ELUCIDATING CYTOTOXIC EFFECTS OF *QUERCUS INFECTORIA* GALL EXTRACT ON TEMOZOLOMIDE-RESISTANT GLIOMA CELLS

NORHAZILAH BINTI MUHAMAD

UNIVERSITI SAINS MALAYSIA

2023

ELUCIDATING CYTOTOXIC EFFECTS OF *QUERCUS INFECTORIA* GALL EXTRACT ON TEMOZOLOMIDE-RESISTANT GLIOMA CELLS

by

NORHAZILAH BINTI MUHAMAD

Thesis submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy

September 2023

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful, Alhamdulillah, Praise to Allah and His blessings and strengths for allowing me to complete this thesis that marked my remarkable journey and experiences through up and downs in the pursuit of knowledge.

First and foremost, I would like to express my greatest gratitude towards my main supervisor, Dr. Tan Suat Cheng for her guidance, encouragement, perseverance, patience and efforts throughout my research project. Special appreciation to my co-supervisors, Assoc. Prof. Dr. Hasmah Abdullah and Dr. Wan Nordiana Wan Abd Rahman for their support and guidance. I would also like to thank the Ministry of Higher Education Malaysia for funding this research under the Fundamental Research Grant Scheme (FRGS) 203/PPSK/6171154. Furthermore, my deepest thank for sponsoring me throughout this study under SLAB scheme funds. I am grateful to Universiti Sultan Zainal Abidin (UniSZA) for approving my study leave and encouraging me to continue my studies.

I sincerely thank the laboratory staff and personnel for providing the necessary materials and instrumental facilities for my research study. My heartfelt gratitude also goes to my close friends Nurul Aini, Kang In Nee, Nurul Fatihah, Hakimah, Alisa, Norlida, Munirah, Siti Fatimah, and Farah Amna, who have offered moral support and assistance on numerous occasions. Last but not least, I would like to express my gratitude to the backbone of my success; my husband Mr. Wan Fadli bin Wan Abdullah as well as my son Adam Fahmi, my parents and the whole family for the endless support, encouragement and countless love. Finally, thank you to everyone who has crossed my path and may the world benefit from this research and writing. Aamiin YRA. Thank you.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS, ABBREVIATIONS AND SYMBOLS

ADAM17	A disintegrin and metalloproteinase 17
AKT	Protein kinase B
ANGPT1	Angiopoietin-1
AO	Acridine orange
APNG	Alkylpurine–DNA–N-glycosylase
ATCC	American Type Culture Collections
ATEs	Arterial thromboembolic events
BAX	Bcl-2-associated X
BBB	Blood-brain barrier
BCM	Bevacizumab
BER	Base excision repair
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CASP3	Caspase 3
CCNU	Lomustine
cDNA	Complementary deoxyribonucleic acid
CD133	Prominin-1
CI	Combination index
CNS	Central nervous system
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRs	Death receptors
EDTA	Ethylenediaminetetraacetic acid
eIF4A	Eukaryotic initiation factor 4A
EMEM	Eagle's Minimum Essential Medium
ER	Endoplasmic reticulum
EGFR	Epidermal Growth Factor Receptor
EtOH	Ethanol
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FT-IR	Fourier Transform Infrared
GB	Glioblastoma

GBP5	Guanylate-binding protein 5
GLOBOCAN	Global Cancer Statistics
GSCs	Glioblastoma stem cells
HPLC	High Performance Liquid Chromatography
IC_{50}	Half maximal inhibition concentration
JNK	c-Jun N-terminal kinase
KOH	Potassium hydroxide
KPS	Karnosky Performance Scale
LC3B	Microtubule-associated proteins 1A/1B light chain 3B
LC-MS	Liquid Chromatography Mass Spectrometry
MCyp	Micellarized cyclopamine
MDM2	Mouse double minute 2 homolog
MEM	Minimum Essential Medium
MGMT	O^6-methylguanine methyltransferase
m-MGMT	Methylated MGMT
MMR	Mismatch repair
MNCRR	Malaysia National Cancer Registry Report
MPG	N-methylpurine-DNA-glycosylase
MRP	Multidrug resistance protein
MSH6	MutS homolog 6
MTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NaCI	Sodium chloride
NaOH	Sodium hydroxide
Nrf2	Nuclear factor erythroid 2-related factor 2
NSC	Neural stem cells
OD	Optical density
OS	Overall survival
PBS	Phosphate-buffered saline
PFS	Progression-free survival
PI	Propidium iodide
PI3KA	phosphatidylinositol 3-kinase alpha
PTEN	Phosphatase and tensin homolog
QI	*Quercus infectoria*
QIA	*Quercus infectoria* aqueous extract
QIAc	*Quercus infectoria* acetone extract

QIE	*Quercus infectoria* ethanol extract
QIEA	*Quercus infectoria* ethyl acetate extract
QIM	*Quercus infectoria* methanol extract
qPCR	Quantitative real-time Polymerase Chain Reaction
RNA	Ribonucleic acid
RNAi	RNA interference
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute Medium 1640
SD	Standard deviation
SEM	Standard error of means
Shh	Sonic-hedgehog
SOC	Standard of care
SOD-1	Superoxide dismutase-1
Sphk	Sphingosine-1-phosphate/sphingosine kinases
TAE	Tris-acetate-EDTA
TAM	Tamoxifen
TBP	TATA box binding protein
TMZ	Temozolomide
TTFs	Tumor-treating fields
UV	Ultraviolet
VEGFA	Vascular endothelial growth factor A
WHO	World Health Organization
et al.	And others
FeCI3	Ferum III chloride
bp	Base pair
CO_2	Carbon dioxide
C_T	Threshold cycle
dH_2O	Distilled water
H_2SO_4	Sulfuric acid
°C	Degree celcius
Δ	Delta
E	Efficiency of primer
g	Gram
h	Hour
µ	Micro

ml	Millilitre
min	Minute
M	Molar
n	Nano
O_2	Oxygen
pH	Potential of hydrogen
rpm	Rotation per minute

PENJELASAN KESAN SITOTOKSIK EKSTRAK BIJI *QUERCUS INFECTORIA* TERHADAP SEL GLIOMA KERINTANGAN TEMOZOLOMIDE

ABSTRAK

Glioblastoma (GB) adalah tumor otak primer yang sangat agresif yang dicirikan oleh kadar kematian yang tinggi dan prognosis yang buruk. Kemoterapi barisan pertama untuk GB ialah temozolomide (TMZ). Walau bagaimanapun, rintangan dadah, kesan sampingan yang buruk dan GB berulang menjadi kelemahan utama kemoterapi. Oleh itu, tumbuhan ubatan digunakan sebagai rawatan alternatif yang menawarkan akses yang luas dan kesan sampingan yang lebih rendah. Daripada kajian terdahulu, ekstrak metanol biji *Quercus infectoria* (QIM) menunjukkan aktiviti antiproliferasi *in vitro* yang kuat terhadap sel glioma manusia. Oleh itu, kajian ini dijalankan untuk mencirikan sebatian antikanser dalam QIM dan mengenalpasti mekanisme antikanser yang mendasari. Selain itu, potensi QIM untuk meningkatkan terapeutik TMZ dalam rawatan kombinasi, terutamanya terhadap sel glioma kerintangan TMZ (DBTRG-05MG) dan TMZ-sensitif (U-87MG) juga dijalankan. Pemeriksaan fitokimia, pencirian fitokonstituen oleh analisis FT-IR dan LC-MS dan asai DPPH telah dilakukan untuk menilai bioaktiviti QIM. Aktiviti antiproliferatif rawatan QIM dan TMZ terhadap sel glioma dan glial (SVG p12) ditentukan menggunakan asai MTT. Nilai IC_{50} kemudiannya ditentukan dari lengkung tindak balas dos dan digunakan untuk rawatan kombinasi. Kesan rawatan kombinasi ditentukan berdasarkan indeks kombinasi (CI) dengan menggunakan perisian *CompuSyn*. Mekanisme antikanser rawatan tunggal dan kombinasi dinilai oleh pewarnaan AO/PI untuk penentuan mod kematian sel, ujian klonogenik untuk

penentuan keupayaan pembiakan, ujian calar luka untuk penentuan anti-invasi dan RT-qPCR untuk menilai profil ekspresi gen yang berkaitan dengan mekanisme apoptosis, autofagi, anti-invasi, angiogenesis, antioksidan dan rintangan dadah. Hasil penemuan mencadangkan bahawa fitokonstituen utama QIM adalah gallotannin dengan kumpulan berfungsi, bernama fenol. QIM menunjukkan aktiviti penghapusan radikal DPPH yang setara (EC_{50} = 16.90 µg/mL) berbanding asid gallik (EC_{50} = 17.33 µg/mL). QIM menunjukkan sitotoksisiti yang lebih baik terhadap U-87MG (IC_{50} = 20.5 µg/mL) berbanding DBTRG-05MG (IC_{50} = 21.4 µg/mL) dan sitoselektif terhadap sel SVG p12 (tiada IC_{50} dikesan). Kerintangan TMZ ditunjukkan oleh DBTRG-05MG (IC_{50} = 480.30 µg/mL) yang memerlukan kepekatan yang lebih tinggi berbanding U-87MG (IC_{50} = 56.14 µg/mL). Rawatan kombinasi dapat mengurangkan percambahan sel glioma berbanding TMZ sahaja dan menunjukkan kesan sinergistik (CI<1). Selain itu, rawatan tunggal dan kombinasi menyebabkan apoptosis dalam sel glioma yang dicirikan oleh morfologi apoptosis dan peningkatan ekspresi gen apoptosis (BAX dan CASP3). QIM juga merencat autofagi dengan menurunkan ekspresi gen LC3B. Rawatan kombinasi juga menghalang penghijrahan dan metastasis sel glioma dengan menghalang penutupan luka dan menurunkan ekspresi gen invasi, (ADAM17). Penemuan ini disokong lagi dengan penindasan penyembuhan luka dan penurunan ketara gen berkaitan angiogenesis (VEGFA dan ANGPT1) yang menyekat bekalan nutrien dan oksigen kepada sel kanser. Rawatan kombinasi juga meningkatkan terapeutik TMZ dengan menurunkan gen rintangan dadah (MGMT, MRP dan PI3KA) yang mengakibatkan perencatan percambahan sel kanser yang lebih besar. Kesimpulannya, QIM menunjukkan aktiviti antikanser terpilih melalui laluan apoptosis dan autofagi dan rawatan kombinasi QIM dan TMZ memberikan kesan sinergistik dalam sel glioma.

ELUCIDATING CYTOTOXIC EFFECTS OF *QUERCUS INFECTORIA* GALL EXTRACT ON TEMOZOLOMIDE-RESISTANT GLIOMA CELLS

ABSTRACT

Glioblastoma (GB) is a highly aggressive primary brain tumour characterized by a high mortality rates and poor prognosis. The first-line chemotherapy for GB is temozolomide (TMZ). However, drug resistance, unwanted side effects and GB recurrence become the major drawbacks of chemotherapy. Hence, medicinal plants are used as alternative treatments which offer wide accessibility and lesser side effects. From a previous study, *Quercus infectoria* gall methanol extract (QIM) has demonstrated its potent *in vitro* antiproliferative activity against human glioma cells. Thus, this study aims to elucidate the anticancer compounds within QIM and explore the underlying anticancer mechanisms. Additionally, QIM's potential to enhance the therapeutic effects of TMZ in combination treatments, particularly against TMZ-resistant (DBTRG-05MG) and TMZ-sensitive (U-87MG) glioma cell lines was conducted. Phytochemicals screening, phytoconstituents characterization by FT-IR and LC-MS and DPPH assay were done to evaluate the bioactivity of QIM. Antiproliferative activity of QIM and TMZ treatment against glioma and glial (SVG p12) cells were determined using MTT assay. IC_{50} values were then determined from a dose-response curve and employed for combination treatment. The combined effect of the treatment was determined based on their combination index (CI) using CompuSyn software. Anticancer mechanisms of single and combined treatment were evaluated by AO/PI staining for determination of the mode of cell death, clonogenic assay for reproductive ability, wound scratch assay for anti-invasion and RT-qPCR to evaluate the gene expression profiles associated with apoptosis, autophagy, anti-

invasion, angiogenesis, antioxidant responses and drug resistance mechanisms. The findings suggested that the major phytoconstituent in QIM was gallotannin with a functional group, named phenols. QIM showed robust DPPH radical scavenging activity (EC_{50} = 16.90 µg/mL) comparable to gallic acid (EC_{50} = 17.33 µg/mL). QIM exhibited better cytotoxicity against U-87MG (IC_{50} = 20.5 µg/mL) compared to DBTRG-05MG (IC_{50} = 21.4 µg/mL) and cytoselective against SVG p12 cells (no IC_{50} detected). Notably, TMZ-resistant was shown by DBTRG-05MG (IC_{50} = 480.30 µg/mL) which require higher concentration compared to U-87MG (IC_{50} = 56.14 µg/mL). Remarkably, combination treatment significantly reduced the proliferation of both glioma cell lines when compared to TMZ alone and it demonstrated a synergistic effect (CI<1). Moreover, both single and combined treatments induced apoptosis in glioma cells, characterized by apoptotic morphological changes and upregulation of apoptosis-related genes (BAX and CASP3). QIM also inhibited autophagy by downregulating the LC3B gene. Additionally, the suppression of wound healing and the downregulation of the invasion gene (ADAM17) indicated that the combined treatment inhibited cell migration and metastasis. The significant downregulation of angiogenesis-related genes (VEGFA and ANGPT1), which restricted the availability of nutrients and oxygen to cancer cells, further supported these effects. Combination treatment also significantly increased TMZ's therapeutic efficacy by downregulating drug-resistant genes (MGMT, MRP and PI3KA), resulting in greater cell proliferation inhibition. In conclusion, QIM exhibits selective anticancer activity through apoptosis and autophagy pathways, and the combination treatment of QIM and TMZ exerted a synergistic effect in glioma cells..

CHAPTER 1

INTRODUCTION

1.1 Background of study

Despite remarkable advances in the discovery and development of new cancer therapeutics, cancer remains the world's second leading cause of death (Sung et al., 2021). According to the World Health Organization (WHO) in its Global Cancer Statistics (GLOBOCAN) report 2020, cancer was responsible for an estimated 9.6 million deaths accounting for approximately one in every six deaths worldwide. Whereby, Malaysia National Cancer Registry Report (MNCRR) 2012-2016 reported a total of 82,601 medically certified and non-medically certified cancer deaths from 2012 to 2016 which was increased from the previous report (Azizah et al., 2019). The increasing trend of cancer death or mortality affect individuals, families, communities, and society as a whole. It leads to increased human suffering and healthcare cost which could impact on psychological and financial strain of individual, families and communities. Indeed, cancer-related productivity losses impose a significant economic burden on transitioning economies (Tabuchi, 2020; Ghoshal et al., 2022). Cancer's economic burden includes spending on primary, outpatient, emergency, and inpatient care, as well as drugs (McCormick, 2018).

Glioblastoma (GB) is the most common malignant, aggressive and deadliest cancer of the brain. GB has a median survival rate of less than 14 months following diagnosis, making it a critical public health concern (Thakkar et al., 2014; Ghoshal et al., 2022). Despite recent advancements in multimodality therapy integrating surgery,

radiation, chemotherapy, targeted therapy and palliative care, treating GB remains the most difficult clinical oncology endeavour. Its radical infiltrative, heterogeneity, therapeutic resistant and tumor recurrence hinder the successful of the treatment and management (Kang et al., 2022).

Chemotherapy employs drug to target and destroy cancer cells has been employed for decades in the cancer treatment. Present chemotherapeutic approach failed to stop GB growth due to two main reasons: (1) impermeability of the chemo-drug by blood-brain barrier (BBB) due the complex vascularized network of GB and (2) enzymatic modification of drug action via O^6-methylguanine-DNA methyltransferase (MGMT) which results in poor efficacy of the chemo-drugs (Lee, 2016; Stavrovskaya et al., 2016; Nagel et al., 2017). To date, temozolomide (TMZ), an oral alkylating agent has long been the first line of chemotherapy drug for GB due to its permeability to the BBB and a well-tolerated safety profile. However, its therapeutic efficacy is futile by its favour of methylation of MGMT status in GB patients and the drug resistant. Numerous interventions have been studied to comprehend the resistance mechanisms and combine TMZ with another therapeutic agent to better benefit the patient. However, each intervention is defeated by GB's adaptability, alteration, and resistance, leaving the research where it began (Arora & Somasundaram, 2019).

Phytotherapy, used of natural product from plant as anti-GB agent has been explored and offered beneficial potential to improve the treatment of GB. A controlled clinical phytotherapy study has reported a reduction of tumour size and an increment of survival rate in GB patients (Trogrlić et al., 2018). Antiproliferative

activity of GB cells by phytotherapy also have been reported vastly in vitro. The positive effects of phytotherapy are mainly due to its (i) chemical diversity that provides a rich source of potential anticancer agents, (ii) natural origin that is less toxic and easier for the body to tolerate than synthetic chemicals, (iii) multitargeting capability in which it is able to target multiple pathways involved in cancer development and progression due to the multiple active phytoconstituents present in a medicinal plant (Soukhtanloo et al., 2020; Alves et al., 2021; Siddiqui et al., 2022; Talib et al., 2022).

Based on our group previous study, 100% methanol crude extract from a potential medicinal plant known as *Quercus infectoria* (QI) possessed antiproliferative activity towards glioma in vitro (Tan et al., 2018). However, the exact mechanism or pathway contributed to its antiproliferative effect was not well investigated. Hence, the aims of this present study were to further characterize the phytoconstituent profile of QI gall extract and its in vitro anticancer mechanism to consolidate the potential of QI gall extract as an alternative treatment for TMZ-resistant glioma cells.

1.2 Rationale of study

Emergence of TMZ resistant and limited efficient chemotherapy drug for GB treatment have inspired the discovery of new anti-glioma agents which offer better efficacy, minimal adverse effects and able to combat the resistant cell. Nowadays, phytotherapy had been in trend for potential anti-glioma agents, supported by positive preclinical evidences. Moreover, the phytotherapy is cost-effective treatment option which could be easily and inexpensively obtained, making them an attractive

option for developing countries where access to expensive chemotherapy drugs may be limited. However, up to date, not many of these phytotherapy options were able to translate into clinical practise. This is due to limited study of the its safety and complete mechanism of action as anticancer agent. Therefore, the comprehensive evaluation of cytotoxicity effects of QI gall extract on TMZ-resistant glioma cells as presented in this study could contribute to the development of novel, secure pharmaceutical drug derived from natural therapeutic plants to overcome the limitation of current GB chemotherapy options.

1.2.1 Hypotheses of study

The hypotheses in this study were:

1. QIM inhibited glioma cell proliferation greater than TMZ.
2. Combination treatment of QIM and TMZ increased the inhibition of chemoresistance glioma cells proliferation than single treatment.
3. Combination treatment of QIM and TMZ enhanced the therapeutic of TMZ on chemoresistance glioma cells.

1.3 Objective of the study

1.3.1 General objective

To determine the anticancer potential of combined therapy using *Quercus infectoria* galls methanol extract (QIM) and temozolomide (TMZ) to enhance the cytotoxic effects in chemoresistance glioma cells, compared to the chemotherapeutic activity of single therapy using QIM and TMZ, respectively.

1.3.2 Specific objectives

1. To characterize phytochemical compound present in QI galls methanolic extract (QIM).
2. To determine the optimum combination index (CI) of QIM and TMZ concentration against chemoresistance glioma cells.
3. To investigate antiproliferative activity by combined treatment of QIM and TMZ on chemoresistance glioma cells.
4. To elucidate cell death mechanisms induced by combined treatment of QIM and TMZ on chemoresistance glioma cells.
5. To elucidate anti-metastasis effects by combined treatment of QIM and TMZ on chemoresistance glioma cells.
6. To elucidate underlying mechanisms against drug resistance by combined treatment of QIM and TMZ on chemoresistance glioma cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Glioma

Glioma is a malignant primary brain tumour that derived from glial cells in brain. Glial cells consisting of astrocytes, oligodendrocytes, microglial and ependymal cells, support the neurons with energy and nutrients and helps maintain the BBB. According to the WHO, cancer of brain and nervous system accounting for 1.6% of the total cancer incidence with 2.5% mortality (Sung et al., 2021). As for Malaysia, a total number of 1199 cases of brain and nervous system cancer were reported in the latest MNCRR (Azizah et al., 2019). Gliomas are the most common primary malignant brain tumour, accounting for over 60% of all adult brain tumours and with a male predominance (Ostrom et al., 2021).

2.2 Glioblastoma (GB)

2.2.1 Incidence and prognosis of glioblastoma

Glioblastoma is the most aggressive and infiltrative form of the glioma. It is presented with a high mortality rate and recurrent risks leads to huge burdens on society and families (Ostrom et al., 2019). In the United States, GB was ranked as the third common intracranial tumours and the most common malignant central nervous system (CNS) tumours (Ostrom et al., 2018). A study by Balqis and colleagues (2017) reported that GB (7.8%) ranked as the second common adult primary brain tumour in the East Coast of Malaysia, overtake by meningioma (32.7%). This finding is similar with reported incidence of GB in Terengganu, Malaysia (Karim Othman et al., 2020).

GB remains as incurable disease due to its detrimental nature and poor prognosis and worsen as the age increases (Rajaratnam et al., 2020). Median overall survival (OS) varies between 12 to 18 months and the 5-year survival is less than 7% (Ostrom et al., 2021). Standard treatment of GB, surgical resection and post-operative radiotherapy and chemotherapy significantly delay the tumour progression but the recurrent rate is still high. The median progression-free survival (PFS) for GB varies between six to nine months of primary treatment prior to the recurrent (Weller & le Rhun, 2020). PFS refers to the amount of time that passes between the start of a specific treatment and the point at which the disease either recurs or the patient dies (Liang et al., 2020).

2.2.2 Classification of glioblastoma

Heterogenetic characteristic of GB tumour leads to its classification diversity over the years. Historically, GB was classified as WHO grade IV astrocytoma, the most invasive and deadly GB. In 2016, GB was known as Glioblastoma multiforme (GBM) based on isocitrate dehydrogenase (IDH) gene mutant status as declared by WHO classification standard (Louis et al., 2016). Later in 2021, WHO renamed GBM to GB and include the IDH-wild type and IDH-mutant type into the classification for this disease. Moreover, it is classified as adult-type diffuse GB which recognized as a disease affected the adult only (Louis et al., 2021).

2.2.3 Aetiology and risk factors of glioblastoma

As complicated as its classification, aetiology of GB also is hard to be determined in most cases of GB. Among many possible causal factors, therapeutic ionizing radiation is the most established and strongest risk factors for GB (Rice et

al., 2015) . Epidemiology studies revealed that prior to the diagnosis of GB, majority of the patients had history of previous radiation treatment such as exposure to the scalp during childhood or receiving one therapeutic dose of ionizing radiation (López et al., 2019). Moreover, though GB is believed to be spontaneous, it has association with rare c such as Li-Fraumeni syndrome, Turcot syndrome and neurofibromatosis type 1 (Ko & Brody, 2021).

2.2.4 Current treatment of glioblastoma

Treatment of GB required a multimodal approach based on the complex pathogenesis of the disease. The standard of care (SOC) in newly diagnosed GB patients includes maximal safe surgical resection followed by external beam radiotherapy with concomitant and adjuvant temozolomide (TMZ)-based chemotherapy (Sastry et al., 2018). However, GB always recurs with poorer prognosis (Audureau et al., 2018).

The aims of surgical resection of GB include relieving mass effect, achieving cytoreduction and providing adequate tissue for histology and molecular tumour characterization (Muller et al., 2019). Karnosky Performance Scale (KPS) index is used to evaluate the eligibility of patient for surgical intervention with intention of good post operative measurement. A KPS index greater than or equal to 70 is the minimum for offering surgical intervention (Liu et al., 2022). Survival rate is increased with high volume of resected tumour (Wang et al., 2019). However, the invasiveness of GB cells towards the adjacent healthy brain cells makes it impossible for complete tumour resection. Thus, radiotherapy and chemotherapy are given to target the remaining tumour cells.

Radiotherapy aims to control the tumour growth and minimize the risk of recurrent reducing symptoms (Tu et al., 2021). Standard radiotherapy regimen for GB following the surgical resection is 60 Gy in 30 fractions in combination with TMZ (Stupp et al., 2018). However, the treatment is limited towards tumour cells residing in the area of hypoxia which are resistant to the treatment (Chédeville & Madureira, 2021). Heterogenity of tumour cell population including the GB stem cells (GSCs) confer adaptive radioresistance in radiotherapy of GB due to its high self-renewal capacity (Osuka et al., 2021). Volume of brain irradiated and the dose to critical structures need to be considered to control the risk of radiation necrosis with concurrent chemotherapy (Tan et al., 2020).

The standard chemo-drug used for GB including temozolomide (TMZ), bevacizumab and carmustine, with TMZ remains as the first liner chemotherapy for GB despite resistance of glioma cells, owing to its efficacy to increase the median survival rate (Fisher & Adamson, 2021). Bevacizumab causes direct inhibition of tumour-associated angiogenesis, a direct anti-GB effect on VEGF receptor-expressing GB cells which leads to disruption of the GB stem cell microvascular niche and improved vascular function. It was also approved as a single agent in recurrent GB (Detti et al., 2021). While, carmustine, an intravenous alkylating agent works by attaching alkyl groups to DNA molecules, preventing them from replicating and repairing themselves. As a result, DNA cross-links form, preventing cancer cells from dividing and eventually killing them (Xiao et al., 2020). All the chemo-drugs provides longer survival times, but neither drug prevents GB recurrences, mainly due to the activation of a mechanism that enables immune evasion and causes drug resistance. Moreover, the main challenge for chemotherapy

in GB are BBB permeability, selective tropism toward cancer cell and with minimum or no adverse effects on the healthy cells.

Apart from the aforementioned SOC of GB, many other innovative therapeutic modalities currently studied extensively to improve the treatment of GB. For example, the use of tumor-treating fields (TTFs) which involve the continuous delivery of low-intensity alternating electric fields to disrupt mitosis of GB cells revealed a promising and improvement of progression free of GB (Fabian et al., 2019). RNA interference (RNAi) therapy which use small RNA molecules to silence specific gene is also a useful novel therapy for cancer treatment. A study by Yu *et al.* (2021) revealed that silencing of guanylate-binding protein 5 (GBP5) by RNAi inhibited the GB cell proliferation, migration and invasion in vitro and in vivo.

Recently, immunotherapy has been employed to tackle the complex immunosuppression microenvironment of GB cells. For example, chimeric antigen receptor (CAR) - T cell therapy which use the genetically modified T cells extracted from GB's patient to express a chimeric receptor that specifically target the GB cells. The modified T cells are then multiplied and reintroduced into the patient's bloodstream, where they can recognise and attack cancer cells. This therapy shows ability to elicit long-lasting immune responses, potential to cross the BBB and target tumour cells in the brain (Maggs et al., 2021).

Lastly, as the noticeable of male predominance in GB which implying a hormonal influence during disease development and progression, hormone treatment become a potential alternative treatment for GB patients. For example, Tamoxifen

(TAM), a selective oestrogen receptor modulator (SERM) promotes blocking of c-Jun N-terminal kinase (JNK) - dependent signalling pathway that result in reduced migration and invasion of GB cells (González-Mora & Garcia-Lopez, 2021).

2.3 Temozolomide (TMZ)

Temozolomide (TMZ) or methyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide is an imidazotetrazine derivative of DNA alkylating agent. It has been approved by the United States Food and Drug Administration (FDA) since 2005 as the first liner chemotherapy for GB (Stupp et al., 2014). It is sold under the brand name Temodal or Temodar.

2.3.1 Temozolomide treatment in glioblastoma

Currently, TMZ works as the standard concomitant and adjuvant chemotherapy with radiotherapy following the post-operative of the GB tumor (Delgado-López & Corrales-García, 2016). The supremacy of TMZ as the first liner chemotherapy is attributed to its permeability through the BBB, oral administration, stability in an acidic environment and no toxicity of superposition with other drugs (Hu et al., 2020).

TMZ is effective in disease control whereby it increased the median survival rate of the patients. The adjuvant chemoradiotherapy with TMZ after surgical resection of the tumor is proven to offer better median overall survival (OS) than the radiotherapy alone (14.6 vs 12.1 months) for the newly diagnosed GB patient. In addition, studies have reported that TMZ treatment increased the 5 years OS to 9.8% compared to radiotherapy alone at 1.9% (Stupp et al., 2009; Thomas et al., 2014).

Therefore, the standard care of adult GB patients includes TMZ concurrent with radiotherapy and maintenance of TMZ for six cycles.

Generally, TMZ is well tolerated and safe with mild to moderate adverse effect such as nausea, vomiting, fatigue, thrombocytopenia and neutropenia. However, cases of severe myelotoxicity such as aplastic anaemia and myelodysplastic syndrome have been reported in some GB patients (Strobel et al., 2019).

2.3.2 Mechanism of action of Temozolomide

TMZ can cross the BBB owing to its lipophilicity thus this chemo-drug can be administered orally (N. Singh et al., 2021). It is stable at acidic pH (<5) whereby at physiologic pH (>7), it is activated and converted to the metabolite 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC) (Stupp et al., 2014). Subsequently, MTIC is hydrolyzed to 5-aminoimidazole-4-carboxamide (AIC) and to methylhydrazine which are electrophilic methylated molecules that cause DNA damage (Reid et al., 1997).

The therapeutic advantage of TMZ is accredited to DNA adducts formation due to methylation of DNA purine bases. TMZ is a monofunctional alkylating agent which contains only one active group; thus, it could methylate the DNA at specific sites, favorably at the N7 position of guanine (N7-methylguanine/N7-MeG), O3 position of adenine (N3-methyladenine/N3-MeA) and O^6 position of guanine (O^6-methylguanine/O^6-MeG) (Wang et al., 2016). Alkylation of the O^6 site on guanine leads to the insertion of thymine instead of a cytosine opposite the methylguanine

during subsequent DNA replication. Consequently, activated the DNA mismatch repair (MMR) pathway to remove the O^6-MeG causing the formation of single- and double-stranded DNA breaks. Eventually, resulting in cell cycle arrest at G2/M and apoptosis (Lee, 2016; Arora & Somasundaram, 2019).

2.3.3 Temozolomide resistance in glioblastoma

Certainly, TMZ has improved the prognosis of GB. However, TMZ resistance is inevitable in patients suffering from early disease recurrence (Chien et al., 2021). A literature study by Hu et al., (2020) reported low efficacy of TMZ in 60-75% of GB patients. Therefore, a new therapeutic approach is urgently needed to improve its efficacy. There are two types of chemotherapy resistance; (i) primary resistance or intrinsic resistance and (ii) secondary resistance or acquired resistance which is (i) due to the inability to respond to the initial therapy and (ii) which is due to the inability to respond after the initial response to the same therapy (Bukowski et al., 2020). Generally, there are five major factors that contributed to the TMZ resistance in GB treatment, namely: MGMT status, mismatch repair (MMR), base excision repair (BER), autophagy activation and GB stem cells (GSCs) (Figure 2.1).

2.3.3(a) O^6-methylguanine-DNA methyl-transferase (MGMT) status

The mechanism of TMZ resistance in GB is tricky, the most notable factor is the O^6-methylguanine-DNA methyl-transferase (MGMT) status (Butler et al., 2020). The MGMT gene, located on chromosome 10q26.3, encodes a DNA repair enzyme that is highly conserved and widely expressed. MGMT functions at the DNA level by removing alkyl adducts from the O^6 position of guanine, thereby counteracting the lethal effects of any alkylating agents including the TMZ. The methyl moiety of the

O^6-methylguanine adduct is transferred to the MGMT protein during the repair process, where it is irreversibly inhibited. In contrast, faulty MGMT function causes the O^6-methylguanine adduct to persist, resulting in base misrepairing and mismatch repair futile cycling during DNA replication, eventually leading to cell cycle arrest and apoptosis. Eventually, methylation of the MGMT gene promoter causes loss/low levels of functional MGMT protein, resulting in inadequate repair of DNA alkylation in response to TMZ chemotherapy.

Therapeutic effects and prognosis of GB is improved in 45% of GB patients with a downregulated MGMT expression (Binabaj et al., 2018). Therefore, MGMT status is a crucial factor in the effectiveness of TMZ therapy (Singh et al., 2021). Clinical trial studies have shown TMZ therapy favoured patients with methylated MGMT promoter (m-MGMT) in their tumours more than those with unmethylated MGMT promoter (Yu et al., 2020). Furthermore, in recurrence GB, tumours with preliminary methylated MGMT promoter demonstrated a reduction of methylation ratio upon recurrence after TMZ therapy. This indicates resistance to TMZ is associated with a reduction in MGMT promoter methylation (Castro et al., 2021).

2.3.3(b) Mismatch repair

Mismatch repair (MMR) is vital for TMZ-induced apoptosis in GB (Ganesa et al., 2022). Mutation of MMR genes such as MutS homolog 6 (MSH6) increased the resistance to TMZ against glioma cell line (Ganesa et al., 2022). This established the association between MMR deficiency and TMZ resistance. Studies also reported that GB with downregulated MGMT expression can activate the MMR pathway and resulted in TMZ resistance (Caccese et al., 2020).

2.3.3(c) Base excision repair

Base excision repair (BER) mechanism detected and repaired the majority of N^7-methylguanine and N^3-methyladenine lesions in GB (Kaina & Christmann, 2019). Protein involved in the BER pathway was revealed to promote TMZ resistance resulting in a poor prognosis for the patients. High expression of N-methylpurine-DNA-glycosylase (MPG) and alkylpurine–DNA–N-glycosylase (APNG), the enzymes for BER mechanism were also reported in GB patients correlated with poorer OS in GB patients (Serrano-Heras et al., 2020).

2.3.3(d) Autophagy

Autophagy is the process by which cells degrade and recycle cellular components such as damaged or abnormal proteins and organelles. It is characterised by the isolation of damaged or unwanted cytoplasmic proteins and organelles, such as mitochondria and endoplasmic reticulum (ER), into autophagosomes, which are then marked for lysosomal degradation. This mechanism for removing damaged cellular contents protects cells from apoptosis. Chemotherapeutic agents and radiation both activate this process in tumour cells. Autophagy triggered by TMZ treatment is primarily a survival and protective mechanism, and it is thought to be a chemoresistance mechanism (Khan et al., 2021). Furthermore, autophagy has been linked to TMZ resistance by altering the cellular response to oxidative stress, which is a common mechanism of chemotherapy-induced cell death. Autophagy can help to reduce oxidative stress and promote cell survival in the face of chemotherapy by increasing cellular antioxidant defence mechanisms. Autophagy has been shown to regulate DNA repair, which is another mechanism by which cells can become

resistant to chemotherapy. Autophagy can help to restore the integrity of damaged DNA and reduce the effects of chemotherapy by promoting DNA repair. Therefore, autophagy, by promoting cell survival, reducing oxidative stress and DNA damage, and restoring cellular homeostasis, may thus contribute to the development of TMZ resistance in glioma cells (Elshazly & Gewirtz, 2023).

2.3.3(e) Glioblastoma stem cells

Comprehensive studies of GB revealed the presence of glioblastoma stem cells (GSCs) or also known as tumor-initiating cells within a heterogeneous cell population of GB tumor tissue in brain. These cells represent the therapy-resistant and slow-dividing malignant cells within the GB tumor. They exhibit the common characteristics of neural stem cells (NSC); in which they are able to self-renewal, proliferation indefinitely, and multipotency (Zhang et al., 2021). They also have NSC markers in common including CD133 and nestin. However, unlike the NSCs, these GSCs are mutated cells with altered genomic stability. Studies have found that the formation, maintenance, invasiveness, and recurrence GB are closely linked to the GSCs (Piper et al., 2021). Numerous studies also supported the concept that non-stem cancer cells (non-GSCs) are sensitive to cancer therapy while the stem cancer cells such as GSCs are relatively resistant to treatment (Torres et al., 2018). Thus, the presence of GSCs in the GB tumour catalysed the poor prognosis, high recurrent and high malignant relapse of GB after the first conventional therapy (Tang et al., 2021)

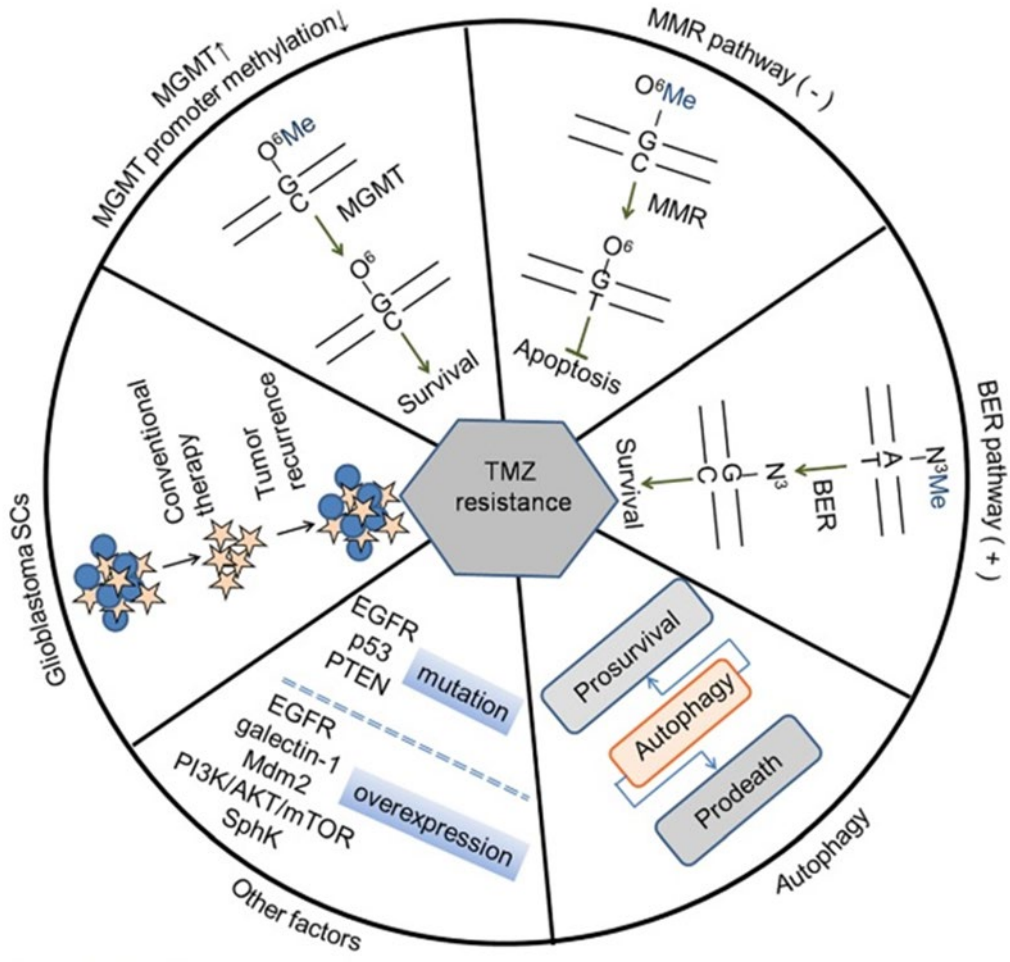

Figure 2.1 Resistance mechanism of Temozolomide (TMZ) in glioblastoma therapy (Yan et al., 2016)

2.3.3 (f) Other factors

A diversity of molecular pathways contributes to the acquired TMZ resistance (Singh et al., 2021). Other factors, in addition to DNA repair systems, have been shown to play important roles in TMZ resistance, including mutation of epidermal growth factor receptor (EGFR), phosphatase and tensin homolog (PTEN) and p53 (Gong et al., 2022). Studies also shown overexpression of EGFR, galectin-1, murine double minute 2 (MDM2), PI3K/AKT/mTOR pathway, and sphingosine-1-phosphate/sphingosine kinases (Sphk) contributed to TMZ resistant (Bahar et al., 2019; Bukowski et al., 2020; Videla-Richardson et al., 2022).

2.3.4 Combination therapy of Temozolomide in glioblastoma treatment

As forementioned, monotherapy using TMZ is incapable to achieve optimum treatment of GB which is rendered by drug resistance and heterogenicity of the tumor. The benefits of TMZ for patients could be improved by probing with combination chemotherapy regimens. Combination therapy or multimodal therapy is a therapeutic intervention that constitutes more than one type of therapy. In combination chemotherapy, there are three factors indicative of a successful therapy:(i) each component of the combination regimen should have a single-agent activity without any cross-resistance, (ii) combination drugs shown synergism in pre-clinical studies, and (iii) each component should have a separate safety criterion (Ghosh et al., 2018).

Advances in molecular profiling of GB and preclinical research discovered a panel of anti-GB agents. Preclinically, TMZ has been combined with small molecule inhibitors that act on their specific target genes. As an example, a study by Liu and colleagues (2017) demonstrated the synergistic cytotoxicity of TMZ combined with

micellarized cyclopamine (MCyp), a Sonic-hedgehog (Shh) signaling pathway inhibitor on glioma cells. In line with it, the Shh inhibitor was also reported to induce autophagy in $CD133^+$ GSCs suggesting it may overcome the chemoresistance of GSCs (Chen et al., 2020). On the other hand, low-dose TMZ combined with morphine (P-gp1 inhibitor) treatment resulted in a significant reduction in tumour development, implying superior long-term therapeutic (Iorio et al., 2017). The combination of TMZ and nutlin3a (a murine double minute 2 (MDM2) protein-protein interaction inhibitor) inhibited the development of p53 wild-type GB cells and results in considerable cell death (Wang et al., 2017). Furthermore, TMZ-induced apoptosis was enhanced when combined with sulforaphane (SFN) via downregulation of miR-21 via Wnt/β-catenin signaling in GB cells (Lan et al., 2015). A PI3K/mTOR dual inhibitor; XL765 in conjunction with TMZ also showed additive cytotoxicity in genetically varied GB xenograft (Zhao et al., 2019).

In clinical trial settings, Phase III randomised trial by Herrlinger (2019) reported combination treatment of lomustine with TMZ is more effective than TMZ monotherapy in GB patients with a median survival of 16.7 months. Whereby, bevacizumab (BCM), an angiogenic agent targeting the vascular endothelial growth factor-A (VEGF-A), also demonstrated an increased survival rate of GB patients when combined with TMZ. However, BCM + TMZ treatment exhibits peculiar toxicities that represent causes of morbidity and mortality. The toxicity profile of this combined treatment includes hypertension, proteinuria, haemorrhage, thromboembolism, arterial thromboembolic events (ATEs) and spontaneous perforation of the gastrointestinal tract (Grill et al., 2018). Therefore, alternative combination regimes with lesser side effects are necessary to help the GB patients.

Table 2.1 summarized the different combination regimens of TMZ in the pre-clinical settings, while Table 2.2 summarized the combination chemotherapy of TMZ in the clinical trial settings.

.

Table 2.1 Combination chemotherapy of TMZ in the pre-clinical development for GB.

Combination treatment	Target	Reference
TMZ + micellarized cyclopamine (MCyp)	Sonic-hedgehog (Shh) pathway	Liu et al., 2017
TMZ + morphine	P-glycoprotein 1(P-gp1)	Iorio et al., 2017
TMZ + nutlin3a	MDM2-p53 associated signaling pathways	Wang et al., 2017
TMZ + XL765	PI3K/mTOR pathway	Zhao et al., 2019
TMZ + SFN	Wnt/β-catenin/TCF4 signaling pathway	Lan et al., 2015
TMZ + SGT-53	Nanomedicine	Kim et al., 2015
TMZ + bromodomain inhibitor (JQ1) via Tf-NPs	Nanodelivery	Lam et al., 2018

Table 2.2 Combination chemotherapy in the clinical trial of TMZ for GB.

Molecule	Target	GB type	Stage of testing	References
TMZ + lomustine	DNA alkylating	GB	Phase III	NCT01149109
TMZ + bevacizumab + tarceva	EGFR inhibitor	GB	Phase II	NCT005255525
TMZ + avastin + irinotecan	VEGF-A + topoisomerase I inhibitor	Unresectable/Multifocal GB	Phase II	NCT00979017
TMZ + LY2228820 (Ralimetinib)	p38 mitogen-activated protein kinase (MAPK) inhibitor	GB	Phase I/II	NCT02364206
TMZ + LX3397 (Pexidartinib)	Colony-stimulating factor 1 receptor (CSF1R)	Recurrent GB	Phase I/II	NCT01790503

2.4 Phytomedicine in cancer therapy

Phytomedicine or phytotherapy or herbal medicine is defined as an herbal-based traditional medical practice that uses a variety of plant components in preventative and therapeutic treatment. Phytochemicals derived from the natural plant are considered to have minimal adverse effects compared to the established chemotherapy agents. Its competency improved the treatment outcome and also lessen the cost of cancer chemotherapy which directly reduces the burden on patients and society (Chaudhary et al., 2015). The efficacy of prominent phytochemicals such as vinca alkaloid (vinblastine and vincristine), taxol analog (paclitaxel) and alkaloid (camptothecin) have been proven in clinical practice for various types of cancers (Habli et al., 2017; Choudhari et al., 2020). In addition, phytochemicals are also targeted to overcome drug resistance in cancer therapy. The anticancer efficacy of medicinal plants has been investigated widely at both in vivo and in vitro levels. Mechanism of action of medicinal plants includes reducing the carcinogenic process by scavenging free radicals (Pandey et al., 2020), inhibiting the cancer cell survival and proliferation (Mamat et al., 2020), and reducing the invasiveness and angiogenesis of the tumors (Majumder et al., 2019).

2.4.1 Safety of phytomedicine

Many medicinal plants were investigated and shown noteworthy potential as anticancer agents. They promote safer, environmentally friendly, cost-effective, and less harmful treatments for cancers. However, their preparation and therapeutic dosage are not well documented which require a genuine understanding of plant qualities, safety, and other pertinent information (Iqbal et al., 2017; Choudhari et al., 2020).

2.5 *Quercus infectoria*

In this study, the anticancer role of medicinal plant known as *Quercus infectoria* (QI) was investigated. Botanical description of QI is described in Table 2.3. It is a deciduous, evergreen small shrub growing about four to six feet tall (1 to 2 meters), crooked, with an ovate-oblong shape, sinuate dentate leaves (Askari et al., 2020). The galls of QI are excrescences formed upon the young branches parasitized by the gall wasps, Cynips gallae-tinctoriae Olivier (Hapidin et al., 2015). The galls are typically round or oblong in shape and can range in size from a few millimetres to several centimetres. The numerous horny protuberances on the smooth outer surface give it a rough touch. The outer surface is greyish brown to brownish black and the inner surface is yellow. The presence of pores on the uneven surface indicates infection as shown in Figure 2.2.

2.5.1 The ethnopharmacological activity of *Quercus infectoria* (QI) gall

QI gall has been used widely for decades, particularly in traditional Chinese medicine (TCM) and Indian traditional medicine (Ayurveda) for various diseases. For example, in TCM, QI gall was used as an astringent for skin sores and inflammation, while in Ayurveda, it has been used as a dental powder, mouthwash, and toothache relief (Elham et al., 2021). It is also used as a home remedy for sore throat and chronic diarrhoea. In Malaysia, the QI gall or manjakani is infamously consumed by females as a health supplement and for postpartum care (Bustami et al., 2020). In traditional Iranian medicine (TIM), it was used as a grey hair-dying agent and as an antiperspirant or deodorant (Askari et al., 2020). Other ethnomedicinal activities of QI gall include antiviral, antidiabetic, larvicidal, antibacterial, anti-ulcer,