TÜRKİYE CUMHURİYETİ
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL
BIOENGINEERING DEPARTMENT

INVESTIGATION OF THE CYTOTOXIC ACTIVITY OF ASTAXANTHIN ON GLIOBLASTOMA CELLS

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M.Sc.

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ÖZET

ASTAKSANTİN'İN GLİOBlASTOMA HÜCRELERİ ÜZERİNDEKİ SİTOTOKSİK AKTİVİTESİNİN ARAŞTIRILMASI

Nur ZARKAVI

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Glioblastoma, beyin tümörlerinin en agresif ve zorlu formlarından birini temsil eder; hızlı ilerleyişi, invaziv doğası ve geleneksel tedavilere direnci ile karakterize edilir. Cerrahi, radyasyon ve kemoterapi gibi tedavi yöntemlerinde önemli ilerlemelere rağmen, glioblastoma hastalarının prognozu hala kötüdür ve genel sağkalım oranlarında sınırlı iyileşmeler vardır. Glioblastoma'nın kompleks moleküler ve hücresel heterojenliği, yüksek derecede infiltratif doğası ve bağışıklık gözetiminden kaçabilme yeteneği, etkili tedavi stratejileri geliştirmekte önemli zorluklar oluşturur.

Astaxanthin, anti-inflamatuar, antioksidan ve antikanser etkileri de dahil olmak üzere çeşitli farmakolojik aktiviteleri nedeniyle dikkat çekmektedir. Astaksantinin umut verici terapötik potansiyeline rağmen, nispeten az sayıda çalışma özellikle glioblastoma hücreleri üzerinde sitotoksik aktivitesini araştırmıştır. Bu bağlamda, mevcut çalışmada, güçlü antioksidan özelliklere sahip doğal bir bileşik olan astakansantin Astaksantinin glioblastoma hücrelerine karşı tek başına ve kemoterpötik ilaçlarla (Etoposide) birlikte sierjistik sitotoksik aktivitesi araştırılmışdır.. Hücre canlılığı analizleri, koloni oluşumu deneyleri ve moleküler analizler gibi çeşitli in vitro deneylerin kombinasyonunu kullanarak, Astaksantinin glioblastoma hücrelerindeki sitotoksik etkilerinin ve altta yatan mekanizmalarının aydınlatılması ve Astaksantin ile etoposidin potansiyel sinerjistik etkilerini değerlendirmesi amaçlamıştur Ayrıca, bu çalışmada Astaksantinin anti mikrobiyal etkinliği araştırılmış lup bu dai alanda az sayıda çalışma yapılmış olmasından dolayı bir ek bakış açısı sunmaktadır.

Bu çalışmanın bulguları, Astaksantin, etoposid ve bunların kombinasyonu ile doza bağlı olarak hücre canlılığında azalmaları göstermiştir, bulgular IC50 değerlendirmeleri tarafından desteklenmiştir. Astaksantin ve etoposid ayrıca U87MG hücrelerinde klonojenik potansiyeli inhibe etmiş ve morfolojik değişiklikler oluşturmuş, aynı zamanda survivin ve c-Myc'in düzeylerini düşürmüş ve Caspase-3 ekspresyonunu artırmıştır, bu da güçlü apoptotik etkileri

işaret etmektedir. Ek olarak, Astaksantin Bacillus subtilis, Bacillus, Staphylococcus aureus ve Escherichia coli'ye karşı antimikrobiyal aktivite göstermiştir.

Anahtar Kelimeler: Astaksantin, Etoposide, Glioblastoma.

ABSTRACT

THE INVESTIGATION OF THE CYTOTOXIC ACTIVITY OF ASTXANTHIN ON GLIOBLASTOMA CELLS

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Glioblastoma is well known among cancer cells to be one of the most formidable and highly proliferative forms of brain tumors. Despite the fact that there are numerous significant milestones in treatment that include chemotherapy, surgery, and radiation, its rapid progression , invasive nature and resistance to conventional therapies characteristics make it difficult to manage the spread of this disease and to enhance the several rates in total. In other words, glioblastoma is thought to have a very complex cellular and molecular heterogeneity coupled with highly infiltrative nature and ability to control immune system mechanisms which present obstacles in achieving suitable treatment approaches.

Astaxanthin (ASX) has received much attention for its anticancer, antioxidant, anti-inflammatory, and anti-aging properties. However, the studies that explore the cytotoxic effects of ASX on glioblastoma cells remain poor. To address this gab, this study aims to investigate the cytotoxic potential of ASX against glioblastoma cells as a standalone treatment and in combination with the chemotherapeutic drug Etoposide.

In order to perform this , a series of in vitro experiments that include cell viability tests, colony formation assays, and molecular analyses was done. Additionally, it was examined whether combining ASX with Etoposide enhances the cytotoxic effects against these cancer cell lines. Furthermore, this research further study the antimicrobial properties of ASX to expand our understanding of its potential applications as relatively few studies were conducted in this area.

The findings of this study indicated dose-dependent reductions in cell viability with ASX, etoposide, and their combination, supported by IC50 assessments. ASX and etoposide also

prevented clonogenic potential and caused morphological changes in U87MG cells, while downregulating survivin and c-Myc and upregulating Caspase-3 expression, indicating potent apoptotic effects. Moreover, the antimicrobial activity of ASX against Bacillus subtilis, Bacillus, Staphylococcus aureus, and Escherichia coli, was demonstrated suggesting further studies into its antimicrobial effectiveness and mechanisms of action.

Overall, this thesis offers new perspective in the use of ASX in treating glioblastoma, highlighting its therapeutic potential importance as a natural substance with promising anticancer properties.

Keywords: Astaxanthin, Etoposide, Glioblastoma.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ASX	: Astaxanthin
GBM	: Glioblastoma
U87MG	: Glioblastoma Cell Lines
TMZ	: Temozolomide
DMEM	: Dulbecco's Modified Eagle's Medium
FBS	: Fetal Bovine Serum
PBS	: Phosphate-Buffered Saline
DAPI	: 4',6-diamidino-2-phenylindole, dihydrochloride
SEM	: Scanning Electron Microscopy
IC50	: Half-Maximal Inhibitory Concentration
BSA	: Bovine Serum Albumin
MICs	: Minimum Inhibitory Concentrations

LIST OF SYMBOLS

µL	**:** Microliter
mL	**:** Milliliter
µM	**:** Micromolar
mm	**:** Milimeter
µg	**:** Micrgram
h	**:** hour

1. INTRODUCTION

Cancer is a serious health concern accounting for one out of every six deaths globally (Debela et al., 2021). In 2020, there were approximately 19.3 million new cancer cases and around 10 million cancer-related deaths worldwide (Debela et al., 2021). It has a highly complicated development of disease conditions marked by an uncontrolled growth. Over many years, individuals had limited treatment choices, such as chemotherapy, surgery, and radiation therapy, either used alone or in conjunction(Debela et al., 2021; Knight et al., 2021).
Breakthroughs in research have developed treatment options and improved outcomes for many cases. However, despite these advancements, challenges remain, and more studies need to be done in order to develop new drugs to combat this disease.

Glioblastoma (GBM) is known as a type of brain cancer that forms from star-shaped cells such as microglia, astrocytes, oligodendrocytes, and ependymal cells, or from a subpopulation of CSCs present in the tissue, it is therefore thought to originate by genetic alterations affecting neuroglial stem or progenitor cells (D'alessio et al., 2019.). Despite extensive experimental research and advancements in therapeutic approaches, GBM remains largely incurable, with an average survival duration of 12 to 18 months, and fewer than 5% of patients survive beyond five years post-diagnosis (D'Alessio et al., 2019). Many features of GBM play an essential role in its resistance to treatment, these features include the blood-brain barrier which prevents drug delivery to the brain, the tumor's heterogeneity that leads to different drug sensitivities among cell populations, and the tumor cells' infiltration into healthy brain tissue which causes recurrence and increased tumor progression. (Molina et al., 2010).

In the past years, there has been an increased attention towards investigating the influence of natural compounds on human health, particularly those showing ability in fighting cancer. (Faraone et al., 2020). Many natural substances have been shown effectiveness in decreasing cancer cell migration and progression.Examples include Vinblastinae, Taxol, and Vincristine , which are currently used in chemotherapy (Faraone et al., 2020).

Among carotenoids, astaxanthin, Lycopene, crocin, β-cryptoxanthin, β-carotene and lutein, among carotenoids, are pivotal plant pigments that exert important roles in the treatment and prevention of various diseases, including cancer. Astaxanthin (ASX), a red xanthophyll carotenoid found in marine animals and various microorganisms, is often referred to as the

'super antioxidant' for its remarkable antioxidant properties among other carotenoids. Recent studies have investigated the ASX's potential in combating cancer and reducing the spread of tumor. ASX operates through at least six different mechanisms that include reducing the proliferation of malignant cells, promoting cell death, decreasing oxidative stress, reducing inflammation, prevention of cancer metastasis and improving communication between cells (L. Zhang & Wang, 2015).

Numerous studies have found that ASX usually exerts several anti-cancer properties on different types of cancer cells including lung, skin, breast, and prostate cancers, these properties include:

1. Antioxidant Effects: ASX is recognized as a strong t antioxidant that neutralizes free radicals and reduces oxidative stress, thereby helping the cells to be protected from DNA damage, which is a crucial factor in cancer development.

2. Anti-inflammatory Effects: Chronic inflammation is strongly linked to cancer progression. ASX has been shown to block inflammatory pathways and reduce the production of pro-inflammatory substances which in turn lead to slowing cancer growth and metastasis.

3. Induction of Apoptosis: ASX was found to induce apoptosis in cancer cells, helping in the removal of abnormal or damaged cells and prevent their uncontrolled proliferation and spread.

4. Inhibition of Cancer Cell Proliferation: Laboratory studies have shown that ASX can stop the proliferation of different cancer cell lines, which may aid in limiting tumor growth

5. Anti-angiogenic Effect: The formation of new blood vessels is known to be defined as angiogenesis, this process plays an essential role in developing tumor growth and metastasis. It was reported that “ASX could inhibit this process, disrupting the blood supply to tumors and reducing their progression” (L. Zhang & Wang, 2015). Despite the ongoing studies on ASX’s effect on cancer cells, recent findings indicate its potential anti-cancer properties as a natural compound. However, studies that imply clinical trials are further needed to deeply understand how its mechanisms of action can affect cancer prevention and treatment (Faraone et al., 2020; L. Zhang & Wang, 2015).

the mechanisms underlying ASX's actions on GBM cells could pave the way for the development of new therapeutic strategies that can control this aggressive cancer. In this study,

the cytotoxic activity of ASX on GBM cell line will be examined to pave the way for the improvement of new strategies and to enrich the literature about the anti-cancer properties of ASX indicating its promise as a therapeutic agent that can help in GBM treatment.

2. LITERATURE REVIEW

2.1. Glioblastoma

GBM is described to be one of the most malignant and aggressive cancers that primarily affects the brain and the central nervous system (Grochans et al., 2022). It accounts for 14.5% of cases and almost half of all malignant tumors in the nervous system. These tumors are thought to arise from star-shaped cells such as oligodendrocytes, microglia, astrocytes, and ependymal cells, or from a subset of Cancer Stem Cells (CSCs) present in the tissue.(Fig.2.1) (Grochans et al., 2022).

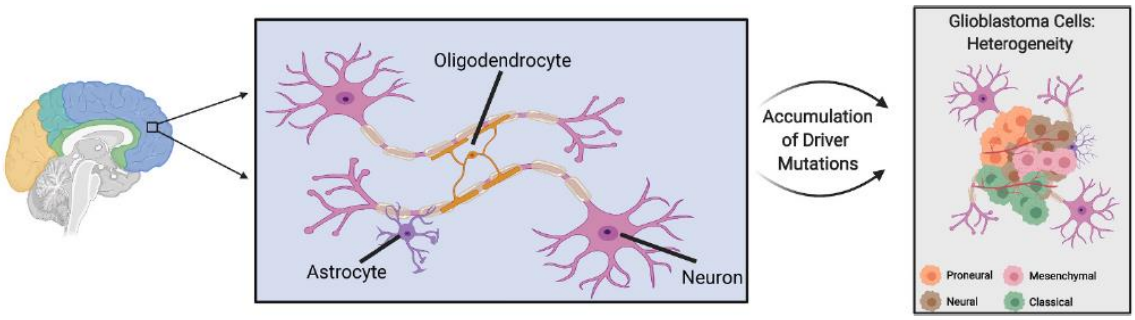


Figure 2. 1 GBM arises from neural stem cells (NSCs), astrocytes derived from NSCs, and oligodendrocyte precursor cells (OPCs) that are present in brain tissue. This figure is taken from (Taylor et al., 2019).

GBM exhibits significant variability both between tumors and within individual tumors, leading to its classification into neural, proneural, classical, and mesenchymal subtypes (Fig. 2.1)(Taylor et al., 2019).

From the early stages of neuro-oncology, numerous discussions and debates have arisen regarding the origin of GBM cells, whether they arise from embryonic cells or dedifferentiated cells. Through various trials and observations, it has been theorized that the cellular source of GBM possesses stem cell characteristics, and this particular cell type has been suggested to be responsible for tumor reoccurrence (Flores Ledur et al., 2017).

The World Health Organization (WHO) classification system is the internationally accepted standard for naming and diagnosing gliomas. In this system, GBM is categorized as a grade IV cancer. The fourth WHO classification, introduced in 2016, evaluates gliomas according to

histopathological criteria to determine their malignancy level, resulting in the recognition of four distinct types of this tumor (Wesseling & Capper, 2018). This classification was determined by studying both the tumor's histological features and the molecular alterations happening within the cells and it includes :

- Glioblastoma, isocitrate dehydrogenase (IDH) wildtype, which respresnts 90% of cases and typically develops spontaneously around the age of 60 (Wesseling & Capper, 2018).

- Glioblastoma with IDH mutation, observed in approximately 10% of cases, typically displays as a secondary GBM. It commonly arises in younger patients with higher-grade gliomas (WHO grades I-III) and is linked with more favorable prognosis compared to the wildtype IDH variant(Wesseling & Capper, 2018).

- Glioblastoma not otherwise specified (NOS) refers to cases where the IDH mutation status remains unknown due to insufficient molecular or histological material for testing(Wesseling & Capper, 2018).

- Not-elsewhere-classified (NEC) GBM is a newer category category that does not fit with established categories in 2016 WHO classification despite extensive efforts that aim to classify it.This scenario may arise due to discrepancies among clinical, histological, immuno histological, and genetic tumor features. Additionally, there may be a GBM subtype with a unique combination of features that has not yet been recognized in the WHO classification(Wesseling & Capper, 2018).

The survival rate for GBM patients is generally low, with a median lifespan of 15 months to 21 months (Grochans et al., 2022). Pinpointing the exact incidence of GBM is challenging due to variations in reporting, ranging from 3.19 to 4.17 cases per 100,000 person-years. In children, the incidence is notably reduced to 0.85 per 100,000, with pediatric glioblastoma (p-GBM) forming a substantial but minority share of primary brain tumors in this age group. Despite being relatively rare, the primary tumors in the central nervous system rank as the second most frequent cancer type in children, and they are the predominant form among solid tumors within this age group (Grochans et al., 2022).

2.1.1. Key Features and Mechanisms of Glioblastoma Chemoresistance:

GBM can develop resistance to current treatment methods such as chemotherapy and radiotherapy. Key features contribute to the resistance of GBM to therapy are , the restriction of drug distribution to the brain by the blood brain barrier, limiting the effectiveness of treatments, the tumor's heterogeneity, which comprises cell populations with varying sensitivities to drugs, complicates treatment strategies, the tumor cells' tendency to infiltrate normal brain tissue contributes to recurrences, complicating therapeutic outcomes even further. (Molina et al., 2010). Beside these, the presence of cancer stem cells (CSCs) plays a significant role (Lathia et al., 2015).

1.0.1.1 Blood Brain Barrier (BBB) :

One of the major challenges in delivering chemotherapy drugs into the brain tumor cells is the existence of the blood brain barrier (BBB). The BBB restricts the entry of chemeotherapeutic drugs into the brain, limiting their effectiveness in reaching tumor cells (Fig.2.2) (Dymova et al., 2021; Ou et al., 2021). BBB is located between the extracellular space and the circulatory system in the central nervous system.BBB is shaped by endothelial cells tightly connected by junctions and helps in the protection of the brain from infectious pathogens and environmental neurotoxic substances (Harder et al., 2018).

In GBM, the blood-brain barrier undergoes heterogeneous interruption resulting in the formation of blood-tumor-brain barrier, marked by irregular pericyte dispersion, diminished tight junctions, absence of astrocytic endfeet, and heightened permeability to circulating immune cells (Abbott et al., 2006; Ou et al., 2021).

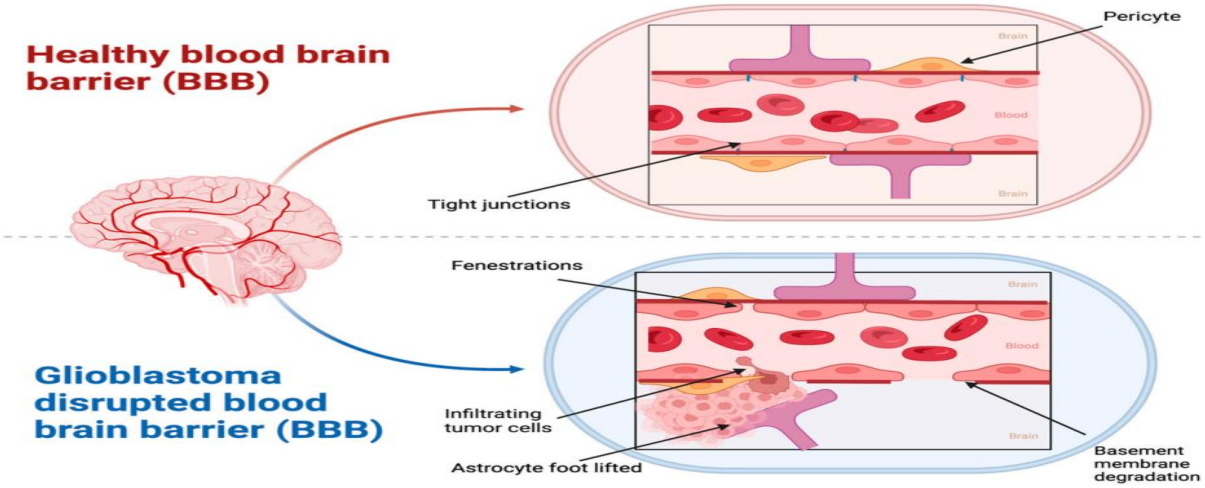


Figure 2. 2 Representation of the features of a healthy BBB and a disrupted BBB in GBM.This figure was taken from (Ou et al., 2021).

1.0.1.2 High Infiltration:

Another obstacle in GBM treatment is the high infiltrative nature of GBM that makes it nearly impossible to complete removal at the cellular level (Lara-Velazquez et al., 2017)(Lara-Velazquez et al., 2017).

Furthermore, abundant hypoxic areas make habitats for glioma initiating cells (GICs). These self- regenerating cells have the capacity to generate aggresively recurrent tumors resilient to both chemotherapy and radiation(Da Ros et al., 2018).

1.0.1.3 Heterogeneity:

GBM tumors consist of diverse cell populations with varying sensitivities to chemotherapy. This heterogeneity makes it challenging to target all tumor cells effectively and envelops both inter-tumor heterogeneity (variations between different populations) and intra-tumor heterogeneity (variations within individual tumors). This wide-ranging diversity presents a substantial obstacle in developing successful therapies. Moreover, cell-mediated immune resistance in cancer adds another layer of complexity to treatment approaches for GBM(Becker et al., 2021).

GBM heterogeneity is elucidated by clonal evolution and the existence of stem cells. The occurence of subsequent mutations in a cell lead to the development of clonal expansions that

proliferate under various selective influences within the tumor microenvironment, such as immune evasion, competition for resources, acidosis, hypoxia and treatments. Initially, a benign neoplasm is believed to form, with malignant transformation occurring only upon the accumulation of mutations. Additionally, inherited modifications in the epigenome could grant beneficial characteristics to diverse subgroups, aiding in their clonal proliferation. It's noted that as tumor advancement progresses, there's an escalation in the appearance of clonal expansions, potentially intensifying inter-clonal diversity (Marjanovic et al., 2013; Yalamarty et al., 2023).

1.0.1.4 Cancer Stem Cells (CSCs):

CSCs within GBM possess inherent resistance to chemotherapy and radiotherapy. These cells can regenerate the tumor even after treatment, leading to recurrence. Tumor-resident CSCs capable of proliferation and differentiation into various types of cells leading to tumor growth and recurrence has a significant role in chemo-radiation resistance in GBM. A major obstacle in administering therapies to GBM CSCs is their positioning in the brain, protected by the blood–brain barrier (BBB). This poses a considerable obstacle for transporting chemotherapeutic agents and other therapeutic substances to the tumor location by effective way (Beier et al., 2011; Yalamart(Beier et al., 2011; Yalamarty et al., 2023)Also, because cancer stem cells have special traits like being really good at surviving cell death, being able to form cancer clusters, and having certain proteins that push drugs out, it's hard to treat them with regular chemotherapy drugs.

1.0.1.5 DNA Repair Mechanism:

GBM cells often have enhanced DNA repair mechanisms, allowing them to repair damage caused by chemotherapy drugs and evade cell death.These repair mechanisms are usually divided in two systems (Taylor et al., 2019).

1.0.1.5.1 Single Strand Break Repair Systems:

Repair mechanisms for single-strand breaks (SSBs) include nucleotide excision repair (NER), mismatch repair (MMR), and base excision repair (BER). These systems start by identifying

and eliminating the damaged segment of the DNA. Afterwards, in order to synthsize a new sequence and close the break , a complementary strand is used (Jackson & Bartek, 2009).

The mutations that are involoved in these SSB repair pathways have a significant role in cell's ability to respond to therapy and also in resistance mechanisms. Deficiencies in mismatch repair (MMR) that is usually caused by mutations in genes like MLH, MSH2, MSH6, and PMS2, results in microsatellite instability and has been linked with resistance mechanism to the chemotherapeutic agents like temozolomide (Hegi et al., 2005).

The mutations in nucleotide excision repair (NER) pathway genes, such as ERCC1, ERCC2 (XPD), and ERCC3 (XPB), usually affect the repair of bulky DNA adducts that are caused by chemotherapy drugs like temozolomide (TMZ) (Hegi et al., 2005).These mutations can contribute to the resistance mechanism in GBM by reducing the efficiency of DNA repair processses. Likewise, the mutations in genes involved in base excision repair (BER) can influence the repair system of single-strand breaks caused by oxidative stress and chemotherapy agents (Reardon et al., 2008). This can lead to alterations in DNA repair system and sensitivity to DNA damaging therapies in GBM.

1.0.1.5.2 Double Strand Break Repair Systems:

The double strand break repair system is more complex becasue it involves both strands, and this arises when DNA polymerase lacks a template for replication, hindering the formation of a new strand due to the absence of DNA formtion from both strands (Jackson & Bartek, 2009)(Jackson & Bartek, 2009). Two different systems are included in the repair system of double-strand breaks (DSBs): non-homologous end-joining (NHEJ) and homologous recombination (HR) (Ferri et al., 2020).

HR becomes activated when cells undergo damage within the cell cycle during the late S/G2 phase, but, when damage occurs in various phases of the cell cycle and a second copy of the DNA is lacking, NHEJ becomes activated (Li & Heyer, 2008).

Mutations in genes included in DSB repair pathways play are essential in GBM resistance mechanism. BRCA1 and BRCA2 mutations, which have important roles in HR repair, are linked to impaired repair of DSBs and increased genomic instability in several cancer types, including glioblastoma ((Brennan et al., 2013). These mutations can contribute to resistance to

therapies that cause DNA damage, such as ionizing radiation and alkylating agents such as temozolomide (Stupp et al., 2005).

Mutations in ATM and ATR which are crucial in regulating DNA damage response pathways, can diminish DSB repair efficiency and contribute to therapy resistance in glioblastoma (Stupp et al., 2005). Loss or mutation of TP53, which plays an essential role in cell cycle regulation and DNA repair, further worsens genomic instability and promotes resistance to DNA-damaging therapies (McLendon et al., 2008).

Moreover, DSB repair mechanisms are also influenced by PTEN, which is a tumor suppressor gene frequently mutated in glioblastoma and affects cell survival and proliferation, thereby affecting therapeutic outcomes (Koul, 2008). Alterations in the epidermal growth factor receptor (EGFR), including amplification and the EGFRvIII mutation, can dysregulate DSB repair pathways and contribute to therapeutic resistance in GBM (Vivanco & Sawyers, 2002).

1.0.1.6 Mechanisms of Drug Efflux in GBM:

GBM cells use active efflux mechanism to develop resistance, this mechanism is based on reducing the effectiveness of chemotherapy drugs by pumping them out of the cells, this is usually achieved by multiple drug resistance proteins through cellular membrane(Haar et al., 2012).

The transportation of drugs across the membrane typically occurs through ATP-independent mechanisms or via ATP-dependent proteins. The ATP-dependent category encompasses the ATP-binding cassette (ABC) transporter family, including resistance protein (MRP), P-glycoprotein (P-gp), and breast cancer resistance protein (BCRP) (Haar et al., 2012; Molnár et al., 2010). The overexpression of P-dp by MDR1 gene is known to be the major cause of resistance to anticancer drugs. When P-glycoprotein (P-gp) is excessively produced, it mainly works by stopping ATP from breaking down, disturbing the lipid membrane, or blocking specific sites. This means P-gp has an impact in both natural and acquired drug resistance (Tsuruo et al., 2003).For instance, erlotinib, a drug that blocks EGFR and could help treat GBM, hasn't worked well in trials because P-gp and Bcrp1 interfere with its absorption (De Vries et al., 2012).

1.0.1.7 Tumor Microenvironment :

The unique microenvironment within GBM, including hypoxia and acidic conditions, can promote chemoresistance by providing a protective niche for tumor cells. Like many solid tumors, glycolysis produces ATP in both oxygen-rich and oxygen-poor (hypoxic) environments. Increased glycolysis in tumors can make the environment more acidic, which might affect how certain drugs are taken up by tumor cells (Harder et al., 2018; Manallack, 2007).

In some regions of tumors, there's often not enough oxygen, a condition called hypoxia. Cells in these areas grow slowly and need more energy, but they survive using other mechanisms. A protein called hypoxia inducible factor 1 (HIF-1) triggers a process that boosts the growth of blood vessels (angiogenesis) and activates a type of self-cleaning process in cells called macroautophagy (autophagy) (Bellot et al., 2009).These survival mechanisms that are exerted by cells contribute to the development of a malignant phenotype and show potential as effective targets in the treatment of GBM (Abdul Rahim et al., 2017; Hu et al., 2012).

2.1.2. Glioblastoma Treatment:

Several GBM therapies have emerged in recent years since its treatment has been a central issue in the enhancement of the lethal impacts resulting from tumor progressiveness (Flores Ledur et al., 2017). However, it remains challenging to develop successful treatments due to the proliferative and diffuse nature and the aggressive behavior of GBM, along with its sensivity to drug resistance and tendecy for reccurence. The typical treatment for GBM usually includes surgical removal followed by radiotherapy and the administration of chemotherapeutic drugs as adjuvant therapy(Minniti et al., 2008; Stupp et al., n.d.). The therapeutic approach and clinical outcome in GBM are defined mainly by two molecular biomarkers including O6-methylguanydil DNA-methyltransferase (MGMT) and isocitrate dehydrogenase (IDH). (Cantidio et al., 2022).

Temozolomide is an oral alkylating chemotherapy agent that is used primarily as the first-line treatment for GBM(Bocangel et al., n.d.). Basically, the mechanism depends on targeting specific locations on individual DNA strands leading to methylation at particular positions such as N7 of guanine, O3 of adenine, and O6 of guanine (Stupp et al., 2005.). Alkylating the O6 site on guanine forms O6-methylguanine adducts, leading to the insertion of thymine instead of cytosine, these genetic changes cause breaks in both single and double DNA strands, leading

to halt of cell cycle progression at the G2/M phase and cell death through apoptosis (Stupp et al., 2005.).

2.1.2.1 Temozolomide Resistance Mechanism:

Although Temozolomide **(**TMZ) is acknowledged the major chemotherapeutic drug for treating GBM, research has indicated that TMZ plays an important role in tumor recurrence and resistance. Studies have shown that resistance toTMZ is correlated with the expression levels of DNA repair enzymes and DNA alkylating proteins.This resistance arises from the pervasive exposure to TMZ and the highly mutation-prone and heterogeneous nature of GBM. As a result, defeating resistance to TMZ remains a significant obstacle in GBM treatment strategies. (Arora & Somasundaram, 2019; Sarkaria et al., 2008.).

According to the type of DNA damage, different DNA repair pathways are triggred due to the response to chemotherapy. These pathways involve mismatch repair (MMR), direct repair (DR), and base excision repair (BER) (Lee, 2016). Studies indicate that each of these pathways has an essential role in cellular responses to temozolomide exposure at different stages (Arora & Somasundaram, 2019).

2.1.2.1.1 Direct repair by MGMT:

In order to maintain genomic stability, MGMT uses mismatch repair mechanism to directly repair DNA damage caused by alkylation which exerts a significant influence on TMZ treatment (Singh et al., 2021). MGMT has been demonstrated to have an effect on TMZ by reducing the DNA damage caused by the drug though its ability to eliminate the methyl group from O6-methlyguanine which in turn leads to reduction in overall effectiveness of TMZ (Feldheim et al., 2019; Rivera et al., 2010).

Moreover, the possibility of alterations changes in MGMT status throughout the course of tumor treatment, progression, or recurrence was suggested by meta-analytical studies (Feldheim et al., 2019). Also, it has been observed that tumors initially displaying MGMT methylation usually show decreased methylation upon recurrence following TMZ treatment. This can be used by tumors as a mechanism to develop resistance to TMZ therapy by decreasing MGMT promoter (Park et al., 2012).

2.1.2.1.2 Base excision repair:

Base excision repair (BER) is identified as a mechanism employed to repair single nucleotide modifications which involve multiple enzymatic reactions carried out by endonucleases, glycosylases, DNA ligases, and polymerases (Arora & Somasundaram, 2019; Singh et al., 2021). BER mechanisms are used to identify and repair most of the N7-guanine and N3-adenine methylation induced by TMZ (Tang et al., 2011; Trivedi et al., 2005). The pathway that is known to promote resistance to TMZ involves various proteins such as DNA glycosylase MPG, high-mobility group A2 (HMGA2) stem cell factor, and DNA polymerase-β (pol-B). Pol-B protects against DNA-induced cytotoxicity through its lyase activity, Poly (ADP-ribose) polymerase 1 (PARP1) that identifies breaks in single-stranded DNA, protecting cells against excessive accumulation of apurinic/apyrimidinic sites. (Agnihotri et al., 2014; Parsons et al., 2005; Tang et al., 2011; Thanasupawat et al., 2017).

2.1.2.1.3 Mismatch repair:

Mismatch repair (MMR) is known as a mechanism utilized by DNA to repair mismatches included in nucleotide base pairs DNA synthesis. As previously discussed, TMZ induces the formation of O6-MeG, which forms incorrect pairs with thymine instead of cytosine during DNA synthesis (Yoshimoto et al., 2012). In the next replication, mismatch occurs again and MMR becomes active, causing breakages in the newly formed DNA strand. The recurrence of DNA breakages triggered by the MMR system in response to TMZ-induced DNA damage leads to the formation of irreparable DNA strands, ultimately leading to apoptosis (J. Zhang et al., 2012).

MMR has been linked to resistance against TMZ due to the emergence of MMR mutations, occurring either spontaneously or in reaction to conventional chemotherapy. These mutations in MMR have been observed to cause increased mutation rates in repeatedly occurring tumors, especially in cases of primary MGMT methylation(Touat et al., 2020). Without MMR, O6MG lesions are accepted by the cell, leading to chemical resistance (Fig.2.3). Mutations in the MMR component of GBM are rare. Nonetheless, recurrent GBM cases have shown MSH6 mutations absent in corresponding primary tumors (Arora & Somasundaram, 2019).

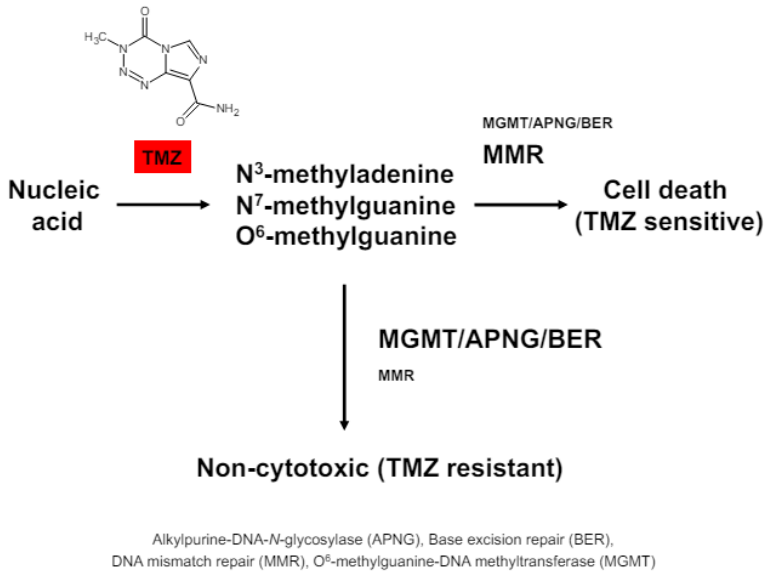


Figure 2. 3 Mechanism of Temozolomide Action and Resistance. Temozolomide (TMZ) induces alterations in DNA or RNA molecules by methylating certain sites, including N7 and O6 on guanine, and N3 on adenine, through the attachment of methyl groups. This figure was taken from (Lee, 2016).

2.2. History of Astaxanthin Molecule and Properties :

Astaxanthin (ASX), a red-orange compound categorized within the carotenoid group, has captured significant interest in recent times due to its remarkable physical attributes, biochemical traits, and physiological impacts(Nishida et al., 2023). Research on ASX began soon after carotenoids were first discovered. Carotenoids have been studied since the early 19th century when they were first found in paprika (1817), saffron (1818), annatto (1825), carrots (1831), and autumn leaves (1837) (Maoka, 2020; Natural Food Colorants, n.d.). However, Back then, carotenoid structures were mostly unknown, and scientists mainly identified them based on how they dissolved and absorbed light. In 1937, the isolation of a protein complex called “ovoverdin” from lobster was done by Stern and Salomon, and in 1938, Kuhn and Sörensen analyzed "ovoverdin" and identified "ovoester" as a xanthophyll carotenoid naming it as ASX (Ovoverdin, 1936; Stern & Salomon, 1938).

2.2.1 Mechanisms of Action of Astaxanthin:

Typically, carotenoids are taken up into body lipids, a process that is facilitated by high cholesterol levels. To facilitate the absorption of ASX, it's often combined with bile acids to create micelles, which are then integrated into chylomicrons. Afterward, ASX is incorporated with lipoproteins and carried to different body tissues, where it acts to shield cells, including skin and lipid-based membranes, from oxidative damage(Ambati et al., 2014). Moreover, ASX's polyene chain and numerous double bonds allow it to neutralize singlet oxygen and radicals, thereby inhibiting oxidative reactions. ASX's antioxidant attributes are ascribed to its chemical and physical interactions with cell membranes, where its polyene chain aids in neutralizing free radicals(Ambati et al., 2014).

A large body of applications and studies have revealed that ASX exerts its activity through various mechanisms. These effects involve different molecules and pathways such as anti-invasive, anti-proliferation, and anti-apoptosis. These effects involve different molecules and pathways, such as anti-proliferation, anti-apoptosis, and anti-invasion, via signal transducers and activator of transcription 3 (STAT 3), nuclear factor kappa light chain enhancer of activated β-cell (NF-κβ), peroxisome proliferator activator receptor gamma, and other multiple mechanism of cancer effects. According to Zhang et al., ASX is believed to protect body tissues from oxidation and ultraviolet (UV) damage by suppressing of NF-κB activation (Nagendraprabhu & Sudhandiran, 2011; Palozza et al., 2009).

ASX additionally inhibits cancer formation by shielding DNA from UV induced oxidative damage. It achieves this by facilitating the early recognition and elimination of cells that have progressed into malignant forms, thereby evading immune system regulation(L. Zhang & Wang, 2015). Moreover, Jacobsson et al, Palozza et al, and Nagendraprablu and Sudhondranm corroborate that ASX hampers tumor metastasis by downregulating the production of tissue-degrading proteins within tumors. Additionally, it impedes the rapid proliferation of tumor cells during their growth phase by disrupting the cancer cell reproductive cycle and inducing apoptosis (Jacobsson et al., 2004; Nagendraprabhu & Sudhandiran, 2011; Palozza et al., 2009).

Recent studies and researches have explored the impact of this molecule in combating cancer and mitigating tumor proliferation. ASX, fundamentally, employs at least six distinct mechanisms to counteract cancer. These mechanisms encompass astaxanthin's capacity to diminish the proliferation of malignant cells, stimulate apoptosis, alleviate oxidative stress,

mitigate inflammation, impede cancer metastasis, and enhance intercellular communication (L. Zhang & Wang, 2015).

2.2.1.1 Inhibition of Cell Proliferation:

Tumor development is governed by the accelerated proliferation and migration of cancer cells into healthy tissues and organs, contingent upon the signaling conveyed by adhesion proteins and growth factors (Ostan et al., 2015; L. Zhang & Wang, 2015).

Many researchers have investigated how ASX affects cell growth in cancer cells. Accorsing to a study,it was found that ASX had a strong anti-proliferative effect on CBRH-7919 (human hepatoma), SHZ-88 (rat breast), and Lewis (mouse lung) cells (Song et al., 2011), it was discovered that the sensitivity to ASX varied among these cells, with CBRH-7919 being the most sensitive, with an IC50 concentration of 39 μM. Moreover, another study have compared the growth inhibitory effects of ASX with other carotenoids on K562 leukemia cells(X. Zhang et al., 2011) , and it was found that ASX was the most effective at inhibiting cell growth at low concentrations, followed by bixin, β-carotene, and capsanthin. ASX also slowed down cell growth in oral cancer in hamsters and decreased cell viability in human HCT-116 colon cancer cells in a dose- and time-dependent manner(Palozza et al., 2009). Additionally, studies showed that ASX had less impact on normal cells compared to cancer cells. For example, although ATX significantly inhibited the proliferation of cancer cells, it had little effect on HL-7702, a normal human hepatocyte line, suggesting that ATX specifically targets cancer cells (Song et al., 2011).

2.2.1.2 Apoptosis:

Apoptosis, alternatively termed programmed cell death, is a regulated process involving various cellular events in multicellular organisms, resulting in cell demise through the packaging of cellular contents into membrane-bound vesicles for clearance by immune cells.While apoptosis occurring in tumor cells leads to a reduction in tumor volume, thereby diminishing tumor burden and increasing life expectancy, cancer cells develop mechanisms to evade apoptosis, allowing them to persist and proliferate (Hormozi et al., 2019). ASX thereby can turn this programmed cell death back on in cancer cells by enhancing antioxidant enzyme activity, decreasing malondialdehyde production, and upregulating apoptosis-related gene expression(Hormozi et al., 2019). It was discovered in one study by Kim et al, " that ASX can

modulate apoptotic molecules prompting the death of breast cancer cells" (Kim et al., 2020). Furthermore, the study by Kim et al indicate that "ASX was discovered to initiate the reduction of mutp53 and cleaved PARP-1, demonstrating its capacity to prompt apoptosis in SKBR3 cells. Additionally, it was observed that astaxanthin increased Bax expression while decreasing Bcl2 levels depending on the dose, suggesting that ASX induces apoptosis in breast cancer cells (SKBR3) using Bcl2/Bax pathway" (Kim et al., 2020).

2.2.1.3 Anti-Inflammation:

The link between cancer development and inflammation was first described by Rudolf Virchow in 1863(Inflammation and Cancer, n.d.). Inflammation is identified as the immune system's reaction to harmful substances and it involves multiple biological responses where the body increases its production of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1), and tumor necrosis factor-alpha (TNF-α), both in the bloodstream and within cells(Franceschi, 2008; Franceschi et al., n.d.).

ASX's impact on inflammation has been also investigated in cancer. For instance, two studies by Franceschelli et al and Speranza et al, in U937 cell line , it was demonstrated that "the activation of nuclear factor-κB , (NF-κB) transcription factor induced by reactive oxygen species (ROS) was inhibited by ATX. This inhibition effectively decreased the production of inflammatory cytokines like IL-1β, IL-6, and TNF-α by restoring the physiological levels of SHP-1" (Franceschelli et al., 2014; Speranza et al., 2012). Moreover, another study by Yasui et al indicated that " dietary ATX notably decreased the occurrence of colonic mucosal ulcers, dysplastic crypts, and colonic adenocarcinoma in mice with colitis-related colon carcinogenesis and colitis, they proposed that the reduction in inflammatory cytokines, such as NF-κB, TNF-α, IL-1β, IL-6, and COX-2, played a role in the anti-cancer efficacy of ATX " (Yasui et al., 2011).

2.2.1.4 Anti-Oxidation:

Oxidative stress arises from imbalance between free radical production and the body's antioxidant capacity to counteract their harmful effects. In cancer, oxidative stress plays a crucial role in initiation and progressing of the disease by increasing DNA mutations, inducing DNA damage, promoting genome instability, and enhancing cell proliferation (L. Zhang & Wang, 2015).

ASX is thought to work as an antioxidant by directly neutralizing reactive chemicals like reactive nitrogen species (RNS) and reactive oxygen species (ROS). It promotes antioxidant enzymes production in living organisms (Nishida et al., 2023).One study demonstrated that astaxanthin exhibits antioxidant properties by reducing the O_2 level in a lipopolysaccharide-stimulated U937 cell line (Franceschelli et al., 2014). This ASX's ability to interact with various radicals plays an imporant role in its antioxidative effect.

Moreover, one study by Hormozi et al has shown that, "ASX was identified as a potent antioxidant as it enhances superoxide dismutase and other enzyme activities, this alteration in enzyme activity possibly affects the cell's antioxidant defense system, resulting in anti-proliferative effects and apoptosis induction in LS-180 colorectal cancer cells" (Hormozi et al., 2019).

2.2.2 Astaxantin in Glioblastoma:

Recently, studies on ASX's effect on GBM have been gaining so much interest as researchers seek new approaches to develop treatment. In one study by Shin et al, " it was found that the antioxidant enzyme SOD2 plays a role in sensitizing GBM cell lines to TRAIL through a pathway involving changes in mitochondrial potential, which enhances the TRAIL apoptosis signal, this indicates that ASX may influence the TRAIL-induced pathway not primarily through its antioxidative properties in the cytosol, but rather via a specific mitochondrial pathway, suggesting that ASX could be efficient in GBM treatment, particularly in low SOD2 activity conditions" (Shin et al., 2020). However, it was suggested that more experiments that include downregulating SOD2, are needed to enhance the results indicating that anticancer drugs are affected by SOD2 expression levels in patients (Shin et al., 2022).

Furthermore, it was showed by Siangcham et al that "ASX treatment reduced the migration and invasion of A172 cells in a manner dependent on both time and concentration, these effects were associated with a decrease in the expression of MMP-2 and MMP-9 following ASX treatment in a concentration-dependent manner"(Siangcham et al., 2020) . Thus, the findings by Siangcham et al study suggested that "ASX can hinder the migration and invasion of A172 cells by reducing the levels of MMP-2 and MMP-9. Moreover, ASX did not exhibit toxicity towards A172 cells, indicating that its anti-invasive effect in GBM is not due to inhibiting cell growth" (Siangcham et al., 2020).

In another study by Shin et al, it was examined that , “ASX affects CRT-MG, astroglioma cell lines U251-MG, and T98G, however, each cell line responded differently to ASX, while U251-MG cells demonstrated a unique response known as hormesis when exposed to ASX, at high doses (20–40 μM), ASX induced cell death in U251-MG cells, consistent with findings in other cancer cell lines. However, at lower doses (4–8 μM), ASX unexpectedly stimulated cell growth. Interestingly, these lower doses did not increase levels of reactive oxygen species (ROS) inside the cells, while the activity of the antioxidant enzyme superoxide dismutase moderately increased” (Shin et al., 2020). The study by Shin et al also revealed that, “treatment with low doses of ASX raised levels of cyclin-dependent kinase (Cdk) 2 and phosphorylated forms of Cdk2/3 while decreasing levels of the tumor suppressor protein p53”(Shin et al., 2020).

2.3. Etoposide and Cancer :

Etoposide arises from podophyllotoxin which is a toxin substance present in the American Mayapple plant. It was chemically synthesized for the first time in 1966 and gained approval from the U.S. Food and Drug Administration for cancer treatment in 1983 (Hande, 1998).It is a potent anti-tumor drug that belongs to the class of topoisomerase poisons. Since the 1980s, numerous studies have shown that Etoposide targets DNA topoisomerase II, causing DNA breaks and effecting cell metabolism. Topoisomerase II (Topo II) is a crucial enzyme responsible for regulating the structure of DNA. It achieves this by guiding a complete double helix through a temporary break in another double-stranded DNA segment, using energy from ATP (Champoux, 2001).

Etoposide mechanism of action occurs during the late S and G2 phases of the cell cycle (Sinkule, 1984). Topoisomerase II cuts both strands of the DNA helix simultaneously, generating and repairing double-stranded DNA breaks crucial for replication. Etoposide disrupts this process by poisoning the topoisomerase II cleavage complexes, thereby blocking the DNA re-ligation step. This disruption triggers a pathway leading to mutations and cell death, particularly effective in tumor cells with elevated levels of topoisomerase II enzymes (Hainsworth & Greco, 1995; Meresse et al., 2004).

Currently, it's widely recognized that Etoposide acts as an inhibitor of Topoisomerase II (TOP2), which is considered a key anticancer mechanism of the drug. TOP2 consists of alpha and beta isoforms and is essential in various DNA processes, regulating DNA winding during

replication. Inhibitors of TOP2 play a crucial role in cancer therapy by inducing apoptosis in cancer cells through the accumulation of DNA breaks. Typically, TOP2 enzymes form a complex with DNA, which is rapidly resealed. However, when Etoposide interacts with TOP2, this complex is trapped, leading to an accumulation of DNA breaks. If these breaks are not repaired promptly, the cell initiates apoptosis, resulting in cell death (Determinants of Response to DNA Top, n.d.; Edwards et al., 1987).

3. MATERIALS AND METHODS:

The purpose of this investigation is to demonstrate the ASX's efficacy in inhibiting the proliferation of glioblastoma U87MG alone or together with the chemotherapeutic drug Etoposide. Figure 3.1 outlines the methodologies employed to achieve this aim in a comprehensive and scholarly manner.

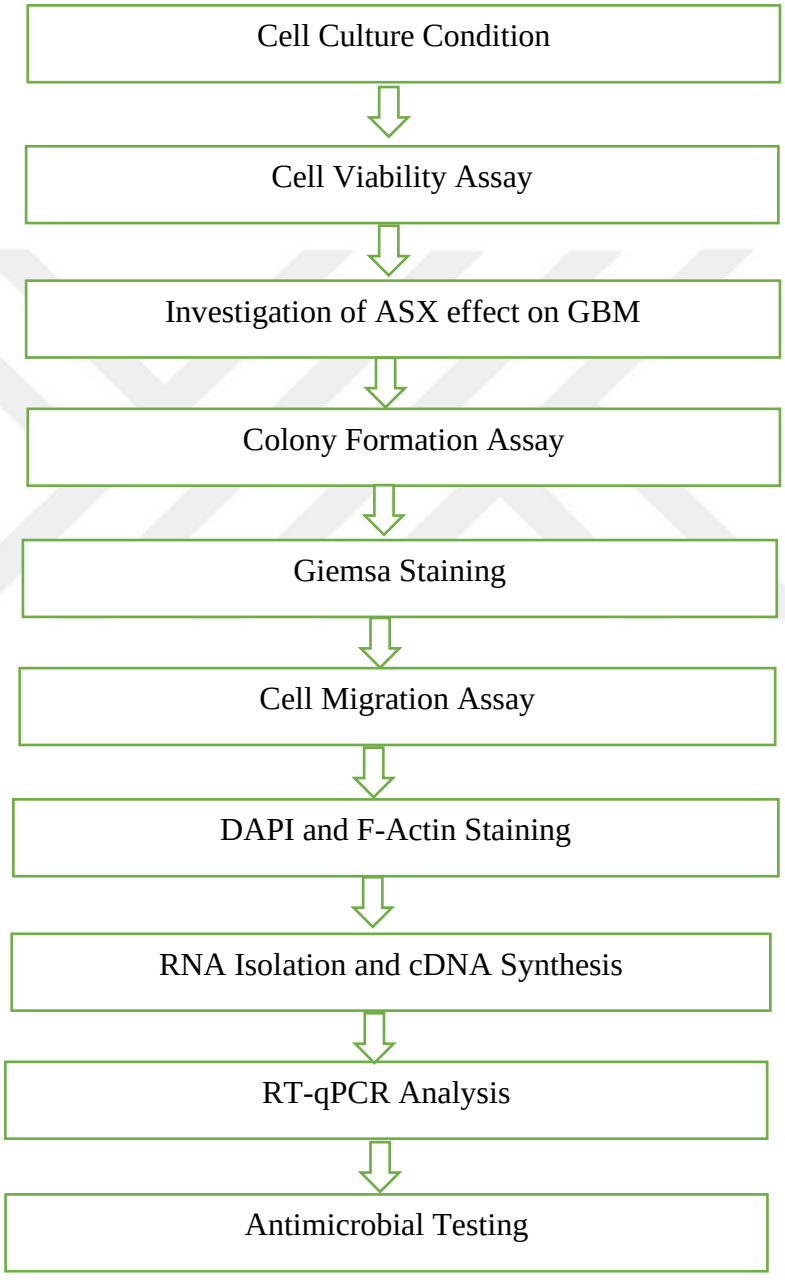


Figure 3. 1 The methodologies applied in this study.

3.1. Cell Culture Condition

The cell line used for this study was the Glioblastoma U87MG (ATCC® Manassas, VA, USA; HTB-14™) cell line. U87 MG cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were kept at 37°C with a stable CO_2 concentration of 5% in a humidified incubator (Nüve EC 160, Nüve, Ankara, Türkiye).

3.2. Cell Viability Assay

In order to investigate the effect of ASX and Etoposide on cell viability, a neutral red cell viability test was applied. The neutral red uptake test is performed to measure quantify xenobiotic-induced cytotoxicity in vitro. It works by examining how much neutral red dye cells can absorb and retain in their lysosomes. This dye absorption decreases when cells are exposed to toxic substances, indicating their level of toxicity. Typically, this test is utilized to assess potential hazards in toxicology studies conducted outside of living organisms (Ates et al., 2017). The nutrient solution utilized for the cultured cells under examination was aspirated, in PBS a neutral red dye (0.033%) was applied to overlay the cells. Subsequently, the cells were allowed to incubate for a minimum of 2 hours. Following the incubation period, the neutral red solution was removed and a solution of neutral red dye dissolved in a mixture of 49% ultra-pure water, 1% acetic acid, and 50% ethanol was introduced. To achieve uniform color distribution, the solution was agitated. The microplate reader was utilized to measure absorbance at 540 nm. (Spectrostar Nano, BMGLabtech, Ortenberg, Germany).

3.3. Investigating the Impact of ASX on Cancer Cells

The IC50 value represents the concentration of ASX required to inhibit 50% of cancer cell growth or viability is determined by analyzing the neutral red dose-response curve and calculating where it intersects with 50% inhibition.

To determine the IC50 value, around 5000 cells were calculated utilizing a Neubauer hemocytometer, then placed into individual wells of a 96-well plate and incybated in a humidified incubator at 37°C with 5% CO_2. The next day, different concentations of ASX (20, 40,60, 80 and 100 ug/mL) were applied to the cells for 24 hours. Cell viability was assessed using a neutral red uptake test to determine the percentage of viable cells at each concentration, with the IC50 value determined based on the test results. Multiple treatments that include ASX

alone, Etoposide alone, and their combination were done on U87MG cells, The results were taken after 24-hour. The calculation of viable cells was obtained by using neutral red test to determine the synergistic impact of the calculated IC50 value for ASX and Etoposide. IC50 value of 40 µM for Etoposide was applied on U87MG as it was previously indentified through experimental investigation by Ozdemir and Gokturk (Ozdemir & Gokturk, 2018).

3.4. Colony Formation Assay

The ability of cells to form colonies was calculated using colony formation test on Glioblastoma U87MG cells. Approximately around ($2x10^5$ cells/well) of cells were grown in six-well plates under four different experimental conditions: control groups without treatment, ASX treated group, Etoposide treated group, and a group treated with both ASX and Etoposide. For U87MG cells, concentration of 40 mM for Etoposide was applied, while ASX was given at a concentration of 90 µg/mL, corresponding to their respective IC50. Following a 24-hour incubation period, 500 viable cells were calculated and subsequently seeded into six-well plates utilizing Neuber hemocytometer.

After the formation of colonies was observed under a microscope (Leica Biosystems, Germany), they were stained with crystal violet for examination. A staining solution for the colonies was prepared, consisting of 6% volume/volume glutaraldehyde (Merck, Germany) and 0.5% weight/volume crystal violet (Sigma-Aldrich, United States). The cell culture medium was aspirated, the cells attached to the culture dish surface were washed with Phosphate-buffered saline (PBS) (Merck, Germany). Subsequently, PBS was removed, and enough colony fixation staining solution (1 mL) to cover the culture surface was added. After being left at room temperature for 30 minutes, the colony fixation staining solution was discarded and washed with distilled water.

3.5. Giesma Staining

Giemsa staining is one of the best known histological stains, coloring the nuclei dark blue and the cytoplasm blue to pink, according to the acidity of the cytoplasmic contents. The staining solution was passed through a 0.22-micron syringe filter for sterile filtration. Experiments on U87MG cell lines were conducted in six-well plates under various situations: untreated control group, Etoposide and ASX treatment seperately , and a combination of them both.

Concentration of 40µM for Etoposide was applied, and 90 µg/mL for ASX, corresponding to their respective IC50 values. Following 24 hours of incubation duration, the medium was aspirated, and cells were rinsed two times with PBS. Subsequently, 1 mL of 60% methanol in PBS was added to the wells individually and kept for 10 minutes at room temperature. Afterward, the medium was aspirated, and the wells were rinsed with pure methanol. After that, 1 mL of Giemsa staining solution was added into each well and incubated for 20 minutes at room temperature. Following incubation period, the staining solution was removed and the cells were rinsed three times with purified water. Finally, in order to observe cell morphology changes LED-inverted microscope (Leica Biosystems, Wetzlar, Germany) was used.

3.6. Cell Migration Assay:

Cell migration assay was assesed in order to see how ASX and Etoposide influence the migration of Glioblastoma U87MG cells following the methodology outlined by Suarez-Arnedo et al. (2020). Firstly, $2x10^5$ cells were seeded in to six-well cell culture plates. The following day, the plates were rinsed with phosphate-buffered saline (PBS), and a linear scratch was made using a 1 mm pipette tip. Treatments, including ASX alone, Etoposide seperately, combination of both, and a control culture medium were tested. Cells were exposed to 40 µM Etoposide corresponding to their respective IC50 concentrations. ASX was applied at (90 µg/mL) as previously determined IC50 levels. The plates were then incubated for 24 hours. Images of the cells were taken by inverted microscope at 0, 8, and 24 hours following treatment by inverted microscope. Quantitative analysis of cell migration and wound closure was performed using ImageJ software (version 1.53t).

3.7. DAPI and F-Actin Staining

To evaluate the viability of U87MG cells and the structural components in this study, two fluorescent dyes, 4',6-diamidino-2-phenylindole (DAPI) and phalloidin were used . Through electrostatic interactions DAPI can bind tightly to adenine-thymine (A-T) rich regions of nuclear DNA, this is utilized to observe nuclear DNA in both living and treated cells. This binding results in fluorescence becasue of the inhibition of internal rotation in DAPI's molecular structure (Tarnowski et al., 1991).

Phalloidin, a molecule derived from mushrooms, specifically binds to filamentous actin (F-actin). This binding cause fluorescence in F-actin filaments for the phalloidin derivatives tagged molecules which can be visualized under microscopy in various research fields(Romani & Auwerx, 2021).

U87MG cell lines were set under four different experimental conditions in 24-well plates: untreated groups (control), ASX alone, Etoposide seperately, and a combined treatment of ASX and Etoposide. Etoposide was added at a concentration of 40 µM, while ASX was given at 90 µg/mL, according to their respective IC50 values. Following 24-hour incubation period, the cells were imaged. The culture medium was aspirated, and the cells were rinsed two times with PBS. Following this, a 4% paraformaldehyde solution was applied to each well and incubated for 20 minutes at room temperature. Subsequently, the cells were washed with two more PBS washes and treated with PBS containing 0.1% Triton X-100 for 10 minutes to permeabilize the cell membrane. Following two PBS washes, DAPI and phalloidin-iFluor™488 were prepared by diluting each in PBS containing 1% Bovine Serum Albumin (BSA) at a ratio of 1:1000 and introduced into the wells. The cells were then allowed to be incubated for 30 minutes in a dark place at room temperature to label the nuclei and F-actin filaments. After that, the cells were washed twice with PBS containing BSA and then observed under the fluorescent light using a Leica DM IL LED inverted microscope (Leica Biosystems, Wetzlar, Germany).

3.8. cDNA Synthesis and RNA Isolation

The cell culture of U87MG cell lines was performed in 75 cm² cell plates. The experimental setup included four different groups, each cultivated in separate plates: a control group with no treatment, a group with ASX treatment, another treated with Etoposide, and a combined treatment group of both Etoposide and ASX were added according to their respective IC50 values. After an incubation period of 24 hours at 37°C with 5% CO_2, RNA extraction was performed by the Invitrogen™ PureLink™ RNA isolation kit from Thermo Fisher Scientific, USA, in accordance with the manufacturer's instructions. This RNA was then utilized to generate cDNA using the RevertAid First Strand cDNA Synthesis Kit, also following the instructions. Thermal cycling for cDNA synthesis was performed using an Agilent SureCycler 8800 PCR thermal cycler with the following conditions: At 25°C for 10 minutes, at 37°C for 120 minutes, and at 85°C for 5 minutes. The quantity and the quality of the resulting cDNA

were evaluated by utilizing NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA)

3.9. RT-qPCR Analysis

We conducted RT-qPCR analysis utilizing a Rotor-Gene Q qPCR system (Qiagen, Hilden, Germany). The reaction protocol entailed an initial activation step at 95°C for 12 minutes, followed by denaturation at 95°C for 15 seconds, annealing at 55°C for 20 seconds, and elongation at 72°C for 20 seconds. Subsequently, the ΔCt values were acquired and normalized to the expression level of the β-actin reference gene.
The primers utilized for this investigation :

- Survivin forward primer: 5'-TCCACTGCCCCACTGAGAAC-3'
- Survivin reverse primer: 5'-TGGCTCCCAGCCTTCCA-3'
- c-myc forward primer: 5'-TACCCTCTCAACGACAGCAG-3'
- c-myc reverse primer: 5'-TCTTGACATTCTCCTCGGTG-3'
- Caspase 3 forward primer: 5'-TGACTGGAAAGCCGAAACTC-3'
- Caspase 3 reverse primer: 5'-AGCCTCCACCGGTATCTTCT-3'

3.10. Antimicrobial Test

The Minimum Inhibitory Concentrations (MICs) of ASX on gram-negative (E. coli) and gram-positive (S. aureus, B. subtilis, B. cereus) bacteria were determined using the microdilution method with a 96-well microtiter plate. The bacteria in the wells were cultured in Mueller Hinton Broth at McFarland 0.5 density. Subsequently, ASX (at concentrations of 10,20,40 and 100 µg/mL) was added to the plates containing the culture medium and microorganisms. The plates were then incubated at 37°C for 24 hours using a Memmert incubator. After incubation, to determine the MIC value as the lowest concentration without visible growth, agar plates with Mueller Hinton Agar were inoculated from the wells without growth along with controls.

3.11. Statistical Analysis

Triplet experiments were performed for each condition. Statistical analysis was conducted using Microsoft Excel 2021, employing both one-way analysis of variance (ANOVA) and t-tests. A significance threshold of $p<0.05$ was utilized for all comparative analyses.

4. RESULTS AND DISCUSSION

4.1. Determination IC50 Value of ASX on U87MG Cells

The cytotoxic effects of ASX were evaluated using a neutral red test and following the equation mentioned in the figure, which showed an IC50 value of 90 µg/mL for the Glioblastoma U87MG cell line (Figure 4.1). As the concentration of ASX increased, the rate of cell death among GBM cells also increased proportionally. These results suggest that higher ASX levels lead to greater effectiveness.

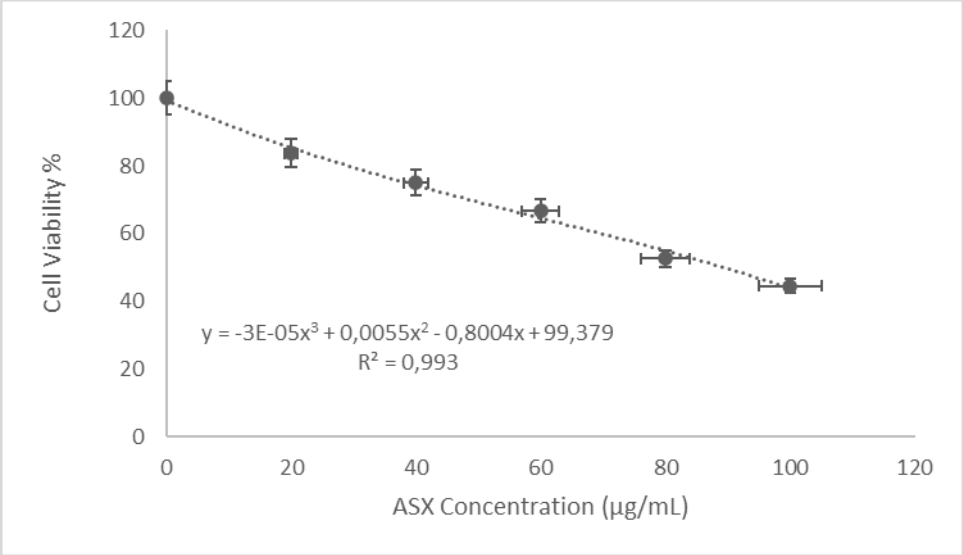


Figure 4. 1 Different concentration of ASX effect on the viability of U87MG cells. The IC50 value of ASX was determined as 90 µg/mL($p<0.001$)..

4.2. The Synergistic Effect of ASX and Etoposide on the Viability of Cells

The determination of the synergistic effect of ASX and Etoposide on U87MG cells was done using neutral red test. These resluts demonstrate that in the presence of ASX, the cytotoxic effect of Etoposide was enhanced. Furthermore, comparing to using either treatment alone under 24-hour and 48-hour culture conditions , a higher occurrence of cell death in U87MG was found when simultaneous administration of ASX and Etoposide was performed. These findinfgs indicate that ASX has the potential to enhance the cytotoxicity of Etoposide which in turn lead to increasing the rate of cell death in U87MG cell lines (Figure 4.2).

These findings support earlier studies indicating the ability of ASX to sensitize cancer cells to chemotherapy-triggered apoptosis and suppress proliferation in various cancer models, including GBM (L. Zhang & Wang, 2015). The findings from this study align with existing literature that show the combined effect of various agents with traditional chemotherapeutics on GBM cells and indicate that combining chemotherapeutic agents with natural compounds can result in synergistic effects, leading to enhanced cytotoxicity and increased cancer cell apoptosis (Aghababaei et al., 2024). For instance, a study done by Liu et al has shown that "combining TMZ with natural antioxidants like resveratrol enhances its efficacy against GBM" (Liu et al., 2020). Similarly, another study by Kluska and Woźniak demonstrated that "Polyphenols, another natural compound, can increase the cytotoxicity of Etoposide when used together" (Kluska & Woźniak, 2021). The current study's validation of ASX enhancing Etoposide's cytotoxic effects supports these findings showing a promising potential for ASX as an adjuvant therapy in GBM treatment. This alignment shows the importance of further investiagtions for these combinations to develop more effective treatment protocols for GBM.

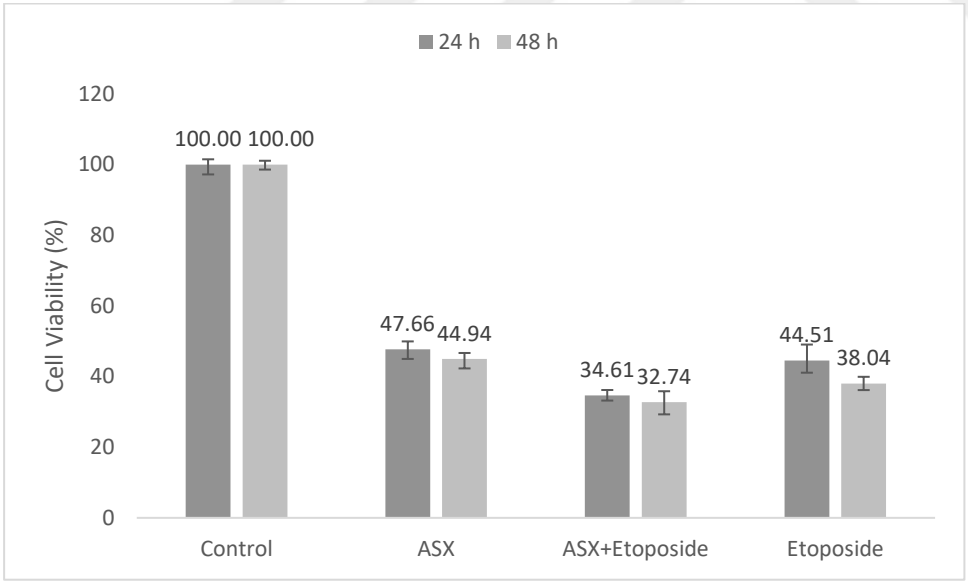


Figure 4. 2 The combined effect of ASX and Etoposide on U87MG cells after 24 and 48 hours ($p<0.001$).

4.3. Colony Formation Assay Results on U87MG Cells after ASX and Etoposide Application:

In order to determine the effect of ASX , Etoposide and their combination on the ability of U87MG cells to proliferate and form colonies, colony formation assay was performed.
The results showed a significant decrease in colony formation in U87MG cells after they are treated with ASX. Following three weeks of incubation, there was a notable reduction in the number of colonies formed by U87MG cells (Figures 4.3). Furthermore, the combined treatment of ASX and Etoposide resulted in a significant decrease of colonies in U87MG cells in comparision to the control groups as illustrated in Figure 4.4.

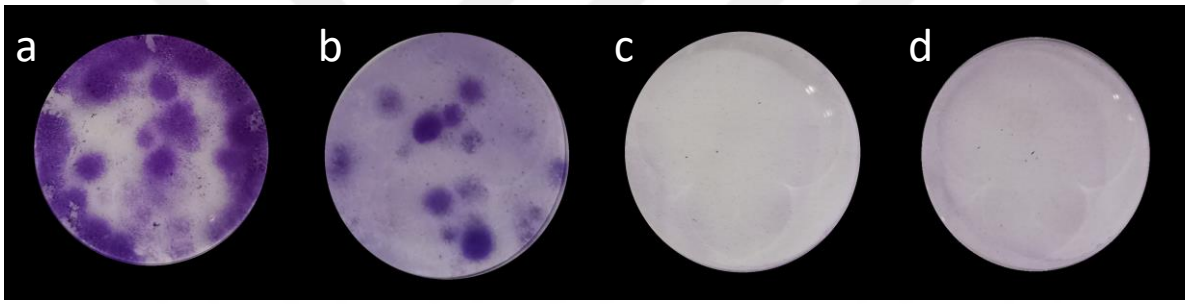


Figure 4. 3 Colonies of U87MG cells (a-control, b- ASX, c- Etoposide, d- ASX+ Etoposide) post 21 days of treatment.

These findings demonstrate that ASX effectively inhibited the ability of U87MG cells to create colonies by decreasing both the number of colonies and their size. This decline in colony growth underscores the inhibitory influence of ASX on the proliferative potential and viability of U87MG cells. Furthermore, it was shown that the effect of ASX and Etoposide is enhanced when they are given together on U87MG cells (Figure 4.4). This aligns with studies demonstrating ASX's ability to suppress the proliferative potential and viability of glioblastoma cells (Siangcham et al., 2020). Importantly, our findings extend this understanding by illustrating a synergistic enhancement of ASX and Etoposide when administered in combination. This synergism suggests a therapeutic efficacy, potentially through complementary mechanisms that further impede U87MG cell colony growth. Collectively, these results underscore ASX's promising role as an anti-proliferative agent in GBM,

particularly when used synergistically with Etoposide, highlighting avenues for enhanced treatment strategies.

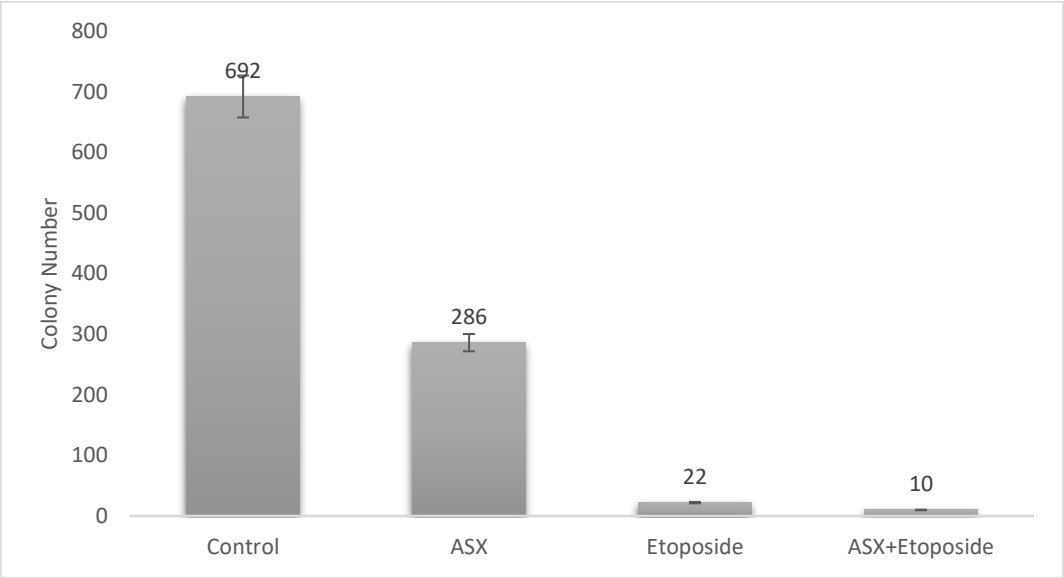


Figure 4. 4 Colonies Number of U87MG cells after 21 days .

4.4. Giemsa Staining Images of U87MG Cells after ASX and Etoposide Administration

Giemsa staining was performed in order to observe Glioblastoma U87MG cells under the treatment of ASX, and Etoposide. The morphological changes in the images indicated signs of structural loss and disappearance of the cells . These results demonstrate that ASX and Etoposide applied as the combined treatment lead to changes in the organisation of the cytoskeleton and adhesion of U87MG cells. In addition, some of the cells had more rounded shape which normally points and associates to the onset of apoptosis and cellular stress. Moreover, the number of the cells was decreased suggesting a notable reduction in the viable cells count because of the cytotoxic effects of ASX and Etoposide. The morphological changes observed after Giemsa staining give visual aspect of the transformations occuring in U87MG cells (Figure 4.5).

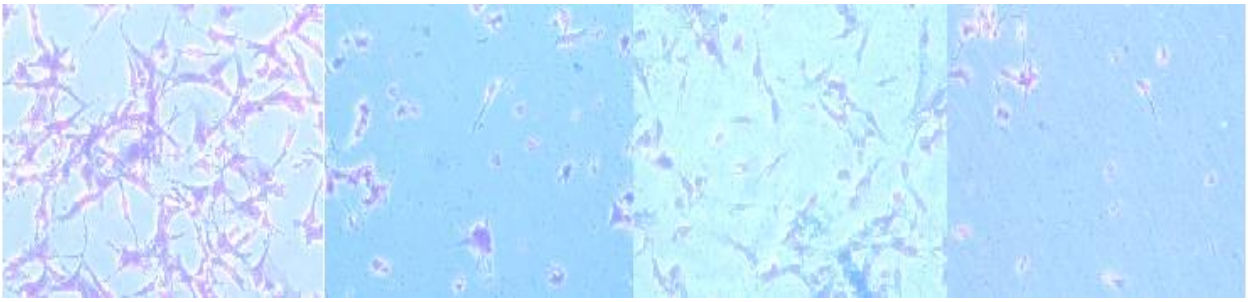
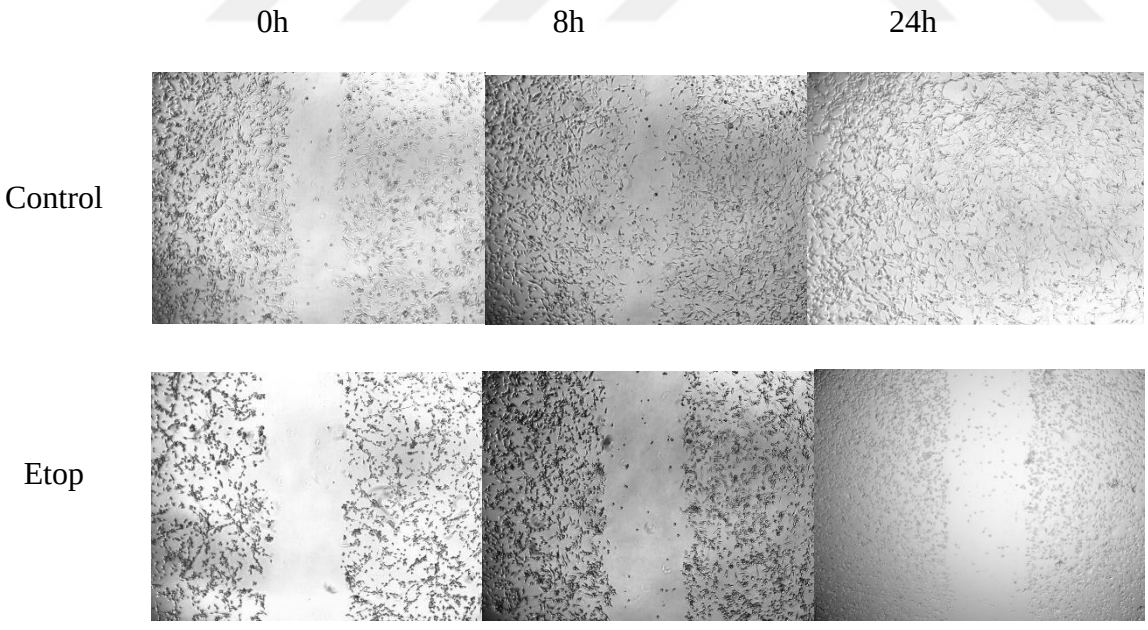


Figure 4. 5 Giemsa staining images of the effect of ASX with Etoposide on U87MG cells.

4.5. Evaluation of the Migation of Glioblastoma Cells after Astaxanthin Treatment:

A wound closure assay was performed for observing the migration behavior of GBM cells in the presence of ASX. After adding IC50 concentrations of Etoposide (40 µM) and ASX (90 µg/mL) to the wells, followed by 24 hours of incubation, ASX effectively impeded the migration of U87MG cells. Unlike the control groups, which exhibited wound closure within 24 hours, the wound left open when treated with ASX and Etoposide, Cell death occured, accompanied by cellular morphological alterations characterized by a transition towards a rounded shape cells and disruption of intercellular adhesion (Figure 4.6).



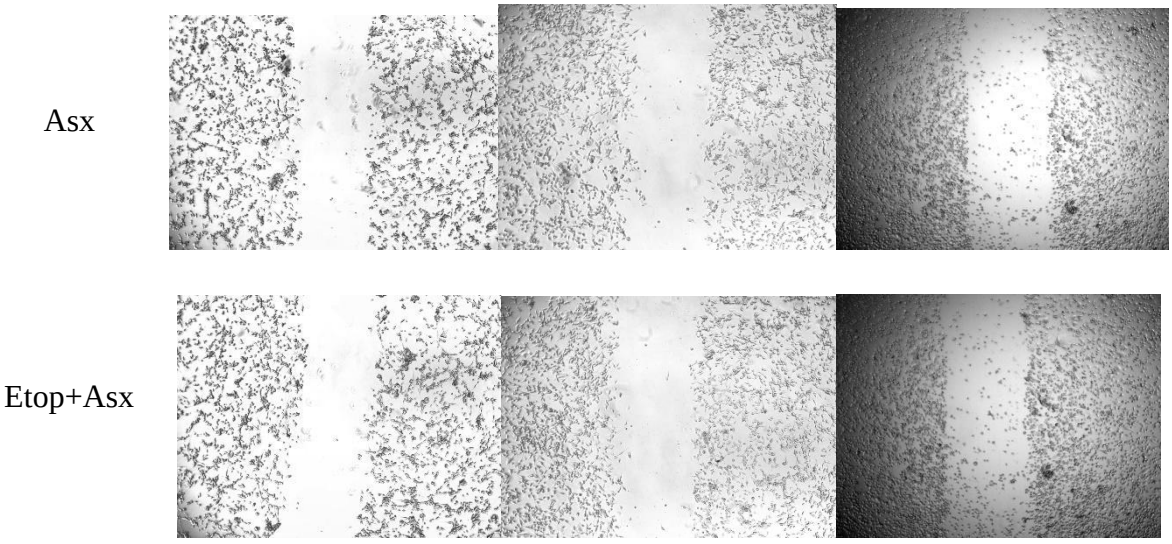


Figure 4. 6 U87MG Migration over 24 h after ASX and Etoposide Treatment ($p<0.001$).

These results show that ASX can prevent cells from migration which is considered a critical factor in cancer treatment. The graphs below demonstrate that ASX, Etoposide and their combination treatment on U87MG cells inhibited wound closure over 24 hours (Figure4.7 and Figure 4.8). Cell death was predicted to be induced due to notable increase in the wound width that was observed in the cells treated with ASX and Etoposide following 24 hours of incubation.

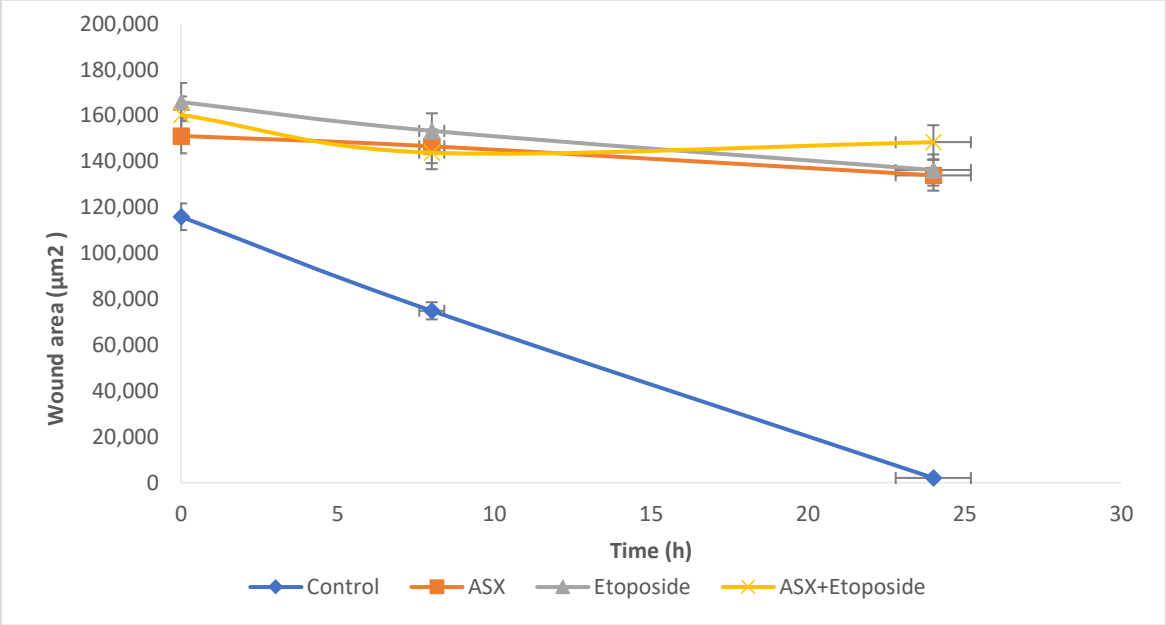


Figure 4. 7 Wound area change in U87MG cells($p<0.001$).

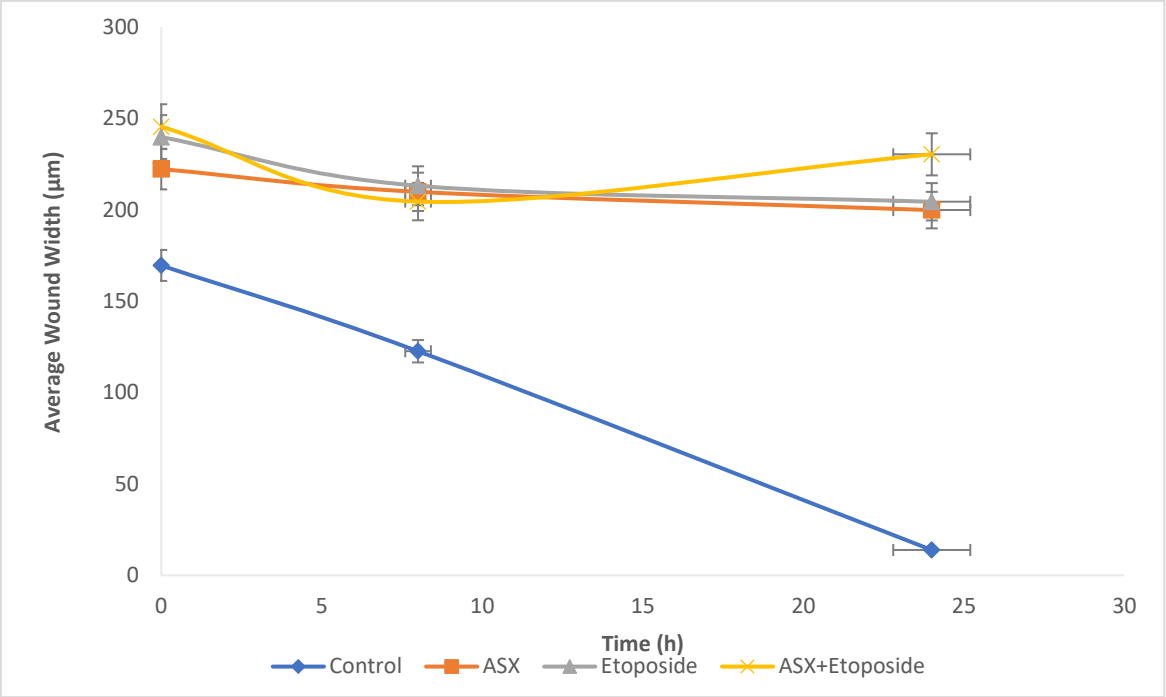


Figure 4. 8 Average Wound Width change in U87MG cells ($p<0.001$).

In comparing to the control groups which could exhibit a notable cell migration leading to wound closure in U87MG cells, the experimental cells treated with Etoposide and ASX on the contrary could successfully inhibit cell migration and wound closure pecentages (Figure 4.9 and Figure 4.10). These observation indicate that treatment with ASX and Etoposide alone or together can signifcantly decrease the number of migrating cells by initiating cell death which in turn participate in the development of cancer treatment.

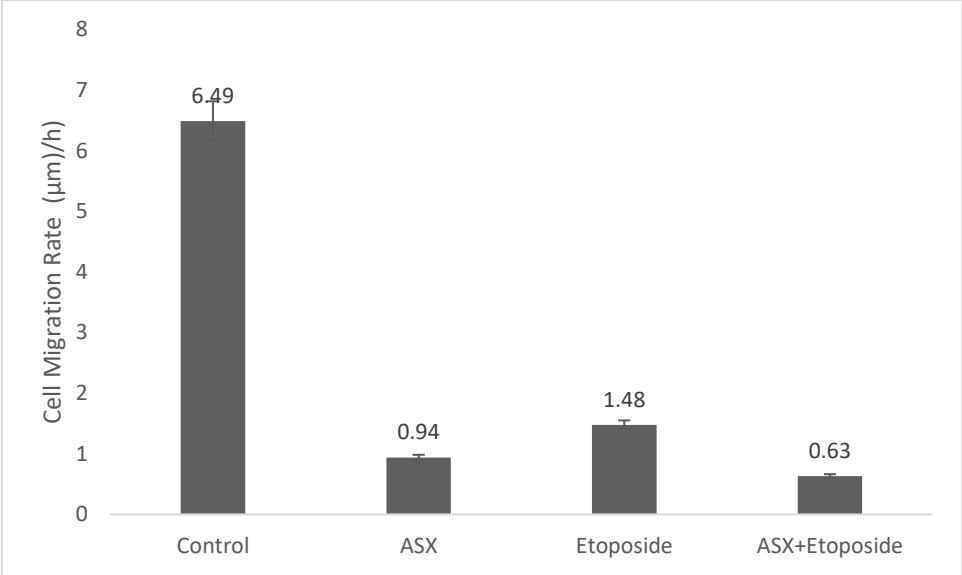


Figure 4. 9 Cell migration rate for U87MG cells ($p<0.001$).

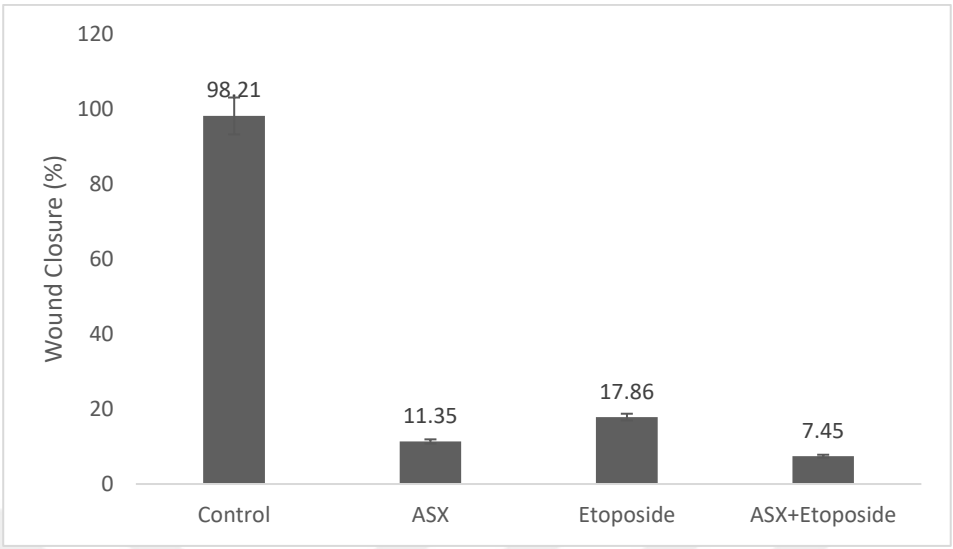


Figure 4. 10 Wound closure rate for U87MG cells ($p<0.001$).

4.6. DAPI and F-Actin Staining Images after ASX and Etoposide Application

Fluorescence staining with DAPI and F-actin was employed to evaluate the nuclear morphology and actin cytoskeleton organization, respectively. U87MG cells were cultured in 24-well plates and treated with ASX and Etoposide for 24 hours. After incubation, cell fluorescence was analyzed using an inverted microscope. ASX treatment led to a noticeable decrease in nuclear population, suggesting potential reductions in cell viability or the induction of apoptotic pathways. Futheromore, a significant decline in F-actin filaments, crucial for cellular structural integrity, was observed. Moreover, the characteristic star-shaped morphology of U87MG cells was no longer seen following ASX and Etoposide treatment, this can be due to structural disruptions and potential alterations in cellular processes.The integrity of fluorescent nuclei and filaments was affected by ASX, Etoposide, and their combination (see Figure 4.11), suggesting possible changes in protein levels, gene expression and cellular responses. Fluorescence staining techniques have illustrated that the anti-proliferative effects of Etoposide was enhanced when it is combined with ASX.

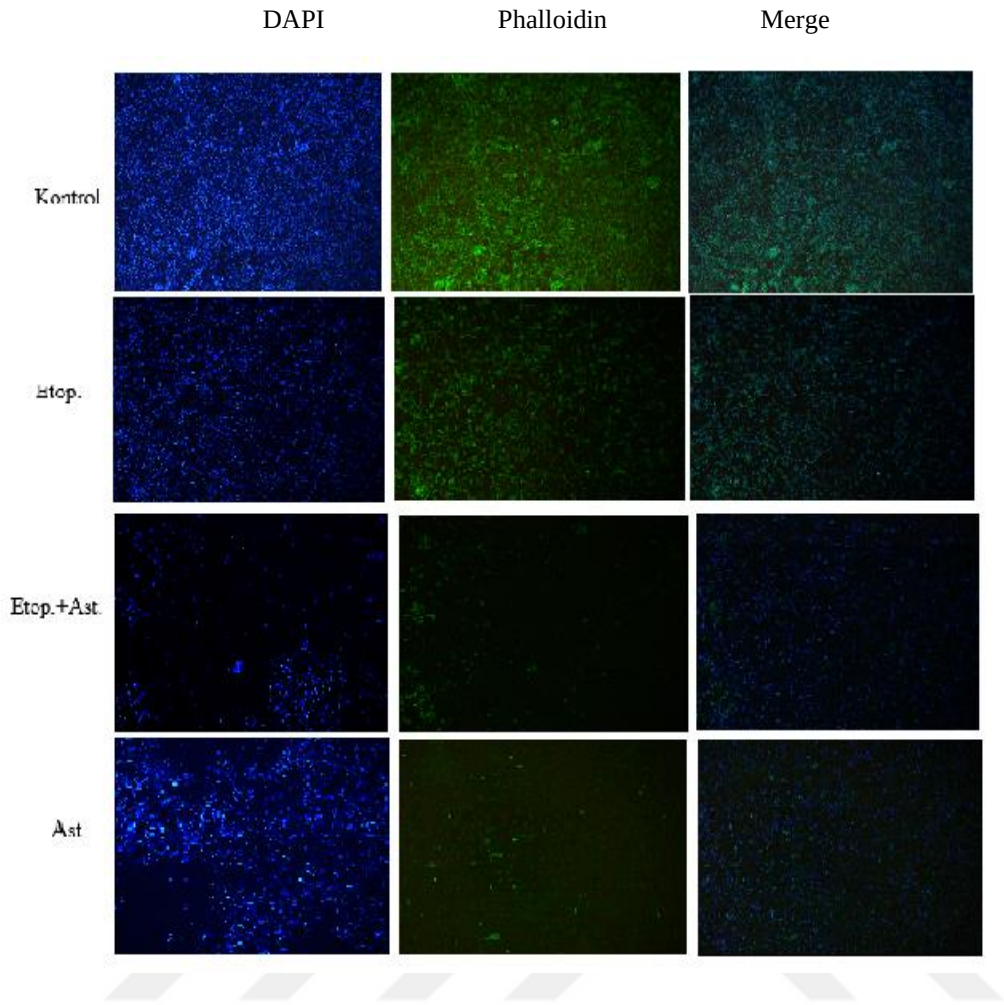


Figure 4. 11 Effect of astxanthin and Etoposide on U87MG cell lines using DAPI and F-Actin staining.

4.7. RT-qPCR Results

RT-qPCR was was used to evaluate to assess the expression levels of Survivin , c-Myc and Caspase-3, genes in U87MG cells subsequent to treatment with Etoposide, ASX, or a combination of both.

One of the key genes that control apoptosis pathways and highly expressed in cancer cells is Survivin (Mobahat et al., 2014). Survivin, also known as BIRC5 (Baculoviral Inhibitor of Apoptosis Protein Repeat-Containing 5), is the smallest member of the inhibitor of the inhibitor of apoptosis protein (IAP) family, comprised of 142 amino acid residues. It is characterized by its single Baculoviral IAP Repeat (BIR) domain (Mobahat et al., 2014).

The overexpression of survivin at both mRNA and protein levels seems to be linked with higher grades of malignacy and decreased survival rates in different types of cancer types including GBM(Shirai et al., 2009). According to our results, mRNA expression analysis in U87MG cells after a 24-hour incubation with ASX and Etoposide rshowed a notable reduction in survivin levels. However, when ASX and Etoposide were administrated in combination, Survivin expression was almost completely suppressed after 24 hours of incubation (Figure 4.12). This reduction in expression levels indicates that these treatments effectively impact the anti-apoptotic pathway by decreasing cell viability and promoting apoptosis.

The c-Myc oncoprotein is well-known for its significant role in the proliferation and growth of cancer cells as it ts dysregulation is commonly observed in various human tumors often indicating advanced malignancy and a poor prognosis including GBM (Vita & Henriksson, 2006; Wang et al., 2008). It was shown in different studies that"the overexpression of c-Myc frequently disrupts essential cellular functions like transcription, translation, and protein synthesis, thereby promoting malignancies development" (Carroll et al., 2018; Van Riggelen et al., 2010).

Similarly, when Etoposide was used alone or with in combination with ASX complete suppression of c-Myc expression was achieved after a 24-hour culture period in U87MG cells (Figure 4.12).

Analysis of Caspase-3 expression levels offers valuable insights into the apoptotic response and the efficacy of treatments aimed at evoking apoptosis. According to our findings, Caspase-3 levels were notably increased when ASX and Etoposide are applied together comparing to the control groups and to the individual treatment of ASX and Etoposide (Figure 4.12).

Comparing to the existing literature, one study on oral cancer in hamsters, it was found that ATX triggers caspase-mediated mitochondrial apoptosis (Kavitha et al., 2013). This occurs through reducing the expression of anti-apoptotic proteins like survivin, while increasing pro-apoptotic proteins such as Bax and Bad. Additionally, in hepatocellular carcinoma cell research, ATX was observed to lower c-myc expression indicating that ASX could induce mitochondria-mediated apoptosis in cancer cells (L. Zhang & Wang, 2015).

Similarily, our findings from RT-qPCR analysis showed that ASX and Etoposide possesses inherent cytotoxicity against U87MG cells. Furthermore, it induces cytotoxic effects by

reducing the expression of crucial genes necessary for cancer cell survival, such as c-Myc and Survivin, while increasing the expression of Caspase-3, a gene pivotal for apoptosis.

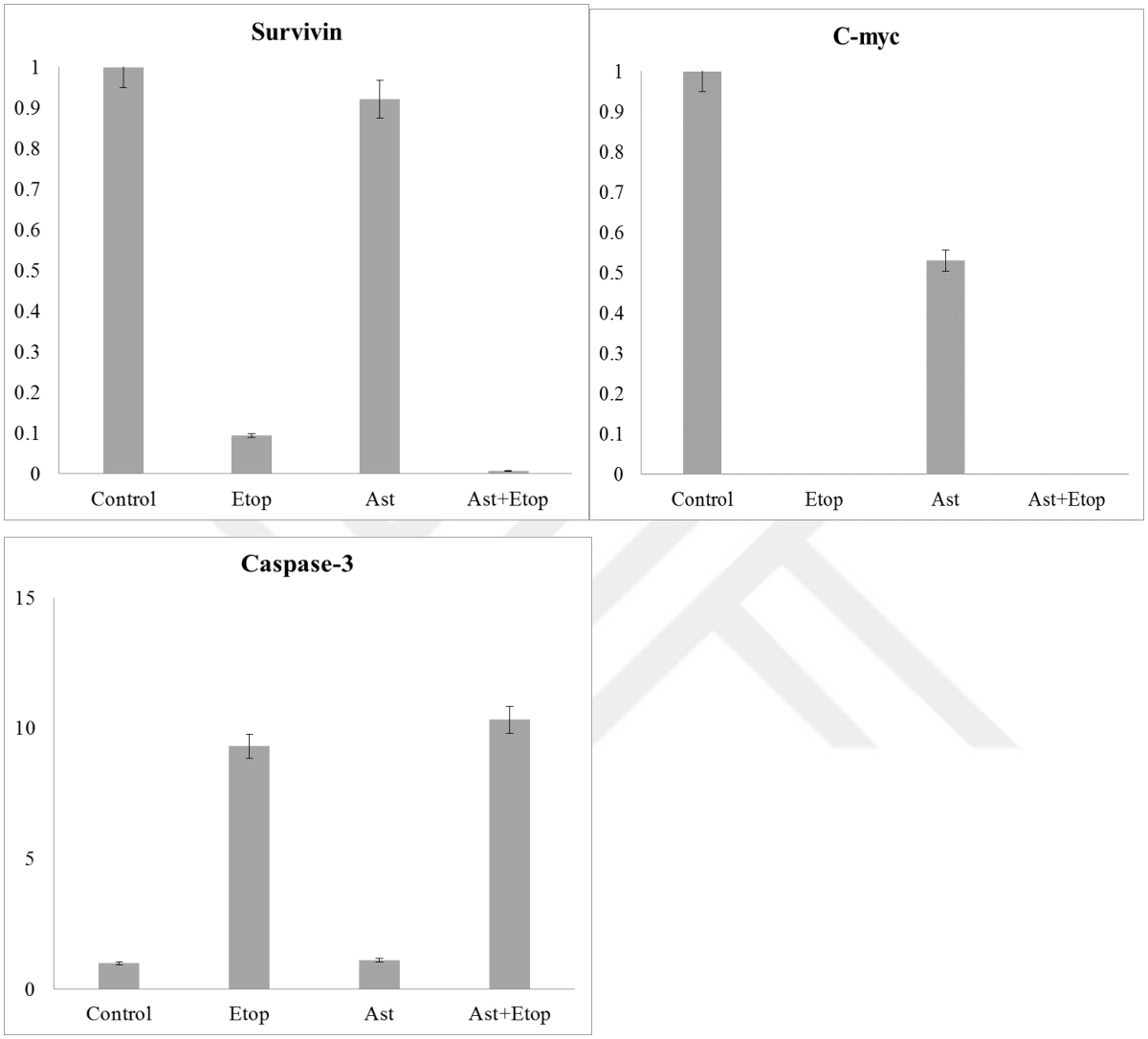


Figure 4. 12 Effect of ASX and Etoposide on the expression of (Survivin, C-myc, and Caspase-3) genes in U87MG cells ($p<0.001$).

4.8. The Results of Anti-Microbial Activity of Astaxanthin

The findings of anti-microbials experiment demonstrated a remarkable antimicrobial potential of ASX against four common gram negative and gram positive bacteria including: Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Bacillus subtilis (B. subtilis), and Bacillus cereus (B. cereus). Comparing to the control groups (Figure 4.13), ASX could inhibit the growth of these bacteria (Figure 4.14). In comparison to the study by Weintraub et al. on ASX-based polymers, which explores structural modifications to enhance antimicrobial properties, our findings demonstrate the potent antimicrobial potential of astaxanthin (ASX) in its natural form (Weintraub et al., 2017). Our experiments highlighted the ASX's effect against Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Bacillus subtilis (B. subtilis), and Bacillus cereus (B. cereus), showing its ability to combat a diverse range of bacterial pathogens and highlighting its promise as a natural antimicrobial agent in comparision to other studies (Ahmed et al., 2022; Weintraub et al., 2017).

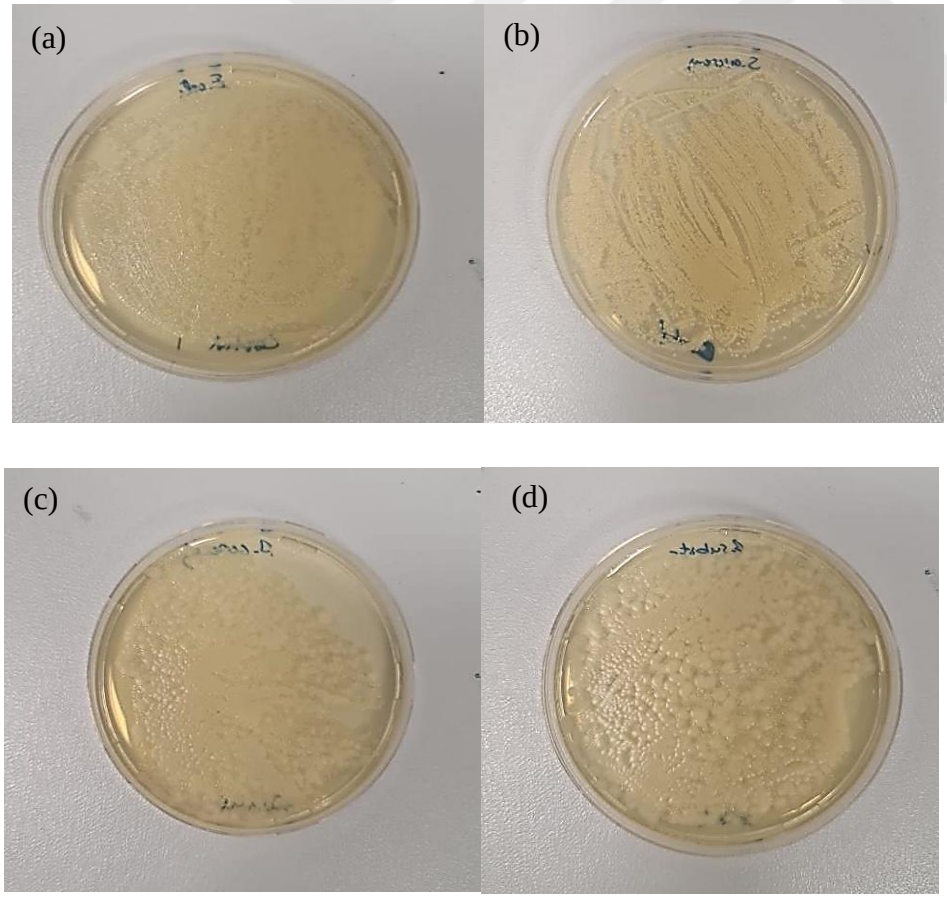
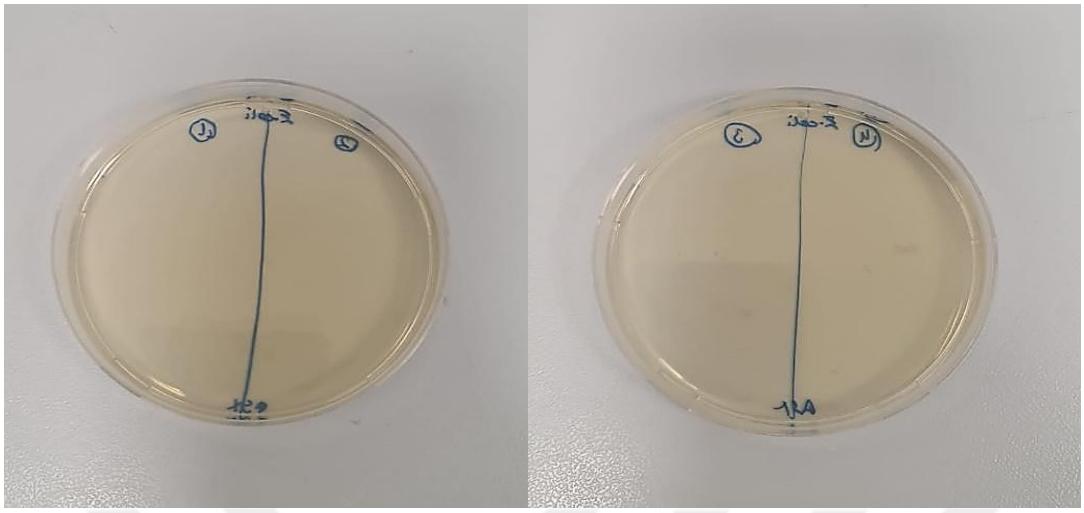
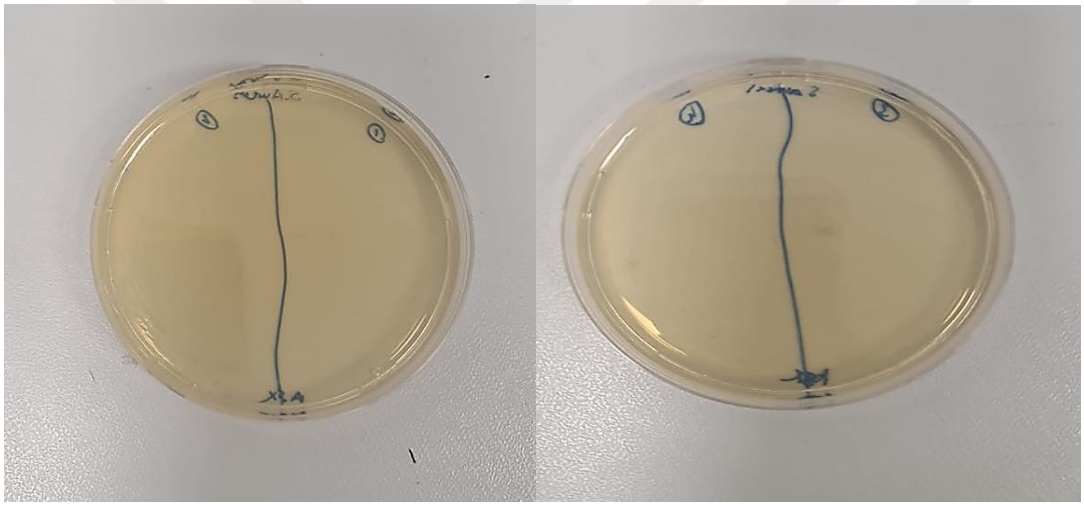


Figure 4. 13 Control goups of (a) E.coli, (b) S. aureus, (c) B. cereus, (d) B. subtilis

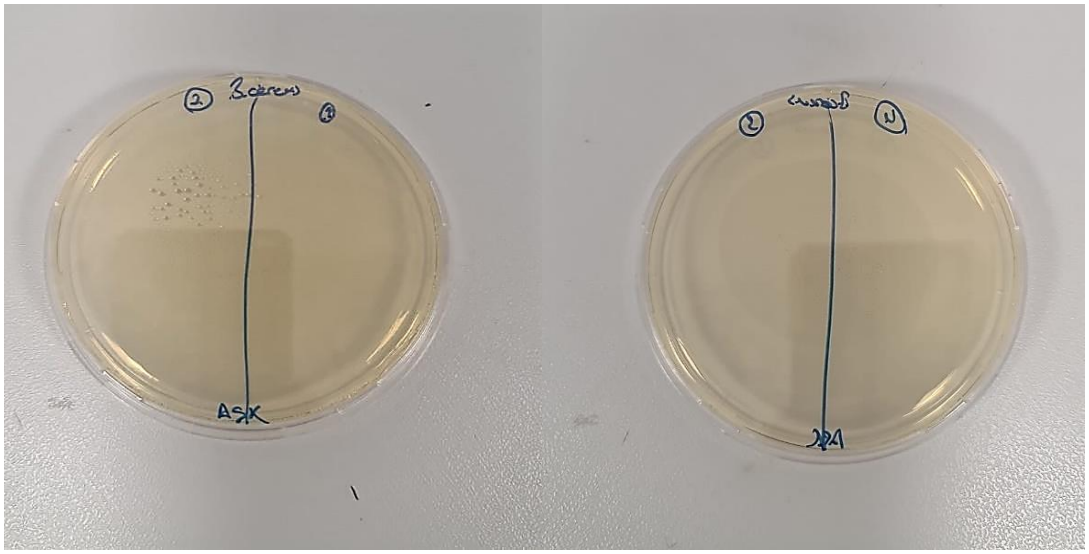
(a)



(b)



(c)



(d)

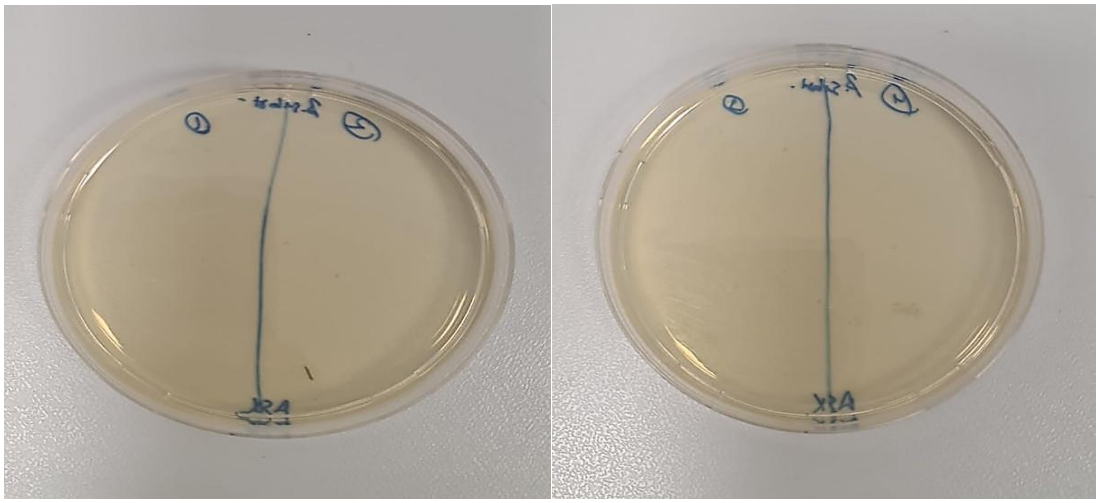


Figure 4. 14 Antimicrobial effect of ASX on (a) E.coli, (b) S. aureus, (c) B. cereus, (d) B. subtilis.

5. CONCLUSION

In conclusion, this study investigated the cytotoxic activity of ASX and Etoposide, individually and in combination, on the GBM cell line U87MG using a variety of assays.The neutral red assay provided initial insights into the impact of ASX and Etoposide on cell viability, indicating a dose-dependent decrease in cell viability upon treatment with astaxanthin, Etoposide, or their combination., while the determination of IC50 values allowed for a quantitative assessment of their cytotoxic effects. Furthermore, the colony formation assay revealed the ability of ASX and Etoposide, alone or in combination, to inhibit the clonogenic potential of U87MG cells, suggesting their potential as anti-proliferative agents. The visualization of cellular morphology through DAPI and F-actin staining provided valuable insights into the morphological changes induced by treatment with astaxanthin, Etoposide, or their combination, further supporting their cytotoxic activity. Furthermore, Giemsa staining facilitated the evaluation of cellular morphology and integrity, offering supplementary insights alongside other assays. The findings also revealed that ASX and Etoposide, whether individually applied or in combination, resulted in the downregulation of survivin and c-Myc expression, both recognized for their anti-apoptotic functions. Moreover, astaxanthin and Etoposide, whether administered independently or in conjunction, led to an increase in Caspase-3 expression, indicating that their combined treatment enhances apoptotic pathways in cancer cells.

Finally, the anti-microbial test showed that astaxanthin could inhibit the growth of Bacillus subtilis, Bacillus , Staphylococcus aureus and Escherichia coli showing a complete decrease in the number of colonies growing, however, while this study provides insights into the potential antimicrobial activity of ASX.

Overall, the findings of this study highlight the promising antimicrobial activity of ASX, suggesting its potential for developing novel antimicrobial agents alongside its cytotoxic effects on GBM cells when used alone or in combination with Etoposide.

Future research efforts could focus on elucidating the specific molecular pathways through which ASX exerts its cytotoxic effects, as well as exploring its efficacy in preclinical and clinical settinig. Morever, future research should focus on understanding how ASX works against microbes as investigating its specific effects on bacterial cells can help us understand exactly how it stops them from growing and spreading. It would also be important to study how ASX works synergistically with other medicines that fight infections.

RECOMMENDATIONS

This study was performed in order to investiagate the cytotoxic activity of ASX and Etoposide on U87MG glioblastoma cells. Based on the findings, it is recommended to further study the molecular pathways that are used by ASX to exert cytotoxic effects, indicating also more detailed exploration on how ASX promote apoptotic pathways in cancer cells as it downregulates survivin and c-Myc expression while enhancing Caspase-3 activity. This understanding is crucial for using ASX-based therapies and developing targeted treatment strategies for glioblastoma. Moreover, taking in consideration the promising antimicrobial activity of ASX against different types of bacteria including Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, and Escherichia coli, it is recommended to delve deeper into understanding how ASX interacts with bacterial cells to inhibit their growth and spread. Furthermore, more studies should focus on evaluating ASX in preclinical and clinical settings to evaluate its effectiveness in therapy. Additionally, investigating synergistic interactions between ASX and other chemotherapeutic drugs that are widely used in the treatment of GBM could enhance treatment outcomes and address emerging challenges to maximize its therapeutic potential in cancer.

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APPENDIX

Appendix A- ANOVA analyses for dose optimization (IC50 determination) assay

Appendix B- ANOVA analyses for cytotoxicity assays of U87MG cells

Appendix C- t-test analyses for cytotoxicity assay of U87MG cells

Appendix A- ANOVA analyses for dose optimization (IC50 determination) assay

Table A.1. ANOVA analyses for dose optimization (IC50 determination) assay of U87MG ($p<0,001$).

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0 µg/mL	3	2,892	0,964	0,000399
20 µg/mL	3	2,418	0,806	0,000516
40 µg/mL	3	2,167	0,722333	0,000196
60 µg/mL	3	1,928	0,642667	0,00011
80 µg/mL	3	1,52	0,506667	3,03E-05
100 µg/mL	3	1,29	0,43	2,8E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,736346	5	0,147269	537,1519	1,13E-13	3,105875
Within Groups	0,00329	12	0,000274			
Total	0,739636	17				

Appendix B- ANOVA analyses for cytotoxicity assays of U87MG cells

Table B.1. ANOVA analyses for cytotoxicity assay of 24 hours cultured U87MG cells ($p<0,001$)

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	2,031	0,677	0,000271
ASX	3	0,968	0,322667	6,53E-05
ASX+Etoposide	3	0,675	0,225	3,6E-05
Etoposide	3	0,968	0,322667	5,03E-05

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,355864	3	0,118621	1122,6	7,75E-11	4,066181
Within Groups	0,000845	8	0,000106			
Total	0,35671	11				

Table B.2. ANOVA analyses for cytotoxicity assay of 48 hours cultured U87MG cells ($p<0,001$)

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	3	1,68	0,56	5,2E-05
ASX	3	0,755	0,251667	3,43E-05
ASX+Etoposide	3	0,531	0,177	4,9E-05
Etoposide	3	0,639	0,213	0,000016

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,2779	3	0,092633	2448,46	3,45E-12	4,066181
Within Groups	0,000303	8	3,78E-05			

Total	0,278203	11

Appendix C- t-test analyses for cytotoxicity assay of U87MG cells

Table C.1. t-test analyses for cytotoxicity assay of 1-day cultured U87MG cells (p<0,001)

t-Test: Paired Two Sample for Means

	Control	*ASX*
Mean	0,677	0,322667
Variance	0,000271	6,53E-05
Observations	3	3
Pearson Correlation	0,939416	
Hypothesized Mean Difference	0	
df	2	
t Stat	66,05161	
P(T<=t) one-tail	0,000115	
t Critical one-tail	2,919986	
P(T<=t) two-tail	0,000229	
t Critical two-tail	4,302653	

t-Test: Paired Two Sample for Means

	Control	*Etoposide*
Mean	0,677	0,301333
Variance	0,000271	0,000152
Observations	3	3
Pearson Correlation	0,204252	
Hypothesized Mean Difference	0	
df	2	
t Stat	35,27045	
P(T<=t) one-tail	0,000401	
t Critical one-tail	2,919986	
P(T<=t) two-tail	0,000803	
t Critical two-tail	4,302653	

Table C.2. t-test analyses for cytotoxicity assay of 2-days cultured MIAPaCa-2 cells (p<0,001)

Table C.2. t-test analyses for cytotoxicity assay of 2-days cultured U87MG cells ($p<0,001$)

t-Test: Paired Two Sample for Means

	Control	*ASX*
Mean	0,56	0,251667
Variance	5,2E-05	3,43E-05
Observations	3	3
Pearson Correlation	-0,071	
Hypothesized Mean Difference	0	
df	2	
t Stat	55,57786	
P(T<=t) one-tail	0,000162	
t Critical one-tail	2,919986	
P(T<=t) two-tail	0,000324	
t Critical two-tail	4,302653	

t-Test: Paired Two Sample for Means

	Control	*ASX+Etoposide*
Mean	0,56	0,183333
Variance	5,2E-05	3,63E-05
Observations	3	3
Pearson Correlation	-0,62117	
Hypothesized Mean Difference	0	
df	2	
t Stat	54,68453	
P(T<=t) one-tail	0,000167	
t Critical one-tail	2,919986	
P(T<=t) two-tail	0,000334	
t Critical two-tail	4,302653	

t-Test: Paired Two Sample for Means

	Control	*Etoposide*
Mean	0,56	0,213
Variance	5,2E-05	0,000016
Observations	3	3
Pearson Correlation	-0,97073	
Hypothesized Mean Difference	0	
df	2	
t Stat	53,97334	
P(T<=t) one-tail	0,000172	
t Critical one-tail	2,919986	
P(T<=t) two-tail	0,000343	
t Critical two-tail	4,302653	