Review Article

Glioblastoma multiforme: molecular biology and updated systematic review on natural extract and phytochemical efficacy *in-vitro*

Abstract

Glioblastoma multiforme (GBM) is the most prevalent primary malignant central nervous system tumours (CNST). Current treatment consists of surgical resection and adjuvant chemoradiotherapy, the gold-standard agent being Temozolomide (TMZ). Despite optimal treatment, GBM has an abysmal associated prognosis of approximately 15 months. The disproportionately high morbidity and mortality necessitate studies which strive to increase knowledge on putative therapeutic agents to confirm or deny their possible use against GBM Therefore, there is an urgent need to develop a deep understanding of GBM molecular biology upon which more effective therapeutic strategies may be developed.

Natural extracts and phytochemicals have shown promise as potential therapeutic agents against GBM. In this review, the molecular biology of GBM and molecular factors which contribute to therapeutic resistance are discussed in depth. The mechanisms by which phytochemicals and natural extracts exhibit can inhibit GBM growth and proliferation are discussed. Moreover, statistical analysis demonstrated that natural extracts and phytochemicals may be superior to TMZ in-vitro.

This review demonstrates that phytochemicals have great potential as novel therapeutic agents against GBM. Further preclinical and clinical studies are needed to determine the optimal doses, routes of administration, and potential toxicities of these agents, but their unique molecular targets and low toxicity profiles make them attractive candidates for further investigation.

Keywords

Glioblastoma Multiforme, molecular biology, intracellular signalling, natural extracts, phytochemicals

Introduction

Gliomas are among the most prevalent central nervous system tumours (CNSTs) and are classified according to their presumed cell of origin. These primarily include tumours stemming from astrocytes (astrocytoma, anaplastic astrocytoma and glioblastoma), oligodendrogliomas, mixed gliomas and ependymomas (1). The most incident and aggressive glioma is glioblastoma multiforme (GBM), which can be distinguished from other gliomas since GBM tumours express necrosis and/or microvascular proliferation coupled with rapid, infiltrating growth. GBM forms highly heterogeneous tumours, with complex molecular biology (2–5). Despite decades of research and current optimal treatment, patients' prognosis is still abysmal. Research striving to discover putative therapeutic approaches against GBM has veered towards assessing the efficacy of naturally derived compounds or extracts (6). This review aims to give an updated and concise overview of recent literature which discusses the classification, epidemiology, molecular biology, and current treatment approaches of GBM.

Method

Relevant literature published in the past 10 years describing the molecular biology of GBM was reviewed. In addition, a systematic literature search of articles assessing the efficacy of natural extracts and phytochemicals against GBM *in-vitro* was conducted according to Systematic Review and Meta-analyses (PRISMA) checklist 2020 (1).

Systematic study identification and selection

PubMed, Google Scholar, Science Direct and Semantic Scholar databases were searched for relevant articles published until week 36-2022. Combinations of free text keywords and MESH terms (were appropriate), identified through a preliminary literature search, were used to develop a database-specific search strategy as outlined in **S-Table1.** Grey literature was not explored, since only articles published in reputable peer-reviewed journals were considered. Original studies which discussed the anti-cancer properties and detailed the IC50 of extracts derived from natural sources against glioblastoma were retrieved from the respective database and stored digitally in dedicated files.

S-Table 1: Database-specific search strategy.

PubMed	Google Scholar	Science Direct	Semantic Scholar
(((phytochemical[MeSH Terms]) OR (phytochemical)) OR ((natural compound[MeSH Terms]) OR (natural compound)) OR ((extract[MeSH Terms])) OR (extract))) AND ((glioblastoma[MeSH Terms]) OR (glioblastoma multiforme) OR (glioblastoma multiforme[MeSH Terms]))	allintitle: "glioblastoma" OR "glioblastoma multiforme" AND "phytochemical" OR "natural compound" OR "extract" biology"	Title, abstract or author-specified keywords: (“glioblastoma” OR “glioblastoma multiforme”) AND (“phytochemical” OR “natural compound” OR extract”)	Title: ("phytochemical" OR "natural compound" OR "extract") AND ("glioblastoma" OR "glioblastoma multiforme")

Data extraction and analysis for systematic review

Data regarding cell lines, IC50 and observed intracellular signalling pathways from studies identified through the systematic review were extracted using data collection tables. Descriptive and inferential statistical analysis (Mann Whitney-U testing) was conducted using JASP Team (2021) JASP (Version 0.16). The significance level was set at $p < 0.05$ for all analyses.

Systematic literature search results

874 tentative studies were identified, with 31 potential records being duplicates. 504 studies were excluded following title and abstract screening using the pre-defined inclusion criteria. Thus, 309 reports were sought for retrieval and their full text was assessed in detail for eligibility. Among these, 42 studies could not be retrieved and 231 studies were excluded following full-text review resulting in a total of 36 studies included in this systematic review (2–37) **(S-Figure 1).**

Identification of studies via databases and registers
Identification
Screening

S-Figure 1: Identification of studies via databases and registers. Prisma 2020 flow diagram for systematic literature search, adapted from Page *et al.* (2021).

Prisma Checklist

S-Table 2: Prisma Checklist for systematic searches.

INFORMATION SOURCES AND METHODS			
Database name	1	Name each individual database searched, stating the platform for each.	2

Multi-database searching	2	If databases were searched simultaneously on a single platform, state the name of the platform, listing all the databases searched.	2
Study registries	3	List any study registries searched.	N/A
Online resources and browsing	4	Describe any online or print source purposefully searched or browsed (e.g., tables of contents, print conference proceedings, web sites), and how this was done.	2
Citation searching	5	Indicate whether cited references or citing references were examined, and describe any methods used for locating cited/citing references (e.g., browsing reference lists, using a citation index, setting up email alerts for references citing included studies).	N/A
Contacts	6	Indicate whether additional studies or data were sought by contacting authors, experts, manufacturers, or others.	N/A
Other methods	7	Describe any additional information sources or search methods used.	N/A
SEARCH STRATEGIES			
Full search strategies	8	Include the search strategies for each database and information source, copied and pasted exactly as run.	2
Limits and restrictions	9	Specify that no limits were used, or describe any limits or restrictions applied to a search (e.g., date or time period, language, study design) and provide justification for their use.	2
Search filters	10	Indicate whether published search filters were used (as originally designed or modified), and if so, cite the filter(s) used.	2
Prior work	11	Indicate when search strategies from other literature reviews were adapted or reused for a substantive part or all of the search, citing the previous review(s).	N/A
Updates	12	Report the methods used to update search(es) (e/g, rerunning search, email alerts).	N/A
Dates of searches	13	For each search strategy, provide the date when the last search occured.	2
PEER REVIEW			
Peer review	14	Describe any search peer review process.	N/A
MANAGING RECORDS			
Total Records	15	Document the total number of records identified from each database and other information sources.	2-3
Deduplication	16	Describe the processes and any software used to deduplicate records from multiple database searches and other information sources.	3

Classification and epidemiology

Since the turn of the 21st century, frameworks for classifying GBM have been continuously revised to keep up with novel insights in GBM molecular biology **(Figure 1).** The World Health Organization (WHO) CNST classification framework has established itself as the international standard for glioma nomenclature and diagnosis (Lee et al., 2018). GBM is

classified as an 'adult-type diffuse glioma' in the WHO 2021 CNST classification, which must occur as a WHO grade-IV isocitrate dehydrogenase (IDH)-wildtype glioma *de-novo*. IDH-mutant WHO grade-IV gliomas, often presenting as secondary glioblastoma stemming from WHO grade II or III astrocytoma, have historically been considered as glioblastoma, since mutant IDH makes the tumours more amendable to treatment. However, such tumours have been reclassified as WHO grade IV astrocytoma in the WHO 2021 CNSTs framework (1). This reclassification invariably questions the validity of glioblastoma research which does not specify the IDH status of grade-IV gliomas being tested, while likely invalidating prior epidemiological research which does not include the IDH-status within patient demographic data. This necessitates clear guidelines concerning protocols which should be followed in pre-clinical research to standardise reporting and gain a clear understanding of the actual global epidemiological trends of GBM.

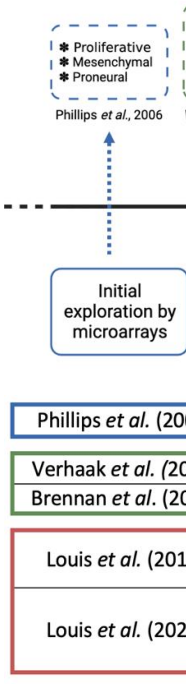

Figure 1: Glioblastoma multiforme classification systems. Advances in molecular biology have led to the development of several classification systems for glioblastoma multiforme (GBM), with initial microarray

analysis identifying three subgroups: proliferative, mesenchymal and proneural (7). High-throughput sequencing has since updated this classification, splitting the proliferative subgroup into neural and classical groups (8). This classification system was further stratified subtypes based on methylation signatures (9). The fourth World Health Organization (WHO) classification system for gliomas is based on variable isocitrate dehydrogenase (IDH) expression, identifying four neoplasms including GBM IDH-wildtype, GBM IDH-mutant, GBM not-otherwise-specified, and not-elsewhere-classified (10). In 2021, WHO updated its classification of central nervous system tumours, reclassifying GBM IDH-mutant as astrocytoma-IDH-mutant and reserving the term GBM only for IDH-wildtype grade-IV *de-novo* tumours (1). Created in Biorender.com.

GBM, as reported before the WHO 2021 CNST classification, is the most common primary CNST, accounting for 14% of all CNSTs, and nearly half of all malignant CNSTs, with an estimated incidence of 3.23 per 100,000 population (11). GBM is most commonly diagnosed in adults over the age of 65, however, this is known to vary between studies (12). GBM has an exceptionally poor prognosis regardless of age, with the median survival in optimally treated patients being less than 15 months, and the 10-year survival estimated at 1% (13). Recent reports show that the annual incidence of GBM is increasing (14,15), adding to the disease's already high burden. As a result, there is an urgent need to understand GBM's molecular biology and investigate novel putative therapeutic strategies to improve patient survival.

Glioblastoma multiforme molecular biology

Predominant genetic alterations

Expression of IDH-wildtype is the defining molecular feature of GBM (1). IDH-wildtype promotes tumour progression and resistance to cell death by scavenging reactive oxygen species (ROS) and producing fatty acids (FAs) (16). Furthermore, GBM tumours frequently show epidermal growth factor receptor (EGFR) and mouse double minute 2 (MDM2) homolog amplification or mutation, telomerase reverse transcriptase (TERT) promoter mutations, chromosome 7 gain, and loss of heterozygosity in the long arm of chromosome 10 (q10) (17).

Altered metabolism: focus on the Warburg effect

Genetic mutations or hypoxia promote metabolic remodelling in GBM by shifting cellular energy metabolism from oxidative phosphorylation to glycolysis (18). The switch to glycolysis allows GBM cells to meet the energy demands of proliferation and invasion while adapting to the surrounding microenvironment (19) and has been linked to a poorer prognosis (20,21).

Pyruvate kinase (PK) is essential for glycolysis because it catalyses the final rate-limiting step of producing pyruvate and generating ATP (22). Despite not being expressed in the normal human adult brain (23), the M2 isoform (PKM2) is significantly expressed in GBM tumours consequent to PKM1 to PKM2 isoform switching (24,25). Increased PKM2 expression is associated with increased glycolytic turnover, tumour growth regulation, and maintenance of mitochondrial function (24,26–28). Furthermore, HIF-1α directly targets

PKM2 to promote tumour progression (29) by suppressing mitochondrial function via reduced ROS production (24,28,30). This results in a positive feedback loop that amplifies HIF-1α expression (29). As a result, PKM2 is a critical promoter of the Warburg effect and has been proposed as a potential biomarker for the metabolic status of GBM cells (31).

Oncogenic receptor tyrosine kinase signalling

Signalling by receptor tyrosine kinases (RTKs) is involved in cell invasion, proliferation, and angiogenesis. As a result, RTK signalling can be considered oncogenic and serve as a potential therapeutic target (32). EGFR signalling (PI3K/AKT/mTOR and Ras/MAPK/ERK pathways) **(Figure 2)**, are the most frequently dysregulated RKT signalling cascades in GBM oncogenesis (5,33).

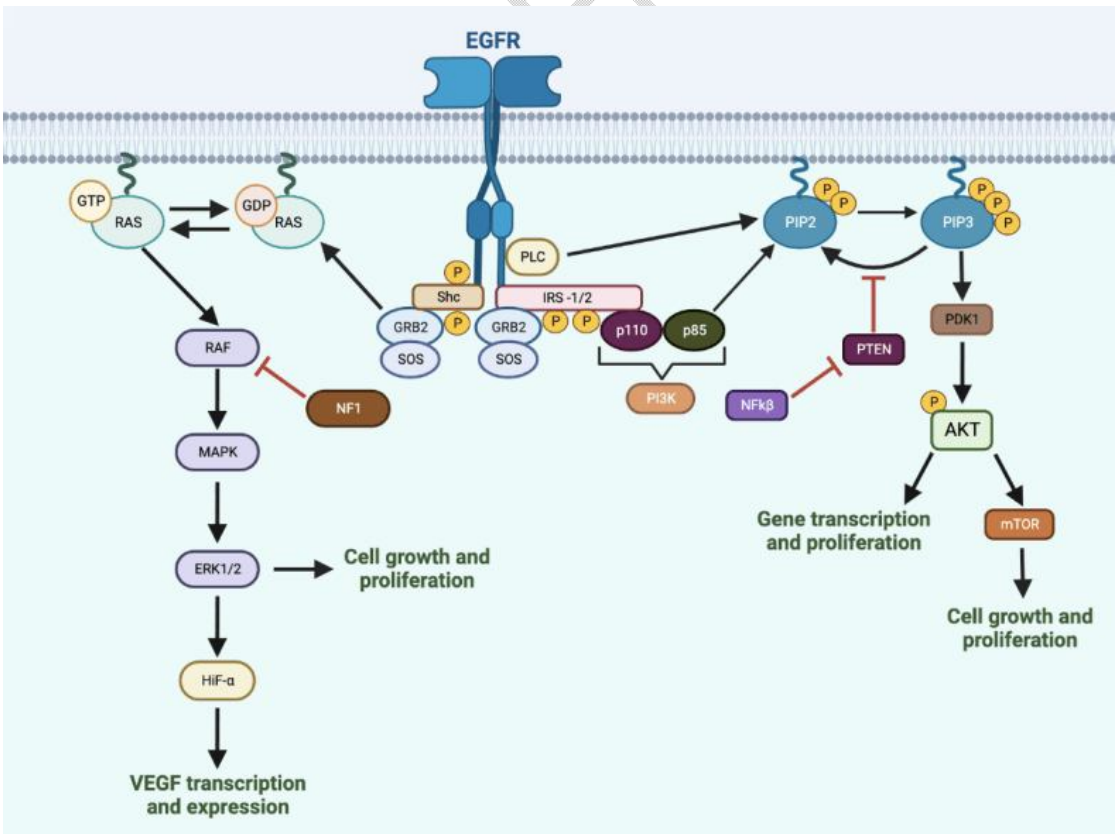

Figure 2: Frequently altered receptor tyrosine kinase signalling cascades in GBM oncogenesis. The PI3K/Akt/mTOR and Ras/Raf/ERK1/2 pathways are the most frequently altered receptor tyrosine kinase (RTK) signalling in GBM (5,33). PI3K/Akt/mTOR signalling regulates cell migration and invasion and also contributes to the regulation of apoptosis. Activation of the pathway occurs through RTK phosphorylation, which produces PIP3 and mediates the activation of Akt. PTEN acts as a negative regulator of this pathway. Active Akt can activate mTOR and inhibit pro-apoptotic proteins (33–35). Ras/Raf/ERK1/2 signalling regulates cell proliferation, migration, and survival. The activation of Raf is regulated by NF1, which acts as a negative regulator of the pathway (36,37). Created in Biorender.com.

PI3K/Akt/mTOR signalling

The PI3K/Akt/mTOR pathway primarily regulates cell migration and is often constitutively activated in GBM oncogenesis. This pathway is altered in roughly 70% of GBM tumours, either through EGFR amplification or loss of PTEN function (38). EGFR overexpression is found in approximately 35% of tumours, and its amplification leads to increased activation of the PI3K pathway. EGFRvIII, a ligand-independent mutant, is also present in approximately 40% of GBM tumours (39). Moreover, 40% of all GBM tumours also demonstrate loss of function PTEN mutations (40), resulting in loss of cascade regulation and subsequent upregulated PI3K/Akt/mTOR signalling leading to uncontrolled cell proliferation (33).

Ras/Raf/ERK1/2 signalling

Ras/Raf/ERK1/2 signalling occurs downstream of EGFR activation to regulate cell proliferation, migration, and survival activity, complementary to the PI3K/Akt/mTOR. Ras/Raf/ERK1/2 pathway mutations are uncommon in GBM, and present in approximately 2% of all GBM tumours. Notwithstanding, increased RAS activity is frequently observed, suggesting the importance of this pathway in the development of GBM (41,42). Furthermore,

18% of GBM patients have NF1 mutations or deletions, allowing constitutive activation of this pathway and promoting uncontrolled oncogenesis (9,41).

Cell death signalling in glioblastoma multiforme.

Apoptosis

GBM cells exhibit intrinsic apoptosis deregulation (43), which has been proposed as one of the mechanisms conferring therapeutic resistance due to the upregulation of anti-apoptotic proteins and downregulation of pro-apoptotic proteins, favouring cell survival (44). Anti-apoptotic Bcl-2 or Bcl-xL overexpression in GBM cell lines promotes migration and tumour invasion via apoptosis resistance (45). Similarly, resistant GBM exhibits Bcl-2 family proteins overexpression and downregulation of pro-apoptotic proteins Bax and Bak (**Figure 3)**, which has been linked to a worse clinical outcome (46–49). As a result, increased Bax expression is likely to confer a survival advantage, whereas increased Bcl-2 expression is associated with a poorer prognosis since it inhibits the release of mitochondrial cytochrome-C and the formation of the apoptosome (50). Increased procaspase-3 expression was linked to increased chemotherapy sensitivity in cells and longer progression-free survival (PFS) in patients (51). Similarly, procaspase-7 and -8 dysregulation in human GBM tissue biopsies was suggested to dysregulate the formation of the death-inducing signal complex (DISC) (**Figure 3)**, and thus downstream caspase activity, leading to decreased apoptosis (52). Together, the downregulation of apoptotic proteins may confer a patient survival advantage (51,52).

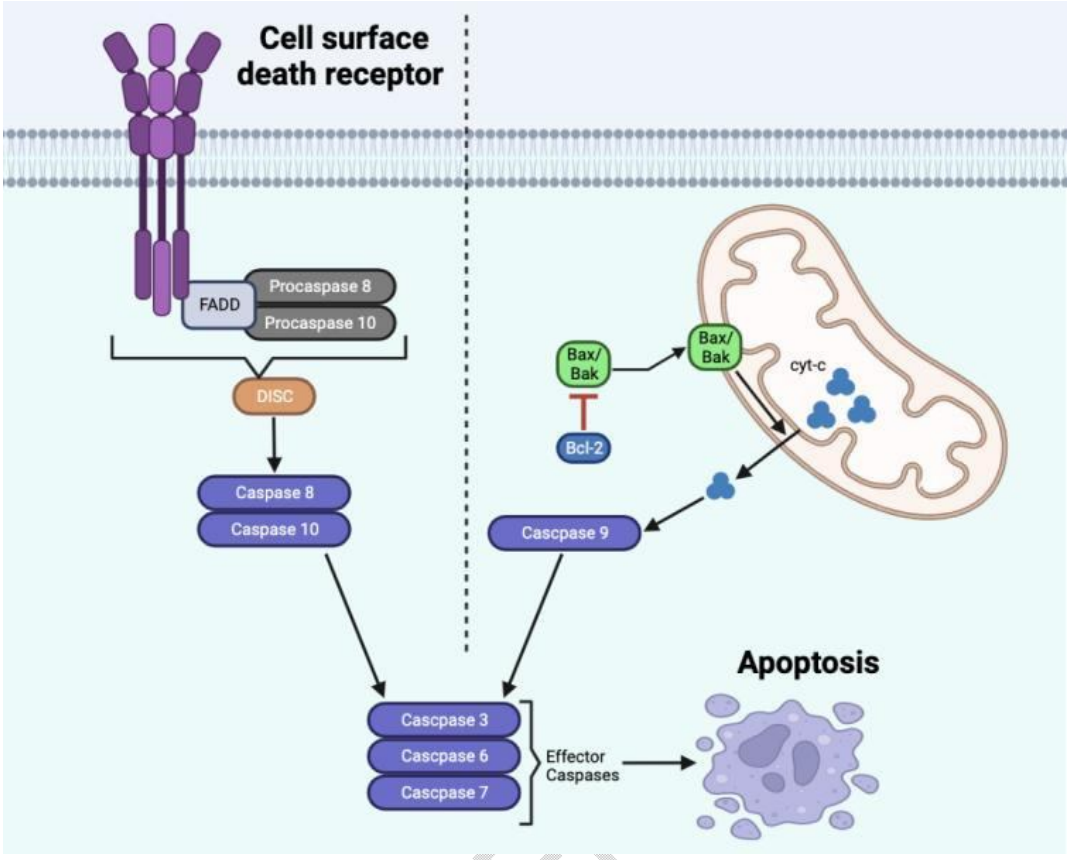

Figure 3: Extrinsic and intrinsic apoptosis. (A) The extrinsic apoptotic pathway is triggered by the docking of extracellular pro-apoptotic ligands on cell surface death receptors, forming a death-inducing signalling complex (DISC). The DISC autoactivates procaspases by dimerization, which yields the active form of the proteins and triggers apoptosis. Procaspase-8 is automatically cleaved to caspase-8, which activates effector caspases until cell death occurs (50,53). (B) The intrinsic apoptotic pathway is activated by severe cell stress and is independent of death receptors. Pro-apoptotic Bcl-2 family proteins located on the inner mitochondrial membrane can either promote or inhibit cell death. Bcl-2 can promote cell death by promoting the release of other pro-apoptotic proteins such as cytochrome-C (cyt-c), or it can prevent cell death by inhibiting the release of cyt-c via pro-apoptotic proteins Bax and Bak (54). Cyt-c then forms an apoptosome with Apaf-1 and procaspase-9, which activates effector caspases to execute apoptosis (43). The release of cyt-c also disrupts the electron transport chain and increases the synthesis of ROS (55). Created in Biorender.com.

Autophagy

Macroautophagy is a vital homeostatic in GBM, as it ensures the bulk removal of defective or no longer needed cellular components, characterised by the formation of autophagosomes **(Figure 4)**. Hypoxia and chemotherapy are the most prominent external stimuli promoting autophagy in GBM. Hypoxia causes starvation, leading to BECN1 phosphorylation activating autophagy via the HIF-1α /BECN1 signalling pathway. This acts to increase nutrient and energy availability through the degradation of redundant cellular components (56). Baseline autophagy is increased in hypoxic areas of GBM tumours with cytosolic BECN1 overexpression (57). This demonstrates the critical adaptive role of GBM survival (58).

Poor prognosis and aggressive clinical behaviour of GBM tumours in response to standard-of-care therapy have been linked to upregulated autophagy, conferring a survival advantage to the pathway (59–62). Despite this, numerous studies have demonstrated autophagy's anti-tumour function. Inducing excessive autophagy results in large-scale autophagic vacuolisation in the absence of chromatin condensation. Notwithstanding, it is still unclear whether the activation of large-scale autophagy contributes to cell death as a result of therapy or represents a last-ditch attempt at survival (63–66).

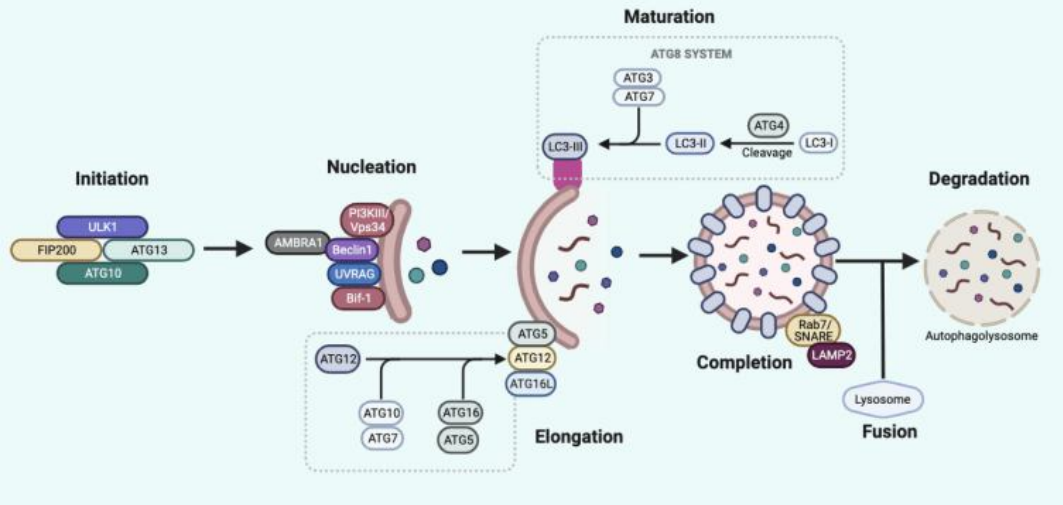

Figure 4: Autophagosome formation. This process can be initiated by various stimuli and requires the inhibition of mTOR1, which negatively regulates autophagy (50). The activation of Unc-51-Like kinase 1/2 (ULK1/ULK2) phosphorylates mATG13 and FIP200, leading to autophagosome formation. Autophagy gating regulator BECN1 binds to different proteins to either promote or inhibit autophagy. The PI3KC3 protein is the master regulator of autophagy and controls its function through two complexes. Autophagosome maturation occurs in two stages, involving Atg8-PE and Atg5-Atg12-Atg16 protein systems. LC3/Atg8 is cleaved to form LC3-I, which is then conjugated with PE by Atg7 and Atg3 to form LC3-II, marking autophagy induction. Autophagosome fuses with lysosomes to degrade and recycle cellular components (67–76). Created in Biorender.com.

Oxidative stress

The imbalance between ROS and the cells' ability to detoxify reactive intermediates or repair subsequent oxidative damage is collectively referred to as oxidative stress (77). The presence of ROS activates several transcription factors in GBM, most notably nuclear factor erythroid 2-related factor 2 (Nrf2) and HIF-1α (78). When oxidative stress is detected, Nrf2 dissociates

from its inhibitor and translocates to the nucleus, where it interacts with antioxidant response elements (AREs) to regulate antioxidant gene expression (79). Nrf2 signalling can mediate the role of HIF-1α regulatory signalling in response to oxidative stress. Moreover, HIF-1α 1 is upregulated in tumours with high Nrf2 activity since Nrf2 binds to the ARE responsible for HIF-1α transcription (80).

Endoplasmic reticulum stress

A buildup of unfolded or misfolded proteins in the ER causes ER stress and activates the unfolded protein response (UPR) to ensure proper protein folding (81). Exogenous stress may also promote ER stress, as can the accumulation of harmful metabolites, particularly ROS, which affect protein and lipid synthesis. To protect cell proliferation, ER stress can induce the expression of ER molecular chaperones (81). However, ER stress can also cause endogenous cell death on its own (82). Continuous UPR in response to cell stress may protect against GBM oncogenesis by inducing cell death via ER stress, preventing the continuous synthesis of misfolded protein (83). Conversely, the induction of ER stress by exogenous agents may result in widespread cell death, making it another potential therapeutic target (84).

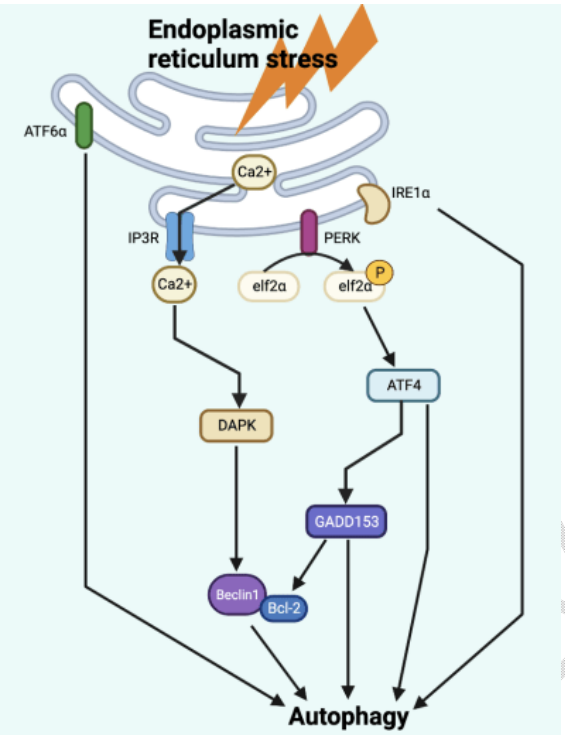

Figure 5: Endoplasmic reticulum stress as a pathway resulting in cell death. Endoplasmic reticulum (ER) stress can also independently induce endogenous cell apoptosis and ultimately affect cell fate, such as adaptation, injury or apoptosis (82). Under continuous ER stress the protein kinase R□like endoplasmic reticulum kinase (PERK), inositol□requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6α) signalling pathways are initiated to transduce the downstream autophagic pathways (85,86), the latter being recognised as a marker for ER stress (87,88). Created in Biorender.com.

Crosstalk between predominant glioblastoma multiforme intracellular signalling pathways

In GBM, apoptosis and autophagy are intricately linked (50). BECN1 regulation by apoptotic components determines whether the autophagic process is inhibited or activated, depending on intra- and extracellular conditions (89). Similarly, Atg proteins have been implicated in the regulation of apoptosis (90–92) **(Figure 6).**

The regulation of autophagy by PI3K/Akt/mTOR signalling is poorly understood (93), with the majority of evidence stemming from autophagy observed after the administration of Akt/mTOR dual inhibitors. Upregulated Akt in GBM induces the upregulation of the protoncogene MDM2, inhibiting Bad downstream. Thus, upregulated PI3K/Akt/mTOR inhibits apoptosis (94). Similarly, when nutrients are abundant, mTOR complex I (mTORC1) activation inhibits autophagy, whereas, under conditions for starvation, AMP-activated protein kinase (AMPK) induces autophagy by inhibiting mTORC1 (94). **(Figure 6)**

Oncogenic EGFR signalling increases transcriptional HIF-1α expression, increasing the rate of cellular glucose utilization (95). PTEN loss in GBM increases HIF-1α transcription and unregulated PI3K/Akt/mTOR activity (96). Similarly, EGFR overexpression or the presence of EGFRvIII causes constitutive signalling, which increases HIF-1α expression (97). Upregulated ERK1/2 also phosphorylates PKM2, causing its translocation to the nucleus, where it upregulates lactate dehydrogenase A (LDHA) and glucose transporter 1 (GLUT1) expression, contributing to the metabolic shift toward glycolysis (98).

Hypoxia also promotes the Warburg effect, resulting in similar HIF-1α upregulation (99). This reduces mitochondrial respiration, which reduces ROS leakage from the ETC and confers tumour survival. Increased PKM2 expression is also associated with a reduction in ROS production (100). Conversely, PKM2 overexpression is linked to increased ROS production (101–103), most likely due to an increase in mitochondrial membrane potential (104).

The interaction between PKM2 and ROS may also make cells more sensitive to ROS (105). PKM2 regulates HIF-1α activity by increasing its binding to hypoxia response elements (HREs), resulting in increased HIF-1α target gene expression. This increases the expression of anti-apoptotic Bcl-2 family members, which helps to prevent cell apoptosis (33). PKM2

translocation to the mitochondria also interacts with and phosphorylates Bcl-2, inhibiting ROS-induced apoptosis (106) **(Figure 6).**

Hypoxia can cause apoptosis directly through ER stress or indirectly through ROS production (107). Hypoxia increases the expression of the ER stress protein ATF6, which inhibits C/EBP Homologous Protein (GADD153), which then inhibits Bcl-2 family proteins and translocates the pro-apoptotic molecule Bax from the cytosol to the mitochondria. As a result, the intrinsic apoptotic pathway is activated (104). Hypoxia also causes cell death through acid stress since it causes an increase in lactate production via anaerobic glycolysis. Acidic stress can contribute to ER stress and directly initiate apoptosis via caspase activation (108–110)

As a consequence of hypoxia, GBM cells also undergo physiological angiogenesis to ensure a sufficient supply of oxygen and nutrients (111). Hence, hypoxic induction of angiogenesis enables GBM to survive under adverse conditions **(Figure 6).**

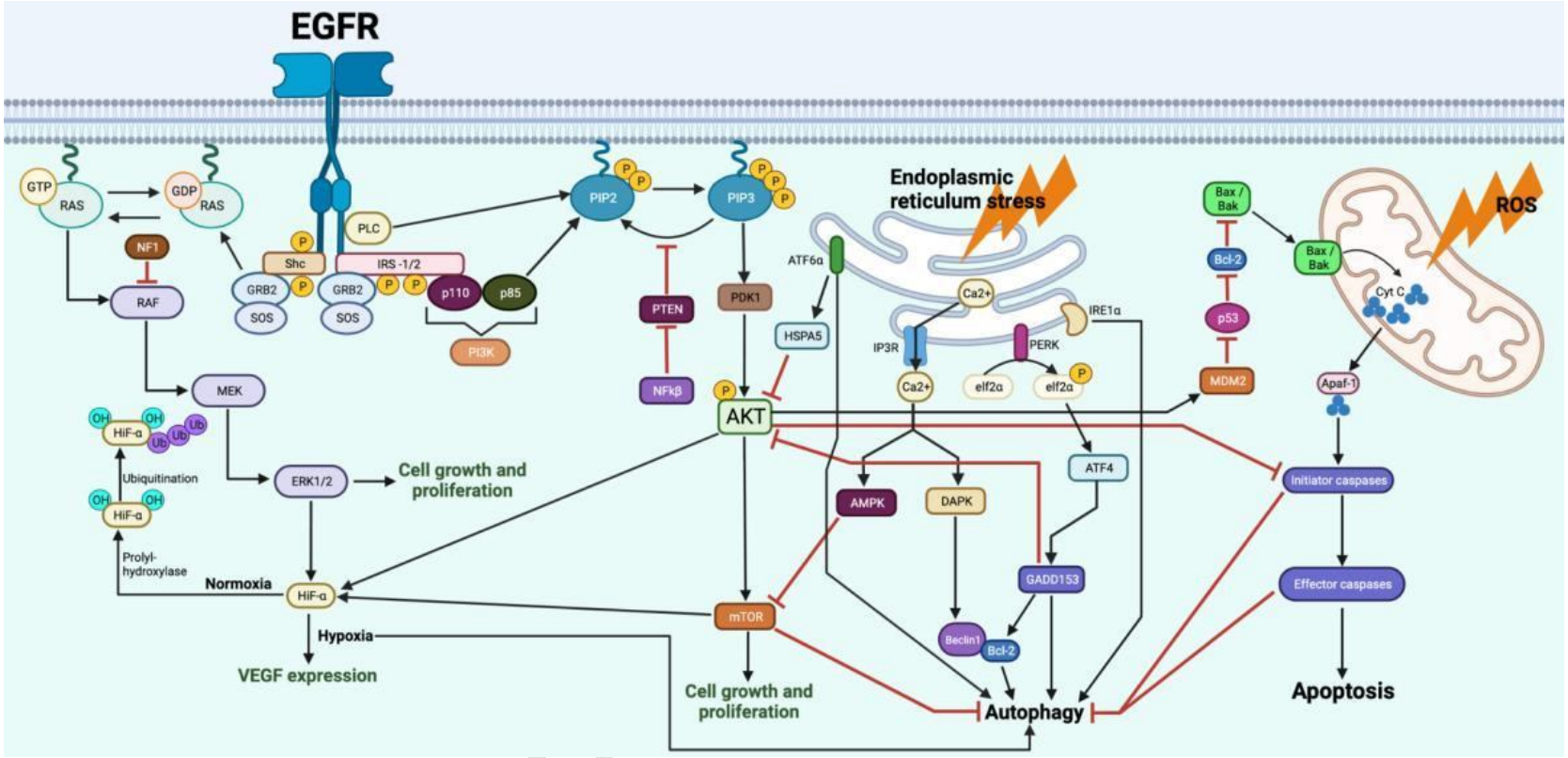

Figure 6: Intracellular cascade crosstalk in glioblastoma multiforme molecular biology.

The current standard of care against glioblastoma multiforme

The gold standard treatment for GBM IDH-wildtype WHO grade IV involves maximal safe resection (MSR), followed by concurrent chemoradiotherapy, which includes TMZ combined with fractionated radiotherapy, and subsequent maintenance TMZ administration (112) **(Figure 7)** (Stupp et al., 2005). TMZ is an orally active alkylating agent, the efficacy of which is determined by the drug's ability to alkylate/methylate DNA, most commonly at the O^6 position of guanine residues (112). For recurrent GBM, resection followed by chemoradiotherapy is also recommended **(Figure7),** where chemotherapy may also include lomustine (CCNU) and carmustine (BCNU), as well as anti-angiogenic targeted antibody therapy bevacizumab (BVZ) (113) **(Table1).**

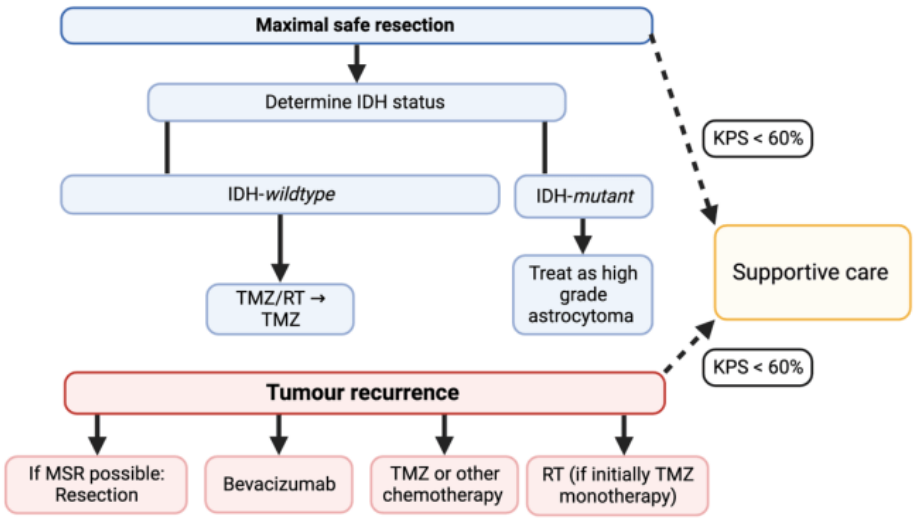

Figure 7: Current treatment pathway for glioblastoma multiforme. Adapted from Weller *et al.* (2019).

Table 1: Approved treatments currently used against glioblastoma multiforme (114,115). Temozolomide (TMZ) is the first-line treatment modality against glioblastoma multiforme (GBM), following maximal safe resection (MSR). Carmustine, lomustine and bevacizumab are being used to aid in the management of recurrent GBM.

	Approved for	**Target molecule/ cellular process**	**Overall survival (months)**	**Progression-free-survival at 6 months**	**Complications**	**Notes**
Temozolomide (TMZ)	All GBM	Non-specific alkylating agent which induces mismatch repair in DNA by methylating guanine at the O^6 position	14.60 – 16.10	53.90%	Haematologic toxicity (16.00%); thrombocytopenia (12.00%); neutropenia (7.00%) and leukopenia (7.00%)	Standard of care
Carmustine (BCNU)	Recurrent GBM	Non-specific alkylating agent which causes DNA and RNA crosslinking in dividing cells, also binds to, and modifies glutathione reductase	11.75	/	Pulmonary toxicity (<30.00%), ocular toxicity (>10.00%) and bone marrow suppression (>10.00%)	No benefit compared to radiotherapy alone

Lomustine (CCNU)	Recurrent GBM	Non-specific alkylating agent which causes DNA and RNA crosslinking in dividing cells	11.50	/	Haematologic toxicity (49.70%)	No benefit compared to radiotherapy alone
Bevacizumab (BVZ)	Recurrent GBM	Therapeutic antibody which binds and inhibits VEGF protein	9.30 (Recurrent GBM)	36% (recurrent)	Hypertension (5.50 – 11.40%), thromboembolic events (3.20 – 11.90%), gastrointestinal perforation (1.50 – 5.40%), cerebral bleeding (2.00 – 5.30%) and proteinuria (2.70 – 11.40%)	Used to treat radiation necrosis and symptomatic oedema

Molecular characteristics of Glioblastoma multiforme which confer therapeutic resistance

GBM demonstrates inter- and intra-tumour heterogeneity, as well as functional and molecular heterogeneity (116), which has been linked to a decrease in patient survival (117). The presence of stem cells and clonal evolution are the most widely accepted paradigms which attempt to explain GBM heterogeneity. GBM stem cells (GSC) are intratumorally proliferating and differentiating cells that are responsible for the initiation and maintenance of tumours after treatment (118). On the other hand, the clonal evolution theory suggests that successive cellular mutations result in clonal outgrowths that proliferate in response to specific stressors such as acidosis, hypoxia, and cytotoxic treatment. Epigenomic changes may also confer advantageous traits to tumour subpopulations, resulting in the faster formation of tumours (119).

Various subtypes of GBM with distinct molecular profiles coexist within the same tumour and exhibit differential therapeutic responses (120), making it extremely difficult to identify appropriate therapy (121). Cells within the same tumour exhibit differential gene expression, resulting in different intratumorally oncogenic signalling, proliferation, and stress response mechanisms (122). Moreover, hypermutation, thought to originate from *de-novo* pathways, is attributed to constitutional defects in DNA polymerase and pathways involving mismatch repair (MMR) genes. These pathways are frequently the result of alkylating agent treatment (123) but hypermutations can also occur as a result of GSCs (121).

Resistance to Temozolomide

The median overall survival for GBM patients receiving optimal TMZ treatment is still around 15 months (115,124). This low efficacy could be attributed to the emergence of drug-resistant phenotypes or intrinsic TMZ resistance (125,126). Intrinsic TMZ resistance occurs due to the presence of already methylated guanine residues, whereas acquired resistance occurs as a result of DNA damage and tumour cell death activating the PI3K/Akt/mTOR pathway (127). Furthermore, TMZ exhibits indiscriminate cytotoxicity, resulting in significant side effects, complicating the drug's application against GBM (128). As a result, it is critical to continue working toward the development of novel treatments with increased efficacy and lower toxicity to overcome GBM chemotherapeutic resistance.

Natural extract and phytochemical efficacy against glioblastoma multiforme *in-vitro*

Due to high therapeutic resistance and poor prognosis with optimum treatment, the ongoing search for potential treatments against GBM has turned to investigate the use of natural substances as standalone chemotherapeutic medicines or as chemotherapeutic adjuvants. Natural substances offer a wide range of commonly available, affordable biologically active chemicals, some of which have shown promising cytotoxic effects on GBM cells (129,130).

Studies identified through systematic review synthesised following PRISMA 2020 guidelines **(Supplementary material A)** demonstrated that extracts against GBM exhibit a broad spectrum of cytotoxicities with non-specific action mechanisms **(Table 2).** Additionally, it is unknown which specific bioactive molecule/s in the extract are responsible for the anti-cancer properties. When compared to extracts, known phytochemicals derived from natural sources had greater efficacy against GBM in-vitro **(Table 3)**, and studies were able to propose distinct mechanisms of action of these biomolecules, which may suggest greater specificity against GBM (131–135).

Table 2: Natural extracts assessed against glioblastoma multiforme *in-vitro.*

Extract	Cell lines	Concentration of -solanine which achieved IC50			Mode of action	Reference
		24 hours	48 hours	72 hours		
Rhazya stricta and *Zingiber officinale* crude alkaloid extract	U251	200.00 g/ml	135 g/ml	80.00 g/ml	● Induction of apoptosis by the intrinsic pathway ● Increases expression of p53, p21 and NOXA	(136)
Rhazya stricta and *Zingiber officinale* flavonoid extract		150.00 g/ml	115.00 g/ml	75.00 g/ml		
Solanum lycocarpum fruit extract	MO59J	18.26 g/ml	/	/	/	(137)
	U343MG	20.04 g/ml	/	/		
	U251	23.58 g/ml	/	/		
Brazillian red propolis extract	SF295	34.27 g/ml	/	/	● Interferes with mitosis by inducing apoptosis	(138)
Abutilon indicum extract	U87MG	/	/	42.2 M	/	(139)
Cedrus atlantica extract	DBTRG-05MG	46.59 g/ml	44.59 g/ml	/	● Triggers G0/G1 cell cycle arrest and apoptosis ● Produces reactive oxygen species and induces DNA damage ● Regulates p53/p21 and CDK4/cyclin D1 protein expression ● Decreased Akt/mTOR signalling	(140)
	G5T/VGH	39.20 g/ml	41.33 g/ml	/		
	GBM8401	49.71 g/ml	43.28 g/ml	/		
	GBM8901	40.55 g/ml	41.71 g/ml	/		
Hexaplex trunculus hypobranchial gland extract	U87MG	/	22 g/ml	/	/	(127)
Danshenn standardised extract	LN-229	50.00 M	/	/	/	(141)
Clerodendrum volubile extract	U87MG	/	120.00 g/ml	/	● Attenuation of redox imbalance inactivating G0/G1 phase ● DNA depopulation in the G0/G1 and G2/M phases ● Cell cycle arrest at S phase leading to apoptosis	(142)
Prunus spinosa L. extract	LN-229	5.25 mg/ml	/	/	/	(143)

	U87	9.78 mg/ml	/	/		
	T98G	5.55 mg/ml	/	/		
Fagara tessmannii bark extract	U87MG	/	/	17.34 g/ml	● Enhanced reactive oxygen species production ● Apoptosis mediated through the intrinsic pathway ● Mitochondrial membrane potential alteration	(144)
Salvia officinalis l. extract	42-GBMA	52.27 g/ml	/	/	/	(145)
Rosmarinus officinalis l. extract		63.59 g/ml	/	/		
Dendrobium transparens extract	U251	75.84 g/ml	/	/	/	(146)
Papilionanthe uniflora extract		2585.88 g/ml	/	/		
Pholidota articulata extract		3170.55 g/ml	/	/		
Vanda cristata extract		163.66 g/ml	/	/		
Araliopsis soyauxii bark extract	U87MG	/	/	18.67 g/ml	● Mitochondrial membrane potential alteration ● Enhanced reactive oxygen species production ● Apoptosis mediated through the intrinsic pathway	(147)
Araliopsis soyauxii root extract		/	/	35.42 g/ml		
Araliopsis soyauxii leaf extract		/	/	33.22 g/ml		
Hexane extract of seaweed Caulerpa lentillifera	A172	22.47 g/ml	/	/	/	(148)
Herba anthrisci cerefolii extract	A172	/	765.21 g/ml	/	/	(149)

Table 3: Phytochemicals assessed against glioblastoma multiforme *in-vitro*

Compound	Cell lines	Concentration of -solanine which achieved IC50			Mode of action	Reference
		24 hours	48 hours	72 hours		
Honokiol	DBTRG-05MG	/	/	30.00 M	● Triggers sub-G0 arrest leading to apoptosis	(150)
Saponin 1	U251	7.40 g/ml	/	/	● Decrease in Bcl-2/Bax ratio, initiated apoptosis by activating casp-3 and casp-9	(133)
	U87MG	8.6 g/ml	/	/		
Solamargine	MO59J	9.59 g/ml	/	/	/	(137)
	U343MG	16.30 g/ml	/	/		
	U251	8.09 g/ml	/	/		
Solasonine	MO59J	21.72 g/ml	/	/	/	
	U343MG	23.09 g/ml	/	/		
	U251	26.21 g/ml	/	/		
Gossypol	U87MG	/	57.00 M	/	/	(151)
Piplartine	SF295	0.8 g/ml	/	/	/	(152)
Deoxypodophyllotoxin (DPT)	SF126	/	/	13.95 nM	● Triggers G2/M arrest	(153)
	U87MG	/	/	15.06 nM		
Olivetoric acid	U87MG	/	17.55 mg/L	/	● Autophagy ● Oxidative DNA damage	(154)
Physodic acid		/	410.72 mg/L	/		
Psoromic acid		/	56.22 mg/L	/		
Kukoamine A.	U251	73.40 g/ml	/	/	● Triggers G0/G1 arrest ● Upregulation of Bax and casp-3, downregulation of Bcl-2	(135)
	WJ1	22.10 g/ml	/	/		
Gastrodin	DBTRG-05MG	25.00 M	/	/	● Oxidative stress-associated apoptosis ● P53 activation	(130)

Dihydrotanshinone	T98G	/	/	1.78 M	/	(155)
	U87MG	/	/	1.50 M		
Cordycepin	U251	175 M	/	/	● Upregulates p53, Bax, casp-3 and casp-9 and downregulates Bcl-2 ● Induced generation of reactive oxygen species and SOD, GPX and catalase	(131)
Tanshinone IIA	LN-229	48.20 M	/	/	/	(141)
Cryptotashinone		51.90 M	/	/		
Bacoside A	U87MG	83.01 g/ml	/	/	● Triggers sub-G0 arrest ● Induced early apoptosis	(156)
Nuciferine	U87MG	/	72.30 M	/	● Triggers G2/M arrest ● Inhibited proliferation, mobility, stemness, angiogenesis and epithelial-to-mesenchymal transition	(157)
	U251	/	59.90 M	/		
Naringin	U87MG	/	15.10 M	/	/	(158)
Cedrol	DBTRG-05MG	118.7 M	101.5 M	107.2 M	● Produces reactive oxygen species resulting in autophagy and apoptotic cell death	(159)
Ascorbic acid	42-GBMA	20.61 g/ml	/	/	/	(145)
PBI-05204	U87MG	/	/	4.90 g/ml	● Induction of apoptosis and suppressed expression of Akt and mTOR	(129)
	U251	/	/	7.30 g/ml		
	T98G	/	/	8.45 g/ml		
Withaferin A	U87MG	/	4.61 M	/	● Triggers G2/M arrest ● Initiates intrinsic apoptosis by endoplasmic reticulum stress through the ATF4-ATF4-CHOP axis	(134)
	U251	/	1.37 M	/		
Matteucinol	U251	/	/	26.57 g/ml	/	(160)
Gallotannin	DBTRG-05MG	/	/	22.50 g/ml	/	(161)
Chalcone derivative	U87MG	19.50 M	16.51 M	18.07 M	● Triggers G2/M arrest	(162)

Zerumbone	U87MG	150 M	/	/	● Decreased expression of MMP-2 and MMP-9 ● Inhibits Akt and ERK1/2 signalling	(163)
Crocin	A172	3.10 mg/ml	2.19 mg/ml	1.72 mg/ml	● Apoptosis by upregulation of Bax	(132)
Dimethylcrocetin	A172	4.73 mg/ml	2.8 mg/ml	1.95 mg/ml	● Apoptosis by upregulation of Bax and downregulation of Bcl-2	
p-coumaric acid	U87MG	/	0.50 mM	/	● Triggers G2/M arrest ● Increase in p53	(164)

The modes of action of the phytochemicals and extracts varied **(Figure 8)**, however, apoptosis was commonly induced (129,130,132–134,150,156,159,164). In addition, cell cycle arrest was noted by several studies, at different stages of the cell cycle, with G2/M arrest (134,142,153,162,164), being most commonly noted, followed by Sub-G0 (133,135,156) and G0/G1 arrest (140,142). A considerable number of the included studies (9/36) observed that extracts and phytochemicals induced cell death by generating ROS low concentrations (130,131,140,142,143,147,154,159). This suggests that the generation of ROS may be a promising therapeutic strategy against GBM. One of the most frequently studied classes of natural molecules against GBM observed was alkaloids. (137,152,157). Alkaloids are among the most important drugs in human history and phytochemicals within this class of drugs which were determined to be exclusively harmful have found new uses as putative anti-cancer drugs (165). Hence, further studies investigating the uses of alkaloids as putative therapeutic agents against GBM are justified.

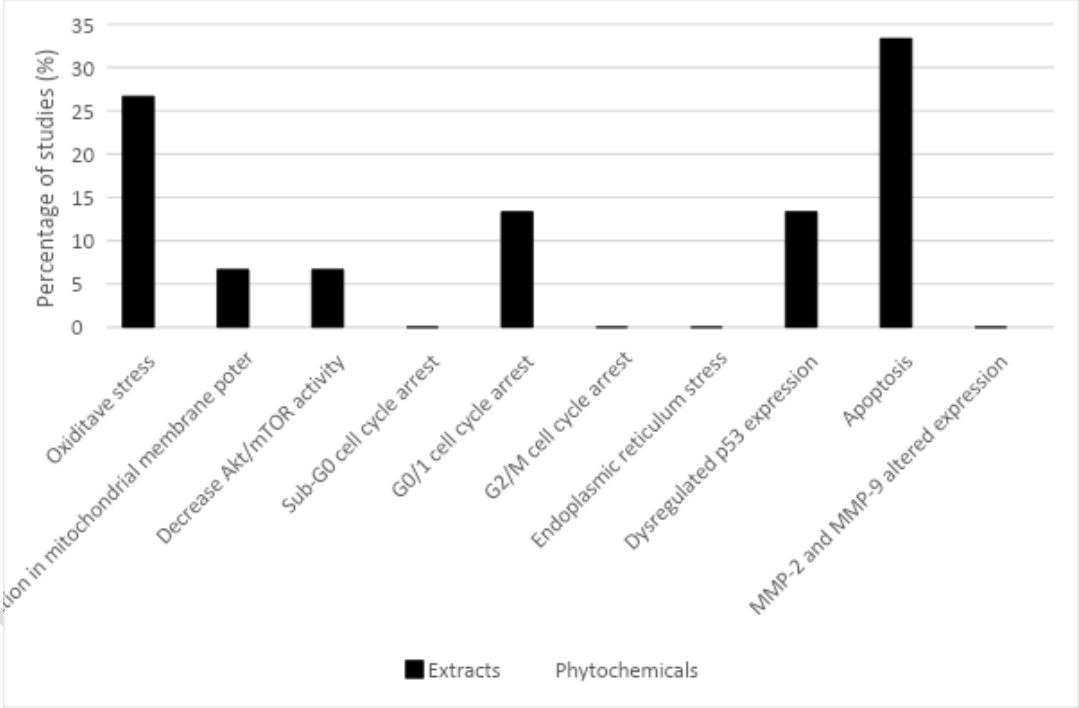

Figure 8: Documented modes of action of natural extracts and phytochemicals against glioblastoma multiforme *in-vitro*.

Several limitations need to be addressed to develop naturally derived molecules into therapeutic strategies. The precise molecule or group of molecules within extracts producing anti-cancer effects is unknown. Further work using chromatographic methods should be

conducted to identify these phytochemicals and identify any overlapping properties. In addition, several phytochemicals investigated against GBM demonstrated significant limitations such as poor water solubility (166), significant toxicity and long-term adipose storage (132), and the need for co-administration with other drugs to produce cytotoxic effects. (131).

Additionally, more studies need to assess the efficacy of phytochemicals against TMZ, the current therapeutic standard of care for GBM. From the studies included in this review, only four studies compared the cytotoxicity of phytochemicals to TMZ (140,159,161). Mann Whitney-U testing demonstrated a statistically significant difference between the concentrations of natural extracts and phytochemicals required to reach IC50 versus TMZ. At 24 and 48 hours of incubation, a statistically significant difference between the concentration of natural extracts and phytochemicals and TMZ was noted, as demonstrated in **Table 4.** Yet, further studies are required to increase statistical power and unequivocally demonstrate the superiority, or otherwise, of natural extracts and phytochemicals against GBM *in-vitro,* over TMZ.

Table 4: Comparison between efficacy of natural extracts and phytochemicals versus Temozolomide (TMZ) against glioblastoma multiforme (GBM) cell lines *in-vitro.* Mann Whitney U-testing revealed that natural extracts and phytochemicals are superior to TMZ at 24 hour and 48 hour incubation.

Time	Cell line	Natural extract/ phytochemical	IC50 (g/ml)	IC50 TMZ (g/ml)	*p*-value	Reference
24 hours	DBTRG-05MG	Cedrol	26.40	189.08	0.029	(159)
	DBTRG-05MG		46.59	180.45		
	G5T/VGH	*Cedrus atlantica* extract	39.20	94.30		(140)
	GBM8401		49.71	166.39		
48 hours	DBTRG-05MG	Cedrol	22.57	68.87	0.029	(159)
	DBTRG-05MG		44.59	95.58		
	G5T/VGH	*Cedrus atlantica* extract	41.33	86.00		(140)
	GBM8401		43.28	95.76		
72 hours	DBTRG-05MG	Cedrol	23.84	23.26	0.2	(159)
	DBTRG-05MG	Gallotannin	22.50	13.90		(161)
	T98G		0.50	121.54		(155)
	U87MG		0.42	62.71		

Conclusion

GBM is a highly complex disease with n abysmal prognosis. Optimal treatment only improves prognosis by a few months. The heterogeneity of GBM tumours coupled with numerous chemotherapeutic resistances significantly complicate research into discovering novel therapeutic strategies. Understanding the different intracellular signalling cascades and how these pathways interact with one another is crucial for evaluating the mode of action of putative treatments. *In-vitro* evidence suggests that naturally derived compounds may be promising agents against GBM, yet further research is needed to consolidate phytochemicals' efficacy over TMZ.

Statements & Declarations

Ethical approval

No ethical approval was required for the synthesis of this review.

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