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# *The impact of Lavender essential oil on the oncogenic properties of human GBM cells*

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# Abbreviations

| | |
|---|---|
| AE | *Aloe-Emodin* |
| AIC | *5-amino-imidazole-4-carboxamide* |
| AIOM | *Italian Medical Oncology Association* |
| AKT | *Protein Kinase B (PKB)* |
| APC | *APC regulator of Wnt signaling pathway* |
| ARTX | *X-linked inheritance, alfa-thalassemia* |
| BBB | *Blood-Brain Barrier* |
| BBR | *Barberine* |
| bLF | *Bovine Lactoferrin* |
| BMDMs | *Bone Marrow-Derived Macrophages* |
| CAR-T | *Chimeric antigen receptor T cell therapy* |
| CCR2 | *C-C motif chemokine receptor 2* |
| CD24/44 | *Class of Differentiation 24/44 molecule* |
| CDF | *Convention-enhanced delivery* |
| CDK2 | *Cyclin- Dependent Kinase 2* |
| CED | *Convention-enhanced and delivery* |
| cIMPACT | *Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy* |
| CNS | *Central Nervous System* |
| CNS5 | *Who Classification CNS Tumor 5* |
| CSF | *Cerebrospinal Fluid* |
| CT | Computed Tomography |
| CTLA-4 | *Cytotoxic T Lymphocyte-related protein 4* |
| E2F | *Transcription factor E2F* |
| EBRT | *External Beam Radiation Therapy* |
| *ECM* | *Extracellular matrix* |
| EGF | Epidermal Growth Factor |
| EGFR | Epidermal Growth Factor Receptor |
| EMT | *Epithelial- Mesenchymal Transition* |
| EPR | *Enhanced Permeability and Retention* |
| FDA | *Food and Drug Administration* |
| FRP | *Frizzled-related protein* |

| | |
|---|---|
| GBM | *Glioblastoma multiforme* |
| GK | *Gamma Knife* |
| GLI1 | *Glioma-Associated Zing Finger Transcription Factor* |
| GSCs | *Glioma stem cells* |
| GSK-3β | *Glycogen Synthase Kinase- 3 beta* |
| $H_2O_2$ | *Hydrogen peroxide* |
| HF-SRS | *Hypofractioned- Stereotactic Radiosurgery* |
| Hh | *Hedgehog* |
| HIF-1α | *Hypoxia-induced factor 1* |
| HIFUS | *High Intensity Focused Ultrasound* |
| HLF | *Lactoferrin* |
| HO· | *Hydroxide* |
| ICIs | *Immune Checkpoint inhibitors* |
| IDH | *Isocitrate dehydrogenase* |
| IL-10 | *Interleukin-10* |
| IL-6 | *Interleukin-6* |
| Iso | *Isoginkgetin* |
| JAK | *Janus Tyrosine Kinase* |
| LCTP | *Lactucopicrin* |
| LDH-A | *Lactate Dehydrogenase-A* |
| LEO | *Lavender Essential Oil* |
| LERS | *Leading-Edge Radiosurgery* |
| LMD | *Leptomeningeal disease* |
| MAPK | *Mitogen activated kinase-like protein* |
| MDM2 | *MDM2 Proto-oncogene* |
| MDSCs | *Myeloid-Derived Suppressor Cells* |
| MEK | *Mitogen-activated protein Kinase Kinase* |
| MGMT | *O6-Methylguanine DNA Methyltransferase* |
| MGs | *Microglial Cells* |
| MHC | *Major Histocompatibility Complex* |
| MITC | *5-(3-methyltrazen-1-yl)-imidazole-4-carboxamide* |
| MITC | *5-(3-methyltriazen-1-yl)-imidazole-4-carboxiamide* |
| M-MDSCs | *Monocytic- Myeloid-Derived Suppressor Cells* |

| | |
|---|---|
| MMP-2 | *Matrix Metalloproteinase-2* |
| MMP-9 | *Matrix Metalloproteinase-9* |
| MNPS | *Magnetic Nanoparticles* |
| MRI | *Magnetic Resonance Imaging* |
| mTOR | *mammalian Target of Rapamycin* |
| mTORC1/2 | *mammalian Target of Rapamycin Complex 1/2* |
| MTX | *Methotraxate* |
| MYB | *MYB proto-oncogene, transcription factor* |
| MYBL1 | *MYB proto-oncogene like 1* |
| Nanog | *Nanog homeobox* |
| NCCN | *National Comprehensive Cancer Network* |
| NEC | *Not Elsewhere Classified* |
| NF1 | *Neurofibromin 1* |
| NFk-$\beta$ | *Nuclear factor kappa B* |
| NOS | *Not Otherwise Specified* |
| NP | *Nanoparticles* |
| NSCs | *Neural Stem cells* |
| O2- | *Superosside* |
| OPC | *Oligodendrocyte progenitor cell* |
| OS | *Oxidative Stress* |
| PARP1 | *Poly (ADP-ribose) Polymerase 1* |
| PAX 7 | *Paired box protein 7* |
| PBT | *Proton Beam Therapy* |
| PDGF | *Platelet-derived growth factor* |
| PDGFR | *Platelet-derived growth factor receptor* |
| PFS | *Progression-Free Survival* |
| PI3K | *Phosphoinositide-3-Kinase* |
| PIK3CA | *Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha* |
| PL | *Phellinus linteus* |
| PLCG1 | *Phospholipase C gamma 1* |
| PMN-MDSCs | *Polymorphonuclear- Myeloid-Derived Suppressor Cells* |
| PT | *Proton Therapy* |
| PTEN | *Phosphatase and Tensin homolog* |

| | |
|---|---|
| PTPN11 | *Protein tyrosine phosphatase non-receptor type 11* |
| RAF | *Rapidly accelerated fibrosarcoma protein* |
| RAPTOR | *Regulatory Associated Protein of MTOR complex 1* |
| RAS | *Ras GTPase protein* |
| Rb | *Retinoblastoma* |
| RIPK1 | Receptor-interacting serine/threonine-protein kinase 1 |
| ROS | *Reactive Oxygen species* |
| RT | *Radiotherapy* |
| RTK | *Receptor Tyrosine Kinase* |
| SRS | *Stereotactic Radiosurgery* |
| STAT1/3/6 | *Signal Transducer and Activator of Transcription 1/3/6* |
| SVZ | *Subventricular zone* |
| TAMs | *Tumor Associated Macrophages* |
| TEM7 | *Tumor Endothelial Marker 7* |
| TERC | *Telomerase RNA* |
| TERT | *Telomerase Reverse Transcriptase* |
| TERTp | *Telomerase Reverse Transcriptase promoter* |
| TGF-β | *Tumor Growth Factor- β* |
| TME | *Tumor microenvironment* |
| TMZ | *Temozolomide* |
| TNFR1 | *Tumor necrosis factor receptor 1* |
| TP53 | *Tumor protein p53* |
| TRADD | *Tumor necrosis factor receptor type 1-associated DEATH domain protein* |
| TTF | *Tumor-Treating Field* |
| TTO | *Tea Tree Oil* |
| VEGF | *Vascular Endothelial Growth Factor* |
| WBRT | *Whole-brain radiation therapy* |
| WHO | *World Health Organization* |

## Abstract

Glioblastoma (GBM) is the most common and aggressive type of brain cancer in adults, known for its high recurrence, fast growth, and significant resistance to standard treatments. Although there have been improvements in surgical methods, radiotherapy, and chemotherapy, managing GBM continues to be a major challenge, with an average survival of just 14 months for patients. The poor selectivity of the antineoplastic drugs to malignant cells over normal cells is the main reason for their toxicity. This grim outlook has led to a rise in interest in alternative and complementary treatment methods, especially the use of natural substances as adjuvant chemotherapic agent for their healing effects in cancer care. *Lavandula angustifolia*, a medicinal plant, has been used as traditional remedies in various cultures for the past thirty years due to its anti-inflammatory and anti-cancer abilities. This healing plant might offer effective treatment choices for several conditions, such as osteoarthritis, pancreatic cancer, breast cancer, liver cancer, and Alzheimer's disease. In addition, it has been proven that the essential oils taken from these healing herbs can disrupt many of the cancer-causing features of tumors. Lavender essential oil (LEO), made from *L. angustifolia* flowers, mentioned earlier, contains active compounds that exert important biological effects affecting key signaling pathways involved in cell death, growth, and differentiation. Recent studies indicate that terpenes like borneol, linalool, and 1,8-cineole, found in essential oils, can slow down the cell cycle and trigger cell death in different cancer cells. Also, terpinen-4-ol has strong antioxidant properties, shown by its ability to help maintain oxidative metabolism balance, both *in vitro* and *in vivo*. There is no information in the literature about the effects of LEO on GBM models, but it was recently reported that terpinen-4-ol and borneol causes cell death and increases the effectiveness of TMZ in glioma cells, respectively. In this context, our study aimed to investigate how LEO and its terpenic component affect the cancerous properties of the human U87MG GBM cell line in an *in vitro* model. The administration of sub-lethal LEO concentrations was employed to exclude non-specific cytotoxicity phenomena. Our research shows that LEO affects U87MG cell growth and migration, and it reduces oxidative stress in the

GBM cell line. Specifically, we showed that LEO slows down growth of U87MG and enhances the effects of TMZ on these cells. Furthermore, the growth inhibition of U87MG is linked to an unusual co-increase of cyclin D1 and p21, two important enzymes that regulate cell cycle. It has been noted that LEO impairs GBM cell migration abilities, and oxidative stress is reduced, as shown by lower levels of oxidized markers, 8-OH(d)G, which indicates oxidative damage to DNA, and 4-HNE, which marks lipid damage. Among the analysed terpenes in LEO, terpinen-4-ol, even though it appears in low amounts, showed significant antiproliferative activity in U87MG cell line, both alone and with TMZ, increasing the expression of cell cycle regulators. Terpinen-4-ol also induces a notable change in GBM cell movement and lowers cell oxidative damage by affecting levels of 8-OH(d)G and 4-HNE. Finally, terpinen-4-ol fully mirrors the effects of LEO on the cancer-related traits of GBM cells, suggesting that it may be, at least in part, responsible for LEO's anticancer activity.

# *Introduction*

# 1. GLIOMA

Glioma is the most common type of Central Nervous System tumor, with various types and levels of malignancy (*Thakkar JP et al., 2014; Jawhari S et al., 2016*). These tumors likely come from glial progenitor cells or neural stem cells that gain glial traits after transformation (*Chen L et al., 2015; Curry RN et Glasgow SM, 2021*). Adult diffuse gliomas are the most common malignant brain tumors, accounting for 27-30% of all primary brain tumors and 80% of malignant ones, often leading to poor outcomes (*Schwartzbaum JA et al., 2006; Hanif F et al, 2017*). Astrocytomas account for 75% of all gliomas, with glioblastoma—grade IV and the most aggressive subtype—being the most common (*Ostrom QT et al., 2017; Tripathy DK et al., 2024*). The Central Brain Tumor Registry of the United States states that glioma patients have a poor prognosis: under 2% of those over 65 survive, and only 30% of patients under 45 live more than two years after diagnosis. High-dose radiation from chemotherapy is the main environmental risk for glioma, affected by genetic susceptibility (*Braganza MZ et al., 2012*). Family history doubles risk (*Campodonico JR et McGlynn SM, 1995*), while neurocarcinogenic effects are linked to N-nitroso compounds, reactive species, and polycyclic aromatic hydrocarbons (*Cao W et al, 2023; Sheweita SA et Mostafa MH, 1996*).

### *1.1 Classification of Glioma*

Gliomas are intrinsic CNS tumors, classified into “diffuse gliomas”, which infiltrate brain tissue, and “focal or circumscribed gliomas” like pilocytic astrocytoma and ependymoma (*Louis DN et al., 2016*). The 2007 WHO classification subdivided diffuse gliomas into oligodendrogliomas, oligoastrocytomas, and astrocytoma, with tumors graded based on increasing malignancy. Astrocytic gliomas are graded into Grade I, II, III, and IV (*Louis DN et al., 2007; Zeng T et al., 2015*)(**Table 1).**

**Table 1- WHO Glioma Classification (2007)**

| Phenotype | Subtype | Grade |
|---|---|---|
| **Astrocytic tumors** | Pilocytic astrocitoma | I |
| | Diffuse astrocitoma | II |
| | Anaplastic astrocytoma | III |
| | Glioblastoma | IV |
| **Oligodendroglial tumors** | Oligodentoglioma | II |
| | Anaplastic oligodendroglioma | III |
| **Oligoastrocytic tumors** | Oligoastrocytoma | II |
| | Anaplastic oligoastrocytoma | III |

Classifying gliomas has been difficult because different observers interpret tumors in various ways (*Wen PY et al., 2010*). To improve accuracy, histological data must now include molecular, genomic, and epigenetic information, leading to a revised classification system. The 2016 WHO update enhanced glioma classification by combining molecular and traditional histological features (*Louis DN et al., 2016*). Genetic profiling is essential for managing diffuse low- and intermediate-grade gliomas (*Gritsch S et al., 2022;Theeler BJ et al., 2012*), focusing on key genetic markers like co-deletion of chromosome arms 1p and 19q, mutations in IDH genes, and alterations in ATRX and TP53 (*Jiao Y et al., 2012; Yan H et al., 2009; van den Bent MJ et al., 2013; Cairncross G et al., 2013; Liu XY et al., 2012*). The updated classification system for lower-grade gliomas categorizes them into IDH-mutant and IDH wild-type (**Figure 1**). Oligodendrogliomas are noted for IDH mutations and 1p/19q co-deletions 19q (*Ghantasala S et al., 2020; Lauber C et al., 2018*). Glioblastomas are also split by IDH mutation status, with wild type linked to worse outcomes. IDH-mutant gliomas often harbour TP53 mutations (1p/19q co-delections) (*Liu XY et al., 2012*). Key mutations in IDH wild-type gliomas include PTEN (23% of cases), EGFR (27%), NF1 (20%), TP53 (14%), PIK3CA (9%), PTPN11 (7%), and PLCG1 (5%) (*Brennan CW et al., 2013;*

*Pienkowski T et al., 2021*). The 2016 WHO classification introduced a "Not Otherwise Specified" (NOS) category for atypical tumors requiring further investigation for precise classification.

The 2021 fifth edition of the WHO Classification of CNS Tumors (CNS5) updates the 2016 version by including findings from the from the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT). It integrates molecular data into diagnoses, revises names for diffuse gliomas, and introduces new tumor types (*McNamara C et al., 2022*). Diffuse gliomas are now split into "adult-type" and "pediatric-type. " Adult-type diffuse gliomas are grouped into three main categories: IDH-mutant astrocytoma, IDH-mutant oligodendroglioma with 1p/19q co-deletion, and IDH-wildtype glioblastoma. Astrocytomas are treated as a single tumor type and are classified as CNS WHO grades 2, 3, or 4. Grade 4 IDH-mutant astrocytomas have specific features like microvascular proliferation or necrosis (*Louis DN et al., 2021*). Oligodendrogliomas contain an IDH mutation and 1p/19q co-deletion, known as "IDH-mutant oligodendroglioma with 1p/19q co-deletion". Testing for the co-deletion in diffuse gliomas with IDH mutations is recommended. Alternative diagnostic methods include immunohistochemical analysis for ATRX loss or TP53 expression. TERT promoter (TERTp) mutations support this classification. IDH-wildtype astrocytomas that look like grade 4 IDH-wildtype glioblastoma can be identified based on molecular markers, even without classic histopathological features (*Komori T, 2022*).

Pediatric diffuse gliomas are less common than circumscribed gliomas but are becoming more recognized. They can show astrocytic or oligodendroglial features and are classified as low-grade or high-grade, all being IDH-

wildtype. Low-grade pediatric gliomas, which have a better outlook, are CNS WHO grade 1 and often have MYB/MYBL1 and MAPK pathway changes (*Louis DN et al., 2021*). In contrast, high-grade pediatric gliomas are characterized by mutations in histone genes, which are linked to a poor prognosis (*Voon HPJ et Wong LH, 2023*). These tumors have different features like necrosis, high cellularity, and blood vessel growth. CNS5 explains "Not Otherwise Specified" (NOS) means not enough molecular info for a diagnosis, while "Not Elsewhere Classified" (NEC) means testing was done but doesn't match WHO criteria. Advances in molecular studies improve diagnosis and decision-making. Including these insights in clinical trials may lead to more personalized treatments.

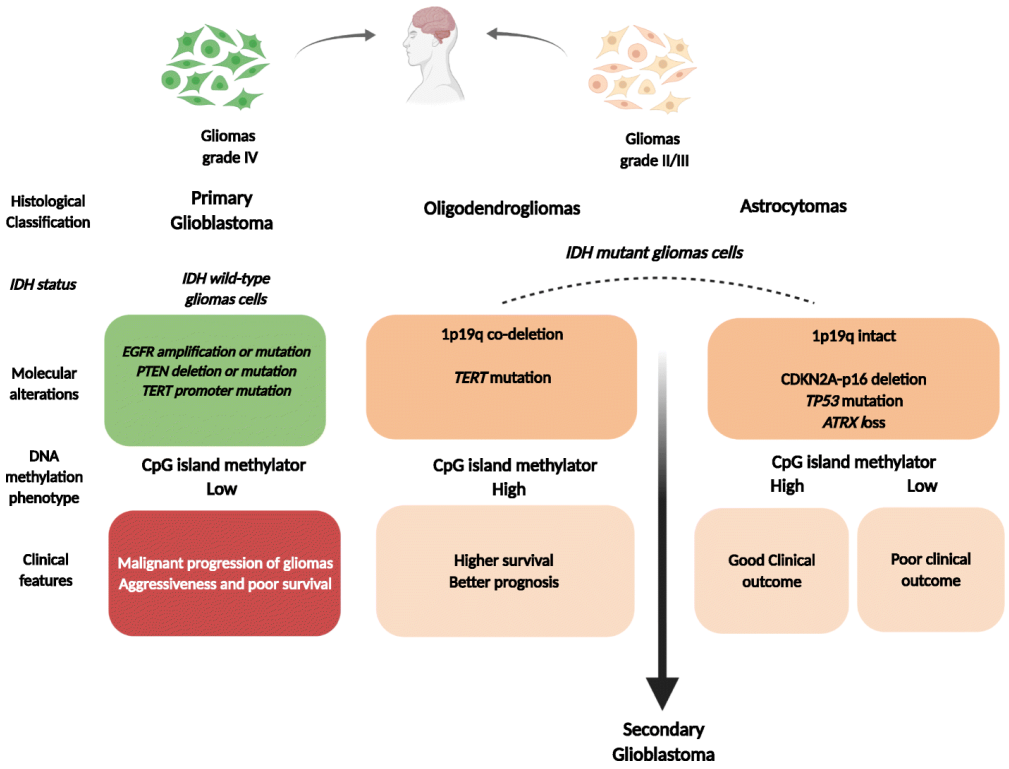



**Figure 1. Gliomas classification regarding the mutation status of isocitrate dehydrogenase 1 (IDH-1) gene.** The main molecular changes in primary GBM include EGFR amplification or mutation, LOH of chromosome 10q at the PTEN locus, and TERT gene promoter mutation. In secondary GBM, common changes include 1p19q codeletion, homozygous CDKN2A-p16 deletion, and IDH1/2 mutations, affecting prognosis *(Image adapted from Alves ALV et al., 2021).*

# 2. GLIOBLASTOMA MULTIFORME

Glioblastoma multiforme (GBM) is the most common and aggressive form of malignant cerebral glioma, accounting for 60-70% of all glial tumors (*Furnari FB et al., 2007; Polivka J et al., 2014*). It occurs more often in men than women and is more prevalent in Caucasians compared to other ethnic groups. In Italy, the Italian Medical Oncology Association (AIOM) reports approximately 1,500 new cases of GBM annually, with a slight male predominance (54% men, 46% women). According to the WHO 2016 classification, GBM is classified as a grade IV tumor and stands for 50% of all gliomas. The median age of diagnosis is 65 years, and the incidence is higher in men (*Sun H et al., 2013; Ostrom QT et al., 2018*). GBM typically presents with nonspecific symptoms, such as headaches and personality changes. However, it is characterized by rapid growth, significant intra-tumor heterogeneity, and an invasive tendency to infiltrate surrounding brain tissue (*Janjua TI et al., 2021*). These features contribute to the disease's rapid progression and poor prognosis, with a median survival of 16-18 months and only 10% of patients surviving beyond five years after diagnosis (*Czapski B et al., 2018; Grochans S et al., 2022*). Recurrence is common, driven by tumor regrowth, difficulties in drug delivery across the blood-brain barrier (BBB), and resistance to radiotherapy (*Uddin MS et al., 2022; Rabah N et al., 2023*). Standard treatment for GBM includes surgical resection followed by radiotherapy and chemotherapy with temozolomide (TMZ) for 6-12 cycles (*Addeo R et al., 2011*). However, due to the aggressive nature of the tumor, complete surgical removal is often not possible, and recurrence is inevitable (*Bonosi L et al., 2023*). Factors like the extent of surgical resection, postoperative recovery, and neurological complications can delay later treatments, often leading to disease progression within weeks or up to two years for most patients (*Biswas C et al., 2024*). With the standard treatment regimen, survival typically ranges from 6-9 months, extending to 12-16 months with the addition of radio-chemotherapy. In cases where only surgery is performed, 50% of patients survive around 12 months, while 20% may live up to 24 months. GBM has two main types: primary or "de novo"

GBM, which is rapid and makes up over 90% of cases, and secondary GBM, which comes from lower grade astrocytoma (grade II or III). Primary GBM mainly affects those over 60, while secondary GBM affects younger patients, and it is associated with a better prognosis. Both types show similar features. Despite their different origins, primary and secondary GBM share significant morphological and histological similarities (*D'Alessio A et al., 2019*).

### *2.1 Genesis and tumor environment*

The origin of glial tumors, particularly GBM, is still a subject of ongoing research, with one widely discussed theory suggesting that GBM arises from a neuronal network originating in the subventricular zone (SVZ). This network extends toward the frontotemporal cortex, forming a distinct "fireworks" pattern (**Figure 2**).

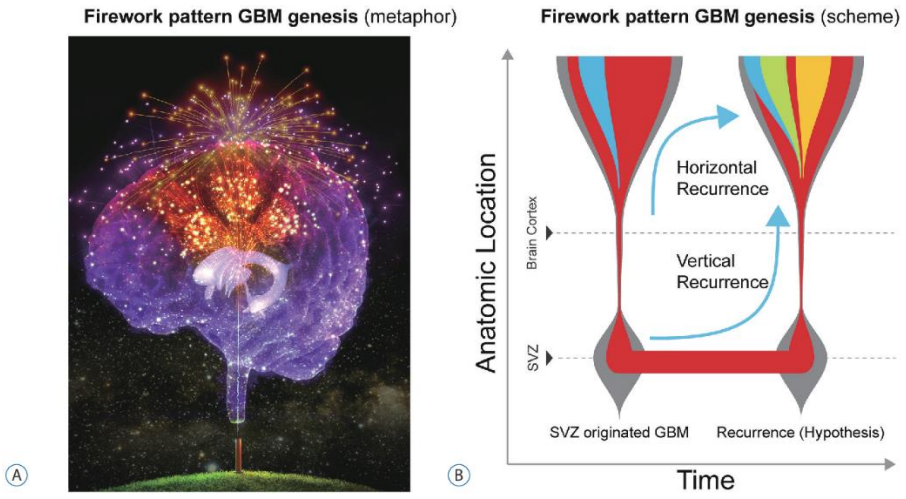



**Figure 2. Firework pattern of IDH-wildtype GBM genesis.** A : Artistic illustration of IDH-wildtype GBM originating from the SVZ (colored in gold). Each firework trail corresponds to a different cancer clone. In this metaphorical depiction, the SVZ is represented as a cannon on the ground, denoting the starting point of GBM genesis. B : Conceptual illustration of the timeline of the genesis of the firework pattern of IDH-wildtype GBM. Horizontal recurrence (or classical model) : GBM recurs from SVZ (red) → tumor (blue) →

recurrence (green), vertical recurrence (hypothetical model) : primary GBM originates from SVZ (red) → tumor (blue), and it recurs from SVZ (red) → recurrent tumor (orange). GBM : glioblastoma, SVZ : subventricular zone, IDH : isocitrate dehydrogenase (*Image adapted from Yoon S et al., 2019*).

The SVZ, a 3-5 mm thick neuroepithelial layer between the lateral ventricle, corpus callosum, and striatum, hosts a high rate of cell proliferation and a significant population of neural stem cells (NSCs). These NSCs are implicated in GBM development, with mutations believed to originate in the SVZ and spread in a fireworks pattern (*Beiriger J et al., 2022*). During early embryonic brain development, neuroepithelial cells elongate to connect with cerebrospinal fluid (CSF) and blood vessels. These elongated cells form radial glia, crucial for brain structure integrity. In GBM, glioma stem cells (GSCs) drive tumor growth, self-renew, and communicate with their environment *(Vieira de Castro J et al., 2020*). GSCs, originating from NSCs in the SVZ, contribute significantly to GBM's heterogeneity and treatment resistance. NSCs are multipotent cells linked to GBM due to their location and characteristics. Mature astrocytes can dedifferentiate into tumor cells, while oligodendrocyte progenitor cell OPC-like cells adapt their transcriptome, enhancing GBM growth through interactions with GSCs. NSCs depend on endothelial cells and pericytes for maintenance, interacting with GSCs and microglia, influencing proliferation and differentiation related to GBM development (*Lin S et al., 2023*). The SVZ is regarded as a unique cancer stem cell niche due to its proximity to CSF, which may influence GBM malignancy. GSCs near the SVZ may receive abnormal CSF signals, causing unchecked growth and genetic changes, potentially driving tumor recurrence through reactivation (*Sistigu A et al., 2020*).

GBMs are histologically defined by features such as cellular and nuclear atypia, frequent mitotic activity, necrosis, and microvascular proliferation (*Brat DJ et al., 2004; Ghosh M et al., 2022*). However, at the molecular level, GBMs exhibit significant

intertumoral heterogeneity, which contributes to the limited effectiveness of conventional therapies. The tumor microenvironment (TME) of GBMs is vital for tumorigenesis, facilitating communication between tumor and host cells. This interaction enhances tumor growth, survival, and treatment resistance, involving diverse cell types and signaling components of the extracellular matrix that collectively influence GBM progression and therapeutic responses (*Broekman ML et al., 2018).*

One hallmark of GBM is microvascular proliferation, which greatly promotes tumor growth through various methods. These methods include angiogenesis, where tumor cells produce factors like VEGF to create new blood vessels; vascular co-option, where tumor cells use existing blood vessels to invade tissue; and vasculogenesis, which brings endothelial progenitor cells from the bone marrow to form new vessels. GSCs can also change into endothelial cells, aiding in vascular formation, while vascular mimicry happens when GSCs create structures that look like blood vessels, imitating pericytes or smooth muscle cells to form vascular-like networks (*Liu ZL et al., 2023; Ahir BK et al., 2020*). GBMs have significant blood supply but face poor circulation, creating low-oxygen areas within the tumor. These hypoxic regions affect various cell types (*Colwell N et al., 2017*). HIF-1α is crucial in these areas, promoting the growth of GSCs and making treatment less effective (*Soeda A et al., 2009*). Hypoxia also changes how tumor cells use energy, helping them survive and grow. Furthermore, HIF-1α boosts tumor cell movement and invasiveness through changes in cell structure and blood vessel permeability.

Immune cells make up about 50% of tumor mass in GBM. Myeloid cells, especially tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), are important for immunosuppression and tumor resistance. TAMs include microglial cells (MGs) and bone marrow-derived macrophages (BMDMs), with microglia found in peri-tumor areas (*Brandenburg S et al., 2020*). In contrast, BMDMs are in perivascular regions

within the tumor (*Chen J et al., 2017*). Upon recruitment, TAMs polarize toward a type II inflammatory phenotype, which supports tumor invasion, angiogenesis, and immunosuppression (*Chanmee T et al., 2014*). The interaction between TAMs, tumor cells, and the TME is eased by factors such as TGF-β, IL-6, and EGF, which further drive tumor growth and progression (*Stuelten CH et al., 2021*). Macrophages are recruited by cytokines secreted by tumor cells (*Cendrowicz E et al., 2021*), and once inside the tumor, microglia adopt a tumor-promoting phenotype, releasing trophic and angiogenic factors that support tumor growth (*Sarkar S et Yong VW, 2014*) (**Figure 3**).

MDSCs also play a critical role in tumor progression by suppressing T-cell activity and promoting regulatory T cells (*Lindau D et al., 2013*). MDSCs are classified into two subtypes: monocytic MDSCs (M-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs), each with distinct molecular and phenotypic characteristics (*De Leo A et al., 2020*). Both subtypes contribute to glioma progression, with higher circulating levels of MDSCs associated with worse prognosis in GBM patients. Within the TME, MDSC activity is regulated by soluble factors such as IL-6, IL-10, TGF-β, CCR2, and VEGF, secreted by both GBM and immune cells. These factors activate signaling pathways mediated by Janus kinase (JAK) and signal transducers and activators of transcription (STAT1, STAT3, STAT6), promoting MDSC proliferation, survival, and the expression of immunosuppressive molecules (*Lakshmanachetty S et al., 2021*).

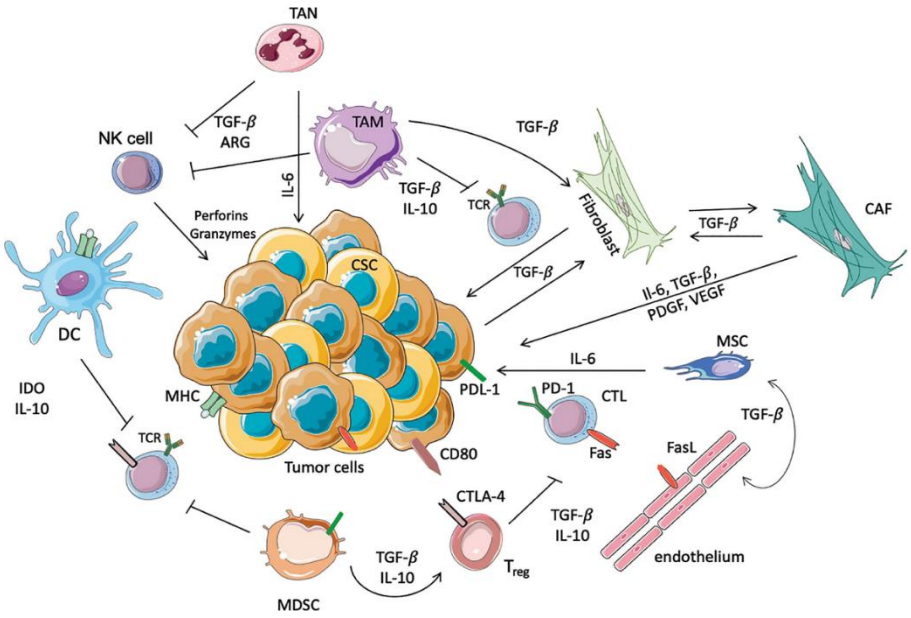



**Figure 3. Schematic representation of immunosuppressive cells in the TME.** In this scheme the major immune cells involved in the anti-tumor or pro-tumor response are highlighted. TANs and TAMs secrete TGF-β, Il-10 and ARG-I, which inhibit the cytotoxic activity of NK cells and T cells. Moreover, TAMs promote the conversion of normal fibroblasts into CAFs, which in turn promote the proliferation of tumor cells. In this immunosuppressive microenvironment DCs, through the secretion of IL-10 and overexpression of IDO, prevent the activation of T cells, avoiding the recognition of the tumoral antigens expressed on MHC. Another immunosuppressive population is represented by MDSCs that inhibit the activation of T cells, and furthermore allow the activation of Tregs, increasing the expression of CTLA4 on the surface of Treg themselves. Moreover, Tregs inhibit the cytotoxic functionality of the CTLs, where PD-1 and Fas are increased to inhibit the anti-tumoral response. Finally, the endothelium contributes to immunosuppression, since these cells express FasL (*Image adapted from Bilotta MT et al., 2022*).

### *2.2 GBM genetic mutations*

GBMs are characterized by genetic alterations that affect critical pathways involved in cell proliferation, apoptosis, and tissue invasion. Primary GBMs often show key genetic changes, such as amplification of the epidermal growth factor receptor (EGFR), mutations in the p53 pathway, loss of the PTEN tumor suppressor, and mutations in the TERT

promoter (*Brennan CW et al., 2013)*. These molecular features are sufficient to classify tumors as high-grade according to WHO criteria. Notably, IDH-wildtype glioblastomas are diagnosed in adults with IDH-wildtype diffuse astrocytic gliomas and are defined by features such as microvascular necrosis, TERT promoter mutations, EGFR amplification, chromosomal alterations (+7/-10), and MGMT promoter methylation (*Gritsch S et al., 2022*).

Telomerase, an enzyme crucial for supporting telomere length and preventing chromosomal damage during cell division, is essential for the survival of adult NSCs by preventing telomere shortening (*Collins K, 2000; Ferrón S et al., 2004*). It consists of TERT, telomerase RNA (TERC), and associated proteins (*Venteicher AS et al., 2008).* While TERC is widely expressed, TERT expression is tightly regulated and dictates telomerase activity (*Eitan E et al., 2012*). TERT promoter mutations, present in over 50% of adult primary GBMs, enhance telomerase activity, thereby promoting NSC self-renewal and increasing the likelihood of accumulating mutations that drive GBM. These mutations are also linked to poorer survival outcomes in GBM patients (*Vinagre J et al., 2013*).

Another critical mutation in GBM involves the TP53 gene, which makes the protein p53. This protein helps start cell death or stop the cell cycle when there is DNA damage (*Hernández Borrero LJ et El-Deiry WS, 2021*). During brain development, p53 manages cell division and differentiation, and in adults, it controls NSC growth and renewal (*Armesilla-Diaz A et al., 2009*). Mutations in TP53, found in primary and secondary GBMs, cause p53 to dysfunction, affecting NSC behaviour and leading to abnormal cell clusters in SVZ (*Zhang Y et al., 2018*).

Loss of the tumor suppressor PTEN is another major driver of GBM progression. PTEN regulates NSC migration, apoptosis, and proliferation in the SVZ. When PTEN function is lost, NSCs are reprogrammed into a GSC-like state, through the upregulation

of PAX7, which drives oncogenic transformation. Elevated PAX7 levels in PTEN-deficient GBMs are associated with more aggressive tumor characteristics, making PTEN-deficient NSCs a key therapeutic target (*Jaraiz-Rodriguez M et al., 2017*).

EGFR amplification and mutations, especially the EGFRvIII variant, are found in 40-50% of GBMs. This receptor tyrosine kinase is activated by growth factors, and mutations cause continuous signaling without a ligand (*Yoshimoto K et al., 2012*). This aberrant signaling activates pathways like RTK/RAS/PI3K, which drive unchecked cell proliferation by disrupting cell cycle checkpoints (*Liu F et al., 2015*). This leads to uncontrolled cell growth and worse patient outcomes, with a median survival of 0. 8 years for those with EGFRvIII mutations (*Shinojima N et al., 2003*). EGFR signaling promotes NSC proliferation and inhibits differentiation in transit-amplifying cells (type C cells), which may contribute to glioma development (*Doetsch F et al., 2002*). Like EGFR, platelet-derived growth factor receptor (PDGFR) is implicated in GBM, particularly PDGFRA overexpression, which is a marker of poor prognosis. PDGF signaling promotes NSC proliferation in the SVZ and contributes to the development of early glioma-like hyperplastic regions by preventing the differentiation of type B cells and promoting the accumulation of type C cells that invade surrounding brain tissue (*Jackson EL et al., 2006*).

The DNA repair enzyme O6-methylguanine-DNA-methyltransferase (MGMT) removes alkyl groups from guanine in DNA. Its expression is controlled by factors like NFκB and is an important prognostic marker in secondary GBMs (*Cabrini G et al., 2015*). Methylation of the MGMT promoter improves TMZ effectiveness, while low methylation and high mRNA levels are associated with resistance to TMZ (*Ramirez YP et al., 2013*).

### *2.3 Classification of GBM*

The classification of GBM distinguishes between primary and secondary forms. Primary GBM (IDH-wildtype, grade IV) arises without a precursor lesion and typically occurs in older adults, accounting for 90% of all GBM cases (*Ohgaki H et al., 2007*). In contrast, secondary GBM, characterized by IDH mutations, constitutes about 5% of cases, occurs in younger patients, and has a more favourable prognosis (*Ohgaki H et al., 2013; Aldape K et al., 2015*). Although primary and secondary GBMs share similar histological features, they show distinct genetic alterations in key oncogenic pathways (*Ohgaki H et al., 2007*). Historically, GBM classification relied primarily on histopathological criteria, particularly for grade IV IDH-wildtype diffuse astrocytomas. However, recent research has shown that even grade II and III IDH-wildtype diffuse astrocytomas, lacking certain histological features of GBM, show similar genomic profiles, clinical behaviour, and prognosis to glioblastoma (*Kibe Y et al., 2023*). This has led to a change in basic assumptions, reflected in the 2021 WHO CNS5 classification, which now incorporates molecular markers—such as EGFR amplification, TERT promoter mutations, and chromosome 7 gain/chromosome 10 loss (+7/-10)—into the diagnostic criteria for IDH-wildtype glioblastoma. According to this classification, any IDH-wildtype diffuse astrocytoma with these molecular features is classified as grade IV glioblastoma, regardless of traditional histopathological grading. This molecular approach has significant implications for prognosis and treatment, underscoring the importance of genetic analysis in the management of high-grade IDH-wildtype astrocytic gliomas (*Brat DJ et al., 2018*).

### *2.4 GBM signaling pathways*

Tumor heterogeneity in gliomas arises from dysregulation of diverse key signaling pathways, including Wnt, Notch, and TGF-β (*Saito N et al., 2019*). The Wnt pathway,

which regulates cell proliferation, migration, and apoptosis, also inhibits glycogen synthase kinase-3β (GSK-3β), influencing inflammation and cell membrane signaling (*Vallèe A et al., 2021*). Epigenetic silencing of this pathway often occurs via hypermethylation of soluble frizzled-related protein (FRP) genes, which normally form a receptor complex that binds Wnt ligands, activating the AXIN/APC/GSK-3β complex. This leads to β-catenin accumulation, triggering receptor tyrosine kinase (RTK) activation and later stimulating HIF-1α via the PI3K/Akt pathway. HIF-1α, a key player in hypoxia response, enhances the Warburg effect, driving overproduction of glycolytic enzymes such as LDH-A. Silencing FRP impairs glucose metabolism in glioma cells (*Roth W et al., 2000*). The Notch pathway regulates processes like migration, differentiation, apoptosis, and self-renewal through four receptors (Notch 1-4) and ligands (Jagged-1, Jagged-2, DII 1-4). Notch1 expression in neurons, astrocytes, and endothelial cells correlates with patient survival, making it a potential prognostic marker in GBM (*Herrera-Rios D et al., 2020*).

In the search for molecular targets in GBM, pathways like RTK/PI3K/Akt/mTOR, JAK-STAT3, RAS/RAF/MEK, and p53-mediated cell cycle regulation are of particular interest (*Le Rhun E et al., 2019*).

The PI3K/Akt pathway plays a leading role in glioma progression by phosphorylating GSK-3β and promoting nuclear translocation of β-catenin, which activates the oncogenic transcription factor STAT3. STAT3 is implicated in GBM angiogenesis, proliferation (via cyclin D1 and c-Myc), and cell invasion (through MMP-2/9 overexpression) (*Paw I et al., 2015*). Targeting the PI3K/Akt axis holds promise for reducing GBM invasiveness (*He Y et al., 2021*). The RTK/PI3K/Akt/mTOR pathway is essential for controlling cell growth, metabolism, and survival in gliomas. In this pathway, mTOR functions in two complexes: mTORC1 (with RAPTOR) regulates growth in response to nutrients, while mTORC2 organizes the cytoskeleton and activates Akt. Loss

of PTEN function in gliomas leads to increased Akt activity, promoting mTOR-driven cell proliferation. RTK activation also triggers PI3K, leading to Akt activation, which drives glioma cell growth and resistance to treatment (*Colardo M et al., 2021*). Hyperactivation of this axis underlies metabolic reprogramming and therapeutic resistance in GBM (*Verdugo E et al., 2022*).

The p53 pathway stays crucial for controlling cell immortality through regulation by MDM2, which inhibits p53's tumor-suppressive functions in mutated cells. Additionally, the retinoblastoma (Rb) pathway regulates cell cycle progression by suppressing E2F, a transcription factor that drives the transition from G1 to S phase. Targeting these pathways offers potential strategies for limiting GBM invasiveness and cell migration (*Wagle N et al., 2020*). The Hedgehog (Hh) signaling pathway, involved in both development and tumorigenesis, also plays a role in tissue repair and regeneration. In gliomas, GLI1, a zinc-finger transcription factor, is a key effector of the pathway. An alternative splice variant, tGLI1, is expressed in most GBM samples but not in normal brain cells. This gain-of-function variant activates genes beyond GLI1's normal targets, including VEGFR1, VEGF-A, VEGF-C, TEM7, HPSE, CD24, and CD44, driving GBM cell proliferation, migration, invasion, and angiogenesis (*Doheny D et al., 2020*)(**Figure 4**).

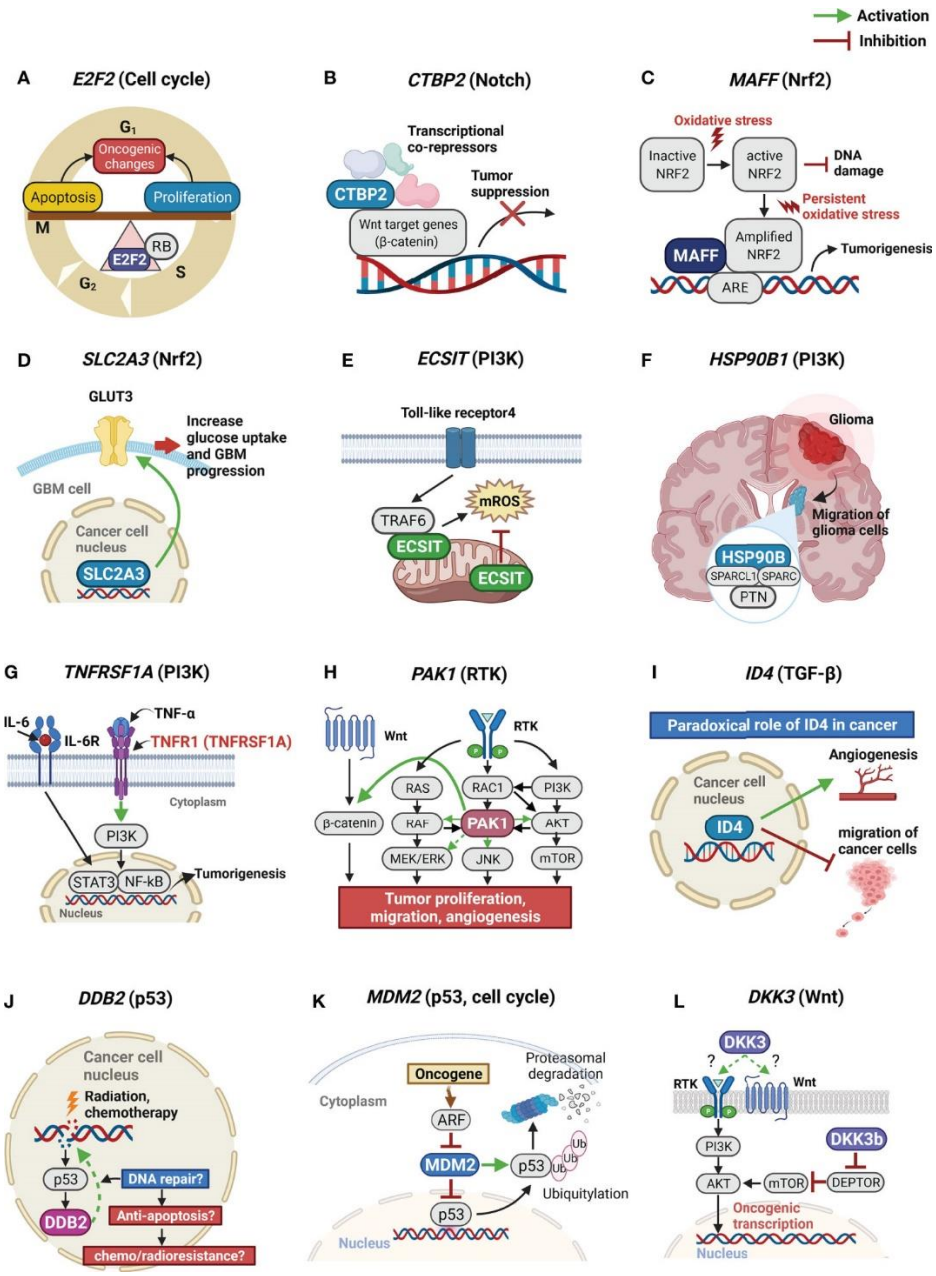



**Figure 4. Schematic illustrations of possible roles of the 12 significant genes in GBM. GBM, glioblastoma**. (A) E2F2 is important for balancing cell growth and stopping the cell cycle or causing cell death. (B) CTBP2 works as a repressor of Wnt target genes, which helps suppress tumors. (C) When MAFF levels are high, it binds to Nrf2, and in stressful times, they work together to activate ARE, which can lead to tumors. (D) SLC2A3 produces GLUT3, and its abnormal levels due to high glucose use in tumor cells are linked to a bad outlook in brain tumors. (E) A TRAF6-ECSIT complex is necessary for making mROS, and ECSIT also helps in this process. (F) A protein complex with PTN, SPARC, SPARCL1, and HSP90B aids glioma cell movement. (G) The TNFRSF1A gene makes the TNFα receptor, which leads to ongoing NF-κB

activity and increases pro-cancer proteins because of TNFα and IL6. (H) PAK1 is a key point for various signaling paths, and its overexpression links to tumor growth and movement. (I) ID4 can change roles between being a tumor suppressor and an oncogene. (J) DDB2 is vital for DNA repair but can help cancer cells resist chemotherapy. (K) MDM2 mainly inhibits the p53 protein, (L) while DKK3 might enhance cancer cell behaviour through interactions with specific receptors and proteins (*Image adapted from Han MH et al., 2022*).

### *2.5 Role of Oxidative Stress in GBM*

Oxygen levels significantly influence cellular functions such as proliferation, differentiation, angiogenesis, and metabolism (*Ortman B et al., 2014; Hubbi ME et al., 2015*). While it remains unclear whether the molecular abnormalities in GBM cells result from elevated reactive oxygen species (ROS) levels, the imbalance in oxygen delivery, consumption, and capacity promotes a proinflammatory environment, fostering cancer cell migration, proliferation, drug resistance, and resistance to cell death (*Salazar-Ramiro A et al., 2016; Krawczynski K et al., 2020*). Oxidative stress (OS), driven by the production of ROS, can damage proteins, lipids, and DNA. However, GBM cells can adapt to hypoxic conditions, enabling them to resist treatment.

ROS play a dual role: physiologically, they regulate signal transduction pathways, transcription factors, and mitochondrial enzymes but they can also induce damage to biomolecules, leading to genomic instability (*Irani K et al., 1997; Halliwell B et al., 2007*). Tumor cells must balance ROS levels and oxidative stress responses to survive. Two hypotheses describe the effects of ROS in cancer. The "threshold concept for cancer therapy" suggests that tumor cells support a controlled ROS-antioxidant equilibrium, and exceeding this threshold leads to cell death or enhanced sensitivity to therapy (*Kong Q et al., 2000*). The second hypothesis posits that tumor cells experience a rapid rise in ROS when exposed to exogenous agents, which reaches a lethal threshold more quickly than in

normal cells (*Wang J et Yi J, 2008*). During tumorigenesis, changes in redox status activate ROS production in tumor cells. ROS, including radicals like superoxide (O−2) and hydroxyl (HO•) and non-radicals such as hydrogen peroxide (H2O2), primarily originate from oxygen used in metabolic processes in organelles such as mitochondria, the endoplasmic reticulum (ER), and peroxisomes. Elevated ROS levels disrupt DNA repair, cause base modifications, DNA crosslinks, and DNA-protein interactions, contributing to genomic instability and promoting tumorigenesis (*Bae YS et al., 2011*). Cancer cells must continuously balance ROS production and oxidative stress responses to survive this genomic instability.

In response to low oxygen levels, pathways like HIFs and ER stress mechanisms are activated. HIFs play a key role in regulating the tumor-initiating ability of GSCs, which respond to hypoxia with elevated HIF levels. Many HIF-regulated genes are selectively expressed in GSCs compared to other GBM cells (*Li Z et al., 2009*). Hypoxia promotes GSC self-renewal and induces stem-like characteristics in non-stem cell populations by upregulating stem cell factors like Oct4 and Nanog (*Heddleston JM et al., 2009*). HIF-2α is notably expressed in GSCs at oxygen levels comparable to normal physiological conditions (2–5%). This ability to adapt to varying oxygen concentrations allows GBM cells to thrive in hypoxic environments, with elevated ROS levels damaging non-malignant cells while allowing tumor cells to survive.

### *2.6 GBM therapeutic approaches*

#### *2.6.1 Surgical methods for resection of the tumor mass*

Treating GBMs is highly challenging due to their complex cellular composition, marked by genetic and epigenetic variability. Additionally, the

BBB hinders drug delivery, and treatments often cause significant side effects. GBM heterogeneity complicates treatment further, with quiescent GSCs coexisting alongside proliferating cells, enabling them to evade standard chemotherapy and radiotherapy (*Tejero R et al., 2019*). Patient survival is intricately linked to the extent of tumor resection, with total resection being the ideal approach when possible. In fact, surgical resection is crucial for treating brain tumors and increasing life expectancy in patients with low- or high-grade gliomas. A 2013 study found that surgical treatment improved life expectancy and quality of life outcomes (*Sanai N et Berger MS, 2008*).

Advanced imaging techniques have become crucial in guiding GBM surgery, helping define tumor margins and preserve critical brain structures both preoperatively and intraoperatively. Fluorescence-guided resection in high-grade gliomas has been shown to enhance the extent of resection, with a higher rate of gross total resection (GTR) and extended overall survival (OS) and progression-free survival (PFS) compared to conventional surgical methods. Studies have also shown that 5-ALA-guided resection is superior to conventional neuronavigation and can increase the extent of resection (EOR) when tumors are fully removable (*Gandhi S et al., 2019; Golub D. et al., 2020*). However, different intraoperative adjuncts during surgical excision of high-grade gliomas can potentially improve EOR and extend OS and PFS (*Golub D. et al., 2020*; Haider SA et al., 2019).

On the other hand, the management of metastatic disease requires invasive surgical techniques for brain metastases. Both surgical resection and stereotactic radiosurgery (SRS) have shown similar safety and efficacy

in treating single metastases (*Fuentes R et al., 2018*). A study by Hatiboglu et al. evaluated the suitability of surgical management and adjuvant SRS, considering patients with larger lesions, neurological deficits, radiographic mass effects, and potential interruption of cerebrospinal fluid flow (*Hatiboglu MA et al., 2020*).

Other method used for GBM resection is the Laser interstitial thermal treatment (LITT), a method that uses a heat-delivering probe to heat tumor tissue, causing targeted hyperthermic damage and coagulative necrosis (*Chaunzwa TL et al., 2018*). The two approved LITT systems, NeuroBlate® and Visualase®, support this method (*Chaunzwa TL et al., 2018; Patel B et Kim AH, 2020*). Real-time MRI thermometry monitors ablation temperatures, and if they rise, treatment is stopped. LITT is effective in treating recurrent brain metastases and recurring metastatic lesions. It has reported local tumor control rates of 77.4%, and when at least 80% of the tumor is eliminated, there is no disease progression (*Shah AH et al., 2020; Chen C et al., 2021*). LITT can also be used to treat high-grade gliomas that cannot be surgically removed. It can also improve adjuvant treatments like systemic chemotherapy by rupturing the blood-brain barrier (*Shah AH et al., 2020; Appelboom G et al., 2016*).

### *2.6.2 Chemotherapy in GBM*

***Temozolomide.*** TMZ, a DNA alkylating drug, is an oral medication used in treating GBM in a variety of clinical settings including inpatient, outpatient or at home (*Fernandes C et al., 2017*). It results in poor DNA repair and cell death. The "Stupp protocol" treatment plan is widely used in patients with

GBM (*Stupp R et Weber DC, 2005*). Because these changes restricted the repair of TMZ-induced DNA damage, patients with methylation MGMT mutations benefited more from TMZ treatment (*Herbener VJ et al., 2020*). However, over 50% of GBM patients do not benefit from TMZ treatment, making it difficult to understand and treat. TMZ resistance can be an intrinsic trait of some cancers or develop after initial treatment (*Wick W et Platten M, 2014; Lee SY, 2016*).

The clinical standard consists of administering TMZ at a daily dose of 75 mg/m² during radiotherapy, followed by six cycles of TMZ at 150-200 mg/m² for five days every 28 days (*Tan AC et al., 2020*). TMZ, a small, lipophilic molecule weighing 194 kDa from the imidazotetrazine class of oral alkylating agents, was approved by the US FDA in 2005 for treating newly diagnosed glioblastoma in adults. When combined with radiotherapy, TMZ has been shown to extend median overall survival to 12 months (*Alonso MM et al., 2007*). Chemically known as 3-methyl-4-oxoimidazo[5,1-d] [1,2,3,5] tetrazine-8-carboxamide, TMZ is rapidly absorbed after oral administration with 100% bioavailability and reaches peak plasma concentration within an hour. Its metabolism and elimination are swift, with a half-life of approximately 1.8 hours, and it is completely cleared from the plasma within 8 hours (*Moody CL et Wheelhouse RT, 2014*). TMZ's primary mechanism of action involves inducing cell cycle arrest at the G2/M checkpoint and triggering apoptosis in GBM cells. After absorption, TMZ is hydrolysed into its active metabolite, monomethyl triazene 5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide (MITC), which further reacts to produce 5-amino-imidazole-4-carboxamide (AIC) and the methyl diazonium cation. The methyl diazonium cation mediates TMZ's

toxicity by methylating DNA, particularly at the N7 and O6 positions of guanine and the N3 position of adenine. Alkylation at the O6 position of guanine disrupts DNA replication, leading to thymine incorporation instead of cytosine, causing cell death (*Lee SY, 2016*). Despite its effectiveness, TMZ therapy faces two major challenges: it can be toxic to hematopoietic cells at high concentrations, and GBM patients often develop resistance to the drug. This resistance is often linked to overexpression of the MGMT protein, driven by promoter demethylation and a dysfunctional DNA repair pathway in GBM cells, which worsens patient outcomes (*Thon N et al., 2013*). TMZ resistance is still a significant obstacle in the treatment of malignant brain tumors, arising from a complex interplay of multiple molecular mechanisms rather than a single cause (*Ortiz R et al., 2021*).

***Virmustine, Procarbazine, and Lomustine.*** Combination therapy with procarbazine, lomustine, and vincristine is recommended for gliomas, as it interferes with mitosis and microtubule production (*Levine VA et al., 1980; Cornetta K et al., 2006; Boyle FM, 2004*). Patients with anaplastic oligodendroglioma and anaplastic oligoastrocytoma who received vincristine in addition to radiation therapy had longer progression-free survival (PFS) than those who received radiation therapy alone (*van den Bent MJ et al., 2013*). However, radiation and TMZ are better tolerated. Understanding the complex interplay of molecular pathways contributing to TMZ resistance is expected to lead to more potent therapeutic methods.

***Bevacizumab.*** Bevacizumab is a monoclonal antibody that prevents tumor formation and is given intravenously once every two weeks (*Fu M et al., 2023*). It has side effects like bleeding, GI perforation, and cytotoxic effects. A phase II trial showed improved radiographic response and median OS in recurrent GBM patients (*Vredenburgh JJ et al., 2007*). Combining bevacizumab with radiation and tumor-treating field treatments may optimize therapeutic efficacy (*Morris SL et al., 2019; Palmer JD et al., 2018; Schernberg A et al., 2018*). However, the European Medicines Agency has rejected its use for treating recurrent GBM due to a lack of positive benefit-risk ratio (*Yu Z et al., 2016*).

***Carmustine.*** Carmustine, a nitrosourea DNA alkylating chemical, is less attractive than temozolomide due to its side effects like myelosuppression, weariness, nausea, and pulmonary damage. With the addition of carmustine or other comparable nitrosoureas, surgery and radiation therapy be beneficial by meta-analysis (*Ueda-Kawamitsu H et al., 2002*). However, it can be incorporated into a biodegradable polymer wafer for improved efficacy and reduced adverse effects, with a median survival of 31 weeks using this method *(Wait SD et al., 2015; Brem H et al., 1995).*

***Methotrexate.*** Methotrexate (MTX) inhibits dihydrofolate reductase, preventing DNA synthesis and repair. It can be administered orally, intravenously, or intrathecally for oncologic therapy. Integrating MTX with systemic chemotherapy may be effective for GBM patients (*Kang X et al., 2022*). However, none of these single-use medications significantly increase

the survival rate of Leptomeningeal disease (LMD) patients (*Birzu C et al., 2020*). MTX also up-regulates CD73 expression in GBM tumor tissue (*Figueiró F et al., 2016*).

### *2.6.3 Radiotherapy*

Magnetic resonance imaging (MRI) is the gold standard for diagnosing brain tumors due to its high-resolution anatomical imaging and soft tissue contrast, often used alongside computed tomography (CT) (*Wu W et al., 2021*). However, due to the infiltrative nature of GBM and its poorly defined margins, including finger-like extensions that current imaging methods cannot detect, achieving complete tumor removal without affecting healthy tissue is almost impossible. GBM cells spread along neuronal fibers (neuropils) without causing visible changes, further complicating complete surgical resection. As a result, surgery alone is rarely curative, requiring added therapies to minimize recurrence risk (*Grochans S et al., 2022*).

Radiotherapy (RT), although gliomas are considered radioresistant, is a critical part of GBM treatment. Administered 4-6 weeks post-surgery, it aims to drop residual tumor tissue and neoplastic cells that stay after resection. It is also employed to manage recurrences and secondary tumors. Common RT techniques include conventional 2D RT, 3D conformal RT, intensity-modulated RT, stereotactic radiosurgery (SRS), brachytherapy, and particle therapy (e.g., proton therapy). Despite differing in delivery, these techniques primarily induce double-stranded DNA damage in cancer cells. Moreover, RT is typically given in two fractions, with a total dose of

40-60 Gy over six weeks. Precision in radiation delivery is essential for successful treatment, and advanced multi-leaf collimators are used to limit radiation exposure to a 1-2 cm margin of healthy tissue (*Urbańska K et al., 2014*).

Whole-brain radiation therapy (WBRT) is increasingly used for patients with brain metastases who are not eligible for surgical or Stereotactic radiosurgery (SRS) interventions. WBRT has been shown to significantly enhance survival rates, but some studies suggest a correlation between WBRT and a loss in neurocognitive function. This decline impacts the long-term cognitive status and overall quality of life of individuals undergoing WBRT (*Tanguturi S et Warren LEG, 2019; Brown PD et al., 2017*). Over the past decade, there has been a shift towards high-dose targeted radiation to enhance tumor control rates and is being explored as a cost-effective substitute for SRS. Systemic medication is used in conjunction with SRS to achieve localized CNS control and impede the dissemination of metastatic disease. The highest level of local control is achieved with the combination of radiosurgery and surgical excision. Hypofractionated stereotactic radiosurgery (HF-SRS) is a viable therapeutic option for patients requiring high radiation doses and reducing negative neurocognitive effects (*Soliman H et al., 2019*). Recent research suggests that radiation therapy possesses immunomodulatory properties, which can alter the microenvironment of tumors and lead to the reactivation of the immune system (*Hamilton AJ et al., 2018*).

***Gamma Knife.*** The Gamma Knife (GK) is a stereotactic radiotherapy technique that improves radiation delivery precision for localized malignancies. It requires one treatment session and has fewer radiation effects on healthy tissue (*Sanders J et al., 2019*). GK-mediated stereotactic radiotherapy, combined with bevacizumab, has safely treated focal GBM recurrence (*Morris SL et al., 2019*). The study shows no radiation injuries and improvements in PFS and OS. Future research should stratify risk factors, explain bevacizumab's benefits, and explore targeted therapy for this patient population. Leading-edge radiosurgery (LER)S is a safe, effective adjunctive therapy for newly diagnosed GBM, reducing toxicity and radionecrosis hazards by conformally targeting remaining or recurrent GBM tissue (*Duma CM et al., 2016; Bunevicius A et Sheehan JP, 2021*).

***Brachytherapy.*** Brachytherapy, a brain implant-based radiation treatment, is used for small cancers like GBM. Iodine and iridium are commonly used, and early detection is crucial (*Barbarite E et al., 2017*). It's often used in combination with resection, chemotherapy, and External Beam radiation Therapy (EBRT) (*Barbarite E et al., 2017*). However, it hasn't proven to offer benefits over traditional radiation therapy. Cesium-131 brachytherapy, a new isotope, has shown promising results in recurrent GBM patients, with a 20-month survival rate, suggesting a potential treatment for recurrent GBM patients with low radiation necrosis risk (*Wernicke AG et al., 2020*).

***Proton Beam.*** Photon beam therapy (PBT) and proton therapy (PT) offer advantages in treating CNS malignancies, particularly in pediatric patients.

PBT provides more localized radiation delivery and can achieve a dose distribution superior to conventional external photon beam radiation (*Weber DC et al., 2020*). PBT reduces the volume of irradiated normal tissue and improves the target area's conformability and quality. However, it is costlier than conventional X-ray therapy but may increase patient quality of life and reduce costs associated with late radiation-related adverse effects (*Tian X et al., 2018*). PBT achieves good local control in some high-grade tumors with reduced toxicity, but toxicity profiles for low-grade tumors need to be analyzed. There is currently limited evidence to demonstrate improved results with PT in GBM *(Goff KM et al., 2022).*

### *2.6.4 Tumor-Treating Field in GBM*

The NovoTTF-100A System, developed by Novocure, Ltd., is a novel therapeutic approach for recurrent GBM therapy approved by the FDA (*Fabian D et al., 2019*). The device uses low-intensity, intermediate-frequency alternating electric fields to inhibit GBM cell proliferation. The system is recommended for patients to use for at least 18 hours per day during each 4-week therapy cycle. The National Comprehensive Cancer Network (NCCN) has incorporated the TTField device into the treatment of freshly diagnosed GBM. Despite FDA approval, uncertainty remains regarding the therapy. A randomized controlled phase III trial showed the efficacy of NovoTTF-100A as comparable to chemotherapy, with a success rate of 62% (*Stupp R et al., 2012; Regev O et al., 2021*). TTFields have been authorized for both freshly diagnosed and recurrent GBM in the US, Europe, and Japan (*Mun EJ et al., 2018*). Despite the advantages of TTF in

the field, there are several limitations, including high price and availability issues (*Fabian D et al., 2019*).

### *2.6.5 Immunotherapy in GBM*

The CNS is an immune-privileged site with limited T cell access due to factors such as the BBB, absence of dedicated lymphatic channels, low basal expression of major histocompatibility complex (MHC) class II molecules, scarcity of antigen-presenting cells, and continuous production of immunosuppressive cytokines (*Jackson CM et al., 2014*). Recent studies have contributed to our understanding of immune processes in the CNS, with a typical lymphatic system found within the CNS facilitating fluid and immune cell transportation (*Louveau A et al., 2015*). Immunotherapy has been increasingly used in cancer research, with advancements in immune checkpoint inhibition and chimeric antigen receptors (CAR)-modified T cells. The FDA has approved immunotherapy medications for cancer treatment, including monoclonal antibodies targeting cytotoxic-T-lymphocyte-related protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and PD-1 ligand 1 (PD-L1) (*Mellman I et al., 2011; Topalian SL et al., 2011; McNutt M, 2013; Topalian SL et al., 2015*). However, immunotherapy in GBM management remains challenging due to tumor-induced immune suppression mechanisms. Addressing molecular heterogeneity in GBM is crucial for developing effective immunotherapeutic approaches. Current advancements in immunotherapy for GBM present a promising trajectory for further investigation and improvement of treatment modalities. Despite encouraging outcomes,

immune checkpoint inhibitors therapy (ICIs) is ineffective for all solid tumors, some cancers have a low response rate, and there are significant side effects (*Medikonda R et al., 2021*). In 2018, only 43.6% of cancer patients were immunotherapy candidates, attributed to tumor heterogeneity and multiple immunosuppressive systems. An analysis of the response rate to six anti-CTLA-4 or anti-PD-1 ICIs was reported by Haslam et al. in 2019 (*Haslam A et Prasad V, 2019*). Preliminary clinical trial findings contributed to understanding GBM immunosuppression functions.

***CAR T Therapy in GBM.*** Chimeric antigen receptor T cell therapy (CAR T)-cell treatment has the advantage of bypassing the need for MHC presentation of antigens and the traditional adaptive immune response (*Guedan S et al., 2019*). It has shown clear success in treating blood cancers (*Beyar-Katz O et Gill S, 2020; Batlevi CL et al., 2016*), prompting efforts to apply this treatment to solid tumors like GBM . Recent studies have looked at CAR T-cell therapy for GBM by targeting specific proteins, including EGFRvIII and IL-13Ra2, with varying results (*Brown MP et al., 2016; Migliorini D et al., 2018*). Notably, a case study by Brown et al. involved a patient with recurrent multifocal GBM who received CAR T-cells targeting IL-13Ra2. This treatment led to complete tumor regression in the brain and spine, evidenced by increased cytokines and immune cells in the cerebrospinal fluid, maintaining effectiveness for 7.5 months. However, relapses occurred, sometimes linked to reduced IL-13Ra2 expression (*Brown CE et al., 2016; Brown CE et al., 2016; Migliorini D et al., 2018*). The CAR T-cells were effective in targeting tumors directly and stimulated an innate immune response, even in cases of IL-13Ra2 evasion

*(Ahmed N et al., 2017).* Additionally, trials have explored HER2-CAR modified T-cells for patients with progressing GBM, showing safety and potential clinical benefits *(Ahmed N et al., 2017)*.

### *2.6.6 GBM and Vaccine Therapy*

Cancer vaccine therapy shows promise for preventing and treating tumors, including GBM (*Saxena M et al., 2021; Hu Z et al., 2018*). These vaccines aim to trigger an immune response against tumor-associated antigens. However, due to the limited number of specific antigens for GBM, patient selection is restricted. Few vaccination strategies have reached phase III clinical trials, while others are still in earlier trial phases. EGFRvIII, a variant found in 25–30% of GBM cases, is a key antigen being researched (*Weller M et al., 2014).* Genetic engineering to modify T cells for targeting tumor antigens is emerging as a hopeful therapy. Current clinical trials for GBM vaccines have not shown strong results, but improvements could lead to effective treatments. More funding is needed for GBM vaccine development. Research is ongoing to identify better targets and approaches, and to find immune modulators that could enhance vaccine effectiveness in GBM patients *(Zhao T et al., 2022).*

### *2.6.7 Nanocarrier-Mediated Therapy in GBM*

A small number of particles can pass through the BBB, making it necessary to create new technologies and delivery systems for getting medications into the brain. Nanotechnology and nanocarriers are promising for drug delivery because they are safe, provide controlled drug release, improve how drugs dissolve, and can easily cross the BBB (*Zhao M et al.,*

*2020; Liao W et al., 2019*). Various types of nanoparticles are used to treat GBM, including liposomes, polymeric nanoparticles, solid lipid nanoparticles, and dendrimers. Liposomes, which are lipid-based vesicles, closely resemble cell membranes and help lipophilic drugs pass through the BBB more easily *(Nsairat H et al., 2022).* Liposomes are beneficial for several reasons: they are easy to make, can hold different anticancer drugs, are biocompatible, and have high effectiveness. They were first used to deliver chemotherapy drugs like doxorubicin over twenty years ago, and new techniques are being explored to improve their effectiveness in treating GBMs (*Zhang Y et al., 2018; Khan AR et al., 2018; Alexander A et al., 2019*). For instance, adding polyethylene glycol (PEG) to liposomes can help them stay in circulation longer and avoid capture by the immune system *(Kuo C et a., 2019*). Researchers have also investigated targeting specific receptors on GBM cells with liposomes to improve treatment outcomes. Studies show that these targeted liposomes can reduce tumor size without increasing toxicity in animals (*Yang CY et al., 2015; Glaser T et al., 2017*). However, there are still challenges, including differences in how liposomal nanoparticles perform in various brain regions and their ability to penetrate the BBB based on the drugs they carry.

Chemoresistant/radioresistant cancer stem cells and biological barriers like the blood-brain barrier hinder conventional therapies against GBM. Nano-theranostics has improved the efficacy of conventional techniques like CHT and radiotherapy by combining therapy and diagnostics into a single nanoplatform *(Ge Y et al., 2018).* Nanoparticles (NPs) have been advocated as a therapy option due to their enhanced permeability and retention (EPR) effect, which allows them to enter tumor

lumen through leaky blood vessels *(Richard S et al., 2016).* NPs have shown better effectiveness in drug delivery, radiosensitizer/photosensitizer delivery, and simultaneous therapeutic and imaging functions, demonstrating incredible potential against GBM. Magnetic Nanoparticles (MNPs) or their composites have proven their mettle as efficient drug/radiosensitizer/photosensitizer delivery platforms *(Wilhelm S et al., 2016).*

### *2.6.8 Limits of traditional therapies*

The structural complexity and intertumoral and intratumoral heterogeneity of GBM present significant treatment challenges, highlighting the need for novel therapeutic approaches. Tumors develop denser and more disorganized vascular networks than healthy tissues, characterized by a disordered, thin, and fenestrated endothelium. Since GBM promotes the formation of new blood vessels to sustain growth and invade surrounding tissues, considerable research has focused on treatments that target tumor angiogenesis (*Zhang AB et al., 2023*). One such strategy is anti-angiogenic chemotherapy, specifically the monoclonal antibody Bevacizumab. Bevacizumab is administered intravenously every two weeks at a dose of 10 mg/kg body weight and works by binding to VEGF-A, inhibiting its activity (*Wirsching HG et al., 2016*). While Bevacizumab does not significantly improve overall survival in newly diagnosed GBM patients, it is primarily used for recurrent cases (*Wu W et al., 2021*).

Another challenge in treating GBM is delivering chemotherapy drugs through systemic routes. Even after extensive tumor removal, GBM

often recurs locally, despite the use of adjuvant chemotherapy, due to the presence of the BBB. The BBB, composed of neurovascular units, prevents harmful endogenous and exogenous molecules from entering the brain. The barrier's effectiveness is due to tight junctions between endothelial cells and interactions with pericytes, the basement membrane, and astrocytes, which collectively limit the effectiveness of cancer therapies (*Cheng Y et al., 2014*). In contrast, the BBB in GBM exhibits increased permeability due to poorly formed, leaky blood vessels (*Urbańska K et al., 2014*). However, this increased permeability is inconsistent throughout the tumor, with diverse areas having highly permeable vessels and others more obstructed. Consequently, even when chemotherapeutic drugs reach the tumor tissue, they often do not achieve therapeutic concentrations uniformly across all regions (*Wu W et al., 2021*). This challenge has led to the development of techniques aimed at enhancing BBB permeability. High-intensity focused ultrasound (HIFUS) is a non-invasive method that temporarily increases BBB permeability, easing the systemic delivery of therapeutic agents to GBM. HIFUS works by combining ultrasonic waves with systemically administered microbubbles, which oscillate within the blood vessels and create shear stress that disrupts the BBB's tight junctions. Another approach to bypass BBB limitations is convection-enhanced delivery (CED), where a microcatheter is implanted directly into the tumor through minimally invasive surgery. CED allows for localized, high-dose drug delivery to large volumes of interest while minimizing systemic side effects (*Cruz JVR et al., 2022*).

# 3. Integrative approaches for GBM care

### *3.1 Effects of phytochemical compounds against GBM*

Given the limited effectiveness of TMZ in inhibiting GBM growth and the variability in patient responses based on MGMT promoter methylation status, there is an urgent need to find new adjuvant agents. These agents could potentially enhance TMZ's efficacy, reduce systemic toxicity associated with higher doses, and improve overall patient outcomes. Current first-line chemoradiotherapy treatments for GBM primarily work by inducing DNA damage in cancer cells (*Vilar JB et al., 2022*). However, despite some success in prolonging median progression-free survival (PFS), the monoclonal antibody bevacizumab (Avastin)—which targets VEGF—has not been shown to extend overall survival when used alone or in combination with chemotherapy as a first- or second-line treatment for GBM (*Lombardi G et al., 2017*). The failure of two major trials (ClinicalTrials.gov identifier NCT01290939; ClinicalTrials.gov number, NCT01290939) to achieve the primary endpoint of increased overall survival is largely due to the high cellular heterogeneity of GBM and the deregulation of multiple signaling pathways, which make the tumor less responsive to single-target therapies (*Qazi M et al., 2017*). To overcome these challenges, a multi-targeted approach may be more effective, whether through a single agent capable of modulating multiple pathways or a combination of compounds with different mechanisms of action. Such strategies have the potential to improve the efficacy of conventional DNA-damaging therapies. The role of natural compounds as adjuvant therapies for GBM has been extensively reviewed, and recent research has found added molecules with the potential to inhibit GBM cell growth, further expanding the range of promising natural adjuvant therapies (*Vengoji R et al., 2018*).

Different natural substances with proven biological benefits have proved anti-tumor effects on GBM in both *in vitro* and *in vivo* studies *(Zhai K et al., 2021)*. These include alkaloids, carboxylic acid derivatives, carotenoids, flavonoids, coumarins, curcuminoids, terpenes, lignans, natural steroids, tannins, and various plant extracts. Alkaloids, a diverse group of nitrogen-containing compounds, show multiple anti-tumor activities, such as inducing DNA damage, cell cycle arrest, ER stress, apoptosis, and autophagy, while inhibiting angiogenesis and tumor cell proliferation (*Tasiu Isah et al., 2016; Mollov NM et al., 1968*). Notably, alkaloids can also overcome drug resistance by inhibiting cellular drug efflux pumps. One particularly promising alkaloid for GBM therapy is berberine (BBR), a quaternary ammonium salt derived from barberry that induces autophagy in GBM by targeting the AMPK/mTOR/ULK1-pathway (*Hattori T et al ., 1992*; Wang J et al., 2016) or BBR suppressed the Progression of Human Glioma Cells by Inhibiting the TGF-β1/SMAD2/3 Signaling Pathway (*Jin Y et al., 2022*). It is recently known that BBR increase detectability of infiltrating glioma stem cells to optimize 5-ALA-guided surgery (*Ohtsuka Y et al., 2024*).

Carboxylic acid derivatives, characterized by their carboxyl (-COOH) functional groups, have shown anti-cancer potential by modulating intracellular second messengers and inhibiting DNA synthesis, transcription, and tumor cell proliferation (*Zhai K et al., 2021*). For instance, cinnamic acid from cinnamon and ferulic acid, a derivative from giant fennel, have shown efficacy against GBM *in vitro*.

Carotenoids, natural pigments responsible for red, orange, and yellow hues, are divided into two main classes: carotenes (hydrocarbon-based) and xanthophylls (having oxygen atoms). Chemically, they are tetraterpenoids with 40 carbon atoms and conjugated double bond systems. Carotenoids show anti-cancer properties by

upregulating apoptotic pathways and inhibiting tumor cell migration and invasion, reducing metastasis (*Meléndez-Martínez AJ et al., 2019; Koklesova L et al., 2020*). Examples include astaxanthin from chlorophyte, adonixanthin (a derivative of astaxanthin), and crocetin from saffron.

Flavonoids, polyphenolic plant secondary metabolites, occur in seven classes: anthocyanidins, flavones, flavanones, flavonols, flavan-3-ols, isoflavones, and chalcones. These compounds, with a three-ring structure, have well-established anti-tumor effects by promoting apoptosis and inhibiting tumor cell migration, invasion, and metastasis (*Abotaleb M et al., 2018*). Additionally, they modulate tumor cell metabolism, reducing the Warburg effect (*Samec M et al, 2020; Liskova A et al., 2021*). Flavonoids with anti-GBM potential include diosmin (from germander), epigallocatechin-3-gallate (EGCG, from green tea), naringin (from grapefruit), quercetin (from oak and kale), and resveratrol (from grapes and red wine)(*Kandaswami C et al., 2005).* Flavonoids nanoformulations showed higher brain drug delivery (*Alshweiat A et al., 2023)*.

Curcuminoids, polyphenolic compounds from turmeric, have shown the ability to inhibit GBM by reducing proliferation, inducing apoptosis, and disrupting mitochondrial function (*Zoi V et al., 2021*). These compounds also significantly modulate tumor angiogenesis and inflammation (*Yahfoufi N et al., 2018*).

Lignans, polyphenolic compounds with a two-benzene-ring structure, inhibit topoisomerases, thereby interfering with DNA synthesis and cell proliferation (*Jain CK et al., 2017*). Arctigenin, from greater burdock, and magnolol, from Houpu magnolia, have shown potential in GBM treatment.

Natural steroids, characterized by a four-ring structure, show cytotoxic effects by inducing apoptosis and cell cycle arrest in tumor cells. Examples include

withaferin A (from Ashwagandha), gamabufotalin (from toad skin extract), and diosgenin (from fenugreek).

Tannins, large polyphenolic compounds classified into hydrolysable, phlorotannins, and condensed tannins, exert anti-cancer effects by promoting apoptosis, autophagy, and inhibiting proliferation and angiogenesis. Tannic acid from oak, a hydrolysable tannin, has shown potential as an anti-GBM agent (*Majewska MP et al., 2022*).

Finally, various crude and purified plant extracts also show anti-GBM activity. Examples include extracts from water hyssop, mushrooms like shaggy ink cap and golden chanterelle, the Johnny berry plant, and Polish propolis. These extracts hold multiple bioactive compounds with potential therapeutic effects against GBM (**Figure 5**).

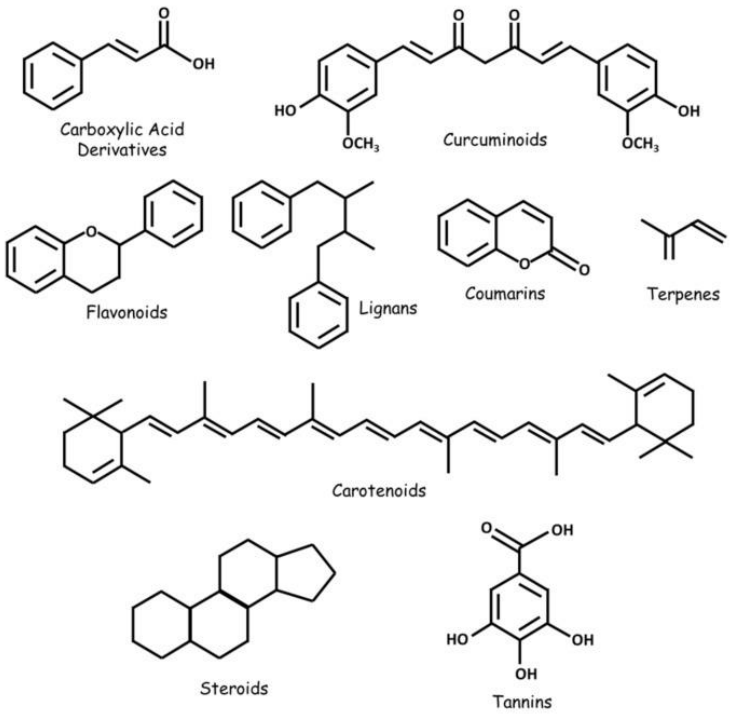



**Figure 5. Classes of natural substances with therapeutic potential in GBM.** The heterogenous substances exert anti-GBM effects by upregulating apoptosis and autophagy, inducing cell cycle arrest, interfering with tumor metabolism (Warburg effect), and inhibiting proliferation,

neuroinflammation, chemoresistance, angiogenesis, and metastasis. Although these beneficial effects are promising, natural substances' efficacy in GBM is constrained by their bioavailability and blood–brain barrier permeability; various chemical formulations are proposed to improve their pharmacological properties *(Image modified from Zhai K et al., 2021).*

### *3.2 Role of Terpenes in cancer*

Terpenes, unsaturated hydrocarbons from plants, are known for their pharmacological properties, including antioxidant, anti-inflammatory, anti-tumor, hepatoprotective, cardioprotective, and neuroprotective effects (*Jakaria M et al., 2018*) by modulating key signaling pathways, inducing apoptosis, and inhibiting angiogenesis and metastasis (*Ashrafizadeh M et al., 2019*; *Quintans JSS et al., 2019*; *Thoppil RJ et Bishayee A, 2011; Kuttan G et al., 2011*; *Kamran S et al., 2022; Li D et al., 2015*). They can protect against neurodegenerative disorders, cardiovascular disease, cancer, diabetes, and aging (*Li Y et al., 2017*; *Gonzalez-Burgos E et Gomez-Serrallinos MP, 2012*). Terpenes like α-pinene, d-limonene, camphene, and linalool reduce oxidative stress, increase enzyme activities, reduce inflammation, promote cell cycle arrest, and modulate cyclin-dependent kinase inhibitors (*Kim T et al., 2020; Baser KHC et al., 2015; Wink M, 2015; Kamran S et al., 2022*; *Sharifi-Rad J et al., 2017*). Linalool reduces inflammation in microglial cells through NF-κB and Nrf2 pathways, promotes cell cycle arrest in leukemia and breast cancer cells, and modulates cyclin-dependent kinase inhibitors (*Li Y et al., 2015; Fernandes J, 2015; Chang MY et al., 2015; Elbe H et al., 2022; Buchbauer G et al., 1991*). Borneol has shown potential in reducing apoptosis in human glioma cells by regulating proteins and autophagy pathways, and in combination with radiation therapy, it reduces HIF-1α and mTORC1 expression (*Wang Z et al., 2020; Li Q et al., 2021*). Moreover, 1,8- cineole and limonene have shown anti-inflammatory and antibacterial properties, induce apoptosis in colorectal cancer

cells, and promote detoxification of carcinogens, inhibiting tumor growth and angiogenesis (*Murata S et al., 2013; Bıçak B, 2024; Araújo-Filho HG et al., 2021; Zhou J et al., 2021*). Terpinen-4-ol, a terpene with growth-inhibitory effects, enhances chemotherapy efficacy and induces cell death, making it a promising cancer treatment option (*Shapira S et al., 2016; Nakayama K et al., 2017; Cao W et al., 2022*).

### *3.3 The biological activity of Lavandula angustifolia*

Lavender, also known as lavender, is a Mediterranean perennial plant cultivated globally (*Boelens MH et al., 1995; Śmigielski K et al., 2009*). It is known for its therapeutic properties, including treating anxiety, insomnia, and neurological disorders (*Akhondzadeh S et al., 2003*). Its sedative and analgesic effects can alleviate depression, headaches, and anxiety symptoms, and may prevent Alzheimer's-related dementia in rats (*Kashani MS et al., 2011; Guillermain J et al., 1989*; *Wolfe N et Herzberg J, 1996*). Lavender, a potent aphrodisiac, has been found to boost blood flow to the penis by 40% when combined with pumpkin dough (*Hirsch A et Gruss J, 1999*). It also promotes hair growth in alopecia areata patients (*Hay IC et al., 1998*).

Lavender essential oil (LEO) has been shown to have analgesic and anaesthetic properties and has been found to lower cholesterol levels (*Martella N et al., 2023; Nikolaevskii VV et al., 1990*), reduce blood pressure (*Romine IJ et al., 1999*), lower heart rate, support digestion, and regulate bowel movements in rats (*Gruncharov V, 1973; Yurkova O, 1999*), protect cell from free radical damage and inhibiting fat oxidation and lipid peroxidation (*Dapkevicius A et al., 1998*; *Economou KD et al., 1991; Lu Hui et al., 2010*). In guinea pigs, it relaxes smooth

muscle and inhibits contractions caused by acetylcholine and histamine (*Lis-Balchin M et Hart SA, 1997; Lis-Balchin M et Hart SA, 1999*).

LEO, used in perfumes and cosmetics, has been shown to reduce chemotherapy-induced side effects (*Bozhanov S et al., 2007*; *Srancikova A et al., 2013; Zhao Y et al., 2017; Shou-Dong S et al., 2009*; *Rodenak-Kladniew B et al., 2018; Sun X et al., 2015*). The terpenes in lavender-propolis extract have been found to enhance the sensitivity of glioma cells to radiation therapy, improving the efficacy of chemotherapeutic agent TMZ (*Li Q et al., 2021; Wang Z et al., 2020; Lin L et al., 2024; Mardani A et al., 2022*). This extract also demonstrated significant cytotoxic effects against the T98 GBM cell line, making it a promising natural remedy *(Keskin S et Çetin E, 2020).*

### *3.4 Natural plant-derived substances as potential adjunctive therapies for GBM*

Despite extensive research, the standard treatment for GBM is still surgery followed by radiotherapy and chemotherapy with TMZ (*Stupp R et Weber DC, 2005*). However, complete tumor removal is difficult, and resistance to TMZ, along with the blood-brain barrier limiting drug delivery, reduces treatment effectiveness. TMZ's efficacy varies based on MGMT promoter methylation status, highlighting the need for new adjuvant therapies to enhance its effects or reduce toxicity. While bevacizumab has improved PFS, it has not extended overall survival in GBM (*Erasimus H et al, 2016; Lombardi G et al., 2017*). A multi-targeted approach using compounds with different mechanisms may improve outcomes. Natural compounds, extensively reviewed for their potential as adjuvants, show promise in inhibiting GBM growth (*Sestito S et al., 2018; Vengoji R et al., 2018).*

*-Aloe-emodin*

The treatment of GBM stays challenging due to its complexity and the multitude of drug resistance mechanisms, including drug efflux, hypoxic tumor regions, glioma stem cells, DNA repair pathways, and the influence of miRNAs (*Haar CP et al., 2012*). Aloe-emodin (AE), a naturally occurring anthraquinone derived from plants such as Aloe vera and Cassia occidentalis, has shown significant anticancer potential, particularly in brain tumors (*Ismail S et al., 2013; Acevedo-Duncan M et al., 2004*). *In vitro* studies on U87MG GBM cells treated with AE proved cell cycle arrest in the S and G2/M phases, accompanied by elevated levels of p53 and p21 and decreased cyclin-dependent kinase 2 (CDK2), resulting in inhibited cell proliferation. AE also reduced Akt phosphorylation, thereby suppressing proliferative signals. Additionally, after 72 hours of AE treatment, cells showed morphological changes typical of apoptosis, such as rounding and volume reduction. AE's pro-apoptotic effect was further confirmed by a decrease in PARP1 expression and the activation of Lamin A (*Manimaran A et al., 2016*). *In vivo* experiments supported these findings, revealing decreased tumor proliferation, as showed by reduced ki67 levels, and increased pro-apoptotic markers like p53 and caspase 6 in tumor tissues (*Michalkova R et al., 2023*).

Importantly, while AE is known to have toxic effects, the concentrations used in these studies were well below the toxic threshold for normal hepatocytes, suggesting that AE could be a safe adjuvant therapy for GBM (*Dong X et al., 2017*). Furthermore, AE was evaluated in combination with TMZ in drug-resistant glioma cell lines, NULU and ZAR. The combination resulted in a significant additive inhibitory effect on cell growth and enhanced cytotoxicity compared to either agent alone. AE and TMZ co-treatment also appeared to modulate the drug-resistance

protein MGMT, suggesting a reversal of resistance in these resistant cell lines. This combination therapy effectively slowed colony formation and reduced GBM cell migration. Collectively, these findings write down that AE has the potential to serve as a powerful natural adjuvant, enhancing the effectiveness of standard treatments like TMZ and overcoming drug resistance in glioblastoma therapy (*Staffieri S et al., 2023*) (**Figure 6**).

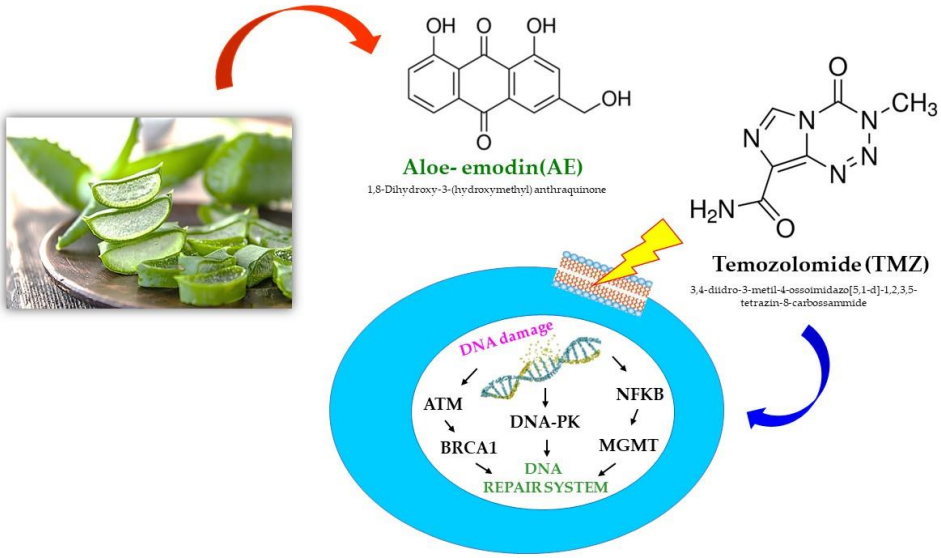



**Figure 6. Aloe-Emodin Overcomes Anti-Cancer Drug Resistance to Temozolomide and Prevents Colony Formation and Migration in Primary Human Glioblastoma Cell Lines NULU and ZAR.** The effect of combined AE (Aloe-emodin) and TMZ (temozolomide) treatment restore drug resistance in both primary resistant cell lines (NULU and ZAR). The expression of MGMT may be regarded as the most significant molecular predictor of TMZ resistance and prognosis in gliomas, provided that the cytotoxic effects of the alkylating agent can be mitigated in cells with a high level of endogenous MGMT (O-6-methylguanine-DNA methyltransferase) activity. Furthermore, mounting data highlight the critical function of the NF-κB (nuclear factor kappa B) signaling system in the treatment resistance of GBM. Moreover, the gene's mRNA levels of ATM (ATM serine/threonine kinase), BRCA1 (BRCA1 DNA repair associated) e DNA-PK (DNA activated protein kinase) rise during treatment because of compensatory mechanisms against MGMT's downregulation *(Image adapted from Staffieri S et al., 2023).*

-*Tea tree Oil*

Tea tree oil (TTO), derived from the leaves of Melaleuca alternifolia, shows a broad spectrum of biological activities, including antimicrobial, antifungal, antiviral, and anti-inflammatory effects (*Akthar MS et al., 2014; Hammer KA et al., 2003; Schnitzler P et al., 2001; Hart PH et al., 2000).* TTO is a complex blend of over 100 compounds, with terpinen-4-ol being the most abundant and primarily responsible for its biological effects. Notably, both TTO and terpinen-4-ol have shown anti-proliferative effects in various cancer cells, including human melanoma and U87MG GBM cells (*Calcabrini A et al., 2004; Moteki H et al., 2002*).

In recent experiments, low concentrations of TTO significantly inhibited cell growth in U87MG glioblastoma cells, with up to 50% reduction in proliferation (*Arcella A et al., 2019*). Furthermore, when combined with the chemotherapy agent TMZ, TTO exhibited synergistic effects, enhancing TMZ's anti-cancer efficacy. The molecular mechanism of TTO's action involves the downregulation of CDK2, a key regulator of the G1-S cell cycle transition, and the upregulation of p27, a tumor suppressor protein, which increased after 8 hours of treatment. Prolonged exposure to TTO for 72 hours led to cell cycle arrest in the G0/G1 phase, effectively halting cell proliferation (*Arcella A et al., 2019*). While apoptosis was not significantly induced, necroptosis—a form of programmed inflammatory cell death—was identified as a potential mechanism for TTO's anti-GBM effects (*Van den Berghe T et al., 2014*). Necroptosis is characterized by organelle swelling, cell membrane rupture, and the release of intracellular contents, and is regulated by death receptors like TNFR1 and proteins such as RIPK1 and TRADD. TTO treatment led to increased expression of TNFR1 and RIP during long-term exposure, while short-term exposure elevated TNFR1 and TRADD levels, further

supporting the involvement of necroptosis (*Sosna J et al., 2014; Shan B et al., 2010*).

*In vivo* studies corroborated these findings, as CD1 nude mice treated with TTO exhibited an 80% reduction in tumor volume compared to control mice. Histological analysis of the tumors revealed extensive necrotic areas, confirming necrosis as a primary mechanism of TTO's anti-GBM activity (*Arcella A et al., 2019*). These results highlight TTO's potential as a natural adjuvant therapy in the treatment of glioblastoma, offering a promising complementary approach to standard therapies.

- *Isoginkgetin*

Isoginkgetin (Iso), a natural bioflavonoid extracted from *Ginkgo biloba* leaves, has demonstrated potent anti-inflammatory, antioxidant, and antitumor properties (*Qaâdan F et al., 2010*). Extensive research on *G. biloba* extracts has shown their inhibitory effects on various cancer types (*Ahmed HH et al., 2017*). Iso specifically exerts antitumor activity by inhibiting tumor metastasis through the modulation of matrix metalloproteinase-9 (MMP-9), a key protein involved in tumor invasion, particularly in fibrosarcoma (*Yoon S et al., 2006; Cao C et al., 2006*). Additionally, Iso has been shown to suppress breast cancer cell growth (MCF-7, MDA-MB-231) and effectively inhibit U87MG GBM cell proliferation in a dose- and time-dependent manner. Colony formation and FACS analyses confirmed that Iso reduces colony formation, induces cell cycle arrest in the S phase, and affects pathways related to autophagy and apoptosis. This study

underscores Iso's potential as an adjuvant therapy for GBM, targeting multiple mechanisms that regulate tumor cell growth and migration (*Oliva MA et al., 2022*).

-*Hispolon*

*Phellinus linteus* (PL), a medicinal mushroom commonly used in traditional Korean, Chinese, and Japanese medicine, is known for its diverse biological activities, including immunomodulatory, antioxidant, anti-inflammatory, and anti-proliferative effects (*Gao Y et al., 2003; Lee JW et al., 2006; Song HH et al., 2012; Ikekawa T et al., 1968; Meneses ME et al., 2016; Huang GJ et al., 2012; Yamac M et al., 2016*). Hispolon, a bioactive compound isolated from PL, has shown particularly promising anticancer properties, especially against GBM (*Arcella A et al., 2017*). In studies on U87MG GBM cells, low doses of hispolon (25–50 µM) significantly reduced cell viability in a dose- and time-dependent manner by inducing G2/M cell cycle arrest. Hispolon upregulated p21 expression and downregulated CDK4, while also activating the tumor suppressor p53 (*el-Deiry WS et al., 1993*). Additionally, hispolon disrupted MAPK signaling by reducing ERK1 phosphorylation and induced apoptosis through the activation of caspase 3 and the cleavage of PARP (*Hannen R et al., 2017; Pellosky CE et al., 2006)*. Hispolon also inhibited GBM cell migration and potentiated the effects of TMZ *in vitro*, suggesting its potential as a valuable adjuvant therapy for GBM care.

-*Lactucopicrin*

Lactucopicrin (LCTP), a natural sesquiterpene lactone extracted from *Lactuca virosa*, has shown promising anticancer effects in various cancer cell lines. Studies have proved its potential in inhibiting the growth of SKMEL-5 human skin cancer

cells (*Zhang X et al., 2018*) and Saos-2 osteosarcoma cells (*Meng Q et al., 2019*). Additionally, LCTP exhibits notable anticancer activity in GBM by specifically targeting U87MG cells. Continuous administration of LCTP resulted in a significant, dose- and time-dependent reduction in GBM cell growth and viability, further supported by a decrease in clonogenic potential and reduced cell mobility, suggesting LCTP effectively hampers GBM cell proliferation and metastasis (*Rotondo R et al., 2020*). On a mechanistic level, LCTP was shown to induce autophagy in U87MG cells by decreasing phosphorylation of key proliferative signaling proteins, including pAKT and pERK. LCTP also triggered cell cycle arrest at the G2/M phase, marked by downregulation of cyclin-dependent kinase 2 (CDK2) and increased expression of tumor suppressor proteins p53 and p21. Apoptosis was enhanced, as showed by a reduction in procaspase-6 levels and an increase in the cleaved/full-length PARP ratio, emphasizing LCTP's role in promoting programmed cell death. Moreover, LCTP's cytotoxic effects appear to be mediated, at least in part, by oxidative stress. Pre-treatment of U87MG cells with the ROS scavenger N-acetylcysteine (NAC) reversed the cytotoxicity of LCTP, saying that oxidative stress is a key mechanism of its action against GBM cells (*Rotondo R et al., 2020*) (**Figure 7**). Significantly, LCTP was found to enhance the sensitivity of U87MG cells to the chemotherapy drug TMZ, showing a synergistic effect that amplifies the therapeutic efficacy. Overall, LCTP exerts its anticancer activity through multiple synergistic mechanisms, including autophagy induction, cell cycle arrest, apoptosis activation, and oxidative stress. These properties make LCTP a compelling candidate as an adjuvant therapy to improve the outcomes of conventional treatments for aggressive and complex cancers such as glioblastoma.

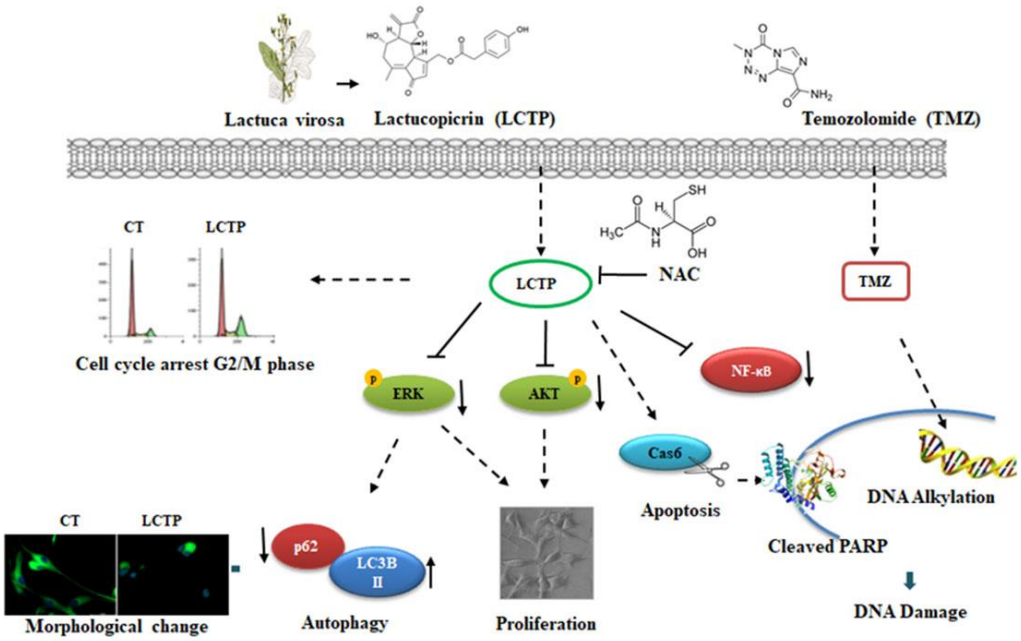



**Figure 7. Implication of Lactucopicrin in Autophagy, Cell Cycle Arrest and Oxidative Stress to Inhibit U87Mg Glioblastoma Cell Growth**. LCTP effectively control the growth of U87MG cells. The compound has a strong cytotoxic effect with daily administration leading to reduced cell growth and viability. LCTP activates autophagy modulating p62 and LC3B II expression levels and causes cell cycle arrest in the G2/M phase. It also stimulates apoptosis and involves oxidative stress. Additionally, LCTP increases the sensitivity of U87MG cells to TMZ *(Image adapted from Rotondo R et al., 2020).*

### *3.5 Lactoferrin, a multifunctional glycoprotein in cancer therapy*

Lactoferrin (HLF), a natural protein found in exocrine gland secretions and neutrophils *(Baker EN et Baker HM, 2009*), has proved significant potential in inhibiting GBM growth in both *in vitro* and *in vivo* studies (*Junes-Gill KS et al., 2014; Arcella A et al., 2015*). Known for its roles in iron metabolism, cell proliferation, and antimicrobial activity, HLF has shown notable antiproliferative effects (*Baveye S et al., 1999; Gifford JL et al., 2005; Iyer S et Lonnerdal B, 1993;*

*Ortensi B et al., 2013; Ward PP et al., 2005*). Bovine lactoferrin (bLF) has been found to reduce colorectal adenomatous polyps and suppress carcinogenesis in various cancers (*Kozu T et al., 2009*). In studies on GBM, HLF combined with TMZ significantly inhibited cell growth in primary GBM cell lines (NMD, FN) as well as the U87MG cell line, with observable effects as early as 24 hours post-treatment (*Arcella A et al., 2015*). Western blot analysis revealed that HLF decreased the expression of cyclins D1 and D4, as well as phosphorylated ERK1/2 (pERK1/2), thereby disrupting cell cycle progression. Flow cytometry further confirmed these findings, showing a reduction in S-phase cells and an accumulation in the G0/G1 and G2 phases, along with decreased BrdU incorporation, all of which show suppressed cell proliferation. *In vivo*, HLF treatment reduced tumor size by approximately 30% in an orthotopic GBM xenograft model. When combined with TMZ, the therapeutic effects were even more pronounced, suggesting that HLF enhances TMZ's efficacy. Given its non-toxic nature, HLF holds considerable promise as an adjuvant therapy for improving the effectiveness of TMZ in GBM treatment by inhibiting tumor growth.

# 4. Role of nutritional adjuvants in the management of gliomas

Although the association of individual foods and nutrients with glioma has been studied, studies on the association of major dietary patterns and glioma are scarce. Factor analysis was used to identify major dietary patterns. Three major dietary patterns were identified using factor analysis: high-protein, vegetarian, and Western dietary patterns. After several adjustments for potential confounders, adherence to the high-protein dietary pattern was inversely associated with glioma risk (OR: 0.47; 95% CI: 0.23, 0.95). Consuming a vegetarian dietary pattern was also associated

with a reduced risk of glioma (OR: 0.16; 95% CI: 0.07, 0.34). Greater adherence to the Western dietary pattern was associated with a greater likelihood of glioma (OR: 3.30; 95% CI: 1.52, 7.17). Therefore, a high-protein, vegetarian and european dietary pattern was significantly associated with the risk of glioma (*Nemati M et al., 2024*). Moreover, recent scientific research has shown that the ketogenic diet may have potential benefits in a variety of medical fields, which has led to the diet receiving considerable attention. Clinical and experimental research on brain tumors has shown that the ketogenic diet has a satisfactory safety profile. Patients with brain tumors who follow a ketogenic diet are more likely to experience better survival rate (*Valerio J et al., 2024*). Specifically, a ketogenic diet had the highest median OS of all the adjuncts (42.6 months) while in recurrent cases, a low copper diet coupled with 1 g penicillamine had the highest median OS (18.5 months). However, no statistically significant difference was observed in OS or progression-free survival (PFS) of newly diagnosed or recurrent gliomas (*Pahwa B et al., 2023*). According to the recent research of The Preston Robert Tisch Brain Tumor Centre 2024, brain tumor patients should focus on:

- **Ketogenic diet** that is a high-fat, low-carbohydrate diet that forces the body to burn fats rather than carbohydrates for energy. This diet can help reduce inflammation and provide a steady energy source for the brain, reduce the growth of tumor cells, can provide an alternative energy source for brain cells and it may improve cognitive function and reduce treatment-related fatigue.
- **Plant-Based Diet**, that emphasizes whole foods derived from plants, which can be rich in antioxidants, vitamins, and minerals. This diet can help reduce inflammation and support overall health.
- **Anti-Inflammatory Diet** that can help manage inflammation, which is often associated with cancer and its treatment.

Certain foods can worsen symptoms or interfere with treatment. Processed foods often contain unhealthy fats, excessive sugars, and artificial additives that can contribute to inflammation and other health issues. High sugar intake can lead to weight gain, increased inflammation, and a weakened immune system. Excessive sodium can lead to high blood pressure and increased fluid retention, which can be problematic for brain tumor patients.

# 5. Drug Repositioning in Glioblastoma

Drug repositioning may be one means of expediting therapeutic drug development for GBM. Drug repositioning is a method of expanding the therapeutic range of FDA-approved drugs to other diseases by identifying novel uses. It is advantageous over novel drug discovery due to the already known pharmacokinetic and safety profiles. In addition, drug repurposing is considerably less costly and less time intensive than novel small molecule discovery. It targets distinct proteins in cells, enhancing efficacy and reducing off-target toxicities (*Tan SK et al., 2018*). Drug repositioning, also known as polypharmacology, involves small molecules targeting distinct proteins in cells, allowing the same molecule to target unrelated pathways in cancer initiation or progression. This approach contrasts with traditional drug discovery, which aims to identify one drug for one target, enhancing efficacy and reducing off-target toxicities.

***Atypical antipsychotics.*** Olanzapine, clozapine, asenapine, lurasidone, quetiapine, risperidone, aripiprazole, brexpiprazole, and ziprasidone, have multiple effects on dopamine, 5-HT2, α-, and H1-receptors, making them attractive for repurposing in GBM. Olanzapine, used in schizophrenia, bipolar disorder, and neurological conditions like Huntington's disease, is an antagonist of serotonin (5-HT2A) and dopamine (D2) receptors. It has antineoplastic capability and has been used to control pain and chemotherapy-associated nausea in the cancer field. It reduces

glioblastoma cell expansion *in vitro* and *in vivo* and has antineoplastic capability (*Karpel-Massler G et al., 2015*). However, the efficacy of olanzapine likely varies among different GBM cell lines due to the heterogeneous nature of GBM. Quetiapine, another atypical antipsychotic, acts as an antagonist at serotonin (5-HT1A and 5-HT2A), dopamine (D1 and D2), histamine (H1), and adrenergic (α1 and α2) receptors. It is FDA-approved for the treatment of schizophrenia, bipolar disorder, major depressive disorder (MDD), and generalized anxiety disorder. Quetiapine suppresses GBM cell growth *in vitro* and *in vivo*, improving survival of mice bearing glioma. Its anti-gliomagenic property is attributed to the finding that well-differentiated cells are more sensitive to chemotherapy than less differentiated ones. Quetiapine controls cell growth via downregulation of the phosphoionositide 3-kinase (PI3K) pathway, a major driver of GBM cell proliferation (*Karbownik MS et al., 2016*).

***Psichiatric drugs and Neuroepiletics.*** Studies have shown that FDA-approved psychotropic agents can inhibit GBM cell proliferation and migration (*Lee JK et al., 2016; Triscott J et al., 2012*). Brain penetrant compounds are particularly promising for reducing GBM growth in humans. The intact BBB allows diffusion of lipid-soluble molecules smaller than 400 Da and naturally transported molecules (*Gan HK et al., 2017*). Compounds enter the brain through active efflux mechanisms due to transporters at the BBB. However, the restriction of the BBB, causing low distribution of therapeutic agents, remains a challenge. Organic anion-transporting polypeptide 1A2 (OATP1A2/SLCO1A2), organic anion transporter 3 (OAT3/SLC22A8), P-glycoprotein (P-gp), multidrug-resistance-associated protein 4 (MRP4/ABCC4), and monocarboxylate transporter 1 (MCT1/SLC16A1) are examples of transporters, which are present at the BBB (*Urquhart BL et Kim RB,*

*2009*). Therefore, equally distributed compounds are attractive for treating GBM patients.

***Neuroleptics***. Antipsychotic drugs, also known as neuroleptics, are used to treat psychosis, schizophrenia, acute mania, bipolar disorder, and Tourette syndrome. Common antipsychotics block dopamine D2 receptors, which are responsible for mitogenic signaling in GBM cells. Antipsychotics have several mechanisms of action for their potential anti-tumor effect. Trifluoperazine binds to a Ca2+-binding protein, calmodulin subtype 2, de-represses the Ca2+ release channel inositol 1,4,5-triphosphate receptor (IP3R) subtype 3, and stimulates the irreversible mass release of Ca2+ in GBM cells (*Oliva CR e al., 2017*). Ca2+ is essential for gene expression and metabolism, and an alteration in Ca2+ levels can result in cell death. Chlorpromazine (CPZ) inhibits mitochondrial cytochrome c oxidase (CcO) in chemoresistant glioma cells and GSCs when CcO subunit 4 isoform 1 (COX4-1) is present, but not COX4-2. Attenuated CcO reduces the efficacy of mitochondrial OxPhos dependent ATP-linked respiration and lowers reactive oxygen species production, lowering glioma progression. Increased CcO activity and increased COX4-1 expression are associated with worse prognosis in GBM (*Oliva CR e al., 2017*). CPZ has been shown to prolong survival in an in vivo preclinical study without adverse behavioral effects, suggesting that similar use in GBM patients could be well-tolerated (*Kang S et al., 2017*).

***Sedative Hypnotics.*** Benzodiazepines, including diazepam, lorazepam, triazolam, temazepam, oxazepam, and midazolam, are commonly used as general anesthetics and for treating anxiety disorders, spasticity, and sleepwalking. They increase the frequency of Cl− channel opening in the central nervous system, facilitating

GABAA receptor complex action (*Chen J et al., 2013*). In GBM patients, diazepam can alleviate post-cancer therapy anxiety and inhibit chemotherapy-associated delayed emesis. However, achieving efficacy in anticancer therapy requires a higher dose. In 2013, researchers found that diazepam can cause cell cycle arrest in human GBM cells by inactivating the cell cycle protein Rb, indicating that it not only sensitizes cells to chemotherapy but also kills tumor cells (*Sarissky M et al., 2005*).

***Non-Psychiatric Drugs.*** Considerable efforts are needed to determine whether combination therapies of these repurposed compounds with the current standard-of-care will either facilitate or inhibit BBB penetrance of these compounds.

***Mebendazole.*** A microtubule inhibitor, Mebendazole is an FDA-approved antihelmintic drug that has a benign safety profile although it has been shown to cause bone marrow suppression and liver toxicity at higher doses (*De Witt M et al., 2017*). Mebendazole, a drug that inhibits protein kinases, has been shown to have anti-tumor effects by inhibiting microtubule polymerization and causing metaphase arrest, like the mechanism of action of vincristine (*De Witt M et al., 2017*).

***Clomifene.*** Clomifene is a selective estrogen receptor modulator used in treating female infertility and hypogonadism in men (*Zheng M et al., 2017*). It acts as an antagonist at estrogen receptors in the hypothalamus, preventing normal feedback inhibition and increasing the release of Luteinizing Hormone and Follicle-Stimulating Hormone, leading to ovulation. Clomifene was identified as an inhibitor of mutant isocitrate

dehydrogenases (IDH) 1, essential for tumorigenesis in multiple cancers. It selectively inhibits mutant IDH1, reducing the accumulation of downstream D-2-hydroxyglutaricacid (D-2HG), which drives carcinogenesis by inhibiting histone demethylases and increasing global methylation of histones and DNA (Zheng M et al., 2017). Clomifene also increases apoptosis of glioma cancer cells with IDH1 mutations without causing hepatotoxicity or nephrotoxicity.

***Metfromin.*** Metformin, a cationic biguanide class drug, is a first-line medication for Type II diabetes mellitus (TIIDM) treatment (*Molenaar RJ et al., 2017*). It is widely available, inexpensive, and safe. Phenformin, a lipophilic analog of metformin, is also used in TIIDM management but was withdrawn in the 1970s due to its lactic acidosis side effect (*Molenaar RJ et al., 2017*). Metformin is believed to inhibit gluconeogenesis, increase glycolysis, and increase insulin sensitivity by promoting peripheral glucose uptake. It has been linked to cancer prevention and is of interest in drug repositioning for cancer treatment (*Adeberg S et al., 2015*). Biguanides have anti-gliomagenic properties by inhibiting GBM cell proliferation, decreasing migration, inducing apoptosis, decreasing angiogenesis, reducing TMZ resistance, reducing self-renewal, and inhibiting stemness of GSCs (*Ferla R et al., 2012*). They also induce tumor regression and prolong survival in xenograft models. Biguanides modulate microRNAs (miRNAs) that regulate cell gene expression, which are critical for energy metabolic pathways, cell cycle, and stemness. They can increase the bioavailability of let-7, inhibit glutamate dehydrogenase, and reduce glutaminolysis in IDH1/2 mutated glioma (*Lee YS et Dutta A, 2007*).

***Statins.*** HMG-CoA reductase inhibitors, such as lovastatin, pravastatin, rosuvastatin, and simvastatin, are widely used lipid-lowering agents in clinics. They inhibit the conversion of HMG-CoA to mevalonate, a cholesterol precursor, and reduce mortality in cardiovascular diseases. The reduction of mevalonate also reduces farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are crucial for cell proliferation and are often mutated in cancers like glioma. Epidemiological studies have shown the pleiotropic effect of pre-operative statin use on the prognosis of GBM patients. However, experimental studies have shown considerable cytotoxic activities in GBM in time- and dose-dependent manners. The mechanisms of these statins in inhibiting GBM include TNF-related apoptosis-inducing ligand (TRAIL)-sensitizing effect, increased expression of pro-apoptotic protein Bim, reduction of MAPK-dependent pathway and GTPase activation, suppression of ERK1/2 and Ras/PI3K/Akt pathway, and activation of JNK1/2 (*Gaist D et al., 2014; Tapia-Perez JH et al., 2011*).

# *Objectives*

# Objectives

The main goal of this PhD thesis was to improve the understanding of how GBM responds to natural substances, considering their possible health advantages. As the most common kind of brain tumor in adults, GBM creates significant treatment challenges due to its quick growth, high chances of coming back, and resistance to usual treatment methods. The spreading nature of GBM makes surgical procedures difficult, rendering complete removal impossible without risking harm to healthy brain cells. Furthermore, the blood-brain barrier (BBB) creates obstacles for the effective use of overall therapies, further reducing their effectiveness. One of the main difficulties in treating GBM is the tumor's resistance to the first-choice chemotherapy drug, TMZ, which is affected by factors like the methylation status of the MGMT gene promoter and higher levels of reactive oxygen species (ROS) in GBM cells. A variety of elements contribute to a tumour's ability to resist treatment. Considering these difficulties, there is a growing interest in exploring natural substances with possible anticancer benefits as additions to current therapies. Essential oils have demonstrated considerable promise in this area. Their active components, like linalool, borneol, 1,8-cineole, limonene, and terpinen-4-ol, have been acknowledged for their anticancer properties. Recent studies indicate that these components may not only hinder the growth of GBM cells and reduce tumour severity but also improve the sensitivity of GBM cells to TMZ, which addresses a significant challenge in effective chemotherapy. By adjusting oxidative stress and affecting cancer cell signaling routes, essential oils and their ingredients may disrupt the processes that promote GBM growth and resistance, especially through the MGMT pathway. The addition of these active substances into GBM treatment plans could provide a fresh strategy for overcoming resistance, enhancing drug effectiveness, and possibly reducing chemotherapy-related side effects.

This research aimed to investigate the effects of LEO and its individual terpenes on the aggressive characteristics of GBM using the U87MG cell model.

The specific objectives included:

• Evaluating the effects of LEO, both independently and alongside TMZ, on the proliferation of GBM cells.

• Analysing how LEO influences cell migration and adhesion.

• Assessing the role of LEO in modulating oxidative stress levels.

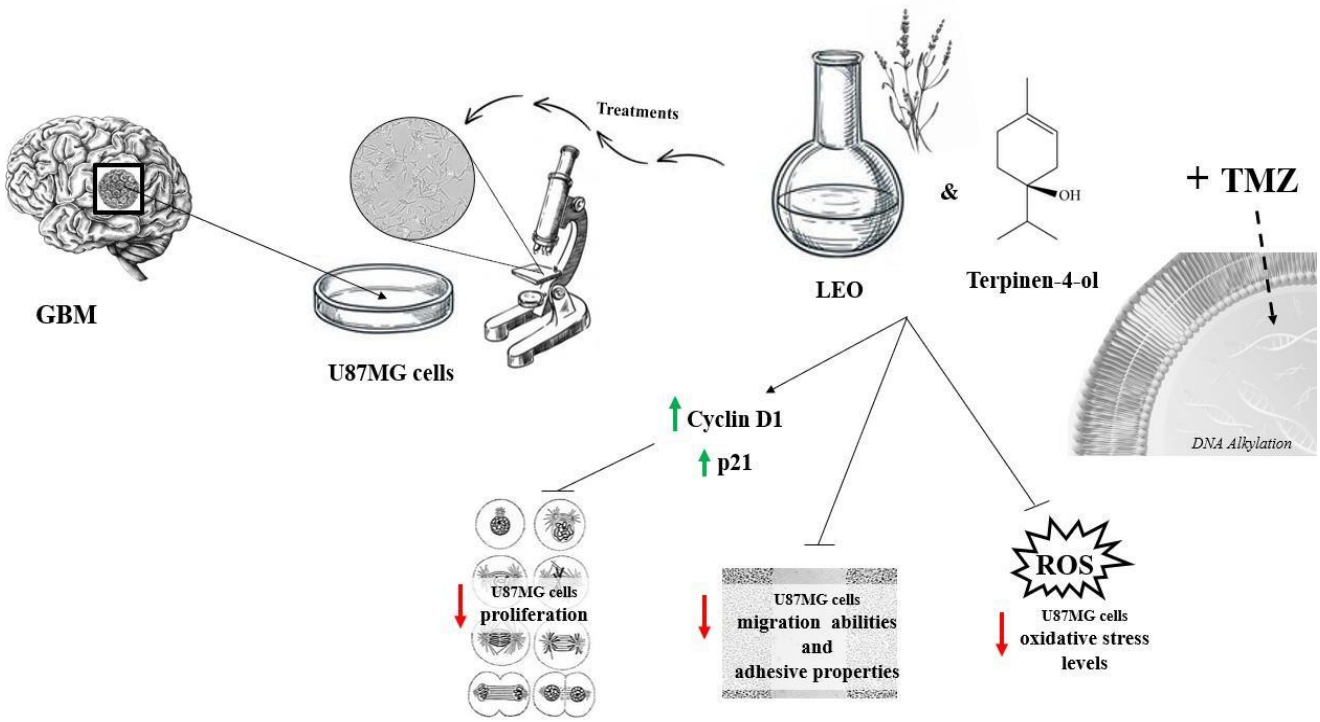
• Exploring the effects of the terpene's linalool, borneol, 1,8-cineole, limonene, and terpinen-4-ol on the proliferation of GBM cells.

• Investigating the combined effects of terpinen-4-ol and TMZ on the growth of GBM cells.

• Examining the impact of terpinen-4-ol on GBM cell migration, adhesion, and oxidative stress levels.

# *Results*

# Graphical Abstract




***Russo M. et al., 2024***

# *Manuscript I.*

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# Lavender Essential Oil and Its Terpenic Components Negatively Affect Tumor Properties in a Cell Model of Glioblastoma

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**Abstract:** Glioblastoma (GBM) is the most common and aggressive form of brain cancer in adults, characterized by extensive growth, a high recurrence rate, and resistance to treatment. Growing research interest is focusing on the biological roles of natural compounds due to their potential beneficial effects on health. Our research aimed to investigate the effects of lavender essential oil (LEO) on a GBM cell model. Chemical characterization using GC-MS analysis indicated that LEO contains several terpenes, compounds that have been found to exhibit anticancer properties by interfering with key cancer-related pathways in several cancer models. By means of cell biology assays, we demonstrated that LEO impairs cell proliferation and migration, and also reduces oxidative stress in U87 cells. We further observed that Terpinen-4-ol, contained in LEO, was capable of reproducing the effects of the oil on GBM cells. Our results suggest that the terpenic molecules present in LEO could be considered valuable allies alongside conventional therapies against GBM.

**Keywords:** GBM; essential oils; *Lavandula angustifolia*; terpenes; cell proliferation; cell migration; oxidative stress



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## 1. Introduction

GBM is the most prevalent and lethal form of brain cancer in adults, with only modest improvements in survival rates over the past thirty years [1,2]. Histologically, GBM is distinguished by its aggressive features: marked mitotic activity, substantial angiogenesis, cellular heterogeneity, necrosis, and rapid proliferation [3,4]. Current treatments for GBM, which include extensive surgical resection followed by radiotherapy and chemotherapy with temozolomide (TMZ), have limited success in preventing tumor progression and infiltration [5,6]. Consequently, the median survival time for GBM patients is approximately 14 months, with most experiencing relapses at various intervals post-treatment [7,8]. In fact, GBM frequently recurs due to its invasive nature and the difficulty of eradicating all cancer cells, including a subpopulation of cancer stem cells. Moreover, the development of TMZ resistance also represents a major obstacle in GBM treatment [9,10]. Considering these limitations, research focuses on unraveling the molecular pathways involved in gliomagenesis and on identifying novel potential therapeutic targets [11–13]. Several natural compounds have also been evaluated in recent years for their antitumor properties, including their ability to inhibit tumor growth and invasiveness, and to improve overall patient prognosis in various cancer models [14–16]. Some of them have shown significant antitumor activities when combined with TMZ in GBM-resistant cells [17,18].

Lamiaceae plants, including *Lavandula angustifolia*, have traditionally been used to alleviate anxiety, insomnia, and various neurological conditions, as well as to combat infections, manage pain, and address a range of other ailments [19]. In particular, lavender essential oil (LEO) has been shown to be effective in preventing some chemotherapy-induced side effects in human leukemia cells and in xenograft models of human prostate cancer [20–22], and in modulating cholesterol metabolism in a cellular model of hepatocarcinoma [23]. LEO's versatile properties underscore its potential as a natural remedy with diverse health benefits, positioning it as an intriguing topic for scientific research and therapeutic investigation.

LEO contains approximately one hundred biologically active compounds, several of which with considerable therapeutic potential [24]. In particular, terpenes, which are prominent in LEO, have become focal points of biochemical and molecular research due to their wide-ranging biological activities, including anti-inflammatory, antimicrobial, and antiviral properties [25,26]. Terpenes interact with specific biological targets such as enzymes, receptors, and ion channels, thereby influencing crucial signaling pathways involved in apoptosis, proliferation, and cell differentiation [27,28]. In line with this evidence, recent studies indicate that terpenic molecules such as borneol, linalool, and 1,8-cineole present in essential oils can suppress cell cycle progression and induce apoptosis in various cancer cells [29–33]. Among these promising bioactive molecules, terpinen-4-ol, a monocyclic monoterpenoid, exhibits notable antitumor properties by inducing ferroptosis and inhibiting cell proliferation in leukemia and melanoma cells [34–37]. Additionally, this monoterpene has significant antioxidant effects, demonstrated by its ability to increase the expression of enzymes that support oxidative metabolism homeostasis, both in vivo and in vitro [36]. No evidence is present in the literature concerning the effects of LEO administration on GBM models, although very recently it has been reported that terpinen-4-ol induces the ferroptosis of glioma cells and that borneol enhances the efficacy of TMZ in vitro [32,34]. In this context, our study aimed to investigate the impact of LEO and of its terpenic components on the properties of GBM cells in an in vitro model. We demonstrated that LEO induces a proliferation slowdown and an impairment of cell migration of GBM cells besides reducing oxidative stress. Furthermore, we observed that terpinen-4-ol mimics the anti-oncogenic properties of LEO on GBM cells.

## 2. Results

### *2.1. Extraction and Characterization of L. angustifolia Essential Oil*

*L. angustifolia* flowers were collected in Pesche (IS, Southern Italy) during the flowering period. After drying, they were hydrodistillated to obtain an essential oil (LEO) in a yield of 4.5%, calculated according to the dry weight of 100 g. The GC-MS analysis found 53 individual components, corresponding to 98.1% of the total peak areas of the chromatogram (Figure 1). The main chemical components of LEO with an area percentage > 1% are reported in Table 1; all compounds, listed according to their elution order on a Rtx®-5 Restek capillary column, are reported in Supplementary Table S1. The complete identification was performed by calculating the experimental retention indices (Exp RI) and comparing them with those found in the literature (Ref RI) [38]. GC-MS analysis confirmed the presence of linalool (33.99%) as the major component and characterizing essence of *L. angustifolia*. The LEO showed high content levels of borneol (13.21%), 1,8-cineole (6.29%), and terpinen-4-ol (5.24%). The oil also contained a mixture of components, mainly oxygenated monoterpenes (73.06%), followed by monoterpenes (15.59%), sesquiterpenes (6.04%), and oxygenated sesquiterpenes (2.81%), as reported in Supplementary Table S2.

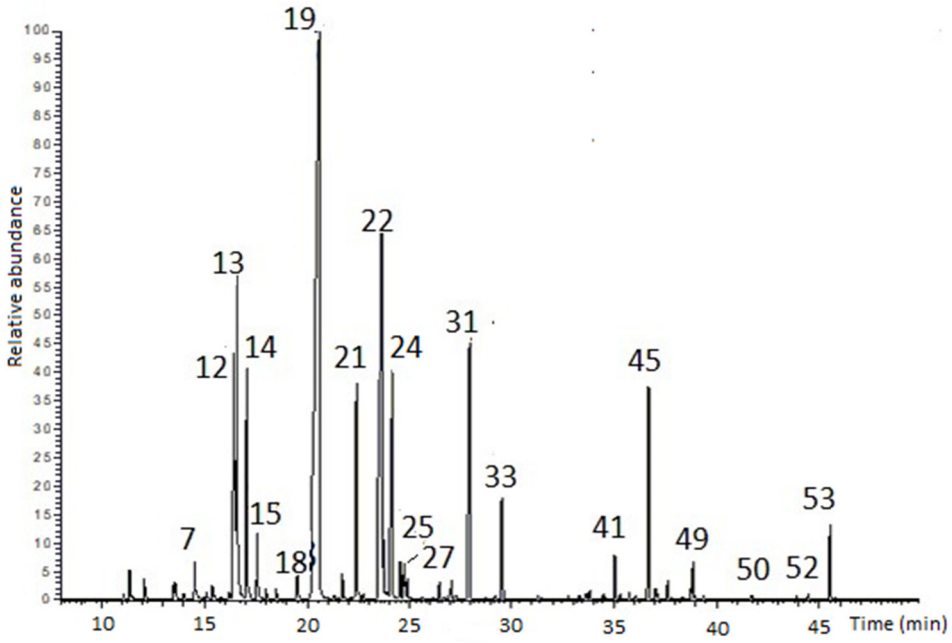


**Figure 1.** The GC-MS TIC chromatogram of LEO. In the graph, each main component of the oil was labeled with a number (N) based on its elution order, as reported in Table S1.

**Table 1.** The main chemical compounds of LEO are listed in descending order by area percentage > 1%. These components were matched to their respective peaks (N) in the chromatogram in Figure 1.

| N | Compounds | Exp RI | Ref RI | Area % ± SD | Abbr. |
|---|---|---|---|---|---|
| 19 | Linalool | 1107 | 1096 | 33.99± 0.23 | AMO |
| 22 | Borneol | 1171 | 1169 | 13.21 ± 0.10 | BMO |
| 13 | 1,8-cineole | 1032 | 1031 | 6.29 ± 0.31 | BMO |
| 12 | Limonene | 1029 | 1029 | 6.12 ± 0.10 | MM |
| 24 | Terpinen-4-ol | 1181 | 1177 | 5.24 ± 0.06 | MMO |
| 31 | Linalyl acetate | 1262 | 1257 | 5.04 ± 0.07 | AMO |
| 21 | Camphor | 1148 | 1146 | 4.36 ± 0.09 | BMO |
| 45 | (E)-β Farnesene | 1460 | 1456 | 4.12 ± 0.17 | AS |
| 14 | cis-ocimene | 1042 | 1037 | 3.59 ± 0.45 | AM |
| 33 | Lavandulyl acetate | 1295 | 1290 | 1.72 ± 0.03 | AMO |
| 53 | α-bisabolol | 1688 | 1685 | 1.64 ± 0.20 | MSO |
| 15 | trans-ocimene | 1052 | 1050 | 1.21 ± 0.01 | AM |
| 20 | allo-Ocimene | 1133 | 1132 | 1.10 ± 0.54 | AM |
| 23 | Lavandulol | 1173 | 1169 | 1.10 ± 0.04 | AMO |

Abbreviations: AM: aliphatic monoterpenes; MM: monocyclic monoterpenes; AMO: aliphatic monoterpenoids; MMO: monocyclic monoterpenoids; BMO: bi- and tricyclic monoterpenoids; AS: aliphatic sesquiterpenes; MSO: monocyclic sesquiterpenoids.

### *2.2. LEO Triggers the Dose-Dependent Inhibition of U87MG Cell Proliferation*

In order to investigate the biological effects of LEO on the U87 GBM cell line, we first evaluated its impact on cell proliferation. Specifically, LEO was administered at concentrations of $5 \times 10^{-4}$, $5 \times 10^{-3}$, and $5 \times 10^{-2}$% in culture medium ($v/v$), and the counting of living cells was performed at 24, 48, and 72 h of treatment. We observed a dose-dependent reduction in cell proliferation, which was statistically significant after 48 h and more evident after 72 h, at all doses used (Figure 2A). In detail, 50% inhibition of cell proliferation was observed when cells were grown in the presence of $5 \times 10^{-3}$% ($v/v$) LEO for 72 h when compared to control cells treated with the vehicle DMSO (Ctrl) (Figure 2A). Based on the results obtained with the different LEO doses tested, we selected the $5 \times 10^{-3}$% ($v/v$) concentration for the subsequent experiments as it proved to be the minimum effective dose in impacting cell proliferation. We also tested the impact of

LEO on U87MG treated with TMZ, the first-line chemotherapeutic agent employed for GBM. As shown in Figure 2B, TMZ began to inhibit cell proliferation at 24 h, and the co-administration of LEO and TMZ resulted in a significant reduction in cell proliferation, by over 50% compared to the control after 72 h, thus indicating that LEO enhances the antiproliferative property of TMZ. Consistent with the inhibitory effect on cell proliferation, we also observed an alteration in the expression level of some proteins involved in cell cycle regulation upon LEO treatment. Specifically, the expression of p21, a key cyclin-dependent kinase inhibitor, increased, starting from 24 h of treatment with $5 \times 10^{-3}$% ($v/v$) LEO (Figure 2C). Additionally, the expression of cyclin D1, a crucial protein in regulating cell cycle progression, significantly increased from 24 h after treatment with $5 \times 10^{-3}$% ($v/v$) LEO, thus suggesting an alteration of the cell cycle machinery induced by LEO (Figure 2C).

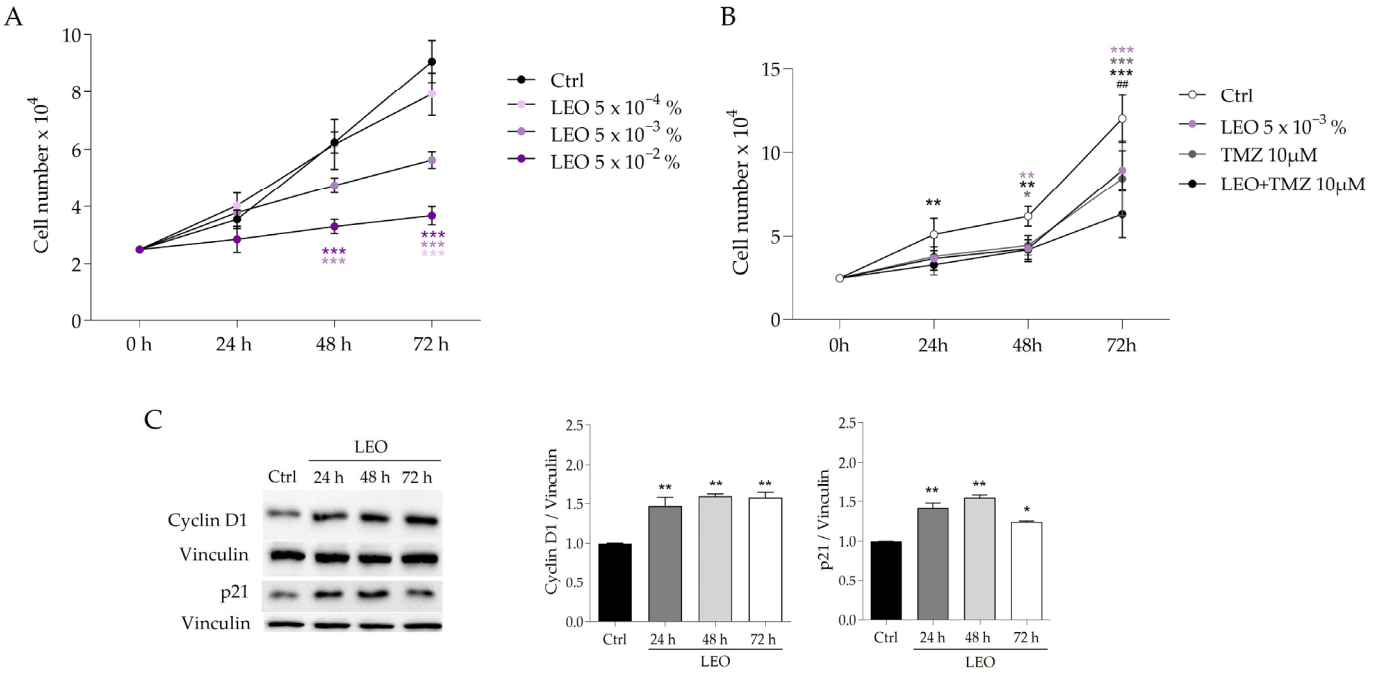


**Figure 2.** LEO administration reduces the proliferation of U87MG cells. (**A**) U87 cells were cultured in complete DMEM with DMSO as vehicle (Ctrl) or treated with of $5 \times 10^{-4}$, $5 \times 10^{-3}$, and $5 \times 10^{-2}$% ($v/v$) of LEO for 24, 48, and 72 h. At the specified time points, the cells were trypsinized and counted in a Blutzählkammer THOMA chamber, and growth curves were plotted. *** $p < 0.001$. (**B**) U87MG cells were cultured in DMEM and treated with $5 \times 10^{-3}$% ($v/v$) LEO, 10 μM TMZ and LEO + TMZ for 24, 48, and 72 h. Cells were then trypsinized and counted as in (**A**). Data are presented as means ± SD from three independent experiments. Statistical analysis was performed using the two-way ANOVA and Bonferroni's post hoc test. Asterisk indicates statistical difference vs. Ctrl group; ## indicates statistical difference vs. TMZ group, $p < 0.01$; * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$. (**C**) Western blot analysis of p21 and Cyc D1 proteins in U87MG cells treated with $5 \times 10^{-3}$% ($v/v$) for 24, 48, and 72 h. Vinculin was used as loading control. Data are presented as means ± SD from three independent experiments. Statistical significance is assessed with one-way ANOVA test, followed by Tukey's post hoc and indicated vs. Ctrl as follows: * $p < 0.05$; ** $p < 0.01$.

### *2.3. LEO Treatment Impairs Migratory Abilities of U87MG Cells*

The migration ability of cancer cells is a key indicator of their degree of malignancy and is often targeted to contrast cancer aggressiveness or to evaluate anticancer drug efficacy. The ability of LEO to affect the migration of U87MG cells was evaluated by Transwell assays. We observed that a 24 h treatment with LEO markedly diminished the migratory capacity of U87MG cells, as shown in Figure 3. In detail, the presence of $5 \times 10^{-3}$% ($v/v$) of LEO led to a significant reduction of the chemotactic response of GBM cells to fetal bovine serum (FBS) stimulus. The impairment of cell chemotaxis highlights the potential

inhibitory effect of LEO on the motility of GBM cells, suggesting its interference with cellular mechanisms essential for tumor spreading.

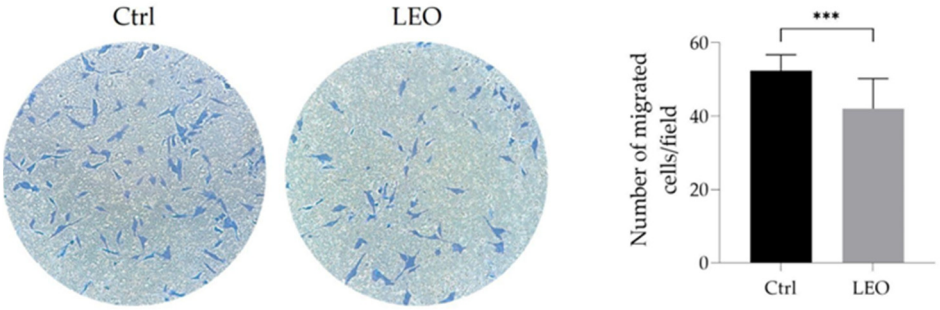


**Figure 3.** LEO treatment impairs U87MG cell migration. Representative images (20x magnification) of Transwell migration assay of Ctrl and $5 \times 10^{-3}$% ($v/v$) LEO-treated U87MG cells and quantitative analysis of the relative number of migrating cells/field. Data are presented as means ± SD from three different experiments. Statistical analysis was performed using the unpaired Student's *t*-test. *** $p < 0.001$.

### *2.4. LEO Reduces Oxidative Damage in U87MG Cells*

A plethora of findings highlighted that cancer cells exhibit high basal levels of ROS due to the deregulation of redox metabolism, which results in the generation of oxidized derivatives of biological macromolecules [39]. Immunofluorescence experiments confirmed the presence of oxidized derivatives in our cellular model; in detail, a high expression of both 8-OH(d)G, a marker of oxidative damage to nucleic acids, and 4-HNE, a marker of lipid peroxidation, was observed in U87MG cells. Notably, we observed a strong reduction in both markers in LEO-treated cells, suggesting that LEO can exert antioxidant properties in GBM cells (Figure 4).

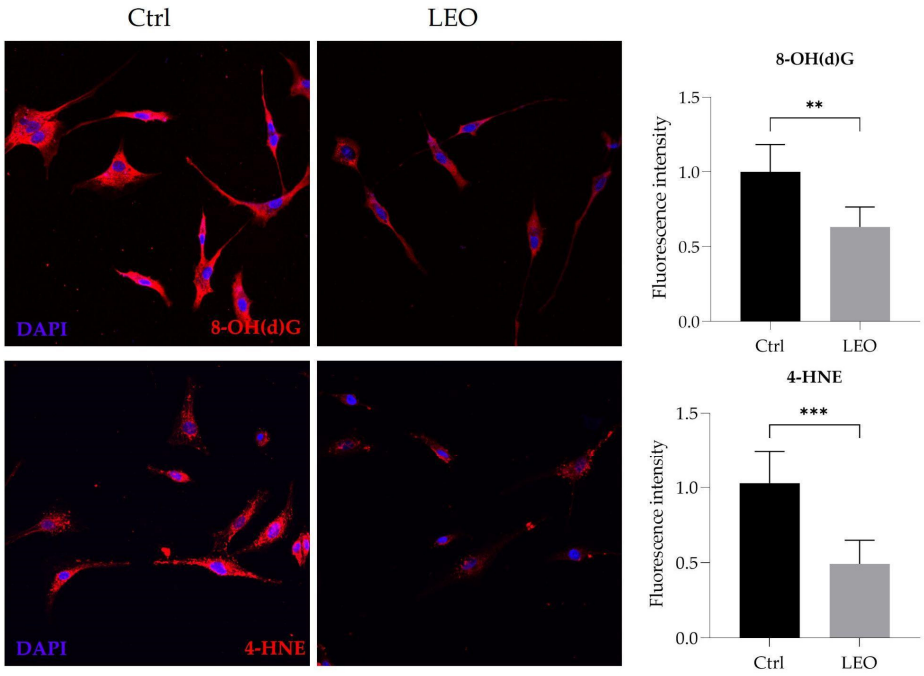


**Figure 4.** LEO reduces oxidative damage in GBM cells. U87MG cells were cultured in DMEM with DMSO (Ctrl) or treated with $5 \times 10^{-3}$% ($v/v$) LEO for 24 h. Representative immunocytochemistry images and respective signal quantification in fixed U87MG cells illustrate the fluorescence intensity of 8-OH(d)G (red) and 4-HNE (red). Nuclei were counterstained with DAPI (blue). Data represent media ± SD. Statistical significance was assessed using the unpaired Student's *t*-test. ** $p < 0.01$; *** $p < 0.001$.

### 2.5. Monoterpenes Present in LEO Affect GBM Cell Proliferation

The essential oil employed in this study is rich in oxygenated monoterpenes, as shown in Table 1, and monoterpenes have been reported to exhibit anticancer properties in several cancer models, including GBM [31,40,41]. To determine whether the observed growth inhibition was due to the monoterpenes and monoterpenoids enriched in LEO, we performed cell proliferation assays on U87MG cells treated with each of the terpenic molecules most represented in LEO. In detail, cells were exposed to each purified compound at the concentration as in a $5 \times 10^{-3}$% (*v*/*v*) LEO solution. Specifically, cells were treated with $1.7 \times 10^{-3}$% (*v*/*v*) linalool, $6.6 \times 10^{-4}$% (*v*/*v*) borneol, $2.6 \times 10^{-4}$% (*v*/*v*) terpinen-4-ol, $3.1 \times 10^{-4}$% (*v*/*v*) 1,8-cineole, and $3.1 \times 10^{-4}$% (*v*/*v*) limonene for up to 72 h. In Figure 5A, the chemical structure of each molecule employed is shown. Figure 5B illustrates that both terpinen-4-ol and borneol markedly reduced the proliferation of U87MG cells after 72 h, to a similar extent as LEO. Although present at the lowest concentration among the monoterpenes analyzed, terpinen-4-ol was able to significantly affect cell proliferation in our model. Therefore, it was selected for subsequent experiments in order to investigate its ability to reproduce the cellular effects obtained with LEO administration.

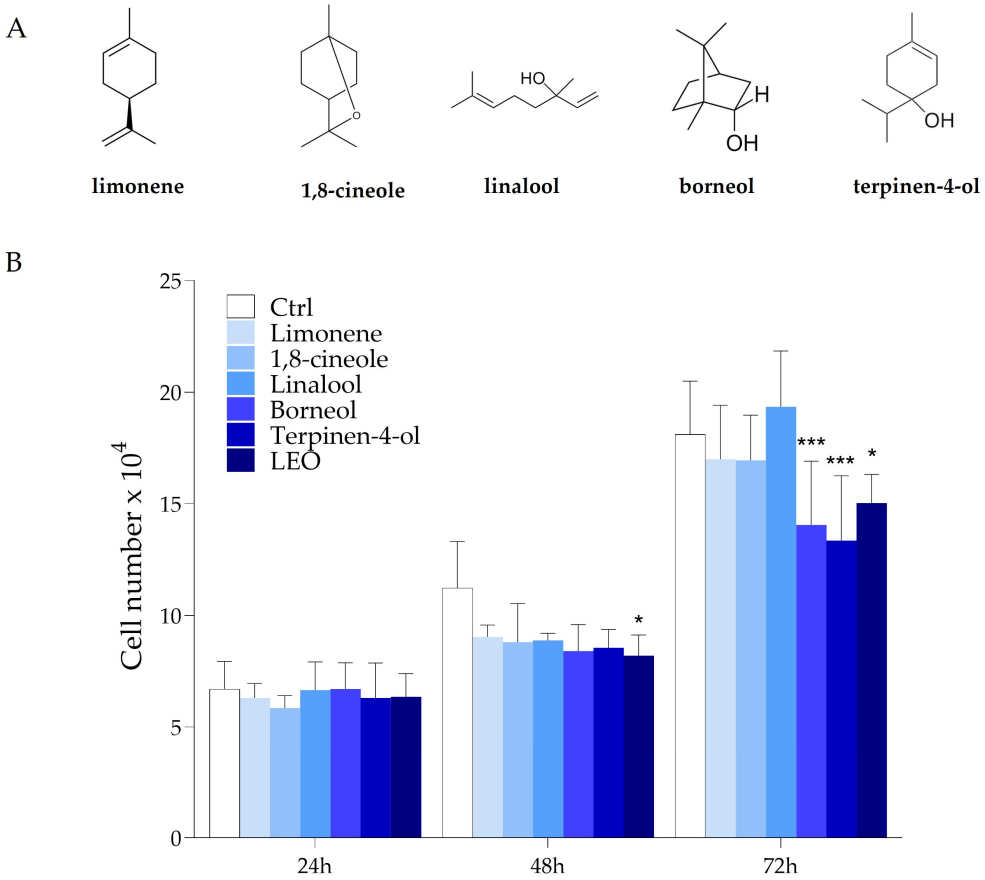


**Figure 5.** Terpenes enriched in LEO affect cell proliferation. (**A**) Chemical structures of the most abundant terpenes identified in LEO; (**B**) U87MG cells were cultured in DMEM with DMSO as vehicle (Ctrl) or incubated with $1.7 \times 10^{-3}$% (*v*/*v*) linalool, $6.6 \times 10^{-4\%}$ (*v*/*v*) borneol, $2.6 \times 10^{-4}$% (*v*/*v*) terpinen-4-ol, $3.1 \times 10^{-4}$% (*v*/*v*) limonene, or $3.1 \times 10^{-4}$% (*v*/*v*) 1,8-cineole for 24, 48, and 72 h. At these time points, the cells were trypsinized and counted, and growth curves were plotted. Data are presented as means ± SD from three independent experiments. Statistical analysis was performed using the two-way ANOVA and Bonferroni's post hoc test. Asterisk indicates statistical difference vs. Ctrl group at 24, 48, and 72 h. * $p < 0.05$; *** $p < 0.001$.

### *2.6. Terpinen-4-Ol Reproduces the Biological Effects of LEO in U87 Cells*

We decided to investigate whether terpinen-4-ol was able to reproduce the effects of LEO on GBM cells. First, we evaluated a possible additive effect of terpinen-4-ol and TMZ on cell proliferation. To this end, U87MG cells were incubated with a combination of $2.6 \times 10^{-4}$% ($v/v$) terpinen-4-ol and 10 µM TMZ for 24, 48, and 72 h. As shown in Figure 6A, the co-administration significantly enhanced the antiproliferative effect compared to TMZ alone. Indeed, the combination of terpinen-4-ol and TMZ induced a significant reduction in cell proliferation at 72 h, suggesting that this monoterpene enhances the anti-proliferative activity of TMZ. We also analyzed the expression levels of key p21 and cyclinD1 proteins and we found that terpinen-4-ol induces the upregulation of the two cell cycle players, as observed in LEO-treated cells (Figure 6B).

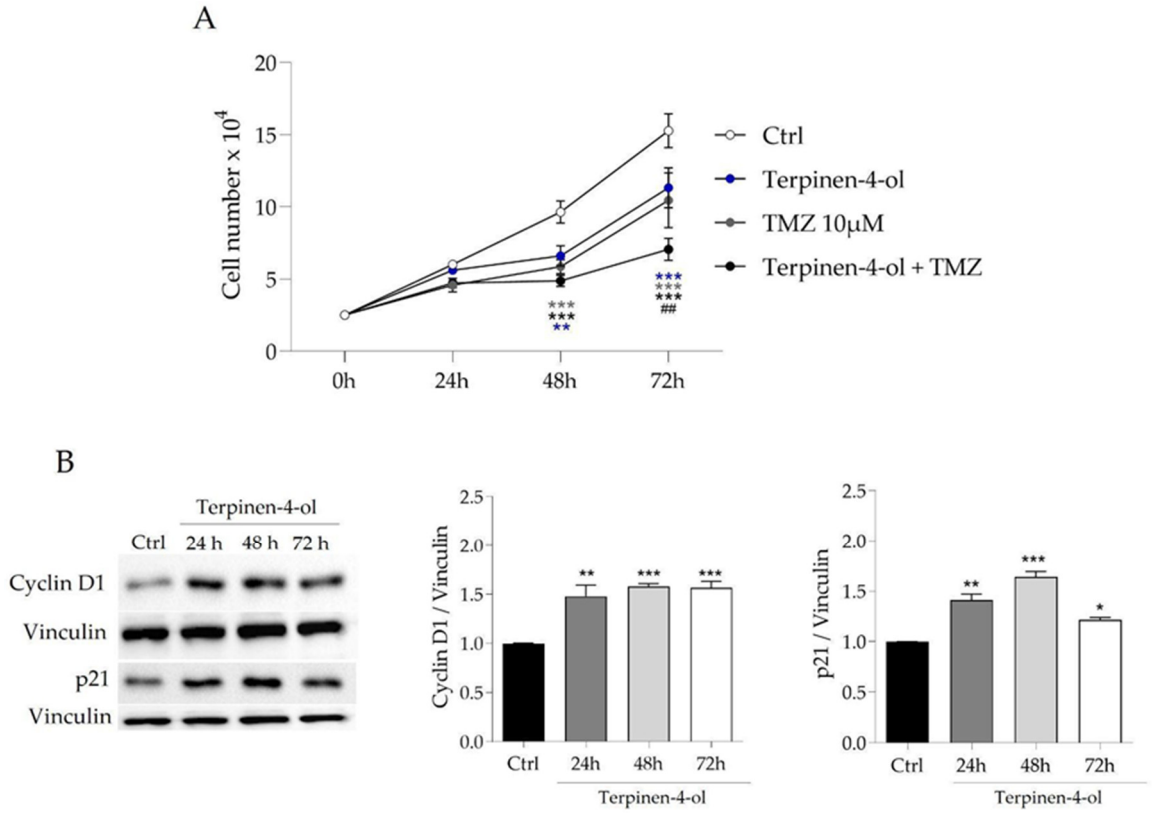


**Figure 6.** Terpinen-4-ol synergizes with TMZ by upregulating p21 and Cyclin D1. (**A**) U87MG cells were cultured in DMEM with DMSO as vehicle (Ctrl) or treated with $2.6 \times 10^{-4}$% ($v/v$) terpinen-4-ol, or 10 µM TMZ and terpinen-4-ol + TMZ for 24, 48, and 72 h. After trypsinization, cells were counted as described and growth curves plotted. Data are shown as means ± SD of three independent experiments. Statistical analysis was performed using the two-way ANOVA and Bonferroni's post hoc test. Asterisk indicates statistical difference vs. Ctrl group; ## indicates statistical difference vs. TMZ group, $p < 0.01$; ** $p < 0.01$; *** $p < 0.001$. (**B**) Representative western blot and densitometric analysis of p21 and Cyc D1 proteins in U87MG cells cultured in DMEM with DMSO (Ctrl) or treated with $2.6 \times 10^{-4\%}$ ($v/v$) terpinen-4-ol for 24, 48, and 72 h. Vinculin was used as loading control. Data are presented as means ± SD of three independent experiments. Statistical significance is assessed with one-way ANOVA test, followed by Tukey's post hoc and indicated vs. Ctrl as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Cell migration was then examined, and, as shown in Figure 7A, a reduction in migrated cells was observed after 24 h of treatment using a Transwell assay. Moreover, we observed a marked reduction in the fluorescence intensity of the oxidative stress markers 8-OH(d)G and 4-HNE. This reduction was observed 24 h post-treatment with terpinen-4-ol, analogous

to what was previously seen with LEO (Figure 7B). The decreased fluorescence intensity can suggest a reduction in cellular oxidative stress, underscoring the potential of terpinen-4-ol as an antioxidant molecule.

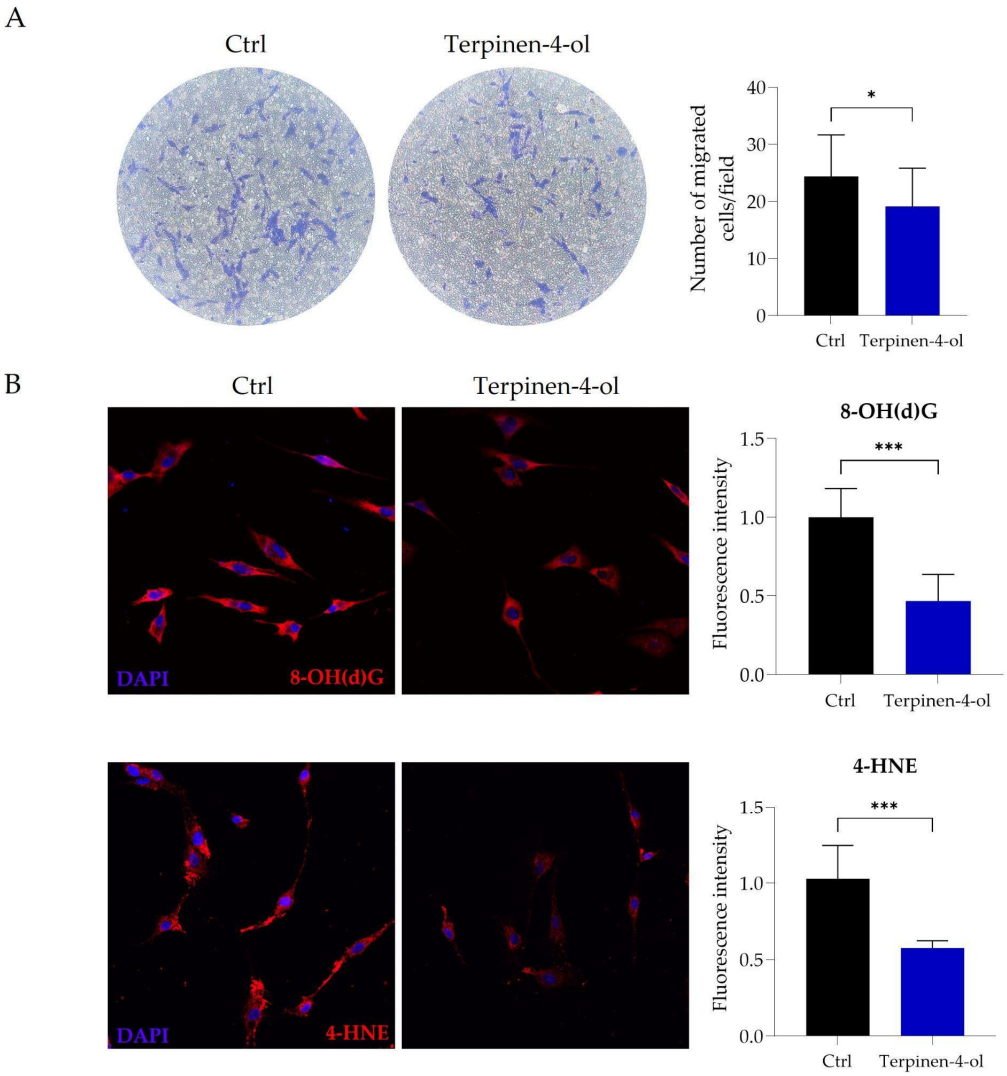


**Figure 7.** Terpinen-4-ol reproduces the effects of LEO in U87 cells. (**A**) Representative images (20× magnification) of Transwell migration assay of Ctrl and 2.6 × $10^{-4}$% ($v/v$) terpinen-4-ol-treated U87MG cells and quantitative analysis of the number of migrating cells per field obtained through a Transwell migration assay, as previously reported. (**B**) U87MG cells were cultured in DMEM with DMSO (Ctrl) or treated with 2.6 × $10^{-4}$% ($v/v$) terpinen-4-ol for 24 h. Representative immunocytochemistry images and respective signal quantification on U87MG fixed cells of 8-OH(d)G (red) and 4-HNE (red). Nuclei were counterstained with DAPI (blue). Data represent median ± SD. Statistical significance was determined using the unpaired Student's *t*-test. * $p < 0.05$; *** $p < 0.001$.

## 3. Discussion

Current standard care protocols for GBM patients typically involve a multimodal approach following surgical resection. This usually includes chemotherapeutic agents such as TMZ in combination with radiotherapy, a regimen commonly known as the Stupp protocol [42,43]. Despite these efforts, the mean survival rate for GBM patients has seen limited improvements over the past decade, with a disheartening 5-year survival rate still below 9.8% [44]. One of the primary challenges in treating GBM is the development of resistance to these therapeutic agents, alongside the significant side effects they induce [45]. Consequently, interest in identifying possible new therapeutic strategies and evaluating the

efficacy of new active molecules is growing in the research community. Several preclinical and clinical studies have highlighted the benefits of integrating numerous phytocompounds with conventional anticancer treatments [46,47]. Essential oils are the key constituents of medicinal herbs, and their biological activities have been known since ancient times and are enormously utilized in pharmaceutical industries. It is noteworthy that essential oils possess important biological properties like antibacterial, antioxidant, antiviral, and insecticidal activities [48,49]. The low toxicity and beneficial effects have contributed to their extensive use in promoting both physical and mental well-being. Essential oils have been also proposed as potential anticancer adjuvants due to evidence demonstrating that they may prevent, inhibit, or even reverse the formation of cancerous cells [50,51]. Our research has explored the effects of essential oil extracted from *L. angustifolia* on an in vitro model of GBM. To start, we evaluated whether LEO administration may affect cell proliferation and viability. To this end, the administration of sub-lethal LEO concentrations was employed to exclude non-specific cytotoxicity phenomena [23,52]. We observed that LEO induced a dose- and time-dependent slowdown of cell proliferation, without leading to cell death phenomena or evident morphological alterations. We also found that LEO administration enhanced the antiproliferative effect exerted by the drug TMZ, thus indicating an additive effect of the two molecules. In line with the effect of LEO on slowing down cell proliferation, we observed an accumulation of the cell cycle regulators p21 and cyclin D1. p21 is a cyclin-dependent kinase (CDK) inhibitor (CKI) that effectively suppresses the activity of cyclin–CDK complexes, including CDK1, CDK2, and CDK4/6, thus regulating cell cycle progression, irrespective of cyclin abundance [53,54]. The dysregulation of cell cycle regulators after treatment with essential oils extracted from several plants such as *Citrus limettioides*, *Origanum onites*, and *Rosmarinus officinalis*, among others, has been observed in both in vitro and in vivo studies on non-small-cell lung cancer [55,56]. In addition to affecting cell proliferation, we observed that LEO led to a significant reduction in the migratory capability of GBM cells, suggesting a less aggressive tumor phenotype. Notably, the capacity of GBM cells to disseminate throughout the surrounding parenchyma is a major contributory factor in the tumor's aggressiveness and is closely associated with poor prognosis [57,58]. Furthermore, due to the well-documented role of essential oils as antioxidant agents, we also investigated the effect of LEO, if any, on the levels of oxidative stress within GBM cells. Indeed, LEO has been demonstrated to enhance glutathione levels and the activity of pivotal antioxidant enzymes including catalase, thereby preventing oxidative stress [59,60]. Similarly to other cancer models, GBM cells display elevated basal levels of ROS compared to normal cells, primarily due to an imbalance between pro-oxidant and antioxidant molecules [61,62], and growing evidence suggests that oxidative stress may represent a crucial aspect in GBM biology, as it may promote cell proliferation and tumor cell survival by activating several oncogenic signaling pathways [61]. Sustained oxidative stress has also been associated with the radio- and chemoresistance of GBM [63]. Our results show that LEO effectively reduces oxidative damage in the cellular environment. Indeed, the decreased levels of 4-HNE, a product of lipid peroxidation capable of chemically modifying proteins, and of 8-OH(d)G, generated following the chemical oxidation of cellular guanosines, suggest a protective role of LEO against oxidative stress that fosters tumor aggressiveness.

In order to discriminate the contribution of the individual molecules contained in the essential oil to the biological effects observed, we decided to incubate GBM cells with LEO-enriched terpenic components. GC-MS analysis performed on Pesche LEO has identified 53 individual components and highlighted the abundance of linalool (33.99%), borneol (13.21%), 1,8-cineole (6.29%), and terpinen-4-ol (5.24%). Terpenes, a heterogeneous group of plant-derived compounds, have been indicated as promising antitumor agents acting at several stages of tumor progression. Indeed, they can suppress early tumorigenesis by inducing cell cycle arrest, inhibiting cell differentiation, and triggering apoptosis in several cancer models [41]. In later stages of cancerogenesis, terpenes may also inhibit angiogenesis and metastasis by modulating key intracellular signaling pathways [27]. We observed

that both borneol and terpinen-4-ol were able to reproduce the effects of LEO on GBM cell proliferation. Among the LEO-enriched terpenes that have been demonstrated to be effective in the inhibition of cell proliferation, terpinen-4-ol is particularly relevant due to its notable antitumor properties and ability to induce cell cycle arrest, as evidenced in models of melanoma, lung, colorectal, pancreatic, prostate, and gastric cancers [64]. Additionally, murine xenograft models of lung tumors highlight the antiproliferative effects of terpenes, which induce G0/G1 cell cycle arrest by regulating the expression of Cdk4, cyclin D1, p21, and p27 [65–67]. In line with results obtained on GBM cells by Cao et al. [34], we observed that, despite being present at low concentrations among the terpenes analyzed, terpinen-4-ol exhibited a significant antiproliferative activity, alone or in combination with TMZ. We decided to explore the impact of terpinen-4-ol on the migration capability of cells and, as for LEO, we observed, for the first time, a significant impairment of GBM cell migration. Moreover, this terpene effectively reduced oxidative cell damage, providing protection against the increased oxidative stress associated with carcinogenesis. As terpinen-4-ol fully reproduces the effects of LEO on the oncogenic properties of GBM cells, we can speculate that it is, at least partially, responsible for the biological activity exerted by LEO. However, we cannot rule out the possibility that other monoterpenes, such as borneol, could elicit a similar biological effect to that observed with terpinen-4-ol on GBM cells. Our study highlights the potential role of monoterpenes as putative adjuvants in the management of GBM. The combination with traditional drugs could also offer the advantage of mitigating the side effects often associated with high-dose chemotherapy.

## 4. Materials and Methods

### *4.1. Plant Material and Isolation of Essential Oil*

*L. angustifolia* flowers were gathered during the balsamic period in August 2022 at the University Garden of Pesche, situated in Molise Region, Italy (41.60003° N, 14.23701° E). The botanical group of the Department of Bioscience and Territory (DiBT) conducted the plant identification, and a voucher specimen was archived in the Herbarium of the DiBT of the University of Molise. The fresh flowers (100 g) were selected, thoroughly cleaned, dried in darkness for one week, and then subjected to hydrodistillation for three hours to extract the essential oil (LEO) according to the standard procedure described in the *European Pharmacopoeia* [68]. The extract was dried over anhydrous sodium sulfate to remove traces of water and then stored in dark vials at 4 °C prior to gas chromatography–mass spectrometry (GC-MS) analysis.

### *4.2. GC-MS Analysis*

The analysis of LEO was performed using a Trace GC Ultra gas chromatography system (Thermo Fisher Scientific, Waltham, MA, USA). It was equipped with an Rtx®-5 Restek capillary column (Restek, Bellefonte, PA, USA) (30 m × 0.25 mm i.d., 0.25 µm film thickness) coupled with an ion-trap mass spectrometry (MS) detector, specifically the Polaris Q (Thermo Fisher Scientific, Waltham, MA, USA). For injection, a programmed temperature vaporizer (PTV) injector was employed in conjunction with a chromatography station Xcalibur on a PC. The ionization voltage was set at 70 eV, with a source temperature of 250 °C. Full scan acquisition in positive chemical ionization mode ranged from $m/z$ 40 up to 400 atomic mass units (a.m.u.) at a scan rate of 0.43 scans per second. The column temperature profile started at 40 °C for 5 min, and then increased gradually to 250 °C at a rate of 3 °C/min and was held isothermally for 10 min. Helium gas served as the carrier at a flow rate of 1.0 mL/min. Before injection, each sample (1 µL) was dissolved in n-hexane (1:500 n-hexane solution). The experiment was replicated three times for validation purposes.

### *4.3. Identification of Essential Oil Components*

The components were named by comparing their mass spectra fragmentation patterns with those stored in the NIST 02, Adams, and Wiley 275 mass spectral libraries [38,69,70].

Additionally, their retention indices were calculated compared to a series of n-alkane C8–C20. The average relative contents (%) of the sample components were decided from peak areas obtained in triplicate without any adjustments [71–73]. All analytical standard components employed (n-alcane C8–C20, linalool, borneol, 1,8-cineole, limonene, terpinen-4-ol, camphor, and lavender oil) were bought from Merck Life Science, Milan, Italy.

### *4.4. Cell Culture and Treatments*

The human GBM U87MG cell line was kindly provided by Prof. G. Velasco from Complutense University, Madrid, Spain. The U87 cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin/streptomycin solution, and supported at 5% $CO_2$. Eight hours after seeding, the U87 cells were treated with $5 \times 10^{-4}$, $5 \times 10^{-3}$, and $5 \times 10^{-2}$% in DMEM ($v/v$) of LEO. Based on the chemical–analytical characterization of the oil and the abundance of each compound, individual terpenes were tested at the same concentration found in LEO, specifically $1.7 \times 10^{-3}$% ($v/v$) linalool (74856, Merck Life Science, Milan, Italy); $6.6 \times 10^{-4}$% ($v/v$) borneol (420247, Merck Life Science, Milan, Italy); $2.6 \times 10^{-4}$% ($v/v$) terpinen-4-ol (86477, Merck Life Science, Milan, Italy); $3.1 \times 10^{-4}$% ($v/v$) limonene (86477, Merck Life Science, Milan, Italy); and $3.1 \times 10^{-4}$% ($v/v$) 1,8-cineole (0002-05-90, HWI pharma services GmbH, Rülzheim, Germany). To facilitate solubilization in the growth medium, the oil and individual compounds were first dissolved in FBS (at a final concentration of 10% in DMEM) before being added to the DMEM. Cells treated with the vehicle (DMSO dilution 1:1000 in cell culture medium) served as the control. For the proliferation assays, 10 μM of Temozolomide (Merck KGaA, Darmstadt, Germany) was used for the time indicated.

### *4.5. Proliferation Assays*

GBM cells were plated at a density of $30 \times 10^3$ cells per well in 24-well plates filled with DMEM supplemented with 10% FBS and then incubated at 37 °C in a 5% $CO_2$ environment. After six hours, the cells were subjected to treatment with the different concentrations of LEO, TMZ as described above, or DMSO (as a vehicle). Cell proliferation was assessed by cell counting using a Blutzählkammer THOMA chamber (Merck Life Science, Milan, Italy) at specific time intervals (0, 24, 48, and 72 h) after trypsinization. Each experiment involved a minimum of three replicates for each condition.

### *4.6. Transwell Migration Assay*

U87MG cells were detached using trypsin, then pre-incubated, in suspension, with $5 \times 10^{-3}$% ($v/v$) LEO, $2.6 \times 10^{-4}$% ($v/v$) terpinen-4-ol or DMSO in invasion medium (DMEM without glutamine supplemented with 100 IU/mL penicillin/streptomycin and 25 mM HEPES, pH 7.4) for 1 h at 37 °C. Subsequently, they were plated at a density of $14 \times 10^3$ cells per $cm^3$ onto Transwell inserts with an 8 μm pore size. A chemotactic FBS gradient was set up between the lower chamber (10% FBS) and the upper chamber (without FBS), where the cells were seeded. Following 24 h of incubation at 37 °C, the cells were fixed with ice-cold 10% trichloroacetic acid (TCA) for 10 min. Cells adhering to the upper side of the filter were removed by scraping, while those migrated through the insert were stained using a solution of 50% isopropanol, 1% formic acid, and 0.5% brilliant blue R 250 ($v/v$). Finally, the U87MG cells were counted in more than 20 fields under a light microscope (Eclipse 7s100; Nikon Europe, Amstelveen, The Netherlands) at a 20× magnification.

### *4.7. Cell Lysis and Western Blotting*

The protein extracts were prepared by lysing cells with the proper amount of RIPA buffer (50 Mm Tris HCl, pH 7.4; Triton 1%; Na Deoxycholate 0.25%; SDS 0.1%; 150 mM NaCl; 1 mM EDTA; and 5 mM $MgCl_2$) supplemented with a protease inhibitor cocktail. After incubation on ice for 20 min, the samples were centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatants were recovered, and the protein concentrations were determined

using a Lowry protein assay (Bio-Rad Laboratories, Milan, Italy). Laemmli buffer 5X (Tris-HCl 315 mM, pH 6.8; 2.5% β-mercaptoethanol; 50% glycerol; 10% sodium dodecyl sulfate; and 0.5% Bromophenol Blue) was added to the supernatants and the samples were boiled at 95 °C for 5 min. The protein extracts were separated on SDS-PAGE and then electroblotted onto nitrocellulose (GE Healthcare, Life Sciences, Little Chalfont, Buckinghamshire, UK) using a turbo Trans-blot Transfer system (Biorad Laboratories, Milan, Italy). After blocking with 5% fat-free milk powder in Tris-buffered saline and 0.1% Tween-20, the membranes were probed overnight at 4 °C with primary antibodies: anti-p21 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-6246, dilution 1:500); anti-Cyclin D1 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-954, dilution 1:500); and anti-Vinculin (Santa Cruz Biotechnology, Dallas, TX, USA, sc-73614, dilution 1:5000). Detection was obtained using horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Milan, Italy) and the protein antibody immune complexes were visualized with an ECL plus system (GE Healthcare, Life Sciences, Little Chalfont, Buckinghamshire, UK). The respective chemiluminescence signals were recorded using a ChemiDoc MP system (Bio-Rad Laboratories, Milan, Italy). Densitometric analysis was performed using Image J software (version 1.53) for Windows (National Institutes of Health, Bethesda, MD, USA).

### *4.8. Immunocytochemistry and Confocal Analysis*

U87 cells were seeded on coverslips and grown in DMEM high glucose with 10% FBS. Cells were treated with $5 \times 10^{-3}$% *(v/v)* LEO, $2.6 \times 10^{-4}$% *(v/v)* terpinen-4-ol, or DMSO for 24 h. Subsequently, the cells were fixed with paraformaldehyde (4% solution) for 10 min followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min at room temperature, and then blocked in 3% Bovine Serum Albumin (BSA) dissolved in 0.1% PBS Triton for 1 h. For 8-OHdG staining, immunofluorescence was performed with one added step of incubation with 2M HCl for 20 min at room temperature. The 8-OHdG (Santa Cruz Biotechnology, Dallas, TX, USA, sc-66036; dilution 1:100) and 4-HNE (Thermo Fisher Scientific, MA5-27570; dilution 1:100) primary antibodies was incubated overnight at 4 °C and visualized by Alexa 555 Fluor secondary antibodies (ThermoFisher Scientific, Waltham, MA, USA). After nuclear staining with DAPI (D9542, Merck Life Science, Milan, Italy), the coverslips were mounted with Fluoroshield mounting medium (F6182, Merck Life Science, Milan, Italy) and examined under a confocal microscope (TCS SP8; Leica, Wetzlar, Germany). Images were captured using Leica TCS SP8 equipped with a 40× magnification and Leica LAS X Software (version 3.5.5) (Leica Camera, Wetzlar, Germany) for Windows 10.

### *4.9. Statistical Analysis*

Each experiment was conducted a minimum of three times. Statistical analysis was performed using GraphPad Prism software, version 5.03 (GraphPad, La Jolla, CA, USA). The results are presented as means ± standard deviations (SDs). An unpaired Student's *t*-test was performed to compare the means between two experimental groups. For three-group comparisons, statistical significance was assessed using either a one-way analysis of variance (ANOVA) test followed by Tukey's post hoc test, or a two-way ANOVA followed by Bonferroni's post hoc test, as specified.

## 5. Conclusions

We have demonstrated that both LEO and its terpenic components could represent promising molecules in addressing the aggressive nature of GBM, with the potential to enhance the effectiveness of TMZ therapy. These natural compounds target key pathological properties of GBM cells, such as proliferation, migration, and oxidative stress, which promote tumor growth and metastasis. The ability of many terpenes to penetrate the blood–brain barrier makes them valuable candidates for GBM treatment, potentially allowing for lower, less toxic chemotherapy doses. By mitigating oxidative damage and protecting healthy cells, LEO and terpinen-4-ol may offer a comprehensive therapeutic approach

combining natural and conventional therapies. This strategy could lead to more effective treatments, improved clinical outcomes, and better survival rates for GBM patients.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/molecules29246044/s1: Table S1: LEO chemical composition reported in GC-MS elution order; Table S2: The components of LEO organized into chemical groups.

**Author Contributions:** Conceptualization, S.D.B. and A.A.; methodology and investigation, M.R., N.M., G.S., C.M., F.F., D.G., V.R. and M.A.O.; data curation, M.R., N.M. and D.G.; software, M.R. and N.M.; resources, G.S., S.D.B. and A.A.; writing—preparation of the original draft, M.R. and N.M.; writing—proofreading and editing, S.D.B., G.S., M.S., A.A. and D.G.; visualization, D.G., V.R. and M.A.O.; supervision, S.D.B., G.S. and A.A.; project administration, S.D.B. and G.S.; acquisition of funding, G.S. All authors have read and agreed to the published version of the manuscript.

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# *Supplementary Materials*

# Supplementary material of

# Lavender Essential Oil and Its Terpenic Components Negatively Affect Tumor Properties in a Cell Model of Glioblastoma

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**Table S1.** LEO chemical composition reported in GC-MS elution order.

| N | Compounds | Exp RI | Ref RI | Area % ± SD | Abbr. |
|---|---|---|---|---|---|
| 1 | α-Thujene | 927 | 930 | 0.12 ± 0.00 | BM |
| 2 | α-Pinene | 933 | 939 | 0.60 ± 0.03 | BM |
| 3 | Camphene | 948 | 954 | 0.44 ± 0.02 | BM |
| 4 | Sabinene | 974 | 975 | 0.01 ± 0.01 | BM |
| 5 | β-Pinene | 976 | 979 | 0.24 ± 0.01 | BM |
| 6 | 1-Octen-3-ol | 983 | 979 | 0.13 ± 0.01 | OT |
| 7 | Myrcene | 992 | 990 | 0.78 ± 0.02 | AM |
| 8 | α-Phellandrene | 1000 | 1002 | 0.11 ± 0.00 | MM |
| 9 | 3-Carene | 1007 | 1011 | 0.28 ± 0.01 | BM |
| 10 | α-Terpinen | 1015 | 1017 | 0.05 ± 0.00 | MM |
| 11 | *p*-Cymene | 1024 | 1024 | 0.11 ± 0.00 | MM |
| 12 | Limonene | 1029 | 1029 | 6.12 ± 0.10 | MM |
| 13 | 1,8-Cineole | 1032 | 1031 | 6.29 ± 0.31 | BMO |
| 14 | *cis*-Ocimene | 1042 | 1037 | 3.59 ± 0.45 | AM |
| 15 | *trans*-Ocimene | 1052 | 1050 | 1.21 ± 0.01 | AM |
| 16 | γ-Terpinene | 1061 | 1059 | 0.21 ± 0.01 | MM |
| 17 | *cis*-Sabinene Hydrate | 1070 | 1070 | 0.28 ± 0.01 | BMO |
| 18 | Terpinolene | 1089 | 1088 | 0.52 ± 0.01 | MM |
| 19 | Linalool | 1107 | 1096 | 33.99 ± 0.23 | AMO |
| 20 | *allo*-Ocimene | 1133 | 1132 | 1.10 ± 0.54 | AM |
| 21 | Camphor | 1148 | 1146 | 4.36 ± 0.09 | BMO |
| 22 | Borneol | 1171 | 1169 | 13.21 ± 0.10 | BMO |
| 23 | Lavandulol | 1173 | 1169 | 1.10 ± 0.04 | AMO |
| 24 | Terpinen-4-ol | 1181 | 1177 | 5.24 ± 0.06 | MMO |
| 25 | Cryptone | 1188 | 1185 | 0.59 ± 0.01 | MMO |
| 26 | α-Terpineol | 1189 | 1188 | 0.59 ± 0.01 | MMO |
| 27 | Exil butanoate | 1193 | 1192 | 0.26 ± 0.01 | OT |
| 28 | Isobornyl formate | 1230 | 1239 | 0.28 ± 0.01 | OT |
| 29 | Cumin aldehyde | 1243 | 1241 | 0.33 ± 0.03 | MMO |
| 30 | Hexyl isovalerate | 1247 | 1244 | 0.12 ± 0.01 | OT |
| 31 | Linalyl acetate | 1262 | 1257 | 5.04 ± 0.07 | AMO |
| 32 | Bornyl acetate | 1288 | 1288 | 0.04 ± 0.01 | BMO |
| 33 | Lavandulyl acetate | 1295 | 1290 | 1.72 ± 0.03 | AMO |
| 34 | Hexyl tiglate | 1334 | 1332 | 0.08 ± 0.00 | OT |
| 35 | Neryl acetate | 1369 | 1361 | 0.08 ± 0.01 | AMO |
| 36 | *trans*-Myrtanol acetate | 1387 | 1386 | 0.11 ± 0.01 | MMO |
| 37 | Hexyl hexanoate | 1389 | 1383 | 0.08 ± 0.01 | OT |
| 38 | Sesquithujene | 1391 | 1391 | 0.16 ± 0.00 | BS |
| 39 | *7-epi*-Sesquithujene | 1405 | 1405 | 0.11 ± 0.01 | BS |
| 40 | Longifolene | 1408 | 1407 | 0.03 ± 0.01 | BS |
| 41 | *(E)* Caryophyllene | 1419 | 1419 | 0.92 ± 0.03 | BS |
| 42 | Linalool butanoate | 1425 | 1423 | 0.09 ± 0.01 | AMO |
| 43 | *trans*-α–Bergamotene | 1436 | 1434 | 0.12 ± 0.01 | MS |
| 44 | Aromadendrene | 1444 | 1441 | 0.06 ± 0.01 | BS |
| 45 | *(E)*-β-Farnesene | 1460 | 1456 | 4.12 ± 0.17 | AS |

| | | | | | |
|---|---|---|---|---|---|
| 46 | Linalool isovalerate | 1468 | 1468 | 0.18 ± 0.01 | ASO |
| 47 | γ-Muurolene | 1482 | 1479 | 0.32 ± 0.02 | BS |
| 48 | *(Z)*-α-Bisabolene | 1510 | 1507 | 0.20 ± 0.02 | MS |
| 49 | Lavandulyl isovalerate | 1513 | 1509 | 0.68 ± 0.04 | ASO |
| 50 | Caryophyllene oxide | 1586 | 1583 | 0.10 ± 0.01 | BSO |
| 51 | α-Muurolol | 1644 | 1646 | 0.09 ± 0.01 | BSO |
| 52 | *B*-Bisabolol oxide | 1659 | 1658 | 0.12 ± 0.02 | BSO |
| 53 | α-Bisabolol | 1688 | 1685 | 1.64 ± 0.20 | MSO |

Abbreviations: AM: aliphatic monoterpenes; MM: monocyclic monoterpenes; BM: bi- and tricyclic monoterpenes; AMO: aliphatic monoterpenoids; MMO: monocyclic monoterpenoids; BMO: bi- and tricyclic monoterpenoids; AS: aliphatic sesquiterpenes; MS: monocyclic sesquiterpenes; BS: bi- and tricyclic sesquiterpenes; ASO: aliphatic sesquiterpenoids; MSO: monocyclic sesquiterpenoids; BSO: bi- and tricyclic sesquiterpenoids, OT: others.

**Table S2.** The components of LEO organized into chemical groups.

| **Chemical group** | **Abbreviation** | **Area %** *L. angustifolia* |
|---|---|---|
| Aliphatic monoterpenes | AM | 6.68 |
| Monocyclic monoterpenes | MM | 7.12 |
| Bi- and Tricyclic monoterpenes | BM | 1.69 |
| **Monoterpenes** | **M** | **15.49** |
| Aliphatic monoterpenoids | AMO | 42.02 |
| Monocyclic monoterpenoids | MMO | 6.86 |
| Bi- and Tricyclic sesquiterpenes | BMO | 24.18 |
| **Monoterpenoids** | **MO** | **73.06** |
| Aliphatic sesquiterpenes | AS | 4.12 |
| Monocyclic sesquiterpenes | MS | 0.32 |
| Bi- and Tricyclic sesquiterpenes | BS | 1.60 |
| **Sesquiterpenes** | **S** | **6.04** |
| Aliphatic sesquiterpenoids | ASO | 0.98 |
| Monocyclic sesquiterpenoids | MSO | 1.64 |
| Bi-and Tricyclic sesquiterpenoids | BSO | 0.19 |
| **Sesquiterpenoids** | **SO** | **2.81** |
| **Others** | **OT** | **0.95** |

Abbreviations: AM: aliphatic monoterpenes; MM: monocyclic monoterpenes; BM: bi- and tricyclic monoterpenes; AMO: aliphatic monoterpenoids; MMO: monocyclic monoterpenoids; BMO: bi- and tricyclic monoterpenoids; AS: aliphatic sesquiterpenes; MS: monocyclic sesquiterpenes; BS: bi- and tricyclic sesquiterpenes; ASO: aliphatic sesquiterpenoids; MSO: monocyclic sesquiterpenoids; BSO: bi- and tricyclic sesquiterpenoids, OT: others.

# *Conclusion*

# Conclusion

The search for clear treatment for glioblastoma multiforme (GBM) continues to face major difficulties, despite extensive research efforts. The main issue is the natural diversity of GBM, which has caused many clinical trials aimed at finding effective therapies to fail. While there are various integrative or alternative treatments and efforts to find new uses for existing drugs, the Stupp protocol, which combines surgery with radiotherapy and chemotherapy using temozolomide (TMZ), remains the established standard of care. Nonetheless, this method is often weakened by the common development of both natural and acquired resistance to TMZ, leading to a chemoresistant form and a group of glioma stem cells, which limits treatment effectiveness and results in poor outcomes for GBM patients. Recent research, however, has uncovered ways to enhance patient results by mixing traditional methods with natural substances, including those obtained from plants. Essential oils, in general, have demonstrated many new health-related uses that are good for human well-being, such as anti-inflammatory, pain-relieving, numbing, and anticancer effects. Among these, lavender essential oil (LEO) taken from *L. angustifolia* flowers has improved the lives of colon cancer patients and triggered cell death in breast cancer studies. There were no major negative effects or issues observed during LEO use in cell models. It is noted that LEO is safe for cell culture and depends on the doses of LEO used, and it may be utilized for drug and biomedical treatments in potential future uses in regenerative medicine. Although beneficial effects on preventing cancer growth have been seen *in vitro* and *in vivo* xenograft models of cancers like prostate, small lung, colon, and breast cancer cells, we have shown for the first time how LEO affects important cancer features of GBM in a controlled cell setting (U87MG, a human glioblastoma cell line frequently used in brain cancer research).

This thesis explores the impact of LEO and terpinene-4-ol on the GBM by examining various critical aspects of tumorigenesis. In our experimental plan, the administration of sub-lethal LEO concentrations was employed to exclude non-specific cytotoxicity phenomena.

The *manuscript I* highlights the significant impacts of LEO on important traits of GBM cells. After treatment with LEO alone or with TMZ, a notable reduction in GBM cell growth was seen without causing cell death or clear changes in cell shape, along with an unusual build-up of cell cycle regulators cyclin D1 and p21. P21 is a cyclin-dependent kinase (CDK) inhibitor (CKI) that effectively hinders the function of cyclin–CDK pairs, such as CDK1, CDK2, and CDK4/6, thus managing cells' cycle progress, regardless of cyclin levels. In addition, LEO treatment led to a significant decline in the migration abilities of U87MG cells, indicating a less aggressive tumor type. Given the well-known role of essential oils as antioxidant substances, we also explored the impact of LEO, if any, on oxidative stress levels in GBM cells. The lower amounts of 4-HNE, a byproduct of lipid peroxidation that can chemically alter proteins, and of 8-OH(d)G, produced from the chemical oxidation of cellular guanosines, indicate a protective function of LEO against oxidative stress that promotes tumor aggressiveness. To determine the function of the molecules found in the essential oil, which have been identified and confirmed using gas chromatography/mass spectrometry analysis, we chose to treat GBM cells with the most common terpene components in LEO, whose helpful qualities are well known. Both borneol and terpinen-4-ol could mimic the effects of LEO on GBM cell growth. We noted that, even though terpinen-4-ol was present in low amounts among the terpenes examined, it showed a notable ability to prevent cell growth, either on its own or together with TMZ. We chose to investigate how terpinen-4-ol affects the migration ability of cells and, regarding LEO, we noticed, for the first time, a notable decrease in GBM cell movement. Additionally, this terpene successfully lowered cell damage caused by oxidation, offering protection against the heightened oxidative stress linked to cancer development. As terpinen-4-ol completely mimics the effects of LEO on the cancer-causing properties of GBM cells, we can suggest that it is, at least partly, responsible for the biological effects produced by LEO. However, we cannot dismiss the chance that other monoterpenes, like borneol, could produce a similar biological effect as seen with terpinen-4-ol on GBM cells. Our research points out the possible role of monoterpenes as potential adjuvants in the treatment of

GBM. The mix with standard medications might also provide the benefit of reducing the side effects often linked to high-dose chemotherapy. Although the abilities of LEO to slow down cancer cell growth in various types of cancer are recognized, the uniqueness of our research project is that we have assessed for the first time how the LEO affects a cell model of brain tumors, where standard treatments have not given a good outcome. Our findings motivate us to focus on the use of LEO, not as a replacement but as an additional treatment that could enhance the success of existing chemotherapies used for GBM. Additional tests, both *in vitro* and *in vivo*, will help us gain a clearer understanding of how and in what way a natural substance from plants can influence tumor growth. It will be intriguing to examine the impact of LEO on healthy cells, a discovery that could back the potential use of LEO in patients.

# List of publications

This thesis is based on the work contained in the following paper:

## Manuscript I.

Russo, M.; Martella, N.; Gargano, D.; Fantasma, F.; Marcovecchio, C.; Russo, V.; Oliva, M.A.; Segatto, M.; Saviano, G.; Di Bartolomeo, S.; et al. *Lavender Essential Oil and Its Terpenic Components Negatively Affect Tumor Properties in a Cell Model of Glioblastoma*. Molecules **2024**, *29*, 6044. https://doi.org/10.3390/molecules29246044

# Other contributions

The list includes author's contributions not related to the thesis work.

## II.

Staffieri, S.; Russo, V.; Oliva, M. A.; Alborghetti, M.; Russo, M.; Arcella, A. *Aloe-Emodin Overcomes Anti-Cancer Drug Resistance to Temozolomide and Prevents Colony Formation and Migration in Primary Human Glioblastoma Cell Lines NULU and ZAR*. Molecules (Basel, Switzerland) **2023**, 28(16), 6024. https://doi.org/10.3390/molecules28166024

## III.

Martella, N.; Colardo, M.; Sergio, W.; Petraroia, M.; Varone, M.; Pensabene, D.; Russo, M.; Di Bartolomeo, S.; Ranalli, G.; Saviano, G.; Segatto, M. *Lavender Essential Oil Modulates Hepatic Cholesterol Metabolism in HepG2 Cells.* Curr Issues Mol Biol **2023**, 45(1):364-378. doi: 10.3390/cimb45010026.

## IV.

Colardo, M.; Gargano, D.; Russo, M.; Petraroia, M.; Pensabene, D.; D'Alessandro, G.; Santoro, A.; Limatola, C.; Segatto, M.; Di Bartolomeo, S. *Bromodomain and Extraterminal Domain (BET) Protein Inhibition Hinders Glioblastoma Progression by Inducing Autophagy-Dependent Differentiation*. Int J Mol Sci **2023**, 24(8):7017. doi: 10.3390/ijms24087017.

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*I hereby declare that this thesis, submitted as partial fulfilment to obtain the academic degree of Doctor of Philosophy (Ph.D.) in Biology and Applied Science, is my own unaided work. I have not used sources other than those indicated, and all direct and indirect sources are acknowledged as references. Parts of this dissertation have been published in international journals and/or conference articles.*

*Date* *Signature*

*06/12/2024*