Clinical and Experimental Health Sciences

Effect of Native Hemp Seed Oil Enriched with Cannabidiol on Epigenetic Stability and Oxidative Stress Status of Colon Cancer Cells

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ABSTRACT

Objective: Hemp is a plant that has become popular among recent research topics due to its wide range of uses and rich content. This study aims to determine the effectiveness of hemp seed oil and CBD on cytotoxicity, genetic stability, and oxidative stress status of cancer cells and to determine whether these two materials have potential in complementary cancer treatment.

Methods: For this purpose, hemp seed oil (Narlı) and combinations of these oils with CBD were applied to the colon cancer cell line (SW480) in vitro. The MTT test was performed to reveal the cytotoxic effects of the applications performed. To determine whether the same applications create epigenetic modifications in parallel with the cytotoxic effect percentages, the levels of epigenetic modifications were determined with histone deacetylase ELISA kit. At the same time, the GSH ELISA kit was used to determine the effects of hemp seed oil and CBD-containing applications on oxidative stress formation. The results were analyzed statistically.

Results: It was observed that both hemp seed oil with Narlı and hemp seed oil with CBD added at a dose of 150 µl/ml significantly reduced colon cancer cell viability compared to the control group. In the glutathione test, which is an important criterion in measuring antioxidant potential, it was determined that doses that showed cytotoxic effects exhibited antioxidant activity. In addition, it was understood that histone deacetylase activity increased at the same doses.

Conclusion: When all findings were evaluated in the light of literature data, it was predicted that hemp seed oil and CBD may have exhibited an effect not by oxidative stress and HDAC inhibition, but by increasing intracellular stress levels via the endoplasmic reticulum.

Keywords: hemp seed oil, cannabidiol, cancer, epigenetics

1. INTRODUCTION

The hemp plant (*Cannabis sativa* L.) has been cultivated for industrial purposes such as fiber, oil, and biomass since ancient times. P roducts obtained from hemp have a wide range of uses, from medicine to cosmetics, from biofuel to animal feed, and from paper to textile products (1). There are studies showing that the hemp plant contains more than 100 compounds and that these compounds inhibit the proliferation, angiogenesis, and metastasis of cancer cells. Additionally, the hemp plant exhibits apoptosis-inducing effects due to the flavonoids, terpenes, and cannabinoids it contains. In addition to these effects on cancer cells, studies have also determined that hemp does not reduce the vitality of healthy cells (2, 3).

Hemp seed oil is one of the main components, constituting approximately 25-35% of the seed. Hemp seed oil contains high levels of polyunsaturated fatty acids (PUFAs) and triacylglycerols (TAGs). In addition, the seed oil contains many minor elements. These minor components, referred to as tocopherols and polyphenols, are known to be the main reason for the use of hemp seed oil for cardiovascular diseases, cancer, cholesterol, blood pressure, and cosmetic purposes (4, 5).

In addition to hemp seed oil, which has been used in traditional treatment for many years, hemp flowers and leaves are also frequently used due to the cannabinoids they contain. Studies have shown that cannabinoids have both *in vitro* and *in vivo* anticancer effects on many types of

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cancer such as glioma, leukemia, lymphoma, liver, cervical, colorectal, prostate, breast, thyroid, and stomach (6). Cannabidiol (CBD), one of the cannabinoids found in hemp leaves and flowers, is a non-psychoactive phytochemical and is widely used to control pain in cancer patients. In addition, it is thought that CBD also has a share in the anti-cancer activity of cannabinoids. Research indicates that CBD can help slow down the growth of cancer cells by affecting the 5HT1A, GPR55, TRPV, and PPAR-ɣ receptors (7).

CBD has been found to exhibit potent anti-proliferative and pro-apoptotic effects on a wide range of cancer types, both in cultured cancer cell lines and mouse tumor models. Anti-tumor mechanisms range from cell cycle arrest to autophagy, cell death, or altered tumor types. Mechanistically, another mechanism of action of CBD on cancer cells is thought to be disruption of cellular redox homeostasis, causing an increase in reactive oxygen species (ROS) and endoplasmic reticulum stress (6).

Human cancer cells contain global epigenetic abnormalities and numerous genetic changes. Mutations that occur due to epigenetics can cause the silencing of tumor suppressor genes and the activation of oncogenes. This situation promotes tumor formation. At the same time, it is possible to reverse epigenetic abnormalities with epigenetic modifications. This situation, which is referred to as epigenetic therapy, can be expressed as benefiting the treatment process by reprogramming DNA methylation, histone modifications, nucleosome positioning and many other processes. The goal of the therapy is not only treatment, but also increasing sensitivity to treatment, suppressing metastasis and reversing chemoresistance. There is no published study investigating the effects of hemp oil and CBD on epigenetic therapy in cancer cells (8). This study aims to investigate whether hemp seed oil and CBD have an epigenetic contribution in the fight against cancer and to determine their relationship with oxidative stress.

2. METHODS

2.1. Hemp Seed Oil Analysis

In a previous thesis study (9), fatty acid analyses of hemp seed structure were conducted. The fatty acid ratios obtained in the relevant study are presented below (Table 1). Narlı hemp seed oil was obtained from Ondokuz Mayıs University Hemp Research Institute.

Table 1. *Fatty acid composition of Narlı hemp seed oil (9).*

	Fatty Acid Type	Fatty Acid Ratio (%)
Saturated Fatty Acids	Palmitic	7.13
	Stearic	2.56
	Behenic	0.31
	Arachidic	0.92
	Total	10.74
Unsaturated Fatty Acids	Oleic	13.75
	Linolenic	18.73
	Linoleic	56.16
	Total Monounsaturated Fatty Acid	13.75
	Total Polyunsaturated Fatty Acid	74.89

2.2. Cell Culture Applications

The human colorectal cancer cell line (SW480, ATCC, USA) was thawed by removing it from the liquid nitrogen tank. To proliferate the cells in an incubator environment containing 5% CO_2 at 37°C; DMEM (Dulbecco's Modified Eagle Medium, Gibco, USA) medium containing 10% FBS (fetal bovine serum, Invitrogen, USA), 1% L-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin (Gibco, USA) was cultured. The cells that reached enough in the flask were cultured in 96-well plates under appropriate environmental conditions. After one day of incubation, various concentrations of hemp seed oil and hemp seed oils with cannabidiol added, listed below, were applied to the cells (Table 2). In the study, each application was performed with 8 repetitions. The relevant doses were determined with the support of preliminary studies and literature information. Paclitaxel was used as a positive control. The SW480 cell line is known as a model line for chemotherapy-resistant, metastatic cancer studies (10). The 10 µM dose chosen in this study is related to the Resistance Index (RI) dose applied to the resistant cell lines. The RI is calculated by dividing the IC50 value of paclitaxel that kills sensitive cells by the dose that kills resistant cells (11).

The cytotoxic effect of the applications was tested with the MTT cell viability test at 24 hours. The same procedures were repeated for GSH (Glutathione) and epigenetic HDAC (Human Histone Deacetylase) (Mybiosource, USA) analyses as in the MTT cell viability test.

Table 2. *Applications and doses tested on SW480 cell line*

Application	Dose
Control	-
Narlı Hemp Seed Oil	50 µg/ml, 100 µg/ml, 150 µg/ml
Narlı Hemp Seed Oil with CBD (3 µM)	50 µg/ml, 100 µg/ml, 150 µg/ml
Cannabidiol (CBD)	3 µM
Negative Control (EtOH)	% 2
Positive Control (Paclitaxel)	10 µM

2.3. MTT Cell Viability Test

This test is based on a water-soluble substance called MTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide from Sigma-Aldrich, USA). Living cells process this substance and reduce it to formazan crystals. The resulting orange dye is measured at relevant wavelengths with the help of a spectrophotometer device.

2.4. GSH Test

In this study, which will be carried out with the aim of directly monitoring the production of reactive oxygen species, procedures were carried out in accordance with the commercial ELISA kit (Elabscience, USA) protocol after hemp seed oils and CBD applications. Samples were diluted with supplemented buffer. Then, the samples were added to the 96-well plate with reaction buffer + inhibitor in each well. After the formation of the yellow color, measurements were made at a 420 nm wavelength using a microplate reader.

2.5. Human Histone Deacetylase (HDAC) ELISA Kit

In this analysis, where the effectiveness of hemp seed oils and CBD applications on the epigenetic profile of cancer cells will be monitored, first the samples were diluted. Then, 100 µl of standard or sample was added to each well and incubated at 37°C for 90 minutes. Washing was done twice. 100 µl of biotin-labeled antibody working solution was added to each well and incubated at 37°C for 60 minutes. Plates were washed 3 times. 100 µl of SABC working Solution was added to each well and incubated at 37°C for 30 minutes. Plates were washed 5 times. 90 µl of TMB substrate solution was added. Incubated at 37°C for 10-20 minutes. 50 µl of stop solution was added. Measurement was made immediately at 450nm wavelength.

2.6. Statistical Analysis

Statistical analysis of all data was performed using IBM SPSS 21.0 software. In the analysis to be performed using the one-way ANOVA test, differences between applications were determined using Tukey's multiple comparison test.

3. RESULTS

In the study conducted with the aim of investigating the effectiveness of hemp seed oil and cannabidiol supplements on cancer cells, Narlı hemp seed oil was tested in different doses with and without CBD supplementation in the SW480 colon cancer cell line. The cytotoxicity test results are given in Figure 1. It was found that all applications containing hemp seed oil exhibited statistically significant data. According to the MTT test results, hemp seed oils increased the proliferation of cell viability at low doses. However, at a dose of 150 µl/ml, it reduced cell viability to 49% compared to the control group. This effect was followed by the application of 150 µl/ml hemp seed oil supplemented with 3 µM CBD. It was determined that this application reduced cell viability to 65%. It was determined that both applications were statistically significant ($p \leq .001$) compared to the control group. CBD applied at a low dose had no effect on cell viability alone compared to the control group. Similarly, the effect of paclitaxel, an anticancer agent, was such that it reduced cell viability to 93%. Both CBD-enriched and hemp seed oil administered alone at doses of 50 and 100 µl/ml were found to increase cell viability. At the same doses, CBD-enriched treatments were found to have a proliferative effect with statistical significance at $p \leq .05$. Furthermore, the increase in cell viability in hemp seed oil treatments alone (50 and 100 µl/ml) was found to be statistically significant at $p \leq .001$.

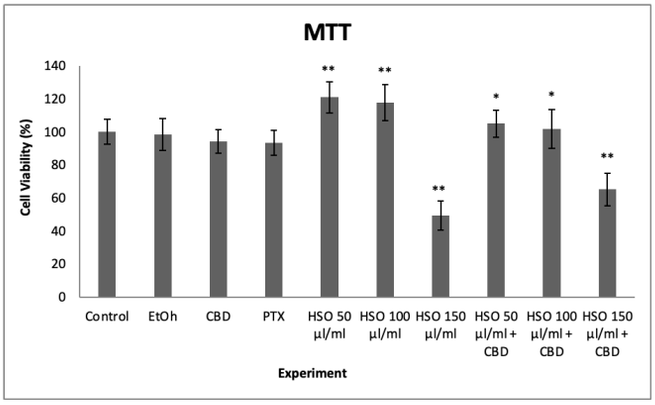

Figure 1. *MTT test results of Narlı hemp seed oil (HSO) and CBD. (* p≤.05, ** p≤.001 compared to the control group.)*

Glutathione (GSH) is an endogenous antioxidant that has the capacity to scavenge reactive oxygen species that cause oxidative stress (12). In our study where Narlı hemp seed oil and CBD were tested, it was determined that hemp seed oil and CBD applications applied at a flat dose on colon cancer cell lines showed antioxidant effects by increasing glutathione levels. It was determined that 150 µl/ml doses of seed oil and CBD-added seed oil did not increase antioxidant activity. It was observed that the antioxidant activities of 50 and 100 µl/ml doses of both applications were statistically significant (Figure 2). While level $p \leq .001$ was significant at the application level of 50 µl/ml of Narlı hemp seed oil, level $p \leq .05$ was significant at the doses of 100 µl/ml of hemp seed oil and 50 and 100 ul/ml of CBD-added hemp seed oil.

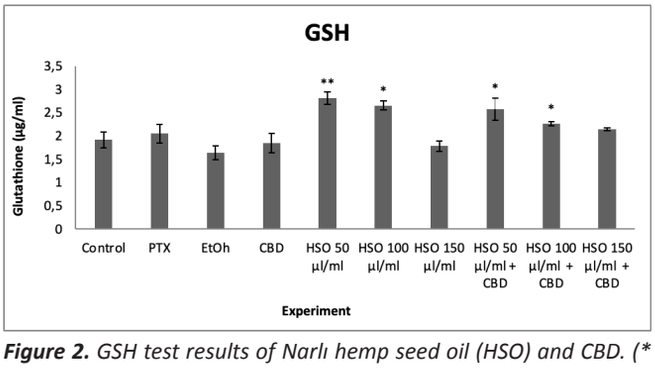

Figure 2. *GSH test results of Narlı hemp seed oil (HSO) and CBD. (* p≤.05, ** p≤.001 compared to the control group.)*

Histone deacetylase (HDAC) activity is a structure that plays a role in the removal of acetyl groups on chromosomes and the regulation of gene expression, allowing DNA to be packaged more tightly by histone proteins (13). There is no previous study examining the effectiveness of CBD and hemp seed oil on HDAC activity in cancer cells. In our study, hemp seed oil and CBD were found to increase HDAC activity to a large extent ($p \leq .05$, $p \leq .001$). It was found that CBD alone had a significant effect on HDAC activity compared to the control group at a $p \leq .001$ level. The highest HDAC activity was observed at a dose of 50 µl/ml of hemp seed oil with added CBD, and this effect was statistically significant ($p \leq .001$). Considering that the application of ethyl alcohol as a solvent also increased HDAC activity, it is thought that the 100 and 150 µl/ml doses of seed oils and the 150 µl/ml dose of CBD-added hemp seed oil may not have significantly affected HDAC activity (Figure 3).

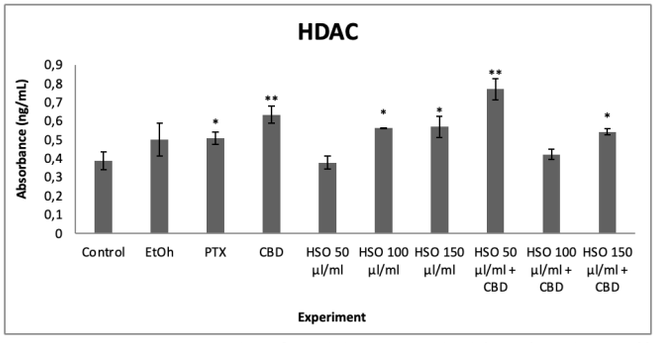

Figure 3. *HDAC test results of Narlı hemp seed oil (HSO) and CBD. (* p≤.05, ** p≤.001 compared to the control group)*

4. DISCUSSION

CBD applied at low doses had no effect on cell viability alone compared to the control group. Similarly, the effect of paclitaxel, an anticancer agent, was such that it reduced cell viability to 93%. It was concluded that the reason for the failure to see the expected anticancer activity in CBD application was the low dose applied. Because in the studies conducted, it was stated that the IC_{50} value of CBD in the colon cancer cell line SW480 started at a dose of 5 µM (14). However, the aim of this study is not to directly test the anticancer activity of CBD but to perform CBD application in a way that will contribute to the therapeutic activity of hemp seed oil.

Our study found that hemp seed oil, at low doses, increased cell viability. Numerous scientific studies have proven that hemp seed oil, with its rich composition, accelerates wound healing. It has been determined that hemp seed oil, especially in low doses, reduces wound size, supports epithelialization and promotes cell proliferation (15). It has been stated that hemp seed oil has a modulatory effect that supports wound healing and proliferation due to the antioxidant phenolic compounds and rich omega 3 and omega 6 potential it contains (16). Besides, our study found that hemp seed oil reduced cancer cell viability at relatively high doses.

There are not many studies that directly examine the effectiveness of hemp seed oil on colon cancer cells. There are more studies in the literature on obtaining seed extracts and determining the effectiveness of this extract. However, because of literature research, it is thought that the reason why a 150 µl/ml dose of hemp seed oil applications reduces cell viability may be that it promotes autophagy. In another study, it was determined that polar oil extract obtained from hemp seeds increased autophagy in HT-29 colon cancer cells within 24 hours and reduced cell development by half. It was stated that hemp seed oil should be applied at 130 µg/ml for this effect to occur (17, 18). These studies are similar to our study in that hemp seed oil in the 130-150 µl/ml dose range reduces proliferation in cancer cells.

In another study conducted on the SW620 colon cancer cell line, the effectiveness of CBD was also examined in addition to different phytocannabinoids. It was determined that CBD, especially at a dose of 6 µM, significantly reduced cell viability. It was stated that this anticancer activity exhibited may be due to the promotion of apoptosis with mild antioxidant activity (19). The reason why the antiproliferative properties of CBD in this study differ from those in our study is related to the CBD dose used. In the relevant literature, CBD was used at a dose twice that used in our study. Despite this, our data suggest that the antiproliferative potential of 3 µM CBD appears to be synergistic when used in combination with hemp seed oil.

It has been reported that the expected increase in glutathione levels was not recorded in fruit fly larvae where hemp seed oil applications were tested (20). In another study where CBD was tested in brain cancer types, it was determined that a 30 µM CBD dose reduced GSH levels compared to the control group. The researchers stated that this effect may be related to the decrease in glutathione levels during autophagy and ferroptosis (21). It is thought that the effectiveness of CBD on cancer cells may have occurred through endoplasmic reticulum stress and may have triggered ferroptosis (iron-mediated necrosis) by accelerating the increase in ferritin levels in the cell. (22).

It has been determined that applications of CBD-added hemp seed oil mediate an increase in antioxidant activity in glutathione pathways in cancer cells and increase the gene expression of the relevant antioxidant enzyme (23). The findings appear to support our research. It is possible that hemp seed oil and CBD may be able to interact with apoptosis independently of the ROS system while exhibiting joint activity, and may be using a different mechanism while promoting the death of cancer cells (23).

Inhibiting HDAC activity for cancer treatment has significant therapeutic potential. To date, four HDAC inhibitors have been approved by the FDA for use in cancer treatment (24). It has been observed that hemp seed oil and CBD do not inhibit HDAC activity in colon cancer cells. The mechanism of action of HDAC inhibitors in cancer cells is generally stated to occur through apoptosis via the p53 protein or oxidative stress by promoting the production of reactive oxygen species (25).

HDAC inhibitors suppress oxidative stress in the cell via the endoplasmic reticulum. This situation is classified among the harmful effects of HDAC inhibitors (26). The reason for the decrease in cancer cell viability despite the increase in HDAC levels observed in our study is that cell death characterized by increased oxidative stress via the endoplasmic reticulum (ER) can be promoted (27). The data obtained revealed an illuminating difference related to the mechanism of action of hemp seed oil and CBD.

Studies have shown that CBD promotes cell death via paraptosis. Paraptosis is a relatively less researched form of the caspase-independent cell death pathway, characterized by widespread cytoplasmic vacuolation and associated with ER swelling. It has been reported that CBD increases gene expressions characterized by paraptosis, and thus may exhibit cytotoxic activity in cancer cells (28). In this project study, it is thought that hemp seed oil and CBD may exhibit their effectiveness via ER stress, and thus may have a cytotoxic effect in colon cancer cells.

Hemp seed oil contains high levels of saturated and unsaturated fatty acids (Table 1). It was thought that this rich fatty acid range could mediate the inhibition of histone deacetylase activity. Because studies have determined that fatty acids have significant activities in HDAC inhibition (29). However, in our study, it was observed that hemp seed oil did not have the expected effect on HDAC (Figure 3). It is thought that the reason for this situation may be the activation of SIRT6 sirtuins, which exhibit protein deacetylase activity, by linoleic and linolenic fatty acids (30).

Additionally, the antiproliferative effect of hemp seed oil at high concentrations suggests that it may have a cytotoxic effect on healthy cells. In vivo studies have demonstrated that hemp seed and oil are effective in improving atherogenic parameters (31). Therefore, further in vivo and clinical studies are needed to understand the potential cytotoxic effect profile. This study did not conduct a large-scale dose study to determine the therapeutic dose range. Therefore, although an antiproliferative effect was observed at the highest dose of 150 µl/ml in the study, the dose at which this effect persists is unknown. Therefore, further research should determine the dose range for potential cytotoxic effects, and in vivo studies should clarify the tissue, organ, and organismal implications of this effect. Therefore, future studies are needed to determine the safe and appropriate therapeutic dose range.

5. CONCLUSION

Having a wide therapeutic effect in terms of medicine has made hemp and its phytocannabinoids popular in drug research. In our research, the effect of the seed oil of the native hemp variety Narlı and the phytocannabinoid cannabidiol (CBD) in its content on colon cancer was examined. According to the findings, it was determined that 150 µl/ml hemp seed oil application and 3 µM CBD application doses added to 150 µl/ml seed oil showed successful effects in reducing colon cancer cell viability. In the glutathione test, which is an important criterion in measuring antioxidant potential, it was determined that the doses showing cytotoxic effect exhibited antioxidant activity. In addition, it was understood that histone deacetylase activity increased at the same doses. When all the findings were evaluated in the light of literature data, it was thought that hemp seed oil and CBD may have exhibited an effect not by oxidative stress and HDAC inhibition, but by increasing the intracellular stress level via the endoplasmic reticulum. Hemp seed oil and CBD may have driven cells to paraptosis by inducing ER stress. This highlights the need for future studies to elucidate the relationship between hemp seed oil and CBD and the mechanisms involved in paraptosis and ER stress.

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Design of the study: ÖB
Acquisition of data for the study: ÖB, MSE
Analysis of data for the study: ÖB, MSE, AKA
Interpretation of data for the study: ÖB, MSE, AKA
Drafting the manuscript: ÖB, BT
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