ANTICANCER EFFECTS OF *APIS CERANA* AND *HETEROTRIGONA ITAMA* HONEYS ON TEMOZOLOMIDE-RESISTANT GLIOBLASTOMA CELLS

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by

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DECLARATION

I hereby declare that this dissertation is the result of my own investigation, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

You Ying Hui

Date: 27 JANUARY 2025

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LIST OF ABBREVIATIONS

A. cerana	*Apis cerana*
Akt	protein kinase B
ATR	attenuated total reflectance
BME	β-mercaptoethanol
BSC	biological safety cabinet
C_2H_5OH	ethanol
$C_6H_8O_6$	L-ascorbic acid
cDNA	complementary deoxyribonucleic acid
CH_3COOH	acetic acid
CH_3COONa	anhydrous sodium acetate
CH_3OH	methanol
CNS	central nervous system
CO_2	carbon dioxide
D-2-HG	D-2-hydroxyglutarate
DBTRG-05MG	Denver Brain Tumor Research Group 05
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial-to-mesenchymal transition
FBS	fetal bovine serum
FDA	The United States Food and Drug Administration
$FeCl_3$	iron (III) chloride
FTIR	fourier transform infrared spectroscopy
GBM	glioblastoma multiforme
H. itama	*Heterotrigona itama*
H_2SO_4	sulphuric acid
HCl	hydrochloric acid
$HgCl_2$	mercuric chloride
HILDA	Honey Interlinked Dehydration and Dispenser Apparatus
I_2	iodine
IC50	half-maximal inhibitory concentration
IDH	isocitrate dehydrogenase
KI	potassium iodide
m^6A	N6-methyladenosine
MDM2	mouse double minute 2
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	sodium hydroxide
NF-κB	nuclear factor-kappa B

NTC	no-template control
OD	optical density
P13K	phosphoinositide 3 kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNase	ribonuclease
TBE	tris-borate-EDTA
TFC	total flavonoid content
TMZ	temozolomide
UV	ultraviolet
VEGF	vascular endothelial growth factor
WHO	World Health Organization
WNT	wingless-related integration site
WNT5A	wingless-related integration site family member 5A
YTHDF2	YT521-B homology N6-methyladenosine RNA binding protein F2

LIST OF SYMBOLS

%	percentage
°C	degree celcius
µL	microlitre
µM	micromolar
μm^2	square micrometre
A	ampere
cm^{-1}	reciprocal centimetre
g	gram
Gy	gray
h	hour
kb	kilobase
mg	milligram
mg/m^2	milligram per square metre
mg/mL	milligram per millilitre
mL	millilitre
mM	millimolar
N	normality
ng/µL	nanogram per microlitre
nm	nanometre
rpm	revolutions per minute
V	volt
W	watt
xg	relative centrifugal force

KESAN ANTIKANSER MADU *APIS CERANA* DAN *HETEROTRIGONA ITAMA* TERHADAP SEL GLIOBLASTOMA DENGAN KETAHANAN TEMOZOLOMIDE

ABSTRAK

Glioblastoma merupakan tumor yang sangat agresif dan mempunyai prognosis teruk dengan kadar kelangsungan hidup median kurang daripada 15 bulan. Disebabkan oleh kesukaran pembedahan untuk mengeluarkan kesemua tumor serta perkembangan ketahanan terhadap rawatan kimia temozolomide (TMZ), apitherapi menggunakan madu menjadi rawatan alternatif yang berpotensi bagi glioblastoma kerana kekayaan sebatian fenoliknya mempunyai sifat antioksidan yang tinggi. Namun begitu, perbezaan antara madu *Apis cerana* dengan madu *Heterotrigona itama* terhadap kesan anti-glioblastoma jarang dikaji. Oleh sebab yang demikian, kajian ini bertujuan untuk membandingkan komposisi fitokimia madu *A. cerana* dan *H. itama* melalui ujian saringan fitokimia serta analisis spektroskopi inframerah fourier transform (FTIR). Selain itu, keupayaan antioksidan antara madu *A. cerana* dan *H. itama* juga telah dibandingkan dengan ujian penyingkiran radikal 2,2-difenil-1-pikrilhidrazil (DPPH). Selepas itu, kepekatan perencatan separuh maksimum (IC50) bagi madu *A. cerana* dan *H. itama* terhadap sel glioblastoma yang mempunyai ketahanan terhadap TMZ (sel DBTRG-05MG) telah ditentukan melalui ujian 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) manakala keupayaan invasif dan kekambuhan glioblastoma telah ditentukan dengan ujian calar dan ujian klonogenik. Kemudian, ekspresi gen antara sel DBTRG-05MG yang dirawat dengan madu *A. cerana* dan *H. itama* telah dibandingkan dengan reaksi rantai polimerase transkripsi balik kuantitatif (qRT-PCR) untuk menjelaskan kesan

mereka terhadap apoptosis (gen *MDM2*), metastasis (gen *WNT5A*) dan ketahanan kimia (gen *YTHDF2*). Analisis melalui kajian ini berjaya menunjukkan bahawa madu *A. cerana* mempunyai kandungan alkaloid dan saponin yang lebih tinggi berbanding dengan madu *H. itama*, menyumbang kepada aktiviti antioksidan yang lebih tinggi seperti yang dibuktikan dengan ujian DPPH. Data berkenaan juga telah disokong dengan nilai IC50nya yang lebih rendah (130.5 ± 33.1 mg/mL) daripada madu *H. itama* (185.8 ± 27.6 mg/mL) dalam rawatan selama 72 jam terhadap sel DBTRG-05MG. Sebaliknya, madu *H. itama* mempunyai kandungan flavonoid yang lebih tinggi berbanding dengan madu *A. cerana*. Madu *A. cerana* dan *H. itama* mengandungi kumpulan berfungsi yang sama seperti yang ditunjukkan dalam analisis FTIR. Madu *A. cerana* memberi kesan perencatan yang kuat terhadap invasif dan migrasi sel DBTRG-05MG dengan peratusan penutupan yang paling rendah sehingga 72 jam manakala madu *H. itama* memberi kesan profilaktik yang kuat terhadap kekambuhan sel DBTRG-05MG dengan jumlah pembentukan koloni yang paling kurang. Namun begitu, tiada perbezaan yang signifikan dapat dikesan dalam ekspresi gen *MDM2*, *WNT5A* dan *YTHDF2* antara sel DBTRG-05MG yang dirawat dengan madu *A. cerana* dan *H. itama*. Hasil kajian ini mencadangkan bahawa madu *A. cerana* lebih berkesan dalam membunuh sel glioblastoma yang mempunyai ketahanan terhadap TMZ manakala madu *H. itama* lebih berkesan dalam mencegah kekambuhan glioblastoma. Dengan ini, kesan anti-kanser bagi setiap fitokimia dalam kedua-dua madu patutlah dikaji dengan lebih mendalam pada masa depan demi penjelasan yang lebih baik terhadap mekanisme apoptosis, metastasis dan ketahanan kimia.

ANTICANCER EFFECTS OF *APIS CERANA* AND *HETEROTRIGONA ITAMA* HONEYS ON TEMOZOLOMIDE-RESISTANT GLIOBLASTOMA CELLS

ABSTRACT

Glioblastoma is characterized by high aggressiveness and poor prognosis with median survival rate of less than 15 months. Due to the complexity of surgery to remove whole tumour and rapid development of chemoresistance towards temozolomide (TMZ), apitherapy using honey emerges as potential alternative treatment for glioblastoma due to its rich phenolic compounds with high antioxidant properties. However, the difference between *Apis cerana* honey and *Heterotrigona itama* honey for anti-glioblastoma effects has not been extensively studied. In this study, the phytochemical composition of *A. cerana* and *H. itama* honey were compared using phytochemical screening test and fourier transform infrared spectroscopy (FTIR) analysis. Their antioxidant capabilities were also compared using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Then, the half-maximal inhibitory concentration (IC50) values of both honeys on TMZ-resistant glioblastoma cell line (DBTRG-05MG cells) were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while the analysis of cancer invasiveness and recurrence were determined through scratch assay and clonogenic assay respectively. After that, gene expressions between both honey-treated DBTRG-05MG cells were compared using quantitative reverse transcription polymerase chain reaction (qRT-PCR) to elucidate their effects towards apoptosis (*MDM2* gene), metastasis (*WNT5A* gene) and chemoresistance (*YTHDF2* gene). The analysis revealed that *A. cerana* honey contained higher levels of alkaloid and saponin as

compared to *H. itama* honey, which contributed to its higher antioxidant activity as evidenced via the DPPH assay. This data was also supported by its lower IC50 value (130.5 ± 33.1 mg/mL) than *H. itama* honey (185.8 ± 27.6 mg/mL) in 72-hour treatment on DBTRG-05MG cells. In contrast, *H. itama* honey contained higher levels of flavonoid than *A. cerana* honey. Both honeys shared similar functional groups as indicated in FTIR analysis. *A. cerana* honey exerted strong inhibitory effect towards invasiveness and migration of DBTRG-05MG cells with its lowest closure percentage up to 72 hours while *H. itama* honey exerted strong prophylactic effect towards recurrence of DBTRG-05MG cells with its lowest colony number formed. However, there was no significant difference in *MDM2*, *WNT5A* and *YTHDF2* expressions between honey-treated DBTRG-05MG cells. These findings suggest that *A. cerana* honey could be more effective in killing TMZ-resistant glioblastoma cells while *H. itama* honey could be more effective in preventing glioblastoma recurrence. The anticancer effect of each phytochemical in both honeys should be further investigated in future for better elucidation towards apoptotic, metastasis and chemoresistance mechanisms.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Glioblastoma is a grade IV primary malignant brain tumour that commonly occurs in adults aged 40 years and above. Being the most common type of brain tumour, it occupies almost half (~49%) of the total brain tumour incidence as compared to other malignant brain tumours such as primary central nervous system (CNS) lymphoma (~7%), ependymomas (~3%) and meningiomas (~2%) (Schaff and Mellinghoff, 2023). Glioblastoma originates from a variety of brain cells, including astrocytes, oligodendrocyte progenitor cells, and neural cancer stem cells. It appears as a large abnormal mass with thick, irregular enhancing margins and central necrotic core containing haemorrhagic component. This disease is characterized by poor prognosis and high aggressiveness with the median survival rate of less than 15 months. According to Schaff and Mellinghoff (2023), in the early stage of glioblastoma, the patients mostly experience consistent and aggravating headache (50%-60%), seizure (20%-50%), neurocognitive impairment (30%-40%) and focal neurologic deficits (10%-40%). Approaching the end stage of disease, hallucinations, alterations in breathing pattern and bluish tint skin appearance exist due to the worsening hypoxic condition within the brain intracranial space (Hansen et al., 2023).

In the treatment for glioblastoma, maximal surgical resection is the first-line therapy given to the patients for removing the whole tumour as much as possible. However, the residual tumour can still exist even after the surgical procedure due to the complexity of

brain structure (Gerritsen et al., 2022). The neurologist needs to achieve balance between maximal safe resection and minimal neurological deficits. Hence, radiotherapy concomitant with chemotherapy is applied to remove the residual tumours. Temozolomide (TMZ) is currently the first-line chemotherapy drug approved by The United States Food and Drug Administration (FDA) for glioblastoma treatment. Nonetheless, at least 50% of glioblastoma patients do not respond to TMZ due to the acquired resistance towards TMZ (Teraiya et al., 2023), resulting in high tumour recurrence rate and rapid cancer progression. In this study, apitherapy using honey as an alternative treatment for TMZ-resistant glioblastoma was investigated.

Apitherapy is a complementary medicine involving the application of bee products such as honey, propolis and royal jelly for treatment in various health conditions. Recent studies have highlighted its potential role in oncology due to its anticancer properties that may support conventional cancer therapies (El-Didamony et al., 2024). Among various bee products, honey is the most extensively studied, renowned for its antioxidant, anti-inflammatory, and antimicrobial properties that contribute to its potential therapeutic effects in cancer care. Honey is unique with its enrichment in phenolic compounds including flavonoids and phenolic acids. These phenolic compounds play important roles in promoting strong antioxidant activities through modulation of oxidative stress and interference of cell cycle to prevent cancer growth (Martinotti et al., 2024). Nonetheless, honey produced by different types of bee species might be different in their chemical composition, resulting in different effects for anticancer therapy. Therefore, this study aimed to compare the inhibitory effects of *Apis cerana* honey and *Heterotrigona itama* honey on TMZ-resistant glioblastoma cells.

A. cerana honey is a type of honey produced by *A. cerana* honeybee species found in East, Southeast and South Asia. This bee species normally forms large colony size and produces greater amount of thick and sweet honey. In contrast, *H. itama* honey is a stingless bee species found native to Malaysia in which it usually forms smaller colony size with lesser production of honey as compared to *A. cerana* honeybee species. Recent studies had found that *H. itama* honey is characterized by high moisture, great acidity, low carbohydrates and strong antioxidant activities (Esa et al., 2022). It had been initially evidenced of inducing apoptosis and inhibiting cell proliferation in cancer. However, due to the less production of *H. itama* honey, the anticancer effect of this type of honey has not been extensively studied. As a result, research gap exists between the anticancer effect of honeybee (*A. cerana*) and stingless bee (*H. itama*) honey. Therefore, in this study, the anticancer effects of *A. cerana* honey and *H. itama* honey towards glioblastoma were investigated especially from the aspects of tumour aggressiveness, recurrence and their underlying gene expressions.

1.2 Objective of Study

Main objective:

To compare the anticancer effect of *A. cerana* honey and *H. itama* honey on TMZ-resistant glioblastoma cells.

Specific objectives:

1) To compare the type of phytochemical present in *A. cerana* honey and *H. itama* honey using phytochemical screening test and fourier transform infrared spectroscopy (FTIR) analysis.
2) To compare the antioxidant capabilities between *A. cerana* honey and *H. itama* honey using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.
3) To determine the half-maximal inhibitory concentration (IC50) of *A. cerana* honey and *H. itama* honey in killing TMZ-resistant glioblastoma cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.
4) To compare the effectiveness of *A. cerana* honey and *H. itama* honey in reducing aggressiveness and recurrence rate of TMZ-resistant glioblastoma cells using scratch and clonogenic assays.
5) To compare the gene expression regulated by *A. cerana* honey and *H. itama* honey in killing TMZ-resistant glioblastoma cells using quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay.

1.3 Significance of Study

Glioblastoma is the most prevalent and aggressive form of primary brain tumour, accounting for approximately 49% of all brain tumours. Despite advancements in treatment, its prognosis remains poor, with a median survival of only 12-15 months following diagnosis. This is due to the limitations of current treatment for glioblastoma such as resistant towards chemotherapy drug - TMZ. Therefore, this study presented an alternative treatment for glioblastoma using apitherapy, particularly the honey which has been reported to effectively inhibit tumour growth, induce apoptosis and enhance the

effectiveness of conventional therapies of various cancers. Through this study, the potential of honey as alternative solution to treat TMZ-resistant glioblastoma was presented. Moreover, this study also presented the comparative analysis of two types of honey produced by two different bee species. Such investigation could provide insight information to identify the unique therapeutic properties of each honey type, helping to determine the most effective bee product for supporting cancer treatment. In addition, this study also investigated the target genes involved in enhancing programmed cell death of TMZ-resistant glioblastoma cells after treated with honey. This data could provide future directions for investigating molecular mechanism regulated by honey in preventing cancer progression, aggressiveness and tumour recurrence of glioblastoma.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a disease involves uncontrollable cell growth which forms tumour and rapid spreading towards other organ systems through blood or lymphatic circulation within the body. It is caused by mutations in genes related to regular cell division, either through inheritance or external environmental factors such as carcinogens and ultraviolet radiation. There are three main types of genes tend to be mostly affected by cancer including proto-oncogenes, tumour suppressor genes and DNA repair genes. Proto-oncogenes are responsible for stimulating cell growth and division while tumour suppressor genes are responsible for decelerating the cell cycle (Chen et al., 2020). On the other hand, DNA repair genes assist in the fixing of damaged DNA. When mutations happen in either of these three genes, additional mutations in other regulating genes will be triggered and ultimately promote the initiation of cancer disease (Torgovnick and Schumacher, 2015).

Initiating from accumulation of multiple changes in chromosomes, the normal cells are able to evolve into mutated cells and subsequently progress into the occurrence of hyperplasia. During hyperplasia stage, the cells multiply faster than normal cells which lead to the buildup of extra cells. As hyperplasia advances, it will eventually develop into dysplasia and cause alterations in both cell morphology and tissue organization. In more advanced circumstances, carcinoma in situ, which happens as the pre-stage of cancer, may progress into invasive cancer cells as the in situ cancerous cells break through the

basement membrane and invade surrounding tissues, leading to the formation of malignant tumours that can spread to other parts of the body (Figure 2.1).

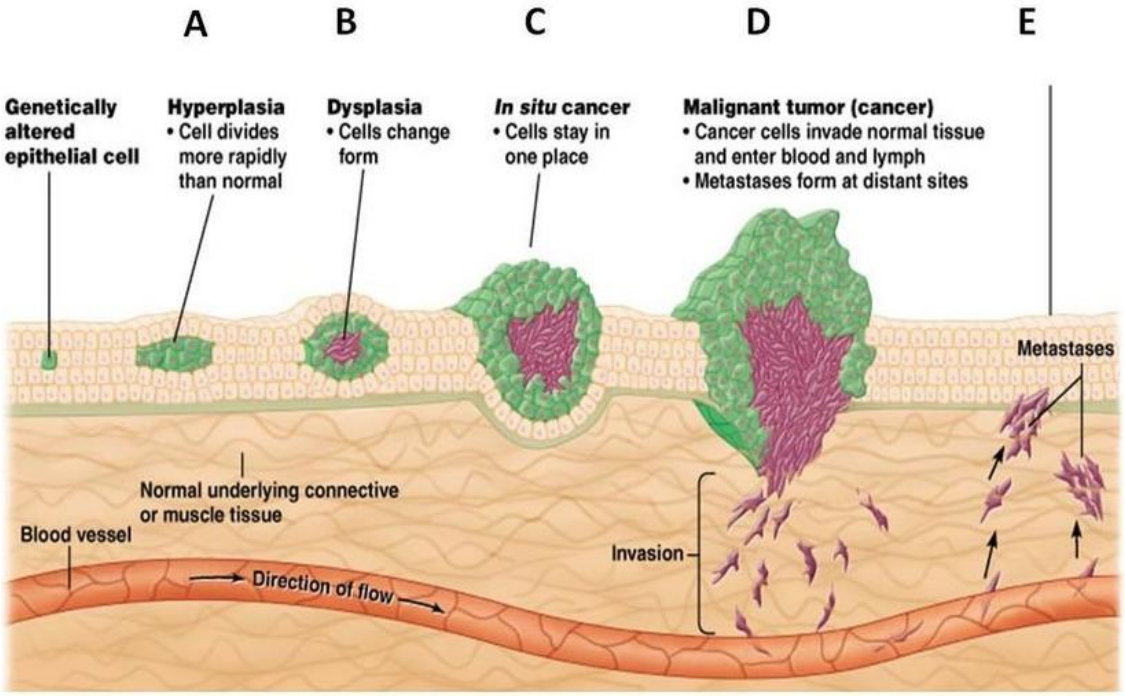

Figure 2.1 Cancer disease initiates from genetic alteration, subsequently progressing into hyperplasia, dysplasia and carcinoma in situ before forming malignant tumours. (modified from Kanwal, 2013)

2.1.1 Hallmarks of Cancer Cells

In cancer, there are several distinct hallmarks that have been acquired by the cancer cells during the development of tumour. Differing from normal cell division, cancer cells sustain proliferative signalling through continuous growth even without the presence of growth signals. Instead, these cells ignore the signals of terminating cell division and inducing programmed cell death. As a result, replicative immortality is enabled in these cancer cells. In addition, cancer cells intend to invade nearby regions and metastasize to other body regions. Throughout the metastatic process, these cells are able to evade host

immune system through various mechanisms such as restricting antigen recognition, activating immunosuppression and inducing T cell exhaustion (Kim and Cho, 2022). In order to adapt themselves within hypoxic hostile microenvironment, cancer cells strategize angiogenesis which enables abnormal growth of blood vessels from existing vasculature to obtain sufficient oxygen for optimal tumour growth. As for the purpose of gaining nutrients to support rapid growth and proliferation, cancer cells undergo metabolic reprogramming mediated by activation of proto-oncogenes and mutation of tumour suppressor genes (Hanahan, 2022).

2.2 Brain Cancer

Brain cancer, or known as glioma, originates from the cells within CNS. In total, there are over 120 different types of brain tumours that are subdivided according to the cell types in which they are initially formed in the CNS. The most common type of brain tumour is astrocytoma. Astrocytoma is a primary brain tumour which arises from astrocytes, the star-shaped glial cells that are mostly found in cerebrum and make up the human CNS. Accounting for 60% of brain tumours, astrocytoma is the most prevalent and common form of glioma. Based on their level of malignancy and aggressiveness, astrocytoma can be further classified into grades I, II, III and IV. Grade I astrocytoma is a non-infiltrating tumour in which this tumour grows slowly and does not expand into surrounding brain tissues. Grade II astrocytoma, also known as low-grade or diffuse astrocytoma, is an infiltrating tumour in which the tumour tends to grow slowly into nearby areas of brain and may develop in a more aggressive manner over time. It is characterized by nuclear atypia in which the abnormal cell nuclei can be seen in the tumour cells. As for grade III astrocytoma, also known as anaplastic astrocytoma, is a

fast-growing malignant tumour characterized by nuclear atypia and poor cellular differentiation. However, prominent proliferation activity and mitoses are present in these tumour cells. In grade IV astrocytoma, also known as glioblastoma, is the most aggressive type of brain tumour characterized by nuclear atypia, prominent mitoses, microvascular proliferation and even necrosis (Ballestero et al., 2024).

2.2.1 Glioblastoma

Glioblastoma, or formerly known as glioblastoma multiforme (GBM), is the most common and aggressive type of primary brain tumour that happens in adults with the peak incidence between 60 to 69 years of age (Tataranu et al., 2024). Glioblastoma is characterized by poor prognosis with the 5-year survival rate of 37.13% (Sabouri et al., 2024). Throughout the world, the incidence of glioblastoma varies across different areas, ranging from 4.5 cases to 9.23 cases per 100,000 persons (Sandhanam et al., 2024).

According to the World Health Organization (WHO) 2007 classification, glioblastoma is also referred to as grade IV astrocytoma since it originates from astrocytes, which are the major star-shaped glial cells that play essential roles in providing structural support to hold the nerve cells in place for proper development and functioning. As glioblastoma research progressed, it is later recognized as a grade IV isocitrate dehydrogenase (IDH)-wild type diffuse glioma in WHO 2021 classification. This refers to the tumours with non-mutated IDH gene which is critical in energy metabolism to avoid production of oncometabolite such as D-2-hydroxyglutarate (D-2-HG) that can influence normal cellular metabolism and epigenetics (Louis et al., 2021).

In glioblastoma, it mostly affects astrocytes located at cerebrum especially the frontal (26%), temporal (19%) and parietal (12%) lobes of the brain. For rare cases, cerebellum (5%), brain stem (4%), occipital lobe (3%) and ventricle (2%) of brain will be affected (Figure 2.2). Among these brain regions, frontal lobe is responsible for executive function, social understanding and voluntary muscle movement while temporal lobe is responsible for memory storage, auditory and language information processing. Meanwhile, parietal lobe is responsible for integration of sensory information which includes pain, pressure, temperature and touch (Jawabri and Sharma, 2023). Hence, the tumour formation within one of these regions can cause the declination and loss of these capabilities especially when the tumour enlarges. The rapid growth of glioblastoma tumour is devastating to the brain region since it can invade and destroy the brain tissue, subsequently exerting pressure on nearby tissue to increase intracranial pressure within the skull (Lipková et al., 2022). The increase of size in tumour can result in the accumulation and blockage of cerebrospinal fluid in the brain tissue (Hong et al., 2018). Bleeding may also occur in serious conditions (Richard et al., 2018).

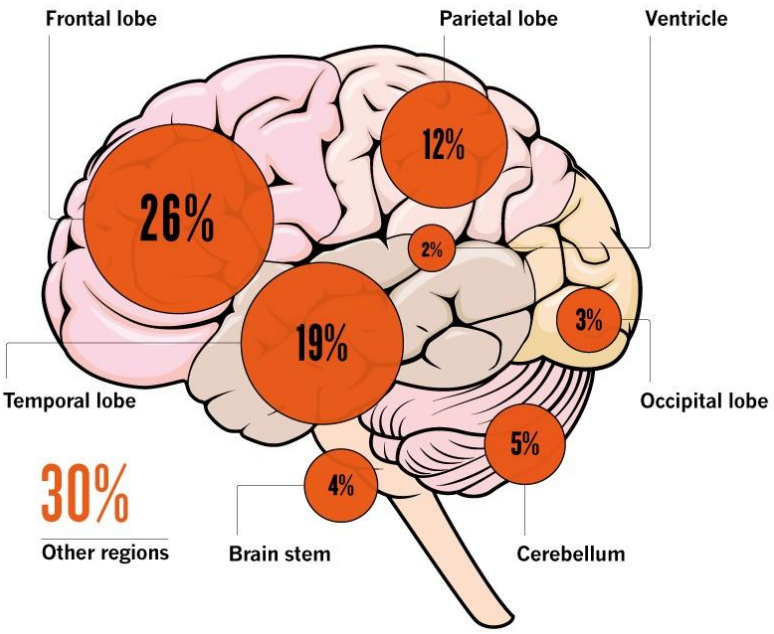

Figure 2.2 Glioblastoma mostly affects frontal (26%), temporal (19%) and parietal (12%) lobes of the brain. Meanwhile, it rarely affects cerebellum (5%), brain stem (4%), occipital lobe (3%) and ventricle (2%) of brain. (Gould, 2018)

2.2.2 Risk Factors and Causal Factors of Glioblastoma

It has been reported that the disease is more common to affect men than women due to the protective effects of female sex hormones on the development of glioblastoma (Carrano et al., 2021). In addition, those living in the urban countries are more likely to suffer from glioblastoma as compared to those living in rural countries due to the living lifestyle including nitrosamines found in tobacco smoke and processed meat products, and exposure to environmental factors including ionizing radiation, heavy metals and pesticides excreted from industries (Ostrom et al., 2020). Although the exact underlying cause of glioblastoma remains unknown in most cases, genetic syndromes such as neurofibromatosis type I, Li Fraumeni syndrome and Turcot syndrome are possible factors for the occurrence of glioblastoma in certain rare cases (Backes et al., 2014).

2.2.3 Signs and Symptoms of Glioblastoma

In the early stage of glioblastoma, headache often appears as the first symptom due to increased intracranial pressure resulting from dura stretching and cerebrospinal fluid blockage with the existence of tumour (Palmieri et al., 2020). Differing from normal headache, headache associated with glioblastoma constantly appears and aggravates during early morning or night especially when the actions of coughing, shouting, straining or bending over exist (Schankin et al., 2007). As glioblastoma progresses, the headache becomes more frequent and may not respond well to over-the-counter pain killer.

In association with glioblastoma headache, there are specific symptoms that appear indicating the location of tumour within functional areas of brain. The frontal lobe tumours can result in impaired judgement, motivation or inhibition, reduced mental abilities, paralysis on one side of the body, behavioural and emotional changes. As for temporal lobe tumours, they can lead to difficulty in speaking and understanding language, increased aggressive behaviour, short-term and long-term memory problems. Moreover, parietal lobe tumours can cause spatial disorders, impaired eye-hand coordination and lack of recognition (Acevedo-Vergara et al., 2022).

During the end-of-life stage for glioblastoma, patients may lose the ability to move, speak and eat. Hallucinations and alterations in breathing pattern accompanied with bluish tint skin appearance caused by decreased oxygen supply indicate increasing lethargy in glioblastoma patients (Sizoo et al., 2010).

2.3 Limitations of Current Treatments for Glioblastoma

2.3.1 Surgical Resection

As the first-line therapy for glioblastoma, surgical resection is usually given to the patients for maximal tumour removal. In order to achieve higher rate of gross total resection, intraoperative magnetic resonance imaging (MRI) is suggested to be carried out simultaneously for assessing the resection in real time (Wang et al., 2019; Sales et al., 2022). Through the surgical procedures, the tumour burden can be reduced and the mass impact of glioblastoma can be relieved. Although current standard surgical resection allows the removal of most of the tumour tissue from glioblastoma patients, the effectiveness of this therapy is limited because in most cases, the tumour is unable to be totally removed due to the complexity of brain structure. The neurosurgeon needs to achieve balance between maximal safe resection and minimal neurological deficits, especially for the tumour locating proximal to eloquent areas of brain which are critical for cognition, language, sensory perception and motor control functions. As a consequence, the possibility of residual tumour is high even after the surgical resection (Patel and Chavda, 2024).

2.3.2 Radiotherapy and Chemotherapy

Due to the limitation of surgical resection, the combination of radiotherapy and chemotherapy is usually given to the patients beyond 4 to 6 weeks of surgery as the concomitant therapy for glioblastoma. In the early years, conventional fractionated radiotherapy is most commonly used for radiation delivery in which the total radiation dose of 60 Gy is given in 30 fractions (Villà et al., 2014). Along the research progress, hyperfractionated radiotherapy and accelerated fractionated radiotherapy have been

introduced. However, both techniques are abandoned for clinical practice since they do not confer significant survival benefit especially to the elderly patients (Cao et al., 2010). Meanwhile in chemotherapy, TMZ as the first-line chemotherapy drug approved by FDA is given to the patients as daily regimen with the dosage of 75 mg/m^2 for consistently 6 weeks together with the radiotherapy. Following by a 4-week break, six cycles of TMZ with the dosage of 150 to 200 mg/m^2 for 5 days in every 28 days are given alone to the patients for removal of residual tumour (Rodgers et al., 2024). Such concomitant therapies possess challenges to patients especially the elderly group due to the risk for deterioration of initially declining mental function within this range of age (Mazarakis et al., 2024). In addition, rapid development of resistance towards radiotherapy and TMZ chemotherapy occurs in most patients can subsequently contribute to local tumour recurrence (Yalamarty et al., 2023). As a result, the therapeutic efficiency of combined radiotherapy and chemotherapy greatly reduces, leading to the rapid progression and high recurrent rate of glioblastoma.

2.4 Apitherapy as Complementary Treatment for Glioblastoma

Apitherapy is the complementary and alternative medicine that involves the application of bee products including honey, propolis and royal jelly for treatment in various health conditions. Among the apitherapy products, honey is most commonly consumed for the purpose of wound healing and body health due to their rich phenolic and antioxidant contents. From cancer aspect, it provides significant antioxidant, anticancer and immunomodulatory effects by inhibiting tumour cell proliferation and metastasis (Waheed et al., 2019). This promotes optimal recovery for patients through prolongation of survival period. Honey has been reported as a promising anticancer agent which is

evidently shown in tumour cells such as bladder cancer, breast cancer, cervical cancer, colon cancer, glioblastoma, kidney cell cancer, leukaemia, oral cancer and prostate cancer (Kavurmacı and Yıldız, 2024).

2.4.1 Honey

Honey is a sweet and viscous substance usually produced by the bees through collection and transformation of flower nectar into simple sugars before storing them into the honeycomb. It is mainly composed of carbohydrates including glucose and fructose which made up 75% of monosaccharide sugars, together with sucrose and maltose which made up 10% to 15% of disaccharide sugars (Afrin et al., 2020). Apart from that, honey also contains other components such as proteins, organic acids and minerals which are beneficial for health. The nutritious honey is also rich in phenolic compounds especially flavonoids and phenolic acids which promote strong antioxidant activity that subsequently prevent cancer growth through its ability to modulate oxidative stress (Erejuwa et al., 2012) In addition, the phenolic compounds of honey possess the ability of interfering cell cycle by blocking G_0/G_1 phase, inhibiting the progression of cell division to lower the proliferation of tumour cells.

2.4.2 Bee Species

Bees are vital pollinators in ecosystems, playing a crucial role in the reproduction of flowering plants and the production of crops. Scientifically, bees are known for their ability to produce a variety of bioactive substances, including honey, propolis, and royal jelly, which have been studied for their medicinal properties.

Bees belong to the Kingdom Animalia, which encompasses multicellular organisms that are typically motile and consume organic material. Within this kingdom, bees are classified under the Phylum Arthropoda, characterized by their exoskeleton, segmented bodies and jointed appendages. They are further categorized into the Class Insecta, distinguished by their three-part body structure, compound eyes and antennae. Within the Order Hymenoptera, bees are part of the Family Apidae, which includes a diverse range of bee species, among which *A. cerana* and *H. itama* are notable (Figure 2.3).

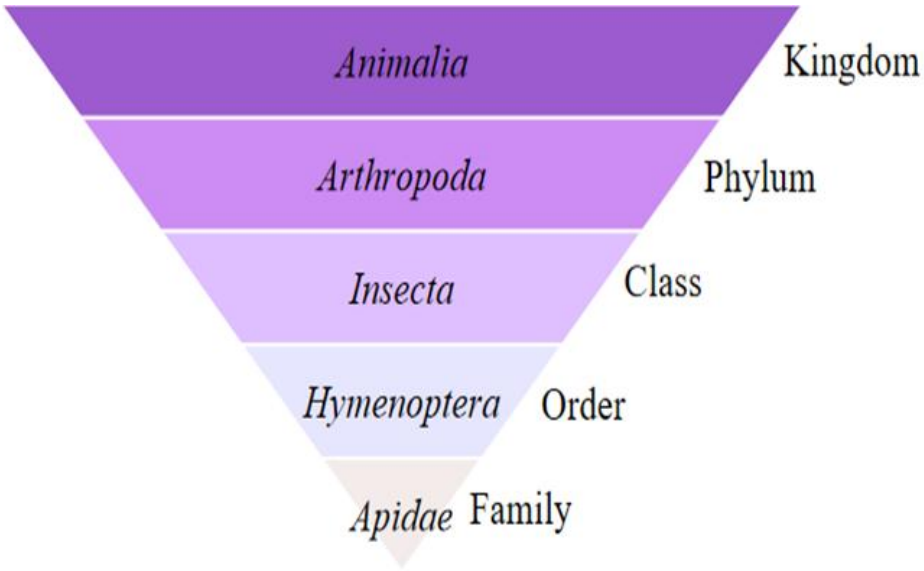

Figure 2.3 Taxonomy of bee.

2.4.2.1 *Apis cerana*

Apis cerana, also known as Eastern honeybee, is a species of honeybee which is native to East, South and Southeast Asia (Figure 2.4). This species contains large colony size with great production of thick and sweet honey. The honey produced by *A. cerana* is usually dark golden in colour. From past studies, it had been found that *A. cerana* honey composes large amount of glucose, making up 59.3% of honey's monosaccharide hence

indicating high glycaemic index in it (Donkersley et al., 2021). The high sugar content within *A. cerana* honey can be associated with its high acidity and low moisture content. Moreover, *A. cerana* honey was found containing high level of quercetin, a bioactive compound renowned for its antioxidant properties (Guo et al., 2023; Huyop et al., 2024). Quercetin is a flavonoid that exhibits proapoptotic effects towards cancer cells through activation of caspase 3 and inhibition of Akt phosphorylation (Lotfi et al., 2023). Hence, *A. cerana* honey possesses anticancer potential, as evidenced in cervical cancer (Zulpa et al., 2021). In this study, the anticancer effect of *A. cerana* honey towards glioblastoma was further investigated.

Figure 2.4 *Apis cerana* is honeybee species native to East, South and Southeast Asia. (Rojas-Sandoval et al., 2022)

2.4.2.2 *Heterotrigona itama*

Heterotrigona itama, also known as Malaysian stingless bee, is a species of stingless bee found native to Malaysia (Figure 2.5). This species contains small colony size with less honey production. The honey produced by *H. itama* is usually light yellow in colour. However, differing from honeybee honey, stingless bee honey contains low pH and high

moisture level, which is tightly correlated to its water content. Furthermore, *H. itama* honey is unique with its lower glycaemic index due to the existence of trehalulose, which is an isomer of sucrose containing unusual α-(1→1) glucose-fructose glycosidic linkage (Fletcher et al., 2020; Zaidi et al., 2023). In glioblastoma, *H. itama* honey had been initially evidenced of inducing apoptosis and inhibiting cell proliferation (Ahmad et al., 2019). However, the physicochemical properties of *H. itama* honey and its contribution towards cancer are currently under researched. Hence, this study is necessary to be conducted for elucidation and comparison with *A. cerana* honey from aspects of tumour invasiveness, recurrence and their underlying gene expressions.

Figure 2.5 *Heterotrigona itama* is stingless bee species native to Malaysia. (Nurul Huda et al., 2015)

2.5 Intracellular Signalling Pathway for Glioblastoma

Among the hallmarks of cancer, inhibition of apoptosis, development of metastasis and chemoresistance are major components that contribute to rapid glioblastoma progression. Hence, key target genes which play vital roles in these three mechanisms were selected in this study to investigate the effects of *A. cerana* and *H. itama* honeys on glioblastoma.

2.5.1 Apoptosis

Apoptosis is the process of energy-dependent programmed cell death which subsequently causes degradation of cell architecture. Under regular conditions, it allows necessary removal of damaged and dysfunctional cells from time to time. However, alterations in apoptotic mechanism leads to tumorigenesis due to excess cell proliferation and reduced cell apoptosis. Within the apoptotic mechanism, mouse double minute 2 (*MDM2*) is a proto-oncogene that encodes a nuclear-localized E3 ubiquitin ligase targeting p53 through ubiquitination for degradation by the proteasome (Pellot Ortiz et al., 2023). Hence, the suppression of *MDM2* enables glioblastoma cell apoptosis with the normal physiological functioning of p53. This phenomenon had also been evidenced across various cancers such as lung adenocarcinoma and triple-negative breast cancer (Sinha et al., 2022; Adams et al., 2023). In this study, the gene expression level of *MDM2* in glioblastoma after treated with *A. cerana* and *H. itama* honeys were studied.

2.5.2 Metastasis and Invasiveness

Metastasis is the process in which the cancer cells break away from the primary tumour, spreading from the initial site to other parts of the body through blood or lymph with the potential of forming new, secondary tumour. Associated with metastatic process, epithelial-to-mesenchymal transition (EMT) has been shown to play its role when the epithelial cells lose their properties and become mesenchymal cells, with altered expression of cell adhesion molecules and remodelling of cytoskeleton (Roche, 2018; Huang et al., 2022; Cai et al., 2024). EMT facilitates the migration of cancer cells by increasing their mobility and invasion towards the secondary site. Within the EMT mechanism of metastasis, wingless-related integration site (WNT) family member 5A

(*WNT5A*) plays a significant role as a proto-oncogene to encode activator for WNT/β-catenin pathway to induce migration of cancerous cells. From past studies, *WNT5A* had been proven driving invasiveness through EMT in glioblastoma cells (Binda et al., 2017). In this study, the roles of *A. cerana* and *H. itama* honeys to inhibit *WNT5A* gene expression and subsequently inhibit the invasiveness of glioblastoma were studied.

2.5.3 Chemoresistance

Chemoresistance is the development of protective mechanism in cancer cells from chemotherapy, which subsequently contributes to potential tumour recurrence. Within the mechanism of chemoresistance, YT521-B homology (YTH) N6-methyladenosine RNA binding protein F2 (*YTHDF2*) is an m^6A-binding protein mainly distributed in cytoplasm and responsible for regulating N6-methyladenosine (m^6A) RNA methylation in tumours, in which the m^6A modification could contribute to chemoresistance through activation of phosphoinositide 3 kinase (P13K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) and nuclear factor-kappa B (NF-κB) pathways. From recent studies, *YTHDF2* had been evidenced promoting temozolomide resistance in glioblastoma (Chen et al., 2022). Thus, the suppression of *YTHDF2* could enable sensitization of glioblastoma cells towards TMZ. In this study, the roles of *A. cerana* and *H. itama* honeys to regulate *YTHDF2* gene expression were studied.

CHAPTER 3

METHODOLOGY

3.1 Study Design

This study aimed to compare the anticancer effects between *A. cerana* honey and *H. itama* honey on TMZ-resistant glioblastoma cell line (DBTRG-05MG). The flowchart of experimental study design is illustrated in Figure 3.1.

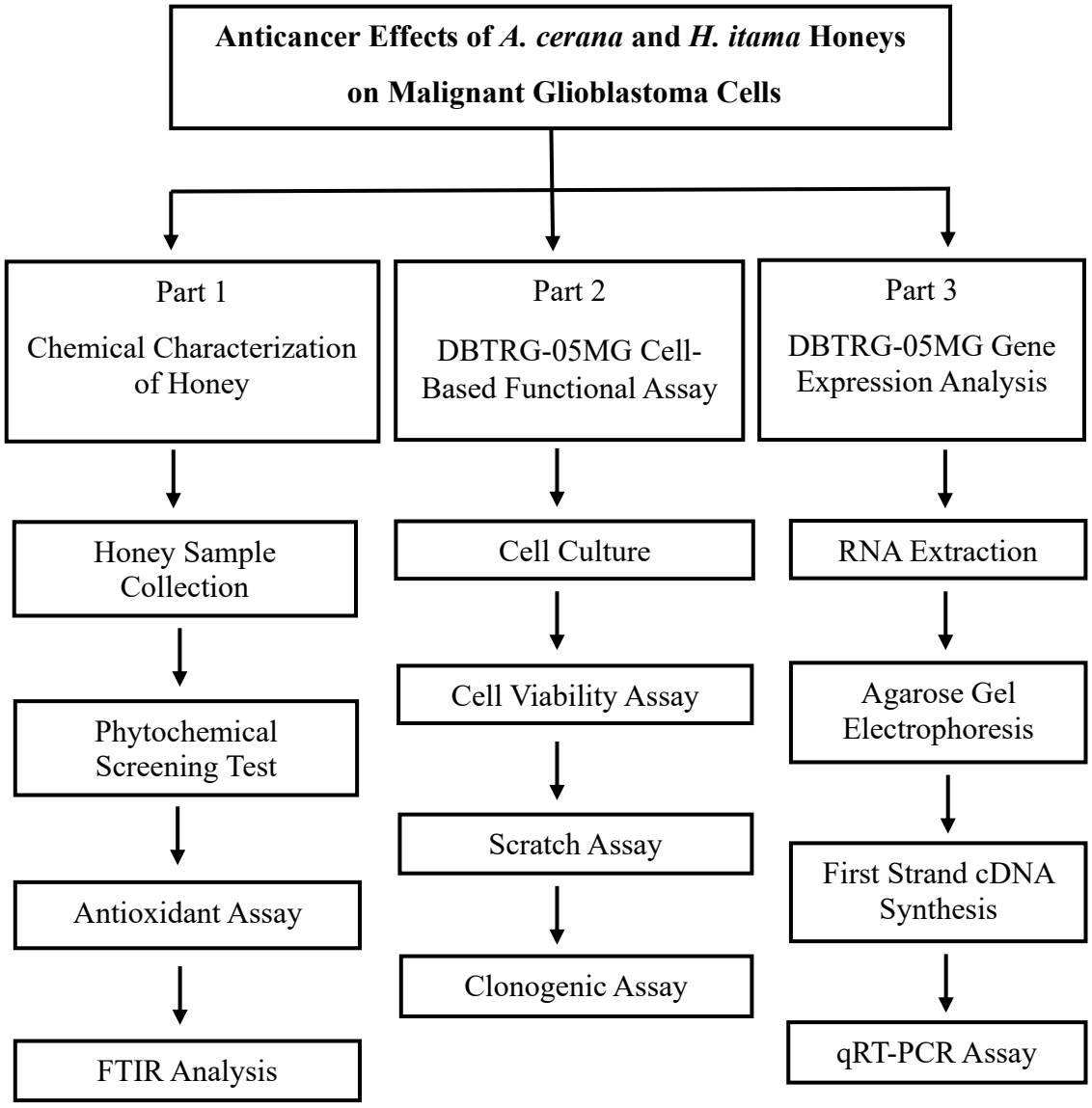

Figure 3.1 Flowchart of experimental study design.

3.2 Materials

3.2.1 General Chemicals and Reagents

Chemicals and reagents used in this study are listed in Table 3.1.

Table 3.1 List of chemicals and reagents used in this study.

Chemicals and Reagents	Brands
0.01% crystal violet	Sigma Aldrich, United States
0.25% trypsin-ethylenediaminetetraacetic acid (EDTA)	Gibco, United States
0.4% trypan blue	Gibco, United States
1Kb DNA ladder	GeneDireX, United States
1X phosphate buffered saline (PBS)	Gibco, United States
2,2-diphenyl-1-picrylhydrazyl (DPPH) powder	Milipore, United States
2 N hydrochloric acid (HCl)	Sigma Aldrich, United States
6X DNA loading buffer	Acmec Biochemical, China
10% neutral buffered formalin solution	Sigma Aldrich, United States
10X tris-borate-EDTA (TBE)	Sigma Aldrich, United States
Absolute ethanol (C_2H_5OH)	Sigma Aldrich, United States
Absolute methanol (CH_3OH)	Sigma Aldrich, United States
Agarose powder	Sigma Aldrich, United States
Anhydrous sodium acetate (CH_3COONa)	Sigma Aldrich, United States
β-mercaptoethanol (BME)	Sigma Aldrich, United States
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, United States
Dulbecco's Modified Eagle Medium (DMEM)	Nacalai Tesque, Japan
Fetal bovine serum (FBS)	Gibco, United States
Glacial acetic acid (CH_3COOH)	Sigma Aldrich, United States
Greensafe DNA gel stain	Canvax, Spain
Iodine powder (I_2)	Sigma Aldrich, United States
Iron (III) chloride powder ($FeCl_3$)	Sigma Aldrich, United States
L-ascorbic acid powder ($C_6H_8O_6$)	Sigma Aldrich, United States
Mercuric chloride powder ($HgCl_2$)	Sigma Aldrich, United States
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder	Milipore, United States
Penicillin-Streptomycin	Gibco, United States
Potassium iodide powder (KI)	Sigma Aldrich, United States
RNase-free water	Thermo Fisher Scientific, United States
Sodium hydroxide (NaOH)	Sigma Aldrich, United States
Sulphuric acid (H_2SO_4)	Sigma Aldrich, United States

3.2.2 General Consumables

Consumables used in this study are listed in Table 3.2.

Table 3.2 List of consumables used in this study.

Consumables	Brands
0.22 µM syringe filter	Bioflow Lifescience, Malaysia
1.5 mL microcentrifuge tube	Labcon, United States
2 mL cryovial	SPL Life Sciences, Korea
3 mL syringe without needle	Muzamal Industries, Malaysia
6-well plate	SPL Life Sciences, Korea
10 µL, 100 µL and 1000 µL pipette	Thermo Fisher Scientific, United States
10 µL, 100 µL and 1000 µL pipette tips	Corning Incorporated, United States
12-well electrophoresis comb	Bio-Rad, United States
15 mL Falcon conical tube	Corning Life Sciences, China
50 mL Falcon conical tube	SPL Life Sciences, Korea
96-well plate	SPL Life Sciences, Korea
Ambient reagent bottle	Pyrex, United States
Beaker	Pyrex, United States
Cell scraper	SPL Life Sciences, Korea
Countess^{TM} Cell Counting Chamber Slide	Invitrogen, United States
Dropper	Corning Life Sciences, China
Electrophoresis tank	Thermo Fisher Scientific, United States
Filter funnel	Pyrex, United States
Filter paper	Thermo Fisher Scientific, United States
Glass rod	Pyrex, United States
Measuring cylinder	Pyrex, United States
Parafilm	Parafilm, United States
Scar scratcher	SPL Life Sciences, Korea
Schott bottle	DURAN, Germany
Serological pipette controller	Thermo Fisher Scientific, United States
Serological pipette	Thermo Fisher Scientific, United States
T25 culture flask	Biologix, China
Test tube	Pyrex, United States

3.2.3 General Instruments

Laboratory instruments used in this study are listed in Table 3.3.

Table 3.3 List of instruments used in this study.

Instruments	Brands
−80°C Freezer	ETS Bio Freeze, Malaysia
Class II Biological Safety Cabinet (BSC)	Esco Lifesciences, Singapore
CO_2 Incubator	Esco Lifesciences, Singapore
Countess™ Automated Cell Counter	Invitrogen, United States
CPX Opus 96 Real-Time PCR System	Bio-Rad, United States
Electronic Balance	Shimadzu Corporation, Japan
FTIR Machine	Bruker, Germany
Hot Plate & Magnetic Stirrer	ERLA® Technologies, Malaysia
Infinite® F50 Absorbance Microplate Reader	Tecan, Switzerland
Inverted Microscope	Leica Microsystems, Germany
Liquid Nitrogen Tank	Air Liquide, French
Molecular Imager® Gel Doc™ XR+ System	Bio-Rad, United States
NanoDrop™ 2000 Spectrophotometer	Thermo Fisher Scientific, United States
PCRmax Thermal Cycler	Cole-Parmer, United States
PowerPac™ Basic Power Supply	Bio-Rad, United States
Sprout® Plus Mini Centrifuge	Healthrow Scientific, United States
Universal 320 Benchtop Centrifuge	Hettich Lab, Germany
Water Bath	Memmert, Germany

3.2.4 Cell Line

Cell line used in this study is listed in Table 3.4.

Table 3.4 List of cell line used in this study.

Cell Line	Brands
DBTRG-05MG	ATCC, United States