ELUCIDATION OF ERK1/2/C-MYC/P53 SIGNALING PATHWAYS INVOLVED IN ANDROGRAPHOLIDE-INDUCED ANTIPROLIFERATIVE ACTIVITY IN HUMAN GLIOBLASTOMA DBTRG-05MG CELL LINE

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2023

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

March 2023

ACKNOWLEDGEMENT

Praise Allah SWT for granting me the strength and opportunity to complete this research project during the COVID-19 pandemic. This research project also has given me an opportunity to improve and implement all my laboratory techniques and scientific knowledge that I had previously learned. Here, I would like to express my sincere gratitude to everyone who assisted me in conducting and completing this research.

Firstly, I would like to express my gratitude to my supervisor Dr. Daruliza Kernain binti Mohd Azman, for the continuous support and for her guidance, encouragement, and knowledge that motivated my master's degree study.

Next, I would like to thank all the lecturers, staff, and laboratory assistants of the Institute for Research in Molecular Medicine (INFORMM) and IPPT, USM, for their support, suggestions, and assistance in accessing all apparatus and equipment while conducting my research study.

Besides, I thank my friends, lab-mates, and research team- Nadia, Deeza, Nanthini, Aina, and Janany for helping me a lot with the experimental setup and analysis. Then, I would like to express my gratitude to my family for their encouragement and support in helping me finish my research project.

Last but not least, I want to thank me for believing in me. I want to thank me for doing all the hardwork. I want to thank me for having no days off, I want to thank me for never quitting, I want to thank me for always being a giver and try to give more than I receive, I want to thank me for trying to do more right than wrong, I want to thank me for just being me at all times.

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LIST OF SYMBOLS

%	Percentage
~	Approximately
µL	Microliter
$2^{-\triangle\triangle CT}$	2 Delta Delta Ct
$A_{t=\Delta h}$	Area measured at *h* hours
bp	Base pair
Cell / mL	Cell over milliliter
Ct	Cycle threshold
E	PCR efficiency
g	G-force or relative centrifugal force
g	Gram
kg	Kilogram
kDa	Kilo dalton
g/mol	Grams per mole
L	Liter
L	Amplicon length
mg	Milligram
mg/mL	Milligram per milliliter
mL	Milliliter
mm	Millimeter
Mm	Millimolar
nm	Nanometers
O. D	Optical density
ºC	Degree Celsius

R^2	Correlation coefficients
T_m	Melting temperature
V	Volts
v/v	Volume over volume
μm	Micrometer
μM	Micromolar

LIST OF ABBREVIATIONS

AIF	Apoptosis-inducing factor
AKT	Protein kinase B
ANOVA	Analysis of variance
APS	Ammonium persulfate
ARF	ADP-ribosylation factor
ATCC	American Type Culture Collection
ATGs	Autophagy-related
AVBB	Annexin V Binding Buffer
b/HLH/LZ	Basic helix-loop-helix leucine zipper
BAX	Bcl-2 Associated X-protein
BBB	Blood-brain barrier
Bcl-2	B-cell lymphoma-2
BiCNU	Carmustine
BMI1	Polycomb complex protein
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CCNU	Lomustine
cdc25A	Cell division cycle 25A
CDK	Cyclin-dependent kinase
c-Myc	MYC proto-oncogene, bHLH transcription factor
CNS	central nervous system
CO_2	Carbon dioxide
co-IP	Co-immunoprecipitation
COX-2	Cyclooxygenase 2
CREB	cAMP response element-binding protein
Ct	Cycle threshold
CTD	C-terminal domain
CXCR4	C-X-C chemokine receptor type 4
DBTRG-05MG	Denver Brain Tumor Research Group 05 Malignant Glioma
DDR	DNA damage response
D-MEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGFR	Mutation of epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
EtBr	Ethidium bromide
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
gadd45	Growth arrest and DNA damage-inducible 45
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GLI1	Glioma-associated oncogene homolog 1
HGG	High-grade glioma
HRP	Horseradish Peroxidase
IAPs	Inhibitors of apoptosis proteins
ID	inhibitors of DNA binding and cell differentiation proteins
IDH	Isocitrate dehydrogenase
IPS	Institut Pengajian Siswazah
IVIS	Real-time in-life fluorescence and bioluminescence imaging
JAK	Janus kinase
LC3	Microtubule-associated protein 1A/1B-light chain 3
LC_{50}	Half-maximal lethal concentration
LDH	Lactate dehydrogenase
LR4	Lipoprotein receptor-
MAPK	Mitogen-activated protein kinase
MAX	Myc-associated factor X
MB	MYC box
MDM2	Murine double minute
MEK	MAPK/ERK kinase
MGMT	O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation
MMPs	Matrix metalloproteinases
mTOR	Mammalian target of rapamycin

MYD88	Myeloid differentiation primary response 88
NANOG	Homeobox protein NANOG
NCBI	National Centre for Biotechnology Information
NCCD	Nomenclature Committee on Cell Death
NCDs	Noncommunicable diseases
NF1	Neurofibromatosis type1
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NTD	N-terminal transactivation domain
NTRK	Neurotrophic tyrosine receptor kinase
O. D	Optical density
OCT4	Octamer-binding transcription factor 4
OLIG2	Oligodendrocyte Transcription Factor 2
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PBS	Phosphate-buffered saline
PDGFR	Platelet-derived growth factor receptor
PE	Plating efficiency
PI	Propidium iodide
PI3KIII	Phosphoinositide 3 kinase
PPI	Protein-protein interaction
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
Raf	Rapidly Accelerated Fibrosarcoma
Ras	Rat sarcoma virus
RIP	Receptor-interacting protein
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RQ	Relative quantification
rRNA	Ribosomal RNA
RT	Room temperature
SDG	Sustainable Development Goal
SDS	Sodium Dodecyl Sulfate

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard error of the mean
SF	Surviving fraction
SMAC	Second mitochondria-derived activator of caspase
Smads	Suppressor mothers against decapentaplegic
SOC	Standard of care
SOX2	SRY-Box Transcription Factor 2
STAT	Signal transducer and activator of transcription
TAD	Transcription activation domain
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline – Tween-20
TEMED	N, N, N', N', -tetraacetylethylenediamine
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
TGF-β	Transforming growth factor β
TME	Tumour microenvironment
TMZ	Temozolomide
TP53	Tumor protein 53
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UKL1	Uridine/cytidine kinase 1
USM	Universiti Sains Malaysia
VEGFR	Vascular endothelial growth factor receptors
WB	Western Blot
WHO	World Health Organization
Wnt	Wingless-related integration site
WST-1	Water Soluble Tetrazolium Salts – 1

LIST OF APPENDICES

PENJELASAN LALUAN PENGISYARATAN ERK1/2/C-MYC/P53 YANG MELIBATKAN AKTIVITI ANTIPROLIFERATIF DIARUHKAN ANDROGRAFOLIDA DALAM TITISAN SEL GLIOBLASTOMA MANUSIA DBTRG-05MG

ABSTRAK

Andrografolida adalah lakton diterpene bicyclic yang disintesis dari *Andrographis paniculata* dengan kesan antikanser dalam pelbagai jenis garis sel barah. Keseluruhan kajian menilai keberkesanan antikanser andrografolida dan jalur molekul berpotensi menggunakan titisan sel glioblastoma multiforme (GBM). Aktiviti antiproliferatif andrografolida ditentukan menggunakan ujian WST-1. Sementara ujian calar dan ujian klonogenik digunakan untuk menilai keberkesanan andrografolida terhadap garisan sel kanser dengan memeriksa penyembuhan luka dan migrasi sel. Seterusnya, flowcytometri juga digunakan untuk memeriksa apoptosis dan penangkapan kitaran sel yang disebabkan oleh andrografolida. Tahap ekspresi yang terlibat dengan jalur isyarat ERK1/2 /c-Myc /p53 kemudian dinilai menggunakan qRT-PCR dan western blot. Interaksi protein-protein antara c-Myc dan p53 ditentukan oleh eksperimen timbal balik co-IP menggunakan jumlah sel lisat DBTRG-05MG. Andrografolida secara signifikan mengurangkan daya maju titisan sel DBTRG-05MG dalam kepekatan dan bergantung pada masa, dengan nilai LC_{50} 42.82 µM (24 jam), 27.21 µM (48 jam) dan 13.95 µM (72 jam). Selain itu, ujian calar dan klonogenik membuktikan bahawa andrografolida mempunyai kecenderungan untuk mengurangkan migrasi sel, pembentukan koloni dan kitaran sel terhenti pada fasa G2 / M, diikuti dengan apoptosis. Menurut kajian qRT-PCR, DBTRG-05MG dirawat dengan kepekatan andrografolida rendah (LC_{50}: 13.95 µM)

dan tinggi ($2LC_{50}$: 27.21 µM), tahap ekspresi c-Myc meningkat dengan ketara dari 1.12 kali ganda menjadi 5.73 kali ganda; ERK1/2 dari 1.72 kali ganda hingga 2.87 kali ganda dan p53 masing-masing dari 1.4 kali ganda hingga 2.25 kali ganda berbanding dengan sel DBTRG-05MG yang tidak dirawat. Oleh itu, ekspresi berlebihan ERK1/2 meningkatkan ekspresi c-Myc dan p53 dengan ketara. Hasil western blot menunjukkan bahawa ekspresi berlebihan c-Myc meningkatkan pengeluaran protein anti-apoptotik p53. c-Myc dan p53 dapat berinteraksi untuk mengaktifkan jalur isyarat apoptotik, seperti yang ditunjukkan oleh hasil co-IP. Kemudian, keputusan co-IP telah menunjukkan bahawa c-Myc dan p53 boleh berinteraksi untuk mengaktifkan laluan isyarat apoptosis. Akibatnya, penglibatan c-Myc dalam laluan isyarat apoptosis melalui penekan tumor p53 telah didedahkan dalam kajian ini.

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ABSTRACT

Andrographolide is a bicyclic diterpene lactone synthesized from *Andrographis paniculata* that has anticancer effects in a range of cancer cell line types. The whole study evaluates andrographolide's anticancer effectiveness and potential molecular pathways using a glioblastoma multiforme (GBM) cell line. The antiproliferative activity of andrographolide was determined using the WST-1 assay. While scratch assays and clonogenic assays were used to evaluate andrographolide's effectiveness against the cancer cell line by examining wound healing and cell migration. Next, flowcytometry was used to examine the apoptosis and cell cycle arrest induced by andrographolide. The expression level of ERK1/2 /c-Myc /p53 signaling pathway was assessed using qRT-PCR and western blot. Then, the protein-protein interaction (PPI) between c-Myc and p53 was determined by a reciprocal co-immunoprecipitation (co-IP) experiment using DBTRG-05MG total cell lysate. Therefore, andrographolide significantly reduced the viability of DBTRG-05MG cell line in a concentration- and time-dependent, with L_{C50} values of 42.82 µM (24 hours), 27.21 µM (48 hours) and 13.95 µM (72 hours). Besides, scratch and clonogenic assay evidence that andrographolide tends to reduce cell migration and colony formation and may induce a concentration-dependent and cell cycle arrest in the G2/M phase, followed by apoptosis. According to a qRT-PCR study, DBTRG-05MG was treated with low (LC_{50} :13.95 µM) and high ($2LC_{50}$: 27.21 µM) concentrations of andrographolide, the expression levels of c-Myc significantly

increased from 1.12-fold to 5.73-fold; ERK1/2 from 1.72-fold to 2.87-fold and p53 from 1.4-fold to 2.25-fold respectively in comparison to untreated DBTRG-05MG cells. Hence, the expression of ERK1/2, c-Myc and p53 were significantly increased. Then, the co-IP results was demonstrated that c-Myc and p53 can interact to activate the apoptotic signalling pathway. As a result, the involvement of c-Myc in the apoptosis signalling pathway via the p53 tumor suppressor was revealed in this study.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Brain cancer is one of the most uncommon cancers, with a low annual incidence compared to other types of cancer. However, brain cancer has gained significant attention since the number of deaths from brain cancer is increasing yearly due to poor prognoses, affecting patients' chances of survival. According to Globocan 2020, the number of deaths from brain cancer will rise from 251k to 304k between 2020 and 2030 (Sung et al., 2021). As a result, to meet the Sustainable Development Goal (SDG) target 3.4.1, which is to reduce premature mortality from noncommunicable diseases (NCDs) by 2030 and to promote mental health and well-being for all ages, especially mortality rate attributed to cancer disease (Prager et al., 2018), current treatment or alternative therapies for brain cancer must be improved.

Glioblastoma multiforme (GBM) is a type of primary malignant brain cancer with a higher mortality rate among individuals aged 45 to 65 years old than other types of brain cancer and is considered the deadliest human cancer. GBM is difficult to treat because it grows quickly after conventional treatment and can infiltrate surrounding normal brain cells resistant to chemotherapy treatments. Furthermore, only 2-3% of patients survive up to two years after major resection surgery, radiation therapy, and chemotherapy since current treatments disrupt the neurological central nervous system and have side effects that can disrupt a patient's quality of life (Bahadur et al., 2019).

Then, researchers started to focus on identifying any factors involved in the GBM tumorigenesis and progression. Therefore, there are few cancer hallmarks that

require intensive study to meet the clinical requirement for personalized and targeted therapies such as on the activation of oncogenes or inactivation of tumor supressor genes within the signalling pathway that leads to uncontrolled growth and proliferation (Torrisi et al., 2022). However, there are few challenges in developing anticancer drugs for specific targeted glioblastoma disease because many promising bioactive compounds can inhibit the GBM brain cancer cell line but cannot pass through the blood-brain barrier (BBB) (Harder et al., 2018) and the mechanism of action is still need to be explored in details.

From that, andrographolide is gaining the attention of researchers due to its potential to treat GBM or diseases related to the central nervous system (CNS) because it is a polar compound with low molecular weight and easy to pass through the BBB (R. Yang et al., 2017) and also acts as neuroprotection by preserving the neuronal structure and function (Lu et al., 2019). More interesting, previous studies has discovered pharmacological properties of andrographolide involving an immunomodulatory, anti-inflammatory, and hepatoprotective agent, implemented in the treatment of diarrhea, cold, fever, and other infectious diseases. Other than that, andrographolide also has anticancer properties against colon cancer, lung cancer, breast cancer, melanoma cancer, and leukemia by involving various mechanism of action such as inhibition of cell cycle progression, induced apoptosis, reduced cell invasion and migration through targeting different targeted genes (Khan et al., 2018).

With more emphasis, recent studies claimed that andrographolide may be a potential cancer therapeutic agent because it can inhibit the growth of various cancer cell lines such as MDA-MB-231, MDA-MB-361, MCF-7, BT549, and T47D, HCT-116, C8161 and A375 Cells (Y. Peng et al., 2018; Khan et al., 2020; Mehta et al.,

2021). However, its effect on human SVGp12 & DBTRG-05MG cell lines remains incomplete. The similar research conducted by Islam et al. (2018), which has shown that andrographolide can be further developed as a new drug to treat and reduce cell cancer growth by involving a few mechanisms such as proliferation, oxidative stress, cell cycle arrest, inhibition of cell adhesion, anti-inflammatory, and immune system-mediated effects, apoptosis, necrosis, autophagy, migration, invasion, anti-angiogenic activity, and other actions.

However, the cell cycle arrest and apoptosis are the most critical strategies for eliminating cancer cells in this investigation. The cell cycle arrest was used by cells to promote DNA repair before cell growth. At the G1 phase, S phase, and G2/M transition are the cell cycle checkpoints that can be activated due to DNA damage response (DDR) (Visconti, Della Monica & Grieco, 2016). This checkpoint allows cells to repair damaged DNA, but if the damage is too prominent, other signalling mechanisms are activated to induce cell cell cycle arrest or apoptosis in order to prevent cancer (O'Grady & Lawless, 2015). While apoptosis, or programmed cell death, may be suppressed by a few molecular mechanism steps which involved extrinsic (dependent on so-called death receptors expressed in the plasma membrane) or via the intrinsic (mitochondria-dependent). Then, any deficiency or abnormality on gene activation in the apoptotic pathways might be a promising benchmark for cancer treatment (Hassan et al., 2014).

In addition, the focus of this study was aimed into the involvement of the andrographolide with the c-Myc gene in GBM to induce cell death mechanism. C-Myc is a transcription effector that encoded gene which controls the expression of over 100 target genes. It is involved in cell proliferation and the formation of cancer.

The c-Myc is the most frequently deregulated oncogenes in human cancer (Ciribilli et al., 2015). Other researchers have claimed that an increase in c-Myc expression may initiate the activation of some downstream genes such as ARF, p53, gadd45, cdc25A, Cyclin A, Cyclin D and CDK4 which trigger cell cycle arrest and apoptosis (Yoshida, 2018). Then, this study aimed to use a specific compound like andrographolide as the important source for small molecule drugs by targeting the specific signaling pathway that is involved in the activation of c-Myc such as Wnt/β-catenin, Hedgehog, Hippo, p53, NF-Kb, STAT, and p13-K/AKT/ERK, which can also encourage cancer cells to undergo cell death via apoptosis through the c-Myc signaling pathway (Mishra, 2015). From that, c-Myc can be a hallmark in cancer therapeutic strategies.

However, there is currently a lack of research and understanding of whether andrographolide can directly target c-Myc or not, as well as the interaction of andrographolide in the upstream and downstream c-Myc signalling pathways in human brain cell lines. As a consequence, the aim of this study was to discover more about the molecular mechanism of andrographolide's cell death effect on the DBTRG-05MG human glioblastoma cell line.

1.2 Problem Statements and rationale of the study

Glioblastoma multiforme (GBM) is the most aggressive and fast-growing type of brain tumor. GBM therapy present therapeutic difficulties due to adverse effects after conventional therapy. Then, many researchers have begun to emphasize on small molecule targeted therapy, which only specifically target the cancer cell, not a normal cell. The targeted therapy option for GBM disease that focused on gene or any specific molecular alterations are needed to improve the therapeutic target identification and diagnostic accuracy. Therefore, there is a need to find a potential

small molecule targeted therapy that may directly target oncogenes or tumour suppressors that are generally involved in specific molecular signaling pathways in GBM cells. Now, andrographolide attracts a lot of interest from researchers who want to explore how this small molecule drug can aid in the battle against different cancer cells by targeting a specific gene within a cancer cell. However, more study is needed because the specific molecular mechanism signalling pathway of andrographolide towards DBTRG-05MG human glioblastoma cell line is still controversial.

1.3 General Objective

This study aims to elucidate the molecular mechanism(s) of andrographolide that leads to antiproliferative activity in DBTRG brain cancer cell line.

1.3.1 Specific Objectives

1. To evaluate the cell proliferation and migration of DBTRG-05MG human brain cell line after treatment with andrographolide

2. To elucidate the mechanism(s) of cell death and apoptosis of DBTRG-05MG cell line via ERK1/2/c-Myc/p53 signaling pathway after treated with andrographolide

3. To determine protein-protein interaction (PPI) of c-Myc with p53 in DBTRG-05MG cell lines after treated with andrographolide

1.4 Experimental Design

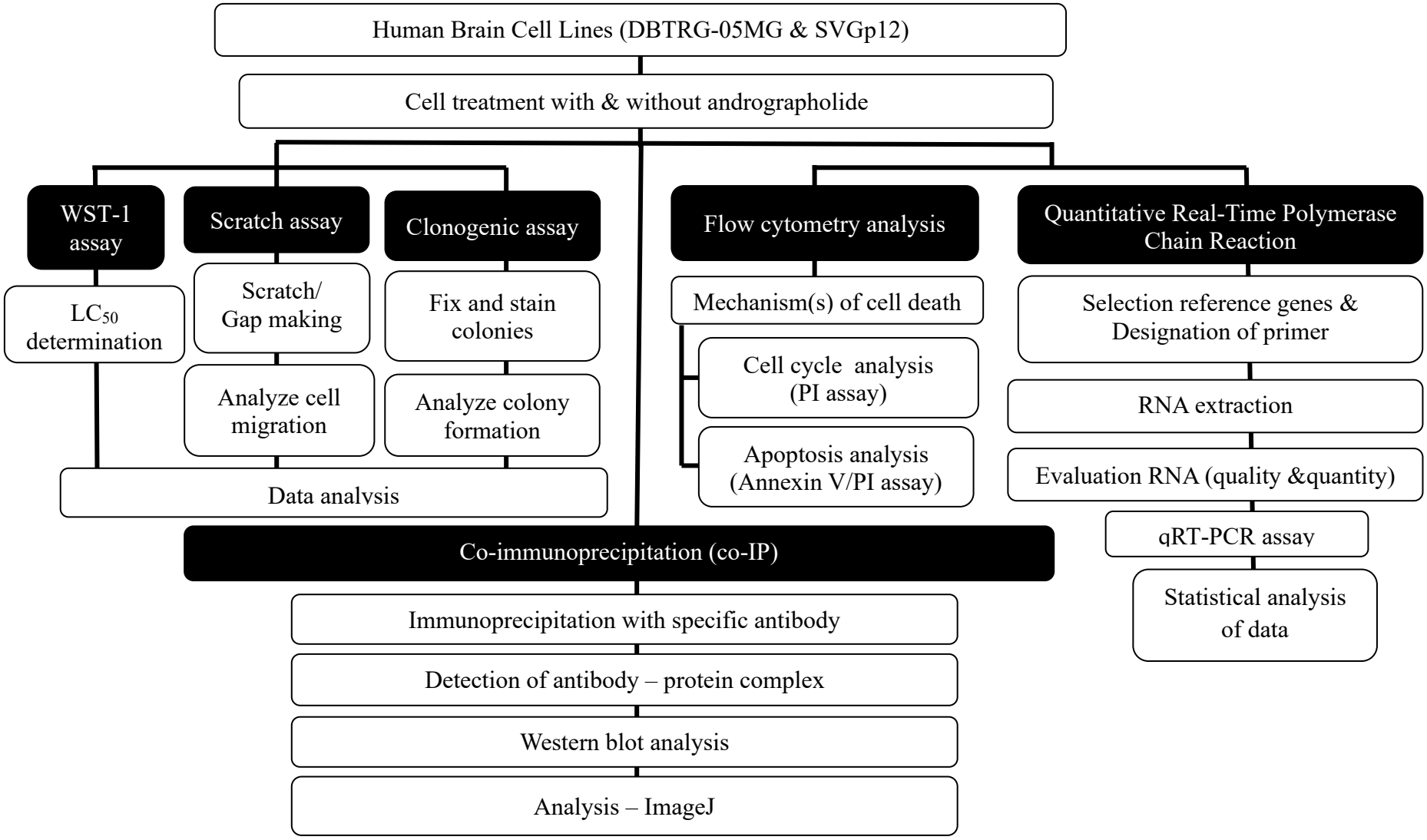

Figure 1.1 Research flowchart

CHAPTER 2

LITERATURE REVIEW

2.1 Brain tumor

The brain is a complex mass of supporting tissues and nerve cells that connect to form the central nervous system (CNS), which carries information throughout the body (Boire et al., 2020). A brain tumor is a collection of unwanted or abnormal tissue that forms in the brain, causing pressure and disrupting normal brain function in patients. Brain tumors are classified as malignant or benign tumors. Many tumor grow rapidly, while others grow slowly. Brain tumors are the deadliest and most common cancers in both children and adults (Rehman et al., 2020). The most prevalent causes of brain cancer include ionising radiation, insecticides, and cyclic aromatic hydrocarbons (Khazaei et al., 2020).

According to the World Health Organisation's Globocan databases from 2012 and 2020, the total number of incidence increase from 786 to 800 cases which includes all Malaysian citizens with ages below 85 years in Malaysia. WHO also estimated the number of new cases for brain cancer in 2020 to 2040 will rise again from 800 cases to 1100 cases (Ferlay et al., 2021 ;World Health Organization, 2021). As a result, actions are required to avoid the country's rising cancer incidence and mortality because brain cancer is becoming more advanced and less treatable with current treatment options (Karim Othman et al., 2020).

2.1.1 Classification of brain tumor

There are two categories of brain tumours: primary and metastatic. The primary brain tumor develops within the brain tissue when a normal cell's DNA is mutated, allowing the cells to proliferate and divide rapidly, resulting in the formation of a tumor (Rehman et al., 2020). Primary brain tumours are classified as benign (non-

cancerous) and malignant (cancerous). Benign gliomas are represented by slow-growing cells that seldom spread, whereas malignant brain tumours are represented by rapidly growing cells that infiltrate normal brain tissue. Apart from that, the malignant brain tumor also can metastasize in other body parts such as the liver, lungs, brain, and bone. As a result, this tumor requires immediate treatment, including chemotherapy or radiotherapy, to prevent it from migrating to other body parts (Patel, 2020).

Gliomas, meningiomas, schwannomas, craniopharyngiomas, germ cell tumors, and pineal area tumors are the primary brain cancers that are classified based on the type of tissue from which the tumor grows. Glioma brain tumours have now been classified into various forms, including oligodendrogliomas, astrocytoma, ependymomas, choroid plexus papilloma, and glioblastoma multiforme (Rehman et al., 2020). The metastatic or secondary brain is a form of cancer that develops in another part of the body and subsequently spreads to the brain. A secondary tumor is more common in persons who have already been diagnosed with lung, breast, colon, or melanoma cancer. These cancers can migrate to the brain via the bloodstream and cause a brain tumor (Iqbal et al., 2017).

2.1.2 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most prevalent aggressive type of astrocytoma, which arises in the brain with star-shaped cells. Astrocytes are cells that provide extensive support for nerve cells, including physical and metabolic support. It is known as the highest-grade glioma, which is grade IV in the WHO grading system. The histologic features that distinguish glioblastoma from all other grades are the presence of necrosis and the increase of blood vessels around the tumor. In 1926, Percival Bailey and Harvey Cushing created the name of glioblastoma multiforme; the

term multiform was intended to reflect the varied appearances of haemorrhage, necrosis, and cysts (Stoyanov & Dzhenkov, 2018).

Basically, the glioblastoma are located in the supratentorial brain (frontal, temporal, parietal, and occipital lobes), with only a few cases occurring in the cerebellum, brain stem, or the spinal cord (Tamimi & Juweid, 2017). The GBM is mostly resistant to therapy and has a very poor prognosis that causes to higher death rate among the patient. The average survival rate of patients that have been diagnosed with GBM is only about 12-15 months after standard therapy (Dorte Schou et al., 2017). The GBM is difficult to treat since it occurs in our body's most critical and complicated organ. As a result, removing tumors surgically without affecting or damaging neurological function was incredibly challenging for neurosurgeons (Hopkin, 2019).

From Table 2.1, a few of the most common genetic alterations in GBM that were rarely or not detectable in normal brain tissue may provide information or can be hallmarks of therapeutic decision-making for GBM tumor. The amplification or mutation of EGFR (22-50%) has the highest frequency incidence in GBM tumor followed by O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation (36%), CDKN2A/B deletions (31%), TP53 mutation (28-31%), PTEN deletions (24-30%), glioma-associated oncogene homolog 1(GLI1) (5-22%), neurofibromatosis type1 (NF1) a deletion or mutations (11 %), TERT mutation and murine double minute 2(MDM2) (7-12%). As a result, the EGFR remains a promising target for GBM therapy (Montemurro, 2020).

Then, recently the amplification or mutation of MYC (c-Myc, L-Myc, S-Myc, and N-Myc) has been found to be present in GBM. The MYC amplification was

approximately 40% in the GBM sample, so it usually forms extrachromosomal DNA, which leads to tumorigenesis and relates to a poor prognosis and aggressive conditions in cancers (Borgenvik et al., 2021). Myc is a challenging oncoprotein to target because of its recurrence of overexpression in cancers and its ubiquitous function in transcriptional regulation. Targeting the MYC oncoprotein seems to be a very difficult but intense requirement for its dual pivotal roles in both normal proliferating cells and cancerous cells. Then, the alternative methods for targeting on MYC in cancer therapy were divided into the following, including MYC transcription, MYC mRNA translation, MYC stability, MYC interaction with Myc-Max, and MYC accessibility to downstream genes. As a response, recent findings have questioned whether targeting MYC and its downstream genes is a particular therapeutic approach for GBM (Wang et al., 2021).

Table 2.1 List of genetic alteration in GBM

Gene Alteration (GA)	**Percentage of mutation in GBM**
Amplification or mutation of epidermal growth factor receptor (EGFR)	22-50%
Cyclin-dependent kinase inhibitor2A/B(CDKN2A/B) deletion	31%
TERT mutation	10%
Amplification of platelet-derived growth factor receptor (PDGFR)	7%
Tumor protein 53(TP53) mutation	28–31%
Phosphatase and tensin homolog (PTEN) mutation or deletion	24–30%
IDH1/2mutation	5%
Telomerase reverse transcriptase (TERT) promoter	10%
O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation	36%
Murine double minute 2(MDM2)	7–12%
Neurofibromatosis type1 (NF1) deletion or mutations	11%
Glioma-associated oncogene homolog 1(GLI1)	5–22%

2.2 Current treatment of GBM

The current standard therapy for GBM is the most challenging and costly cancer therapeutic option. Misdiagnosis of brain tumors leads to ineffective therapy and reduces patients' chances of survival. Accurate brain tumour detection is critical for effective therapeutic interventions to cure and improve the lives of individuals with brain tumours. The treatment options may differ based on the location, size, classification, and WHO grading of the tumor. Since GBM malignancies are incurable and often resistant to therapy, oncologists will frequently employ a combination of treatments to keep the tumour from spreading (Carbone, 2020).

2.2.1 Surgery

Surgery is one of the primary treatments for higher-grade GBM tumors, along with daily radiation and oral chemotherapy (Jovcevska, 2019). The tumor can be removed as much as possible during surgery without affecting neuro logical abilities such as speaking, walking, vision, or hearing. During surgery, the neurosurgeon will do a stereotactic biopsy, which entails drilling a small opening in the patient's skull to allow the neurosurgeon to locate the abnormal location. This will be followed by a craniotomy, in which the patient's skull will be opened to access the tumour and remove as much of it as possible (Lara-Velazquez et al., 2017). Moreover, the surgery procedure is the most challenging in oncology therapies because the tumor is located in the most critical area of the central nervous system and sometimes a second surgery will be performed if the cancer grows again.

2.2.2 Radiotherapy

Radiotherapy is used after surgery if the tumor cannot be removed or is impossible to remove due to its location. Normally, neurosurgeons recommend

radiotherapy to eradicate the tumor by employing high-energy x-rays, gamma rays, or other particles to shrink or stop the tumor from growing again and completely remove it (De Ruysscher et al., 2019). It has been discovered that combining radiotherapy with surgery extends the life of patients from 3-4 months to 7-12 months (Mann et al., 2018).

2.2.3 Chemotherapy

Chemotherapy is one of the current standard treatment to combat glioblastoma diseases. The patient undergoing this treatment is commonly administered the special drug to kill cancer. Based on the standard of care (SOC) for glioblastoma multiforme, the combination of chemotherapy and radiation treatment has improved patient survival and reduced the effect of surgery after treatment. The combination treatment can be either radiotherapy and chemotherapy alone or both before surgical procedure. However, limited drugs are still used in chemotherapy (Ozdemir-Kaynak et al., 2018).

Many researchers are trying to explore and develop any potential compound that can treat the high glioma grade. Table 2.2 lists a few FDA-approved chemotherapeutic drugs (Fisher & Adamson, 2021). Temozolomide is the most preferred by the FDA as the chemotherapy drug for standard care (SOC) for all high-grade glioma (HGG), such as glioblastoma multiforme disease. Furthermore, bevacizumab, lomustine, and carmustine, which are also frequently used in the treatment of recurrent HGG and tumour symptoms. Each drug has a different mechanism for treating GBM, but there is still a need to find another treatment that can target a new mechanism, because GBM appears to be the most aggressive tumor resistant to current chemotherapeutic agents. Apart from the four FDA-approved drugs, which are lomustine, carmustine, temozolomide, and bevacizumab, a few compounds are still being evaluated in clinical trials due to the urgent need for new

promising compounds to overcome treatment resistance in GBM. As a result, numerous research began to concentrate on identifying any bioactive substance, particularly those derived from natural resources, as a possible small molecule targeted treatment for GBM.

2.2.4 Diagnostic approaches in GBM

The current conventional treatments for GBM are currently facing a problem due to poor prognosis after treatment because patients with GBM only survive up to 12 months following a standard treatment. Moreover, patients with GBM mostly suffer due to the molecular heterogeneity of GBM and the penetration of bioactive agents through the BBB, which can lead to a poor prognosis and create tumor resistance. Then, many researchers try to identify the molecular biomarker in GBM through next-generation sequencing methods for a better understanding of the molecular pathogenesis of this disease.

There are few molecular markers still being studied, but several are commonly tested as part of the routine clinical interrogation of GBM patients including O6-methylguanine DNA methyltransferase (MGMT), isocitrate dehydrogenase (IDH) (Weller et al., 2013), epidermal growth factor receptor (EGFR) (Ramaiah & Kumar, 2021), VEGF, tumor suppressor protein TP53, phosphatase and tensin homolog (PTEN), p16INK4a gene, phospholipid metabolites, cancer stem cells, and recently also imaging biomarkers (Brito et al., 2019;Korfiatis et al., 2016). As a result of this new technology that allows for the detailed characterization of molecular biomarkers, many researchers are beginning to focus on personalised therapies, which could lead to a new generation of anti-GBM therapies. For example, molecular inhibitors targeting growth factor receptors, vaccines, antibody-based drug conjugates, and, most recently, immune checkpoint blocking inhibitors (Szopa et al., 2017).

Table 2.2 List of chemotherapy drug for HGG that approved by FDA

Drug Name	**Drug Mechanism**	**Common Toxicities**	**Year Approved**
Lomustine (CCNU)	Nonspecific alkylating agent that causes crosslinking of DNA and RNA in dividing cells triggering cell death	Hematologic toxicity (49.7%)	1976 for recurrent HGG
BiCNU / Carmustine	Nonspecific alkylating agent that causes crosslinking of DNA and RNA in dividing cells; also binds to and modifies glutathione reductase	Pulmonary toxicity (<30%), ocular toxicity (>10%) and bone marrow suppression (>10%)	1977 for recurrent HGG
Temozolomide /Temodar	Nonspecific alkylating agent that causes mismatch repair in DNA by methylation at the O6 position of guanine	Hematologic toxicity (16%): thrombocytopenia (12%), leukopenia (7%), and neutropenia (7%)	2005 for ALL HGG (Standard of care)
Avastin /Bevacizumab	VEGF/VEGFR inhibitors	Hypertension (5.5–11.4%), thromboembolic events (3.2–11.9%), gastrointestinal perforation (1.5–5.4%), cerebral bleeding (2–5.3%), wound healing complications (0.8–3.3%), and proteinuria (2.7–11.4%)	2009 for recurrent HGG

2.2.5 Limitation of current treatment

The present treatments for GBM, such as surgery, chemotherapy, and radiation, have limitations since they affect healthy tissue or organs, causing adverse effects in the patient. The post-treatment effect of surgery is related to the neurologic effect, but the side effects of radiation are malaise, skin changes, and alopecia, with long-term effects being cognitive alterations and endocrine difficulties. Furthermore, the current treatment with TMZ, carmustine, lomustine, and bevacizumab causes the undesirable side effects described in table 2.2, such as nausea or vomiting, hematologic effects (thrombocytopenia and lymphopenia), wound healing complications, and others (Serventi & Behr, 2018). Hence, there is a need to urgently find other approaches that only specifically target the cancer cell, not a normal cell. Moreover, many researchers were facing the same problem because of the presence of the blood-brain barrier (BBB) that prevents drug entry from the blood to the brain region, causing hard to target the specific tumor in the brain. Researchers need to find a compound that has a low molecular with a high degree of lipid solubility to overcome the BBB.

2.2.6 Hallmark of glioblastoma multiforme

According to a recent study conducted by Hanahan D. and Weinberg R.A in 2022, 14 cancer hallmarks are acquired throughout the multistep development of cancer and can be one of the treatment approaches. Then, over the same years, researchers tend to investigate and suggest GBM hallmarks by referring to the review of Hanahan D. and Weinberg. As a result, seven major hallmarks of GBM have been identified, including enhanced growth and proliferation, altered stress response, sustained angiogenesis, tissue invasion and metastasis, metabolic reprogramming or

modification, immunological modulation, and tumor microenvironment (TME) stimulation (Torrisi et al., 2022).

Based on existing GBM hallmarks, this study focused on targeted therapy for uncontrolled proliferation caused by genes and dysregulation of signalling pathways which involved P16, p53, EGFR, WNT, Cyclin-dependent kinase inhibitor 2A (CDKN2A), N-Myc downstream regulated gene 2 (NDRG2) (Uddin et al., 2022). Many researchers have begun to emphasize targeted therapy, which targets cell growth and proliferation as a potential treatment for cancer. This targeted therapy employs small molecule drugs to target specific genes or proteins related to tumor progression, as well as monoclonal antibodies to inhibit cell growth and lead them to self-destruct, such as apoptosis. Targeted therapy has the advantage of limiting harm to normal tissue since it is directly focused on cancer. It can be used alone or in combination with normal chemotherapy, surgery, and radiation therapy to improve survival and eliminate unwanted side effects (Lee et al., 2018).

Next, the Ras-Raf-MEK-ERK pathway is the major mitogen-activated protein kinase (MAPK) pathways that regulate the nucleus and cytoplasm protein involved in cell proliferation, survival, and metastasis. The MAPK pathway is activated then leads to cell growth and proliferation via direct (such as changes in the cell cycle) and indirect effects (the formation of a beneficial TME and new angiogenesis, ensuring the accessibility of nutrients and growth factors) (Krishna et al., 2021). The previous study also suggests that one of the novel therapies for malignant gliomas involves targeting the MAPK pathways and their important downstream (Cheng & Guo, 2019; K. Banerjee et al., 2021).

Apart from that, there are a few emerging targeted therapy options for GBM disease based on gene alteration (GA) studies, such as EGFR, PI3K/AKT/mTOR, MET, EGFR, BRAF, NTRK, VEGF, Integrins, and TGF. Then there is the fact that GBM is highly resistant to conventional therapies like radiation and chemotherapy due to EGFR signalling pathway dysregulation. Many studies have begun to focus on on EGFR biomarker to overcome GBM therapeutic resistance (Le Rhun et al., 2019a). Furthermore, it is important to do an intensive study on the other molecular-targeted genes involved in GBM. This is because the molecular-targeted genes may trigger or activate a wide range of signals within the cell and identify the potential downstream signals that drive biological responses like cell proliferation, motility, apoptosis, and cell cycle arrest.

Moreover, the previous study also targeted ligands or receptors that initiate extracellular signaling and prevent adaptive resistance after chemotherapy and radiotherapy. Now, several new approaches still need to be explored by targeting the transcription factors using a small molecule drug in GBM therapy. The GBM has triggered a variety of gene expressions, most of which occur at the transcription level when information contained inside the gene's DNA is passed to mRNA. Thus, oncogenes such as c-Myc, OLIG2, BMI1, SOX2, NANOG, OCT4, and ID may activate numerous extracellular signaling pathways by activating transcription factors, which in turn activate numerous downstream signaling pathways involved in the regulation of cellular metabolism, proliferation, differentiation, and programmed cell death (Osuka & Meir, 2017). These oncogenes binds to gene regulatory regions, where they either increase or decrease transcription levels. As a result, this approach can determine when and how much protein product a gene produces, which can be considered a potential strategy for GBM treatment.

2.3 Andrographis paniculata

Andrographis paniculata (A. paniculata) is classified as a herbaceous plant and belongs to the Acanthaceae family. *A. paniculata is* known in Malaysia as "Hempedu Bumi," which is also named as “King of Bitter” due to the bitter tastes of the leaves (Figure 2.1). *A. paniculata* was traditionally used to treat diabetes, hepatitis, skin infections, snake bite, fever and other human diseases in India, Sri Lanka, Malaysia, China, Indonesia, and Thailand (Rahmi et al., 2022: Mussard et al., 2019). In China, *A. paniculata* is one of their Traditional Chinese Medicine (TCM) and is available at their herbal medicine market in three types which are aerial part (stems and leaves), leaves, and stems. They also believed that *A. paniculata* leaves are more bitter than stems and have more medicinal properties compared to stems.

The modern pharmacological study revealed that *A. paniculata* (aerial part) has various medical properties such as antibacterial, antiviral, anticancer, anti-inflammatory, antioxidant, and hepatoprotective (X. Zhang et al., 2018). The active compound found in *A. paniculata* extract (stems and leaves) was found in more than 55 labdane diterpenoid lactone and 30 flavonoids, 8 quinic acids, and 4 xanthones (Loureiro Damasceno et al., 2022: Dai et al., 2019). Based on this finding, the major bioactive compound in *A. paniculata* has been reported as diterpene lactones, known as andrographolide.

Figure 2.1 Andrographis paniculata

2.3.1 Andrographolide

Andrographolide is the main bioactive compound isolated from *A. paniculata* and was firstly isolated by Gorter in 1911. Based on Figure 2.2 shows the *A. paniculata* extract that contains 98% andrographolide appears as a crystalline solid with a white square prism (Y. Yan et al., 2018b). Recently, andrographolide was reported to exhibit anticancer, antibacterial (L. Zhang et al., 2020), antiviral (Tan et al., 2022), antioxidant (Mussard et al., 2019b), hepatoprotective (Mondal et al., 2022), neuroprotective (M. Y. Yang et al., 2019), anti-fibrosis (Karkale et al., 2018), anti-obesity (Islam et al., 2020), immunomodulatory (Gupta et al., 2021), and hypoglycemic properties (H. Zhang et al., 2021).

Moreover, few clinical trials registered at ClinicalTrials.gov were ongoing to confirm the effectiveness of andrographolide in treating various human diseases. One clinical trial still in phase 2 involves multiple sclerosis (MS), while phase 4 is acute tonsillitis and bronchitis disease (Zeng et al., 2022). These clinical trials have increased andrographolide's credibility in preventing and treating diseases, which will hasten the clinical development of its products. Hence, andrographolide has attracted many researchers to study the mechanism action of this compound toward various human diseases, especially cancer.

Figure 2.2 Andrographis paniculata extract powder (98% andrographolide)

2.3.2 Chemical structure of andrographolide

Based on the National Center for Biotechnology Information (NCBI) on compound summary data reported that the chemical structure name of andrographolide (Figure 2.3) is 3α,14,15,18-tetrahydroxy-5ß,9ßH,10α-labda-8(20),12-dien-16-oic acid γ-lactone with the molecular formula and weight are $C_{20}H_{30}O_5$ and 350.4 g/mol (*Andrographolide | C20H30O5 - PubChem*, 2022). Andrographolide is a gamma-lactone, primary alcohol, secondary alcohol, a C_{20} labdane diterpenoid, and a carbobicyclic compound (Islam et al., 2018).

The structure and purity of andrographolide were extensively studied in 1-D and 2-D nuclear magnetic resonance (NMR), Infrared (IR), Raman, Ultraviolet (UV) spectroscopy, mass spectrometry (MS), and chromatography. From that, andrographolide was reported to be sparingly soluble in water but dissolvable in solvents such as methanol and ethanol but most stable in trichloromethane or DMSO. Lastly, it is most stable at lower temperatures and has a pH value of 3-5 (Y. Yan et al., 2018a).

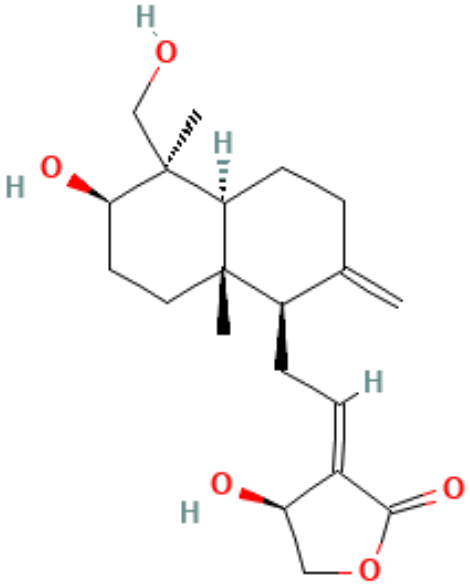

Figure 2.3 Chemical structure of andrographolide.

The diagram is from National Center for Biotechnology Information (https://pubchem.ncbi.nlm.nih.gov/com accessed on 14 September 2021