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Cannabidiol and Aloe vera Extract to Human Cells: Bioactive Molecules as a Promising Anti-Lung Cancer Drug

Cruz-Rodriguez,L[1,3*], Dilsiz, N[2*] and Martinez, J[3]

[1]*Elidan Dreams, LLC, Elidan Dynamic, LLC, 7117 Robindale Rd, 33619 Tampa, FL, USA*

[2]*University of Health Sciences, Experimental Medicine Application and Research Center (EMARC) Validebag Research Park, Istanbul, Turkey*

[3]*Elidan Gold Label LLC, 1802 Turkey Creek Rd, Plant City, FL 33566, USA*

***Correspondence author**
Dr. Luis CRUZ RODRIGUEZ, PhD
Elidan Dynamic, LLC,
7117 Robindale
Rd, 33619 Tampa,
FL, USA

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Abstract

According to World Health Organisation data, cancer is one of the most influential diseases that goes head to head with heart diseases in the ranking of causes of death, causing approximately 10 million deaths annually and accounting for 20% of all deaths. Lung cancer is a leading cause of cancer-related deaths globally for both men and women. In some pre-clinical studies in cancer cell line in vitro experiments, some results have been obtained that Aloe Vera Extract (AVE) obtained from Aloe vera plant and Cannabindiol (CBD) obtained from Cannabis sativa may have anticancer effect against cancer, but more analysis is required for the reliability of these results. In this study, the cytotoxic and apoptotic effects of AVE and CBD in human lung cancer (A549 and BEAS) cells were studied in a multifaceted manner. Diagnosis of lung cancer disease is approximately 15% in the early stage and 85% in the late stage or metastatic stage. Therefore, despite the use of targeted drugs today, the 5-year survival rate of patients can only be 5-10%. In this study, the cytotoxic and apoptotic effects of AVE and CBD on human lung cancer cells A549 and healthy normal BEAS-2B cells were studied in a versatile and comparative manner. Cytotoxic effect of AVE and CBD was evaluated by xCELLigence RTCA System and AnnexinV-FITC/PI Apoptosis Assay by Flow Cytometry. In the current investigation, we discovered that AVE and CBD might incite a relative higher ratio of cell death in lung cancer cells (A549) than in non-cancer human epithelial lung cell line BEAS-2B, which may be achieved through regulating mitochondrial metabolism. In summary, our research is under Patent submission and shows that CBD and AVE have the potential to develop into a potent new anti-lung cancer drug.

Keywords: Patent submission, Aloe vera (AV), Cannabis sativa, cannabidiol (CBD), cytotoxicity, apoptosis, anti-Lung cancer drug.

Introduction

The *Aloe vera* (AV) belongs to the family of *Xanthorrhoeaceae* and that includes about 500 xerophyte plant species (Figure 1). Most of these species are grown around the world and originated in South Africa [Klopper and Smith 2007]. Combinations of active compounds isolated from several Aloe species have been shown to provide a range of biological effects with various mechanisms of action [Sánchez et al., 2020]. *Aloe vera*, scientifically known as A. *barbadensis*, has been utilized as a kind of medicine from ancient times and is the Aloe species that has been studied the most. Preclinical research has increasingly shown that the chemicals in *Aloe vera* have anticancer, anti-inflammatory, wound-healing, and immune-modulating properties [Budai et al., 2013; Lopez et al., 2019; Majumder et al., 2019].

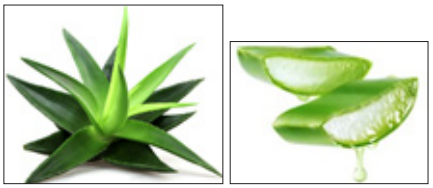

Figure 1: Aloe vera plant.

The *Cannabis sativa* plant is used as a medicinal herb for thousands of years by Chinese, Indian, and Japanese people, although its use in western nations like the USA, Britain, and other European countries started much later, especially in the nineteenth century [Abel, 1980; Bonini et al., 2018; O'Brien et al., 2021; Pamplona and Takahashi, 2012; Touwn, 1981]. The genus *Cannabis* is a member of the *Cannabaceae* family and it is subdivided into three species, *Cannabis sativa*, *Cannabis ruderalis*, and *Cannabis indica*. *Cannabis plant* comes in hundreds of different "strains" or "cultivars," and each one has a unique chemical composition.

Cannabis sativa is a plant with medical and psychotropic

characteristics that has long been utilized for its seed oil, oleoresin, and textile fibers [Chandra et al., 2017]. The two primary phytochemicals found in *Cannabis sativa* L., cannabidiol (CBD) with high levels (up to 5%), Δ^9-tetrahydrocannabinol (THC) in very low levels (up 0.5%), have received a lot of attention in recent years. CBD has non-psychoactive effects in comparison to psychoactive compound THC, which is advantageous for therapeutic uses of anti-tumor properties in different types of tumors [Dilsiz and Cruz-Rodriguez, 2022; Chen et al., 2021; Kis et al., 2019; Appendino et al., 2011; Andre et al., 2016] (Figure 2).

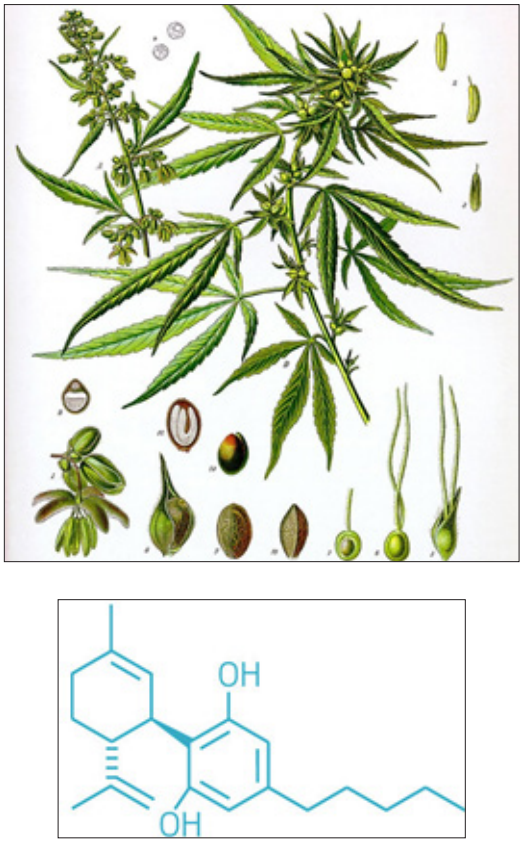

Figure 2: *Cannabis sativa plant and chemical structure of its compound cannabidiol* (CBD).

Three main categories of cannabinoids can be distinguished: natural cannabinoids derived from the *Cannabis sativa* plant, endogenous cannabinoids, which are synthesized by the body (endocannabinoids), and synthetic cannabinoids which are analogues of naturally occurring cannabinoids [Tran, 2021; Gertsch et al., 2010]. The main phytocannabinoid that have received the most attention and study are cannabidiol which is present in hundreds of secondary metabolites derived from *Cannabis sativa* L. There is evidence to suggest that this phytocannabinoid may be useful in the overall treatment of cancer. This evidence consists of its relevant signaling pathways, proof of its effectiveness in the treatment of cancer, symptoms related to the treatment of cancer, and proof that they might be used in addition to traditional cancer therapies.

Anticancer Mechanisms of Aloe vera gel extract and CBD

One of the main causes of death that is rapidly rising worldwide is cancer. Lung cancer and breast cancer are the most common types of cancer diagnosed and the main causes of cancer-related death in men and women, respectively [Sung et al., 2021]. Small cell lung cancer (SCLC, 13%) and non-small cell lung cancer (NSCLC, 84%) are the two types of lung cancer [Seltzer et al., 2020; Heider al., 2022]. Adenocarcinoma, squamous cell carcinoma, and giant cell carcinoma are further subtypes of lung cancer. The development of innovative anticancer medications with specific targeting for cancer cells and low toxicity for normal healthy cells continues to be a major area of study given the continually rising burden of cancer death. There is still a lack of effective treatments, despite the fact that cancer diagnosis and therapy have made enormous strides in reducing mortality.

It is known that novel drugs for the treatment of cancer disorders can be developed using a variety of plants. It has been demonstrated that the anticancer agents from plants have anti-proliferative and pro-apoptotic activities with less side effects [Seltzer et al., 2020; Ahmad et al. 2020]. Despite improvements in drug development, there is still a need to look for new therapeutic drugs made from plants. One of the oldest medicinal herbs, *A. vera*, has the pharmacological potential to be effective against many diseases, including cancer [Maan et al. 2018]. Gao et al. 2019 noted that *A. vera* has expanded notable consideration for exploring the novel chemotherapeutic drug due to its various therapeutic properties. According to a number of studies, *A. vera* leaf extract has the ability to kill various cancer cells, including melanoma and colorectal cancer [Majumder et al. 2022; Laux et al. 2022; Tong et al. 2022].

In numerous preclinical studies, including cancer cell line experiments and rodent models of cancer, extract of Aloe vera and a variety of cannabinoids, including CBD of Cannabis sativa, have been shown to have anti-cancer activity, addressing many of the "hallmarks of cancer" [Velasco et al., 2012; Pecere et al. 2000; Kabbas et al. 2008; Pan et al. 2013; Seltzer et al., 2020]. It has been demonstrated that cannabinoids have pro-apoptotic and anti-proliferative effects in numerous cancer types [Seltzer et al., 2020; Moreno et al., 2020; Scheau et al., 2020]. A few of the actions that cannabinoids can have include cell cycle arrest, inducing apoptosis, suppression of chemotaxis, adhesion, angiogenesis, invasion, and metastasis [Seltzer et al., 2020; Ramer and Hinz, 2008; Shrivastava et al., 2011]. The regulation of reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, and immunological modulation by CBD accounts for a large portion of its anti-tumor efficacy (all important in tumor development) [Seltzer et al., 2020].

Cell cycle arrest, apoptosis, and autophagy are just a few of the several ways by which CBD inhibits the proliferation of breast cancer cells [Seltzer et al., 2020; Ligresti et al., 2006; McAllister et al., 2011]. Many bioactive substances derived from *A. vera* have been linked to the possible anticancer properties [Grimaudo et al. 1997; Kim et al. 1999; Pecere et al.

2000; Kabbas et al. 2008; Pan et al. 2013]. Many therapeutic capabilities, including antioxidant, anti-inflammatory, antibacterial, antiviral, anti-cancer, and wound healing, are known to be present in it [Gao et al. 2019; Fatima et al. 2022; Majumder et al., 2019]. Although *A. vera* is known to be a potent and useful therapeutic plant, the precise mechanism(s) through how it induces cancer cell death have not yet been precisely defined. *Aloe vera's* bioactive components, such as its polysaccharides and anthraquinones, may function in concert to inhibit the growth of cancer cells. According to Liu et al. [2019], polysaccharides, particularly ace-mannan, have immunomodulatory action. This activity includes anti-inflammation [Budai et al., 2013], immunological activation [Harlev et al., 2012; Kumar and Tiku, 2015], and macrophages, which are pluripotent effector cells.

Emodin and aloin are two anthraquinones whose anticancer properties have been extensively studied. This has brought attention to their chemopreventive, radioprotective, anti-proliferative, and apoptosis-inducing capabilities with or without anticancer drugs [Haddad et al., 2013; Hamiza et al., 2013]. [Hussain et al., 2015; Im et al., 2016; Sanders et al., 2017]. The current study examined the ethanolic extract of *A. vera's* cytotoxic and apoptotic effects in human lung cancer.

Due to the critical role that abnormalities in the apoptotic pathway play in the conversion of healthy cells into mutated cells and in the evasion of planned cell death, apoptosis is a highly effective pathway for preventing tumor growth [Wong, 2011]. The majority of cell death is controlled by mitochondria, that is known as the center for maintaining the perfect balance of numerous signaling pathways, including energy production, biosynthesis, redox homeostasis, calcium, and cell fate regulation. It is well known that many cancer cells prefer aerobic glycolysis (Warburg effect), bypassing the tricarboxylic acid (TCA) cycle with oxidative phosphorylation and electron transport with damaged mitochondria or mitochondrial uncoupling [Vander Heiden et al., 2009]. Since abnormal or malignant cells with damaged mitochondria are more likely to suffer apoptosis in these circumstances, mitochondrial recoupling has emerged as a novel strategy for cancer therapy [Baffy et al., 2011].

The permeabilization of the mitochondrial outer membrane and the loss of the mitochondrial membrane potential ($\Delta\Psi m$) initiate the intrinsic or mitochondrial apoptosis pathway, which is in turn mediated by the oligomerization of Bcl-2-like protein 4 (BAX), or Bcl-2 associated agonist of cell death. Moreover, reactive oxygen species (ROS) may result in depolarization of the mitochondrial membrane and the opening of BAX/Bcl-2 homologous antagonist killer (BAK) channels [Redza-Dutordoir et al., 2016]. Following membrane disruption, mitochondrial cytochrome c is released into the cytoplasm where it combines with the protein apoptotic protease activating factor 1 (Apaf1) to create the apoptosome and activate the caspase cascade, causing apoptosis [Jin and El-Deiry et al., 2005]. In other study, it was found that, CBD shifts the mitochondrial voltage-dependent anion channel (VDAC) from a fully open to a major subconductance state at a reasonably high concentration of 30 μM therefore preventing this channel from entering its Ca^{2+} -permeable state, causing a severe oxidative stress, mitochondrial Ca^{2+} excess, Cyto-c release into the cytoplasm, stimulation of LC3-phosphatidylethanolamine conjugate (LC3-II), and activation of caspase in leukemia cells [Olivas-Aguirre et al., 2019].The anti-apoptotic Bcl2 family of proteins, which under normal physiological conditions are attached to the BAX/BAK channels to suppress their function, inhibit mitochondrial outer membrane permeabilization [Green and Llambi et al., 2015]. Caspases are thought to cleave poly(ADP-ribose) polymerase [PARP-1, 116 kDa) when the caspase cascade is activated, which is the evidence of apoptosis [Kaufmann et al., 1993; Chaitanya et al., 2010]. It was shown that CBD decreased Bcl-2, p-Akt, p-PI3K, p-mTOR, p-Erk1/2, and MMP9 levels while increasing Bax, Cyto-c, and caspase 7 levels. Together, CBD's effects on the PI3K/Akt pathway can stop bladder cancer cells from growing, migrating, and inducing apoptosis [Kim et al., 2019; Chen et al., 2021].

Purified chemicals taken from *A. vera* effectively triggered the cancer cells' intrinsic apoptotic mechanism. Unfortunately, little research has been done on the effects of unprocessed Aloe extracts. In the human hepatoblastoma HepG2 cell line, the lyophilized AVE has been shown to have the ability to decrease Bcl-2 gene expression [Shalabi et al., 2015]. Aloe emodin has been shown to activate apoptosis by upregulating the protein expression of BAX and caspases 9 and 3, while downregulating that of Bcl-2, leading to the loss of $\Delta\Psi m$, the release of mitochondrial cytochrome c into the cytoplasm, and an increase in ROS levels which results in the mitochondrial-dependent pathway being used to induce cell death.

It was indicated that emodin and anthraquinone chrysophanol possess stimulatory effects on the intrinsic apoptotic pathway, including the suppression of Bcl-2 expression and the upregulation of pro-apoptotic proteins, including BAX, caspase-3, caspase-9 and PARP-1 [Wei et al., 2011; Ren et al., 2018; Zhang et al., 2020]. In the human gastric cancer cell lines BGC823 and HGC27, aloin was able to significantly decrease the formation of ROS [Wang et al., 2020], while also boosting the dose-dependent cleavage of PARP-1 and caspase-3 in HGC27 cells [Cruz Rodriguez et al., 2020; Tao et al., 2019]. Aloin also significantly increased the protein expression levels of BAK, BAX, p53i cleaved caspase-3 and caspase-9 were reported to be significantly increased in the human liver cancer cell lines HepG2 and Bel-7402 following aloin treatment [Zhang et al., 2017; Wan et al., 2017; Sun et al., 2020]. In HepG2 cells, a lyophilized AVE has been shown to boost tumors suppressor p53 mRNA expression in a time, and dose-dependent manner [Shalabi et al., 2015]. Moreover, emodin caused apoptosis in A549 cells via activating the ROS-induced ATM/p53/BAX signaling pathway [Lai et al., 2009].

On the other hand, the activation of the death receptor family, which includes the tumor necrosis factor (TNF) receptor 1 (TNFR1), Fas/CD95, DR3, DR4, and DR5, initiates the

extrinsic pathway of apoptosis. This leads to the assembly of a platform for activating caspase called the death-inducing signaling complex. This platform participates in the extrinsic apoptotic cascade by recruiting and activating caspase 8 through the adaptor protein Fas-associated protein with death domain (FADD) [Green and Llambi et al., 2015]. However, there is very little information about how chemicals from A. vera affect the extrinsic apoptotic pathway [Byun et al., 2018].

On the other hand, a number of reports demonstrate the ability of CBD to induce apoptosis in tumor cells, little work has been done demonstrating tumor suppressive mechanisms of CBD. Massi et al. found that apoptosis in human glioma cell lines following exposure to CBD was mediated through cannabinoid receptor CB2 and the generation of reactive oxygen species (ROS). The generation of ROS can play an important role in the induction of apoptosis in T cells undergoing either activation induced cell death (AICD) or activated T cell autonomous cell death (ACAD) [Massi et al., 2004; Hildeman et al., 2003; Sultan et al., 2018].

Cannabis contains more than 500 different chemical compounds that have been identified. More than 100 terpenophenols originating from *Cannabis sativa* are currently referred to as "cannabinoids" each with 21 carbon atoms, in addition to synthetic substances that either directly or indirectly interact with cannabinoid receptors [Daniller, 2023]. In addition, cannabinoids are divided into phytocannabinoids (subclassified into various categories based on their chemical structures, such as 9-THC, D8-THC, cannabinol, CBD, and cannabicyclol), synthetic compounds that act on cannabinoid receptors such as JWH-133, WIN 55,212-2, and SR141716, and the natural endogenous cannabinoids such as N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG). However, the undesirable psychoactive side effects of Δ^9-THC and other synthetic agonists frequently restrict their clinical usage [Massi et., 2013]. For this reason, interest in non-psychoactive phytocannabinoids, such as CBD, has significantly expanded in recent years. In addition to helping with the treatment of pain, spasticity, and other CNS disorders, numerous studies have shown that CBD has anti-proliferative effects, increases apoptosis in cells, and inhibits the migration, adhesion, and invasion of cancer cells.

By prolonged activation of caspases-3, -7, and -9 and targeted control of the extracellular signal-regulated serine/threonine protein kinase, ERK, combination therapy's synergistic effect implies in *vitro cell* cycle arrest, ROS production, and cell apoptosis [Marcu et al., 2010]. Due to the aggressive biological character of lung cancer and its poor response to currently available therapies, a number of targets and novel therapeutic approaches are now being researched [Molina et al., 2006].

Recently, Hinz and Ramer examined the impact of CBD on the invasive capabilities of human lung cancer cell A549 that express seven-transmembrane domain G protein-coupled receptors, known as type 1 and 2 cannabinoid receptors (CB1 and CB2, respectively) as well as agonist of transient receptor potential vanilloid (TRPV) (type 1 and type 2) that are responsible for ions' transmission [Hinz and Ramer, 2022]. CB1 receptors are generally found in the nerve cells of the spinal cord and brain, whereas CB2 receptors are most abundantly expressed in peripheral tissues and immune system cells. The release of chemotransmitters after activation is controlled by both CB proteins. In humans, CB1 is encoded by the CNR1 gene and consists of 472 amino acids. On the other hand, CB2 is derived from the CNR2 gene and composed of 360 amino acids. The amino acid sequences of CB1 and CB2 proteins share 44% sequence homology in general [Heider et al., 2022; Kitdumrongthum and Trachootham, 2023]. When CB1 and CB2 receptors are activated, adenylyl cyclase is inhibited, and signaling cascades that control cell survival, growth, and proliferation are modulated. In addition, CB1 receptor can modulate certain types of ion channels, which plays a crucial role in neuromodulatory actions of the endocannabinoids. The maintenance of bone mass and the reduction of inflammation are made possible by CB2 activation, which is connected to neuro-defense processes. Neurodegenerative disorders including Huntington's and Alzheimer's disease can also be slowed down by CB2 agonists [Shahbazi et al., 2020; Abd-Nikfarjam et al., 2023].

Material and Methods

The freshly harvested aloe leaves were cleaned with distilled water, the water was drained under aired conditions, and the leaves were peeled. The *Aloe vera* extract (AVE) was made by homogenizing fresh gel, and the debris was removed by centrifuging at 300g for 3 min. The AVE was then combined with 3 vol of water and stirred at low speed for 3 times for a total of 2 hours at 60 °C, sterilized in an autoclave, concentrated to the proper volume under reduced pressure, and filtered once more. As we found that, cannabis flowers have a considerably greater CBD concentration than other cannabis parts, only the flowers were used for CBD extraction. CBD was extracted from the cannabis flowers in ethanol (EtOH) by using High-Performance Liquid Chromatography (HPLC) after 21 days of fermentation and kept in olive oil as it is highly hydrophobic. AVE produced from *Aloe vera* and CBD from *Cannabis sativa* had a yield of 3.0 g/L, and was kept at 4 °C. After freeze drying, HPLC-MS/MS was used to determine the compositions of AVE and AVE+CBD. The stock solution was diluted to the proper concentration for the cell culture using the matching culture medium, and it was then sterilized before use by passing through a 0.22 µm sterilizing membrane filter.

Culturing/Sub-Culturing of Cells

Human lung adenocarcinoma epithelial cell line (A549) and normal human epithelial lung cell line (BEAS-2B) were obtained from the ATCC (American Type Culture Collection). The cryovial of each cell (A549 cell and BEAS-2B cell) was obtained from liquid nitrogen storage. The cryovial was then warmed quickly to thaw cells within a water bath at 37 °C. Once the cell sample is thawed, the cells were added dropwise into a centrifuge tube containing 10 ml of pre-warmed complete growth medium. The cell suspension was centrifuged at 800 rpm for 5 minutes. After 5 minutes the centrifuge tube was

visually checked for a pellet and clarity of supernatant. The supernatant was aseptically decanted without disturbing the cell pellet. Cells were then re-suspended in growth medium and transferred into the appropriate culture flasks and incubated at 37°C, 5% CO_2 atmosphere.

All cells were maintained in Dulbecco's Modified Eagles Medium High Glucose (4.5 g/L) (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-Glutamine and 1% non-essential amino acids (NEAA) (complete medium). Cells were cultured in tissue flasks and incubated at 37°C, in a humidified 5% CO_2 incubator and subculture carried out by washing the cell monolayers twice with calcium and magnesium-free phosphate buffered saline (PBS) followed by addition of 1x Trypsin/EDTA solution (Sigma Aldrich) and incubation at 37°C until the cells detached. Trypsin was inactivated by the addition of growth medium before seeding into fresh flasks at densities of 1.5-2x10^4 cells/cm^2. For the long term 25 day cultures A549 cells were seeded into replicate T25 flasks and medium changed every 2–4 days. Inverted phase contrast microscopy images were captured of the monolayers throughout the time course. Cell numbers, viability and size were assessed by Trypan Blue staining using the TC20 Automated cell Counter (BioRad).

Cells grow adherent to the base of tissue culture flasks. As the cells grow, the base of the culture flasks becomes fully occupied, which results in reduced cell growth due to contact inhibition. Therefore, in order to maximize growth of healthy cells, it is necessary to split the cells within culture flasks.

Anticancer potency of AVE and AVE+CBD in cell lines and determination of IC50 value

The anticancer activity of AVE and AVE+CBD was investigated using the BrdU Cell Proliferation ELISA (BCPE). The results were compared with Paclitaxel that was used as positive control. Cell suspension containing 3×10^3 cells in 100 µL was transferred to wells of 96-well cell culture plate.

Cancer cell and control cell were treated with various concentration of molecules (0, 1.5, 3.0, 6.0, 12, 24 µg/mL and DMSO) as triple wells for 24 h and the final volume of the wells was adjusted to 200 µL by DMEM. Then, 20 µL of BrdU solution was added to every single well. The plates were incubated for 4 h. The control cells were received only media without AVE and AVE+CBD. After that, cells were treated 1:100 diluted anti-BrdU-POD for 90 min at room temperature and substrate solution was added to each well. The absorbance of 450-650 nm was measured by microplate enzyme-linked immunosorbent assay (ELISA) reader by Synergy Neo2 Multimode Reader (BioTek). The different absorbance in both treated and the untreated groups will be calculated to determine the IC_{50} value with linear regression analysis using Microsoft Excel. Meanwhile, the survival rates of the treated cells will be calculated by comparing the absorbance with that of untreated control ones. IC_{50} values will be calculated by nonlinear regression-curve fitting of log concentration vs percentage cell survival using GraphPad Prism.

Cell Proliferation Experiment using xCELLigence RTCA System

When the cells (cancer cell and control cell) reached 80% confluency in a T75 flask, the cells were washed with PBS. Then, the cells were treated with 0.05% trypsin/EDTA. After 2 min, 5 mL full media was added to the flask. The cell resuspension was centrifuged at 800 x g for 5 min. The pellet was resuspended with 5 mL media, and the cells were counted by using a TC20 automated cell counter (BioRad). Before seeding cells in 96-well plate, a standard background was measured by adding 100 µl of full media at 37 °C to wells. Then, 4000, 8000, and 15.000 cells per well was seeded in 96-well plate, and the total volume of wells was adjusted to 200 µl with media. The well plate was incubated for 30 min in a cell culture incubator. Finally, the cells were monitored every 15 min for 96 hours.

Cytotoxicity Experiment using xCELLigence RTCA System
Optimum cell cumber for cytotoxicity experiment was determined from the proliferation experiment of cells; 15.000 cells/well are seeded to each well of 96-well plate. Then, the cells were monitored every 15 min. After 24 h, the 100 µL of total volume of wells were discarded and the cells were treated with 0, 1.5, 3.0, 6.0, 12, 24 µg/mL and DMSO. After that and the total volume of wells was adjusted to 200 µL with media. Cells were monitored for another 48h. Data was presented as a normalized cell index and normalized just after each extract treatment (CI; normalized at 23h).

Normalized Cell Index (NCI) = CIoriginal/CInormalize time

AnnexinV-FITC/PI Cell Apoptosis Assay (Flow Cytometry Analysis)

Annexin V-FITC/propidium iodide (PI) assay kit (Millipore) was used to distinguish normal, apoptotic, and necrotic cells. The translocation of phosphatidylserine, an apoptosis marker, from the inner to the outer leaflet of the plasma membrane was detected by the binding of fluorescein isothiocyanate (FITC-conjugated annexin V. Briefly, DLD-1 cells, which had been untreated or treated with cannabidiol, TRAIL, or a combination of these two agents, were resuspended the binding buffer provided with the Annexin V-FITC Apoptosis Detection Kit.
The Annexin V-FITC/PI double staining assay was performed according to the manufacturer's instructions. Briefly, the A549 cells were incubated with the selected concentration of IC50 value of AVE and AVE+CBD for 24 to 48 h in the incubator at 37°C with 5% CO_2. Approximately 1×10^6 A549 cells were collected by centrifugation and the cells were resuspended in 1 mL of 1X Annexin Binding Buffer. 5 µl of Annexin V-FITC and 5 µl of propidium iodide (PI, 50 µg/ml) was added to the 100 µL of resuspended cells and incubated on ice for 5 min in the dark. The staining was then terminated, and the fluorescence intensity of the cells was measured using the CytoFLEX Flow Cytometer (Beckman Coulter, CA, USA) to assess the relationship between dose and effect (Figure 3).

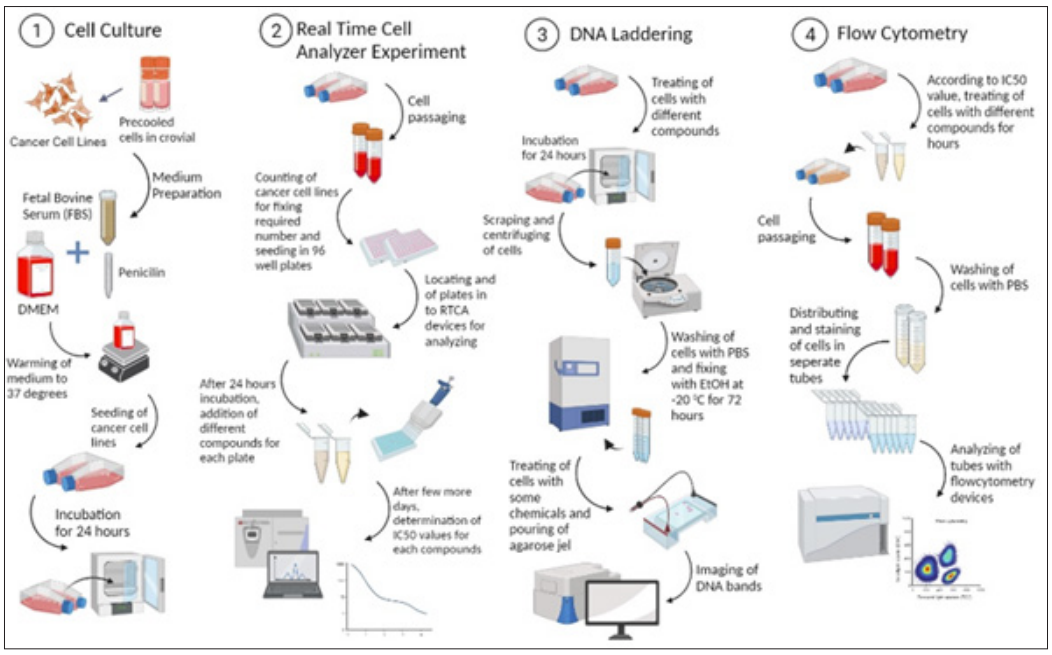

Figure 3: Experimental stages within the scope of the study. 1. Culturing/sub-culturing of cells, 2.Cell Proliferation and cytotoxicity experiment using xCELLigence RTCA system, 3. AnnexinV-FITC/PI cell apoptosis assay using Flow Cytometry Analysis. Created by BioRender

Statistical Analysis

The experiments were conducted at least three times. The data were presented as the mean ± standard deviation. Statistical analyses were performed using the SPSS 21.0 software package (version 21.0, SPSS Inc., Chicago, IL, USA). Student's t-test or one-way ANOVA was used to calculate the statistical differences. Differences with $p < 0.05$ were considered statistically significant.

Results

This research investigated the effects of AVE and AVE+CBD to determine whether they have any possible anti-tumor effects on lung cancer A549. Our results suggest that AVE and AVE+CBD can reduce the viability of human A549 cancer cell lines.

Cytotoxic Activity

The results showed that AVE and AVE+CBD (1.5, 3.0, 6.0, 12, 24 µg/ml) significantly inhibited the proliferation of lung cancer cell A549 in a concentration-dependent manner, with an IC_{50} value of 6.0 µg/ml after 40 h of treatment (Figure 4). The detected inhibition rate was significantly higher in A549 supplemented with AVE+CBD cells after 43 h of treatment compared to AVE extract alone. The cytotoxic effects of AVE extract and AVE+CBD (0, 1.5, 3.0, 6.0, 12, 24 µg/mL and DMSO) on BEAS-2B and A549 cells given as percentage of viable cells are shown in Figure 4 A-D.

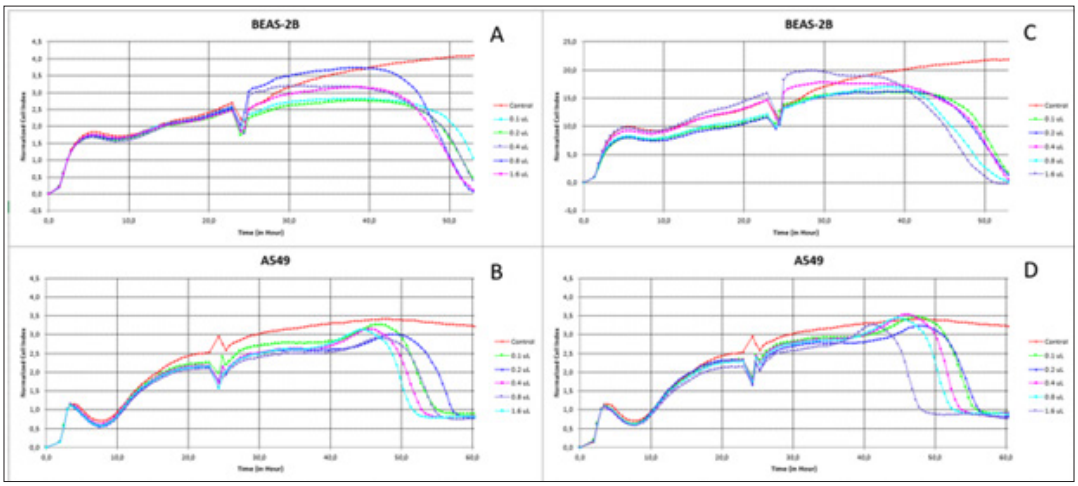

Figure 4 : Determination of antiproliferative effect of AVE and AVE+CBD in BEAS-2B and A549 cell lines.

Cell index value of BEAS-2B (A) and A549 (B) treated with different doses of AVE. Cell index value of BEAS-2B (C) and A549 (D) treated with different doses of AVE+CBD. The values indicate that all AVE and AVE+CBD doses had antiproliferative effect on the cell lines.

Annexin-V/PI apoptosis assay

We also investigate the ability of AVE and AVE+CBD to specifically cause cell death in non-cancer cell lines (BEAS-2B). And we discovered that 20% more A549 cells underwent apoptosis after a 24-hour treatment with 6 µg/ml AVE and AVE+CBD, and the ratio of apoptotic cells was much lower than that of cancer cells (Figure 5 and 6). Analysis of apoptosis and necrosis revealed that AVE and AVE+CBD caused time-dependent cell necrosis in A549 cells. According to this, AVE+CBD and AVE are toxic to cancer cells that have achieved cell apoptosis.

A549 cells stained with Annexin V-FITC and PI were studied using flow cytometry to quantitatively detect the apoptosis induced by exposure to 6 µg/ml AVE and AVE+CBD. Values are shown as mean ± SD of three independent experiments (*** $p < 0.001$ compared to the control).

The apoptosis of A549 cells was considerably increased after being treated for 40 hours with 6 µg/ml AVE and AVE+CBD. Time-course analysis showed that the 6 µg/ml AVE and AVE+CBD treatment highly induce apoptosis in A549 cells within 26 and 40 hours. This result demonstrated that AVE and AVE+CBD induce apoptosis (Figure 5 and Figure 6 below).

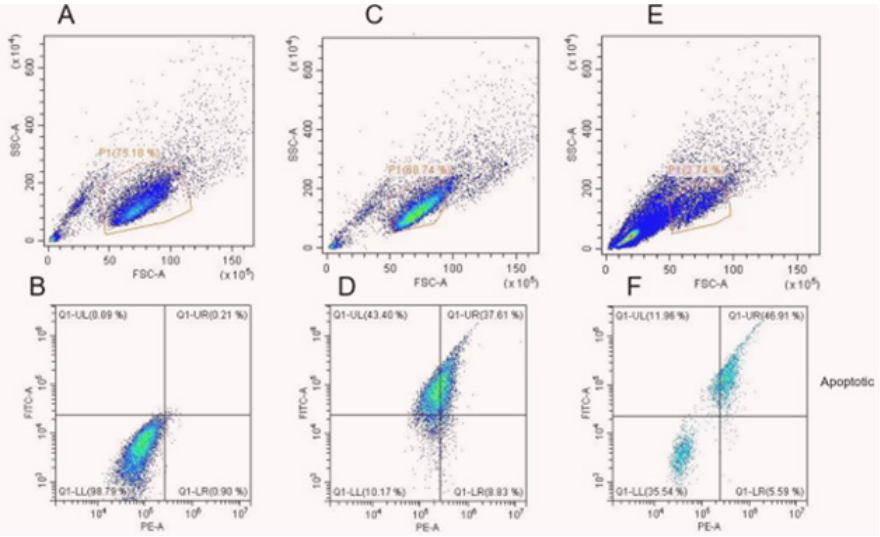

Figure 5: Annexin V-PI Stain Assay. Control, without staining (A-B), Control with staining (C-D), Apoptosis determined by flow cytometry in A549 cells treated with 6 µg/mL of AVE for 40 h after staining with Annexin V-FITC (E and F).

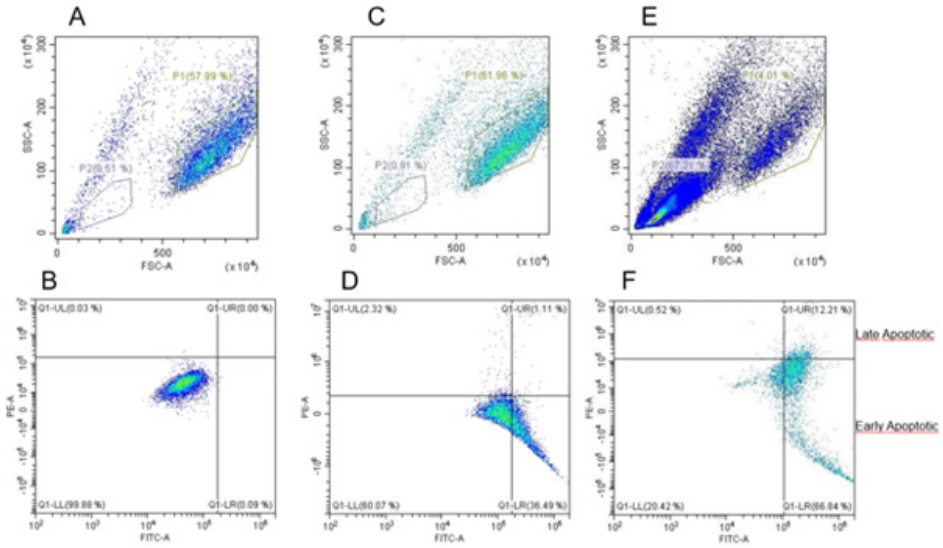

Figure 6: Annexin V-PI Stain Assay. Control without staining (A-B), (C-D) Control with staining (C-D), Apoptosis determined by flow cytometry in A549 cells treated with 6 µg/mL of AVE+CBD for 26 h after staining with Annexin V-FITC (E and F).

The cytotoxic effect of AVE started at 47 h of incubation period and almost all cells were destroyed at 58 h of incubation. On the other, the cytotoxic effect of AVE+CBD started at 45h of incubation period and almost all cells were destroyed at 53 h of incubation (Figure 7 and figure 8 below).

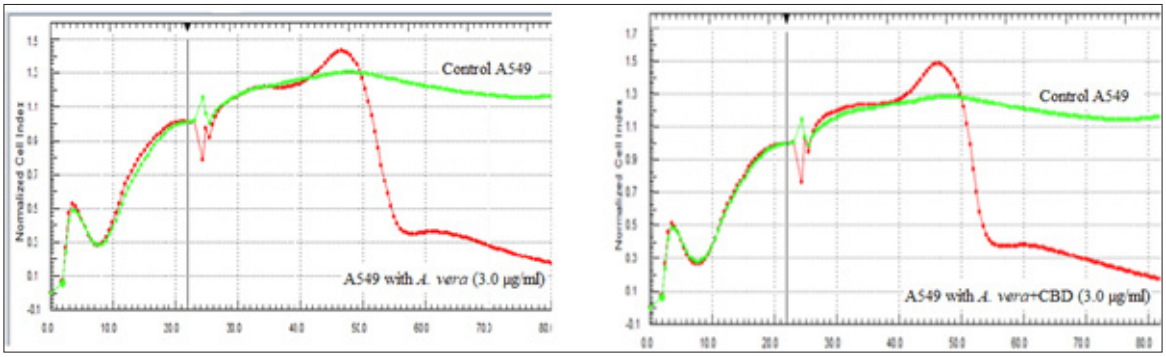

Figure 7: The cytotoxic effects of AVE and AVE+CBD (6.0 µg/mL) on A549 cells xCELLigence MP Real Time Cellular Analysis system. 1. a) control A549 cells without any supplement (green color), b) A549 cells (5.000 cells/well) with AVE (red color). 2. a) control A549 cells without any supplement (green color), b) A549 cells (5.000 cells/well) with AVE and AVE+CBD (red color).

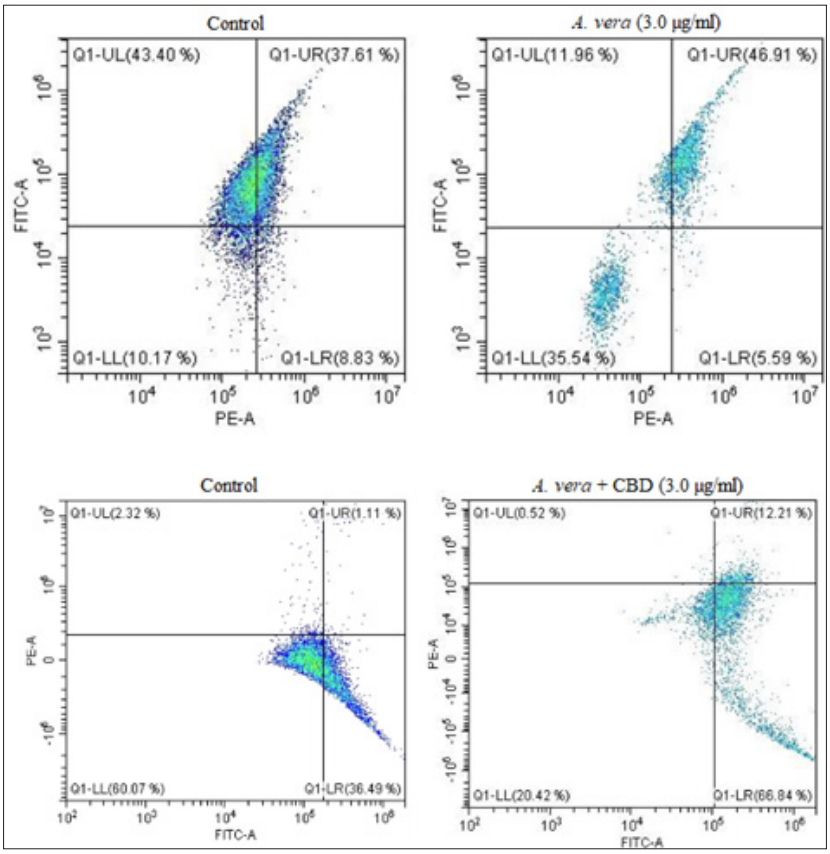

Figure 8: Apoptotic assay by using CytoFLEX Flow Cytometer (Beckman Coulter). The percentage of apoptosis in control was 37.61%. It was increased to 46.91% with AVE supplement (top). The percentage of apoptosis in control was 36.49%. It was increased to 66.84% with AVE+CBD supplement (bottom). Following a 26-hour incubation period with A549 cells, this indicates that an AVE+CBD supplement is more effective than an AVE supplement alone.

Annexin-V-FITC/PI double-staining assay was used to evaluate the percentage of apoptotic and necrotic cells to confirm that the cytotoxic effect on A549 cells, with their own IC50 doses of AVE 6 µg/mL for 24-48 h, was associated with apoptosis (46.91%). The cells with the most change in apoptotic cell percentage were identified as A549 cell with AVE+CBD (66.84%).

These results indicated that both AVE and CBD effectively induced apoptosis in A549 cells. To explore whether the observed inhibition of the proliferation effect of AVE and CBD is related to cell apoptosis, we analyzed the apoptotic rate of AVE+CBD-treated A549 cells using an Annexin V FITC/PI double staining kit and flow cytometry. In summary, our research is under patent

submission and shows that CBD and AVE have the potential to develop into a potent new anti-lung cancer drug.

Discussion

Aloe vera is frequently utilized as a therapeutic agent and functional food. It is typically used as a laxative and is only occasionally utilized in official therapeutic applications. Therefore, it is necessary to conduct controlled and thorough research to examine the side effects, toxicity, and effectiveness of *Aloe vera*.

In summary, the effects of AVE treatment on increased cellular ROS and decreased ATP production indicated that AVE had an impact on mitochondrial function. More research is needed to determine how mitochondria activity affects cancer cell apoptosis in response to AVE treatment. Chemotherapy side effects are primarily caused by the low or non-selectivity of anticancer drugs, which results from the fact that these drugs are meant to kill cells with higher rates of reproduction, such as cancer cells and immune cells found in the bone marrow. While the number of cancer cells (A549 cells) that underwent apoptosis was significantly higher than that of healthy, normal cells (BEAS-2B cells), we saw that AVE significantly altered the degree to which cells underwent apoptosis.

By the generation of ROS, it has been shown that AVE inhibits the growth of human squamous carcinoma cells [Chiu et al. 2009]. It is known that a number of chemopreventive substances, including emodin, doxorubicin, sorafenib, bleomycin, and platinum complexes, cause the production of ROS in cells [Marullo et al. 2013; Di Giacomo et al., 2019]. Moreover, ROS contributes to the depolarization of the mitochondria and the advancement of apoptosis [Redza-Dutordoir and Averill-Bates 2016]. An early stage of apoptosis is the loss of mitochondrial membrane potential [Ly et al. 2003]. According to several investigations, plant extracts could cause cytotoxicity via depolarizing the mitochondria [Al-Oqail et al. 2021].

The mitochondrial apoptosis pathway is dependent on interactions between pro- and anti-apoptotic genes, such as p53, bax, capsase-3, and caspase-9, and is predicated on the activity of the Bcl-2 family. Proapoptotic genes such as p53, bax, capsase-3, and caspase-9 were expressed at higher levels in A549 cells treated with 6.0 µg/mL of AVE for 24 hours, while the expression of the antiapoptotic gene Bcl-2 was downregulated. In the past, Shalabi et al. [2015] shown that treatment with AVE increased p53 gene activity and decreased Bcl-2 gene activity in HepG2 cell death. Similar to this, Jiang et al. [2020] described how Aloe-emodin, a key component of *A. vera*, causes breast cancer cell death by downregulating Bcl-2 expression and is mitochondria dependent.

So, the purpose of this work was to assess the *in vitro* cytotoxicity of ethanolic extract of *A. vera* against human cancer cell lines. Due to their speed, sensitivity, simplicity, dependability, and low cost, cytotoxicity assays using grown cells are commonly used to evaluate the cytotoxic potential of plant extracts [Farshori, 2021]. The outcomes demonstrated that A549 cells are subjected to AVE for 24 hours and are cytotoxic in a dose-dependent manner. With an IC50 of 6.0 µg/mL, AVE exhibited high cytotoxic activity against A549 cells. On the other hand, the non-psychoactive cannabinoid CBD extracted from plants exhibits pro-apoptotic and anti-proliferative effects in several tumor types. Our findings demonstrate that CBD induces apoptosis in lung cancer cells, and these findings are consistent with those seen in breast and bladder cancer types by other researchers (Tomko et al. 2022; Whynot et al., 2023). It may also have anti-migratory, anti-invasive, anti-metastatic, and maybe anti-angiogenic effects. Based on the findings, evidence is mounting that CBD is a powerful inhibitor of cancer growth and metastasis [Hinz and Ramer et al., 2022]. According to a recent study, CBD significantly decreased the proliferation of gastric cancer SGC-7901 cells in a concentration-dependent manner, with an IC50 value of 23.4 g/mL after 24 hours of treatment [Zhang et al., 2019].

The most prevalent CBD effect is an increase in ROS generation, which appears to be the key factor in starting CBD's anti-tumor effects in all cancer cell types taken into consideration. In addition to the *in vitro* evidence, experimental animal models using CBD showed that it was effective in slowing tumor growth and, in some cases, metastasizing. Yet, there are some things to think about while using CBD as a potential medicinal treatment for cancer. Its low toxicity is undoubtedly a smart place to start. In fact, oral administration of 700 mg of CBD per day for six weeks did not exhibit any overt toxicity in humans [McCartney et al., 2022], suggesting that CBD may be used for long-term treatment.

However, there is still much work to be done, particularly in regards to the bioavailability of these plant-derived substances, before we completely comprehend the potential benefits of the Aloe and Cannabis polypharmacy in a way that might be employed for the treatment of cancer in humans. The majority of the research conducted up to this time have been used in cell lines (*in vitro*) or animal models (*in vivo*). Also, to determine whether some of these chemicals (single or in combination with other anti-cancer medicines) might be helpful in anti-cancer therapy, more clinical research is required.

Conclusion

According to the current study, AVE and AVE+CBD extract has the ability to induce death of lung cancer cells and prevent their growth. Furthermore, our findings demonstrated that AVE and CBD may have caused ROS generation and disruption of the mitochondrial membrane in A549 cells. In summary, the ability of AVE and cannabinoids, in particular, to cause suppression of cell proliferation and inducing apoptotic pathway, supports the use of these drugs as an additional therapy option for tumors. Consequently, the next logical step in the development of AVE and CBD as an anticancer drug is to conduct thorough clinical trials to ascertain its safety and efficacy in various cancers.

Moreover, downregulation of the Bcl-2 gene and overexpression of the genes p53, bax, capsase-3, and -9 may both contribute

to AVE+CBD -induced cell death. This in vitro study proves that AVE and CBD could be a useful therapeutic agent for the management for anticancer treatment with excellent safety and efficacy. Hence, more research is required on the molecular processes underlying the anticancer potential of AVE and CBD in vivo settings. We may conclude that AVE and CBD may serve as an alternative cancer treatment due to their selective cytotoxic and apoptotic effects. Furthermore, it should be noted that much work needs to be done in terms of the efficacy, appropriate dosage, administration pathways, drug safety, and anticancer mechanism of AVE and CBD in tumor therapy, particularly with regard to the need for clinical trials, which are the only way to determine the advantages and disadvantages for cancer patients as well as possible risks.

Informed Consent Statement

Not applicable.

Data Availability Statement

Not applicable.

Conflicts of Interest

The authors declare no conflicts of interest for this article.

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