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DELTA-9 TETRAHYDROCANNABINOID AND GEMSITABIN COMBINATION ON NON-SMALL CELL (SQUAMOUS CELL, SK-MES-1) LUNG CANCER CELL LINE

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ABSTRACT

Squamous cell carcinoma falls within the category of non-small cell lung cancers, presenting a considerable challenge in terms of treatment management. There has been growing interest in the potential anti-cancer properties of Delta-9 tetrahydrocannabinol. Gemcitabine, a nucleoside analog, has shown effectiveness against diverse cancer types. This study aimed to assess the combined efficacy of Delta-9 tetrahydrocannabinol and Gemcitabine in treating cancer. The squamous lung cancer cell line required for our study was provided by the stem cell unit. The doses were determined as 5µm/L and 10µm/L for Delta-9 tetrahydrocannabinol, 20 and 40 µm/L for Gemcitabine by performing a literature review. In our study, Cell Viability Analysis by MTT, Xcelligence Real-Time Cell Analysis, Annexin V Apoptosis Flow Cytometry Analysis, Total Oxidant, Antioxidant Status, and Caspase-3 Detection Analysis was performed. Upon evaluating the rates of apoptotic cell death, it was observed that the THC 5 and THC 10 treatment groups exhibited a 30% and 60% increase, respectively, compared to the alcohol group. A significant difference in cytotoxic effect, as determined by MTT, was found between the control group and the Gemsitabin 20, Delta-9 Tetrahydrocannabinol 5µm/L, and Gemsitabin 20+Delta-9 Tetrahydrocannabinol 5µm/L groups ($p<0.001$). There was a statistically significant difference between the groups regarding TOS ($p<0.001$). All experimental groups exhibited a higher level of caspase 3 activations, in comparison to the control group. We observed a significant cytotoxic effect of Gemcitabine on squamous cell lung cancer cells. Delta-9 tetrahydrocannabinol, when used alone, exhibited a relatively low cytotoxic effect. However, no significant difference was observed in the groups where Delta-9 tetrahydrocannabinol was combined with Gemcitabine.

KEYWORDS: Cancer, THC, Gemcitabine, Cytotoxicity, Squamous Cell Cancer, Combined Therapy.

INTRODUCTION

Cancer is a clonal complex disease with a multi-step development process (1). About two million new cases of lung cancer occur each year. Lung cancer originates from the bronchial epithelium (2). Squamous cell carcinoma (SCC) is the second most prevalent form of lung cancer, falling under the category of non-small cell lung cancers (NSCLC) in the classification of lung malignancies. The complicated biology of SCC is now being better understood through studies, which have yielded new potentially actionable molecular targets and their associated therapeutic uses (3).

The treatment regimen in SCC is determined as surgery, chemotherapy, radiotherapy, or simultaneous chemotherapy and radiotherapy (4). Acquired resistance in chemotherapy treatment is an important clinical problem. Comparative studies focusing on the number of medications used in the treatment regimen have shown that employing multiple treatment alternatives leads to improved treatment response and survival outcomes when compared to using a single agent alone (5).

Delta-9 tetrahydrocannabinol (THC) is a prominent cannabinoid present in the Cannabis sativa plant (6). Over the past few years, there has been a surge in interest regarding the potential anti-cancer properties of THC (7). Research studies have indicated that THC can trigger cancer cell death (8), impede tumor growth (9) and metastasis (10), and augment the efficacy of other anti-cancer treatments (11). The mechanisms responsible for these effects involve the activation of cannabinoid receptors (12), modulation of signaling pathways associated with cells survival and

proliferation (13), and regulation of the tumor microenvironment (14). Furthermore, THC has demonstrated its ability to alleviate cancer-related symptoms including pain, nausea, and loss of appetite, thereby improving the overall quality of life for cancer patients (15). Despite these promising findings, additional research is needed to comprehensively comprehend the anti-cancer mechanisms of THC and optimize its therapeutic potential effect (7,11). The potential of THC as an anti-cancer agent signifies a captivating realm of research that holds promise for the development of innovative and efficacious cancer therapies.

Gemcitabine (GEM) is a nucleoside analogue that has shown effectiveness in treating different types of cancer, such as pancreatic, lung, and breast cancer (16). The anti-cancer effects of GEM are primarily attributed to its capacity to inhibit DNA synthesis (17) and induce apoptosis (18) in cancer cells. GEM is metabolized intracellularly into an active triphosphate form (19), which competes with the natural nucleotide substrates for incorporation into the DNA chain during replication (17), leading to chain termination and DNA damage. Moreover, GEM has demonstrated the ability to enhance the sensitivity of cancer cells to radiation therapy (20), indicating its potential role in combination therapy. Ongoing research efforts are focused on identifying biomarkers (21) that can predict patient response to GEM and developing strategies to overcome resistance (22) to this drug.

Combination therapy has emerged as a promising approach in cancer treatment, as it enables the targeting of multiple pathways (23) and reduces the likelihood of developing resistance (24). This strategy involves the simultaneous use of two or more drugs with distinct mechanisms of action, which work synergistically to enhance therapeutic efficacy (25). The rationale behind combination therapy stems from the recognition that cancer cells frequently exhibit diverse genetic and molecular abnormalities, enabling them to evade the effects of single-agent therapies (23). Ongoing research is focused on identifying biomarkers and developing predictive models to guide the selection and optimization of combination therapies for cancer patients.

The objective of this study was to assess the effectiveness of combining Delta-9 tetrahydrocannabinol and Gemcitabine in the SK-MES-1 (squamous cell carcinoma) cell line.

MATERIAL AND METHOD

Chemicals

- Chlorogenic acid (HWI Analytical GMBH Product No: 0050-05-90)
- Gemcitabine (2,2-difluorocytidine-2-deoxycytidine, dFdC),
- Delta-9 Tetrahydrocannabinoid (Δ-9THC),
- Dimethyl Sulfoxide (Sigma, D8418),
- Sodium- pyruvate (Na-pyruvate) (Sigma S8636),
- Dulbecco's Modified Eagle's Medium (DMEM) (Sigma D5546),
- Fetal Bovine Serum (FBS) (Sigma Lot040M3396),
- Dulbecco's Phosphate Buffered Saline (PBS) (Sigma D1408),
- Mem Non-essential Amino Acid Solution (Sigma, M7145),
- L-glutamine (Sigma G 7513),
- Penicillin streptomycin (Sigma P0781),
- TrypsinEDTA (Sigma Lot 11C508),
- RPMI-1640 Medium (Sigma, Lot 116K2416),
- Fetal calf serum (FCS),
- Histopaque-1077 (Sigma, RNBC 6075),
- Cell Counting Kit-8 (WST-8) (Sigma Lot BCBN 61904),
- Tox-1 Kit (Thiazoly Blue Tetrazolium Sigma Lot MKBH9792V, MTT solvent solution Sigma Lot 051M8719V),
- Cell culture flask (T-25 and T-75 cm2) (Corning),
- Micropipette, plate with 24, 48 and 96 wells (Well Plate) (Corning), 15 ml and 50 ml falcon tube with cap (ISOLAB) and disposable sterile pipette (Corning)

Devices Used

- Cell culture cabinet (HeraSafe Type II laminator Flow),
- Inverted light microscope (Olympus CK40-SL),
- Centrifuge (Hettich Rotina 38R),
- Cell culture incubator with CO2 (Thermo Hepa Class 100 Steri- cycle),
- Electric pump (Boeco)
- Multiplate reader spectrophotometer (Thermo labsystems Multiskan Spectrum)

Supply of Cell Series

The Squamous lung carcinoma cells (SK-MES-1) were obtained from the Stem Cell Unit of the Research and Development Center of the University of Health Sciences. Manuplation of the cells was performed in the same laboratory under suitable conditions.

Cell Line and Culture Condition

The cells were cultured following standard cell culture principles in a sterile laminar flow cabinet (HeraSafe Type II laminar flow). A total of 1×10^7 cells were seeded in T75 culture flasks and allowed to proliferate. The cells were maintained in Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12) (Sigma D5546) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. The culture flasks were placed in an incubator with 100% humidity and 5% CO2 at 37ºC. The growth of the cells was monitored until they reached a confluence level of 70-80%. Throughout this period, the culture media were refreshed every 2 days. Trypan Blue Method, which is a classical method, was used to both count cells and detect dead cells in the counting process after passage.

Experimental Groups

In the analyzes we conducted in our study, the groups and treatment doses indicated in the table below were determined (Table 1). The dose of THC and GEM was determined after examining similar models in the literature review. Alcohol used as a solvent for THC was added to all analyzes to observe its cytotoxic effect. In order to observe the cytotoxic effect of GEM and THC on the SK-MES-1 cell line, the doses were determined as 5µm/L and 10µm/L for THC, 20 and 40 µm/L for GEM by performing a literature review (26,27).

Cell Viability Analysis by MTT Method

The MTT solution was prepared by dissolving it in FBS-DMEM medium at a concentration of 5 mg/ml. The solution was then filtered using a 0.22 µm filter to ensure its sterility and make it ready for use. Cells were prepared by counting 400 cells per mm^3 using a hemocytometer and 100 µl of cell solution (cell + medium with 10% FBS) was dispensed into each well with a multi-channel pipette. Cells in 100 µl of FBS solution were removed to the incubator for 24 hours incubation. After 24 hours of incubation, the agents specified in the groups were added to 100 µl of 10% FBS and the final volume of each well was made up to 200 µl. Following the 24-hour incubation period, 20 µl of MTT solution was added to each well of the plates. Subsequently, the plates were incubated for an additional 4 hours. After completing the incubation, the supernatant was carefully aspirated from each well. Subsequently, 100 µl of isopropyl alcohol was added to each well, and the plate was covered with foil.

Groups Analyzes	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
MTT Analysis	GEM 20µg	THC 5µg	GEM 20µg + THC 5µg	Alcohol	Control					
Xcelligence (RTCA) Analysis	GEM 20µg	THC 5µg	GEM 20µg + THC 5µg	GEM 40µg + THC 5µg	Alcohol	Control				
Flow Cytometry Apoptosis	GEM 20µg	GEM 40µg	THC 5µg	THC 10µg	GEM 20µg + THC 5µg	GEM 20µg + THC 10µg	GEM 40µg + THC 5µg	GEM 40µg + THC 10µg	Alcohol	Control
TAS and TOS Analysis	GEM 20µg	GEM 40µg	THC 5µg	THC 10µg	GEM 20µg + THC 5µg	GEM 40µg + THC 5µg	Alcohol	Control		
Caspase-3 Analysis	GEM 20µg	GEM 40µg	THC 5µg	THC 10µg	GEM 20µg + THC 5µg	GEM 20µg +THC 10µg	GEM 40µg + THC 5µg	GEM 40µg + THC 10µg	Alcohol	Control

Table 1: Experimental Groups of Analysis

The plate was then incubated for 3-4 hours in the dark at room temperature to dissolve the formazan crystals. After the incubation, the plate was gently shaken to ensure thorough mixing, and the absorbance was measured using an ELISA plate reader at a wavelength of 570 nm. The experiment was performed in triplicate (n=3).

Xcelligence Real Time Cell Analysis (RTCA) Method

SK-MES-1 cells multiplied in culture medium were placed in special wells that provide impedance measurement together with the medium determined as 100 cells in each well. After observing the optimal rate of adherent cells after 12-24 hours incubation of the cells. SK-MES-1 cells were placed on the "E-plates" of the real-time cell analysis system. GEM and THC were added individually and in combination to the wells containing cells and nutrient media at the determined doses and amounts and incubated for 72 hours. The effects of drugs on cell indices were read on the RTCA control panel with the software.

Annexin V Apoptosis Flow Cytometry Analysis

To determine the level of cell apoptosis, Annexin V flow cytometry analysis was conducted. The cells under investigation were washed twice with phosphate-buffered saline (PBS). Subsequently, 5 µl of Annexin V dye solution and 10 µl of propidium iodide solution were added to the cells, and they were stained in the dark at 4°C for 30 minutes. Flow cytometry was then employed to measure the apoptosis rate.

Determination of Total Oxidant and Antioxidant Status

Before the total oxidant status (TOS) and total antioxidant status (TAS) measurement, in the preparation phase, the cells in the medium were taken into falcon tubes and centrifuged after the treatment and required incubation period. TOS level was measued in the supernatant according to the method developed by Erel. using Rel Assay ® commercial kits (9). Trolox, a water-soluble analog of vitamin E, was used as the calibrator, the results were expressed as mmol Trolox equivalent /L for TAS measurement (10).

Caspase-3 Detection Analysis

Analysis was performed using the Caspase-3/CPP32 Fluorometric Kit. According to the protocol, after the cell lysates which were prepared with lysis buffer solution, protein measurements were made and calculated as 20-200µg cells in 50µl cell lysis buffer. Again, as stated in the protocol, the result in protein determination was divided by 100000, the value obtained was subtracted from 50, and the amount of lysis buffer and sample to be used was calculated. Subsequently, the samples from each group were incubated on ice for a duration of 10 minutes. A solution composed of 10 µl of DTT and 1 ml of 2x reaction buffer was added to the samples in a volume of 50 µl. Subsequently, 5 µl of 1 mM DEVD-AFC substrate was added to achieve a final concentration of 50 mM. The samples were then incubated at 37 ºC for one hour. After incubation, the samples were passed through 400 nm excitation and 505 nm emission filters and loaded onto the plates. Readings were taken three times at specific time intervals: after 1 hour, 1 hour 30 minutes, and 2 hours.

Statistical Analysis

Statistical Program for Social Sciences (SPSS) 15.0 program was used for statistical analysis. Statistical analyzes of cytotoxicity rates on lung cancer cells in all groups were performed with the 'One-Way Anova' test. In this evaluation, values with $p<0.05$ were considered significant. The "Post Hoc Tukey" test was used for pairwise comparison of groups with significant results. Paired statistical comparisons of damage levels in all groups were made with this test. Bonferroni correction was made since $n<30$ in pairwise comparison analyses. As a result, values with $p<0.005$ were considered statistically significant.

RESULTS

MTT Analysis Findings

A significant difference was observed among the five groups, as demonstrated in the table, in terms of MTT analysis during the statistical evaluation. In the paired analyzes (Post Hoc Tests), it was determined that a significant distinction existed in terms of cytotoxic effect ($p<0.001$) between the control group and the GEM 20, THC 5, and GEM 20+THC 5 groups. It was also determined that there was a significant difference between the alcohol group, which is thought to have a cytotoxic effect, and the gem 20, Thc 5 and gem 20+Thc 5 groups ($p1<0.001$; <0.005; <0.001 respectively). (Figure 1).

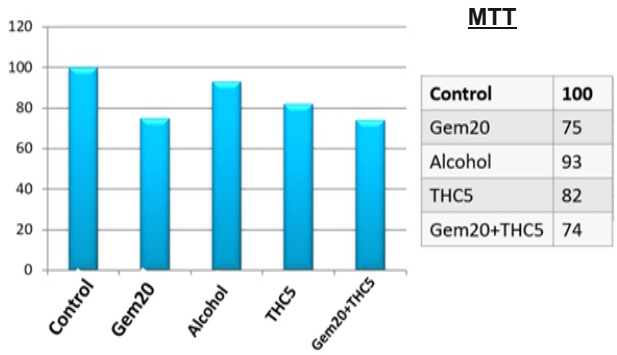


Fig. 1: MTT Cell viability measurement analysis. There was a significant difference between the control group and the GEM 20, Thc 5 and GEM 20+Thc 5 groups in terms of cytotoxic effect (p<0.001).

Xcelligence Real Time Cell Analysis Findings

Cells exposed to GEM, THC and combinations of these two agents were analyzed at the 72nd hours by real-time cell analysis system. While there was no significant difference between alcohol and control and THC groups, a significant difference was found between alcohol and GEM20, GEM40 ($p<0.13$), GEM20+THC, GEM40+THC groups in the form of increased cytotoxic effect ($p<0.001$). Other results have been presented in Figure 2 A-C.

Annexin V Apoptosis Flow Cytometry Analysis

Flow Cytometric Annexin V-FITC/PI staining was performed after 24 h incubation of SK-MES-1 cells exposed to GEM, THC agents.In the GEM40 µg and THC 10 µg combination group, the level of cells that lost their viability increased to the highest level among all groups with 56.6%. In this combination, 53.1% of the cells died by apoptotic activation, while 43.5% suffered necrotic death, and the rates recorded in this combination exceeded the rates in other groups (Figure 3 A-G).

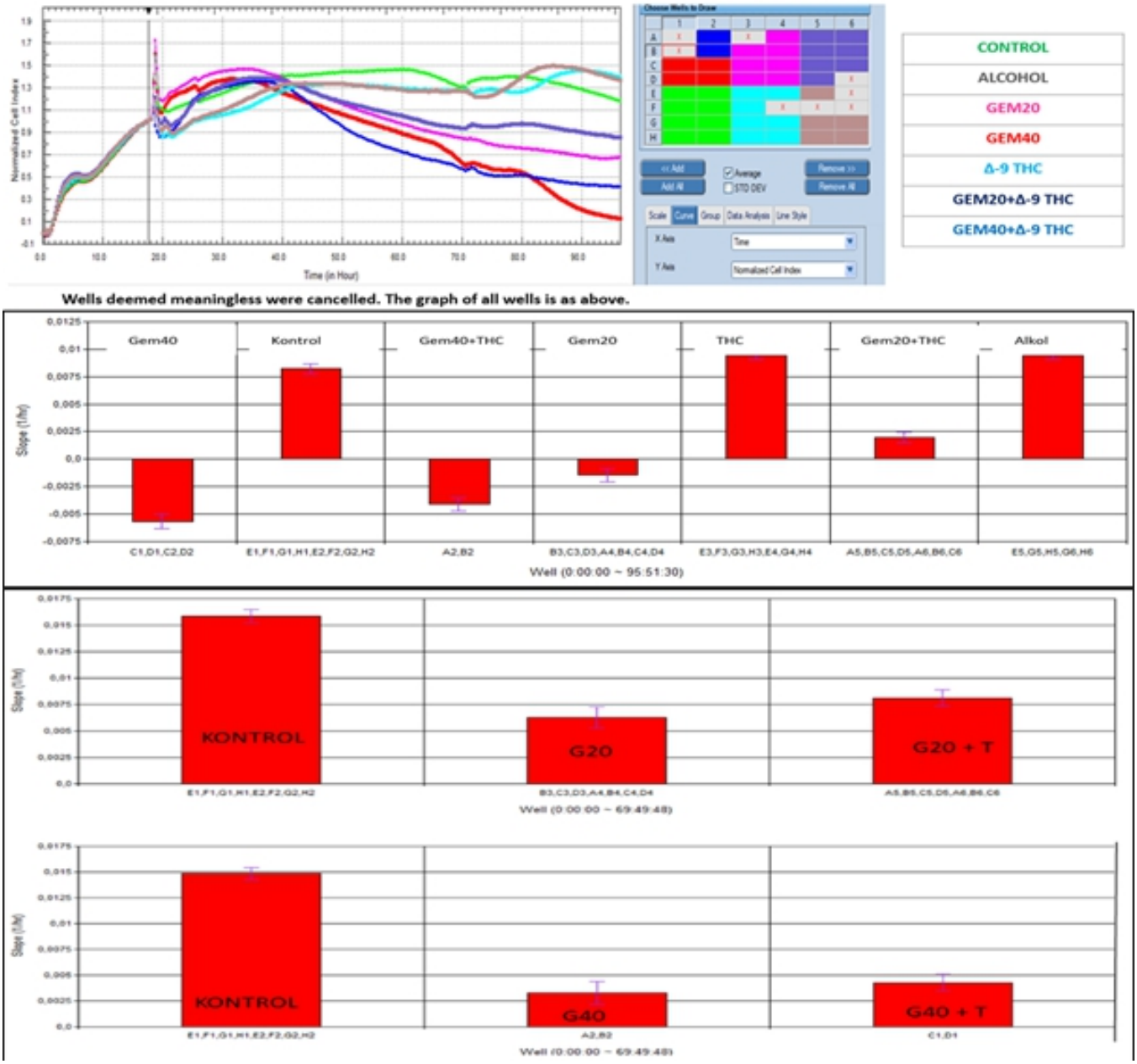


Figure 2. A: Real-Time Graph of xCELLigence Cell Viability Analysis. GEM, THC and combinations of these two agents were applied to cancer cells. Cells were exposed to the indicated agents for 72 hours. Also, SK-MES-1 cells not exposed to any agent were used as control. B: xCelligence Cell Viability Assay Cytotoxicity Levels of the Groups. C: Comparative Cytotoxicity Levels of the xCelligence Cell Viability Assay Treatment Groups

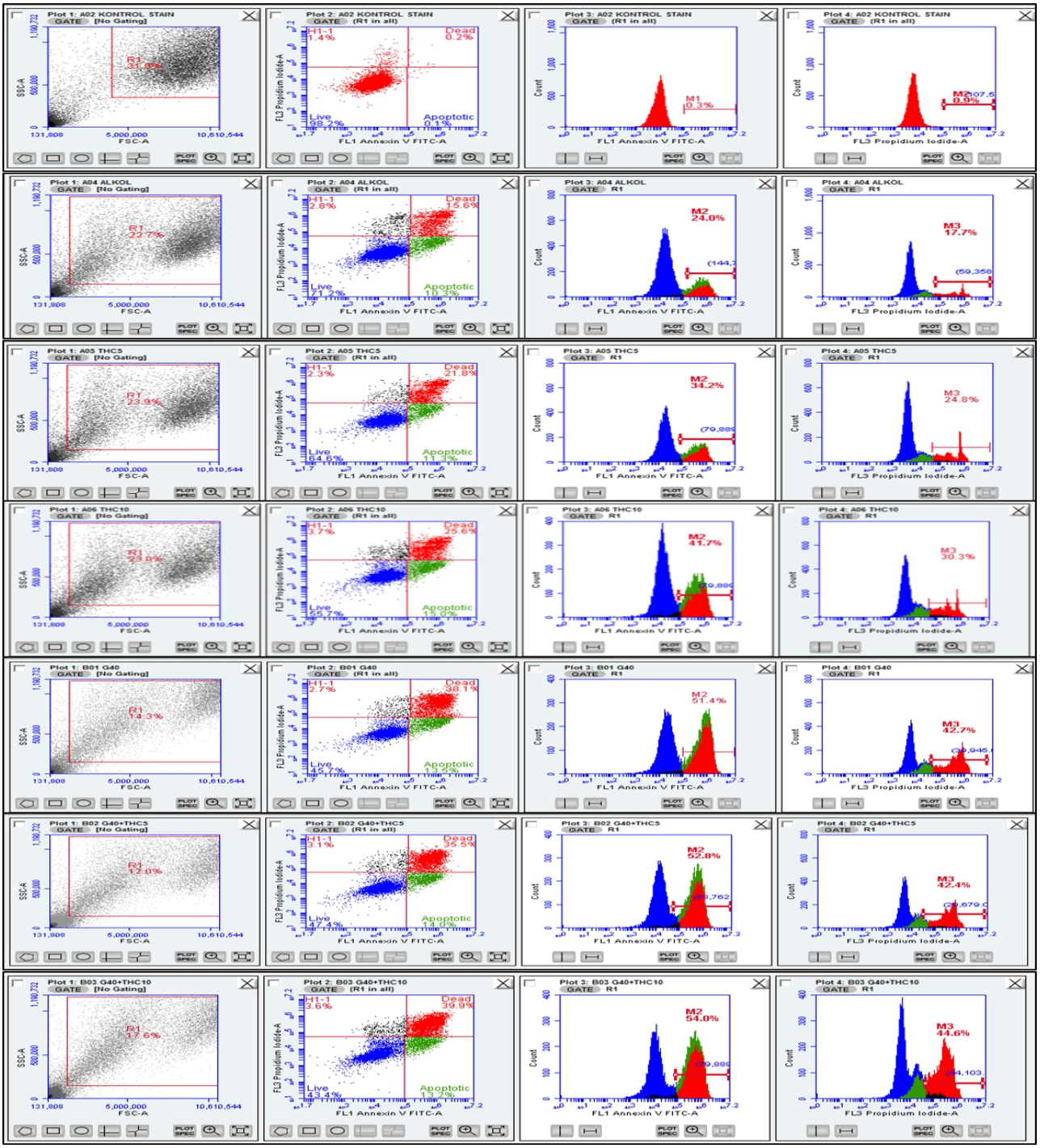


Fig. 3: A. Flow Cytometry Analysis Graph of the Control Group. Only 1.9 percent of cell death was detected in control group. B. Flow Cytometry Analysis Chart of Alcohol Group. There was an increase in the alcohol group compared to the control group. The rate of cells undergoing apoptosis in the alcohol group was 25.9%. It was observed that alcohol used as a solvent for THC was significantly toxic to cells. C.: Flow Cytometry Analysis Chart of THC5µg Group. There was a slight increase in apoptotic cell death compared to the alcohol group. D: Flow Cytometry Analysis Chart of THC10µg Group. Apoptotic cell death in the THC10µg group was 40% with an increase of 7% compared to the THC5µg group. E: Flow Cytometry Analysis Graph Belonging to G40 Group. The apoptotic cell level seen in the GEM40 group was 51.6%, leaving behind the THC groups used as treatment. F: Flow Cytometry Analysis Chart of GEM40+THC5 Group. In the GEM40+THC5 group, the cytotoxicity level was 49.5%. This ratio showed that the expected cytotoxic effect from the combination did not occur. G: Flow Cytometry Analysis Chart of GEM40+THC10 Group. The GEM40+THC10 group achieved the highest rate of cytotoxic effects in this assay. However, this increase was not significant when compared to other treatment groups.

When all the rates of apoptotic cell death were evaluated, it was seen that the rates seen in our treatment agent THC 5 and THC 10 groups increased by 30% and 60%, respectively, compared to the alcohol group. Apoptosis level of 51.6% in the GEM40 group, which is another treatment group, was an expected rate in the study. However, the expected synergistic effect did not occur in the groups in which the two treatment agents were combined.

Total Oxidant Capacity (Tos) And Total Antioxidant Capacity (Tas) Analysis

While a statistically significant difference ($p<0.001$) was observed between the groups regarding TOS, no statistically significant difference was found in terms of TAS, as depicted in Figure 4. The outcome of the aforementioned statistical analyses indicates a significant reduction in total oxidant levels.

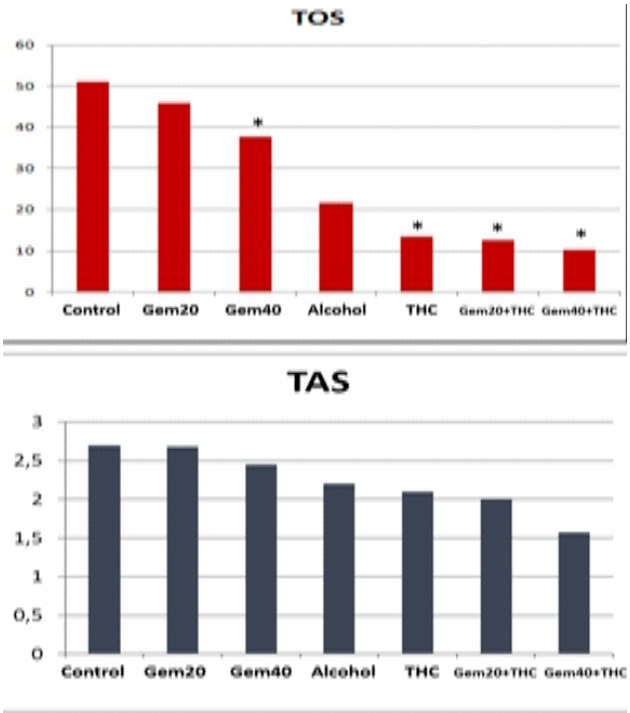
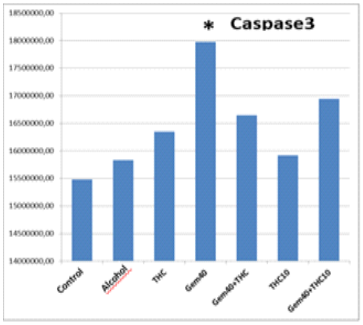


Fig. 4: A: Analysis chart of total oxidant capacity determination. B: Analysis Chart of Total Antioxidant Capacity (TAS) Determination. No statistically significant difference was observed in terms of total antioxidant capacity. B. Analysis Chart of Total Antioxidant Capacity (TAS) Determination. No statistically significant difference was observed in terms of total antioxidant capacity.

Findings from Caspase-3 Analysis

It was observed that there was more caspase 3 activation in all groups compared to the control group. The statistical analysis revealed a significant increase in the caspase 3 level measured in the GEM40 group compared to the other treatment groups ($p=0.027$) (Figure 5).



	Mean	SD
Control	15481737,29	1294542,46
Alcohol	15828566,63	820938,15
THC	16345407,38	1203326,90
Gem40	**17979630,71**	**1623959,25**
Gem40+THC	16648247,67	1631141,91
THC10	15917047,33	1055909,85
Gem40+THC10	16947591,17	1882854,30

Fig. 5: Chart and Table showing caspase-3 levels of all groups. A statistically significant increase was observed in the level of caspase 3 measured in the GEM40 group.

DISCUSSION

GEM has demonstrated valuable results for the treatment of NSCL. GEM provided an objective regression in approximately 25% of the patients. Combination chemotherapy based on GEM and platinum has accepted as the prevailing standard of treatment for patients with non-small cell lung cancer (NSCLC) who are in a favorable clinical condition (28). Reports on the biological role of the endocannabinoid system in cancer pathophysiology are not yet comprehensive. Opinions have begun to be expressed that it can accompany combined therapies in clinical use(29).

It was observed in an animal study that genetically desensitization of CB1 receptors caused colon tumor to grow more (30). In another study conducted for this purpose, it was observed that azoxymethane-mediated precancerous lesions in the colon decreased in an animal model with increased endocannabinoid levels(31).

Utilizing combined anticancer therapies offers several advantages over single-agent strategies. Consistent with this theory, recent studies have suggested that the combination of cannabinoids with other anticancer drugs may exert synergistic effects, leading to a reduction in tumor growth (29). It has been determined that THC and temozolomide administration have strong antitumor effects in glioma (32) xenograft models as well as in many temozolomide resistant tumors. Likewise, a recent study demonstrated that the combined treatment of GEM with different cannabinoid agonists resulted in synergistic reduction of pancreatic cancer cell viability (Reference 33). Furthermore, other reports have indicated that anandamide and HU-210 increased the anticancer activity of paclitaxel and 5-fluorouracil, respectively (References 34, 35).

Targeted therapies has been considered as a crucial in strategies aimed at reducing cancer mortality (36).

Consequently, the emphasis in the development of anticancer drugs has shifted towards molecularly targeted inhibitors (Reference 37). However, despite the extensive preclinical research conducted on the mechanisms of action of these compounds, their clinical applications remain limited (References 37-39).

In our study the anticancer activity of GEM is examined in combination with Delta-9 Tetrahydrocannabinoid which gives important results in combined therapies.

According to our MTT results highest rate of living cells in our 24-hour groups was in the control group. 25% cytotoxic effect was observed in the GEM 20μM group. In the study of Chen et al. to measure the effectiveness of apoptosis signal-regulating kinase 1 (ASK1) pathway on cell death on different pancreatic cancer cells (Bxpc-3, Mia PaCa-2 and Panc-1), they obtained similar results in the GEM group (23).

The cytotoxicity level in the THC 5 μM group is 18%, which is significantly higher than that observed in the control and alcohol groups, indicating a toxic effect. However, no superiority was observed against the GEM 20μM and GEM+THC groups. Caffarel et al. detected a significant cytotoxicity level in the THC 5 μM group in breast cancer (MCF-7) cells (40). A similar study conducted on glioblastoma cell lines (U251) showed a distinct cytotoxic effect (41). The cytotoxicity level in the GEM20+THC group (74%) and the GEM20 group (75%) indicates that the synergistic activity of the GEM+THC combination does not show superiority over other groups. It can be inferred that the combination of GEM with THC did not have a reinforcing effect on cytotoxicity based on MTT analysis.

Xin Zhang et al. have hypothesized that the administration of low-dose chemotherapy to natural killer cells isolated from lung cancer cells would increase the effectiveness of chemotherapy. The xCELLigence analysis results from their study showed that GEM caused significant toxicity in lung cancer cells (42). In another study conducted by Shao et al., the xCELLigence analysis on pancreatic cancer cells (PANC-1 GR) determined that GEM showed significantly high activity (43). Lukhele et al. reported in their xCELLigence analysis that cannabinoid activity was effective only in Me-180 cell lines, and significant cytotoxicity was not observed in other cell lines (44). In our study, significant cytotoxic effects of GEM were observed on SK-MES-1 cell line at two different treatment doses, 20 μM and 40 μM. When we combined GEM 20 μM and 40 μM groups with THC 5μM, the result did not exceed the cytotoxic effect of the GEM groups alone; in fact, it limited it.

It is well known that cancer cells are characterized by high levels of ROS and intrinsic oxidative stress (45). In some cases, inducing an increase in ROS in malignant cells with appropriate cytotoxic agents may be a solution to kill cancer cells. Fiorini et al.'s (46) study on pancreatic cancer cell lines, Donadelli et al.'s (47) study on pancreatic adenocarcinoma, and Deng et al.'s (48) study on breast cancer all demonstrated that the results of their cytotoxicity studies indicate that cell death increases as a secondary effect of ROS. In our analysis to determine the total oxidant and antioxidant capacity, we found that in the study groups where the oxidant level significantly decreased with GEM 40μM, THC 5μM, and GEM+THC groups, there was no significant change in antioxidant level.

The effect of THC, a natural compound we used in our study, on oxidative stress has been the subject of a limited number of studies, with different results obtained. Goncharov et al. showed in their study on glioma cells that THC increased oxidative stress (49). Marcu et al. investigated the anticancer activity of cannabidiol, another natural compound found in the cannabis sativa plant and used in research on glioma cells (41). The cytotoxic effects of cannabidiol and THC were evaluated together and separately in the study. It was determined that THC was not as effective as cannabidiol in generating oxidative stress and, thus, ineffective.

In our study, we evaluated the level of apoptosis through AnnexinV flow cytometry analysis. We found that approximately 52% of cells in the GEM 40 μM group underwent apoptotic cell death. Vertrees et al. (50) and Cartee (51) have investigated the efficacy of GEM on NSCLC cells and ovarian cancer cells, respectively. Similar results have been obtained. In the study by Greenhough et al. (27) on colorectal cancer cells (Sw480), a significant level of apoptosis was determined at a dose of 10 μM THC. Ruiz et al. (52) have obtained a significant apoptotic effect at a dose of 10 μM THC in their analyses on prostate cancer cells (PS3).

In our study, we obtained apoptosis levels of 34% and 41% for THC treatment doses of 5 and 10 μM, respectively. Considering the apoptotic level of 24% in the alcohol group, it is believed that the 10 μM THC dose yields results similar to the literature and may be clinically meaningful. The GEM 40 μM and THC 5 and 10 μM groups were combined. The cytotoxic effect in the GEM 40+ THC 10 μM group was 3% higher than in the GEM 40+ THC 5 μM group. This increase was not considered significant.

In our analysis for the determination of caspase-3, a critical protein in apoptotic cell death, an increase was observed in all groups compared to the control group,

but a significant increase in the treatment groups was observed in the GEM40 μM group. In the combination of GEM and THC 5 and 10 μM groups, lower caspase-3 activity was observed compared to the GEM40 μM group. Chandler et al. ((53)) in their study on pancreatic cancer cells (BxPC3) observed that caspase-3 levels increased in the GEM group. Vertrees et al. (50) determined that GEM induces cell death by causing caspase-3 activity. In the study of Marcu et al. (41) on glioma cancer cells, it was stated that THC increased caspase-3 activity to a very limited extent.

THC is the most effective cannabinoid of Cannabis sativa plant. Many studies have been conducted to reveal the mechanism of THC. It has been observed that cytotoxic activity occurs through different mechanisms in various types of cancer. In a study, the THC receptor active in the lungs was found to be largely the CB1 receptor(54).

In our study, we observed the individual anticancer effects of GEM and THC in the MTT and Xcelligence analyzes we performed to examine the cytotoxicity. When we evaluate the results of the analysis, we revealed that there was no synergic effect. Donadelli et al. (47) evaluated the effects of GEM and cannabinoid combination on pancreatic cancer. In this study, it was determined that the ceramide synthesis mechanism in the cell inner membrane was induced by GEM.

ROS production of GEM is an important factor in anticancer mechanism as in other chemotherapeutics. In the studies, a positive relationship between GEM and ROS production was revealed. Daniel Lopez and his friends argued that the studies on the anticancer efficiency of the THC were largely carried out in neuronal glyoma cells and that ROS activity may be specific to these cells, showing oxidative stress caused by dopamine metabolism (55). In another study, Donadelli investigated the effectiveness of the combination of GEM and cannabinoid on the pancreas cell sequence (33). They stated that the effectiveness of the GEM and the combined group depends on ROS activity and that basal ros levels prevented cytotoxic effects. Although it is revealed that the stability of the oxidant and antioxidant capacity is not compatible with the cytotoxic mechanism created by conventional chemotherapeutics, our study is the first cytotoxicity study on GEM and THC and the need for different molecular studies.

In our apoptosis and caspase 3 analysis, we observed that although the activity in the GEM40 group was significant, the apoptosis in the THC groups was limited. It was mentioned that cannabinoids affect a large number of cellular molecules and signaling networks, and that THC is important for the effect of CB1 receptors, which are found in excess in the lung. Two cell signaling pathways, P38/MAPK and PI3K/AKT signaling pathways, are negatively regulated in cancer by stimulation of these receptors. The PI3K-AKT-mTOR signaling pathway has been shown to regulate various cell processes such as cell growth, apoptosis, and autophagy, and has been identified as one of the most problematic pathways in various human malignancies(56).

In the study of Leelawat et al by creating inhibition on the PI3K/AKT signaling pathway in cholangiocarcinoma cell line, it was seen that they provided AKT inhibition at a dose of THC 40 μM (57). In the literature, a dose of 3-15 μM THC was preferred in studies on various cancer cells (58,59). The fact that a dose of THC 40 μM caused AKT inhibition on cholangiocarcinoma cells suggested that THC did not have the desired effect for apoptosis activation at doses of 5 Considering the involvement of the PI3K/AKT signaling pathway in autophagy-mediated apoptotic pathway activation, it has been postulated that the analysis results may not fully reflect the cellular death. THC, apart from the apoptotic pathway, can induce cell death by inhibiting the phosphorylation of proteins through cyclin-dependent kinase inhibitors p21 and p27, which play a role in cell cycle regulation. The limited activation of caspase-3 in the THC and combination groups compared to the GEM40 group can be explained by the aforementioned cell cycle activity.

CONCLUSION

When we evaluated our analysis results, we observed the positive cytotoxic effect of GEM on squamous cell lung cancer cells. In addition, we found that THC alone had a low cytotoxic effect and that there was no significant difference in the groups in which it was combined with GEM. It has been determined in the literature that THC has different results in various cancer cells. Further studies at the molecular level are needed to determine at what concentrations and through which mechanism it may exert activity in lung cancer cells, and what results it will bring in different combinations. Considering that cancer studies are aimed at a multi-step and complex organization, we hope that our study will make a positive contribution to the literature.

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