amount. Vemurafenib was used as the positive control. Data indicated that the tested high THC extracts (#4, #14, and #132) reduced the melanoma cells growth more efficiently than their corresponding THC amount in the concentration of 15 µg/ml. However, for the high CBD extract (#20) the MTT results revealed that the pure CBD is more effective in suppressing the growth of A375 cells comparing to the extract #20.

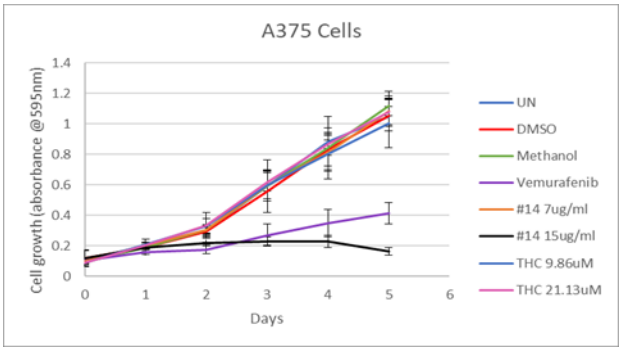
**Table 3. The chosen high THC and high CBD *Cannabis sativa* extracts and their corresponding amount of THC and CBD respectively**. The data were calculated based on the HPLC profile of each extract.

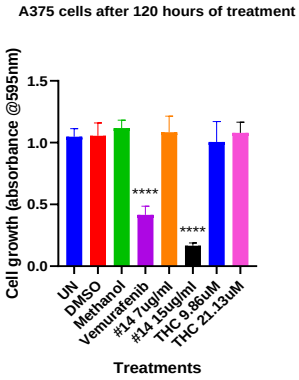
| | 7 µg/ml | | 15 µg/ml | |
|---|---|---|---|---|
| High THC | THC µM | CBD µM | THC µM | CBD µM |
| #4 | 7.48 | | 16.03 | |
| #14 | 9.86 | | 21.13 | |
| #132 | 9.53 | | 20.43 | |
| High CBD | | | | |
| #20 | | 7.51 | | 16.1 |

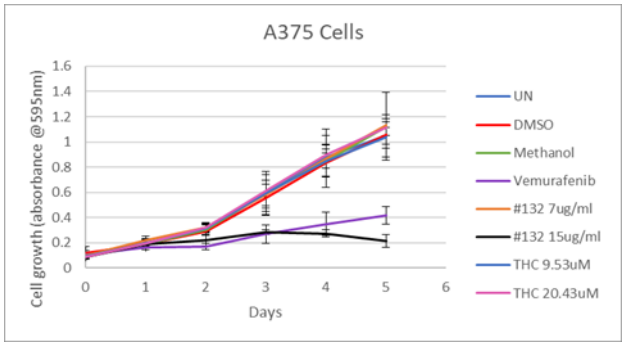
A.

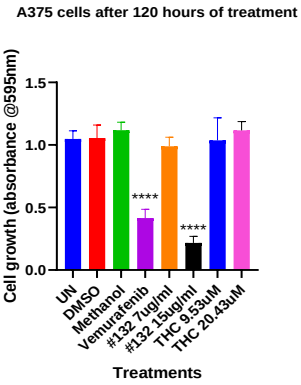

A375 Cells
Cell growth (absorbance @595nm)
Days
UN
DMSO
Methanol
Vemurafenib
#4 7ug/ml
#4 15ug/ml
THC 7.48uM
THC 16.03uM


A375 cells after 120 hours of treatment
Cell growth (absorbance @595nm)
****
****
UN
DMSO
Methanol
Vemurafenib
#4 7ug/ml
#4 15ug/ml
THC 7.48uM
THC 16.03uM
Treatments


A375 Cells
Cell growth (absorbance @595nm)
Days
UN
DMSO
Methanol
Vemurafenib
#14 7ug/ml
#14 15ug/ml
THC 9.86uM
THC 21.13uM


A375 cells after 120 hours of treatment
Cell growth (absorbance @595nm)
****
****
UN
DMSO
Methanol
Vemurafenib
#14 7ug/ml
#14 15ug/ml
THC 9.86uM
THC 21.13uM
Treatments


A375 Cells
Cell growth (absorbance @595nm)
Days
UN
DMSO
Methanol
Vemurafenib
#132 7ug/ml
#132 15ug/ml
THC 9.53uM
THC 20.43uM


A375 cells after 120 hours of treatment
Cell growth (absorbance @595nm)
****
****
UN
DMSO
Methanol
Vemurafenib
#132 7ug/ml
#132 15ug/ml
THC 9.53uM
THC 20.43uM
Treatments

B.

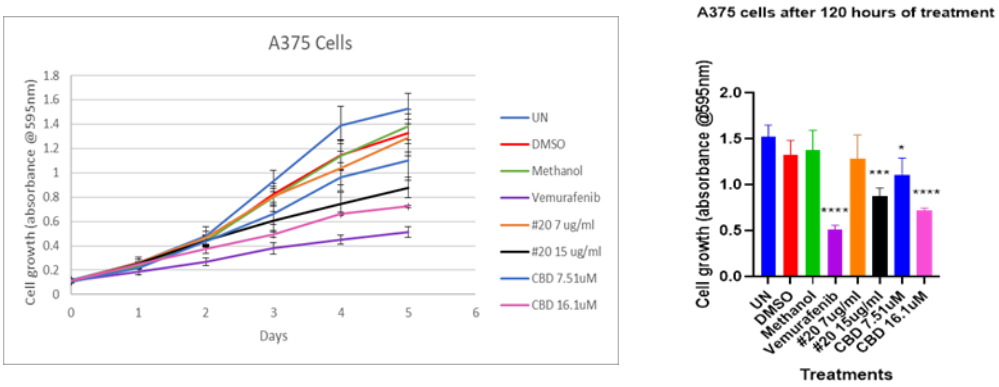


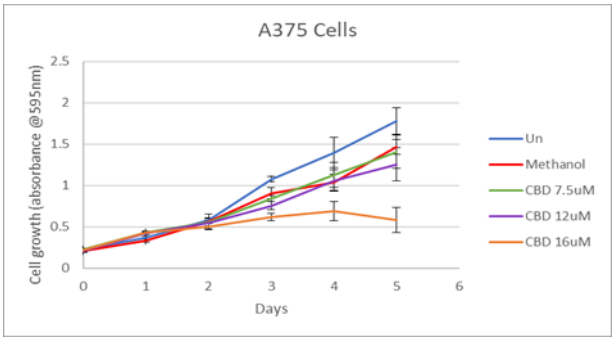

**Figure 7. MTT analysis to compare the effect of cannabis extracts and their corresponding cannabinoids amount on A375 cells.** (A) MTT analysis for the tested high THC extracts in two concentrations of 7 µg/ml and 15 µg/ml, and their corresponding THC amount for each concentration. (B) MTT analysis for the tested high CBD extract in two concentrations of 7 µg/ml and 15 µg/ml, and the corresponding CBD amount for each concentration. Vemurafenib was used as the positive control in its $IC_{50}$ value concentration for A375 cells. Data is expressed as means of calculated cell growth ± standard deviations of each group (N=3). Significant differences between groups are marked with: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

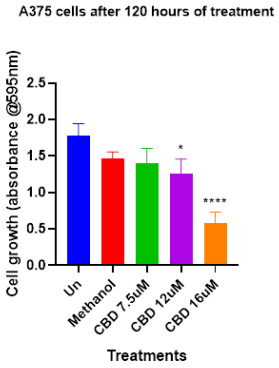
### 4.9 A375 cells are more sensitive to pure CBD than BJ5ta fibroblasts

As the pure CBD has shown to be effective in reducing the growth of melanoma cancer cells, we have tested three different concentrations of pure CBD (7.5 µm, 12 µm, and 16 µm) on A375, HEMa, and BJ5ta cells to also check the cannabidiol effect on normal cells. After five days, the cell growth of A375 cells treated with 12 µm of cannabidiol was reduced significantly (Figure 8A). For the normal melanocytes, CBD was cytotoxic from 7.5 µm (Figure 8B), however, for fibroblasts CBD have reduced the cell growth only at 16 µm (Figure 8C). MTT results indicated that while 12 µm of CBD is cytotoxic for A375

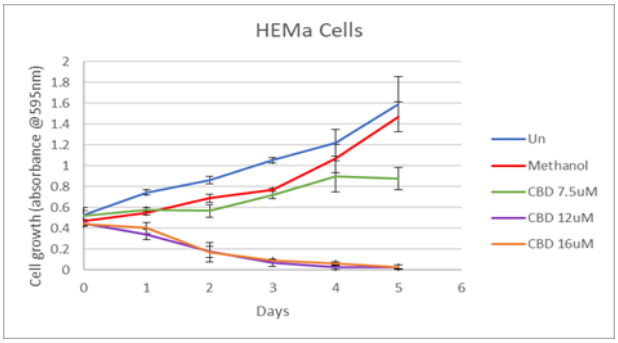
cells, it doesn't reduce the cell growth of BJ5ta cells significantly in comparison to the untreated cells.

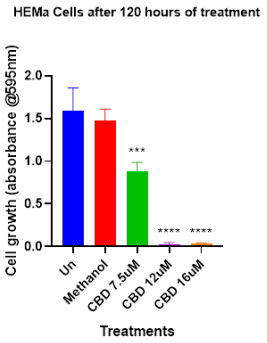
A.



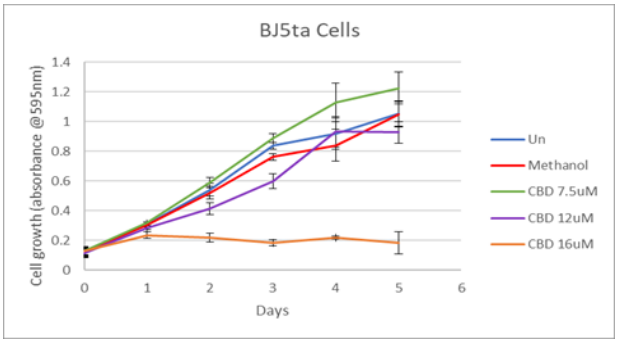




B.




C.



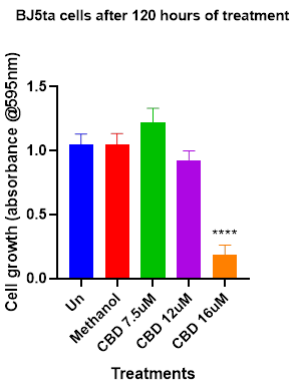



**Figure 8. MTT analysis to check the effect of pure CBD on A375, HEMa, and BJ5ta cells.** (A) pure CBD effect on A375 cells after 5 days (120 hours) of incubation with three concentrations of 7.5 μM, 12 μM, and 16 μM. (B) pure CBD effect on HEMa cells after 5 days (120 hours) of incubation with three concentrations of 7.5 μM, 12 μM, and 16 μM. (C) pure CBD effect on BJ5ta cells after 5 days (120 hours) of incubation with three concentrations of 7.5 μM, 12 μM, and 16 μM. Data is expressed as means of calculated cell growth ± standard deviations of each group (N=3). Significant differences between groups are marked with: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

### 4.10 The cannabis extract #14 inhibits proliferation of A375 cells via ERK1/2 and AKT pathways

The high THC extract #14 was the most cytotoxic extract for the melanoma cells with the $IC_{50}$ value of 10.26 ug/ml. Thus, we used this extract to treat melanoma cells and check the probable mechanisms through which cannabis extracts inhibit the proliferation of A375 melanoma cells. Cells were treated with 10.26 ug/ml of extract #14 and after different time points of 24, 48, 72, and 96 hours the whole cellular lysate was subjected to western blot analysis. The quantification of pERK/ERK ratio showed that phosphorylation of ERK1/2 increases after treatment with the cannabis extract and this upregulation is long term. The pAKT/AKT ratio indicated that while there is no change after 24 hours of treatment with the high THC extract, the phosphorylated AKT is decreased after 48 hours, and this downregulation is time dependent and significant after 96 hours of treatment.

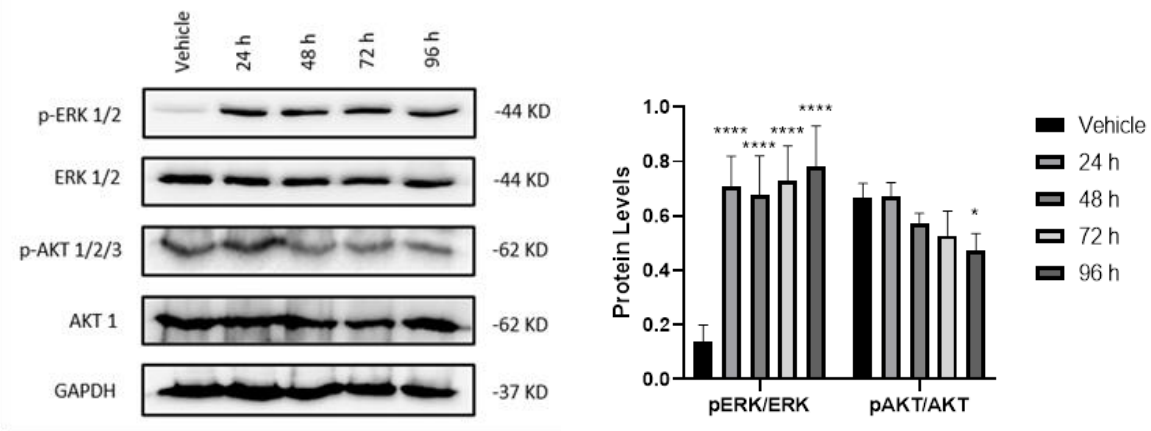



**Figure 9.Western blot analysis of the pERK/ERK and pAKT/AKT ratio after treating A375 cells with extract #14.** Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparisons test. Data are expressed as the mean ± SD (N=3). Significant differences between groups are marked with: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

### 4.11 The terpenes profile of the two most effective *Cannabis sativa* extracts

Extracts #14 and #132 showed the most cytotoxicity for the melanoma cells with the lowest $IC_{50}$ value among all the tested extracts (figure 5 A). We have sent these two extracts to Canvas Labs to determine the terpenes profile of them (table 4). The results showed that both of these extracts contain some of the most abundant terpenes among cannabis plants like α-Pinene, β-Pinene, β-Myrcene, Limonene, and Linalool. Extract #132 has also high amount of Sabinene Hydrate and β-Caryophyllene, 2.75 and 1.41 mg/g, respectively. Generally, while extract #14 has lower $IC_{50}$ than extract #132 for melanoma cells (10.26 ug/ml for #14 and 11.90 ug/ml for #132), the total terpenes of extract #132 was higher than

total terpenes of extract #14. The terpenes profile results showed that extract #132 has total terpenes of 18.63 mg/g, while this number is only 1.75 mg/g for extract #14.

**Table 4. The terpenes profile of extracts #14 and #132.**

| **Terps in mg/g** | **#14** | **#132** |
|---|---|---|
| α-Pinene | 0.04 | 0.29 |
| β-Pinene | 0.06 | 0.64 |
| β-Myrcene | 1.2 | 2.83 |
| Limonene | 0.26 | 3.56 |
| Linalool | 0.19 | 1.59 |
| Sabinene Hydrate | NT | 2.75 |
| β-Caryophyllene | NT | 1.41 |
| α-Bisabolol | 0.06 | 0.01 |
| α-Cedrene | NT | 0.62 |
| Cedrol | NT | 0.55 |
| Geraniol | NT | 0.34 |
| Camphene | 0.05 | 0.07 |
| α-Humulene | 0.13 | 0.94 |
| Menthol | NT | 0.18 |
| Borneol | 0.01 | 1.91 |
| trans-Nerolidol | 0.18 | 0.23 |
| Fenchone | 0.03 | 0.06 |
| cis-Nerolidol | ND | ND |
| Caryophyllene oxide | ND | 0.52 |
| α-Terpinene | ND | ND |
| γ-Terpinene | ND | ND |
| Isopulegol | ND | 0.01 |
| Valencene | ND | ND |
| Terpineol | 0.05 | ND |
| Ocimene | ND | ND |
| Total | 1.75 | 18.63 |

# 5. DISCUSSION

Melanoma is the deadliest form of skin cancer, and its mortality continues to rise. The Canadian cancer society had estimated that 1,250 Canadians will die of melanoma in 2021 [124]. In the early stages, melanoma is treatable with surgery, but when the cancer becomes advanced, the survival rate will decrease significantly [157]. Generally, the effectiveness of conventional therapies such as surgery, chemotherapy, and immunotherapy are limited because of the multiple resistance mechanisms and the short response duration, besides the severe side effects of some of these therapies [157]. There is an increasing need to develop novel agents and therapies for the management of melanoma cancer.

A large body of evidence shows that cannabinoids have potential medical uses such as reducing chemotherapy-induced nausea and vomiting, stimulating appetite, and pain relief in cancer patients [1, 3]. Besides being potential cancer co-treatment, in vitro and in vivo research have demonstrated that cannabinoids could produce antitumor responses in different models of cancer including, gastric, pancreatic, melanoma, and colorectal, among others [74, 79, 91, 93]. Most of the conducted studies have focused on pure cannabinoids, mainly THC and CBD. In the cannabis plants, however, there is a great chemical diversity (other cannabinoids, terpenoids, flavonoids, etc.) that have shown therapeutic properties even though they have been studied much less [16, 17, 111-113]. It is believed that these secondary metabolites might have a positive contribution on the therapeutic capacity of cannabis plants. In this context, we aimed at testing the anti-cancer effects of ten *Cannabis sativa* extracts with different compositions of compounds (four high THC extracts and six high CBD extracts), on melanoma cancer cells. We demonstrated that even though not all

the tested extracts were effective in the lowest tested concentration (7 μg/ml), all of them regardless of being high THC or high CBD, could reduce the cell proliferation of melanoma cells in the higher concentration (30 μg/ml). The results showed that while not all the extracts have similar inhibitory effects on melanoma cells, all of them could reduce the cell growth in a time and dose-dependent manner (figure 2A, 2B). There was also a correlation between the total level of cannabinoids in each extract and its ability to reduce melanoma cells growth. For instance, cell death induction of extract #45 for melanoma cells was 60.33 % after 120 hours of treatment with 30 μg/ml of concentration. This extract has the lowest total cannabinoids among all which is 25.63%. On the other hand, the total cannabinoid of the most effective extracts (#14, #132) with the cell death induction of 100% after 120 hours of treatment with 30 μg/ml of concentration is 45.63% and 44.86%, respectively (table 2). These extracts have the highest total cannabinoids percentage among all the tested extracts. Whereas there was no significant difference between the cytotoxicity of high THC and high CBD extracts (figure 2C), the high THC ones showed better inhibitory effects. For instance, three of the high THC extracts reduced the cell proliferation of A375 cells significantly in the 15 μg/ml (#4, #14, and #132; figure 2A middle panel), but only one high CBD extract was effective in the 15 μg/ml (figure 2B middle panel). This is in line with another study that showed a high THC extract was more effective than high CBD extracts for A375 cells [118].

Terpene profile of the extracts #14 and #132 shows that they contain some of the most common terpenes fund in *Cannabis sativa* that have been shown to be effective in reducing melanoma cancer progression. Extracts #14 and #132 contain limonene and α-pinene (table 4). These two monoterpenes appear to have tumor suppressive effects and

apoptosis induction ability in different *in vivo* studies of melanoma cancer [110, 158-160]. Extracts #14 and #132 also have camphene which is another monoterpene that was reported to induce apoptosis *in vitro* and *in vivo* in different melanoma cancer models [161]. Moreover, these two extracts have β-myrcene, linalool, and α-humulene (table 4). Several studies have highlighted that these terpenes have anti-cancer effects [16, 113]. Myrcene is cytotoxic for cervical, lung, and colon cancer, and linalool induces cell apoptosis in oral cancer by inhibition of PI3K/AKT signaling pathway [16, 162]. Humulene also induces synergistic inhibitory effects when combined with conventional drugs in colon and ovarian cancer cells [16]. The reported anti-cancer effects of these terpenes and the possibility that they might act synergistically with the phytocannabinoids, may explain the high cytotoxicity of *Cannabis sativa* extracts #14 and #132 for melanoma cancer cells.

In agreement with a recent study that demonstrated that high the THC extract exhibited more potent antitumor effects than pure THC in breast cancer cells [119], three of our tested high THC extracts also showed higher cytotoxicity for melanoma cells compared to their corresponding amount of pure THC in two concentrations of 7 μg/ml and 15 μg/ml (figure 7A). Extract #14 had the lowest $IC_{50}$ value (10.26 ug/ml), after that extract #132 with $IC_{50}$ value of 11.90 ug/ml and extract #4 had the highest $IC_{50}$ value among high THC extracts (15.61 ug/ml; figure 5A). According to the HPLC profiles, the THC percentage of extracts #14, #132, and #4 are 44.3%, 42.82%, and 33.6%, respectively. It could be concluded that the half maximal inhibitory concentration of these high THC extracts (#4, #14, and #132) in melanoma cells, have an inverse correlation with their THC levels.

Notably, pure THC didn't have any inhibitory effect on melanoma cells up to 21.13 uM (figure 7A middle panel). Many studies have reported the ability of pure THC to reduce viability of different cancer cell lines, including A375 melanoma cells, however, they were carried out under low or no serum conditions (less than 1% of FBS) [56, 79, 107, 163].In agreement with our result, there were other studies that didn't find THC being effective in the presence of serum in glioblastoma multiforme, melanoma, and colorectal cancer cells [164-166]. Previous reports have shown that cannabinoids bind with a high affinity to proteins of human blood samples [165]. It could be said that the presence of serum will mimic the in vivo situation better and provides cells with the nutrition needed for their growth and the lack of it will slower the cells growth. However, the presence of serum could change the effective concentration of THC.

All the tested high CBD extracts impacted the cell growth of melanoma cells dose-dependently (figure 2B), and our result indicated that a high CBD extract (#20) significantly reduced the cell viability of A375 cells at a concentration of 15 μg/ml (figure 7B). This is in correlation with previous studies that have shown that cannabis extracts enriched in cannabidiol are a potent inhibitor of cancer cells growth [167-169]. 7 μg/ml of extract #20 contains 7.51 μM of CBD (table 2). The viability of cells treated with 7.51 μM of CBD was not significantly lower than those treated with 7 μg/ml of extract #20 (figure 7B). However, unlike 7 μg/ml of extract #20, 7.51 μM of pure CBD significantly reduced the cell growth of A375 cells (figure 7B). In agreement with this, Ligresti (2006), claimed that cannabidiol has a more potent effect on breast, colorectal, and gastric cancer cells than a high CBD extract with the same amount of CBD [120]. The anti-cancer potential of cannabidiol was shown in both in vitro and in vivo studies in different cancer models [93,

97, 101, 165, 170]. On the other hand, CBD doesn't have any psychoactive effect which is an advantage for clinical applications. As we showed that cannabidiol is not cytotoxic for BJ5ta cells at concentrations less than 16 μM (figure 8C), a number of studies also reported that CBD doesn't impact the growth of normal cells [120, 167, 171], including human epidermal melanocytes [172]. All this together makes CBD a promising compound for the treatment of cancer. However, based on our data, normal melanocytes are sensitive to cannabidiol (figure 8B). It should be noted that in our experiment, cannabidiol reduced the viability of epidermal melanocytes at 7.5 μM (figure 8B), while Hwang (2017) has tested the CBD up to 6 μM for epidermal melanocytes [172]. So, our data might not conflict with that. The fact that the tested cannabis extracts were cytotoxic for normal cells may prevent their systemic use, however, the local use for melanoma may still be justified. More studies are needed to confirm the beneficial anti-melanoma effects of cannabis extracts and to test potential adverse effects of the use of cannabinoids orally [173]. The topical use of up to 10% of isolated CBD in a simple grapeseed oil vehicle, however, didn't cause any irritation or sensitization on human healthy skin; and the cream product featuring hemp seed oil only caused mild phototoxic reactions [173]. This shows the importance of the delivery method for cannabinoids or cannabis extracts.

Treatment of melanoma cells with $IC_{50}$ value of our high THC extract #14 (10.26 ug/ml) for up to 96 hours showed that cannabis extract increases the phosphorylation of ERK1/2 (figure 9). In parallel with our result, it was reported that activation of cannabinoid receptors could result in a long-term increase of phosphorylated ERK1/2 in melanoma [174], gastric [92], malignant gliomas [175], and in prostate [176] cancer cells. Abnormalities in the mitogen-activated protein kinase (MAPK) pathway occur frequently

in different cancers, and it has a critical role in the development and progression of cancer. These dysregulations are mainly due to mutations in RAS and RAF genes or because of other genetic or epigenetic modifications [177]. Thus, MAPK/ERK pathway was always a potential drug target, and several known inhibitors of this pathway are used in clinical therapies. Some of the known inhibitors of MAPK/ERK pathway are trametinib, cobimetinib, dabrafenib, and vemurafenib which are FDA approved drugs to be used in combination or as a single therapy agent [178].

Activation of cannabinoid receptors could modulate MAPK pathway members [179]. The members of this pathway (ERK, JNK and p38/MAPK) are serine/threonine protein kinases involved in the control of cell proliferation and survival through the phosphorylation of specific targets [71]. Activation of ERK could be resulted in different and sometimes opposite responses. It is generally accepted that ERK activation causes cell proliferation, but interestingly, the duration of the activation can be fundamental for the type of cellular response caused by the ERK pathway [71]. It has been demonstrated that transient stimulus of ERK leads to cell proliferation, however, prolonged ERK stimulus is pro-apoptotic and can mediate cell cycle arrest and cell death [175, 180, 181]. There is a pathway through which cannabinoid receptors activation leads to cell death by increasing ERK. Mangal (2021) reported that CB receptors activation could stimulate ERK1/2 signaling that activates p27 and p21 and decrease cyclin D1, cyclin E, cdk2, cdk4, and cdk6 through an increase in pRb [180]. This will result in cell cycle arrest at G0/G1 phase and apoptosis as was shown before in gastric cancer cells [92]. Upregulation of pERK1/2 may contribute to cannabis extract-induced cell death in A375 melanoma cells.

One of the other pathways that is dysregulated in many types of human cancer is PI3K/AKT pathway. The serine/threonine protein kinase (AKT) is widely known as a key mediator of cell survival, growth, proliferation, angiogenesis, and plays an anti-apoptotic role in the cancer cells. AKT is constitutively active in different types of cancer [71]. Thus, inhibition of AKT may result in reducing cancer development. Our result showed that treatment of melanoma cells with cannabis extract decreases the protein level of phosphorylated AKT in a time-dependent manner (figure 9). Many studies indicated that downregulation of AKT is involved in cannabinoids anti-cancer ability. Parallel to our result, inhibition of AKT was reported in melanoma [107], breast [56], glioma [182], colorectal [163], and prostate [176] cancer cells treated with cannabinoids or cannabinoids agonists. Inhibition of PI3K/AKT pathway will result in cell cycle arrest and apoptosis by increasing the cyclin-dependent kinase inhibitors p21 and p27 and increasing the pRb [56, 107].

We have shown that treatment of melanoma cells will result in a long-term over activation of MAPK/ERK pathway. Usually, the activation of death pathways should be followed by the inhibition of survival factors. We have also seen the downregulation of AKT in addition to the overactivation of ERK after treating cells with cannabis extracts. The same alterations in these two pathways was also reported in prostate [176], gastric [92], and in colorectal [163] cancer cells treated with cannabinoids or cannabinoids agonists. In conclusion, our findings indicated that *Cannabis sativa* extracts might have anti-tumor activity in melanoma cells via activating the MAPK/ERK pathway and downregulating the PI3K/AKT pathway.

## 6. LIMITATIONS AND FUTURE STUDIES

This study has focused on investigating the anti-tumour effect of different *Cannabis sativa* extracts on melanoma cancer cells and two normal cell lines. We have first demonstrated that all the tested cell lines express CB1 and CB2 receptors. The presence of these receptors makes our cell lines good models for investigating cannabis extracts effects as they are the main cannabinoid receptors. The first limitation of our study was that besides CB1 and CB2, cannabinoids could bind to non-classical receptors, including the transient receptor potential vanilloid receptors 1 and 2 (TRPV1 and TRPV2) and certain orphan G protein-coupled receptors (GPR55 and GPR18). It is important to check the expression level of these receptors in the studied cell lines, as the expression level of targeted receptors might be one of the reasons that cells react differently to cannabis extracts. One other limitation was that to better understand receptors contributing to the anti-proliferating effects of cannabis extracts, it is necessary to knock out those receptors or treat the cells with each receptor selective antagonism before treating cells with cannabis extracts. This can help to check whether the tumour suppressive effect was mediated through that receptor or was independent of that specific receptor.

In this study, we used normal human epidermal melanocytes form an adult donor to check any probable cytotoxic effects of cannabis extracts on normal melanocytes. Our result showed that HEMa cells are sensitive to extracts. One of the limitations of our study was that we used HEMa cells at passage 6 and 7; in other studies, however, melanocyte cells are usually used in lower passages (passage 3) [183]. There is a possibility that one of the reasons that HEMa cells are sensitive to cannabis extracts is the high passage of cells that we have used in our study. On the other hand, we only used one melanoma cell line in this study. A375 cells are one of the most studied cell lines in melanoma cancer cell

research, however, it is important to check different melanoma cell models with a variety of mutations in order to better determine the ability of cannabis extracts to inhibit melanoma progression.

We checked the protein levels of ERK and AKT as they are key proteins of the two main dysregulated pathways in various types of cancer, and A375 cells harbour BRAF mutation and have dysregulated MAPK/ERK pathway. We showed that cannabis extracts might exert anti-mitogenic effects via regulation of pERK/ERK and pAKT/AKT ratio and thus, modulation of MAPK/ERK and PI3K/AKT pathway. However, to better study the changes in these pathways and to confirm that these regulations result in cell cycle arrest and apoptosis it is necessary to check the other downstream proteins like p27, p21, cyclin D1, cyclin E, cdk2, cdk4, and cdk6. Future studies may include checking more cell cycle arrest biomarkers and performing the cell cycle and apoptosis analysis via flow cytometer.

We analysed the anti-proliferative effects of different high THC and high CBD cannabis extracts on melanoma cancer cells. We have demonstrated that all the tested extracts reduce the growth of A375 melanoma cells in a time- and dose-dependent manner. Our study was conducted in vitro. Like every other study, it is necessary to perform in vivo study in future studies to confirm the ability of cannabis extracts as tumour-suppressive agents.

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