CELL DEATH EFFECTS OF *GARCINIA ATROVIRIDIS* EXTRACTS ON DBTRG-05MG AND U-87MG HUMAN BRAIN CANCER CELL LINES

BY

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CERTIFICATE

This is to certify that dissertation entitled “Cell death effects of *Garcinia atroviridis* extracts on DBTRG-05MG and U-87MG human brain cancer cell lines” is bonafide record of the research work done by Bebe Syakinah Binti Hashim during the period from September 2016 to July 2017 under my supervision.

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ABSTRAK

Tujuan kajian ini adalah untuk mengkaji kesan-kesan kematian sel yang diaruh oleh ekstrak minyak pati daripada daun (EO-L) dan kulit kayu pokok (EO-B) *G. atroviridis* terhadap sel-sel kanser otak manusia DBTRG-05MG dan U-87 MG. Kesan anti-proliferatif EO-L dan EO-B terhadap sel-sel DBTRG-05 MG dan U-87 MG ditentukan dengan menggunakan ujian sel pertumbuhan MTT dan diukur melalui pembaca mikroplat. Mod kematian sel yang diaruh oleh EO-L ke atas tititsan sel U-87 MG ditentukan dengan menggunakan ujian pewarna Annexin-V FITC dan propidium iodida dan diukur melalui aliran sitometri. Manakala aktiviti autofagi ditentukan menggunakan ujian perencat NVP- BEZ235 dan bacaan diukur menggunakan pembaca mikroplat. Kesan EO-L pada potensi membran mitokondria sel U-87 MG pula ditentukan menggunakan ujian pewarnaan JC-1 dan pemerhatian dilakukan menggunakan mikroskop pendarfluor dan bacaan diukur melalui pembaca mikroplat pendarfluor.

Berdasarkan keputusan ujian MTT, EO-L menunjukkan potensi perencatan pertumbuhan terhadap kedua-dua sel DBTRG-05MG dan U-87 MG berbanding EO-B. Aktiviti perencatan pertumbuhan oleh EO-L juga dilihat lebih signifikan pada sel U-87 MG berbanding sel DBTRG-05MG. EO-L juga mengaruh kematian sel sacara apoptosis terhadap sel U-87 MG. Setelah 24 jam sel U-87 MG dirawat, apoptosis telah diaruh secara signifikan berbanding sel yang tidak dirawat pada semua kepekatan EO-L. Kehilangan potensi terhadap membran mitokondria merupakan indikator awal bagi mekanisme kematian sel secara apoptosis. EO-L didapati mengaruh kehilangan terhadap potensi membran mitokondria pada sel U-87 MG. Hasil kajian ini mendapati sel U-87 MG diwarnakan hijau oleh pewarna JC-1 yang menunjukkan kehilangan potensi pada membran mitokondria, dan keputusan ini adalah selari dengan bacaan yang diperoleh daripada pembaca mikroplat pendarflour. Hasil kajian ini juga mendapati

gabungan rawatan EO-L bersama dengan perencat NVP-BEZ235 berpotensi mengaruh kematian sel secara autofagi di dalam sel U-87 MG.

Kesimpulannya, kajian ini mendapati EO-L berpotensi merencatkan pertumbuhan sel kanser otak manusia U-87 MG dengan berupaya mengaruh kehilangan potensi membran mitokondria yang akhirnya mendorong kepada kematian sel secara apoptosis dan autofagi.

ABSTRACT

The aim of this study was to investigate the cell death effects of the essential oils extracted from the leaf (EO-L) and stem bark (EO-B) of *G. atroviridis* on DBTRG-05MG and U-87 MG human brain cancer cells. The anti-proliferative effects of EO-L and EO-B were determined using MTT assay by microplate reader. The mode of cell death induced by EO-L on U-87 MG cell line was determined using Annexin-V FITC and propidium iodide staining assay by flow cytometry. Whilst autophagy activity was determined using NVP-BEZ235 inhibitor assay by microplate reader. The effect of EO-L on mitochondrial membrane potential of U-87 MG cells was determined using JC-1 staining assay by fluorescence microscopy and fluorescence microplate reader.

Based on the MTT assay result, EO-L potentially inhibited both DBTRG-05MG and U-87 MG cells in comparison to EO-B. The growth inhibition induced by EO-L was significantly observed in U-87 MG when compared to DBTRG-05MG cells. The cell death induced by EO-L on U-87 MG cells was via apoptosis. At 24 h post-treatment, apoptosis was significantly induced in U-87 MG cells treated with EO-L at all concentration tested in comparison to the untreated cells. The loss of mitochondrial membrane potential is an early indicator for the mechanism of apoptotic cell death. EO-L has induced the loss of mitochondrial membrane potential in U-87 MG cells. The findings herein showed that U-87 MG cells were stained with JC-1 green fluorescence, which indicated the loss of mitochondrial membrane potential, and this observation was correlated with the readings obtained by fluorescence microplate reader. This study also found that co-treatment of EO-L and NVP- BEZ235 inhibitor potently induced the autophagic cell death in U87 cells.

In conclusion, EO-L potently inhibited the growth of U-87 MG human brain cancer cells, by inducing the loss of mitochondrial membrane potential which ultimately leads to the apoptotic and autophagic cell death

. **CHAPTER 1**

INTRODUCTION

1.1 Epidemiology of Brain Tumor/Cancer

One of the leading causes to brain cancer is brain tumor and brain metastases. Brain tumors are among the most serious and complicated disease in comparison to other cancer diseases like lung cancer, breast cancer and skin cancer (Teplyuk *et al.*, 2012). The primary tumor of the central nervous system is glioma. Treatment such as standard chemotherapy and other types of therapy could not totally cure or inhibit the proliferation of the glioma cells (Zülch, 2013).

1.2 Histology and Molecular Genetics of Brain Tumors Types.

Initiation of brain cancer in the glioblastoma (GBM) cells is either through gene mutation or DNA damage when compared to the other types of brain cells such as meningiomas, medulloblastomas, ganglioglioma, schwannomas and chordomas (Cohen-Inbar and Sheehan, 2016). Despite the many advances in tumor treatments, only a small percentage of GBM patients are fully cured. The high invasiveness of GBM cells causes complications to the surgical treatment, chemotherapy and radiotherapy. Furthermore, an abnormality in apoptosis as well as cancer stem cells may contribute to chemo resistance as well (Wong, 2011).

1.3 Natural Products Derived from Plant as an Alternative Treatment for Cancer.

Naturally-derived bioactive compounds from plants had showed potential treatment in inhibition of the growth of the glioma cells in addition to synthetic drugs. Plants have been reported as an alternative therapy in curing cancers as a result of their anti-proliferative and anti-fungal properties (Wang *et al.*, 2012). In this study, essential oils from the leaf and bark of *Garcinia atroviridis* (*G. atroviridis*) were used in determining their cytotoxic property. Flavonoids and 4-methylhydroatrovirinone

are among the phytochemicals of the plants, *G. atroviridis* present in the plant which has anti-inflammatory effect (Tan *et al.*, 2013). However, its cytotoxic activity against cancer has yet to be affirmed. Hence, this is the first study to investigate and report the cytotoxic effects of *G. atroviridis* against the brain cancer cells. Two model of GBM cells were used in this study, namely DBTRG-05MG and U-87MG. Assessments on the cell death effects of essential oils extracted from the leaf (EO-L) and stem bark (EO-B) of *G. atroviridis* were examined and investigated using several number of *in vitro* analyses, including anti-proliferative study using MTT reagent, the mode of cell death via apoptosis or necrosis using Annexin-V FITC and propidium iodide staining assay, mitochondrial membrane potential using JC-1 assay and autophagy by using inhibitor assay.

1.4 Objectives.

Main objective

To investigate the cell death effects of *G. atroviridis* extracts on DBTRG-05MG and U-87MG human brain cancer cells.

Specific objectives

The essential oils extracted from the leaf (EO-L) and stem bark (EO-B) of *G. atroviridis* were used to treat DTBRG-05MG and U-87MG cell lines. The following *in vitro* assays as listed below were conducted:

1. To determine the anti-proliferative effects of EO-L and EO-B on DBTRG-05MG and U-87MG cells using (MTT) assay by microplate reader
2. To determine the mode of cell death induced by EO-L on U-87MG cells using Annexin-V FITC and propidium iodide staining assay by flow cytometry
3. To investigate the cell death effect of EO-L on mitochondrial membrane potential of U-87MG cells using JC-1 staining assay by fluorescence microscopy and fluorescence microplate reader

4. To determine the cell death effect of EO-L on autophagy activity in U-87MG cells using inhibitor assay by microplate reader

CHAPTER 2

LITERATURE REVIEW

2.1 Brain Tumor

An abnormal growth of cells in the brain tissues can lead to a brain tumor. Brain tumors can be either benign without metastatic or malignant with cancer cells that is actively growing and invading the adjacent tissue (Tarin, 2012). In the brain, primary brain tumors can be formed and it can be metastasized to other parts of the human organs.

2.2 Stages of Brain Cancer.

Brain tumors are not categorized in the same group of disease although it derives from the same type of brain tissue. Tumors stages are dependent on how the cells in the tumor appear microscopically.

The primary brain tumors are from gliomas, meningiomas, pituitary adenomas, vestibular schwannomas and medulloblastomas. Glioma is segregated into four types which are astrocytomas, oligodendrogliomas, ependymomas, and choroid plexus papillomas (Chen *et al*., 2010). However, even benign tumors can cause serious problems if they propagate which may cause intracranial dysfunction (DeAngelis and Posner, 2008).

Brain cancers severity depends on their cell type and grade. However, it rarely spread to other organs in comparison to other types of cancer such as breast or lung cancer. Both cancers can be detected according to TMN staging, cancer staging notation system that describes the stage of the cancer which the letter represent as T (Tumors), N (nodes) and M (metastasis) that can detect the location and spread of cancer cells (Edge and Compton, 2010).

U-87 is a human primary glioblastoma cell line formally known as U-87 (Uppsala 87). These cells arise from the brain (glioblastoma astrocytoma) and can cause tumor in the brain (Jaszberenyi *et al.*, 2013). DBTRG-05MG (Denver Brain Tumor Research Group 05 Malignant Glioma) cell line is a malignant glioma cell which can caused tumor in the brain from the anaplastic astrocytoma (Ali *et al.*, 2011).

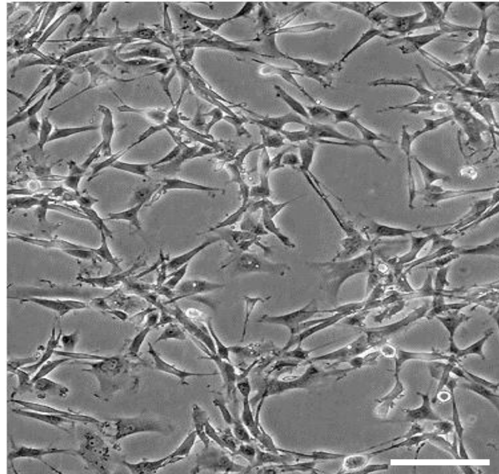

Figure 2.2.1 shows the U-87MG cell line morphology observed under inverted microscope with magnification of 10 X.

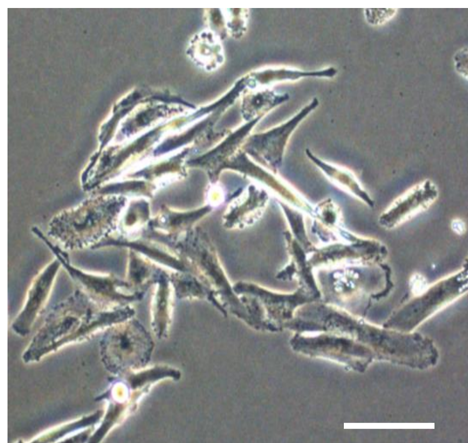

Figure 2.2.2 shows the DBTRG-05MG cell line morphology observed under inverted microscope with magnification of 10 X

2.3 Metastatic Brain Cancer

Cancer cells which developed in the body organ such as the breast can spread in the body system such as the bloodstream as well as to the brain. Tumors formed by such cancer cells metastasize to other the organs are called metastatic tumors. Metastatic brain cancer is a mass of cells that derived from other body organ which then spread into the brain (Roy and Bandyopadhyay, 2012). Metastatic tumors in the brain are more common than primary brain tumors (Chaffer and Weinberg, 2011).

2.4 Factors Lead to Brain Cancer

People exposed hazards are more likely to develop brain cancer. Individuals with higher risk to obtain brain cancer are those who work in an oil refinery, aircraft company and rubber-industry in comparison to the non-industrial. Furthermore, others factors have been suggested to cause brain cancer such as head injury, pesticides, smoking, radiation and viral infection however an extensive should be carried out to further confirm the risk factors (Baldi and Loiseau, 2012). In addition, International Agency for Research on Cancer (IARC) stated that usage of mobile phones may also increase in initiating the formation of gliomas to human. The IARC classified cell phones as group 2b carcinogens (possibly carcinogenic) however, further investigation should also be carried out to validate this hypothesis.

2.5 Treatment of brain cancer.

Many factors such as age, health, medical history, tumor diagnosis, allergy to any medications or treatment are important factor to cause brain tumors (DeSantis *et al*., 2014). Next, grade I and II of tumors are treated by closed monitoring or surgery. But, grade II tumors are observed more closely after surgery to make sure there is no relapse. Grade tumors of grade III and IV are malignant and difficult to remove

and require complicated treatments such as radiation and chemotherapy because the tumor cells still can grow back.

2.6 Types of Cell Death

A balanced homeostasis in the body is a crucial factor in avoiding development of cancer cells. Thus, activation of cell death mechanism is vital in maintaining the homeostasis of proliferative of healthy and normal cells (Walter and Ron, 2011). Cell death can be occurred in cancer cells to those individuals who undergo chemotherapy and radiotherapy (Ouyang *et al.*, 2012). Cell death consists of two types which are typical cell death (apoptotic, autophagy, necrosis and cornification) and atypical cell death (mitotic catastrophe, pyronecrosis and anoikis). Both modes of cell death may involve in the mechanism of apoptotic, autophagy or mitotic catastrophe properties in the cells (Wlodkowic *et al.*, 2011).

2.6.1 Apoptosis

A structured or programmed process of cell death is known as apoptosis when changes occurs in the cell or when the cells induced by either physiological or pharmacological agents (Wong, 2011). The cell morphology changes include nuclear fragmentation and blebbing of plasma membrane. The apoptotic cells will be engulf by macrophages through the phagocytosis process (Underhill and Goodridge, 2012) .

2.6.2 Autophagy Cell Death

It is a programmed self-degradative process which is important for balancing sources of energy in development and in response to nutrient stress. Autophagy also plays a role in removing misfolded or aggregated proteins, clearing damaged organelles and removing intracellular pathogens. Autophagy promotes cellular

senescence, act as cell surface antigen, protects against genome instability and prevents cell damage (Ouyang *et al*., 2012).

2.6.3 Necrosis

Unprogrammed type of cell death mode is called necrosis which can cause injury and induce inflammation to the cell. It causes morphological changes to the cells by bursting the cell content and organelles (Chen *et a*l., 2014). Therefore, it does not follow the apoptotic signal transduction pathway and results in the uncontrollable release of products of cell death into the extracellular space. This will trigger the inflammatory response to the damage area and adjacent tissues compartment (Green *et al*., 2011).

2.7 Sustaining Proliferative Signaling

Tumors are masses of highly proliferative cells or known as cancer cells. Cancer cells have a unique ability to sustain chronic proliferation. In normal cells, they can control the production and release of growth-promoting signals and provide homeostasis in the cells. But in cancer cells, they interrupt the function and mechanism pathway of normal cells (Hanahan and Weinberg, 2011).

2.8 The Mechanism of Apoptosis

Apoptosis aids in maintaining the balance between cell proliferation and cell death. There are two types of apoptotic pathways which are the extrinsic pathway and the intrinsic pathway (Schlossmacher *et al*., 2011). Cancer is one of the consequences of defective in apoptosis mechanism which lead to the formation of proliferative of malignant cells. The mechanism of apoptosis is complicated by many pathways however any interruption along the pathways will lead to malignant transformation as well as resistance to chemotherapy drugs. Nevertheless, apoptosis plays an important role in the treatment of cancer. Mechanism of apoptosis depend on the

stimuli received such as stress, inhibitor of cyclin dependent kinase and p53 pathway (Vurusaner *et al.*, 2012).

2.9 Essential oils and their bio-activities

Essential oil extracted from plants have been used in perfume industry, processed food preservative and flavoring in drinks for many years (Gautam *et al.*, 2014). Moreover, the phytochemicals found in plants can be extracted from various parts of plants such as in flowers, leaves, fruits and barks (Krishnaiah *et al.*, 2011). Recently, plant essential oils play an important role in cancer treatment as an alternative medicine and natural therapies. The essential oil has potential therapeutic benefits because it contains many natural antioxidant phytochemicals such as phenols, flavonoids and tannins which are potent to kill cancer cell (Doughari, 2012). Many research had shown that essential oil from plants contain high antioxidant, act as detoxifying agent as well as anti-inflammatory (Zhang *et al.*, 2011).

An antioxidant can be referred to any entities that interrupt the oxidative damage to a target molecule. The antioxidant is able to scavenge the free radical with the help of the natural (plants) or synthetic antioxidants (Halliwell and Gutteridge, 2015). The natural antioxidants are safer and are without any side effects.

G. atroviridis also known as “Asam Gelugur”, “Asam gelugo” or “Asam keeping” in Malay. The sundried slices of the fruits from this plant are used for culinary purposes and as traditional medicine (Aiemsaard and Punareewattana, 2017). In previous study, Garcineflavanone and Garcineflavonol were isolated from *G. atroviridis* have been used to cure the Alzheimer’s disease (Tan *et al.*, 2013).

The total volatiles obtained by hydrodistillation of the fruit of *G. atroviridis* have showed stronger activity against Gram-positive bacteria and selective inhibitory activity against COX-2 (Tan *et al.*, 2013). But, the cytotoxicity analyses of essential oils obtained from *G. atroviridis* is yet not to be determined. Thus, this study aims to investigate the potential role of total volatiles of *G. atroviridis* in inducing the death in DBTRG-05MG and U-87MG human brain cancer cell line by modulating the activities of apoptosis and autophagy *in vitro*.

CHAPTER 3

METHODOLOGY

3.1 Materials and Chemicals

3.1.1 Chemicals and Reagents

All chemicals and reagents used in this study are listed in Table 3.1

Table 3.1: List of chemicals and reagents

Chemicals and reagents	Supplier
5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethyl-benzamidazolocarbocyanin iodide (JC-1) dye	Invitrogen, USA
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich, USA
Distilled water	Merck, Germany
Accutase ™ Solution	Millipore, USA
Trypsin	Millipore, USA
5-diphenyltetrazolium bromide dye (MTT)	Merck, Germany
Dulbecco Modified Eagle’s Medium (DMEM)	GIBCO BRL, UK
Ethanol (95% v/v)	Merck, Germany
Fetal bovine serum (FBS)	GIBCO BRL, UK
Trypan blue	Sigma-Aldrich, USA
Penicillin Streptomycin Glutamine (100X)	Gibco BRL, UK
Phosphate buffered saline (PBS)	Amresco, USA
Tamoxifen Citrate Salt	Nacalai tesque, Japan

3.1.2 Consumables.

All consumables used in this study are listed in Table 3.2

Table 3.2: List of consumables.

Consumables	Supplier
96-well micro-titer plates	Nunc, Denmark
Chamber slides	Becton Dickson, USA
Cover slips (Menzel-Glaser)	Deckglaser, Germany
Cryogenic vials and Cryoboxes	Nalgene, USA
Luer-Lok™ Syringes (25 ml and 50 ml) and sterilised –filter 0.22 µm	BD Plastipak™, Spain
Micropipette tips (10 µl, 100 µl, 200 µl and 1000 µl)	Axygen® Scientific, USA
Microscope slides	Sail Brand, China
Serological pipettes (10 ml and 25 ml)	Nunc, Denmark
Syringe membrane filter (0.22 µm)	Milipore, UK
Tissue culture flasks (25 cm^2 and 75 cm^2)	Becton Dickinson™, USA

3.1.3 Kits

All kits used in this study are listed in Table 3.3

Table 3.3: List of kits

Kits	Supplier
Annexin-V-FLUOS Detection Kit	Roche, Germany
PI3K inhibitor kit	Roche, Germany

3.1.4 Laboratory Equipment

All of laboratory equipment used in this study are listed in Table 3.4

Table 3.4: List of laboratory equipment

Name and Brand	Supplier
Airstream Class II Biohazard Safety cabinet	Nuaire, USA
Cell counter (Tagoshi RS-4)	Soon Hoong Seng PTE LTD, Singapore
Centrifuge 5415R	Eppendorf, Germany
Centrifuge 5810R	Eppendorf, Germany
Flow cytometer (FACS Calibur)	Becton Dickinson, USA
Fluorescence Microscope	Nikkon, Japan
Haemacytometer (Neubauer Improved)	LO-laboroptik Ltd., UK
Incubator (Binder)	Thermo Scientific Fisher, USA
Inverted microscope (OLYMPUS CKX41)	OLYMPUS, USA
Microcentrifuge (Minispin plus)	Eppendorf, USA
Microplate Reader (Versamax)	Molecular Devices, USA
Vortex mixer (MSI)	IKA, Germany
Water bath (Memmert)	Camlab, UK

3.1.5 Isolation of Essential Oils

Stem bark and leaf of *G. atroviridis* were bought from Herbagus Trading, Kepala Batas, Pulau Pinang. A voucher specimen (USM 11201) has been deposited with the herbarium of Universiti Sains Malaysia, Penang, Malaysia. The dried stem bark collected of *G. atroviridis* was grinded into powder and the leaves were washed with distilled water and cut into small pieces prior to hydrodistillation. The hydrodistillation was carried out for 5 h using a Clevenger-type apparatus. The extracts were carefully concentrated using a gentle stream of nitrogen gas at room temperature, yielding pale yellow oils. The oils isolated from leaf (EO-L) and barks (EO-B) were kept at 4 °C until analysis. A stock concentration of 10 000 µg/ml was prepared by

dissolving the oils in dimethyl sulphoxide (DMSO) and kept at -20 °C until use. Both EO-L and EO-B were diluted in an appropriate growth culture medium before use. The final concentration of DMSO in the medium was less than 0.1 % (v/v).

3.2 Cell Culture

3.2.1 Human Brain Cancer Cell Lines

Two types of brain cancer cell lines were used throughout this study.

a) DBTRG-05MG (Denver Brain Tumor Research Group 05 Malignant Glioma)

b) U-87MG (Upssala 87 Malignant Glioma)

Both cell lines were obtained from by Dr. Agustine Nengsih from Department of Chemical Pathology, School of Medical Sciences, Universiti Sains Malaysia (PPSP, USM). Both cells were originally purchased from the American Type Culture Collection (ATCC) (Rockville, USA). These cells were grown in suitable condition and the medium was changed every 2 days. All cell handling and medium preparation was carried out using aseptic technique in class II safety cabinet.

3.2.2 Reagents for Cell Culture Work

3.2.2.1 Medium

The growth medium used in this study was Dulbecco Modified Eagle's Media (DMEM) (Gibco BRL, UK) for both cell lines and stored at 4 ^{o}C. The reagents were thawed before used and filtered-sterilised with 0.22 μm disposable filter units.

3.2.2.2 Heat-inactivated Fetal Bovine Serum (FBS)

A bottle of FBS (Gibco BRL, UK) (100ml) was stored at -20 °C. The serum was filtered-sterilised with 0.22 μm disposable filter units and aliquoted into sterile 15 ml tubes and kept at -20 ^{o}C. The FBS was thawed in water bath at 37 ^{o}C prior to use.

3.2.2.3 Phosphate-Buffered Saline (PBS)

Ten times Phosphate-buffered saline (PBS) (Gibco BRL, UK) was filtered-sterilised with 0.22 µm disposable filter units and aliquoted into 50 ml Falcon tube with distilled water added to produce 1x working concentration. The PBS was stored at room temperature.

3.2.2.4 Penicilin/Streptomycin (PenStrep) Antibiotics Solution.

A bottle of ready-made containing penicilin/streptomycin (PenStrep) solution of 10 mg/ml (Gibco BRL, UK) was filtered-sterilised with 0.22 µm disposable filter units and aliquoted into sterile 15 ml tubes before being stored at -20 °C. The antibiotic was thawed prior use at 37 °C in the water bath.

3.2.2.5 Complete Growth Medium.

A complete growth medium was used in this study to culture and maintain the cell lines. The medium consists of Dulbecco's Modified Eagle's Medium (DMEM) solution supplemented with 10 % (v/v) FBS and 1 % (v/v) of PenStrep. The 50 ml medium was prepared by adding 5 ml FBS, 50 µl of PenStrep and 45 ml of DMEM media. All prepared media were kept at 4 °C and thawed before used.

3.2.2.6 Trypsin (0.25 %, w/v)/EDTA (0.03 %, w/v) Solution.

A ready- made Trysin of 0.25 %, EDTA 0.03% (Gibco BRL, UK) was filter-sterilised, aliquoted and stored at - 20 °C until use. The trypsin was thawed in 37 °C prior to use.

3.2.2.7 Cryogenic Medium

The medium contained 10 % Dimethyl sulphoxide (DMSO) in FBS was prepared and used to store ampoules of cells in -80 °C storage or liquid nitrogen. The cryogenic medium was prepared fresh and kept cold prior to use.

3.2.3 Culture Procedures and Conditions

All tissue culture procedures were carried out in sterile conditions (Class II biohazard cabinet) using aseptic techniques to avoid any contamination. Cells were cultured in growth medium and maintained in a humidified incubator at 37 °C in an atmosphere of 5 % CO2 and 95 % air.

3.2.4 Thawing of Cells from Frozen Storage

The frozen cells was retrieved from the -80 °C storage and thawed at room temperature for 5 minutes. The entire frozen cells were gently pipetted into a 15 ml centrifuge tube containing 4 ml of complete growth medium. The content was centrifuged at 1000 rpm for 5 minutes to remove the DMSO. The supernatant was aspirated and 2 ml of PBS was then added and re-suspended to wash the cell pellet. The cell pellet was re-centrifuged at 1000 rpm for 5 minutes to make sure DMSO residue or any debris was completely discarded. The cell pellet was then re-suspendered in 1-2 ml of complete growth media. The cell suspension was then slowly pipetted into a 25-cm^2 tissue culture flask containing 4-5 ml of culture media. The cell morphology was observed using an inverted phase contrast microscope to check for cell viability. The culture flask was incubated in a humidified atmosphere containing 5 % CO2 at 37 °C. The growth medium was replaced every 2-3 days.

3.2.5 Sub-Culturing of Cells

The cells were sub-cultured into a new flask when they reached about 70-80 % confluency in the flask to avoid nutritional deficiency and microbial contaminants. The old medium from the flask were discarded and cells were rinsed with 2 ml PBS. After that, the PBS was discarded and 1 ml trypsin-EDTA solution was added into the flask. The flask was left for about 5 min in the CO_2 incubator for allowing the adherent cells to detach from the surface of flask. The flask was gently tapped to ensure complete cells detachment and the process was followed by observing the

flask using the inverted microscope. Then, 1 ml of fresh culture medium was added into the flask to inactivate the trypsin activity. The medium (containing the cells) was aspirated and transferred into a sterile 15 ml centrifuge tube. The tube was centrifuged at 1000 rpm for 5 min. The supernatant was then removed and the cell pellet was re-suspended in 1 ml growth medium. The mixture containing cells was aspirated and dispensed at a 1:4 dilution (normally into 4-5 flasks). The medium volume in each new 75-cm^2 flask was made up to 7-10 ml by adding fresh growth media and the cultures were incubated in humidified atmosphere containing 5 % CO_2 at 37 °C.

3.2.6 Cell Counting for Seeding

The number of viable cell was counted using Equation 1:

$$\text{Number of viable cell/ml} = \frac{\text{A } \times \text{dilution factor } \times 10^4}{4}$$

A = Total of unstained cell in the calculated grid

3.3 Assessment of Cell Viability using (MTT) Assay

Cell proliferation was determined using following formula as noted in Equation 2:

$$\frac{O.D.treatment - O.D.blank}{O.D.untreated\ cell - \ O.D.blank} \times 100\ \%$$

DBTRG-05MG and U-87MG were grown in complete medium. DBTRG-05MG and U-87MG cells were seeded at density of 5 x 10^3 per well and is incubated overnight for the cells to adhere. All cells were cultured at 37 °C in a humidified condition with 5% CO_2. Cells were treated with either essential oil isolated from leaf (EO-L) or bark (EO-B) of *G. atroviridis*. The concentrations of EO-L or EO-B used in this study were 25, 50, 75 and 100 µg/ml. Tamoxifen at 35 µM of concentration was used as positive control in this study. At each time point of 24h, 48h and 72h, 10 µl (5 mg/ml)

of MTT reagent (Merck Millipore, USA) was added to each well and the plate was incubated for 4 hours at 37 °C. The medium containing MTT solution was discarded from each well and 100 µl of DMSO was added. The absorbance at 570 nm was measured by microplate reader. The dose response effect and IC_{50} value of *G. atroviridis* was calculated using formula listed in Equation 2 based on the absorbance reading obtained at time point of 24 h, 48 h and 72 h.

3.4 Apoptosis Detection

Apoptosis was detected using the Annexin-V FLUOS Detection Kit (Roche, Germany). The two-stain kit containing Annexin-V-fluorescein antibody and propidium iodidie (PI) dye was used to determine the mode of cell death induced by the agent.

The phosphatidylserine (PS) on the outer leaflet of apoptotic cell-membranes was detected using Annexin-V FITC which emitted green fluorescence with wavelength of excitation/emission = 488/518 nm whereas PI staining enters the plasma membrane of the necrotic dead cells which emitting red fluorescence wavelength of excitation/emission = 488/617 nm.

3.4.1 Preparation of Annexin-V Staining Solution

Staining solution was ready-to-use for Annexin-V-fluorescein labelling reagent and PI which were provided by the manufacturer (Roche, Germany).

3.4.2 Treatment of Cells

EO-L or EO-B at increasing concentrations with positive and negative controls were also included in the assay and cells were incubated with all treatments for 24 h, 48 h and 72 h. Following incubation, the cells were then observed under microscope and

further analysed for apoptosis detection by fluorescence microscopy and flow cytometry.

3.4.3 Detection of Apoptosis by Flow Cytometry

For flow cytometric analysis, the floating cells were collected in 15 ml tubes and adherent cells were detached as by using Accutase (Millipore, USA). Both floating and adherent cells were pelleted by centrifugation at 300 x *g* for 2 min. The supernatant was then removed and 1 ml of PBS was added to wash the cells. The cells were centrifuged 300 x g for 2 min and PBS was discarded. This step was repeated twice. The cell pellet was re-suspended in 1ml of incubation buffer and transferred out 100 µl of the mixture into Falcon round bottom tube. Then, 2 µl of the two-stain kit were added in each flow tube and incubated at room temperature with no light for 10 min. Following incubation, 400 µl incubation buffer was added to each flow tube before the cells were analysed using the FACSCalibur (Becton Dickinson, USA).

For flow cytometric analysis, 10 000 cells per sample were acquired and analysed with CELLQuest Pro Software (USA). The percentage of apoptotic cells (total late apoptosis + total early apoptosis) was calculated based on the quadrant readings.

3.5 Analysis of Mitochondrial Membrane Potential (∆ψm)

An early feature of apoptosis is via depolarization of the mitochondrial membrane potential (ΔΨm). Loss of the ΔΨ can be detected using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl- benzamidazolocarbocyanin iodide (JC-1) dye. In this study, the effect of EO-L (25-100 µg/ml) in U-87MG cells was investigated using JC-1 dye by which the qualitatively observed by fluorescence microscopy and quantitatively measured by fluorescence microplate reader. Both positive and negative controls were also included in the assay. The ∆ψm is indicated by a decrease in the

red/green fluorescence intensity ratio, where the red fluorescence represents the J-aggregates (high $\Delta\psi$), while the green fluorescence represents the monomer (low $\Delta\psi$). Knowledge of the $\Delta\Psi$ and how it changes during apoptosis may help to clarify the actions of EO-L on the $\Delta\Psi m$ of U-87MG cells.

3.5.1 Preparation of JC-1 Dye and Assay Buffer

JC-1 was supplied in liquid form (Invitrogen, USA). The dye was aliquoted and kept at -20 °C, protected from light according to the manufacturer's guideline. One table of Cell-Based Assay Buffer was diluted in 100 ml distilled water and the mixture can be kept for one year at room temperature.

3.5.2 Preparation of Working Solution

A working solution of JC-1 dye was freshly prepared prior use where a stock solution was diluted in ratio 1:10. The solution was mixed thoroughly to avoid precipitation of the JC-1 dye and it was protected from light. The JC-1 Working solution was immediately used for staining the cells.

3.5.3 Detection of Alterations in $\Delta\Psi m$ using JC-1 Dye by Fluorescence Microscopy.

U-87MG cells were seeded in a 96-well plate with cell density of 1x 10^5 cells/ml. The cells were cultured in a CO_2 incubator overnight at 37 °C. The cells were exposed to different concentrations of EO-L for 24 h to 72 h of incubation periods. The JC-1 Staining Solution was added to each well of the plate. Then, the sample was incubated in CO_2 incubator at 37 °C for 15 min. The plate was carefully centrifuged at 400 x g and then the supernatant was aspirated from each well. Next, 200 µl of Assay Buffer was added to each well in the plate. The supernatant was removed and 200 µl of Assay Buffer was added again at each well of the plate and this step was repeated one more time. The supernatant was discarded again and added 100 µl of Assay Buffer. The cells were observed under the fluorescence microscopy.