TÜRKİYE CUMHURİYETİ
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
BIOENGINEERING DEPARTMENT

INVESTIGATION OF CYTOTOXIC ACTIVITY OF BETA CAROTENE ON GLIOBLASTOMA CELLS

AYTEN BURCU EFE ÖZKAN

M.Sc.

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ADANA, 2024

DECLARATION OF CONFORMITY

In this thesis study, which was prepared following the thesis writing rules of Adana Alparslan Türkeş Science and Technology University Institute of Graduate School, I declare that I provide all the information, documents, evaluations and results in accordance with scientific ethics and moral codes without resorting to any means or assistance that would be contrary to scientific ethics and traditions. I also declare that I refer to all of the articles I used in this study with appropriate references and accept all moral and legal consequences if a situation is found contrary to my statement regarding my work.

..../..../20..

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Ayten Burcu EFE ÖZKAN

ABSTRACT

INVESTIGATION OF CYTOTOXIC ACTIVITY OF BETA CAROTENE ON GLIOBLASTOMA CELLS

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M.Sc., Department of Bioengineering

Supervisor: Assoc. Prof. Dilek GÖKTÜRK

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Glioblastoma is a type of cancer that occurs in the central nervous system. It is highly lethal, and spreads rapidly. The survival time of patients varies depending on the effectiveness of the treatment methods, and the extent of the disease, but on average, it is around 15 months. The standard treatment protocol currently includes surgical intervention, radiation therapy and chemotherapy. Obstacles in treatment include difficulty in completely removing tumours because of their placement in sensitive areas of the brain and close proximity to vital structures. Other challenges are the blood-brain barrier preventing drugs from reaching brain tissue, and the potential of glioblastoma cells to develop resistance to treatment.

Because of the side effects of chemotherapeutic drugs (especially Temozolomide) used in glioblastoma treatment and the ability of tumor cells to develop resistance to these drugs, alternative or supportive treatment methods are being researched.

β-carotene is a natural antioxidant carotenoid. The impact of β-carotene on glioblastoma cells was examined in this study. β-carotene, etoposide, and temozolomide were applied to U87MG glioblastoma cells, and the survival of the glioblastoma cells was assessed. The results indicated that β -carotene alone significantly reduced the glioblastoma cell viability. When combined with etoposide, it exhibited a synergistic effect, decreasing the number of glioblastoma cells and their colony formation potential. However, it was found that β-carotene had an antagonistic relationship with temozolomide when used together, and that glioblastoma cell viability and colony-forming potential were greater under these conditions than when etoposide and β-carotene were administered alone.

Keywords: glioblastoma, β-Carotene, temozolomid, etoposid, carotenoids

ÖZET

BETA KAROTENİN GLİOBLASTOMA HÜCRELERİ ÜZERİNDEKİ SİTOTOKSİK AKTİVİTESİNİN ARAŞTIRILMASI

Ayten Burcu EFE ÖZKAN

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Glioblastoma merkezi sinir sisteminde görülen, ölümcül ve hızlı yayılan bir kanser türüdür. Hastaların sağ kalım süresi tedavi yöntemlerinin etkinliğine ve hastalığın yayılma düzeyine bağlı olarak değişebilmekle birlikte, ortalama 15 aydır. Günümüzde mevcut tedavi protokolü, cerrahi müdahale, ışın tedavisi ve kemoterapiyi içerir. Tedavide karşılaşılan güçlükler, tümörlerin beyinde hassas bölgelere ve önemli yapıların yakınına yerleşebilmesi nedeniyle tamamen çıkarılmasının zorluğu, ilaçların beyin dokusuna ulaşmasını engelleyen kan-beyin bariyeri ve glioblastoma hücrelerinin tedaviye direnç geliştirme potansiyeli olarak özetlenebilir.

Glioblastoma tedavisinde kullanılan kemoterapötik ilaçların yan etkileri ve tümör hücrelerinin bu ilaçlara direnç geliştirebilmesi sebebiyle, alternatif ya da destekleyici tedavi yöntemleri araştırılmaktadır.

Bu çalışmada doğal bir antioksidan olarak karotenoidlerden β-karotenin, glioblastoma hücreleri üzerinde etkisi araştırılmıştır. U87MG glioblastoma hücreleri etoposid, temozolomid ve β-karoten ile muamele edilerek hücrelerin sağ kalımı ölçülmüş ve β-karotenin tek başına glioblastoma hücrelerinin büyük ölçüde ölümüne sebep olduğu, etoposidle kombinasyonunda sinerjistik etki göstererek glioblastoma hücrelerin sayısını azalttığı, koloni oluşturma potansiyelini düşürdüğü sonucuna ulaşılmıştır. Bununla birlikte çalışmada β-karotenin temozolomid ile birlikte kullanıldığında antagonistik ilişkiye sahip olduğu ve bu koşullarda glioblastoma hücre canlılığının ve koloni oluşturma potansiyelinin, etoposit ve β-karotenin tek başına uygulandığı koşullara göre daha fazla olduğu tespit edilmiştir.

Anahtar Kelimeler: glioblastoma, β-karoten, temozolomid, etoposid, karoteoidler

To my precious family

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LIST OF ABBREVIATIONS

GBM	: Glioblastoma
GSC	: Glioblastoma Stem Cells
TMZ	: Temozolomid
PBS	: Phosphate Buffered Saline
DMEM	: Dulbecco's Modified Eagle's Medium
FBS	: Fetal Bovine Serum
IC50	: Half – Maximal Inhibitory Concentration
MTT	: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
SFE	: Super Fluid Extraction
β-carotene	: Beta Carotene
TR	: TMZ Resistant
TERT	: Telomerase Reverse Transcriptse gene
EGFR	: Epidermal Growth Factor Receptor
MMP-10	: Matrix Metalloproteinase
HNC	: Head and Neck Cancer
DMSO	: Dimethyl Sulfoxide
MGMT	: O-6-methylguanine-DNA methyltransferase
CNS	: Central Nervous System
WHO	: World Health Organization

LIST OF SYMBOLS

mL : Milliliter

μL : Microliter

nM : Nanometer

rpm : Revolutions per Minute

μM : Micromolar

mM : Millimolar

1. INTRODUCTION

Glioblastoma (GBM) is classified as grade IV in the World Health Organization's (WHO's) glioma classification (Louis et al., 2021) and GBMs are the most prevalent tumors of the central nervous system. They grow quickly and often lead to patient death within a short period (Cao et al., 2021). Traditional treatment involves surgical resection, radiation, and chemotherapy. Issues with these conventional methods include incomplete tumor removal due to the difficulty in defining tumor borders at the microscopic level, side effects from chemotherapeutic drugs, and the development of drug resistance in tumor-initiating cells (Wu et al., 2021). For these reasons, alternative treatment methods are being investigated. Herbs and plant compounds have long been thought to prevent and treat many diseases. One study investigating the effect of herbal teas in treating GBM used naturally grown herbal mixtures (Phytoteraphy) in addition to standard medical treatment, resulting in significant improvement in the condition of GBM patients who participated in the trial (Trogrlić et al., 2018).

Carotenoids are compounds present in fruits, vegetables, some seafood, microalgae and fungi. Since animal organisms cannot synthesize these compounds, they must be obtained externally through diet. Carotenoids are categorized into two subgroups: carotenes, which lack oxygen, and xanthophylls, which contain oxygen. Examples of carotenes include β-carotene, lycopene, and torulene. β-carotene, an oxygen-free carotenoid, has the.chemical formula C40H56. It consists of a polyene chain with 9 conjugated double bonds connecting two β-ionone rings. β-carotene, a precursor of vitamin A, is fat-soluble and prone to oxidation when exposed to air, light and heat (Rapoport et al., 2021; Saini et al., 2022).

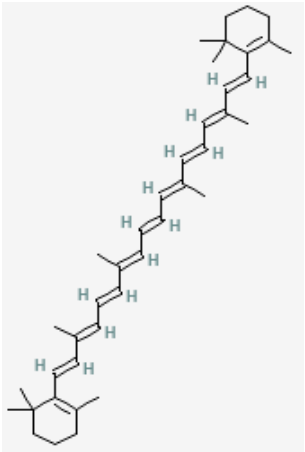

Figure 1.1. Chemical structure of β-carotene (National Center of Biotechnology Information, 2024)

β-carotene is present in carrots, pumpkins and green leafy vegetables. Microalgae like *Dunaliella salina* and the fungus *Blakeslea trispora* are the other source of β-carotene. Research has focused on β-carotene's potential anti-cancer properties because of its antioxidant characteristics, similar to other carotenoids (Saini et al., 2022). β-carotene is thought to be a protector for diseases because of antioxidant properties. Some studies have focused on the anti-cancer effect of β-carotene on gastric cancer, colon cancer, lung cancer etc. it may reduce the risk of stomach cancer. However more research is needed to confirm this, studies have shown that it reduced the risk of gastric cancer. On the other hand, the dietary intake of β-carotene is inconclusive on lung cancer. In a colon cancer study, β-carotene was found to decrease the number and size of tumors. Studies are ongoing to explore β-carotene's protective effects against cancers (Lee et al., 2022; Chen et al., 2021; Kordiak et al., 2022).

This study investigated, the therapeutic potential of β-carotene in the GBM, a type of brain cancer known with its poor prognosis and high fatality rates.

2. THEORETICAL FOUNDATIONS AND LITERATURE REVIEW

2.1. An Overview Of Cancer Diseases Gliomas And Glioblastoma

A statistical study conducted in 2022 by International Agency For Research on Cancer (IARC) that covered 36 types of cancer across 185 countries, there were 20.000.000 new cancer cases globally, with approximately half of these resulting in death due to cancer. It is thought that cancer cases will reach 35.000.000 in 2050. The most common type of cancer is lung cancer, followed by breast cancer in women. This situation poses a significant indicator globally, both in terms of public health, and economically (Bray et al., 2024). The annual global incidence of gliomas is approximately 6 per million, with men having an incidence rate approximately 1.6 times higher than that of women (Weller et al. 2021).

The general nomenclature of central nervous system (CNS) tumors originating from non-neoplastic glial (precursor) cells is 'Glioma' , and the most aggressive and lethal subtype of malignant gliomas is GBM (Kristensen et al., 2019; Ren et al.,2021).

The World Health Organization's (WHO's) classification of central nervous system (CNS) tumors has significantly evolved with improvement in molecular characterization, and the biomarkers' identification. IDH wild type diffuse astrocytic glioma is classified as GBM, IDH-wild type, WHO grade 4 by the latest classification updated with recommendations from the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT). EGFR amplification, which promotes cell proliferation, invasion, and apoptosis resistance; TERT promoter mutation presence, enhancing cell longevity and proliferation; and cytogenetic signatures such as +7 / -10, indicating chromosomal alterations are the key biomarkers for IDH Wild Type GBM. O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation is also crucial as it signifies the potential benefit of alkylating chemotherapy in IDH wild-type GBM patients (Louis et al., 2020; Louis et al., 2021; Weller et al., 2021).

Genomic analysis of GBM began in 2008, focusing on 20,661 protein-coding genes across 22 human GBM samples. The genetic characteristics of these GBMs were investigated, and as a result, genetic alterations were detected in IDH1 gene located on chromosome 2q33, which plays an important role in cellular oxidative damage control by catalyzing isocitrate to alpha-

ketoglutarate and producing NADPH. Mutant IDH1 produces 2-hydroxyglutarate (2HG) when its metabolism is impaired. This situation is also seen in 2-hydroxyglutaric aciduria disease. 2-hydroxiglutarate production is connected with brain tumor risk (Parsons et al., 2008).

2HG production targeted by therapeutic strategies may benefit the GBM patients. Additionally, GBM stem cells (GSCs), constituting about 1% of tumor cells, are resistant to radiotherapy, suggesting a need for understanding molecular pathways in cancer stem cells (CSC) for effective treatment design. There is research on the inhibition of GBM cell motility achieved through membrane signaling proteins such as aquaporins and glutamate receptors. Factors like ionizing radiation exposure, family brain tumor history, and certain health conditions influence GBM risk (Dang et al., 2009; Sasaki et al., 2012; Jin et al., 2013; Yin et al., 2020; Mirzaei et al., 2022).

Various approaches have been put forward to limit the invasive nature of GBM cells. Targeting membrane signaling proteins that facilitate high motility is one of these approaches. Studies have shown that pharmacological interventions on aquaporin water channels, glutamate receptors, and ion channels of GBM cells can potentially restrict tumor cell movement during treatment (Varricchio et al., 2023).

Studies of atomic bomb survivors have shown that moderate to high doses of ionizing radiation increase the risk of brain tumors. Additionally, a family history of brain tumors elevates the cancer risk among adults with glioma. Conversely, conditions like allergies or eczema are associated with a 30% reduction in brain tumor risk. Interestingly, genotypes associated with higher risk of asthma have shown a decreased susceptibility to GBM (Melhem et al., 2022; Khabibov et al., 2022).

Temozolomide

Temozolomide, which is particularly used in the treatment of brain tumors such as GBM, is a chemotherapy drug classified as an alkylating agent. This drug has the ability to cross the blood-brain barrier and induces apoptosis (programmed cell death) in tumor cells by adding a methyl group to purines and pyrimidines in the DNA structure and acts through a mechanism that can allow tumor cells to evade cytotoxic effects and develop resistance, facilitated by the DNA repair system called MGMT (O-6-methylguanine-DNA methyltransferase) (Karachi et al., 2018). In GBM, the biomarker MGMT first came into clinical use (Kristensen et al., 2019).

In 2005, a clinical trial demonstrated that administering temozolomide alongside radiotherapy resulted in a significant survival benefit for patients. The study included 573 newly diagnosed GBM patients from 85 centers, with a median age of 56 years. 287 of these patients, received a combination of radiotherapy and temozolomide, while 286 received radiotherapy alone. The results showed that combining chemotherapy with radiotherapy significantly prolonged survival in newly diagnosed GBM patients. The addition of chemotherapy to treatment reduced the risk of death in newly diagnosed patients by 37% (Stupp et al., 2005). Following this study, the FDA approved the use of temozolomide in combination with radiotherapy for standard clinical practice.

Hematological toxicity is the most common side effect of temozolomide treatment. Morover, the infiltrative nature of tumor cells has been found to contribute to repetitions originating from GBM stem cells (GSCs) that survive in the area following surgical resection have been observed to develop temozolomide resistance (Wu et al.,2021).

It was reported that the slncRNA, known as XLOC013218, overexpression significantly increases temozolomide resistance, preventes cell death and promotes cell proliferation and XLOC013218 was identified as a potential target for overcoming temozolomide resistance in GBM (Zhou et al., 2022).

The temozolomide resistance of GBM cells has led researchers to explore alternative drugs. Studies have showed that azole, and benzimidazole compounds have potential anti-tumor effects on GBM cells (Torres et al., 2015). Specifically, benzimidazoles such as Flubendazole, Fenbendazole, and Mebendazole have been found effectively inhibit the growth ,and spread of U87MG GBM cells. Additionally, comparision of the effects of temozolomide and flubendazole on tumor shrinkage, flubendazole has provided similar benefits to temozolomide was shown. This research findings suggest that alternative drugs, like benzimidazoles, may offer potential therapeutic options for temozolomide resistant patients. Additionally, the combination of temozolomide with benzimidazoles is highlighting a promising treatment strategy for patients exhibiting low responsiveness to temozolomide (Ren et al., 2021).

2.1.1. Etoposide

Etoposide which is a chemotherapy drug works by disrupting the function of topoisomerase II whichis an enzyme critical for DNA repair and replication, leading to DNA strand breaks and consequently causing cell death in rapidly dividing cancer cells. It is used to treat various

cancers, including lymphoma, testicular cancer, lung cancer, and brain tumors such as GBM (Wei et al., 2021; Franceschi et al., 2004).

A study towards the end of 1900s demonstrated that etoposide (VP16-213) effectively penetrated the cerebro spinal fluid following high-dose intravenous administration, highlighting its potential for treating central nervous system metastases (Postmus et al., 1984). Etoposide enhance survival rates, and address recurrent therapy-resistant tumors, researchers have explored combination therapies using carboplatin, an alkylating agent capable of crossing the blood-brain barrier , and effective against various solid tumors, similar to etoposide (Franceschi et al., 2004).

Several clinical studies have been conducted in order to explore ways to enhance the etoposide effectiveness. These studies have focused on describing substances that work synergistically with etoposide, besides the development of etoposide-related nanoparticles (Engelhard et al., 2020; Wei et al., 2021; Franceschi et al., 2004). The usage of a rotating magnet to guide magnetic etoposide nanoparticles in delivering etoposide externally tested in a recent study. It was found that this approach was effective in targeting cancer cells found in the cerebrospinal fluid, especially in the early stages of the disease (Engelhard et al., 2020).

2.1.2. Challenges of Conventional Treatment Methods

Conventional treatment strategies for GBM involve surgical resection of the tumor followed by chemoradiotherapy. However, several challenges block effective treatment outcomes. One of the important difficulty is that the tumors can’t be removed completely due to their infiltrative nature, where tumor cells spread into surrounding tissues resembling finger-like projections. This infiltrative behavior causes incomplete resection of cancerous cells during surgery (Wu et al., 2021).

Another major problem is the development of resistance by GBM cells to both radiotherapy and chemotherapeutic drugs like temozolomide, which are generally administered post-surgery. This resistance is often attributed to GBM stem cells that survive initial treatments and contribute to the recurrence of tumors (Mattei et al., 2021; Yin et al., 2020). Morover, the blood-brain barrier limits the effectiveness of many chemotherapeutic substances, preventing them from reaching therapeutic concentrations within the brain (Altshuler et al., 2020).

Inspite of advancements, current treatment approaches have not significantly improved overall survival rates for GBM patients. The exploration of new methods and therapeutic strategies aimed to enhance survival times, and outcomes in this challenging disease continues (Wu et al., 2021; Altshuler et al., 2020; Mattei et al., 2021; Yin et al., 2020).

2.1.3. Recent Advances in GBM Therapy

To enhance the benefits of conventional treatment methods and overcome treatment challenges, new combined and alternative treatment strategies are being evaluated. In some of these studies, the interaction of drugs previously used for different disorders with temozolomide (TMZ) and their potential effects on treating GBM is investigated by researchers. In a study by Yadav et al. (2022), the impact of "stiripentol", a medication administered to patients with Dravet syndrome (an epilepsy form), on TMZ-resistant (TR) U87MG - GBM cells was investigated. The researchers indicated that stiripentol exhibited a synergistic effect with temozolomide and also effectively reduced cell viability, proliferation, cloning ability, and cell migration in TR GBM cells. Moreover, a study examining how Celastrol, an herbal medicine used for diseases such as rheumatoid arthritis and psoriasis, interacts with temozolomide in GBM treatment, found that Celastrol inhibited vasculogenic mimicry, and angiogenesis by regulating the PI3K/Akt/mTOR pathway, which is closely related with angiogenesis, and the vasculogenic mimicry formation (Zhu et al., 2020).

Suppressing the invasive character of the tumor through membrane signaling mechanisms (pharmacological inhibition such as aquaporin water channels, glutamate receptors, and ion channels) affecting the mobility of GBM cells was also reported (Varricchio et al., 2023).

With advancements in nanotechnology, biodegradable nanoparticles are utilized for various applications increasingly. The cisplatin-alginate nanocomplex related to GBM treatment, induced apoptosis in U87MG GBM cells by enhancing their radiosensitivity (Mousavi et al., 2023). Furthermore, near-infrared fluorescence-guided resection of GBM using a biodegradable nanoimaging agent, polymalic acid chlorotoxin nanoconjugate, was reported (Patel et al.,2019).

Experiments have been conducted on hypericin-mediated photodynamic therapy and photobiomodulation aiming to increase treatment effectiveness and reduce damage to surrounding healthy tissues (Pevna et al., 2021). Studies on immunotherapy continue to

strengthen the immune system suppressed by chemotherapeutic drugs (Karachi et al., 2018; Hotchkiss et al., 2021).

Emerging methods such as immunotherapy, synthetic molecules, natural compounds, and inhibition of GBM stem cells are anticipated to be effective in the GBM management (Rodríguez-Camacho et al., 2022).

2.1.4. Alternative Compounds Previously Judged for GBM Treatment

Currently, there is remarkable interest in exploring the potential anticancer properties of natural compounds in the treatment of GBM and other nervous system cancers. In research, substances like phospholipase A2 and mellitin present in honey bee venom, which have demonstrated effectiveness against cancer cells were being actively investigated. Honey bee venom is especially promising due to its ability to penetrate the blood-brain barrier, making it an important candidate for treating the central nervous system disorders (Malek et al., 2022).

Curcumin is a compound known for its anticancer properties. However, curcumin has obstacles in crossing the blood-brain barrier effectively. To overcome blood-brain barrier limitation, researchers have investigated packaging curcumin with dendrimers, which has enabled it to penetrate the blood-brain barrier. Studies have demonstrated that this approach enhances curcumin's effectiveness in inducing apoptosis in GBM cells (Gallien et al., 2021).

One interesting substance for GBM treatment is the venom of a scorpion species *Leiurus quinquestriatus*. Researchers have identified that chlorotoxin, present in this venom, binds to chloride ion channels in GBM cells. This discovery holds promise for targeted therapies in the treatment of GBM (Varricchio et al., 2023; Cohen et al., 2018).

Studies are also investigating the anticancer properties of plants used in Chinese traditional medicine. Wedelia chinensis, a herbal remedy from Chinese alternative medicine, has demonstrated effectiveness against various types of cancer. Studies indicate that it induces apoptosis in GBM cells (Chen et al., 2021).

2.2. Carotenoids and β-carotene

The total number of natural carotenoids exceeds 1000 and they are classified into two subgroups: carotenes and xanthophylls. Out of these, approximately 50 are provitamin A. Carotenoids are present and synthesized in all photoautotrophic organisms. Since animals

cannot synthesize carotenoids, they must obtain them from their diet. Carotenes are a group of carotenoids that lack oxygen in their chemical structure, while xanthophylls are the group that contains oxygen. The chemical structures of some common carotenoids are depicted in Figure 2.1.

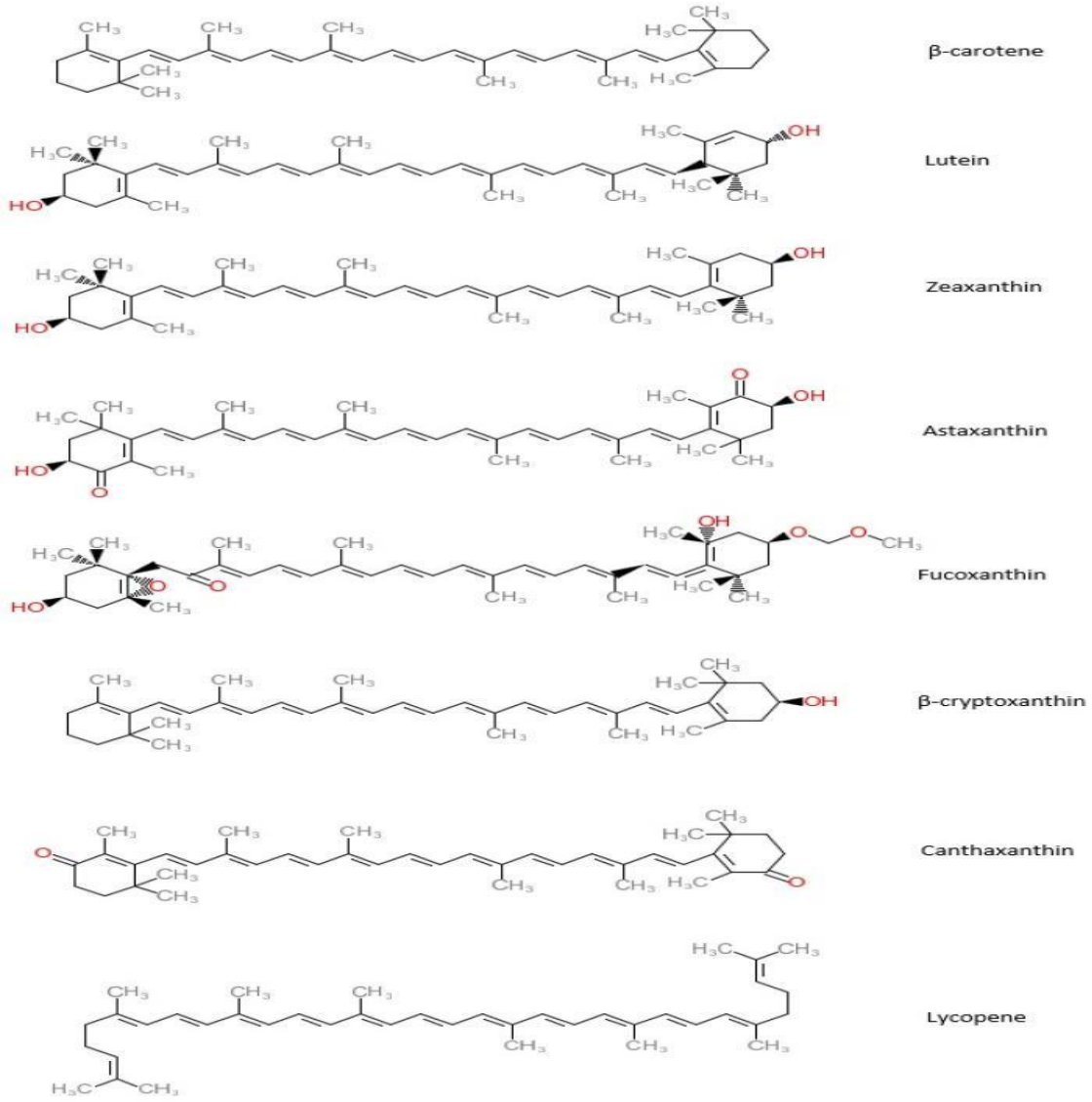

Figure 2.1. Chemical structures of some carotenoids (Brewczyński et al.,2021)

Research on carotenoids focuses on their natural production, encapsulation, and health benefits. Clinical and epidemiological studies have demonstrated that consuming carotenoid-rich fruits and vegetables can extend lifespan. Carotenoids are believed to be effective in preventing and treating cancer due to their antioxidant properties, and numerous studies have been conducted on this subject. Carotenoids are susceptible to decomposition when exposed to heat, light, oxygen, and catalysts. Carotenes, in particular, are prone to oxidation, hydrolysis,

isomerization, and degradation. Therefore, it is recommended to use carotenoids that are free from peroxides in biological studies (Wang et al., 2022; Saini et al., 2022).

Carotene species, β-carotene, lycopene and torulene are unsaturated hydrocarbon structures consisting of 40 carbons and exhibit red, yellow, and orange colors. In plants, carotenes are stored in plastids and play roles such as detoxification, photoprotection, and the synthesis of phytohormones (Maoka, 2020; Saini et al., 2022; Wang et al., 2022).

β-carotene, classified under carotenes, is the most abundant provitamin A. Dietary β-carotene is absorbed in the duodenum of the small intestine. Its antioxidant effect has been proven, leading to the promising idea that it can prevent many chronic diseases, most importantly cancer. However, study results did not show a significant relationship between β-carotene supplementation and cancer prevention, despite highlighting its antioxidant activity (e.g., lung cancer, colorectal cancer, head and neck cancers) (Kordiak et al., 2022; Brewczyński et al., 2021; Jiang et al., 2022). While most studies emphasize the antioxidant properties of β-carotene, it also has pro-oxidant properties. β-carotene can shift from antioxidant to pro-oxidant behavior under changing oxygen concentrations, leading to inconsistent results (Black et al., 2020).

For these reasons, the extraction of β-carotene and other carotenoids aims for maximum efficiency and long-term preservation to maintain their antioxidant properties (Patel et al., 2019; Parniakov et al., 2015; Wang et al., 2023).

2.2.1. Studies on the Effect of β-Carotene in Various Cancer Types

β-carotene is a precursor of Vitamin A (retinol) with antioxidant effects. It exhibits anticancer properties by reducing the production of reactive oxygen species mediated by NADPH oxidase. Retinol is converted to retinoic acid in cells. All-trans retinoic acid increases apoptosis by blocking the cell cycle in cancer cells (Chen et al., 2021).

Carotenoids serve as precursors for retinoids. β-carotene is the precursor of provitamin A, and retinoids are derived from carotenoids by carotenoid-cutting dioxygenases (Moise et al., 2022; Milani et al.,2017). The opinion that retinoids can impact cancers has been long-standing. This idea is based on the consept that carcinogenesis is a multi-step process that tranforms normal cells into malignant tumors. Hereby, it has been suggested that retinoids could potentially be used as anti-cancer agents at any stage (Dragnev et al., 2000).

The connection between carotenes and cancer has been extensively researched, especialy in combination with consumption of fruits and vegetables. These studies have shown that β-carotene has anti-cancer properties, mainly because of its antioxidant activity (Chen et al., 2021; Brewczyński et al., 2021; Kordiak et al., 2022). β-carotene , a precursor of Vitamin A (retinol) with antioxidant properties, demonstrates anti-cancer effects by reducing the production of reactive oxygen species mediated by NADPH oxidase (Chen et al., 2021).

In a study conducted by Lee et al. in 2022, mice injected with CD133+, CD44+, HCT116 colorectal cancer cells, it was found that β-carotene reduced tumor size and number, and delayed tumor onset in mice. Vemurafenib (PLX4032), an approved targeted therapy for melanoma —a type of skin cancer with a poor prognosis can face resistance from melanoma cells. A study investigated the effects of Coenzyme Q10 and β-carotene, both alone and in combination with vemurafenib, on the vemurafenib-resistant melanoma cell line A2058 and the vemurafenib-sensitive melanoma cell line SK-MEL-28. The results showed that β-carotene suppressed the anti-cancer effects of vemurafenib, while coenzyme Q10 exhibited a synergistic effect with the drug (Hu et al., 2021). Additionally, a 2022 study on drug combinations, found that Se-methylselenocysteine, D-alpha-tocopheryl succinate, β-carotene, and L-lysine enhanced the therapeutic efficacy of breast cancer chemotherapy drugs (Cheng et al., 2022).

In a study on ovarian cancer stem cells, a combination of retinoid 9cUAB130 and carboplatin was proved to be the most effective. This combination determined more effective than carboplatin alone, leading to reduced cell viability, decreased CSC marker expression, and tumorigenesis inhibition (Whitworth et al., 2012).

Retinol, derived from its precursor β-carotene, is further converted into retinoic acid within cells. All-trans retinoic acid promotes apoptosis by blocking cell cycle in gastric cancer cells (Chen et al., 2021). All-trans retinoic asid also inhibits tumor growth by targeting ALDH1 activity, a marker of malignant cancer stem cells, including those in ovarian cancer. Morover, retinoids enhance antitumor immunotherapy by increasing the expression of interleukin-2 receptors, which play a key role in activation of the immune system to combat cancer. Retinoic acid regulates signaling pathways by exerting inhibitory effects on Telomerase Reverse Transcriptase Gene (TERT), and Epidermal Growth Factor Receptor (EGFR) (Wierzbowska et al., 2024).

A gram-negative bacterium Helicobacter pylori, is linked to the gastric cancer development. In gastric astrocytoma cells, the expression of Matrix Metalloproteinase-10 (MMP-10), which is derived from H. Pylori is elevated (Hardbower et al., 2014). A study researching the effects of β-carotene on gastric astrocytoma cells found that MMP-10 expression is inhibited, and cell invasion is reduced by β-carotene (Bae et al., 2021).

A study involving 540 head and neck cancer (HNC) patients undergoing radiotherapy, indicated that dietary intake of β-carotene was connected to reduced negative effects of thereatment. Additionally, patients with higher plasma β-carotene levels experienced less frequent local recurrence (Brewczyński et al., 2021). In contrast, another study on β-carotene supplementation on lung cancer suggested an increased risk of developing the disease (Kordiak et al., 2022)

2.2.2. β-Carotene Characteristics and Delicate Attributes

β-carotene is highly sensitive to degradation when exposed to oxygen, light, and elevated temperatures, so maintaining a light-free environment, minimizing oxygen exposure, and keeping temperatures below 70 °C are essential to preserve β-carotene during research and studies.

Various efforts are underway to optimize the extraction of β-carotene for maximum efficiency. While organic solvents have traditionally been employed for extraction, CO_2-based supercritical fluid extraction (SFE) method has emerged as a promising alternative. SFE method successfully extracted β-carotene with high efficiency in a study utilizing ripe bitter melon pericarp. The optimal conditions for maximizing β-carotene content of extract were determined to be 393.32 bar pressure, a flow rate of 36.98 mL/min, and a temperature of 69.15 °C (Patel et al., 2019).

Numerous studies focused on maximization of the extraction of carotenoids while maintaining their antioxidant properties. β-carotene has lipophilic characteristics. Historically, dimethyl sulfoxide (DMSO) has proven effective as a solvent for lipophilic pigments (Parniakov et al., 2015). A 2023 study on the extraction of carotenoids and other valuable compounds from microalgae, the combined use of DMSO and Presured Liquid Extraction (PLE) at concentrations exceeding 50% was successful in obtaining carotenoids while preserving their antioxidant properties (Wang et al., 2023).

Excessive intake of the fat-soluble vitamin A can cause hypervitaminosis because excess of them is stored in tissues and can cause significant side effects. β-carotene is a precursor of vitamin A. This situation should be taken into consideration when using of β-carotene for therapeutic purposes (Chen et al., 2023).

3. MATERIALS AND METHODS

This study aimed to evaluate the cytotoxic effects of β-carotene on GBM cellsThe workflow of the methods utilized in the study was shown in Figure 3.1.

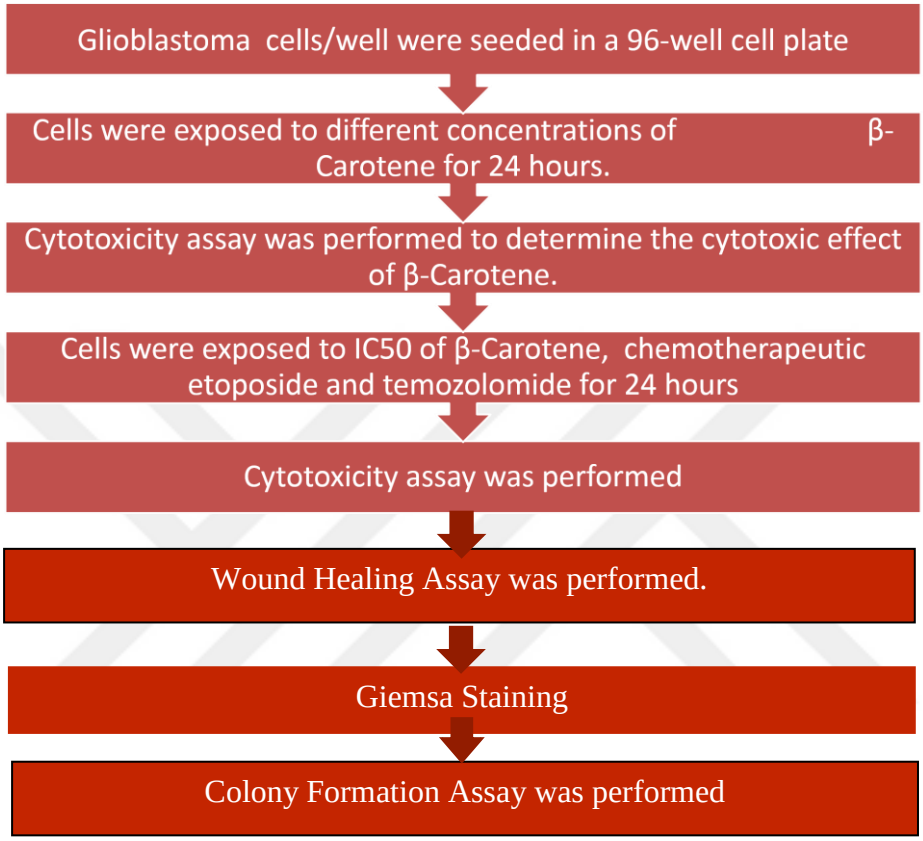

Figure 3.1. Workflow of the Methods

3.1. Cell Thawing and Cell Culture Conditions

U87MG (ATCC HTB-14 TM) GBM cells frozen at –80 °C in a solution of 10% DMSO and FBS were rapidly thawed in a 37 °C water bath to minimize cell loss. Following thawing, the cells were centrifuged in nutrient medium at 1000 rpm for 5 minutes, and the supernatant was discarded to remove freezing substances. The resulting cell pellet was then resuspended in growth medium. The cells were incubated in DMEM supplemented with 10% FBS, 1% L-Glutamine, and 1% penicillin-streptomycin at 37 °C with 5% CO_2 to promote cellular proliferation. After incubation, the medium was aspirated, and cells were washed with PBS. Trypsinization was subsequently performed, with cells remaining in the incubator for 2-3 minutes. A 10 µL aliquot was taken and placed in a Neubauer chamber for cell counting under

a microscope. The average number of viable cells per mL was determined, and 15,000 cells were then seeded into each well of 24-well plates.

3.2. Cell Counting Methodology

Cell counting is performed by using a Neubauer chamber. Initially cell samples were prepared in suspension and mixed thoroughly. A coverslip was then placed over the chamber, and the cell suspension was pipetted into it. Cells were counted under a light microscope (Leica Biosystems, Wetzlar,Germany).

The average number of cells was determined by counting cells in the four corner squares and the central area of the Neubauer slide, followed by calculating the overall average. The number of cells in 1 mL was calculated using the given formula:

Number of cells in 1mL= Average of cells counted x Dilution factor x 10^4

Based on this cell concentration, the volume of cell suspension required per well was calculated to achieve the desired number of cells for growth.

3.3. Determination of IC50 Values for β-Carotene, Etoposide, and Temozolomide

The IC50 values of β-carotene, etoposide, and temozolomide on GBM cells were determined by exposing the cells to various concentrations of each compound individually for 24 hours. Cell viability was then evaluated using of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test to establish the concentration at which each substance exhibited a 50% inhibitory effect on cell growth.

3.4. MTT Cell Viability Assay

To determine cell viability, the MTT method, which relies on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by the mitochondria of living cells, was used.

A 5 mg/mL MTT solution was diluted 1/20 in PBS. β-carotene, etoposide, temozolomide and their combinations were administered to the cells. After incubation, the medium was removed from the wells and the cells were washed with PBS. Then, 500 mL of MTT was added to each well. Following a 1-hour incubation, the MTT solution was removed from the wells and 1 mL of isopropanol was added to solubilize the formazan crystals.. A shaker (Everlast Rocker 147)

was used to mix the samples homogeneously (Figure 3.2). After 30 minutes of incubation, the samples were transferred to cuvettes and analyzed using a spectrophotometer (Spectrostar Nano, BMG Labtech, Ortenberg, Germany) at a wavelength of 570 nm.

Figure 3.2. Dissolving samples. Samples were shaken on Everlast Rocker 247 after treatment with isopropanol.

3.5. Scratch Assay

To evaluate the effects of β-carotene, etoposide, temozolomide, and their combinations on GBM cell migration and invasiveness, scratch assay was performed.

U87MG (ATCC HTB-14 TM) GBM cells were seeded in 6-well plates, and following incubation, a wound was created using a pipette tip, as illustrated in Figure 3.3. The cells were then treated with β-carotene, etoposide, temozolomide, and their combinations. Wound closure was monitored under a microscope and photographed to assess the effects of the treatments.

Figure 3.3. Scratch assay. Each well was scratched with a fine tip pipet. Measurements of wound closure and cell migration were performed.

3.6. Giemsa Staining

Giemsa staining was performed to visualize how β-carotene, temozolomide, etoposide and combinations of these substances affected the proliferation, morphology and communication of GBM cells.

Giemsa solution was sterile filtered through a 0.22 micron syringe filter. U87MG GBM cells were grown in six-well plates under 6 different conditions: control (no treatment), β-carotene treatment, etoposide treatment and temozolomide treatment, etoposide + β-carotene combination treatment, and temozolomide + β-carotene combination treatment. Drugs were administered at their IC50 values. (The 24-h IC50 values of β-carotene, etoposide, and temozolomide on GBM cells had been determined as 2.24 μM for β-carotene, 24 μM for etoposide, 1.5 mM for temozolomide.) After 24 h of incubation, the cell culture medium was removed, and the cells were washed twice with PBS.

A total of 1 mL of 60% methanol in PBS was added to each well and incubated for 10 minutes at room temperature. The solution was then removed, and the cells were washed with pure methanol. Giemsa staining solution (0.01 g/mL) was added to each well (1 mL per well) and left at room temperature for 20 minutes. After incubation, the Giemsa staining solution was removed, and the cells were washed three times with distilled water. Cell morphology was observed using an LED inverted microscope (Leica Biosystems, Wetzlar, Germany). Cells stained with Giemsa were visualized as shown in Figure 3.4.

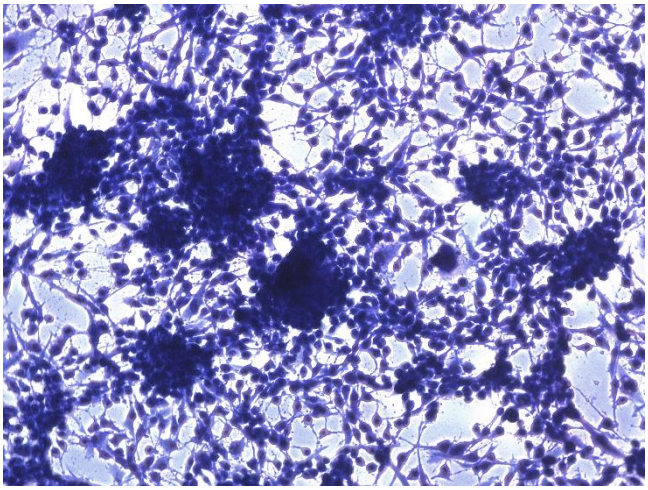

Figure 3.4. Visualization of cells post- Giemsa staining

3.7. Colony Formation Assay

A colony formation assay was performed to evaluate the effect of β-carotene, etoposide, and combination of β-carotene and etoposide on the colony-forming ability of U87MG GBM cells. Cells were seeded at low density (500 cells/well), cultured in six-well plates, and treated with four different conditions: control (no treatment), β-carotene treatment, etoposide treatment, and combination of β-carotene and etoposide, and allowed to grow into colonies, which were later stained and counted to assess their proliferative capacity.

The substances were used at concentrations corresponding to their IC50 values for U87MG cells: 2.24 μM for β-carotene, 24 μM for etoposide, and 1.5 mM for temozolomide.. For colony formation, cells were incubated for 21 days at 37°C in a humidified atmosphere containing 5% CO_2 Cells were then fixed in 4% paraformaldehyde and stained with 2% crystal violet. Colonies were counted using a Leica EZ50 dissecting microscope (Leica Biosystems, Wetzlar, Germany). An example of a colony image is shown in Figure 3.5.

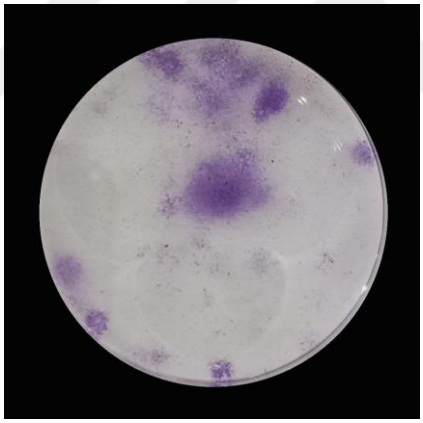

Figure 3.5. Colony formation assay to evaluate the colony-forming ability of U87MG cells treated with β-carotene, etoposide, temozolomide, and their combinations for 24 hours, followed by colony counting.

4. RESULTS AND DISCUSSION

4.1. Cytotoxic Effect of β-Carotene and Chemotherapeutic Drugs: Etoposide and Temozolomide

The cytotoxic effect of β-carotene alone and in combination with the chemotherapeutic drugs etoposide and temozolomide on GBM cells was investigated. The 24-hour IC50 values of β-carotene, etoposide, and temozolomide on GBM cells were determined by exposing the cells to various concentrations of these compounds individually and evaluating their viability. The 24-hour IC50 value of β-carotene for U87MG GBM cells was calculated as 1.2 µg/mL (2.24 µM) based on the equation derived from the graph presented in Figure 4.1.

In a study about the cytotoxic effects of neutraceuticals on lymphoblastic cancer and, colon cancer cell lines, β-carotene 48-hour IC-50 values for HCT116 and HCT116 P53 colon cancer cell lines were determined as 139.53± 5.72 µM and 34.94±0.47 µM, respectively. In the same study, β-carotene IC50 values for CEM/CCRF and CEM/ADR5000 lymphoblastic leukemia cell lines were determined as 34.46±1.2 µM and 220.58±12.25 µM (Nibret et al.,2021). In our study, the IC50 value of β-carotene determined for GBM cells (2.24 µM) was lower than the IC50 value determined for colon cancer and lymphoblastic cell lines. This suggests that β-carotene has a higher cytotoxic effect on GBM cells.

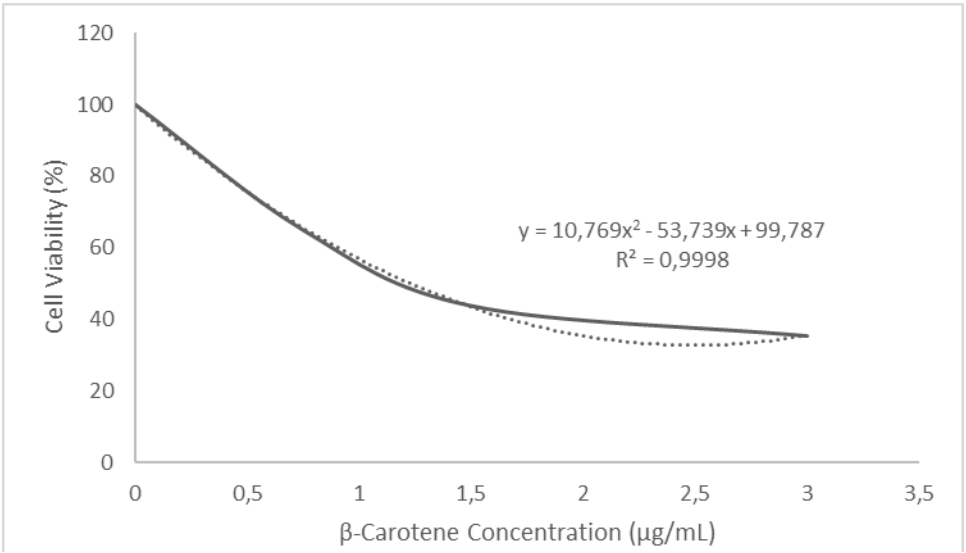

Figure 4.1. The effect of various concentration of β-carotene on GBM cells for 24 hours ($p<0,01$).

The IC50 values of etoposide and temozolomide, calculated based on the equations obtained from the graph shown in Figure 4.2.a and b, are 24 µM and 1.5 mM, respectively.

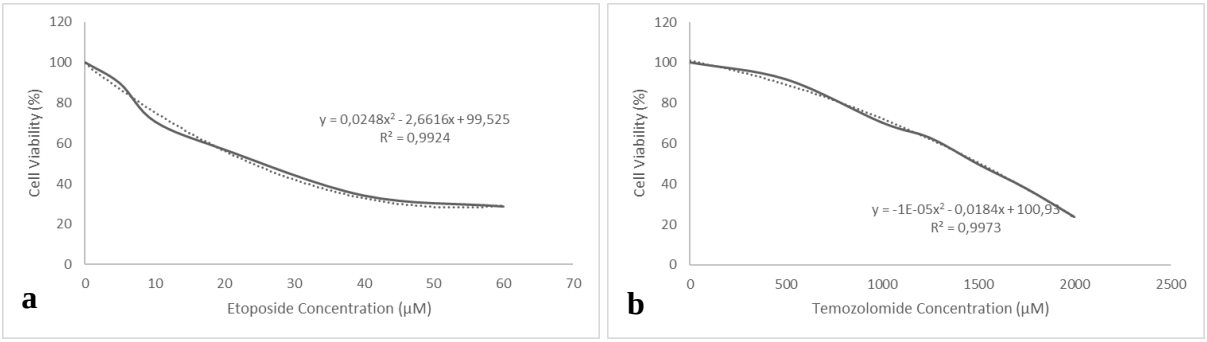

Figure 4.2. The effect of various concentrations of **a)** etoposide and **b)** temozolomide on GBM cells for 24 hours ($p<0,01$).

4.2. Cytotoxic Effect of β-carotene, Etoposide, Temezolomide, and Their Combinations on GBM Cells

β-carotene, etoposide, and temozolomide at calculated IC50 values were were administered to GBM cells individually and in combination at their calculated IC50 values. The effects of these substances on cell viability after 24- and 48-hours were determined using the MTT cell viability test. The results are presented in Figure 4.3.

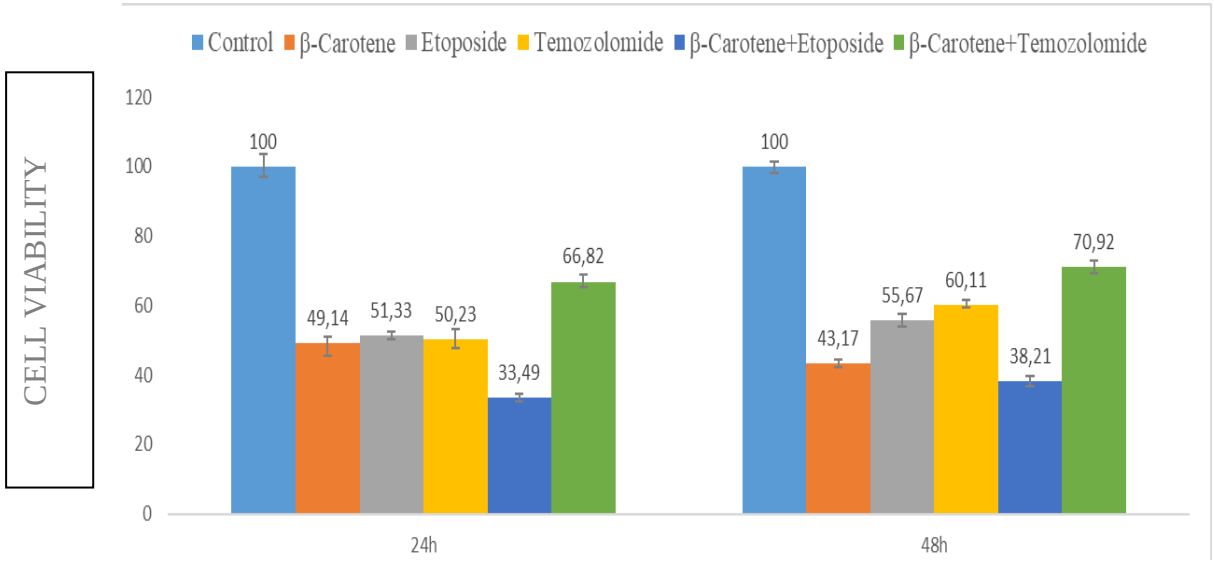

Figure 4.3. Cytotoxic effect of β-carotene, etoposide, temezolomide, and their combinations on GBM cells ($p<0.01$)

According to the cell viability test results, β-carotene alone induced cell death at both 24 and 48 hours, with these effects increasing over time. At 24 hours, cell viability was 49.14%, which

decreased further to 43.17% at 48 hours, indicating a continuous decline in the percentage of viable cells.

There are studies in the literature which are supporting our findings. In a study investigating the apoptotic effects of β-carotene and lycopene on human breast cancer, it was noted that the apoptotic effect of β-carotene continued to increase at 48 hours and 96 hours in MCF-7, MDA-MB-231 and MDA-MB-235 human breast cancer cell lines (Gloria et al.,2014). The results of this study on breast cancer are consistent with our findings on GBM cells. In our study, at 24 hours, cell viability was 49.14%, which decreased further to 43.17% at 48 hours, indicating a continuous decrease in the percentage of viable cells.

Additionally, It was observed that β-carotene significantly enhanced the cytotoxic effect of etoposide, reducing in cell viability to 33.49% at 24 hours and 38.21% at 48 hours. However, an antagonistic effect was observed when β-carotene was combined with temozolomide (Figure 4.3). The results were statistically confirmed with the 24 and 48 hour t test analyses in Table 1 and Table 2.

Table 4.1. Statistical Analyses of 24h Cytotoxicity for Temozolomid, Etoposid, β-carotene, and the Combinations of these substances ($p<0,001$)

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	0,639	0,213	5,2E-05
β-Carotene	3	0,314	0,104667	4,43E-05
Etoposide	3	0,328	0,109333	6,33E-06
Temozolomide	3	0,321	0,107	3,1E-05
β-Carotene+Etoposide	3	0,214	0,071333	6,33E-06
β-Carotene+Temozolomide	3	0,427	0,142333	1,73E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,03572	5	0,007144	272,4377	6,43E-12	3,105875
Within Groups	0,000315	12	2,62E-05			
Total	0,036034	17				

Table 4.2. Statistical Analyses of 48h Cytotoxicity (p<0,001)

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	1,128	0,376	3,9E-05
β-Carotene	3	0,487	0,162333	1,73E-05
Etoposide	3	0,628	0,209333	4,23E-05
Temozolomide	3	0,678	0,226	0,000019
β-Carotene+Etoposide	3	0,431	0,143667	3,03E-05
β-Carotene+Temozolomide	3	0,8	0,266667	4,43E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,105399	5	0,02108	657,6042	3,37E-14	3,105875
Within Groups	0,000385	12	3,21E-05			
Total	0,105784	17				

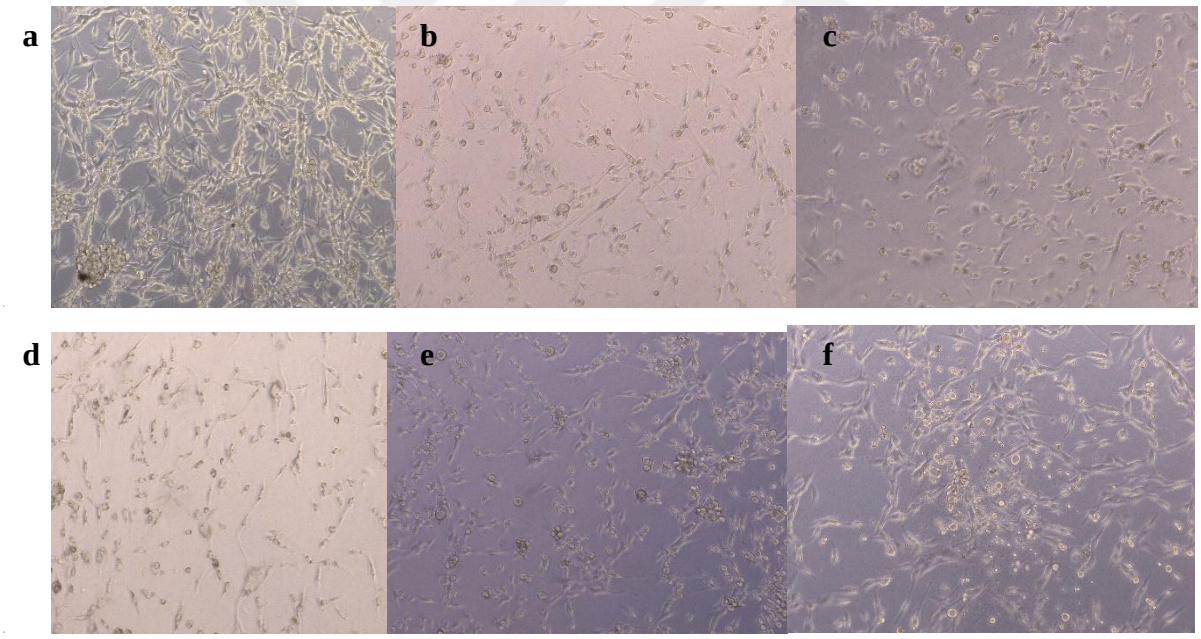

Figure 4.4. Microscope images (a-control, b-etoposide, c-temozolomide, d- β-carotene, e- β-carotene+etoposide, f- β-carotene+temozolomide)

Figure 4.4. illustrates the images under different conditions before giemsa staining. In Image a cell cultures designated as the control group appear to show high proliferation, communication and motility. In Image b,c,d,e it is seen that etoposide, temozolomide β-carotene and the combination of β-carotene and etoposide reduce proliferation and motility. In image f, it is seen that the combination of β-carotene and temozolomide cause higher proliferation than b, c, d and e.

The data presented in the graph in Figure 4.3 that β-carotene and the combination of β-carotene and etoposide caused a significant reduction in the number of U87MG GBM cells was also confirmed by the unstained cell images presented in Figure 4.4. The individualization of GBM cells, the weakening of communication and bond between them, and the rounded shape of existing cells are indicators of cell death. The cytotoxic effect of the etoposide + β-carotene combination on U87MG GBM cells is demonstrated in these images, too.

4.3. Giemsa Staining

Giemsa staining technique is used to stain viable cells purple, allowing visualization of chromatin, nuclear membranes, and various cellular components (Ipek et al., 2023). In this study, Giemsa staining was used to evaluate the effect of etoposide, temozolomide, β-carotene, and their combinations on the U87MG GBM cells. Morphological changes were observed in GBM cells, and a significant dicrease determined in cell numbers in the group treated with the etoposide + β-carotene combination. The appearance of rounded cells observed, and it was noted that cellular individualization increased, leading to weakened intercellular communication and bonding.

Among the GBM cells treated with Giemsa, viable cells were stained purple. The fewest viable cells were observed in the group treated with the combination of β-carotene and etoposide. In contrast, the control group exhibited the most purple staining, indicating the highest number of viable cells. When used individually, etoposide, temozolomide and β-carotene showed lower cell viability compared to the temozolomide and β-carotene combination, as shown in Figure 4.5.

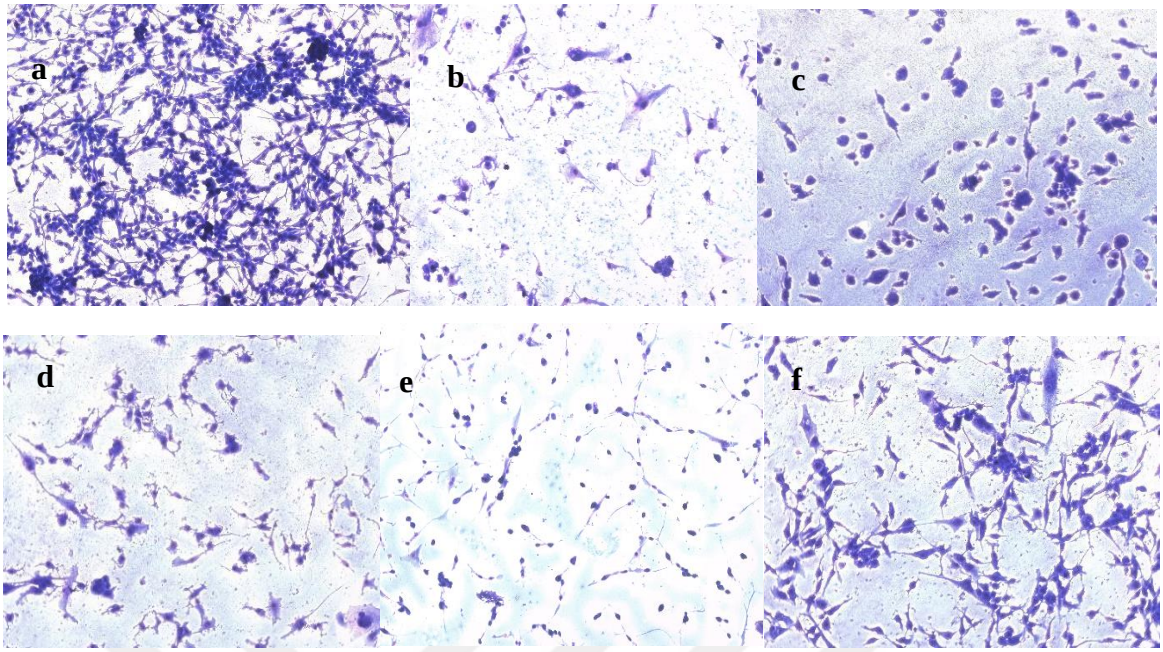

Figure 4.5. Giemsa Staining images (a-control, b-etoposide applied, c-temozolomide applied, d- β-carotene applied, e- β-carotene+etoposide applied, f- β-carotene+temozolomide applied.

The effects of the substances on cell viability are also evident in Giemsa staining and unstained cell images. The data presented in Figure 4.3 is confirmed with the microscope images in Figure 4.4 and images after Giemsa staining in Figure 4.5. The distinctive characteristics of GBM cells in b, c, d, and e images, such as the disrupted communication and connections between them, as well as the spherical shape of the cells, suggest the presence of apoptosis within the cells.

There are other studies in the literature that support our findings that β-carotene and other carotenoids induce cell death and apoptosis in cancer cells. In a study investigated the effect of β-carotene on colon cancer cell lines it was determined that β-carotene had a similar effect on colorectal cancer cells, too. In the study, it was determined that β-carotene inhibited the self-renewal capacity and colon cancer stem cell markers, including CD44, CD133, ALDH1A1, NOTCH1, Sox2 and β-catenin in vitro. In addition, it was indicated that β-carotene supplementation reduced the number and size of tumors and delayed the tumor onset time in xenograft mice injected with CD133, + CD44, + HCT116 cells (Lee et al., 2022).

Morover, in another study in which 1 μM β-carotene treatment was applied to MCF-7 human breast cancer cell line, β-carotene was associated with the induction of apoptosis and increased caspase-3 activity. The study concluded that 1 μM concentration of β-carotene effectively reduced the expression of anti-apoptotic proteins Bcl-2 and PARP and survival protein NF-kB, and also inhibited the activation of intracellular growth signaling proteins Akt and ERK1/2, and

down-regulated the antioxidant enzyme SOD-2 and its transactivation factor (Nrf-2) and the endoplasmic reticulum (ER) stress marker XBP-1 at protein levels. These findings revealed the anticancer effect of β-carotene on this cell line even at low physiological concentrations (Sowmya Shree et al., 2017). The study proved that low concentrations of β-carotene cause MCF-7 human breast cancer cell death. The pro-oxidant effects of carotenoids in triggering apoptosis of cancer cells are known and investigated. Pro-oxidant activity has been reported to promote cancer cell death via reactive oxygen species (Shin et al., 2020).

In the study examining the induction of apoptosis by fucoxanthin, one of the carotenoids in the xanthophyll group, in U251 human glioma cells, it was concluded that fucoxanthin triggered reactive oxygen species-mediated oxidative DNA damage in a time-dependent manner, and also caused dysfunction of MAPKs and PI3K-AKT pathways in a time-dependent manner and induced apoptosis (Wu et al.,2019).

4.4. Scratch Assay

The Scratch assay is an experiment designed to assess the inhibitory effects of various components on the migration ability of cancer cells, providing insights into how treatments may limit the invasive characteristics of these cells. In this experiment, cells seeded in a 6-well plate were scratched using a pipette tip to create a wound. The effects of β-carotene and drugs alone and in combination on the invasion capacity of U87MG cells was examined for 0, 12, 24 hours by using an inverted microscope (Leica Biosystems, Wetzlar Germany) and photographed (Figure 4.6).

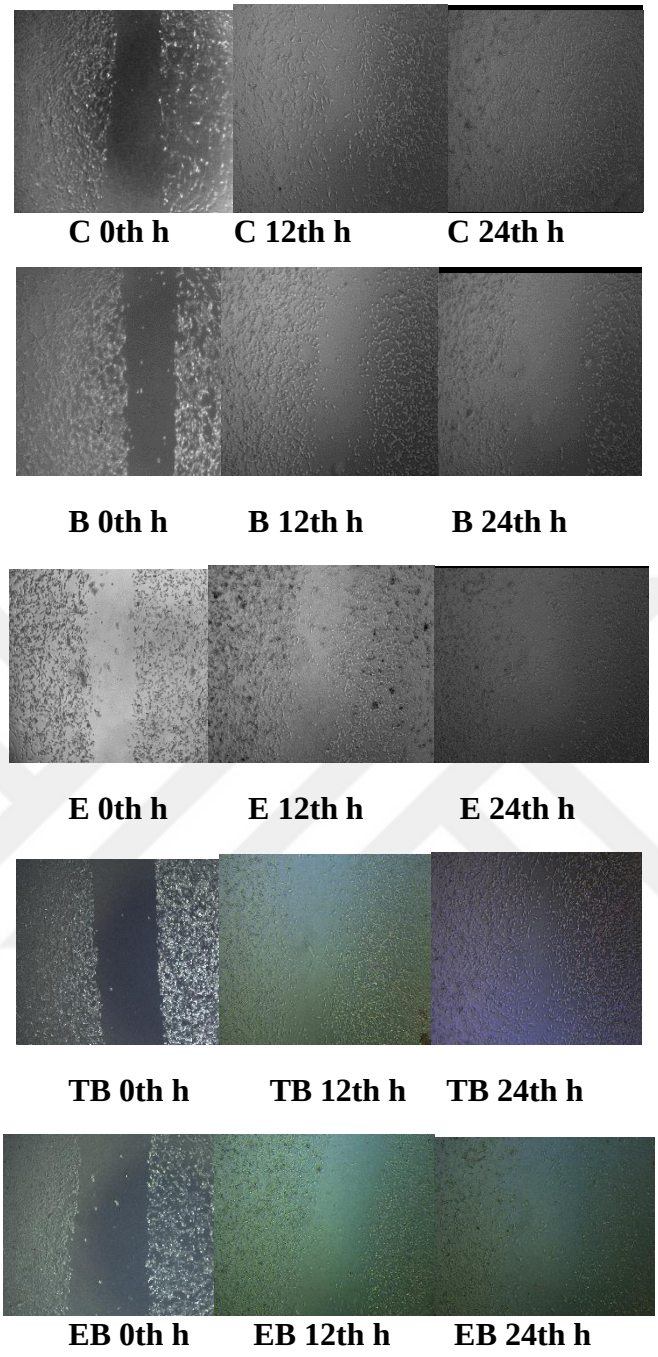

Figure 4.6. Cell Migration Images (C: Control group, B: β-carotene applied, E: Etoposide applied, TB:Temozolomide + β-carotene applied, EB: Etoposid + β-carotene applied; at 0th, 12th, 24th hours)

The migration *rate* and wound closure based on the captured images were quantified using ImageJ software (version 1.53t) and graphically represented to quantify the effects of these treatments. Cell migration images indicated that the combination of etoposide and β-carotene not only induced cell death but also reduced the motility and invasiveness of the cells.

In the graph depicting cell migration speed (Figure 4.7.), it was observed that β-carotene alone significantly decreased the cell diffusion rate -4.63 μm/h. The combination of β-carotene with etoposide further reduced the diffusion rate to -7.85 μm/h. However, the combination of temozolomide and β-carotene was less effective than the other combination, resulting in a diffusion rate of +3.16 μm/h when evaluated individually.

This situation suggests that the β-carotene and etoposide combination treatment have caused cell death, leading to a decrease in the number of migrating cells and hindering wound closure due to the high cytotoxic effects of β-carotene and etoposide.

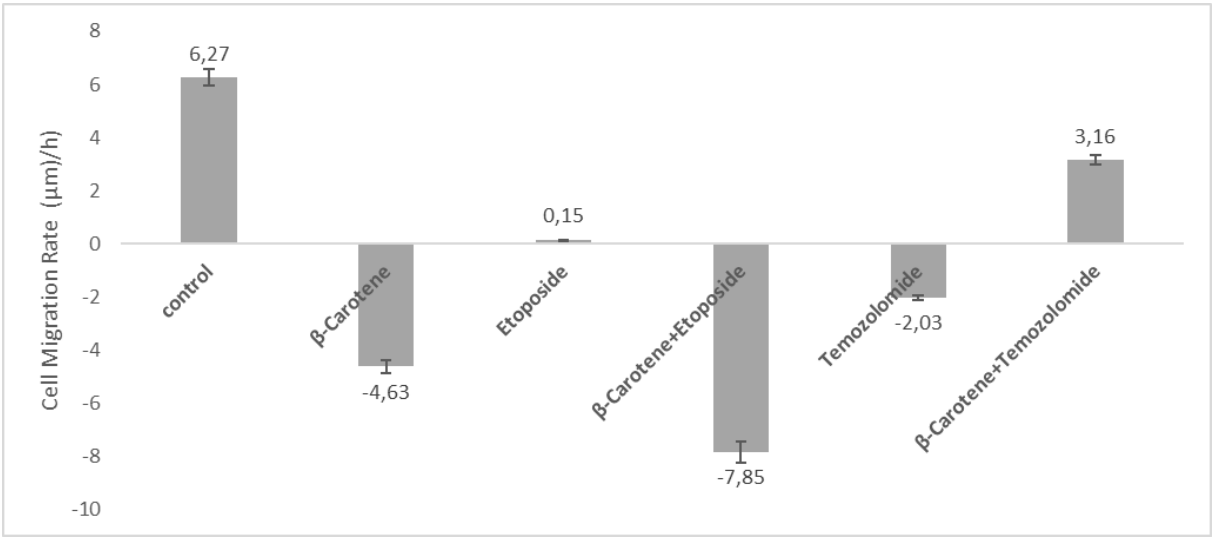

Figure 4.7. Effects of β-carotene, Etoposide, "Temozolomide+ β-carotene", "Etoposid+ β-carotene" on Cell Migration Rates

Wound closure percentages were calculated by measuring the initial wound area and comparing it to the area at following time points described in Figure 4.8. The wound closure percentages were found to be compatible with the cell migration rate data presented in Figure 4.7. The lowest wound closure percentage is indicated in the β-carotene and etoposide combination (-72.20%), while the highest is seen in the β-carotene and temozolomide combination.(+58,82%). When administered alone, wound closure percentages are respectively: -69.93, for β-carotene , +2.38 for temozolomide, and +7.43 for etoposide. The lowest wound closure percentage is indicated in the β-carotene and etoposide combination (-72.20%), while the highest is seen in the β-carotene and temozolomide combination.(+58,82%).

When administered alone, wound closure percentages are respectively: -69.93, for β-carotene , +2.38 for temozolomide, and +7.43 for etoposide.

The individual wound closure percentages for each substance treatment were: β-carotene: -69.93%, temozolomide: +2.38%, and etoposide: +7.43%. The combined use of β-carotene and etoposide further reduced the wound closure percentage to -72.20%, while the combination of temozolomide and β-carotene increased the wound closure percentage to +58.82%. According to these results, it was determined that the combination of β-carotene with etoposide reduced the wound closure percentage, while the combination of β-carotene and temozolomide increased the wound closure percentage.

As a result, the obtained cell migration rate and wound closure percentage findings were consistent with our cytotoxicity findings, which indicated that cell proliferation was reduced when β-carotene administered alone, and cell viability was further reduced when β-carotene was co-administered with etoposide.

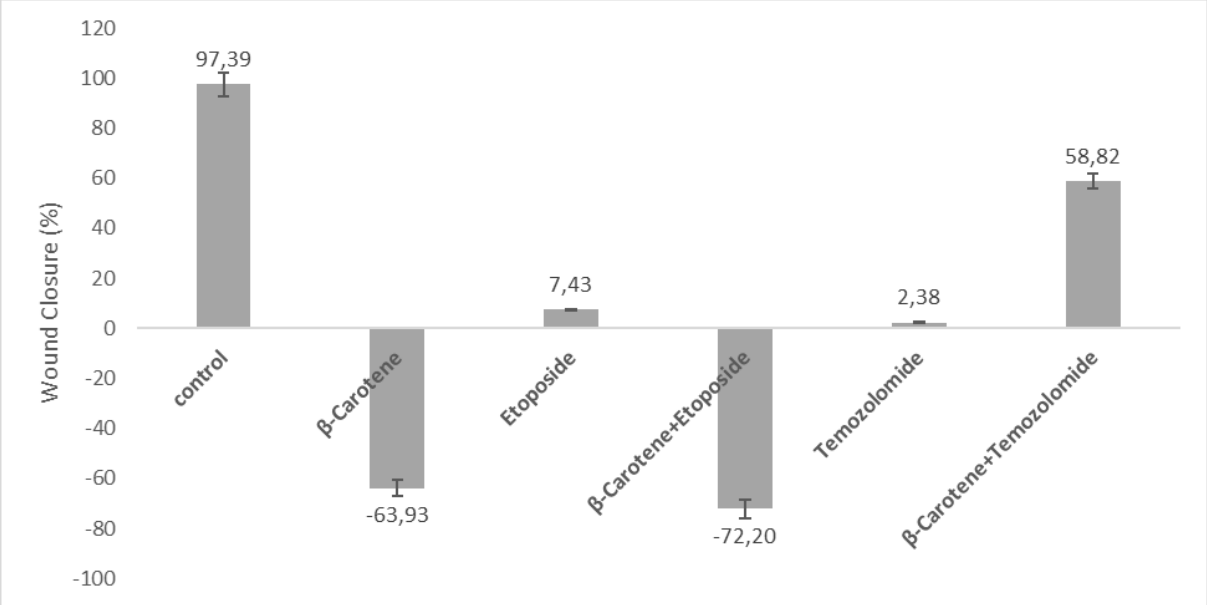

Figure 4.8. Wound Closure Percentages From Scratch Assay

The wound area and wound width are presented in figure 4.9. and figure 4.10.

As shown in the graphical results of wound area measurements, the largest wound area was observed in the etoposide and β-carotene combination, while the greatest reduction in wound area was seen with the β-carotene temozolomide combination application (Figure 4.9).

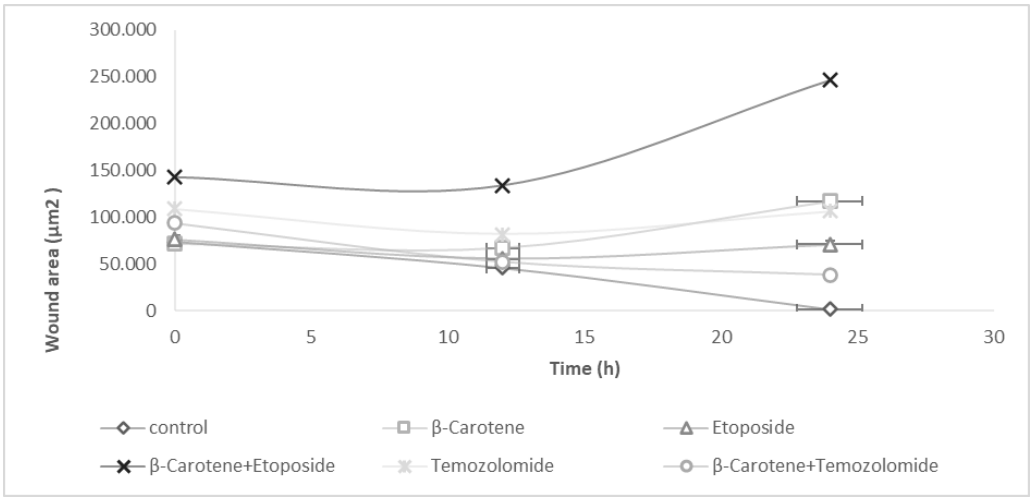

Figure 4.9. Measurements of Wound Area Over Time

The wound width measurements from the graphical results indicate that the widest wound width was observed in the combination of etoposide and β-carotene. Conversely, the most significant reduction in wound width was noted in the combination of β-carotene and temozolomide (Figure 4.10). These findings suggest that β-carotene has an inhibitory effect on metastasis activity of GBM cells and also its combination with etoposide increases it.

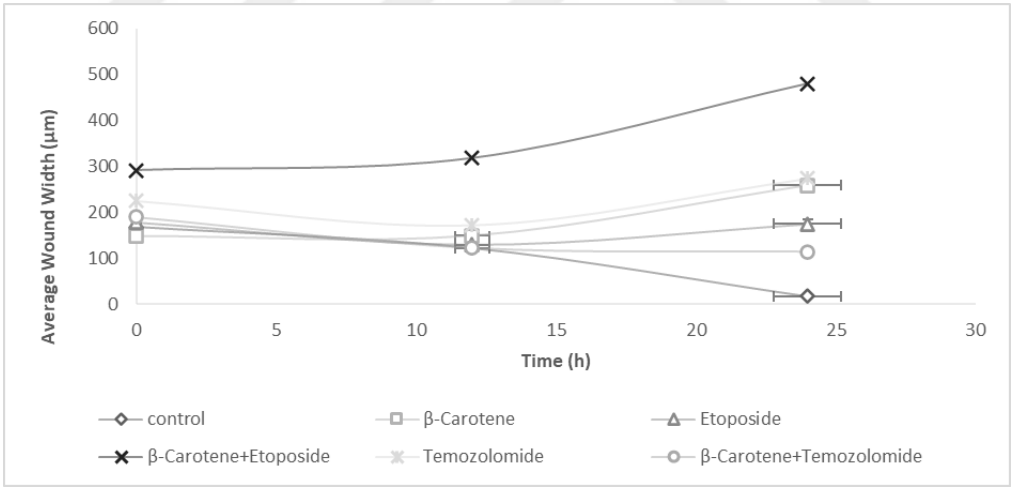

Figure 4.10. Measurements of Average Wound Width Over Time

There are studies in the literature investigating the effects of β-carotene and other carotenoids on metastasis of cancer cells and a few of these studies are sumarized below.

In a study investigating the antimetastatic effects of astaxanthin, a natural carotenoid derivative, it was shown that astaxanthin at concentrations of 50 µM and 100 µM inhibited the migration and invasion ability of A172 GBM cells by reducing the expression of MMP-2 and MMP-9

(Siangcham et al., 2020). In a separate study on GBM, the mouse GBM cell line GL261, and the human GBM cell line U251 MG cell line antitumor effects of astaxantin, and adonixantin were reported (Tsuji et al., 2020). These studies support that the carotenoid family is effective on the inhibition of motility of glioblastoma cells.

The impact of β-carotene on the lung metastasis by B16F10 melanoma cells was investigated in C57BL16 mice. As a result of this study, it was found that simultaneous administration of β-carotene after tumor induction reduced tumor nodule formation by 71.36%. In addition, increased lung collagen hydroxyproline (22.37 μg/mg protein) in the lungs of metastasized control animals decreased to 4.19 μg/mg protein in β-carotene -treated animals. High levels of uronic acid (355.83 μg/100 mg tissue) in metastasized control animals decreased significantly to 87.87 μg/100 mg tissue in β-carotene -treated mice. Also, lung hexosamine content in β-carotene treated mice (1.58 mg/100 mg lyophilized tissue) was significantly decreased compared to untreated control animals (4.2 mg/100 mg lyophilized tissue). The results confirmed the antimetastatic effect of β-carotene (Pradeep et al.,2003).

4.5. Colony Formation Assay

Colony formation experiments aimed to investigate and compare the effects of β-carotene, etoposide, and β-carotene-etoposide combination on the colony formation ability of GBM cells. The colony formation capacities of cells under the following conditions were presented in Figure 4.11: a) control (no drug), b) β-carotene, c) etoposide, and d) β-carotene combined with etoposide.

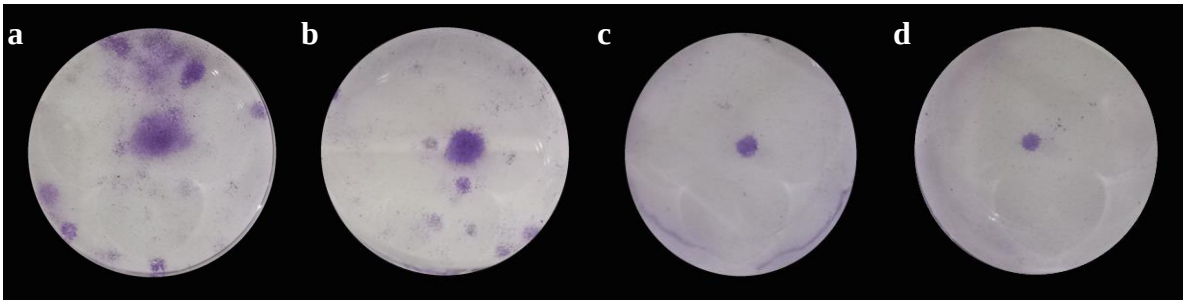

Figure 4.11. Colony Images under the following conditions: (a) control, (b) β-carotene applied, (c) etoposide applied, and (d) β-carotene + etoposide applied.

When administered independently, β-carotene has been shown to diminish the colony-forming capacity of GBM cells and reducethe size of the size of pre- existing colonies. Furthermore, it

has been noted that β-carotene enhances the reduction of colony numbers when combined with etoposide. The combination of etoposide and β-carotene resulted in the fewest colonies.

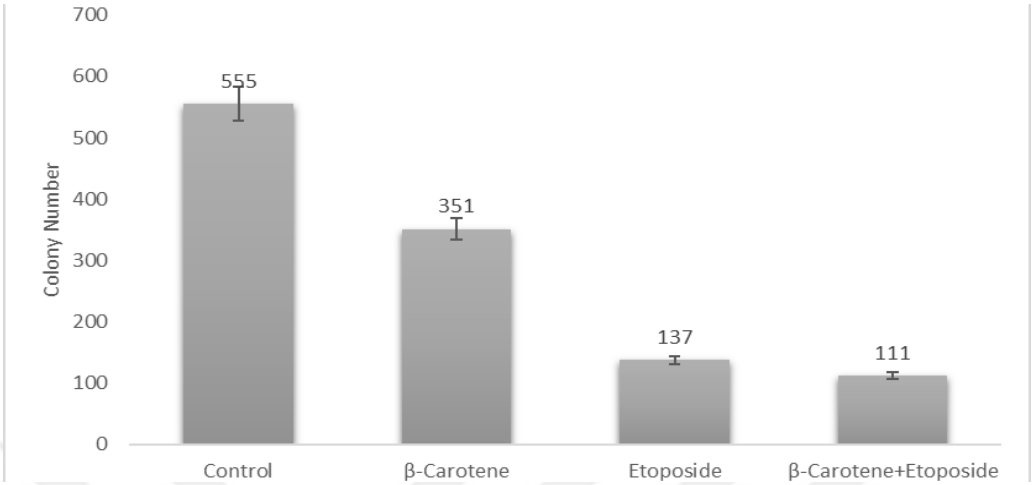

Figure 4.12. Colony Counts Following Treatment with β-carotene, Etoposide, Temozolomide, and Their Combinations.

The colony counts were determined following the administration of β-carotene, etoposide, temozolomide, and their combinations, as illustrated in Figure 4.12. The colony counts for the various treatments are as follows: control: 555 colonies, β-carotene: 351 colonies, etoposide: 137 colonies, and the combination of β-carotene with etoposide: 111 colonies, respectively.

Results of the experiments indicate that β-carotene alone has a lethal effect on U87 MG GBM cells, and its combination with etoposide enhances cell death and decreases colony formation capacities. However, the combination of β-carotene with temozolomide resulted in an increase in cell numbers and colony forming capacities. This situation suggests an antagonistic interaction potential between β-carotene and temozolomide.

5. CONCLUSION

This study aimed to investigate the cytotoxic effects of β-carotene on U87 MG GBM cells when administered alone and in conjunction with the chemotherapeutic agents etoposide and temozolomide. β-carotene alone induces cell death in GBM cells was concluded by experiments. It has also been found to increase cell death and reduces colony formation when combined with etoposide. Conversely, a negative interaction was indicated in cell viability, colony formation, and wound closure experiments when β-carotene was combined with temozolomide. These findings highlight the potential importance of β-carotene as a valuable component in GBM treatment. Furthermore, the observed synergistic effect of β-carotene when combined with the conventional chemotherapeutic drug etoposide shows promise in improving the prognosis of GBM patients, especially in addressing chemotherapeutic resistance challenges.

REFERANCES

Altshuler, D. B., Kadiyala, P., Nuñez, F. J., Nuñez, F. M., Carney, S., Alghamri, M. S., Garcia-Fabiani, M. B., Asad, A. S., Nicola Candia, A. J., Candolfi, M., Lahann, J., Moon, J. J., Schwendeman, A., Lowenstein, P. R., & Castro, M. G. (2020). Prospects of biological and synthetic pharmacotherapies for glioblastoma. *Expert opinion on biological therapy*, *20*(3), 305–317. https://doi.org/10.1080/14712598.2020.1713085

Bae, S., Lim, J. W., & Kim, H. (2021). β-Carotene Inhibits Expression of Matrix Metalloproteinase-10 and Invasion in *Helicobacter pylori*-Infected Gastric Epithelial Cells. *Molecules (Basel, Switzerland)*, *26*(6), 1567. https://doi.org/10.3390/molecules26061567

Black, H. S., Boehm, F., Edge, R., & Truscott, T. G. (2020). The Benefits and Risks of Certain Dietary Carotenoids that Exhibit both Anti- and Pro-Oxidative Mechanisms-A Comprehensive Review. *Antioxidants (Basel, Switzerland)*, *9*(3), 264. https://doi.org/10.3390/antiox9030264

Bray, F., Laversanne, M., Sung, H., Ferlay, J., Siegel, R. L., Soerjomataram, I., & Jemal, A. (2024). Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians*, *74*(3), 229–263. https://doi.org/10.3322/caac.21834

Brewczyński, A., Jabłońska, B., Kentnowski, M., Mrowiec, S., Składowski, K., & Rutkowski, T. (2021). The Association between Carotenoids and Head and Neck Cancer Risk. *Nutrients*, *14*(1), 88. https://doi.org/10.3390/nu14010088

Cao, W., Chen, H. D., Yu, Y. W., Li, N., & Chen, W. Q. (2021). Changing profiles of cancer burden worldwide and in China: a secondary analysis of the global cancer statistics 2020. *Chinese medical journal*, *134*(7), 783–791. https://doi.org/10.1097/CM9.0000000000001474

Cohen, G., Burks, S. R., & Frank, J. A. (2018). Chlorotoxin-A Multimodal Imaging Platform for Targeting Glioma Tumors. *Toxins*, *10*(12), 496. https://doi.org/10.3390/toxins10120496

Chen, G., Weiskirchen, S., & Weiskirchen, R. (2023). Vitamin A: too good to be bad?. *Frontiers in pharmacology*, *14*, 1186336. https://doi.org/10.3389/fphar.2023.1186336

Chen, J., Hsu, C., Yeh, J., Yow, J. L., Chang, L., Lin, H., & Tzang, S. (2021). Differential Effects of Wedelia chinensis on Human Glioblastoma Multiforme Cells. *Integrative Cancer Therapies*. https://doi.org/10.1177/15347354211000119

Chen, Q. H., Wu, B. K., Pan, D., Sang, L. X., & Chang, B. (2021). Beta-carotene and its protective effect on gastric cancer. *World journal of clinical cases*, *9*(23), 6591–6607. https://doi.org/10.12998/wjcc.v9.i23.6591

Cheng, Y., Lian, S., Li, S., Lu, Y., Wang, J., Deng, X., Zhai, S., & Jia, L. (2022). Combination of Se-methylselenocysteine, D-α-tocopheryl succinate, β-carotene, and L-lysine can prevent cancer metastases using as an adjuvant therapy. *Journal of Zhejiang University. Science. B*, *23*(11), 943–956. https://doi.org/10.1631/jzus.B2200232

Dang, L., White, D. W., Gross, S., Bennett, B. D., Bittinger, M. A., Driggers, E. M., Fantin, V. R., Jang, H. G., Jin, S., Keenan, M. C., Marks, K. M., Prins, R. M., Ward, P. S., Yen, K. E., Liau, L. M., Rabinowitz, J. D., Cantley, L. C., Thompson, C. B., Vander Heiden, M. G., & Su, S. M. (2009). Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature*, *462*(7274), 739–744. https://doi.org/10.1038/nature08617

Dragnev, K. H., Rigas, J. R., & Dmitrovsky, E. (2000). The retinoids and cancer prevention mechanisms. *The oncologist*, *5*(5), 361–368. https://doi.org/10.1634/theoncologist.5-5-361

Engelhard, H. H., Willis, A. J., Hussain, S. I., Papavasiliou, G., Banner, D. J., Kwasnicki, A., Lakka, S. S., Hwang, S., Shokuhfar, T., Morris, S. C., & Liu, B. (2020). Etoposide-Bound Magnetic Nanoparticles Designed for Remote Targeting of Cancer Cells Disseminated Within Cerebrospinal Fluid Pathways. *Frontiers in neurology*, *11*, 596632. https://doi.org/10.3389/fneur.2020.596632

Franceschi, E., Cavallo, G., Scopece, L., Paioli, A., Pession, A., Magrini, E., Conforti, R., Palmerini, E., Bartolini, S., Rimondini, S., Esposti, R. D., & Crinò, L. (2004). Phase II trial of carboplatin and etoposide for patients with recurrent high-grade glioma. *British journal of cancer*, *91*(6), 1038–1044. https://doi.org/10.1038/sj.bjc.6602105

Gallien, J., Srinageshwar, B., Gallo, K., Holtgrefe, G., Koneru, S., Otero, P. S., Bueno, C. A., Mosher, J., Roh, A., Kohtz, D. S., Swanson, D., Sharma, A., Dunbar, G., & Rossignol, J. (2021). Curcumin Loaded Dendrimers Specifically Reduce Viability of Glioblastoma Cell Lines. *Molecules (Basel, Switzerland)*, *26*(19), 6050. https://doi.org/10.3390/molecules26196050

Gloria, N. F., Soares, N., Brand, C., Oliveira, F. L., Borojevic, R., & Teodoro, A. J. (2014). Lycopene and beta-carotene induce cell-cycle arrest and apoptosis in human breast cancer cell lines. *Anticancer research*, *34*(3), 1377–1386.

Hardbower, D. M., Peek, R. M., Jr, & Wilson, K. T. (2014). At the Bench: Helicobacter pylori, dysregulated host responses, DNA damage, and gastric cancer. *Journal of leukocyte biology*, *96*(2), 201–212. https://doi.org/10.1189/jlb.4BT0214-099R

Hotchkiss, K. M., & Sampson, J. H. (2021). Temozolomide treatment outcomes and immunotherapy efficacy in brain tumor. *Journal of neuro-oncology*, *151*(1), 55–62. https://doi.org/10.1007/s11060-020-03598-2

Hu, C., Huang, Y., Luo, P., & Yang, Y. (2021). Effect of antioxidants coenzyme Q10 and β-carotene on the cytotoxicity of vemurafenib against human malignant melanoma. *Oncology letters*, *21*(3), 208. https://doi.org/10.3892/ol.2021.12469

İpek, S. L., Özdemir, M. D., Göktürk, D. (2023) Cytotoxic Effect of L-Methioninase from *Brevibacterium linens* BL2 in Combination with Etoposide against Glioblastoma Cells. *Applied Sciences*. 2023; 13(16):9382. https://doi.org/10.3390/app13169382

Jiang, Z., Chen, H., Li, M., Wang, W., Fan, C., & Long, F. (2022). Association of Dietary Carrot/Carotene Intakes With Colorectal Cancer Incidence and Mortality in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. *Frontiers in nutrition*, *9*, 888898. https://doi.org/10.3389/fnut.2022.888898

Jin, G., Reitman, Z. J., Duncan, C. G., Spasojevic, I., Gooden, D. M., Rasheed, B. A., Yang, R., Lopez, G. Y., He, Y., McLendon, R. E., Bigner, D. D., & Yan, H. (2013). Disruption of wild-type IDH1 suppresses D-2-hydroxyglutarate production in IDH1-mutated gliomas. *Cancer research*, *73*(2), 496–501. https://doi.org/10.1158/0008-5472.CAN-12-2852

Karachi, A., Dastmalchi, F., Mitchell, D. A., & Rahman, M. (2018). Temozolomide for immunomodulation in the treatment of glioblastoma. *Neuro-oncology*, *20*(12), 1566–1572. https://doi.org/10.1093/neuonc/noy072

Khabibov, M., Garifullin, A., Boumber, Y., Khaddour, K., Fernandez, M., Khamitov, F., Khalikova, L., Kuznetsova, N., Kit, O., & Kharin, L. (2022). Signaling pathways and therapeutic approaches in glioblastoma multiforme (Review). *International journal of oncology*, *60*(6), 69. https://doi.org/10.3892/ijo.2022.5359

Kordiak, J., Bielec, F., Jabłoński, S., & Pastuszak-Lewandoska, D. (2022). Role of Beta-Carotene in Lung Cancer Primary Chemoprevention: A Systematic Review with Meta-Analysis and Meta-Regression. *Nutrients*, *14*(7), 1361. https://doi.org/10.3390/nu14071361

Kristensen, B. W., Priesterbach-Ackley, L. P., Petersen, J. K., & Wesseling, P. (2019). Molecular pathology of tumors of the central nervous system. *Annals of oncology : official journal of the European Society for Medical Oncology*, *30*(8), 1265–1278. https://doi.org/10.1093/annonc/mdz164

Lee, K. E., Kwon, M., Kim, Y. S., Kim, Y., Chung, M. G., Heo, S. C., & Kim, Y. (2022). β-carotene regulates cancer stemness in colon cancer *in vivo* and *in vitro*. *Nutrition research and practice*, *16*(2), 161–172. https://doi.org/10.4162/nrp.2022.16.2.161

Lee, J., Heo, S. C., & Kim, Y. (2024). Combination of oxaliplatin and β-carotene suppresses colorectal cancer by regulating cell cycle, apoptosis, and cancer stemness *in vitro*. *Nutrition research and practice*, *18*(1), 62–77. https://doi.org/10.4162/nrp.2024.18.1.62

Louis, D. N., Perry, A., Wesseling, P., Brat, D. J., Cree, I. A., Figarella-Branger, D., Hawkins, C., Ng, H. K., Pfister, S. M., Reifenberger, G., Soffietti, R., von Deimling, A., & Ellison, D. W. (2021). The 2021 WHO Classification of Tumors of the Central Nervous System: a summary. *Neuro-oncology*, *23*(8), 1231–1251. https://doi.org/10.1093/neuonc/noab106

Louis, D. N., Wesseling, P., Aldape, K., Brat, D. J., Capper, D., Cree, I. A., Eberhart, C., Fouladi, M., Fuller, G. N., Giannini, C., Haberler, C., Hawkins, C., Komori, T., Kros, J. M., Ng, H., Orr, B. A., Park, H., Paulus, W., Perry, A., . . . Ellison, D. W. (2020). CIMPACT-NOW update 6: New entity and diagnostic principle recommendations of the cIMPACT-Utrecht meeting on future CNS tumor classification and grading. *Brain Pathology*, *30*(4), 844-856. https://doi.org/10.1111/bpa.12832

Małek, A., Kocot, J., Mitrowska, K., Posyniak, A., & Kurzepa, J. (2022). Bee Venom Effect on Glioblastoma Cells Viability and Gelatinase Secretion. *Frontiers in neuroscience*, *16*, 792970. https://doi.org/10.3389/fnins.2022.792970

Maoka T. (2020). Carotenoids as natural functional pigments. *Journal of natural medicines*, *74*(1), 1–16. https://doi.org/10.1007/s11418-019-01364-x

Mattei, V., Santilli, F., Martellucci, S., Delle Monache, S., Fabrizi, J., Colapietro, A., Angelucci, A., & Festuccia, C. (2021). The Importance of Tumor Stem Cells in Glioblastoma Resistance to Therapy. *International journal of molecular sciences*, *22*(8), 3863. https://doi.org/10.3390/ijms22083863

Melhem, J. M., Detsky, J., Lim-Fat, M. J., & Perry, J. R. (2022). Updates in IDH-Wildtype Glioblastoma. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics*, *19*(6), 1705–1723. https://doi.org/10.1007/s13311-022-01251-6

Milani, A., Basirnejad, M., Shahbazi, S., & Bolhassani, A. (2017). Carotenoids: biochemistry, pharmacology and treatment. *British journal of pharmacology*, *174*(11), 1290–1324. https://doi.org/10.1111/bph.13625

Mirzaei, T., Sheikholeslami, S. A., Bereimipour, A., Jalili, A., Zali, A., Sharbati, S., Kaveh, V., & Salari, S. (2022). Molecular pathways in glioblastoma-derived stem cells to identify effective drug agents: A bioinformatics study. *Journal of family medicine and primary care*, *11*(6), 2856–2864. https://doi.org/10.4103/jfmpc.jfmpc_1436_21

Moise, A. R., Bandara, S., & von Lintig, J. (2022). Carotenoid modifying enzymes in metazoans. *Methods in enzymology*, *674*, 405–445. https://doi.org/10.1016/bs.mie.2022.05.005

Mousavi, M., Koosha, F., & Neshastehriz, A. (2023). Chemo-radiation therapy of U87-MG glioblastoma cells using SPIO@AuNP-Cisplatin-Alginate nanocomplex. *Heliyon*, *9*(3), e13847. https://doi.org/10.1016/j.heliyon.2023.e13847

National Center of Biotechnology Information (2024) PubChem Compound Summary for CID 5280489, Beta Carotene. Retrieved June23,2024 from https://pubchem.ncbi.nlm.nih.gov/compound/Beta-Carotene.

Nibret, E., Krstin, S., & Wink, M. (2021). In vitro anti-proliferative activity of selected nutraceutical compounds in human cancer cell lines. *BMC research notes*, *14*(1), 18. https://doi.org/10.1186/s13104-020-05435-1

Parniakov, O., Barba, F. J., Grimi, N., Marchal, L., Jubeau, S., Lebovka, N., & Vorobiev, E. (2015). Pulsed electric field assisted extraction of nutritionally valuable compounds from microalgae Nannochloropsis spp. Using the binary mixture of organic solvents and water. *Innovative Food Science & Emerging Technologies*, *27*, 79-85. https://doi.org/10.1016/j.ifset.2014.11.002

Parsons, D. W., Jones, S., Zhang, X., Lin, J. C., Leary, R. J., Angenendt, P., Mankoo, P., Carter, H., Siu, I. M., Gallia, G. L., Olivi, A., McLendon, R., Rasheed, B. A., Keir, S., Nikolskaya, T., Nikolsky, Y., Busam, D. A., Tekleab, H., Diaz, L. A., Jr, Hartigan, J., … Kinzler, K. W. (2008). An integrated genomic analysis of human glioblastoma multiforme. *Science (New York, N.Y.)*, *321*(5897), 1807–1812. https://doi.org/10.1126/science.1164382

Patel, A. S., Kar, A., Dash, S., & Dash, S. K. (2019). Supercritical fluid extraction of β-carotene from ripe bitter melon pericarp. *Scientific reports*, *9*(1), 19266. https://doi.org/10.1038/s41598-019-55481-4

Pevna, V., Wagnières, G., & Huntosova, V. (2021). Autophagy and Apoptosis Induced in U87 MG Glioblastoma Cells by Hypericin-Mediated Photodynamic Therapy Can Be Photobiomodulated with 808 nm Light. *Biomedicines*, *9*(11), 1703. https://doi.org/10.3390/biomedicines9111703

Postmus, P. E., Holthuis, J. J., Haaxma-Reiche, H., Mulder, N. H., Vencken, L. M., van Oort, W. J., Sleijfer, D. T., & Sluiter, H. J. (1984). Penetration of VP 16-213 into cerebrospinal fluid after high-dose intravenous administration. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, *2*(3), 215–220. https://doi.org/10.1200/JCO.1984.2.3.215

Pradeep, C. R., & Kuttan, G. (2003). Effect of beta-carotene on the inhibition of lung metastasis in mice. *Phytomedicine : international journal of phytotherapy and phytopharmacology*, *10*(2-3), 159–164. https://doi.org/10.1078/094471103321659870

Rapoport, A., Guzhova, I., Bernetti, L., Buzzini, P., Kieliszek, M., & Kot, A. M. (2021). Carotenoids and Some Other Pigments from Fungi and Yeasts. *Metabolites*, *11*(2), 92. https://doi.org/10.3390/metabo11020092

Ren, L. W., Li, W., Zheng, X. J., Liu, J. Y., Yang, Y. H., Li, S., Zhang, S., Fu, W. Q., Xiao, B., Wang, J. H., & Du, G. H. (2022). Benzimidazoles induce concurrent apoptosis and pyroptosis of human glioblastoma cells via arresting cell cycle. *Acta pharmacologica Sinica*, *43*(1), 194–208. https://doi.org/10.1038/s41401-021-00752-y

Rodríguez-Camacho, A., Flores-Vázquez, J. G., Moscardini-Martelli, J., Torres-Ríos, J. A., Olmos-Guzmán, A., Ortiz-Arce, C. S., Cid-Sánchez, D. R., Pérez, S. R., Macías-González, M. D. S., Hernández-Sánchez, L. C., Heredia-Gutiérrez, J. C., Contreras-Palafox, G. A., Suárez-Campos, J. J. E., Celis-López, M. Á., Gutiérrez-Aceves, G. A., & Moreno-Jiménez, S. (2022). Glioblastoma Treatment: State-of-the-Art and Future Perspectives. *International journal of molecular sciences*, *23*(13), 7207. https://doi.org/10.3390/ijms23137207

Saini, R. K., Prasad, P., Lokesh, V., Shang, X., Shin, J., Keum, Y. S., & Lee, J. H. (2022). Carotenoids: Dietary Sources, Extraction, Encapsulation, Bioavailability, and Health Benefits-A Review of Recent Advancements. *Antioxidants (Basel, Switzerland)*, *11*(4), 795. https://doi.org/10.3390/antiox11040795

Sasaki, M., Knobbe, C. B., Munger, J. C., Lind, E. F., Brenner, D., Brüstle, A., Harris, I. S., Holmes, R., Wakeham, A., Haight, J., You-Ten, A., Li, W. Y., Schalm, S., Su, S. M., Virtanen, C., Reifenberger, G., Ohashi, P. S., Barber, D. L., Figueroa, M. E., Melnick, A., … Mak, T. W. (2012). IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics. *Nature*, *488*(7413), 656–659. https://doi.org/10.1038/nature11323

Shin, J., Song, M. H., Oh, J. W., Keum, Y. S., & Saini, R. K. (2020). Pro-Oxidant Actions of Carotenoids in Triggering Apoptosis of Cancer Cells: A Review of Emerging Evidence. *Antioxidants (Basel, Switzerland)*, *9*(6), 532. https://doi.org/10.3390/antiox9060532

Siangcham, T., Vivithanaporn, P., & Sangpairoj, K. (2020). Anti-Migration and Invasion Effects of Astaxanthin against A172 Human Glioblastoma Cell Line. *Asian Pacific journal of cancer prevention : APJCP*, *21*(7), 2029–2033. https://doi.org/10.31557/APJCP.2020.21.7.2029

Sowmya Shree, G., Yogendra Prasad, K., Arpitha, H. S., Deepika, U. R., Nawneet Kumar, K., Mondal, P., & Ganesan, P. (2017). β-carotene at physiologically attainable concentration induces apoptosis and down-regulates cell survival and antioxidant markers in human breast cancer (MCF-7) cells. *Molecular and cellular biochemistry*, *436*(1-2), 1–12. https://doi.org/10.1007/s11010-017-3071-4

Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R. C., Ludwin, S. K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J. G., Eisenhauer, E., Mirimanoff, R. O., European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups, … National Cancer Institute of Canada Clinical Trials Group (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *The New England journal of medicine*, *352*(10), 987–996. https://doi.org/10.1056/NEJMoa043330

Torres, F. C., García-Rubiño, M. E., Lozano-López, C., Kawano, D. F., Eifler-Lima, V. L., von Poser, G. L., & Campos, J. M. (2015). Imidazoles and benzimidazoles as tubulin-modulators for anti-cancer therapy. *Current medicinal chemistry*, *22*(11), 1312–1323. https://doi.org/10.2174/0929867322666150114164032

Trogrlić, I., Trogrlić, D., Trogrlić, D., & Trogrlić, A. K. (2018). Treatment of glioblastoma with herbal medicines. *World journal of surgical oncology*, *16*(1), 28. https://doi.org/10.1186/s12957-018-1329-2

Tsuji, S., Nakamura, S., Maoka, T., Yamada, T., Imai, T., Ohba, T., Yako, T., Hayashi, M., Endo, K., Saio, M., Hara, H., & Shimazawa, M. (2020). Antitumour Effects of Astaxanthin and Adonixanthin on Glioblastoma. *Marine drugs*, *18*(9), 474. https://doi.org/10.3390/md18090474

Varricchio, A., Khan, S., Price, Z. K., Davis, R. A., Ramesh, S. A., & Yool, A. J. (2023). Pharmacological Inhibition of Membrane Signaling Mechanisms Reduces the Invasiveness of U87-MG and U251-MG Glioblastoma Cells In Vitro. *Cancers*, *15*(4), 1027. https://doi.org/10.3390/cancers15041027

Wang, M., Morón-Ortiz, Á., Zhou, J., Benítez-González, A., Mapelli-Brahm, P., Meléndez-Martínez, A. J., & Barba, F. J. (2023). Effects of pressurized liquid extraction with dimethyl sulfoxide on the recovery of carotenoids and other dietary valuable compounds

from the microalgae Spirulina, Chlorella and Phaeodactylum tricornutum. *Food chemistry*, *405*(Pt B), 134885. https://doi.org/10.1016/j.foodchem.2022.134885

Wang, J., Hu, X., Chen, J., Wang, T., Huang, X., & Chen, G. (2022). The Extraction of β-Carotene from Microalgae for Testing Their Health Benefits. *Foods (Basel, Switzerland)*, *11*(4), 502. https://doi.org/10.3390/foods11040502

Wei, H. J., Upadhyayula, P. S., Pouliopoulos, A. N., Englander, Z. K., Zhang, X., Jan, C. I., Guo, J., Mela, A., Zhang, Z., Wang, T. J. C., Bruce, J. N., Canoll, P. D., Feldstein, N. A., Zacharoulis, S., Konofagou, E. E., & Wu, C. C. (2021). Focused Ultrasound-Mediated Blood-Brain Barrier Opening Increases Delivery and Efficacy of Etoposide for Glioblastoma Treatment. *International journal of radiation oncology, biology, physics*, *110*(2), 539–550. https://doi.org/10.1016/j.ijrobp.2020.12.019

Weller, M., van den Bent, M., Preusser, M., Le Rhun, E., Tonn, J. C., Minniti, G., Bendszus, M., Balana, C., Chinot, O., Dirven, L., French, P., Hegi, M. E., Jakola, A. S., Platten, M., Roth, P., Rudà, R., Short, S., Smits, M., Taphoorn, M. J. B., von Deimling, A., … Wick, W. (2021). EANO guidelines on the diagnosis and treatment of diffuse gliomas of adulthood. *Nature reviews. Clinical oncology*, *18*(3), 170–186. https://doi.org/10.1038/s41571-020-00447-z

Whitworth, J. M., Londoño-Joshi, A. I., Sellers, J. C., Oliver, P. J., Muccio, D. D., Atigadda, V. R., Straughn, J. M., Jr, & Buchsbaum, D. J. (2012). The impact of novel retinoids in combination with platinum chemotherapy on ovarian cancer stem cells. *Gynecologic oncology*, *125*(1), 226–230. https://doi.org/10.1016/j.ygyno.2011.12.425

Wierzbowska, N., Olszowski, T., Chlubek, D., Kozłowski, M., & Cymbaluk-Płoska, A. (2024). Vitamins in Gynecologic Malignancies. *Nutrients*, *16*(9), 1392. https://doi.org/10.3390/nu16091392

Wu, W., Klockow, J. L., Zhang, M., Lafortune, F., Chang, E., Jin, L., Wu, Y., & Daldrup-Link, H. E. (2021). Glioblastoma multiforme (GBM): An overview of current therapies and mechanisms of resistance. *Pharmacological research*, *171*, 105780. https://doi.org/10.1016/j.phrs.2021.105780

Wu, H. L., Fu, X. Y., Cao, W. Q., Xiang, W. Z., Hou, Y. J., Ma, J. K., Wang, Y., & Fan, C. D. (2019). Induction of Apoptosis in Human Glioma Cells by Fucoxanthin via Triggering of ROS-Mediated Oxidative Damage and Regulation of MAPKs and PI3K-AKT Pathways. *Journal of agricultural and food chemistry*, *67*(8), 2212–2219. https://doi.org/10.1021/acs.jafc.8b07126

Yadav, A., Alnakhli, A., Vemana, H. P., Bhutkar, S., Muth, A., & Dukhande, V. V. (2022). Repurposing an Antiepileptic Drug for the Treatment of Glioblastoma. *Pharmaceutical research*, *39*(11), 2871–2883. https://doi.org/10.1007/s11095-022-03399-4

Yin, N., Xie, T., Zhang, H., Chen, J., Yu, J., & Liu, F. (2020). IDH1-R132H mutation radiosensitizes U87MG glioma cells via epigenetic downregulation of TIGAR. *Oncology letters*, *19*(2), 1322–1330. https://doi.org/10.3892/ol.2019.11148

Zhou, J., Xu, N., Liu, B., Wang, C., He, Z., Lenahan, C., Tang, W., Zeng, H., & Guo, H. (2022). lncRNA XLOC013218 promotes cell proliferation and TMZ resistance by targeting the PIK3R2-mediated PI3K/AKT pathway in glioma. *Cancer science*, *113*(8), 2681–2692. https://doi.org/10.1111/cas.15387

Zhu, Y., Liu, X., Zhao, P., Zhao, H., Gao, W., & Wang, L. (2020). Celastrol Suppresses Glioma Vasculogenic Mimicry Formation and Angiogenesis by Blocking the PI3K/Akt/mTOR Signaling Pathway. *Frontiers in pharmacology*, *11*, 25. https://doi.org/10.3389/fphar.2020.00025

APPENDIX

APPENDIX A. Statistycal Analyses of IC50 β-carotene , Etoposid and Temozolomide

Table A.1. Statistycal Analyses of IC50 of β-carotene

Table A.2. Statistycal Analyses of IC50 of Etoposid

Table A.3. Statistycal Analyses of IC50 of Temozolomide

APPENDIX B. Statistical Analyses and t-Tests for 24h and 48h Cytotoxicity

Table B.1. 24h Cytotoxicity Statistical Analyses

Table B.2. t-Test Analyses of 24h Cytotoxicity for Temozolomid, Etoposid, β-carotene, and their Combinations

Table B.3. Statistical Analyses of 48h Cytotoxicity

Table B.4. t-Test Analyses of 48h Cytotoxicity for Temozolomid, Etoposid, β-carotene, and their Combinations

APPENDIX A

Table.A.1. Statistycal Analyses of IC50 of β-carotene (p<0,001)

Groups	Count	Sum	Average	Variance
0	3	0,511	0,170333	3,23E-05
0,75	3	0,332	0,110667	5,03E-05
1,5	3	0,224	0,074667	9,33E-06
3	3	0,181	0,060333	3,73E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0,021635	3	0,007212	223,0447	4,77E-08	4,066181
Within Groups	0,000259	8	3,23E-05			
Total	0,021894	11				

Table A.2. Statistycal Analyses of IC50 of Etoposid (p<0,001)

Groups	Count	Sum	Average	Variance
0	3	0,999	0,333	5,7E-05
5	3	0,895	0,298333	4,93E-05
10	3	0,707	0,235667	5,03E-05
20	3	0,568	0,189333	9,23E-05
40	3	0,341	0,113667	2,63E-05
60	3	0,287	0,095667	5,63E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0,139094	5	0,027819	503,2533	1,67E-13	3,105875
Within Groups	0,000663	12	5,53E-05			
Total	0,139757	17				

Table A.3. Statistycal Analyses of IC50 of Temozolomide (p<0,001)

Groups	Count	Sum	Average	Variance
0	3	0,885	0,295	5,7E-05
500	3	0,811	0,270333	2,03E-05
1000	3	0,622	0,207333	1,63E-05
1250	3	0,555	0,185	3,6E-05
1500	3	0,44	0,146667	2,03E-05
1750	3	0,334	0,111333	5,83E-05
2000	3	0,21	0,07	0,000067

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0,119968	6	0,019995	508,339	1,48E-15	2,847726
Within Groups	0,000551	14	3,93E-05			
Total	0,120519	20				

APPENDIX B. Statistical Analyses for 24h and 48h Cytotoxicity

Table B.1. Statistical Analyses of 24h Cytotoxicity (p<0,001)

Groups	Count	Sum	Average	Variance
Control	3	0,639	0,213	5,2E-05
β-Carotene	3	0,314	0,104667	4,43E-05
Etoposide	3	0,328	0,109333	6,33E-06
Temozolomide	3	0,321	0,107	3,1E-05
β-Carotene+Etoposide	3	0,214	0,071333	6,33E-06
β-Carotene+Temozolomide	3	0,427	0,142333	1,73E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0,03572	5	0,007144	272,4377	6,43E-12	3,105875
Within Groups	0,000315	12	2,62E-05			
Total	0,036034	17				

Table B.2. 24h Cytotoxicity for Temozolomid, Etoposid, β-carotene, and their Combinations t-Test Analyses (p<0,001)

t-Test: Paired Two Samplefor Means			t-Test: Paired Two Sample for Means			t-Test: Paired Two Sample for Means		
	Control	*δ-Carotene*		*Control*	*Etoposide*		*Control*	*Temozolomide*
Mean	0,213	0,104667	Mean	0,213	0,109333	Mean	0,213	0,107
Variance	5,2E-05	4,43E-05	Variance	5,2E-05	6,33E-06	Variance	5,2E-05	3,1E-05
Observations	3	3	Observations	3	3	Observations	3	3
Pearson Correlation	0,166618		Pearson Correlation	-0,38573		Pearson Correlation	-0,92155	
Hypothesized Mean Difference	0		Hypothesized Mean Difference	0		Hypothesized Mean Difference	0	
df	2		df	2		df	2	
t Stat	20,93509		t Stat	21,11206		t Stat	14,65267	
P(T<=t) one-tail	0,001137		P(T<=t) one-tail	0,001118		P(T<=t) one-tail	0,002313	
t Critical one-tail	2,919986		t Critical one-tail	2,919986		t Critical one-tail	2,919986	
P(T<=t) two-tail	0,002274		P(T<=t) two-tail	0,002236		P(T<=t) two-tail	0,004625	
t Critical two-tail	4,302653		t Critical two-tail	4,302653		t Critical two-tail	4,302653	

t-Test: Paired Two Sample for Means			t-Test: Paired Two Sample for Means		
	Control	*δ-Carotene+Etoposide*		*Control*	*δ-Carotene+Temozolomide*
Mean	0,213	0,071333	Mean	0,213	0,142333
Variance	5,2E-05	6,33E-06	Variance	5,2E-05	1,73E-05
Observations	3	3	Observations	3	3
Pearson Correlation	-0,38573		Pearson Correlation	0,99926	
Hypothesized Mean Difference	0		Hypothesized Mean Difference	0	
df	2		df	2	
t Stat	28,85088		t Stat	40,06423	
P(T<=t) one-tail	0,0006		P(T<=t) one-tail	0,000311	
t Critical one-tail	2,919986		t Critical one-tail	2,919986	
P(T<=t) two-tail	0,001199		P(T<=t) two-tail	0,000622	
t Critical two-tail	4,302653		t Critical two-tail	4,302653	

Table B.3. Statistical Analyses of 48h Cytotoxicity (p<0,001)

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	1,128	0,376	3,9E-05
β-Carotene	3	0,487	0,162333	1,73E-05
Etoposide	3	0,628	0,209333	4,23E-05
Temozolomide	3	0,678	0,226	0,000019
β-Carotene+Etoposide	3	0,431	0,143667	3,03E-05
β-Carotene+Temozolomide	3	0,8	0,266667	4,43E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,105399	5	0,02108	657,6042	3,37E-14	3,105875
Within Groups	0,000385	12	3,21E-05			
Total	0,105784	17				

Table B.4. t-Test Analyses for 24h Cytotoxicity for Temozolomid, Etoposid, β-carotene, and their Combinations (p<0,001)

t-Test: Paired Two Sample for Means			t-Test: Paired Two Sample for Means			t-Test: Paired Two Sample for Means	
Control	*β-Carotene*		*Control*	*Etoposide*		*Control*	*Temozolomide*
0,376	0,162333	Mean	0,376	0,209333	Mean	0,376	0,226
3,9E-05	1,73E-05	Variance	3,9E-05	4,23E-05	Variance	3,9E-05	0,000019
3	3	Observations	3	3	Observations	3	3
0,038462		Pearson Correlation	0,947517		Pearson Correlation	0,165312	
0		Hypothesized Mean Difference	0		Hypothesized Mean Difference	0	
2		df	2		df	2	
50,20699		t Stat	138,675		t Stat	37,11537	
0,000198		P(T<=t) one-tail	2,6E-05		P(T<=t) one-tail	0,000363	
2,919986		t Critical one-tail	2,919986		t Critical one-tail	2,919986	
0,000396		P(T<=t) two-tail	5,2E-05		P(T<=t) two-tail	0,000725	
4,302653		t Critical two-tail	4,302653		t Critical two-tail	4,302653	

t-Test: Paired Two Sample for Means			t-Test: Paired Two Sample for Means		
Control	*ß-Carotene+Etoposide*		*Control*	*ß-Carotene+Temozolomide*	
0,376	0,143667	Mean	0,376	0,266667	
3,9E-05	3,03E-05	Variance	3,9E-05	4,43E-05	
3	3	Observations	3	3	
0,188982		Pearson Correlation	0,553134		

Hypothesized Mean Difference	0		Hypothesized Mean Difference	0	
df	2		df	2	
t Stat	53,61538		t Stat	30,99309	
P(T<=t) one-tail	0,000174		P(T<=t) one-tail	0,00052	
t Critical one-tail	2,919986		t Critical one-tail	2,919986	
P(T<=t) two-tail	0,000348		P(T<=t) two-tail	0,001039	
t Critical two-tail	4,302653		t Critical two-tail	4,302653	