INVESTIGATION OF QUERCUS INFECTORIA (QI) METHANOLIC EXTRACT CYTOTOXICITY EFFECT ON GLIOMA AND NEURAL STEM CELL

By

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LIST OF ABBREVIATONS

APS	Ammonium Persulfate
BSA	Bovine serum albumin
BSC	Biosafety cabinet
Ca^{2+}	Calcium
cDNA	Complementary DNA
CO_2	Carbon Dioxide
C_t	Cycle threshold
DBTRG-05MG	Denver Brain Tumor Research Group 05
DMSO	Dimethylsulfoxide
DNase	Dioxyribonuclease
dNTP	Dioxyribonuclease triphosphate
DPBS	Dulbecco's phosphate buffer saline
FBS	Fetal Bovine Serum
H9-hNSC	GIBCO® H9 Human Neural Stem Cell
Mg^{2+}	Magnesium
NSCs	Neural Stem Cell
PBS	Phosphate Buffer Saline

PKM2	Pyruvate Kinase Muscle 2
PPIA	Peptidylprolyl isomerase A
QI	*Quercus Infectoria*
qRT-PCR	Qualititative real time polymerase chain reaction
RNase	Ribonuclease
RNA	Ribonucleic acid
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel electrophoresis
SFM	Serum Free Medium
TAT	Targeted Anti-Cancer Therapy
TEMED	Tetramethylethylenediamine

LIST OF UNITS

L	Litre
ml	Mililitre
µl	Microlitre
g	Gram
mg	Miligram
µg	Microgram
ng	Nanogram
cm	Centimetre
mm	Milimetre
µm	Micrometre
M	Molar
mM	MiliMolar
%	Percent
°C	Degree Celcius
A	Absorbance

Penyiasatan Terhadap Kesan Ekstrak Metanolik *Quercus infectoria* (QI) ke Atas Sel Glioma dan Sel Stem Neural

ABSTRAK

Glioblastoma Multiforme (GBM) adalah sub-jenis kanser otak yang paling berbahaya. Rawatan standard pada masa kini adalah terhad dari segi keberkesanan selain kemungkinan untuk menerima kesan sampingan. *Quercus infectoria* (QI) dilaporkan mempunyai aktiviti antioksidan serta bersifat anti-kanser. Dalam kajian ini, kesan ekstrak QI ke atas titisan sel GBM (DBTRG-05MG) dan sel stem neural (H9-hNSC) dinilai. Kaedah Soxhlet digunakan untuk mengekstrak QI menggunakan 70% methanol and 100% methanol sebagai pelarut. Aktiviti antioksidan ekstrak ditentukan dengan teknik DPPH penangkap radikal bebas. Sel dikultur dan kepekatan optimum ditentukan melalui teknik MTT assay. Analisis genomik dan proteomik ke atas sel yang dirawat dengan QI dilakukan menggunakan tindak balas rantai polimerase masa nyata (qPCR) serta Western Blot. Ekstrak 100% metanolik QI (100%-Met) didapati menjana hasil yang lebih banyak dan signifikan (78.46%) berbanding 70% metanolik QI (70%-Met) (43.7%). Walaupun tidak signifikan, 100%-Met menunjukkan aktiviti antioksidan yang lebih tinggi berbanding 70%-Met (94.62% vs 91.33%). 100%-Met (IC_{50}:70.76 µg/ml) dan 70%-Met (IC_{50}:71.55 µg/ml) didapati menunjukkan kesan anti-pembahagian ke atas DBTRG-05MG. Selepas rawatan, DBTRG-05MG menunjukkan penurunan ekspresi gen penanda sel kanser, PKM2. Gen CASP3, gen utama dalam dalam laluan apoptotik sel, diekspres lebih banyak dan signifikan oleh DBTRG-05MG yang dirawat dengan 100%-Met berbanding kumpulan yang tidak dirawat. Bagaimanapun, ekspresi gen CASP3 dalam H9-hNSC yang dirawat dengan 100%-Met tidak berbeza secara signifikan dengan kumpulan kawalan yang tidak dirawat, menunjukkan bahawa ekstrak QI tidak

mempunyai kesan apoptotik ke atas H9-hNSC. Analisis Western Blot juga menghasilkan jalur jelas pada 35 kDA yang mewakili caspase-3 oleh DBTRG-05MG yang dirawat oleh QI, tetapi tidak ditemui dalam H9-hNSC yang dirawat dengan QI serta kumpulan kawalan yang tidak dirawat oleh QI. Kesimpulannya, ekstrak QI berpotensi menjadi ubat anti-kanser di samping tidak mempunyai kesan toksik terhadap sel bukan kanser seperti sel stem neural. Namun, kajian selanjutnya diperlukan bagi menerangkan kesan penurunan ekspresi CD133 dari sudut aktviti dan integiriti sel H9-hNSC.

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Investigation of *Quercus infectoria* (QI) Methanolic Extract Effect on Glioma and Neural Stem Cell

ABSTRACT

Glioblastoma multiforme (GBM) is the most malignant subtype of brain cancer. Current standard treatment are limited in effectiveness and impose additional side effect. *Quercus infectoria* (QI) was reported to be anti-carcinogenic and exhibit inherent antioxidant activity. This study evaluated the effect of QI extract on GBM cell line (DBTRG-05MG) and neural stem cell line (H9-hNSC). Soxhlet extraction was performed using 70% and 100% methanol solvent. Antioxidant activity was measured using DPPH free radicals scavenging assay. Cells were cultured and optimal extract concentration was determined *via* MTT assay. The genomic and proteomic analysis of the QI-treated cells were performed using real-time PCR and Western Blot respectively. We observed that 100% methanolic QI extract (100%-Met) produced significantly higher yield (78.46%) compared to 70% methanolic QI extract (70%-Met) (43.7%). Despite not significant, 100%-Met showed higher antioxidant activity compared to 70%-Met (94.62% vs 91.33%). 100%-Met (IC_{50}:70.76 µg/ml) and 70%-Met (IC_{50}:71.55 µg/ml) were found to show anti-DBTRG-05MG proliferation. Following treatment, DBTRG-05MG showed reduced cancer cell marker (PKM2) gene expression. CASP3 gene, a key player in apoptotic pathway was expressed significantly higher in 100%-Met-treated DBTRG-05MG compared to non-treated group. However, CASP3 expression in 100%-Met-treated H9-hNSC was not significantly different to the non-treated control, indicating QI extract did not possess apoptotic effect on H9-hNSC. Similarly, Western Blot analysis produced clear band at 35 kDA which represent caspase-3 protein for treated DBTRG-05MG, but absent in treated H9-hNSC and non-treated control. In conclusion, QI

extract is a potential anti-cancer drug with negative cytotoxicity response towards non-cancerous cell such as NSCs. Further study is warranted to elucidate the precise effect of reduction of CD133 expression on H9-hNSC activity and cell integrity.

CHAPTER 1: INTRODUCTION & LITERATURE REVIEW

1.1 Glioma

Glioma is a general term used to describe any tumor that derived from the supportive cells or glial cells of the brain. The main functions of glial cells are to provide protection, nutrients, oxygen and insulation for the nerve cells neurons. The occurrence of glioma can therefore affect the neurons and consequently damage the brain. A glioma can be either benign or malignant (cancerous). Glioma comprises more than 70% of all brain tumors which happen in adults as well as in paediatric population wordwide (Ohgaki & Kleihues, 2005). The general age-adjusted incidence for all gliomas stretches from 4.67 to 5.73 per 100 000 persons (Ostrom et al., 2014). Ohgaki & Kleihues (2005) stated that, mortality and incidence rates in both male and female populations are similar for most places. In Malaysia, malignant glioma or brain cancer is the second most frequent cancer in children behind leukaemia. Also, it is among ten most frequent cancer in adults (Ministry of Health, 2007).

According to American Brain Tumor Association (2014), there are three types of glial cells that can give rise to glioma, namely astrocytes, oligodendrocyte and ependymal cells. They can give rise to astrocytoma, oligodendrogliomas and ependymoma respectively (Figure 1.1). Mixed gliomas are those with a mixture of these different gliomas. Astrocytoma is the most common type of glioma, which account for about 75% of all gliomas (CBTRUS, 2012). World Health Organization (WHO) grading system is generally used to classify astrocytoma,

based on the specific histology features and malignant behaviour. The grading includes pilocytic astrocytoma (Grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma multiforme (grade IV). Currently, glioblastoma multiforme (GBM) or also known as Glioblastoma is the most common glioma histology and at the same time is the most malignant subtype of brain cancer (Ehtesham, Stevenson & Thompson, 2005). Due to its high degree of malignancy and devastating outcome, GBM is always the top concern in various ongoing scientific researches or clinical trials related to brain cancer, so as in present study.

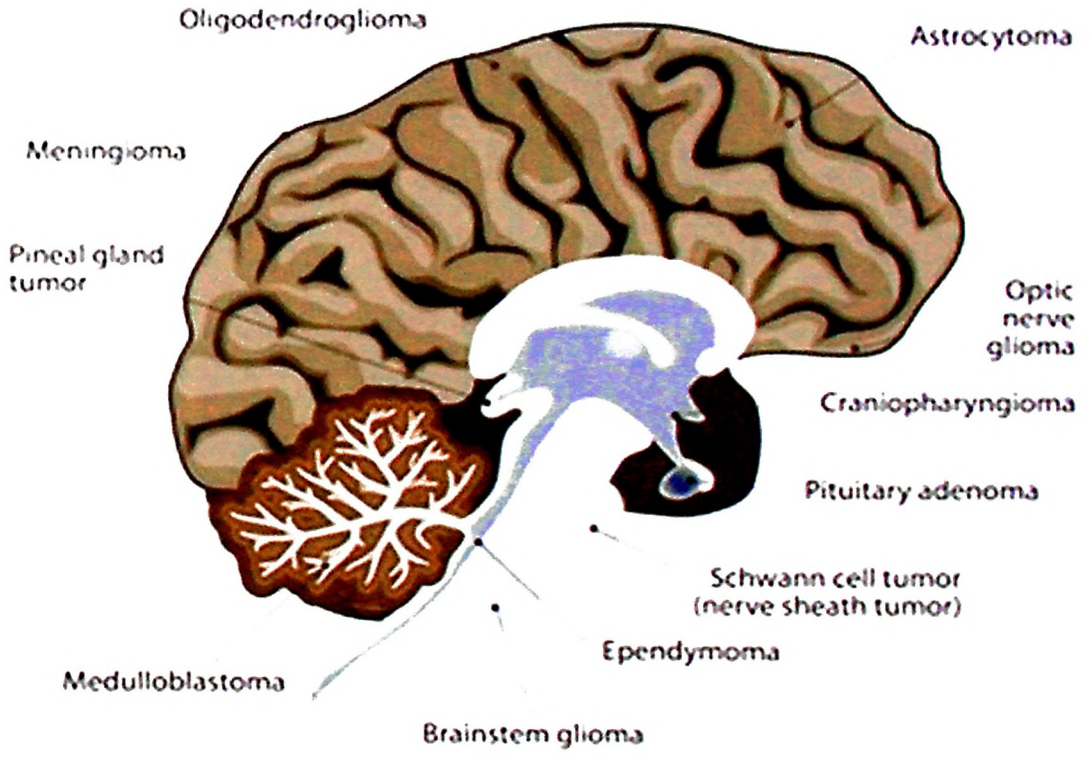

Figure 1.1 Different types of primary brain tumors. Astrocytoma is the most common primary brain tumor with GBM being the most malignant grade of astrocytoma. (Source: https://www.uniklinik-freiburg.de)

1.2 Glioblastoma Multiforme (GBM)

1.2.1 Causal Factor of GBM

Despite GBM has been studied widely, the cause of GBM until now remains unclear and more extensive researches are still ongoing. Some of the possible factors are genetic abnormalities, exposure to ultraviolet rays and stress. However, more studies are required to verify these. The tumor is very malignant due to high invasive rate as well as supported by huge network of blood supplies.

1.2.2 Clinical Outcome of GBM

Since they can grow very rapidly, the symptoms of GBM are generally caused by the pressure increase in the brain. Some of the related manifestation includes persistent headache, nausea, and vomiting. Additional symptoms can develop in relation to where the tumor is such as blurred vision, personality changes, weakness, and speech difficulties (American Brain Tumor Association, 2014).

1.2.3 Incidence and Prognosis of GBM

With a yearly age-adjusted incidence of 3.0 to 3.6 per 100,000 persons, GBM is the leading primary intra-axial glioma across the world (Helseth et al., 2010). Despite its high prevalence, the prognosis of patients with GBM is very poor. By using the existing standard of care, life expectancy of a GMB patient is estimated to be 14 months following diagnosis (Van Meir et al., 2010). Ehtesham, Stevenson & Thompson (2005) also stated that the median survival period of GBM is below a year, and almost no chance of surviving for more than two years.

1.3 Current Treatment of GBM

At present, the standard treatment for glioma and GBM relies on the effectiveness of surgery and chemo-radiation therapy. During surgery, the surgeons perform maximal surgical resection of the tumor within safe margins depending on its location. This is to remove as much tumor tissue as possible. Following surgical procedure, the patient will undergo radiotherapy over a period of weeks. The aim is to kill the remaining tumor using high energy beams. It was found that radiotherapy can improve cancer-specific survival and overall survival of elderly GBM patients who underwent the treatment (Scott et al., 2011). As a follow-up, chemotherapy will be employed. Generally the drug temozolomide is used for the treatment of GBM. It is reported patients who received adjuvant temozolomide with radiotherapy for glioblastoma shows greater survival than those who received the radiotherapy treatment alone (Stupp et al., 2009).

1.4 Limitation of Current GBM Treatment

The current standard treatment of GBM can help to control the tumor but they are not perfect. In order to treat GBM ideally all of the tumor has to be completely removed through surgical procedure. However due to its invasive nature, this task is very difficult even for a skilled surgeon. The tumors often located at the sensitive areas of the brain, causing thorough surgical removal very challenging. Besides, neurosurgery is a very sensitive procedure. The patients can be susceptible to the risk of infection, bleeding, as well as nerve damage. Radiotherapy and chemotherapy are good addition to the treatment of GBM following a surgery. However, they can only control but not totally kill the gliomas. GBM is a highly

aggressive tumor and often develop resistance to the treatment. This allows them to regrow and consequently lead to recurrence. Moreover, the chemo-radiation treatment are known to produce unwanted side effects on the patients such as fatigue, headaches, nausea, vomiting, hair loss, and weakness. These will not only place extra burden on the patients, but also reduce compliance to the treatment. As a result, the tumor cannot be completely healed, leading to possible recurrence. Thus, an alternative treatment targeting GBM specifically to reduce unwanted side effects and increase cancerous cell killing efficiency is necessary.

1.5 Targeted Anti-Cancer Therapy (TAT)

In order to address the limitation of the current treatment for cancerous cells, targeted anti-cancer therapy (TAT) is introduced as a superior alternative. In contrast to standard surgery and chemo-radiation therapy, TAT employs systemic administration of drugs with specific mechanisms or delivery agents that specifically act on cancer cells. When activated, it can trigger deterioration of the malignant process while minimizing unwanted effects on the normal tissues (Junjie et al., 2012). Since the therapy particularly targeting cancel cells, normal healthy cells will be unaffected which therefore lead to reduction of side effects as produced by the conventional radio-chemotherapy treatment. This alternative treatment can increase effectiveness of the anti-cancer drug and simultaneously reduce the burden place on the treated patients. It is recently found that neural stem cell can be a suitable delivery agent for TAT in treating gliomas (Aboody et al., 2000).

1.6 Neural Stem Cells (NSCs)

Neural stem cells (NSCs) are group of stem cells in the nervous system. They are capable of self-renewing and differentiating into neurons and glias, such as astrocytes and oligodendrocytes. These cells show promise for neural repair after injury or brain disease. NSCs can migrate extensively throughout a pathologic brain (Shah et al., 2005). Aboody et al. (2000) proved that NSCs exhibit significant tropism toward glioma. This finding implied that NSCs might able to be used as vehicle to deliver anti-cancer drug and specifically targeting the glioma, which can help to minimize side effects resulting from the effects on normal healthy cells. Therefore, NSCs are proposed as a potential biological vehicle to carry anti-cancer drug toward glioma in the brain for TAT. However, prior to applying NSCs for TAT, cytotoxicity test of the drug on the NSCs and also the glioma cell are necessary to ensure that the anti-cancer drug can efficiently kill the glioma but at the same time does not possess cytotoxicity effect to the NSCs.

1.7 *Quercus infectoria* (QI) as a Potential Anti-Cancer Compound

A variety bioactive compounds in traditional plants have gained interest among researchers such as flavonoids, tannins, alkaloids, saponins, and phenolic compounds due to their potential medicinal effects (Hill, 1952). *Quercus infectoria* (QI) is also known as oak tree or Manjakani by the locals (Figure 1.2). QI galls are round-shaped and grow on the young branches of the oak tree (Figure 1.3). Study on the QI galls have been going for few years and they are taken as health supplement during post-delivery care. It is one of the traditional plants that is reported to produce antioxidant (Nur Syukriah et al., 2014). The major bioactive

compound found in QI gall extract are tannic acid and gallic acid which contribute to the antioxidant activity of the plant (Nur Syukriah et al., 2014). This inherent antioxidant activity of tannic acid and gallic acid also reported to contribute to the anti-carcinogenic effect which can be useful in treating tumor (Hasmah et al., 2010). The potential anti-proliferative activity possessed by QI hold promises for development of alternative anti-cancer drug to treat different type of tumor. In this study, the effectiveness of QI gall extract to treat glioma was investigated.

Figure 1.2 QI Oak tree. (Source: http://www.klactive.com)

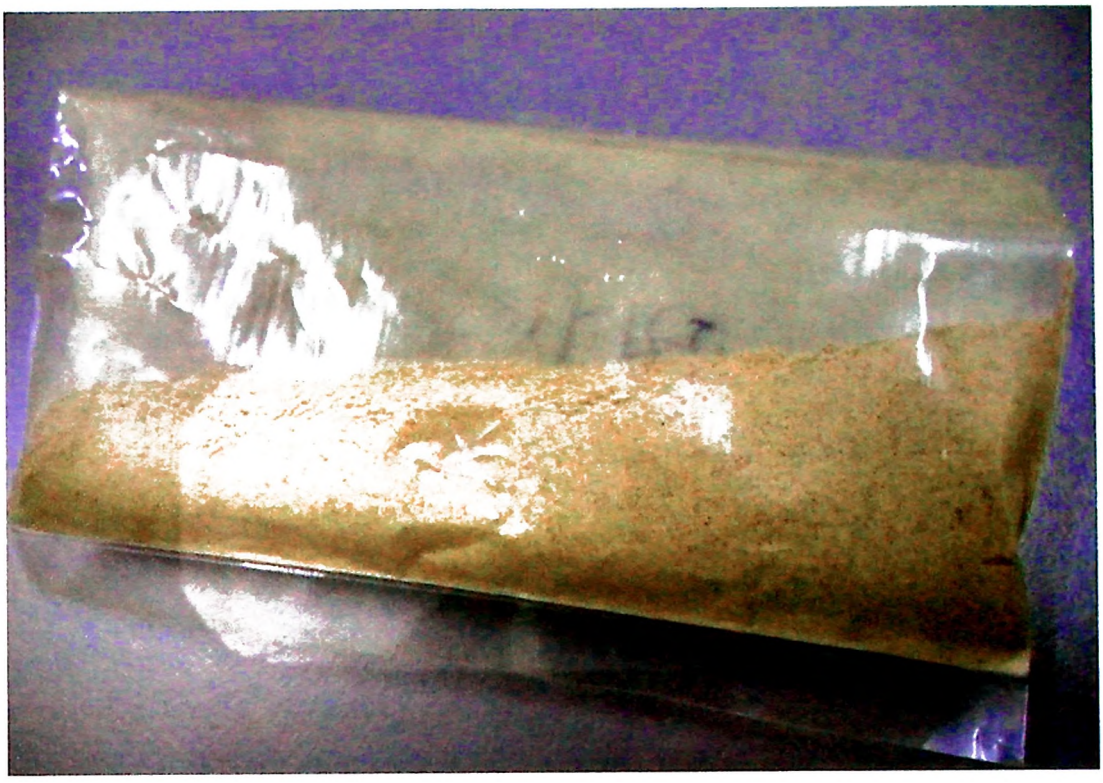

Figure 1.3 QI powder.

1.8 Extraction of Phytochemical Constituent in QI

Polar compound can be simply extracted using polar solvent. For that reason, the extraction of active compounds greatly depend on the polarity of the solvent (Goli, Barzegar, & Sahari, 2005). Choosing the most suitable solvent for the bioactive compounds extraction procedure is critical because it will affect the quantity as well as the quality of the extract. Polar solvent such as methanol, ethanol, acetone and aqueous are commonly used for plant extraction. Moreover, difference in the percentage of the solvent content could also impose diverse effect on the final extract yield. From previous study by Nur Syukriah et al. (2014), it was shown that 70% methanol and 100% methanol yield among the highest quantity of bioactive

compounds from the QI extraction procedure. The result was shown as the diphenylpicrylhydrazyl (DPPH) free radicals scavenging (%) and the total phenolic content of the extract produced by different type of solvents. This suggest that 70% methanol and 100% methanol are suitable solvent for QI extraction. Thus, in this study, active compound of QI were extracted using 70% and 100% methanol respectively.

Soxhlet extraction method is widely used to harvest extract from plant sample when the compound of interest has a low solubility in a solvent (Figure 1.4). The advantage of this method is that the extraction can be performed with minimal monitoring. Furthermore, instead of using multiple portion of solvent, only one batch of the solvent is efficiently recycled throughout the process. The boiling point of methanol solvent is around 64.7°C. This temperature is much lower than the boiling point of aqueous solvent, which further ease the extraction process. The solvent is eliminated using rotary evaporator to yield the extracted compound. The final dried QI extract is in the form of crystals.

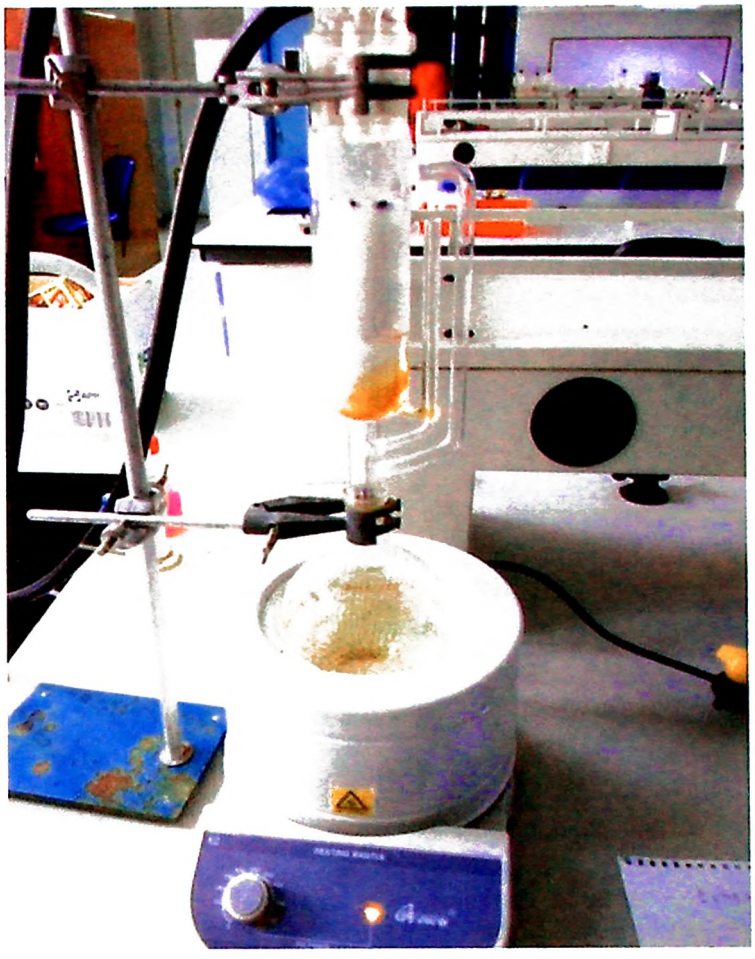

Figure 1.4 Soxhlet extraction method of QI extract.

1.9 Caspase-3 in cell apoptosis regulation

Apoptosis is a physiological process of cell death (Vaux & Korsmeyer, 1999). It is very important for the survival of multicellular organisms by removing infected or damaged cells which can affect normal function (Portt, Norman, Clapp, Greenwood, & Greenwood, 2011). Caspases, the main effector enzyme of apoptosis, present in normal cells as inactive precursor. Their activation is regulated by proteolytic processing between the p20 and p10

subunits (Figure 1.5). Caspase-3 is a caspase protein that act together with caspase-8 and caspase-9. It is encoded by the CASP3 gene. The presence of this gene will mark greater killing efficiency of tumor cells. Therefore in this study, we expect that more caspase-3 will be expressed in the drug-treated glioma cell lines than the non-treated control cells. However at the same time, the NSCs must remain unharmed. This can be tested using real-time polymerase chain reaction (qPCR) and Western Blot, to detect RNA and protein expression respectively.

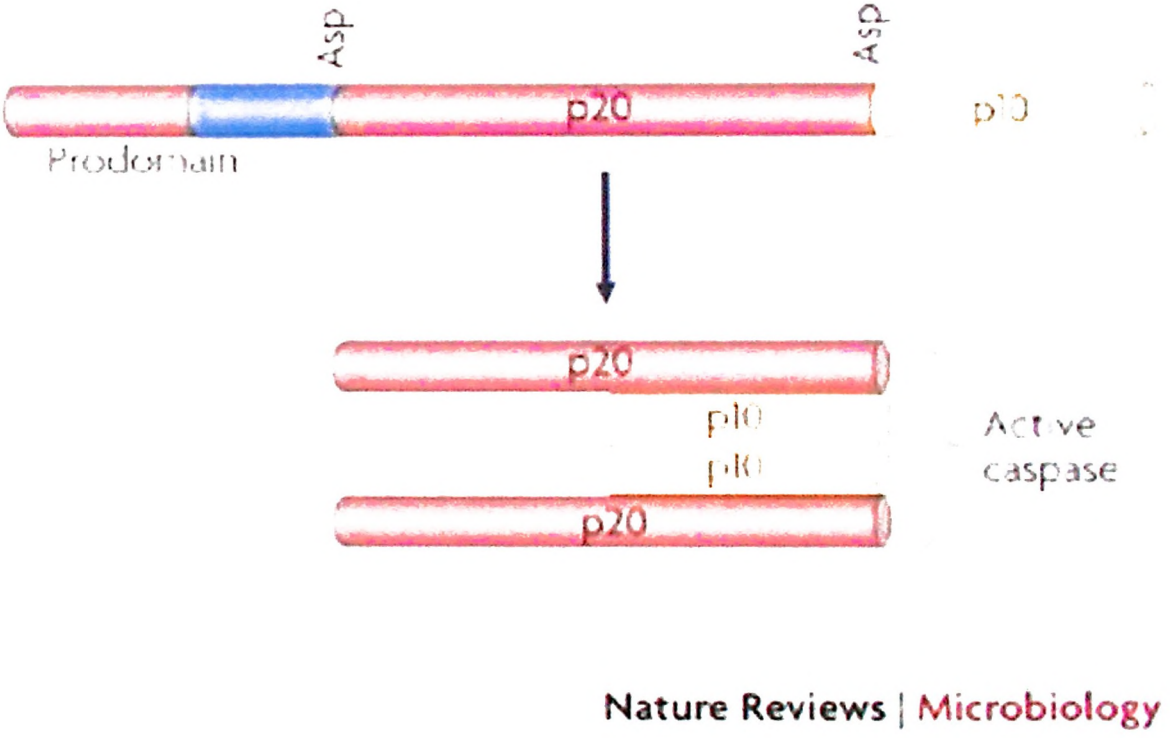

Figure 1.5 Activation of caspase (Clarke & Tyler, 2009)

1.10 Pyruvate Kinase Muscle 2 (PKM2)

Pyruvate Kinase Muscle 2 (PKM2) is a protein that catalyzes the final step in the glycolytic pathway. One of the main characteristic of cancer cells is the enhanced conversion of glucose to lactate which stimulates cell growth. Increased PKM2 level accelerates lactate production in cancer cells (Luo & Semenza, 2012). The protein is encoded by the PKM2 gene which is regulated by oncogenes as well as tumour suppressors (Wong, De Melo, & Tang, 2013). It is previously found that PKM2 gene expression is higher in various human cancers, compared to their normal counterparts (Bluemlein et al., 2011). Therefore, the quantification of PKM2 expression could be used to differentiate cancer cells from normal cells.

1.11 Prominin-1 (CD133)

Prominin-1 (CD133) is a pentaspan membrane glycoprotein, encoded PROM1 gene. It is commonly expressed as the cell surface protein on many stem cell populations. Thus, CD133 has been largely exploited as stem cell biomarker for the identification of stem cells from a various type of pathological or normal tissues (Li, 2013). In this study, the quantification of PROM1 or CD133 expression could be used to differentiate stem cells from normal or cancerous cells.

1.12 Real-time Polymerase Chain Reaction (qPCR)

Real-time Polymerase Chain Reaction (qPCR) (Figure 1.6) is a molecular biology technique which is a modification from the conventional PCR. It is used to quantify the DNA of interest in real-time, allowing the researchers to pinpoint the beginning of exponential phase of the

DNA replication process. A commonly used detection method is by the mean of SYBR Green, a florescent dye that binds specifically to the double stand of DNA molecules. During the amplification of the target DNA, the doubling of the molecules lead to the increase of SYBR Green binding and eventually increase the detection (fluorescence).

qPCR is very sensitive as little different in the concentration of the sample mixture can be hugely multiplied, following the amplification of the target DNA. Once the reading increase significantly above background level, qPCR will detect the specific cycle of the amplification and record it. This reading is called threshold cycle (CT) in which the DNA quantity has reach the threshold level, and the exponential phase of the amplification has started. The lower the CT values, the earlier the threshold level is reached, and the higher amount of target DNA is expected in the sample. Conversely, the higher the CT values, the later the threshold level is reached, and the lower amount of target DNA is expected in the sample. In this study, we expect lower CT values of CASP3 gene in the drug-treated glioma cell line than the untreated control due to the expression of CASP3 gene in drug-treated glioma. Also, we expect PKM2 gene expression in glioma cell and CD133 in NSC cell line for characterization of respective cell lines.

Figure 1.6 Real-time PCR (qPCR).

1.13 Western Blot

Western blot is an analytical technique used to detect specific protein expression from the sample, by exploiting the specificity of the antibody-antigen interaction. It employ gel electrophoresis to separate proteins based on molecular weight (Mahmood & Yang, 2013). The protein are then transferred or 'blotted' to a nitrocellulose membrane. This generates protein band on the membrane which will be incubated or stained with specific antibodies, related to the desired target protein. The result of Western Blot can be viewed using digital imaging system or by development of photographic film.

1.14 Research Objectives

General Objective:

To study the capability of *Quercus infectoria* (QI) extract in killing glioma cell lines without affecting neural stem cells.

Specific Objective:

1. To perform crude extraction of QI using Soxhlet extractor.
2. To determine antioxidant activity of the QI extract using DPPH antioxidant assay.
3. To determine optimum half-maximal inhibition concentration (IC_{50}) of QI extract via MTT assay and cell culture technique.
4. To analyze RNA from the cultured cell lines following treatment through qPCR.
5. To analyze protein from the cultured cell lines following treatment through Western Blot.

1.15 Significance of Study

Prevention of GBM is extremely difficult due to the unclear causal factor. The current treatment available for this condition has limited effectiveness as a result of highly aggressive nature of glioblastomas. Complete removal of cancerous cells from the brain is almost impossible. The locations of the gliomas are normally around the sensitive areas of brain, leading to increased susceptibility to the risk of infection, bleeding, and nerve damage. Complementary treatment such as radiotherapy and chemotherapy can present unwanted side effects, causing extra burden to patients. The use of NSCs as alternative treatment is a hope to address these limitation posed by the current approaches. Autologous transplantation can

potentially deliver anticancer drug or compound specifically to the glioma due to the inherent tropism, without generating significant side effect from the rejection and interaction with the normal healthy cells. Thus, in this study, investigation on the effectiveness of QI extract to kill glioma cells without affecting the NSCs was proposed.

CHAPTER 2: METHODOLOGY

2.1 Quercus Infectoria (QI) Crude Extraction

The extraction procedure was carried out by using Soxhlet Extractor set. First, 15 g of powdered galls were weighed. The powder was then placed in a cellulose thimble. Four hundred and fifty ml of 70% methanol was placed at the bottom of the apparatus. The extraction process was done for 6 hours at the boiling point (60°C) of the methanol. After that, the crude extract solution was placed in the rotary evaporator at 40°C to remove the solvent, leaving partially dried extracted yield. The crude extract was further air-dried in fume hood for 3 days. Finally, the yield was collected and weighed. All these steps were then repeated using 100% methanol. The amount of yield of the extracted samples were calculated using the following equation:

$$\text{Percent of yield extraction} = \frac{\text{Final weight (g)}}{\text{Initial weight (g)}} \times 100\%$$

The final extract yield was placed in a 15 ml Falcon tube, sealed and stored in -20°C refrigerator for the following steps.

2.2 DPPH Free Radicals Scavenging Assay

First, extract solution was prepared by dissolving 0.025 g of dry 70% methanolic QI extract in the 10 ml methanol in order to produce the final concentration of 2.5 mg/ml. Next, 77 µl of the extract solution was mixed with 3 ml of 6×10^{-5} M methanolic solution of DPPH. Then,

the mixture was shaken vigorously and placed in dark area for 30 minutes at room temperature. After 30 minutes, absorbance of the mixture was read at 517 nm in triplicates by using spectrophotometer. Radicals scavenging activity of the extract was calculated by using the formula:

$$\text{Radicals scavenging activity} = \frac{(A_{control} - A_{extract})}{A_{control}} \times 100\%$$

$A_{control}$ is the absorbance reading of control solution, given by the spectrophotometer and $A_{extract}$ is the absorbance reading of the mixture containing the QI extract. All these steps were then repeated using 100% methanolic QI extract.

2.3 Cell Culture

2.3.1 Denver Brain Tumor Research Group 05 (DBTRG-05MG) Cell Lines

2.3.1.1 Revival from Cryopreserved Stock

Denver Brain Tumor Research Group 05 (DBTRG-05MG), a GBM cell line was stored in liquid nitrogen storage before use. For cell revival, the cryo vial was removed and place temporarily in the water bath at 37°C. The cells were thawed by swirling movement of the vial until most of the ice melted. Next, the cells in the cryo vial were pipetted out and transferred into a 15 ml centrifuge tube. Roswell Park Memorial Institute (RPMI) medium was used for DBTRG-05MG. One ml warm complete RPMI medium (Table 2.1) was slowly added into the centrifuge tube. Another 1 ml of complete RPMI medium was added and the tube was centrifuged for 5 minutes at 1500 rpm to remove the Dimethyl Sulfoxide (DMSO)

from the cryopreservation. After centrifugation the supernatant was carefully removed, leaving the cell pellet. One ml of fresh complete RPMI medium was added to the tube and the cell pellet was resuspended in medium. After that, the 1 ml DBTRG-05MG cell suspension was transferred into the T25 flask, containing 4 ml of fresh complete RPMI medium. The revived cells were incubated at 37°C incubator with 5% CO_2 and humidity. They were left to properly attach as well as to multiply inside the flask.

Table 2.1 Complete RPMI medium for DBTRG-05MG.

Components	**Volume (ml)**
RPMI	44.5
Fetal Bovine Serum (FBS)	5
Penicillin-Streptomycin (Penstrep)	0.5
Total	**50**

2.3.1.2 Passaging DBTRG-05MG Cells

DBTRG-05MG cells were cultured in T25 flasks. All cell culture procedure were performed under sterile condition inside the Biosafety Cabinet (BSC) in the Cell Culture Laboratory. Pipette tips were autoclaved before use to ensure sterility. The instruments such as pipettes, 15 ml centrifuge tubes and waste container were first disinfected using 70% ethanol spray solution and exposed to the ultraviolet (UV) light for 10 minutes. At the same time, the related reagent such as the RPMI media and Trypsin-EDTA were pre-warmed in the water bath pre-set to 37°C. After 10 minutes, the reagent were wiped with 70% ethanol and placed inside the BSC.

The old media in the T25 flask was remove and pipetted into a centrifuge tube. The flask was then rinsed using 1 ml of prepared Phosphate Saline Buffer (PBS). Then, 1 ml of Trypsin-EDTA was pipetted into the flask and the flask was incubated for 3 minutes at 37°C incubator with 5% CO_2 and humidity. After 3 minutes, the flask was removed and observed under microscope to view cell detachment. If cells did not detach, wall of the flask was gently knocked to help detach the cells. The old media was then re-inserted into the flask to inactivate Trypsin-EDTA. After a brief rinsing and pipette splashes, all the content of the flask was transferred into the centrifuge tube. The tube was capped tightly and centrifuged for 5 minutes at 1500 rpm.

Next, the supernatant (containing media and Trypsin EDTA) was removed, leaving the pellet of cells in the tube. One ml complete RPMI medium was added onto the pellet and the cells were thoroughly re-suspended inside the media to ensure mixing and produce single cell suspension. The single cell suspension was then divided and pipetted into two new flasks, containing 3 ml of fresh complete RPMI medium. The cap of the flasks were sealed using parafilm tape. The two flasks were observed under microscope to ensure that the condition of the cells were appropriate in term of the sterility, cell number and absence of unwanted microorganism. The apparatuses were removed from the BSC and the BSC was wiped well using 70% ethanol to ensure sterility. After that, the UV light was turn on for 5 minutes. The flasks were incubated at 37°C, 5% CO_2 and humidity for proper growing.

2.3.1.3 Cryopreservation of DBTRG-05MG Cells

The cultured DBTRG-05MG cells were cryopreserved to store for future use. Old media in the T25 flask was removed and pipetted into a centrifuge tube. The flask was then rinsed using 1 ml of PBS. 1 ml of Trypsin-EDTA was pipetted into the flask and the flask was incubated for 3 minutes in 37°C incubator with 5% CO_2 and humidity. After 3 minutes, the flask was removed and observed under microscope to view cell detachment. If cells did not detach, wall of the flask was gently knocked to help detach the cells. The old media was then re-inserted into the flask to inactivate Trypsin-EDTA. After a brief rinsing and pipette splashes, all the content of the flask was transferred into the centrifuge tube. The tube was capped tightly and centrifuged for 5 minutes at 1500 rpm.

Next, the supernatant (containing media and Trypsin EDTA) was removed, leaving the pellet of cells in the tube. 1 ml complete RPMI medium was added onto the pellet and the cells were thoroughly re-suspended inside the media to ensure complete mixing in order to produce single cell suspension. The 10 µl of the 1 ml DBTRG-05MG cells suspension was obtained to perform cell counting using Countess® Automated Cell Counter with 10 µl trypan-blue dye. After that, the cell suspension was centrifuged again for 5 minutes at 1500 rpm. Following centrifugation, the cell pellet was resuspended in 950 µl of FBS and transferred into the cryovials. 50 µl of DMSO was then added into each vials to make up the volume of 1 ml per vial. DMSO acted as cryoprotective agent. The vials were deposited in the 'Mr. Frosty' container and stored in -80°C freezer overnight. After that, the vials were removed from the 'Mr. Frosty' container and placed in the labelled box inside the liquid nitrogen for storage.

2.3.2 GIBCO® H9 Human Neural Stem Cell (H9-hNSC) Lines

2.3.2.1 Coating the Flask for Adherent Cell Culture

For GIBCO® H9 Human Neural Stem Cell (H9-hNSC) culture, the T25 flask must first be coated with 3ml of GIBCO® CELLstart™ Cell Therapy System (CTS) substrate. CELLstart™ substrate was diluted in D-PBS with calcium and magnesium in 1:100 ratio and was pipetted into a T25 flask. The flask was incubated for an hour at 37°C, 5% CO_2 and humidity. The flask must be wrapped due to the light-sensitive nature of the mixture. After the incubation, the coating mixture was removed and replaced with equal amount of D-PBS without calcium and magnesium It was followed by incubation at 37°C, 5% CO_2 and humidity until use. Before using for culture, the D-PBS without calcium and magnesium was removed from the flask.

2.3.2.2 Revival from Cryopreserved Stock

GIBCO® H9-hNSC was stored in liquid nitrogen storage before use. For cell revival, the cryovial was thawed by swirling movement in water bath at 37°C until most of the ice melted. Next, the cells in the cryovial were pipetted out and transferred into a 15 ml centrifuge tube. StemPro® NSC Serum Free Medium (SFM) was used for H9-hNSC. 1 ml of warm complete StemPro® NSC SFM (Table 2.2) was slowly added into the centrifuge tube. Another 1 ml of complete SFM medium was added and the tube was centrifuged for 5 minutes at 1500 rpm to remove DMSO from the cryopreservation. After centrifugation the supernatant was carefully removed, leaving the cell pellet. 1 ml of fresh complete SFM medium was added to resuspend the cell pellet. After that, the cell suspension was transferred into the

CELLStart™-coated T25 flask, containing 4 ml of fresh complete SFM medium. The revived cell was incubated at 37°C incubator with 5% CO_2 and humidity and left to properly attach as well as to multiply inside the flask.

Table 2.2 Complete StemPro® NSC SFM medium for H9-hNSC

Component	**Volume (ml)**
KnockOut™ DMEM/F-12	48.5
StemPro® Neural supplement	1
GlutaMAX™-I supplement	0.5
EGF	0.01
bFGF	0.01
Total	**50**

2.3.2.3 Passaging H9-hNSC Cells

H9-hNSC cells were cultured in CELLStart™-coated T25 flasks. All cell culture procedure were performed under sterile condition inside the BSC in the Cell Culture Laboratory. Pipette tips were autoclaved before use to ensure sterility. The instruments such as pipettes, 15 ml centrifuge tubes and waste container were first disinfected using 70% ethanol spray solution and exposed to the ultraviolet (UV) light for 10 minutes. At the same time, the related reagent such as the SFM media and TrypLE™ Express were pre-warmed in the preset water bath at 37°C. After 10 minutes, the reagent were wiped with 70% ethanol and placed inside the BSC.

The old media in the T25 flask was removed and pipetted into a centrifuge tube. The flask was then rinsed using 1 ml of the prepared D-PBS (without Calcium and Magnesium). 1 ml of TrypLE™ Express was pipetted into the flask and the flask was incubated for 3 minutes at 37°C incubator with 5% CO_2 and humidity. After 3 minutes, the flask was removed and observed under microscope to view cell detachment. If the cells did not detach, the wall of the flask was gently knocked to help detach the cells. The old media was then re-inserted into the flask to inactivate TrypLE™ Express. After a brief rinsing and pipette splashes, all the content of the flask was transferred into the centrifuge tube. The tube was capped tightly and centrifuged for 5 minutes at 1500 rpm.

Next, the supernatant (containing media and TrypLE™ Express) was removed, leaving the cell pellet in the tube. 1 ml complete SFM medium was added onto the pellet and the cells were thoroughly re-suspended inside the media to ensure mixing, producing single cell suspension. The 1 ml cell suspension was then divided into two new flasks, containing 3 ml of fresh complete SFM medium. The cap of the flasks were sealed using parafilm tape. The two flasks were observed under microscope to ensure that the condition of the cells were appropriate in term of the sterility, cell number and absence of unwanted microorganism. The apparatuses were removed from the BSC and the BSC was wiped well using 70% ethanol to ensure sterility. After that, the UV light was turn on for 5 minutes. The flasks were incubated at 37°C, 5% CO_2 and humidity for proper growing.