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AN ALTERNATIVE AND THERAPEUTIC APPROACH FOR HEPATOCELLULAR CARCINOMA

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ABSTRACT

AN ALTERNATIVE AND THERAPEUTIC APPROACH FOR HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma is among of the most frequently forms of liver cancer, with a high death rate, emphasizing the need for improved treatment techniques. Current research has focused on the anti-cancer effects of natural compounds. The most common therapy for HCC varies according to stage: patients in the early stages may require surgical resection or liver transplantation, whereas intermediate-stage patients may get Transarterial chemoembolization (TACE). In advanced stages, immunotherapy combined with targeted therapies, especially Atezolizumab-Bevacizumab, has emerged as the standard. CBD (cannabidiol), a non-psychoactive cannabinoid, has gained attention in cancer research because of its anti-inflammatory, anti-proliferative, anti-angiogenic, and apoptosis-inducing properties. Studies suggest CBD may inhibit tumor growth, induce cell death, and reduce metastasis. Additionally, CBD is being explored as a complementary treatment for alleviating chemotherapy side effects. Vitamin D is also recognized for its potential function in HCC treatment, given its impact on immune regulation and cell growth control. Vitamin D insufficiency has been associated to HCC progress, emphasizing its potential therapeutic value. The current research tested the anti-cancer impacts of single and combination treatments of Vitamin D and CBD in HEP3B and HUH7 cell lines, focusing on apoptosis, autophagy, cell cycle regulation, and DDR. The combination treatment of Vitamin D and CBD demonstrated a synergistic effect, significantly reducing cell survival in both HEP3B and HUH7 cell lines. Notably, the combination induced apoptosis and autophagy in HEP3B cells and inhibited the cell cycle in HUH7 cells, suggesting anti-tumorigenic and anti-cancer effects. These findings indicate the possibility of Vitamin D and CBD as a novel treatment approach against HCC, requiring additional in vivo studies and clinical trials to assess efficacy, safety, and mechanisms in more complex biological systems.

ÖZET

HEPATOSELÜLER KARSİNOM İÇİN ALTERNATİF VE TEDAVİ EDİCİ BİR YAKLAŞIM

Hepatoselüler karsinom (HCC), en sık görülen karaciğer kanseri türlerinden biridir ve yüksek ölüm oranına sahiptir; bu durum, tedavi yöntemlerinin geliştirilmesi gerekliliğini vurgulamaktadır. Mevcut araştırmalar, doğal bileşiklerin anti-kanser etkilerine odaklanmıştır. HCC için en yaygın tedavi, hastalığın evresine göre değişmektedir: erken evredeki hastalar cerrahi rezeksiyon veya karaciğer nakli gerektirebilirken, orta evredeki hastalar transarteriyel kemoembolizasyon (TACE) alabilmektedir. İleri evrelerde ise, özellikle Atezolizumab-Bevacizumab kombinasyonu olmak üzere, immünoterapi ve hedefe yönelik tedavilerin kombinasyonu standart hale gelmiştir. Psikoaktif olmayan bir kannabinoid olan CBD (kannabidiol), anti-inflamatuar, anti-proliferatif, anti-anjiyojenik ve apoptoz indükleyici etkileri nedeniyle kanser araştırmalarında dikkat çekmektedir. Araştırmalar, CBD'nin tümör büyümesini inhibe edebileceğini, hücre ölümünü indükleyebileceğini ve metastazı azaltabileceğini öne sürmektedir. Ayrıca CBD, kemoterapinin yan etkilerini hafifletici tamamlayıcı bir tedavi olarak da incelenmektedir. D vitamini de bağışıklık düzenlemesi ve hücre büyüme kontrolü üzerindeki etkileri nedeniyle HCC tedavisindeki potansiyel rolüyle tanınmaktadır. D vitamini eksikliği, HCC'nin ilerlemesiyle ilişkilendirilmiş olup, bu da terapötik potansiyelini vurgulamaktadır. Bu çalışma, D vitamini ve CBD'nin tekli ve kombinasyon halinde HEP3B ve HUH7 hücre hatlarındaki anti-kanser etkilerini; apoptoz, otofaji, hücre döngüsü düzenlenmesi ve DNA Hasar Yanıtı üzerinden test etmiştir. D vitamini ve CBD'nin kombinasyon tedavisi, her iki hücre hattında da hücre yaşamını önemli ölçüde azaltan sinerjik bir etki göstermiştir. Özellikle bu kombinasyon, HEP3B hücrelerinde otofaji ve apoptozu indüklemiş, HUH7 hücrelerinde ise hücre döngüsünü baskılamıştır; bu da anti-tümörijenik ve anti-kanser etkileri işaret etmektedir. Bu bulgular, D vitamini ve CBD'nin HCC'ye karşı yeni bir tedavi stratejisi olabileceğini göstermekte olup, daha karmaşık biyolojik sistemlerde etkinlik, güvenlik ve etki mekanizmalarının değerlendirilmesi amacıyla ek in vivo çalışmalar ve klinik araştırmalar gerektirmektedir.

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LIST OF ABBREVIATIONS

AP-1	Activator Protein-1
ATCC	American Type Culture Collection
ATG5	Autophagy Related 5
ATM	Ataxia telangiectasia mutated
ATM	Ataxia telangiectasia mutated
ATP5B	ATP Synthase Subunit Beta
ATR	Ataxia telangiectasia and Rad3 related
ATR	Ataxia Telangiectasia and Rad3 related
BAK	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell Lymphoma 2
BECLIN-1	Marker of Autophagy Beclin-1
BIRC5	Baculoviral inhibitor of apoptosis repeat-containing 5
BRCA1	Breast Cancer Gene 1
BRCA2	Breast Cancer Gene 2
c-Myc	Cellular Myelocytomatosis oncogene
Cas7	Caspase 7
Cas8	Caspase 8
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
CBD	Cannabidiol
CDK1	Cyclin Dependent Kinase 1
CDK2	Cyclin Dependent Kinase 2
CDK4	Cyclin Dependent Kinase 4
CDK6	Cyclin Dependent Kinase 6
CO_2	Carbon Dioxide
CTNNB1	Catenin Beta 1
DDR	DNA Damage Response
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribonucleic Acid
ECS	Endocannabinoid System
EMT	Epithelial-Mesenchymal Transition
FBS	Fetal Bovine Serum
FLC	Fibrolamellar Carcinoma
GLOBOCAN	Global Cancer Observatory
IC50	The half-maximal inhibitory concentration
HBV	Hepatitis B Virus
HBX	Hepatitis B virus X protein
HCC	Hepatocellular carcinoma
HCC-CCA	Hepatocellular-Cholangiocarcinoma
HCV	Hepatitis C Virus
HPV	Human Papillomavirus
iCCA	Intrahepatic Cholangiocarcinoma
KI67	Marker of Proliferation Ki-67
LC3B	Microtubule-Associated Proteins 1A/1B Light Chain 3B
mRNA	Messenger RNA
NAFLD	Non-Alcoholic Fatty Liver Disease
NF-κB	Nuclear Factor Cappa B
NS3	Non-Structural Protein 3
NS5A	Non-Structural Protein 5A
PARP1	Poly (ADP-ribose) polymerase 1
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PEI	Percutaneous Ethanol Injection
PI	Propodium İodide
PI3K	Phosphoinositide 3-Kinase
PLC	Primary Liver Cancer
PSA	Penicillin/Streptomycin/Amphotericin
RFA	Radiofrequency Ablation
RPL30	Ribosomal Protein L30
SERCA1	Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase 1

SSB	Single-Strand Breaks
TACE	Transarterial chemoembolization
TERT	Telomerase Reverse Transcriptase
THC	Δ9-tetrahydrocannabinol
TP53	Transcription Factor
UV	Ultraviolet Radiation
UVB	Ultraviolet B
VDBP	Vitamin D-Binding Protein
VDR	Vitamin D Receptor
Wnt	Wingless/Integrated

1. INTRODUCTION

1.1. CANCER

Cancer, referred to as a malignant neoplasm or tumor, encompasses a diverse spectrum of diseases distinguished by the unregulated proliferation of atypical cells, that can originate in virtually every organ or tissue within the human body [1]. Under normal physiological conditions, healthy cells in the human body proliferate during a regulated approach identified as cell division, ensuring the generation of new cells as required for tissue maintenance and repair. Aged or damaged cells undergo programmed cell death (apoptosis) and are systematically replaced by newly formed, functional cells to preserve cellular homeostasis [2]. In certain instances, the tightly regulated process of cellular proliferation is disrupted, leading to the unregulated growth and accumulation of aberrant or damaged cells in inappropriate locations. This results in the formation of tumors, which are tissue masses [3]. Tumors can be divided as malignant (cancerous) and benign (noncancerous). Malignant tumors have the potential to originate in any tissue or organ and possess the ability to invade adjacent structures. Through a process known as metastasis, malignant cells can disseminate via the circulatory or lymphatic systems, establishing secondary tumors in distant body regions [4].

Cancer cells exhibit numerous distinctions from normal, healthy cells, as shown in Figure 1.1. While normal cells rely on extracellular signaling cues to regulate proliferation, cancer cells demonstrate uncontrolled growth independent of these signals [5]. They evade programmed cell death, or apoptosis, a tightly regulated process that eliminates dysfunctional or excessive cells through specific inhibitory signals [6]. Unlike healthy cells, which cease proliferation upon contact with neighboring cells and generally remain stationary within their designated tissues, cancer cells exhibit invasive properties, infiltrating adjacent structures and metastasizing to distant sites [7]. Additionally, cancer cells induce angiogenesis by signaling blood vessels to extend toward the tumor, ensuring a continuous supply of oxygen and nutrients to sustain their growth. Under normal conditions, the immune system recognizes and eliminates abnormal or damaged cells; nevertheless, malignancies use sophisticated mechanisms to evade immune surveillance, enabling their sustained survival and uncontrolled proliferation [8].

Fundamentally, cancer is a genetic disorder arising from mutations in genes that govern critical cellular functions, particularly those regulating growth and division.

Oncogenic genetic alterations can arise from multiple factors, including errors during cellular division, DNA damage induced by environmental carcinogens, such as toxic compounds in tobacco smoke and ultraviolet radiation from sunlight, and inherited mutations passed down from parents [9]. In normal biological circumstances, cells with damaged DNA are efficiently detected and eliminated by the body's regulatory mechanisms before they undergo malignant transformation. However, with aging, the efficacy of these protective processes decline, leading to an increased likelihood of accumulating genetic alterations [10]. This decline in cellular surveillance and repair mechanisms is a key factor contributing to the elevated cancer risk observed in older individuals [11]. The genetic changes that contribute to the creation of malignant cells in the body affect several types of mutated genes. These genes are typically categorized into two primary classes: oncogenes, which promote uncontrolled cell proliferation when activated, and tumor suppressor genes, which typically moderate cell growth and avoid malignant transformation [12]. Proto-oncogenes are essential cell growth, division, and differentiation regulators, encoding proteins that mediate key cellular activities such as cell cycle control and signal transduction [13]. As proto-oncogenes encounter genetic changes, like mutations, gene amplification, or chromosomal translocations, they transform into oncogenes, driving unregulated cell proliferation and contributing to tumorigenesis. The activation of oncogenes disrupts normal cellular homeostasis, promoting malignant transformation and cancer progression [14]. Oncogenes, often referred to as cancer-related genes, become activated within a cell due to various external factors, including tobacco exposure, excessive ultraviolet (UV) radiation, ionizing radiation, and carcinogenic substances. Once activated, these oncogenes cause a normal cell to become cancerous by promoting uncontrolled proliferation and disrupting regulatory cellular mechanisms [15]. Tumor suppressor genes function as critical cell growth and division regulators, preventing normal cells' malignant transformation. When specific genetic alterations occur in these genes, their regulatory capacity is compromised, resulting in uncontrolled cellular proliferation and the potential development of cancer [16]. DNA repair genes, recognized as a distinct class of cancer-associated genes, are responsible for maintaining genomic integrity by repairing damaged DNA.

Mutations in these genes impair the DNA repair mechanisms, accumulating additional genetic alterations, including mutations in other critical genes and structural chromosomal abnormalities such as fragment duplications or deletions [17].

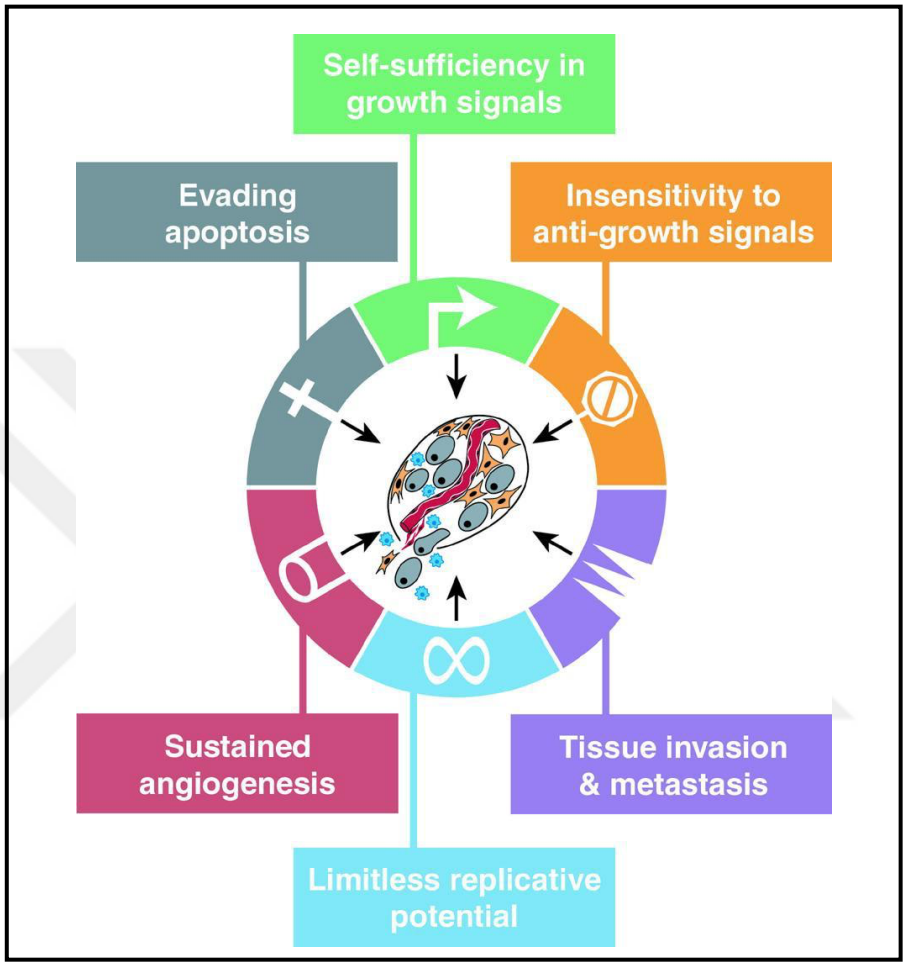

Figure 1.1. Developed characteristics of cancer cells [18].

Cancer continues to be among the most significant global health issues today. According to the GLOBOCAN 2022 report, about 20 million new cancer instances were detected globally, accounting for approximately 9.74 million deaths. The lifetime chance of acquiring cancer is thought to be one in five, with one in nine men and one in twelve women losing their lives to the disease [19]. Lung cancer continues to be the leading common type, accounting for 2.48 million cases (12.4%), closely surpassed by breast cancer (2.31 million instances, 11.6%). Stomach (4.9%), colorectal (9.6%), and prostate (7.3%) cancers are also among the most frequently diagnosed. In terms of mortality, lung cancer remains the deadliest, responsible for 1.82 million deaths (18.7%), with colorectal, liver, breast, and stomach cancers also leading to significant numbers of fatalities [20].

Cancer incidence and mortality rates vary significantly by region. Australia and New Zealand report the highest incidence rates, while Western Africa and South-Central Asia have the lowest. When it comes to cancer-related deaths, Eastern Europe sees the highest mortality among men, while Melanesia records the highest mortality among women. Cancer is highly prevalent in Asia, leading to over half of all global cases (49.2%) and more than half of all deaths (56.1%). Europe follows with 22.4% of cases and 20.4% of deaths. Meanwhile, Africa faces disproportionately high cancer mortality due to limited healthcare access and late-stage diagnoses [21]. Cancer prevalence varies between men and women. In men, the most prevalent diagnosed malignancies are liver, colorectal, lung, prostate, and stomach cancer. Breast cancer is the most extensive between women, ensued by colorectal, lung cervical, and stomach carcinomas [22]. Reducing disparities in cancer care, improving early detection, and enhancing treatment options are crucial steps in the fight against cancer. Implementing effective prevention strategies, addressing key risk factors, and advancing medical infrastructure can help lower cancer incidence and mortality rates, ultimately leading to better global health outcomes. Cancer is a challenging disorder influenced by behavioral, environmental, and biological elements. The studies show that several modifiable risk factors play a significant role in cancer development. Among them, smoking stands the top cause, responsible for 21% of all cancer caused deaths worldwide [23]. Alcohol intake, a low-fruit and vegetable diet, and obesity also play significant roles. In countries with high incomes, tobacco use, drinking, and obesity are the primary reasons for cancer, while in low- and middle-income regions, additional factors such as unsafe sex (leading to HPV infections) and environmental pollutants significantly contribute [24]. Exposure to indoor smoke from household fuel use and urban air pollution are further risk factors, particularly in developing regions.

Despite advances in cancer treatment, preventive measures such as lifestyle modifications and early screening remain the most effective strategies for reducing cancer-related mortality [25]. Cancer treatment involves various approaches, including chemotherapy, radiation therapy, immunotherapy, and targeted therapies, each designed to combat malignancies effectively [26]. According to the studies, chemotherapy remains a cornerstone of cancer treatment but carries the risk of secondary malignancies, particularly with alkylating agents and topoisomerase inhibitors. Radiation therapy, another common approach, induces DNA damage to kill cancer cells but may also lead to long-term complications such as secondary cancers, including sarcomas and hematologic malignancies [27].

Immunotherapy has developed as a revolutionary treatment by harnessing immune system of the body to attack malignant cells, particularly in malignancies with high mutational loads including melanoma and lung cancer [28]. Additionally, advances in molecular medicine have led to targeted therapies that specifically inhibit cancer-promoting pathways, offering more precise and effective treatments with fewer side effects.

Cancer is an extensive illness encompassing more than 100 distinct forms, categorized based on the tissue or cell type where they originate. Carcinomas, the most common type, arise from epithelial cells and include adenocarcinoma (breast, colon, prostate), squamous carcinoma (skin, lungs, bladder), basal carcinoma (skin), and transitional cell carcinoma (bladder, ureters, kidneys) [29]. Sarcomas develop in bones and soft tissues [30]. Leukemias affect blood-forming tissues, leading to abnormal white blood cell buildup, while lymphomas originate in lymphocytes, with Hodgkin and non-Hodgkin lymphoma [31]. Multiple myeloma impacts plasma cells located within the bone marrow, and melanoma develops in melanocytes, primarily in the skin. Brain and spinal cord tumors vary by cell type and location, affecting the central nervous system [32].

1.2. LIVER CANCER

Primary liver cancer (PLC) is characterized by the formation of cancerous cells within hepatic tissues [33]. Among the largest organs in the human system, the liver is responsible for a variety of physiological activities, including bile production for lipid digestion, glycogen storage for energy metabolism, and detoxification of harmful substances from the bloodstream [34]. The proliferation of malignant cells within hepatic tissue disrupts these essential functions. Primary liver cancer arises as a consequence of chronic liver injury, persistent inflammation, and genetic alterations, leading to impaired hepatic function and disease progression [35]. PLC is a major worldwide health issue, accounting for the 6th most frequently confirmed cancer and the third greatest reason of cancer-linked fatalities. In 2020, roughly 905,700 additional instances of liver cancer were detected worldwide, leading to approximately 830,200 deaths [36]. The disease is widespread in Northern Africa, Eastern Asia, and Southeast Asia, with Eastern Asia alone accounting for over 54% of global cases and deaths [37]. Liver cancer disproportionately affects men, with incidence and mortality rates consistently higher than those seen in women across all regions.

If current trends continue, the cases could increase by 55% by 2040, reaching 1.4 million additional diagnoses and 1.3 million fatalities annually [38]. Since many contributing factors, such as Hepatitis B and C infections, too much alcohol intake, obesity, and aflatoxin absorption, are avoidable, enhancing early detection and broadening public health efforts will be essential in alleviating the increasing impact of this illness [39]. PLC, generated from liver epithelial cells, refers to a wide range of cancerous tumors with diverse tissue characteristics and a generally bad outcome [40]. These neoplasms originate from hepatocytes or intrahepatic bile duct cells and include hepatocellular carcinoma, intrahepatic cholangiocarcinoma, and rarer subtypes such as combined hepatocellular-cholangiocarcinoma, fibrolamellar carcinoma, and hepatoblastoma, a childhood liver malignancy [41]. Primary liver cancer consists of several malignant tumor types, each with unique characteristics and risk factors. The most common form, hepatocellular carcinoma (HCC), makes up nearly 75–85% of cases and is closely linked to chronic HBV and HCV infections, alcohol intake, cirrhosis, and aflatoxin poisoning. Unfortunately, due to late diagnosis, HCC has a devastating outcome, duration of survival less than 10% [42]. Intrahepatic cholangiocarcinoma (iCCA), the second most prevalent type, originates from the bile ducts and is associated with conditions like primary sclerosing cholangitis and liver fluke infections [43]. A rarer subtype, mixed hepatocellular cholangiocarcinoma (HCC-CCA), shares characteristics of both HCC and iCCA, making treatment more complex. Fibrolamellar carcinoma (FLC), which typically affects young adults without prior liver disease or cirrhosis, tends to have a slightly better outlook than standard HCC [44]. The particularly prevalent liver cancer in kids is hepatoblastoma, which is frequently associated with hereditary diseases [45].

The therapy of PLC, especially HCC, is complex and based on tumor stage, liver function, and overall patient well-being. While curative treatments are available for early-stage cases, advanced liver cancer often requires palliative or systemic therapies. Treatment options for patients discovered at the outset include surgical removal, liver donation, and targeted ablative treatments [46]. Surgical resection is the preferred approach for those with good liver function and localized tumors, but recurrence is a significant challenge, with more than 70% of cases recurring within five years [47].

Liver transplantation offers a curative solution by addressing all the cancer and fundamental liver illness, but the limited availability of donor organs remains a significant obstacle [48]. For individuals who are not suitable for operation, regional ablative treatments consisting efficient, particularly for small tumors [49]. The most common treatment for intermediate-stage HCC is transarterial chemoembolization (TACE). This procedure administers chemotherapy straight to the cancer cells while cutting off its blood circulation, thereby shrinking the malignancy and slowing its development [50]. In advanced-stage liver cancer, systemic therapies become the primary option, as surgery and locoregional treatments are frequently ineffective. Sorafenib, was the first targeted therapy to show survival benefits in patients with severe HCC [51]. Despite ongoing advancements in treatment, liver cancer remains difficult to manage, particularly in its later stages, where long-term survival rates remain low [52]. A multidisciplinary approach, improved early detection, and continued research into new therapies are essential for improving outcomes for liver cancer patients.

1.3. HEPATOCELLULAR CARCINOMA (HCC)

Malignant tumors that occur in hepatocytes, the primary cell type in the liver, are known as hepatocellular carcinoma [53]. HCC, the most prevalent PLC type, constitutes one of the top causes for death from cancer globally. HCC is the 5th most prevalent cancer globally and ranks 9th in the United States [54]. Due to the poor prognosis and lack of effective treatments for hepatocyte carcinoma, the five-year survival outcome is lower than 5%. Any chronic inflammatory liver disease can trigger HCC, but the pathophysiological process found in up to 80% of cases is cirrhosis [55].

1.3.1. Epidemiology and Ethiology of HCC

HCC is the 5th most widespread malignancy in men and the 9th most prevalent malignancy in women. With around 500,000 to 1 million instances reported each year worldwide, the prevalence of HCC is low in the Western world and high in Southeast Asia and Sub-Saharan Africa.

Still, it has increased in the US, Japan, the UK, and France [56]. Hepatocellular carcinoma is often considered a disease that primarily impacts older persons, with the greatest prevalence reported in people aged 65 to 69. The chance of acquiring HCC rises with age, particularly in high-risk groups like Hispanics, Native Americans, and Asians [57]. Cirrhosis and chronic liver disease are the most significant reasons for developing HCC. Cirrhosis resulting from alcoholic liver disease, HCV infection, and HBV infection are the three leading important risk causes for developing HCC throughout the United States. HCC can develop in people with HCV or HBV chronic liver disease throughout about 10 to 30 years [58]. The most prevalent forms of persistent liver disease worldwide are Hepatitis B and C [59]. HBV is a circular, double-stranded DNA virus that has eight genotypes (A to H) [60]. A and D genotypes are more widespread in the Middle East and Europe, whereas B and C genotypes are more prevalent in Asia [61]. Hepatitis B infects 5% of the global population via infected blood donations, parenteral injections, and sexual intercourse [62]. Unlike the Hepatitis B virus, the HCV is a tiny, single-stranded RNA virus with high genetic diversity. There are six HCV genotypes, with I, II, and III genotypes are dominate in the Far East, while IV dominating in the Middle East [63]. Alcohol intake is another significant risk variable for developing HCC. The relation among alcohol and liver disease is proportional to the amount of alcohol drank throughout a lifespan, with excessive alcohol usage being the most significant risk factor for HCC [64]. Chronic medical disorders including diabetes and obesity can raise the risk of forming HCC [65]. Diabetes has a direct impact on the liver since it is associated with glucose metabolism. As a result, diabetes can induce chronic hepatitis, fatty liver, liver damage, and cirrhosis, and it is a separate risk indicator for HCC [66]. On the other hand, obesity may contribute to HCC progression because it is related with a diversity of hepatobiliary illnesses, including non-alcoholic fatty liver disease, cryptogenic cirrhosis, and steatosis [67].

1.3.2. Molecular Biology Of HCC

Hepatocellular carcinoma, a complex developmental process, is triggered by the increase of multiple genetic alterations [68]. TERT, TP53, and CTNNB1 genes are examples of mutated genes frequently seen in the formation of HCC [69].

The telomerase reverse transcriptase (TERT) gene, that produces the restricting catalytic element of telomerase required for length of telomere support and serves a significant part in stem cell function, getting older, and cancer, is primarily suppressed in somatic cells, with the exception of self-renewing populations including stem cells [70,71]. In contrary to somatic cells, 70-90% of cancer cells contain the TERT gene, which is required for their unrestricted proliferation; thus, TERT is frequently reactivated during carcinogenesis [72]. The TP53 gene, the second commonly altered gene in HCC, appears in 30% of cases [73]. The TP53, a tumor suppressor gene, causes apoptosis, cell cycle arrest, and ageing in reaction to numerous cell-based tensions such as oncogene stimulation, DNA damage, and hypoxia [74]. Catenin beta 1 (CTNNB1) is a frequently mutated gene in hepatocellular carcinoma (HCC). It produces β-catenin, a part of the cadherin protein family at the cell interface and a signaling molecule in the Wnt (Wingless/Integrated) signaling pathway [75]. Abnormal β-catenin expression is seen in 20-30% of patients suffering from HCC [76].

In addition to these genetic alterations, ongoing infections with HCV and HBV have a notable role in the progression of HCC via unique molecular processes [77]. Integrating the HBV of DNA into the human genome can result chromosomal aberrations, rearrangements, or deletions. Endogenous genes like cyclin A and SERCA1, that functions essential roles in regulating division of cells or survival, can be activated by insertion mutations caused by genome integration of HBV viral DNA at particular locations, and this activation can result in uncontrolled cell proliferation [78]. Another method by which HBV causes HCC is the production of viral proteins with carcinogenic characteristics, particularly the x protein (HBX). HBX stimulates transcription factors NF-κB and AP-1, affecting gene expression associated to cell cycle, proliferation, and apoptosis via transaction activity [79]. HBX inactivates p53, a crucial tumor suppressor gene, by directly binding to it. This prevents p53 from carrying out its normal functions, including triggering apoptosis. This interaction allows HBV-infected liver cells to progress into hepatocellular carcinoma (HCC) [80]. Compared to HBV, HCV is an RNA virus which cannot be incorporated into the host's DNA. However, it triggers HCC by interacting with host proteinsNS3 and NS5A proteins are HCV proteins linked to hepatocyte carcinogenesis, and they have been demonstrated to block the post-transcriptional production of p21. This cyclin-dependent kinase inhibitor is critical for controlling the cell cycle [81].

HCV core proteins, which have transcriptional regulatory roles on various cellular genes associated with the controlling of cell growth, comprising the proto-oncogene c-Myc, play a crucial part in HCV hepatocarcinogenesis by regulating cellular proliferation, apoptosis, and immune response [82].

1.4. CANNABIDIOL (CBD)

Cannabis sativa plant, which has been utilized for healthcare for thousands of decades by numerous civilizations including Chinese, Japanese, Indian, and Egyptian, contains about 540 secondary metabolites, more than 120 of which are phytocannabinoids classified into 11 groups [83]. Many published research has explored phytochemicals produced from Cannabis sativa L., particularly phytocannabinoids and non-phytocannabinoids, in current years, owing mostly to the legalization of Cannabis in various nations and areas [84]. Cannabis forms are classified according to levels of the two main phytocannabinoids, Δ9-tetrahydrocannabinol (THC, possibly intoxicated) and cannabidiol (CBD, harmless) [85]. THC and CBD, the two widely investigated types of cannabis compounds, share many medicinal properties but their processes that operate are distinct. CBD, unlike THC, which is accountable for the possibly intoxicating effects of cannabis, is not possibly stimulating and is not connected with the classic symptoms of cannabis intoxication, rendering it a more plausible candidate for treatment [86]. *Cannabis* spp., frequently referred to as marijuana or commercial hemp, has low amount of THC but significant CBD levels. On the other hand, *Cannabis* spp. that has a significant THC content is grown for both medical and recreational purposes [87].

A key pathway through which CBD exerts its anticancer effects is by interacting with the endocannabinoid system (ECS) [88]. Endocannabinoid receptors mediate the most commonly recognized mode of action for cannabinoids. Cannabinoids exhibit their anticancer properties by binding to G protein-coupled receptors known as CB1 and CB2 [89]. The only difference between both CB1 and CB2 receptors, which exhibit pro-apoptotic, anti-inflammatory, and anti-proliferative functions that can aid in cancer treatment, depends on where they are distributed in the human body [90]. About the nervous system, CB1 receptors located at the ends of axons can suppress neurotransmitter function and enhance the control of pain. In contrast, the CB2 receptor, associated with immune functions, is found in high concentrations in peripheral tissues.

CB2 receptors, which are comprised in the regulation and control of cytokinesis in immune cells, contribute to anti-cancer aspects associated to separation of cells, like antiproliferation [91]. CB1 receptors have been shown to affect an array of biological processes, including starvation, metabolism, and body weight, and can be utilized to treat chemotherapy-related symptoms [92]. CBD is known for its pro-apoptotic and antiproliferative effects on various types of cancer. It can cause cell cycle arrest, apoptosis, and the reduction of chemotaxis, cancer cell movement, adhesion, invasion, angiogenesis, and metastasis. As research advances, CBD is increasingly recognized as a promising anticancer drug [93].

1.5. VITAMIN D

Vitamin D is a fat-soluble hormone that regulates blood calcium levels and promotes skeletal mineralization [94]. Vitamin D, generated in the epidermis via ultraviolet B (UVB) through sunlight, demonstrates additional functions in many cancer cell structures, comprising antiproliferative, prodifferentiation, pro-apoptotic, anti-angiogenesis, and anti-invasive qualities [95]. To create calcitriol, vitamin D goes through two steps of metabolism in the liver and kidneys. This biologically relevant version interacts to the Vitamin D Receptor (VDR), triggering its many cellular processes [96]. Vitamin D3 and Vitamin D2 are two leading isoforms of vitamin D, originating from two different sources. Vitamin D2, which is derived from plant-based foods like mushrooms, is created by ergosterol in plants, yeasts, and fungi using ultraviolet B radiation. Vitamin D3, which is generated from 7-dehydrocholesterol via UVB radiation in the outer skin layer, is obtained through a diet rich in animal-derived foods like cod liver oil [97]. Each of the forms of vitamin D are attached to Vitamin D-binding protein (VDBP) in the bloodstream before being delivered to the liver [98].

Vitamin D metabolism is a multistep process, as explained in Figure 1.2. Vitamin D is physiologically inactive, and Vitamin D metabolism begins in the liver. Once in the liver, Vitamin D is broken down by D-25-hydroxylase (25-OHase) to produce 25-hydroxyvitamin D [25(OH)D] (calcidiol), the predominant version of vitamin D seen in serum [99]. Calcidiol is produced in the liver and converted by 25(OH)D1α-hydroxylase in the kidney's proximal tubule to 1α,25-dihydroxyvitamin D [1α,25(OH)2D, calcitriol], the most physiologically active form of Vitamin D.

Calcitriol is synthesized, penetrates the blood flow, binds to VDBP, and is subsequently transported to select tissues including the intestine, bone, and kidney, wherein vitamin D controls calcium and phosphate absorption, mobilization, and reabsorption [100]. Another key kidney enzyme, 25(OH)D-24-hydroxylase, breaks down 1a,25(OH)2D to create 1a,24,25(OH)3D, thus stopping its functions. Furthermore, when there is a high level of 25(OH)D, 24-OHase in the kidneys may change it to 24,25(OH)2D14, avoiding overproduction of 1a,25(OH)2D [101].

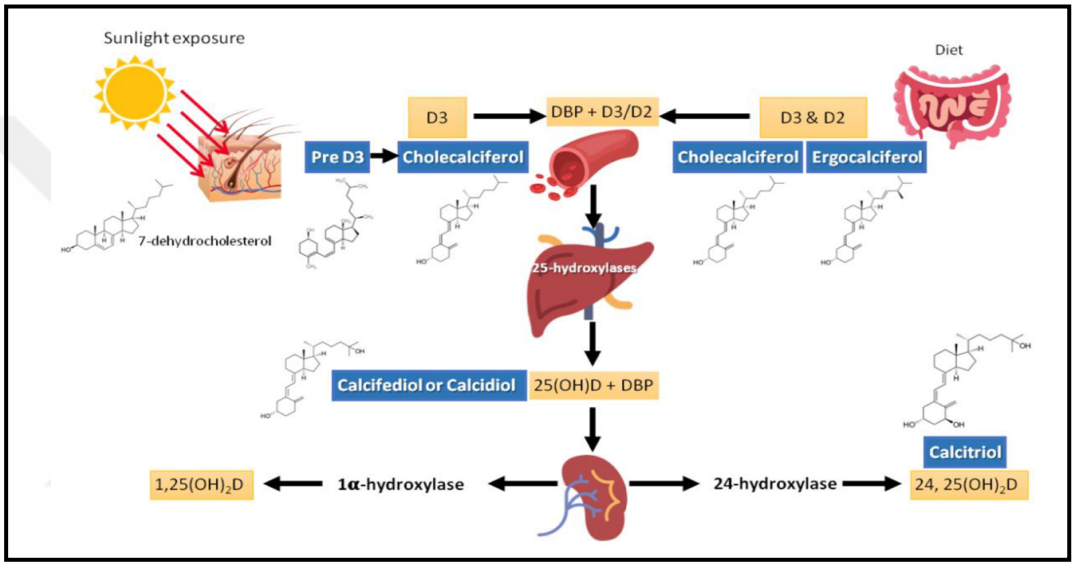

Figure 1.2. Metabolic pathway of Vitamin D in human body [102].

Epidemiological and preclinical research have shown that vitamin D has a favorable influence on cancer prevention and treatment. Vitamin D, which can control the entire step from the beginning of tumor formation to metastasis and cell-microenvironment interactions, provides the modulation of cell activities including differentiation, proliferation, apoptosis, autophagy, and EMT and regulation of cell-microenvironment associations including angiogenesis, antioxidants, inflammation and the immune system [103].

2. MATERIALS AND METHODS

2.1. CELL LINES AND CELL CULTURE CONDITIONS

Hep3B (HB-8064, human hepatocellular carcinoma) and Huh7 (human hepatocellular carcinoma) were bought from the American Type Culture Collection (ATCC, Rockville, MD). Both cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, #41966-029; Invitrogen, Gibco, UK). The medium contained 1% Penicillin/Streptomycin/Amphotericin (PSA) and 10% Fetal Bovine Serum (FBS, #10500-064, Invitrogen, Gibco, UK). In a humidified incubator, cells were incubated at 37°C with 5% CO2.

2.2. CYTOTOXICITY ASSAY

The *in-vitro* cytotoxicity of Vitamin D and CBD was applyed using the MTS cell survival test on Hep3B and Huh7 cell lines. Hep3B and Huh7 cells were planted in 96-well plates at densities of 3000 and 5000 cells/well, respectively. The following day, cells were treated with Vitamin D (doses ranged from 100 µM to 3.12 µM) and CBD (doses ranged from 200 µM to 6.25 µM) for both Hep3B and Huh7 cells. To analyze the synergistic effect of CBD and Vitamin D in both cell lines, the CBD dose was chosen based on cell viability, while Vitamin D was supplied at doses ranging from 100 µM to 3.12 µM. After cells were treated with distinct substance concentrations for 24, 48 and 72 hours, cell viability was done by MTS assay (3- (4,5-dimethyl-thiazol-2) -5 -(3-carboxy-methoxy-phenyl) -2-(4-sulfo-phenyl)-2- (4-sulfo-pheny- 2H-tetrazolium salt (MTS) (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK) based on the manufacturer's directions. The absorbance was measured at 490 nm using an ELISA plate reader (Biotek, Winooski, VT). IC-50 values were detected via the GraphPad Prism software. Finally, calculation of synergism analysis or combination index was done by applying Chou-Talalay theorem [104].

2.3. ANNEXIN V ASSAY

Hep3B and Huh7 cancer cell lines were stimulated with CBD, Vitamin D, and their combined tretament for 72 hours (treatment doses were chosen according to IC-50 values). First, Hep3B and Huh7 cells were seeded in T-25 flasks at densities of 130,000, and 300,000 cells/ml, respectively. Following 72 hours of treatment, the Annexin-V protocol was carried out per the manufacturer's instructions (#sc-4252AK, Santacruz Biotechnology, USA). The cells were then harvested, rinsed with ice-cold PBS, resuspended in Annexin V binding buffer, and divided into four groups: Annexin V, propidium iodide (PI), Annexin V + PI, and a negative control (NC). The data was examined via flow cytometry on a FACS Calibur (BD Biosciences) instrument.

2.4. CELL CYCLE ANALYSIS

Cell cycle profiling was applied through flow cytometric analysis using PI staining in Hep3B and Huh7 cancer cell lines. Hep3B and Huh7 cells were initially seeded into flasks, and treatments were administered 24 hours post-seeding, followed by a 72-hour incubation at 37°C. Before evaluation, cells were collected, rinsed with PBS, and fixed in 70% ice-cold ethanol for a minimum of two hours at -20°C. Cell pellets were permeabilized with 0.1% Triton X-100, then treated with 20 µg/ml RNase at room temperature for 30 minutes. Lastly, cells were treated with propidium iodide and examined immediately using a 488 nm single laser emitting flow cytometer.

2.5. REAL-TIME PCR

RNA was extracted by utilizing an RNA isolation kit (#740955.250, Macherey-NAGEL, Düren, Germany) as instructed in the user handbook. Subsequently, the isolated total mRNAs were transformed into cDNAs with QuantiTect Reverse Transcription Kit (#205313, QIAGEN, Hilden, Germany). SYBR Green (#4309155, Thermo Fisher, Waltham, ABD) was used for Q-PCR, which was tested using the iCycler q-PCR detection equipment (Bio-Rad, Hercules, CA, USA). Expression levels were adjusted in relation to the ribosomal protein RPL30 gene. The genes analyzed in this study, along with their corresponding primer sequences, are presented in Table 2.1. The fold changes for each sample were determined using the 2[-Delta C(T)] method.

Table 2.1. Forward and Reverse Primer Sequences (5′–3′) Designed Using NCBI Primer-BLAST for Real-Time PCR

Gene	Side	Sequence
TP53	F	5’ GCCCAACAACACCAGCTCCT 3’
	R	5’ CCTGGGCATCCTTGAGTTCC 3’
ATM	F	5’ TGTTCCAGGACACGAAGGGAGA 3’
	R	5’ CAGGGTTCTCAGCACTATGGGA 3’
ATR	F	5’ GGAGATTTCCTGAGCATGTTCGG 3’
	R	5’ GGCTTCTTTACTCCAGACCAATC 3’
Caspase 7	F	5’ TCAGTGGATGCTAAGCCAGACC 3’
	R	5’ CGAACGCCCATACCTGTCAC 3’
Caspase 8	F	5’GCCACCCGGCTTCAGAATGGC 3’
	R	5’TATGGGCCATCTGCTGTTGGCAGT 3’
Survivin	F	5’TCATGCTGAGGCTCCAGAGTTC3’
	R	5’ACAGTCTTGCCAACTCCAGCAC3’
BAX	F	5’TGCAGAGGATGATTGCCGCCG3’
	R	5’ACCCAACCACCCTGGTGTTGG3’
BCL-2	F	5’ AACGGAGGCTGGGATGCCTTTGTG 3’
	R	5’ ACCAGGGCCAAACTGAGCAGAGT 3’
PCNA	F	5’ CAAGTAATGTCGATAAAGAGGAGG 3’
	R	5’ GTGTCACCGTTGAAGAGAGTGG 3’
CDK1	F	5’ CACTTGGCTTCAAAGCTGGCTC 3’
	R	5’ ATGGGTATGGTAGATCCCGGC 3’
CDK2	F	5’ CTGGACACGCTGCTGGATG 3’
	R	5’ ATGCCAGTGAGAGCAGAGGC 3’
CDK4	F	5’ GTCTATGGTCGGGCCCTCTG 3’
	R	5’ CAGATCAAGGGAGACCCTCACG 3’
CDK6	F	5’ GTCTGATTACCTGCTCCGCGA 3’
	R	5’ TCCAGAATCATTGCACCTGAGGG 3’
Ki-67	F	5’ GAAAGAGTGGCAACCTGCCTTC 3’
	R	5’ GCACCAAGTTTTACTACATCTGCC 3’
BECLIN	F	5’GGTGTCTCTCGCAGATTCATC3’
	R	5’TCAGTCTTCGGCTGAGGTTCT3’
ATG5	F	5’AAAGATGTGCTTCGAGATGTGT3’
	R	5’CACTTTGTCAGTTACCAACGTC3’
LC3B	F	5’AAGGCGCTTACAGCTCAATG3’
	R	5’CTGGGAGGCATAGACCATGT3’
BRCA1	F	5’GAACCAGGAGTGGAAAGGTCA3’
	R	5’GCTGTTGCTCCTCCACATCA3’

BRCA2	F	5’AGACTGTACTTCAGGGCCGTACA3’
	R	5’GGCTGAGACAGGTGTGGAAACA3’
PARP1	F	5’CCAAGCCAGTTCAGGACCTCAT3’
	R	5’GGATCTGCCTTTTGCTCAGCTTC3’
BAK	F	5’TTACCGCCATCAGCAGGAACAG3’
	R	5’GGAACTCTGAGTCATAGCGTCG3’
RPL30	F	5’ ACAGCATGCGGAAAATACTAC 3’
	R	5’ AAAGGAAAATTTTGCAGGTTT 3’

3. RESULT

3.1. THE SINGLE AND COMBINATION EFFECT OF VITAMIN D AND CBD ON CELL SURVIVAL

The Hep3B and Huh7 cancer cell lines were administrated with Vitamin D, CBD, or a combination of the two for 24, 48, and 72 hours, respectively. The IC50 values for treatment groups were determined using an MTS-based cytotoxicity test on both the Hep3B and Huh7 cell lines (Table 3.1).

Considering the results, the single treatment of Vitamin D and CBD reduces the cell viability of Hep3B cell lines in a dose-responsive way (Figure 3.1a, Figure 3.1b). When the single effects of Vitamin D and CBD on the cell line were detected, the IC-50 was 43.70μM and 62.85 μM, respectively, as found in Table 3.1. CBD was administered at a maximum dose of 50 μM for combination therapy, with Vitamin D levels ranging from 100 μM to 3.12 μM. The IC50 dose of Vitamin D alone in Hep3B cancer cell lines is 43.70μM, while Vitamin D coupled with CBD is 15.48 μM. When the IC50 values of single and combination Vitamin D treatments were evaluated, the Vitamin D dose was found to be 2.8 times lower (Table 3.1). According to these results, the synergistic effect between Vitamin D and CBD was found in Hep3B cell lines (Figure 3.1c).

The Huh7 cell line was treated with Vitamin D and CBD alone and in combination at various dosages for 24, 48, and 72 hours. Table 3.1 shows the IC-50 values for Vitamin D and CBD on the cell line, which were 52.35 μM and 51.44 μM, respectively. In combination therapy, Vitamin D at doses ranging from 100 μM to 3.12 μM and CBD at 50 μM, the maximum level that kept the cells alive, were delivered. The IC-50 value of Vitamin D in Huh7 cell lines was thus established as 52.35 μM, while the dose of Vitamin D in combination with CBD was found to be 26.42 μM. In conclusion, the vitamin D dosage was lowered by 1.98 times when the IC-50 values of the single and combined treatments were compared. Based on the findings, combination of Vitamin D and CBD demonstrated the synergistic effect on Huh7 cell lines (Figure 3.2c).

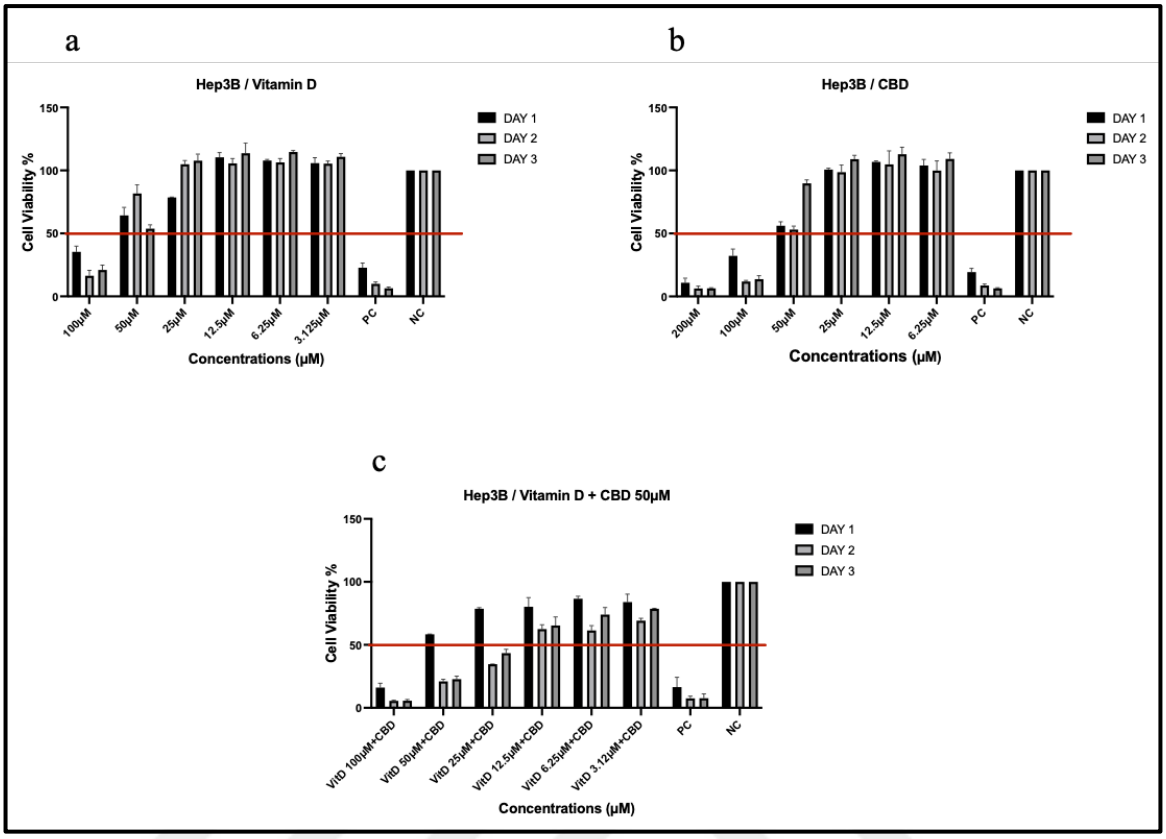

Figure 3.1. The cell survival levels (percentages) of Hep3B cell line at 24h, 48h, and 72h after treatment with (a) Vitamin D, (b) CBD, and (c) Combination of Vitamin D and CBD. Each group was compared to the equivalent negative control.

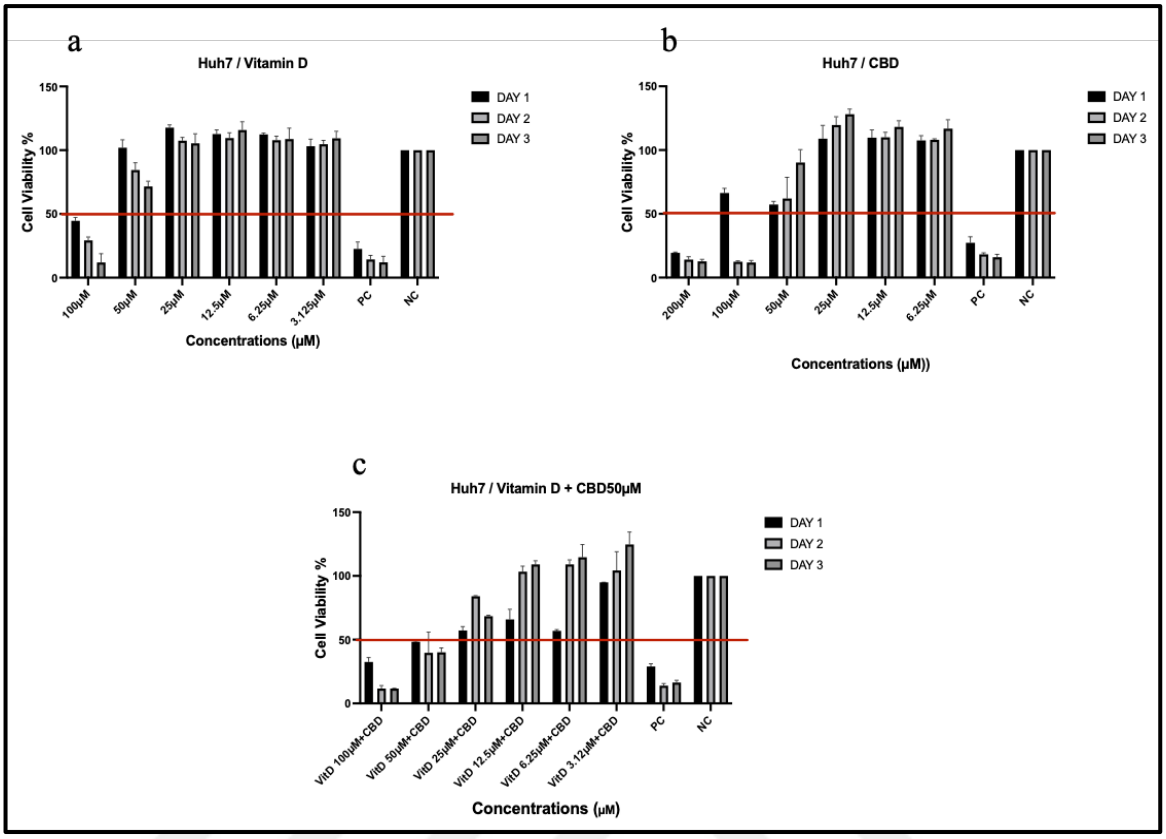

Figure 3.2. The cell survival levels (percentages) of Huh7 cell line at 24h, 48h, and 72h after treatment with (a) Vitamin D, (b) CBD, and (c) Combination of Vitamin D and CBD. Each group was compared to the equivalent negative control.

Table 3.1. The IC 50 values of Hep3B and Huh7 cell lines for the treatment.

TREATMENT	IC-50 Values of Hep3B	IC-50 Values of Huh7
Vitamin D	43.70 µM	52.35 µM
CBD	62.85 µM	51.44 µM
Vitamin D + CBD	15.48 µM + 50 µM	26.42 µM + 50µM

3.2. THE EFFECT OF SINGLE AND COMBINATION TREATMENT OF VITAMIN D AND CBD ON APOPTOSIS

The effect of individual or combined substances on apoptosis was investigated in HEP3B and HUH7 cell lines using the Annexin V staining procedure. Vitamin D at its IC50 concentration and CBD at a non-toxic dose were applied in combination to both cell lines for 72 hours. While the lower left quadrant of the cytogram resulting from the Annexin V assay shows live cells, the upper left quadrant shows PI-positive necrotic cells (Figure 3.3, Figure 3.4). Because of the absence of PI inclusion, early apoptotic cells are placed in the lower right quadrant of the cytogram, while late apoptotic cells are positioned in the upper quadrant (Figure 3.3, Figure 3.4).

Vitamin D and CBD treatments alone induced apoptosis in HEP3B and HUH7 compared to the negative control. However, CBD alone or combined with Vitamin D did not affect apoptosis or necrosis in HEP3B and HUH7 cell lines (Figure 3.3, Figure 3.4). Furthermore, Vitamin D and CBD combination treatment led to a significant induction of early apoptosis in both cell lines found in Figure 3.3 and 3.4 (Vitamin D+CBD on HEP3B and HUH7: %29.375, and %34,175 for early apoptosis marker, respectively).

The expression levels of important genes correlated with programmed cell death were examined to investigate further how single and combined treatments with vitamin D and CBD affect the apoptosis mechanism in HEP3B and HUH7 cell lines. The expression level of the Casp7 significantly increased in CBD and its combination with Vitamin D for HEP3B cell line compared to negative control (≈1.281 fold for CBD and ≈1.288 fold for the combined treatment of Vitamin D and CBD), while the expression level of Caspase 8 significantly increased only in vitamin D treatment (≈1.646 fold for Vitamin D) (Figure 3.3a). However, for the HUH7 cell line, the expression level of Caspase 7 significantly decreased in a single treatment of Vitamin D and combination with CBD (≈0.0161 fold for Vitamin D and ≈0.256 fold for a combination of Vitamin D and CBD) (Figure 3.3b). As found in Figure 3.3b, the gene expression level of Caspase 8 extensively decreased in only the treatment of Vitamin D (≈0.047 fold for Vitamin D).

In the HEP3B cell line, the expression level of the BAX gene was substantially increased relative to the negative control for both single and combination treatments with vitamin D (≈1.602 fold for Vitamin D and ≈1.995 fold for combination of Vitamin D and CBD). However, the expression level of BAK significantly decreased for single treatments of CBD and Vitamin D (≈0.321 fold for CBD and ≈0.520 fold for Vitamin D). In contrast, expression levels of these genes importantly decreased in the HUH7 cell line under the single treatment of Vitamin D and its combination with CBD (BAX: ≈0.011 fold for Vitamin D, and ≈0.586 fold for the combined treatment of Vitamin D and CBD; BAK: ≈0.025 fold for Vitamin D, and ≈0.489 fold for the combined treatment of Vitamin D and CBD). The expression level of the TP53 gene, significantly increased for treatments of Vitamin D and its combination with CBD in HEP3B cell lines (≈1.384 fold for Vitamin D and ≈1.750 fold for the combined treatment of Vitamin D and CBD). On the contrary, the expression level of TP53 gene significantly decreased for all treatment groups in the HUH7 cell line (≈0.503 fold for CBD, ≈0.007 fold for Vitamin D, and ≈0.0841 fold for combination of Vitamin D and CBD).

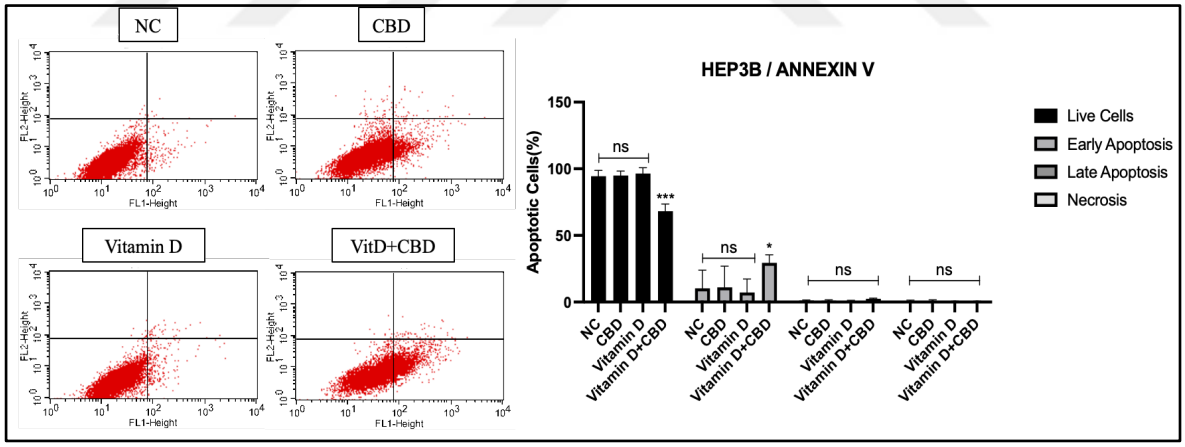

Figure 3.3. Demonstrative Annexin V-Fitc/PI staining results for HEP3B cancer cell lines at 72h.

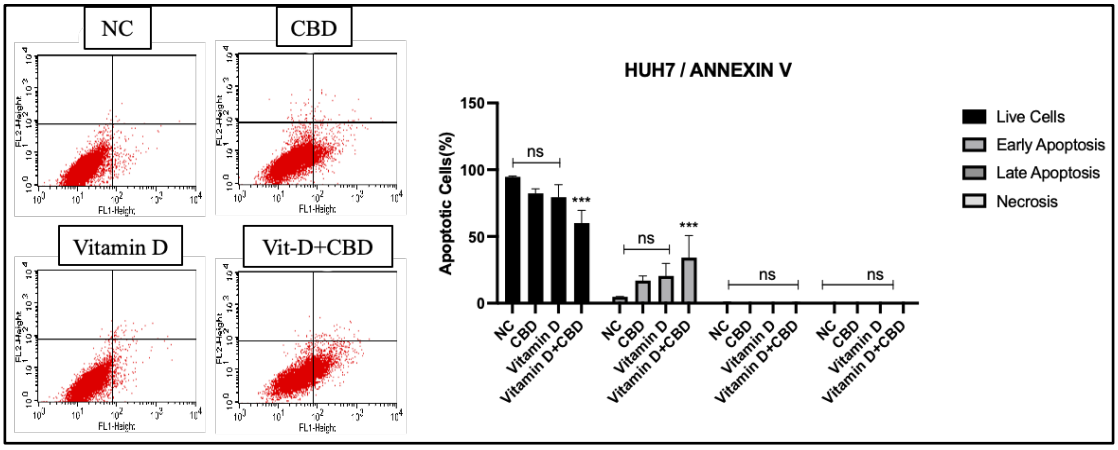

Figure 3.4. Demonstrative Annexin V-Fitc/PI staining results for HUH7 cancer cell lines at 72h.

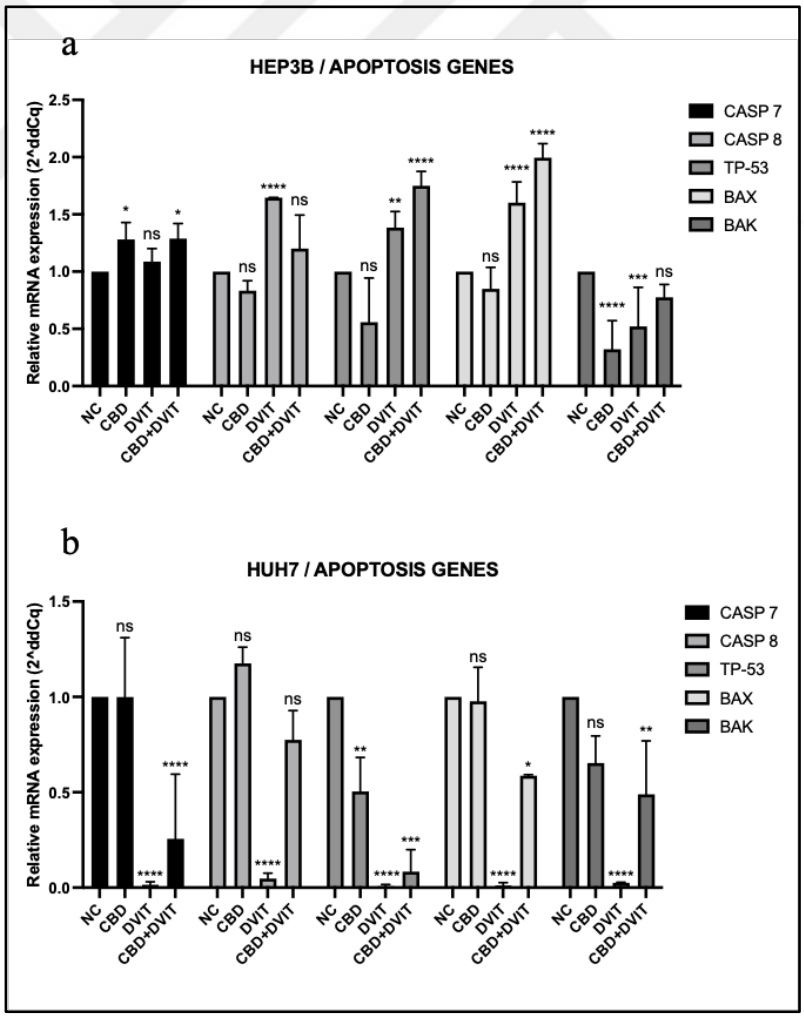

Figure 3.5. Representative graph of Apoptotic genes' expression profiles on (a) HEP3B and (b) HUH7 cell lines after 72 h.

3.3. THE EFFECT OF SINGLE AND COMBINATION TREATMENT OF VITAMIN D AND CBD ON CELL CYCLE AND CELL PROLIFERATION

To confirm the cell cycle effects of single and combination treatments of Vitamin D and CBD in HEP3B and HUH7, the results were obtained using the same treatment procedure as in the Annexin V assay. Figures 3.6 and 3.7 indicate the arrangement of cell cycle phases. The initial, middle, and final phases correspond to the G0/G1, S, and G2 phases, respectfully. The results display the cell population percentages of HEP3B and HUH7 in the G0/G1, S, and G2 phases, relative to NC. The observed positive effects of the chemicals on cell survival could be attributed to enhanced the death of cells (cytotoxicity) or slowdown of cell growth (cytostatic) caused by cell cycle arrest.

In the HEP3B cell line, combined treatment of Vitamin D and CBD resulted in a notable increase in the cell population in the G1 phase (84.61%). This indicates that the cells were arrested in the G1 phase, preventing their progression to the S phase (Figure 3.6). Additionally, as shown in Figure 3.6, the HEP3B cell line exhibited a considerable increase in cell numbers in the S phase following treatment with Vitamin D alone (11.86%), CBD alone (15.22%), and the combination of Vitamin D and CBD (12.88%). Similarly, in the HUH7 cell line, administration with the combination of Vitamin D and CBD resulted in an increase in the G1 phase (%73.1) and a decline in the S phase compared to the negative control, indicating that the cells were unable to advance to the S phase (Figure 3.7).

Furthermore, the mRNA expression levels of cell cycle regulators CDK1, CDK2, CDK4, and CDK6 were investigated in both HEP3B and HUH7 cell lines administered with Vitamin D, CBD, or a combination of the two, as found in Figure 3.8. In the HEP3B cell line, among all genes, only the mRNA expression level of the CDK4 gene significantly increased for the treatments of Vitamin D (≈1.931 fold), CBD (≈1.672 fold), and their combination (≈2.196 fold) (Figure 3.8a). In addition, the expression level of CDK1 significantly decreased for treatment of Vitamin D-CBD combination (≈0.589 fold). On the contrary, the expression levels of CDK1, CDK2, CDK4, and CDK6 notably decreased for all treatment groups, except treatment of Vitamin D in the CDK4 gene in the HUH7 cell line (Figure 3.8b).

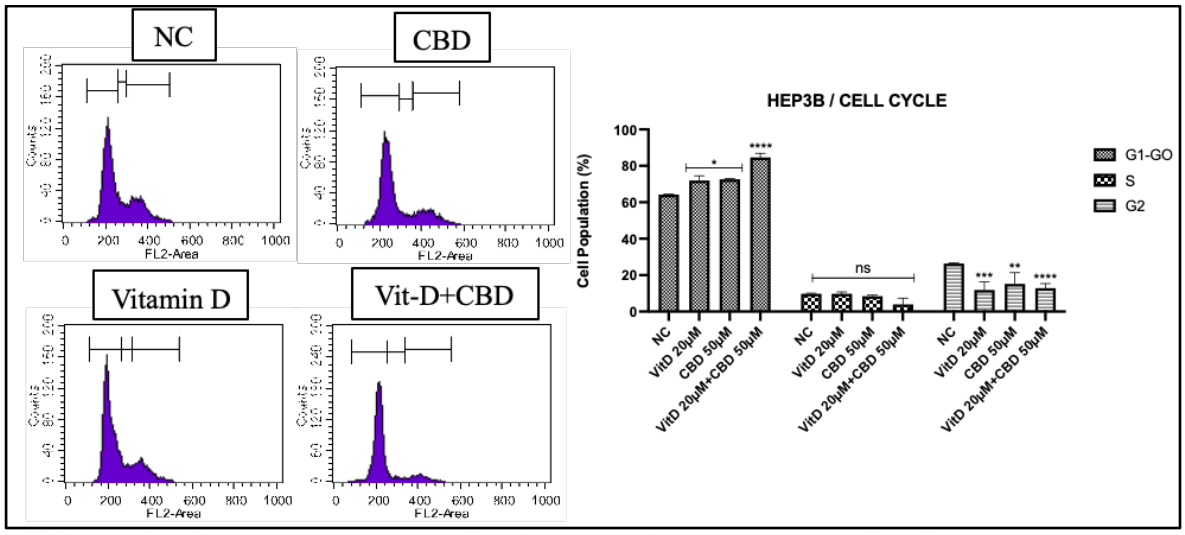

Figure 3.6. The distribution of cell cycle phases by flow cytometry for HEP3B cell lines.

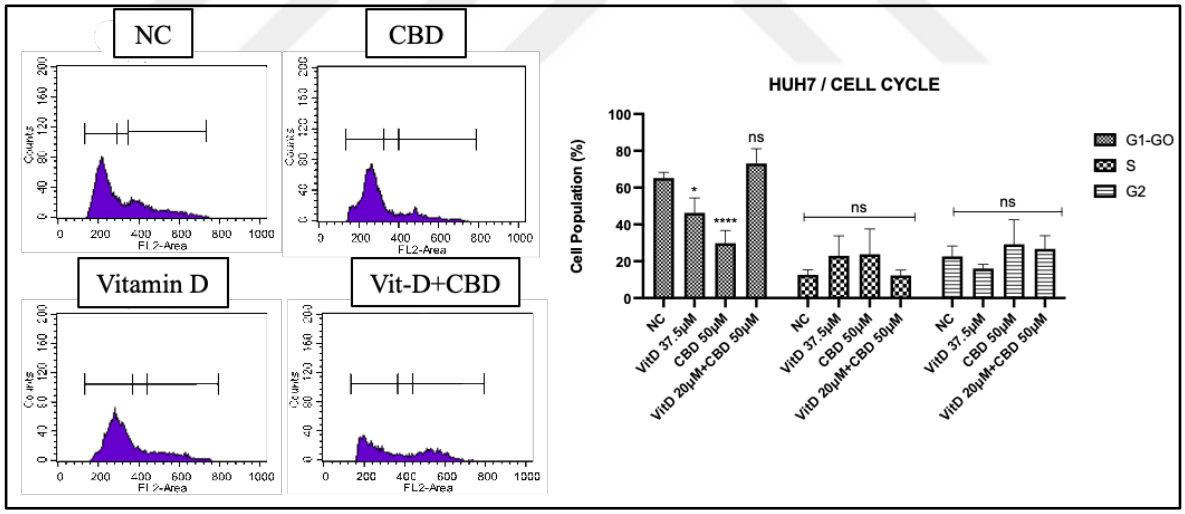

Figure 3.7. The distribution of cell cycle phases by flow cytometry for HUH7 cell lines.

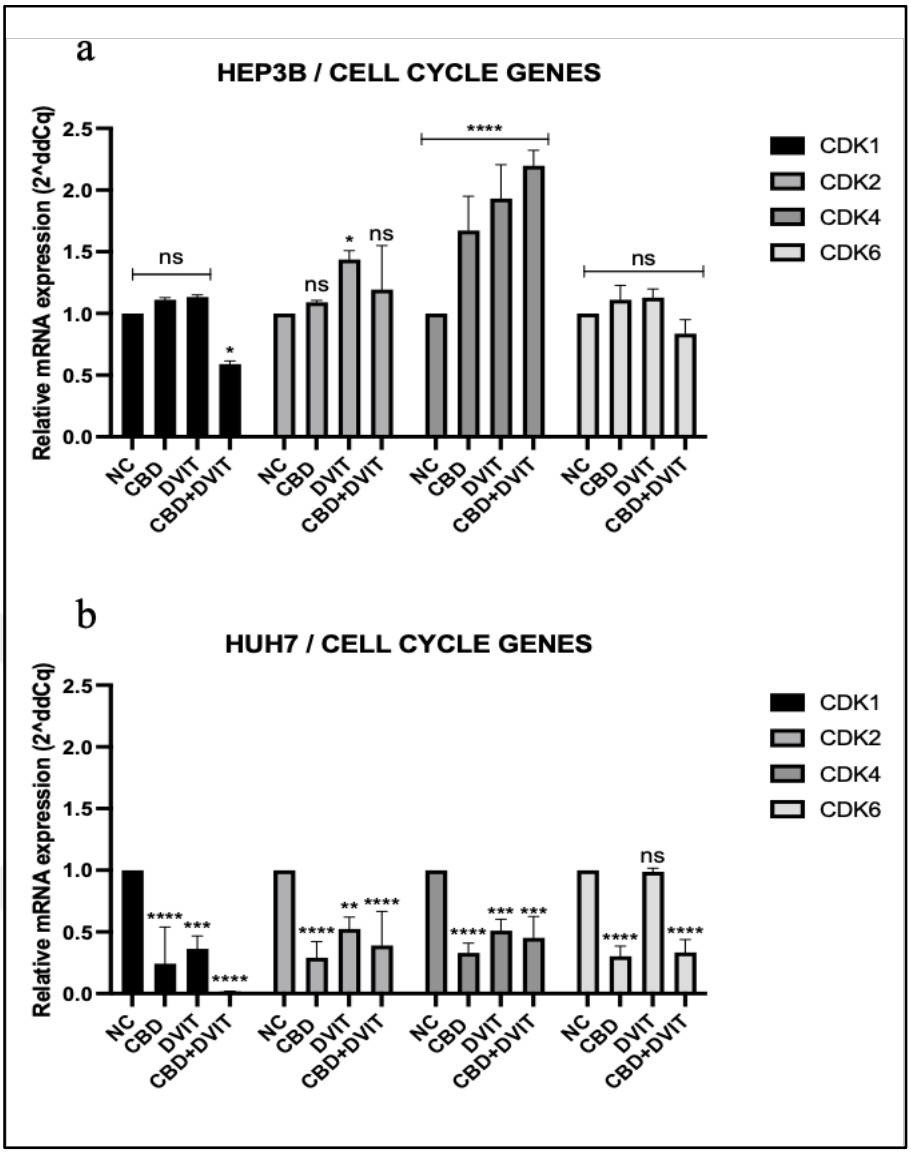

Figure 3.8. Representative graph of Cell Cycle genes' expression profiles on (a) HEP3B and (b) HUH7 cell lines after 72 h.

The expression levels of Ki-67, PCNA, and Survivin were analyzed in both HEP3B and HUH7 cell lines to investigate the fundamental causes of cell proliferation. In the HEP3B cell line, the expression levels of all three genes significantly decreased for the treatment of the combination of Vitamin D and CBD (≈0.666 fold for PCNA, ≈0.184 fold for Survivin, and ≈0.213 fold for KI-67) (Figure 3.9a). However, PCNA, Survivin, and KI-67 expression levels were significantly downregulated for all treatment groups in the HUH7 cell line (Figure 3.9b).

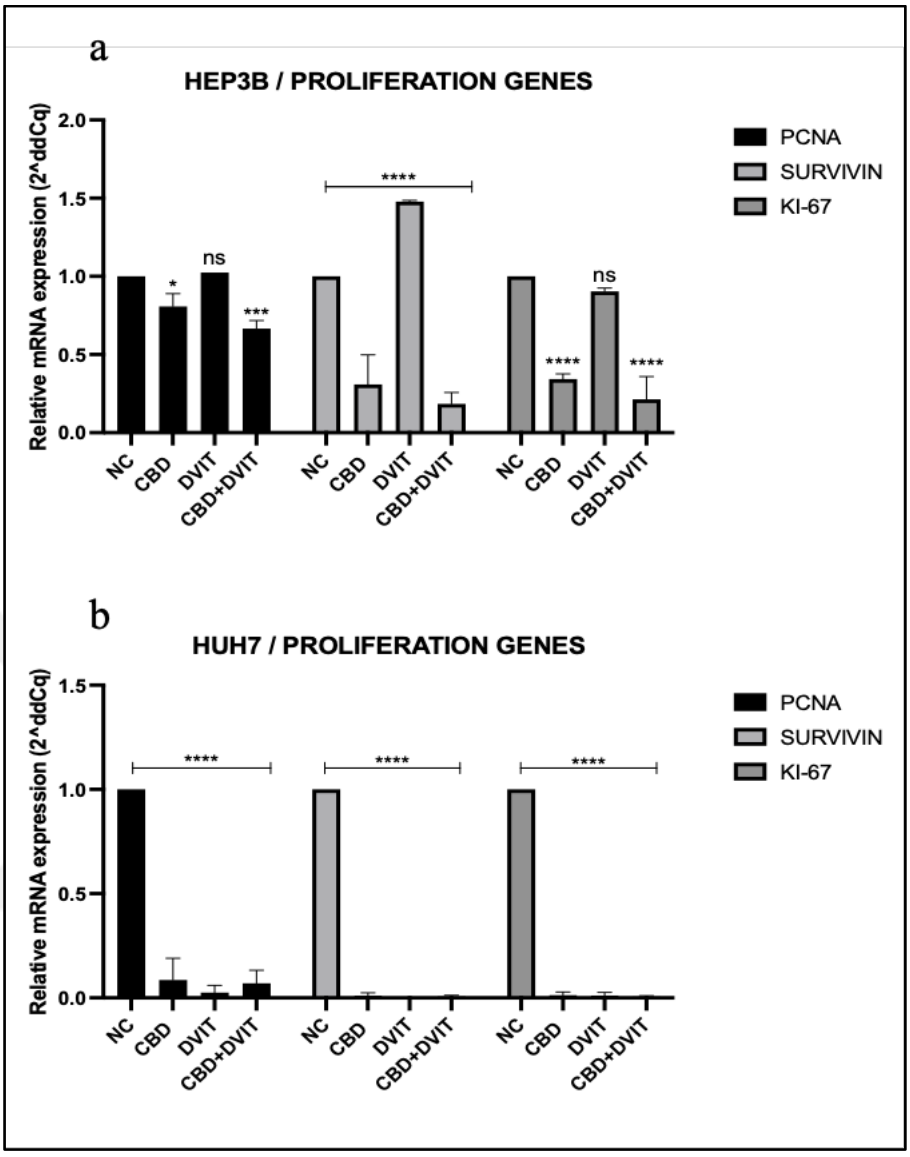

Figure 3.9. Representative graph of Cell Proliferation genes' expression profiles on (a) HEP3B and (b) HUH7 cell lines after 72 h.

3.4. THE EFFECT OF SINGLE AND COMBINATION TREATMENT OF VITAMIN D AND CBD ON AUTOPHAGY CELL DEATH AND DNA DAMAGE MECHANISMS

To investigate the autophagy cell death mechanism, the expression levels of important genes in autophagy, which are ATG-5, BECLIN-1, and LC3B were examined in both cell lines by Real-Time PCR. ATG5 is involved in autophagosome formation, facilitating membrane elongation [105], while Beclin 1 triggers membrane formation as component of a group III PI3K complex that activates autophagy [106]. LC3B serves as a marker to monitor the progress of autophagy and contributes to its maturation by binding to the autophagosome membrane [107]. The expression levels of all three genes were elevated for the Vitamin D-CBD combination treatment (≈2.071 fold for ATG-5, ≈1.710 fold for BECLIN, and ≈2.595 fold for LC3B) in the HEP3B cell line (Figure 3.10a). On the contrary, the expression levels of all three genes significantly decreased for all treatment groups in the HUH7 cell line (Figure 3.10b).

The expression levels of DNA damage genes were detected for both cell lines to understand how CBD, Vitamin D, and their combination affect DNA damage signaling pathways. In the HEP3B cell line, the expression level of BRCA-1 was noticeably upregulated in the combination of Vitamin D and CBD treatment (≈1.250 fold) found in Figure 3.11a. For the combination of Vitamin D and CBD, the expression levels of ATM (≈0.778 fold), ATR (≈0.547 fold), and BRCA-2 (≈0.689 fold) genes significantly decreased compared to the negative control. However, the expression levels of all DNA damage genes significantly downregulated for the treatments of CBD, Vitamin D, and their combination in the HUH7 cell line (Figure 3.11b).

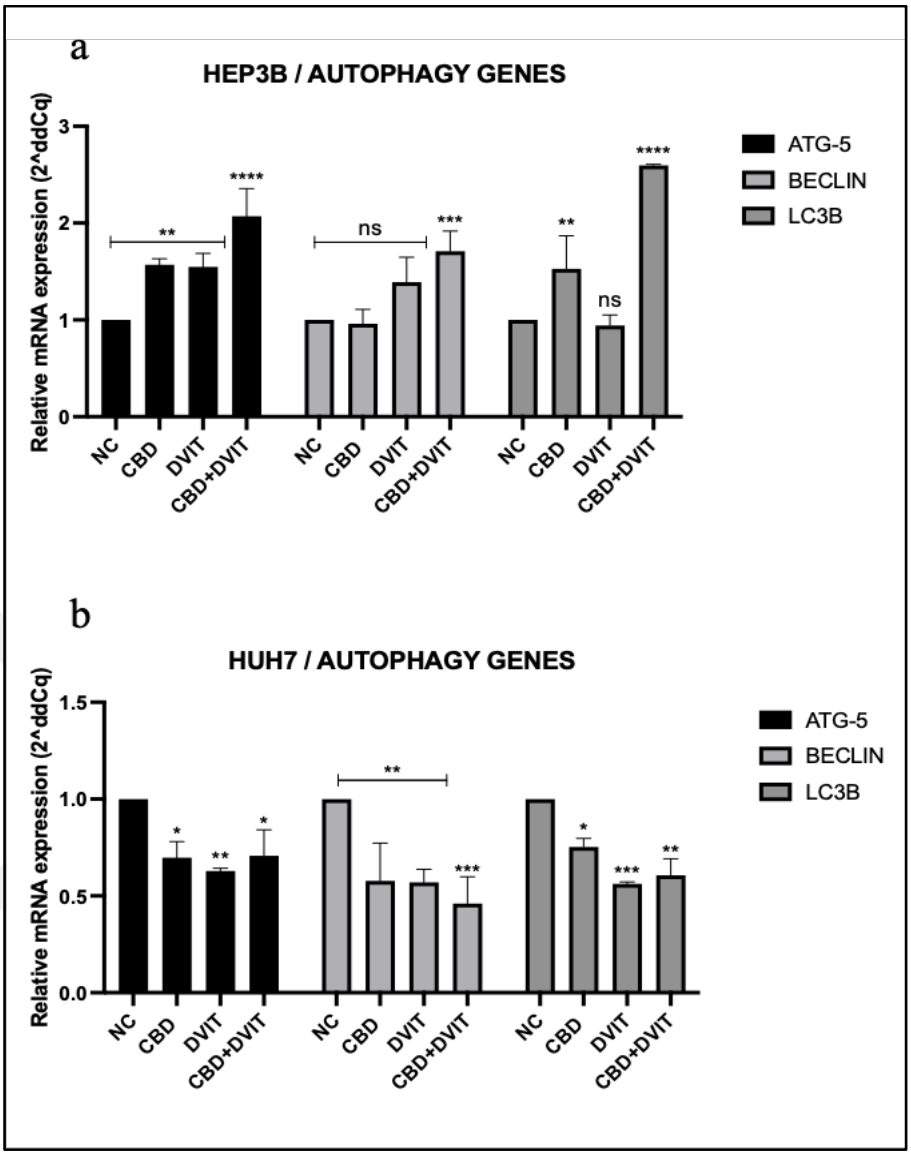

Figure 3.10. Representative graph of Autophagy genes' expression profiles on (a) HEP3B and (b) HUH7 cell lines after 72 h.

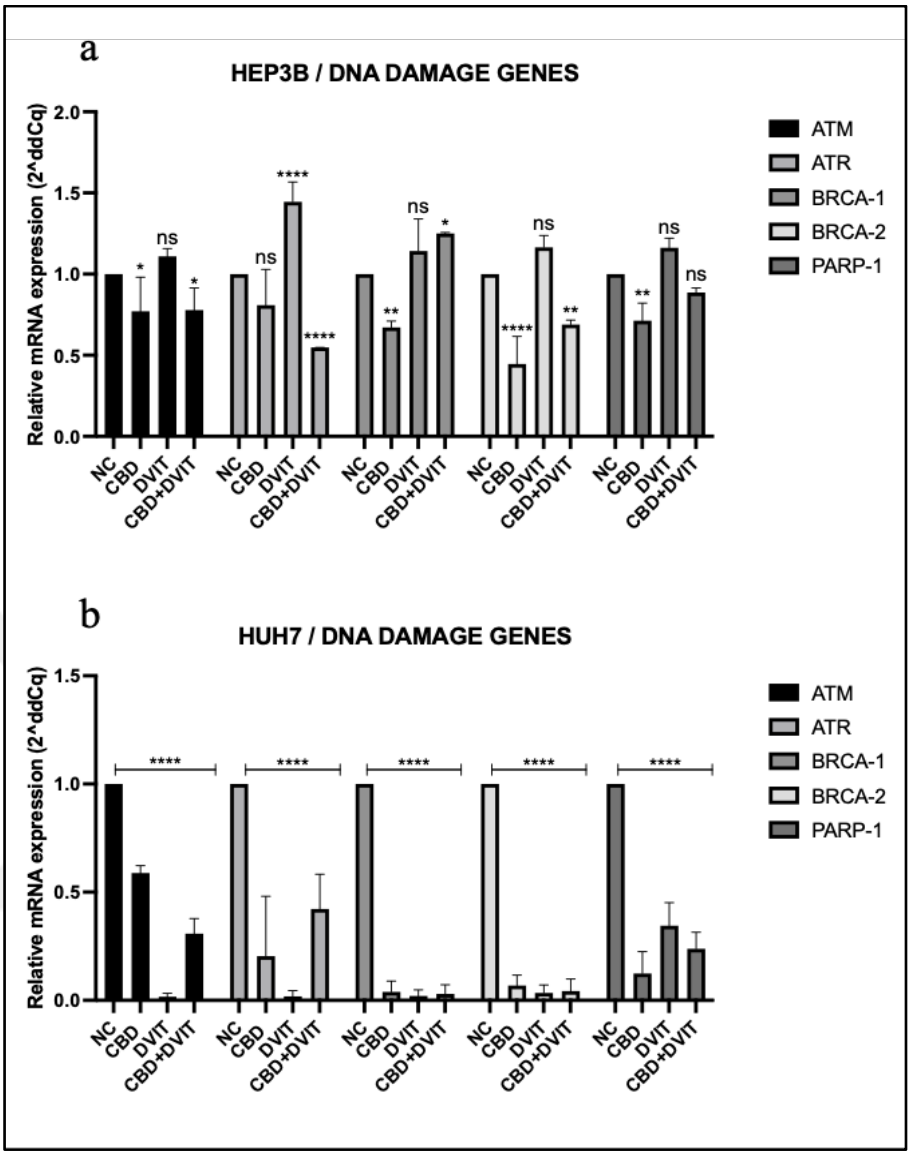

Figure 3.11. Representative graph of DNA Damage genes' expression profiles on (a) HEP3B and (b) HUH7 cell lines after 72 h.

4. DISCUSSION

Cancer, a disease in which abnormal cells with potentially unlimited growth can grow out of control and invade other tissues, is an important worldwide health issue today and the second biggest cause of death, with roughly 20 million new cases confirmed globally, cause to approximately 9.74 million deaths, according to the GLOBOCAN 2022 report [108]. Despite significant advances in cancer treatment over time, there is still a necessity for more efficient and targeted therapies for different types of cancer. Targeting individual molecular anomalies or cancer pathways has been shown to affect mortality in some cancers positively, but addressing a single hallmark or pathway with a single drug has not been an effective solution for cancer treatment [109]. Therefore, instead of single therapy, drug combinations against cancer hallmarks may be an exciting therapeutic method to manage cancer in the foreseeable future.

PLC, the sixth most prevalent cancer in the globe, is a significant health problem with great geographical variation and poor survival rates worldwide [110]. HBV and HCV, along with tobacco, alcohol and aflatoxin, are significant risk factors for primary liver cancer. The most frequent type of primary liver cancer is Hepatocellular carcinoma making up around 75% of cases [111]. Despite the availability of several treatment options, liver cancer remains among the most challenging cancers to manage. Although surgery, local disruptive treatments, and liver transplantation have the ability to cure patients with early HCC, recurrence continues a substantial issue even subsequent to successful treatment, with a frequency of over 70% after 5 years [112]. On the other hand, in clinical practice, the use of Sorafenib, which is limited due to side effects, significantly extends mortality in patients with advanced HCC. Also, new chemotherapeutic agents, including immunotherapy-based regimes for HCC treatment, are essential treatment methods; there is no definite treatment for HCC [113].

Vitamin D's principal roles include skeletal mineralization and calcium homeostasis, but it can also conduct pro-apoptotic, anti-angiogenetic, pro-differentiation, anti-proliferative, and anti-metastatic actions [114–116].

Vitamin D is essential for human health, functioning as a marker of HCC prognosis and assisting in estimating mortality rates in HCC patients; yet, its depletion has quickly become a global public health issue [117]. Some researches have revealed a connection regarding with the Vitamin D deficiency and HCC development [118]. Pathological conditions such as HCC's progress and liver cirrhosis are related to the insufficiency of VD; therefore, it has been suggested that 25 (OH) D is linked to the prognosis of weak liver disease [119]. Although various results support vitamin D's contributions to HCC therapy, many *in vitro* and *in vivo* data, along with controlled clinical studies, suggest that the Vitamin D-related mechanism in HCC progression is critical [120]. Therefore, this study may be essential in working on Vitamin D and its combination with CBD by targeting VD's critical genes and paths in non-classical functions.

For HCC therapy, Cannabidiol (CBD), which is identified in this study as a potential alternative therapeutic agent, has attracted notable interest in cancer treatment. *In vivo* and *in vitro* experiments using diverse cancer cell lines have demonstrated that CBD exhibits inhibitory impacts on cancer cell proliferation, migration, and angiogenesis [121–124]. To sum up, this thesis aims to propose Vitamin D and Cannabidiol, two agents proven by studies to hold significant potential in HCC treatment, as an alternative therapeutic approach, both individually and in combination.

At the beginning of the study, the MTS assay was performed to determine whether single and combination CBD and Vitamin D treatments had a cytotoxic effect on HEP3B and HUH7 cell lines. When single treatments of CBD and Vitamin D at different concentration ranges were applied to HEP3B and HUH7 cell lines, both agents were found to have cytotoxic effects for these cell lines, and half-maximum inhibitory concentrations were determined, as seen in Table 3.1. Although vitamin D monotherapy produced cytotoxic effects up to specific doses, 2.8 times decrease in the IC50 dose was observed for the HEP3B cell line when administered in combination with a non-toxic dose of CBD. Considering this result, it was observed that applying Vitamin D in combination with CBD created a synergistic effect in the HEP3B cell line and reduced cell viability. Although a single treatment of Vitamin D exhibited a cytotoxic effect up to a specific dose in the HUH7 cell line, it was found to have a lesser impact on cell viability than the HEP3B cell line.

However, when Vitamin D was combined with CBD, the treatment caused in a notable decrease in HUH7 cell viability, and the Vitamin D dose decreased 1.9 times in the combination. In conclusion, although single treatments of Vitamin D and CBD had an effect on both cell lines, their combination was determined to have a synergetic effect on cell viability and may be considered an alternative method for HCC treatment.

Apoptosis, a morphologically distinct cell death mechanism designed to destroy unwanted and potentially dangerous cells rapidly, is regulated by complex signaling networks of several factors. Annexin-V assay and qPCR experiments applied to evaluate the influence of Vitamin D and CBD on the activation of apoptotic mechanisms in HEP3B and HUH7. The results indicated that the combination of Vitamin D and CBD activated key genes participated in the apoptotic pathway in the HEP3B cell line, effectively suppressing cell proliferation and promoting cell death. In other words, the combined treatment significantly induced apoptosis, and early apoptosis was detected in the HEP3B cell line through enhancing the levels of effector Casp7, TP53, and BAX gene from the BCL-2 family. Although combination treatment induced apoptosis in the HUH7 cell line, the expression levels of Caspase 7, TP-53, BAX, and BAK genes were significantly decreased.

The different responses to the combined treatment of Vitamin D and CBD in the two cell lines may be attributed to their distinct origins, variations in genetic structures, growth rates, and mutation types. Hep3B and Huh7 cell lines respond differently to apoptosis mechanisms due to different mutations in the TP53. In Hep3B cells, the p53 protein cannot be produced because the TP53 gene is deleted, and p53-dependent apoptosis pathways are disabled [125]. However, p53-independent apoptosis pathways (extrinsic and mitochondrial pathways) remain active, and apoptosis can still be induced. In Huh7 cells, the p53 protein is partially dysfunctional due to the Y220C mutation in the TP53 gene [126]. This results in a weakened p53-dependent apoptosis response, but p53-independent apoptosis mechanisms can still function.

To determine the treatments' effects on the cell cycle, the expression levels of CDK1, CDK2, CDK4, and CDK6 which control the advancement of the cell cycle and the proliferation markers PCNA, SURVIVIN, and Ki-67 were investigated applying qPCR in both cell lines. Additionally, a Cell Cycle Assay was performed to examine in which phase of the cell cycle the cells were arrested.

As seen in Figure 3.6, HEP3B cells exposed to Vitamin D and CBD and their combinations were halted in the G1 phase and were unable to pass to the S stage because they could not complete DNA synthesis preparations. Consequently, population of cells significantly increased in the G1 phase while decreasing in the S phase in the HEP3B cell line for all treatment groups. The expression levels of the CDK4 gene, which serves a crucial part in controlling the G1 phase, particularly in the advancement from the G1 to the S phase, were examined across all treatment groups, and a considerable increase in CDK4 expression was observed. This increase is supported by the simultaneous rise in the population of HEP3B cells in the G1 phase, suggesting a correlation between CDK4 upregulation and G1 phase arrest. In addition, the significant decrease in CDK1 gene expression by combining vitamin D and CBD may be related to the 12.88% decrease in the HEP3B cell population in the G2 phase. The mRNA expression levels of cell proliferation markers PCNA, Ki-67, and SURVIVIN genes significantly reduced by the combined treatment, and this decrease helps to understand that the combined treatment has a significant impact on cell proliferation for the HEP3B cell line. The HUH7 cell population significantly decreased in the G1 phase for the single treatments of Vitamin D and CBD, but there was no specific change in their combination treatment. However, when examined at the mRNA level, it was examined that the combination of Vitamin D and CBD significantly decreased the gene expression levels of CDK1, CDK2, CDK4, and CDK6. The simultaneous reduction of cyclin-dependent kinase genes indicated that the combination treatment significantly inhibited the HUH7 cell cycle and led to cell cycle arrest. However, the combination of vitamin D and CBD considerably decreased the expression levels of CDK1, CDK2, CDK4, and CDK6. The simultaneous downregulation of these cyclin-dependent kinase genes indicates that the combined therapy has a substantial inhibitory impact on the HUH7 cell cycle, causing an arrest in cell cycle at the mRNA level. Furthermore, the significant downregulation of PCNA, SURVIVIN, and Ki-67 cell proliferation genes further confirms that the combination of vitamin D and CBD substantially affects the HUH7 cell line.

Autophagy is a self-digestive mechanism which eliminates unnecessary or damaged organelles and misfolded proteins through lysosomal activity. This mechanism is critical in maintaining cellular metabolism, homeostasis, and overall cell health [127]. Autophagy is vital in protein and organelle quality control, as low basal autophagy levels prevent the gradual accumulation of damaged proteins and organelles in tissues that become toxic over time [128].

ATG5, BECLIN, and LC3B genes are key regulatory genes that participate in various stages of the autophagy process. ATG5 is required to form the autophagosome membrane and lipidation of LC3B through its complex with ATG12 and ATG16L1 [129]. BECLIN initiates autophagy with the class III PI3K complex and regulates the process according to cellular stress conditions by interacting with the Bcl-2 family [130]. LC3B integrates into the autophagosome membrane, interacts with both carrier receptors, and serves as a biomarker in determining the level of autophagy [131]. Dysfunctions of these genes may participate in the development of numerous illnesses, especially cancer and neurodegenerative diseases. To investigate the effects of Vitamin D and CBD, individually and in combined treatment, on autophagy, the expression levels of key autophagy-related genes were analyzed in both cell lines. The combination treatment of Vitamin D and CBD increased the levels of ATG5, LC3B, and BECLIN in the HEP3B cell line. These results suggest the HEP3B cells responded positively to autophagy activation with the combination treatment. On the other hand, ATG5, BECLIN, and LC3B expressions were down-regulated in all treatment groups in the HUH7 cell line, indicating that the HUH7 could not undergo autophagy.

The DNA damage response is a defense response which attempts to recognize and repair DNA damage in the cell [132]. When the cell detects a problem in the DNA, it first detects the damage through the ATM and ATR genes. Then, genes like p53 stop the cell cycle and initiate the repair process [133]. Pathways such as BER (base excision repair) come into play for minor damages, and the PARP1 gene plays a vital role in this process. PARP1 recognizes Single-Strand Breaks (SSB) and attracts the necessary proteins to the site for repair [134]. In cases of more serious double-strand breaks, the homologous recombination pathway, in which the BRCA1 and BRCA2 genes are involved, is used. If the damage cannot be fixed, the cell either destroys itself programmatically (apoptosis) or stops (senescence) [135]. If the genes responsible for this system do not function properly, DNA damage accumulates, mutations increase, and this can lead to diseases such as cancer.

So, the expression levels of ATM, ATR, BRCA-1, BRCA-2, and PARP1 were analyzed to examine the impect of single and combination Vitamin D and CBD treatments on the DDR mechanism in both HEP3B and HUH7 cell lines. The combined treatment of Vitamin D and CBD suppressed key DNA repair genes such as ATM, ATR, and BRCA2 while increasing BRCA1 expression in HEP3B cells, resulting in a complex and unbalanced DDR response.

This suggests that the cell's capability to repair DNA damage is reduced; however, this loss is partially compensated by the upregulation of BRCA1, which is likely insufficient. These findings indicate that the treatment may sensitize cancer cells by targeting DNA repair pathways and could represent a potential anti-cancer strategy. In contrast, the expression levels of all examined genes were downregulated across all treatment groups in the HUH7 cell line, suggesting that Vitamin D and CBD influence the DDR mechanism in HUH7 cells.

5. CONCLUSION

The outcomes of this research propose that the combined treatment of Vitamin D and CBD may represent a promising new therapeutic alternative for treating Hepatocellular carcinoma (HCC). It was observed that the combination therapy significantly reduced cell viability in both HEP3B and HUH7 cell lines. Additionally, *in vitro* researchs demonstrated that the therapy induces apoptotic and autophagic pathways while inhibiting cell survival and proliferation in a cell line-dependent approach. Based on these findings, the combination of these compounds exerts an enhanced therapeutic effect with anti-tumorigenic and potentially preventive roles in the tested cell lines. In conclusion, the results emphasize the promise benefit of using Vitamin D and CBD in combination for HCC treatment. Further research is required to assess their therapeutic possible in *in vivo* models of this aggressive malignancy.

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