HE EFFECT OF CRUDE EXTRACT OF *Centella asiatica* ON VOLTAGE-GATED SODIUM CHANNEL (VGSC) MEDIATED MOTILITY ON MDA-MB-231 BREAST CANCER CELL LINES

by

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SYMBOLS AND ABBREVIATIONS

cDNA	Complementary deoxyribonucleic acid
CO2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dH_2O	Distilled water
ddH_2O	Double distilled water
EtBr	Ethidium bromide
FBS	Fetal Bovine Serum
gDNA	Genomic deoxyribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
SEM	Standard error of mean
TAE buffer	Tris-acetate-EDTA buffer
VGSC	Voltage-gated sodium channel

ABSTRAK

Saluran natrium voltan berpagar (VGSC) didapati memainkan peranan yang penting dalam metastasis kerana membantu dalam proses kanser melekat, motiliti, rembesan serta serangan. Kanser payudara juga menunjukkan sifat ini yang mana kadar ekspresi neonatal $Na_v1.5$ adalah tinggi. Hal ini menunjukkan VGSC, neonatal $Na_v1.5$ khususnya boleh digunakan sebagai sasaran bagi penyelidikan ubat-ubatan baru untuk rawatan kanser payudara dan ubat anti-metastasis. *Centella asiatica* merupakan salah satu tumbuhan atau herba yang digunakan dalam perubatan tradisional dan alternatif untuk membantu menyembuhkan penyakit tertentu termasuk sawan dan gangguan kognitif serta kanser. Oleh itu, dalam projek ini, kesan *Centella asiatica* terhadap VGSC dan motiliti MDA-MB-231 sel kanser payudara telah dikaji. Mekanisma bagaimana ekstrak tumbuhan boleh menunjukkan kesan kepada metastasis juga dikaji dengan melakukan kajian genetik ke atas neonatal $Na_v1.5$. Empat ujian yang berbeza telah digunakan dalam menentukan kesan ekstrak tumbuhan ini antaranya MTT assay, Trypan Blue assay, ujian motiliti dan kajian genetik. Tiada kesan ekstrak tumbuhan pada percambahan sel dan ekstrak tersebut juga didapati tidak toksik terhadap sel namun terdapat kesan ke atas motiliti sel. Oleh kerana tiada kesan ekstrak tumbuhan pada percambahan sel dan ketoksidan, kesan yang dapat dilihat pada motiliti adalah benar-benar dihasilkan oleh ekstrak tumbuhan pada motiliti sel bukan kerana ia juga memberi kesan kepada perkembangan sel. Daripada kajian genetik, terdapat penurunan dalam ekspresi neonatal $Na_v1.5$ dan ini membuktikan bahawa ekstrak tumbuhan tersebut dapat mengurangkan kadar penghijrahan serta motiliti dengan mengurangkan ekspresi neonatal $Na_v1.5$. Kesimpulannya, ekstrak tumbuhan ini

mempunyai potensi yang besar untuk diketengahkan dalam pembangunan ubat anti-metastasis

ABSTRACT

Voltage-gated sodium channel (VGSC) has been found to plays a role in metastasis as it help in the cancer cells adhesion, motility, secretion as well as invasion. Breast cancer also shows these properties with neonatal $Na_v1.5$ to be highly expressed and it is one of the isoform of the VGSC. This suggests VGSC, specifically neonatal $Na_v1.5$ to be used as a target for a new drug development for breast cancer treatment and for anti-metastasis drug. *Centella asiatica* is one of plant or herb that being used in traditional and alternative medicine to help cure certain disease or disorder including epilepsy and cognitive disorder as well as cancer. Thus, in this project, the effect of the *Centella asiatica* on the voltage-gated sodium channel activities and motility of the MDA-MB-231 breast cancer cell lines have been studied. The mechanism on how the plant extract shows an effect to metastasis also being explored by doing a molecular study on the expression of the neonatal $Na_v1.5$. Four different assays are being used in the determination of the effect which includes MTT assay, Trypan Blue assay, motility assay and molecular study. There is no effect of the plant extract on the proliferation and viability and toxicity assay but there is an effect on the motility of the cells. As there is no effect of the plant extract on the cells proliferation and viability, the effect that can be seen on the motility is genuinely produce by the plant extract on cell's motility not because of it also affect proliferation and cells viability. From a molecular study, it has been shown that there is a decrease in the neonatal $Na_v1.5$ expression and this support the fact that the plant extract reduce the migration rate by decreasing or down-regulating the neonatal $Na_v1.5$ expression. In conclusion, this plant crude extract has a great potential to be used in the anti-metastasis drug development specifically on breast cancer which expressed neonatal $Na_v1.5$.

CHAPTER 1

INTRODUCTION

Recently our understanding of cancer has advanced in the realization that a lot of proteins and the genes play an important role in cancer invading and metastasis. Mutation and genetic changes show some control and have a profound effect on the malignant phenotype where the changes that occur lead to a progression of a tumor by expressing certain genes and protein that help in the cancer invading and metastasis. It is now clear that some oncogenic mutations disrupt apoptosis, leading to tumor initiation, progression or metastasis. However, up-regulation and down-regulation of certain gene also give a big impact on the cancer progression, invasion and metastasis.

Involvement of voltage-gated sodium channel (VGSC) in the cancer and metastasis had become one of the issues nowadays especially in breast cancer. Breast cancer is a type of cancer which originating from breast tissue and cancer can spread from its primary site to other part of the body by metastasize and this is usually tissue specific. VGSC help the cancer cells to metastasize by giving a way to detach, invade the blood vessel and start to metastasize in new target organ. High expression of VGSC in cancer cell, the cancer will become more aggressive and this will decrease the patient survival.

Besides, some failure in the regulation of the gene and protein of the VGSC lead to cancer aggressiveness and invasion especially with those who have negative estrogen receptor. This group of patients who did not show any effect toward the anti-cancer drug had been proves to be negative estrogen receptor as the drug develop specifically act on the estrogen receptor. For the patients with negative estrogen receptor and who had been

shown to have high expression of VGSC, a new drug should be develop and this VGSC can be used as a new target for the drug development.

Lots of study had been shown that this VGSC plays a role in the cancer metastasis and cancer aggressiveness and different locality of cancer express different VGSC isoforms. For an example, it had been found that the isoform expressed in prostate cancer is a foetal splice variant of the $Na_v1.7$ ($fNa_v1.7$). For breast cancer, it had been reported that the mainly isoform expressed in breast cancer highly metastatic cells was the $Na_v1.5$. By founding this information, a specific drug targeting specific isoform of VGSC can be developed specifically to cure different locality of cancer in the future.

Furthermore, plants have a long history in cancer treatment where more than 3000 species have been known for their anti-cancer potential. Over 60% of currently used anti-cancer agents are derived in one way or another from higher plants. Indeed, compounds derived from natural sources, including plants, have played, and continue to play, a dominant role in the discovery of leads for the development of conventional drugs for the treatment of most human diseases and disorder especially cancer.

One of the arising plants that being proposed can be used as a cancer treatment is *Centella asiatica* which possess anti-cancer potential where in South Africa. *C. asiatica* is known anecdotally to treat various forms of cancers. The anti-cancer activity of *C. asiatica* has been studied in some part of India. However, despite of anti-cancer properties, *C. asiatica* also had been shown to produce analgesic effect and antidepressants and it is due to VGSC blockers properties that it has. As *C. asiatica* has a VGSC blockers property, we can use this property for further drug development which can help in reducing cancer

invasion and metastasis. The relationship between the VGSC and metastasis and how far VGSC plays an important role in cancer metastasis need to be proved.

The involvement of the VGSC in cancer invasion and metastasis is newly found and lot of study had been done to identify the relationship between both of them. Several of these studies have assessed the activity of Na_v and have shown that it participates in the invasion of the cells *in vitro*. As the Na_v is a channel, thus by blocking it, we can reduce the aggressiveness and prevent it from being metastasize. *C. asiatica* which show properties as VGSC blocker can be suggest and used for the drug development so that breast cancer metastasis and aggressiveness of the cancer can be control.

Rationale of study

Breast cancer is currently controlled by surgery and radiotherapy and it is frequently supported by chemotherapy or hormonal therapy. However, the drug or hormonal therapy available now is only effective on person with positive estrogen receptor as the drug is targeted the estrogen receptor itself. Thus, the drug will not give any effect on the person with negative estrogen receptor and chemotherapy itself give more negative impact on the cancer patient and new target should be find and the target should be specific against the cancer cells. Hence, there is a need to study the VGSC as a potential target for metastasis as there are a lot of studies that shown this VGSC can be a new target in cancer drug development. Therefore, one approach is to screen potential medicinal plant or herb that can block VGSC and have anti-metastatic properties.

Objectives

Main objective:

To study the effect of crude extract of *Centella asiatica* on voltage-gated sodium channel (VGSC) mediated motility on MDA-MB-231 breast cancer cell lines

Specific objective are:

1. To determine the effect of the *Centella asiatica* on cancer cell's toxicity, viability and proliferation.
2. To evaluate the effect of the *Centella asiatica* on the motility of cancer cell.
3. To determine the effect of the *Centella asiatica* on the gene expression of the VGSC.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast cancer

Breast cancer is the first human tumor for which targeted therapies have been developed and it is the most common malignancy among women which affect approximately one out of ten women (Imyanitov, 2004). Breast cancer is a type of cancer which originates from breast tissue, and most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk (Sariego, 2010). Breast cancer commonly affects women but it also can affect men as well but only in a small number.

In past years, carcinomas of the breast have been classified into six subgroups based on gene expression patterns. Each of the subgroup show a different clinical outcome and patients with tumours bearing the basal-like subtype showed the shortest overall survival (Perou *et al.*, 2000). Triple-negative breast cancer with oestrogen receptor negative (ER-), progesterone receptor negative (PR-) and HER2- negative (HER2-) specimens exhibit the basal-like phenotype in 91% of all cases. Therefore, triple negativity can be used as a clinical surrogate for the genotypically defined basal-like phenotype (Kreike *et al.*, 2007).

HER2-overexpression is associated with a more aggressive course of disease and according to ER status (Slamon *et al.*, 1987), HER2-overexpressing breast cancer can be classified into two subgroups. The first is the HER2 subtype (ER-negative) and the second is Luminal B subtype (ER-positive) (Sørlie *et al.*, 2001). Breast cancer patients which expressed ER-negative likely to show and express high number of VGSC and thus, provide

a clear statement why this VGSC can be used as a target for new drug development for breast cancer.

2.2 Metastasis

Metastatic cancer is cancer that has spread from the place where it first started to another place in the body. In a simple way metastasis is a process where the cancer cells migrate from the primary tumor site to other site. A tumor formed by metastatic cancer cells is called a metastatic tumor or a metastasis and the process by which cancer cells spread to other parts of the body is also called metastasis. Metastatic cancer has the same name and the same type of cancer cells as the original, or primary, cancer. For example, breast cancer that spreads to the lung and forms a metastatic tumor is metastatic breast cancer, not lung cancer.

Metastasis is frequently a final and fatal step in the progression of solid malignancies where tumour cell intravasation, survival in circulation, extravasation into a distant organ, angiogenesis and uninhibited growth constitute the metastatic process (Minn *et al.*, 2005). The molecular requirements for some of these steps may be tissue specific for an example breast cancer like to metastasize in bone and lung. As we know, early detection and treatment of cancer have increases survival and improved clinical outcome and the development of metastases is often associated with poor prognostic of survival (Roger *et al.*, 2006). Thus, finding early markers of metastasis and developing new therapies against their development might help a lot in cancer treatment and increasing survival.

Under a microscope, metastatic cancer cells generally look the same as cells of the original cancer. Moreover, metastatic cancer cells and cells of the original cancer usually

have some molecular features in common, such as the expression of certain proteins or the presence of specific chromosome changes. In general, the primary goal of the treatments is to control the growth of the cancer or to relieve symptoms caused by it. Most tumors and other neoplasms can metastasize and the degree of ability to spread, however, varies between different types of tumors. For example, basal cell carcinoma rarely spreads.

Some organs are more prone than others to metastasis of primary tumors. This was first discussed as the "seed and soil" theory by Stephen Paget over a century ago in 1889. For example, bones are the favoured site for prostate cancer, colon cancer spreads to liver, stomach cancer can metastasize to the ovaries and is then called Krukenberg tumor. The theory states that cancer cells are difficult to survive in outside environment thus it need to find a location with similar characteristics as the primary site. For example, breast cancer cells need calcium ions from breast milk to proliferate. Hence, they may prefer the bones as a site of spread as bones are rich in calcium.

2.3 Voltage-Gated Sodium Channel (VGSC)

2.3.1 Background

Voltage-gated sodium channels (VGSC) are ion channel which are regulated by voltage and it allows movement of sodium ions through it. It is a complex transmembrane proteins that is involved in the depolarising upstroke of an action potential generated in an excitable sensory neuron that occur once a rapid flow of sodium ions across the membrane and creating a current (Cummins *et al.*, 2007). Ten genes which encode α-subunits have been identified and nine of these have been functionally studied in expressions system.

Among the ten isoforms, nine of them constitute a single family name Na_v1 which given according to their phylogeny and designated $Na_v1.1$ to $Na_v1.9$. The nine isoforms

can be divided into two classes according to their sensitivity to tetrodotoxin (TTX) where six of them are tetrodotoxin-sensitive (TTX-S) and another three are tetrodotoxin-resistant (TTX-R). The TTX-S channels are inhibited by nanomolar concentration of TTX making it to be known as TTX-sensitive channel as only small amount of the TTX are required to block it. Meanwhile, for TTX-R channels, they are inhibited by micromolar or even greater concentration of TTX and thus, making them different from the TTX-S. The isoforms which are listed under the TTX-S are $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.4$, $Na_v1.6$, and $Na_v1.7$ while the isoforms which are listed under the TTX-R are $Na_v1.5$, $Na_v1.8$, and $Na_v1.9$ (Jean-Yves *et al.*, 2007).

Besides, VGSC are also classically described as crucial elements of action potential initiation and propagation in excitable cells as it is normally expressed in excitable cells. However, these channels are also known to be expressed in non excitable cells like T-lymphocytes, fibroblast, endothelial cells and some cancerous cells where their role is still not clearly understand. Some of the study shows that the properties of normal human T-lymphocytes which express functional VGSCs necessary for their intravasation or extravasation and inflammatory response (Gaspar and Falasca, 1992) and this support and explain the role of the VGSC in human T-lymphocytes.

Other normal cells such as fibroblasts and endothelial cells which capable of invasive behaviour also had been found to have functional VGSCs (Traub *et al.*, 1999) and these prove that the VGSC plays a role in the motility of the cells. Additionally, several different cancers such as prostate, breast, small-cell lung carcinoma and neuronal tumours had also been found to express functional VGSCs (Fraser *et al.*, 2003) that have been

shown to potentiate cell invasion in prostate and breast cancer cell lines (Laniado *et al.*, 1997; Fraser *et al.*, 2003) and this strongly shown the role of VGSC in metastasis.

2.3.2 Structure of VGSC and its subunits

The VGSC as stated have ten genes which encode for the α-subunits and nine of them encode the sodium selective ion channels $Na_v1.1$ to $Na_v1.9$ and the other one is known as Na_x. This Na_x though structurally related to VGSCs, it is not activated by membrane depolarization, but rather by altered sodium concentrations (Goldin *et al.*, 2000). These VGSC channel comprises of highly processed α-subunits which is approximately 260 kDa and sometimes associate with at least two auxiliary β-subunits as displayed in figure 2.1. The number of the β-subunits and the type of it is depending on the site of it being expressed where for an example, in adult central nervous system it have been found to contain β_2 plus either β_1 or β_3 subunits. The pore-forming α-subunit is sufficient for functional expression, but the channel and current density, kinetics and voltage-dependence of gating are modified by the β-subunits (Isom, 2001).

The α-subunit contains four homologous domains DI–DIV, with each domain containing six transmembrane segments known as S1-S6. Functionally, the structure of the VGSCs or the transmembrane segments can be divided into two parts with the transmembrane domains S4 contributing to the voltage sensor and S5–S6 arranging to form the sodium selective pore (Stühmer *et al.*, 1989; Catterall *et al.*, 2005). The molecular mechanism by which changes in membrane voltage confer a conformational change on voltage-gated ion channel proteins is through the movement of modular voltage sensors contained within the S4 segment of domains I–IV as shown in figure 2.1 (Alabi *et al.*, 2007). The voltage sensors contain repeated motifs of positively charged amino acids

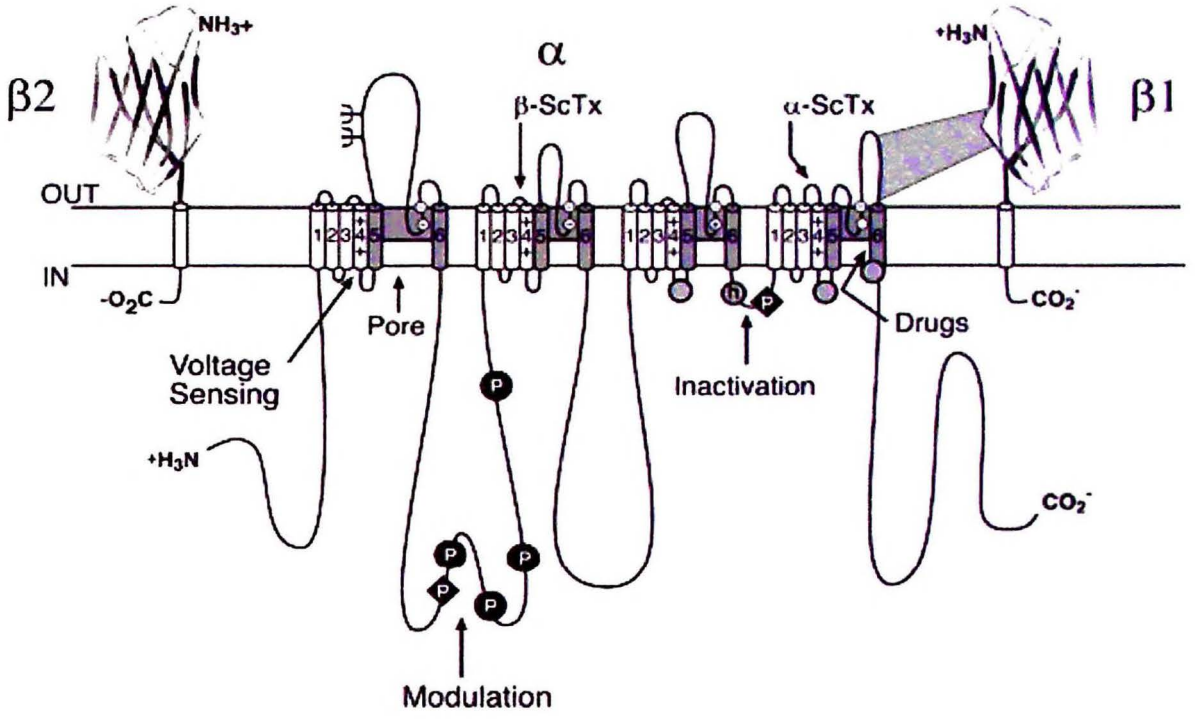

Figure 2.1 The structure of voltage-gated sodium channel (Catterall *et al.*, 2005)

followed by hydrophobic residues arranged in an α-helix with a linear array of positively charged residues.

2.3.3 VGSC and metastasis

Ion channel activity is involved in several basic cellular behaviors that are integral to metastasis such as proliferation, motility, secretion, and invasion. As lots of study have been shown that VGSC plays a role in the metastasis and interestingly, VGSC upregulation has been associated with several strongly metastatic carcinomas, leading to the hypothesis that VGSC up-regulation may 'switch' the cancerous cell to a highly invasive state (Onkal and Djamgoz, 2009). Some cancers express embryonic or neonatal VGSC splice variants, for example, a neonatal isoform of $Na_v1.5$ with seven amino acid differences is the predominant being expressed (480%) in human metastatic breast cancer (Fraser, 2005) as well as neuroblastoma.

The nine different VGSC α-subunits are differentially expressed in different type of cells, and disruption of VGSC function can lead to a broad range of pathologies. The role of VGSCs in epilepsy and pain has been well established. However, there is increasing evidence of a role for VGSCs in other disorders including multiple sclerosis, muscle and immune disorders, autism, neurodegeneration, cardiovascular complications as well as cancer which being discuss in this project (Eijkelkamp *et al.*, 2012). All of the disorders are related to VGSC but different isoform of it are involved in different disorder. Furthermore, recent genetic studies have also expanded the role of sodium channels in health and disease, to include autism, migraine, multiple sclerosis, cancer as well as muscle and immune system disorders (Eijkelkamp *et al.*, 2012) and this will further explore the role of VGSC in cancer development.

By overexpression of VGSC alone, it was sufficient to increase *in vitro* cellular invasive potential, which can conclude that VGSC activity is necessary and sufficient for cancer cell invasiveness (Bennett *et al.*, 2004). This can be applied to breast cancer as well, as VGSC had been found to be highly expressed in highly metastatic breast cancer cells. The isoform of the VGSC which has been expressed in highly metastatic breast cancer cells is the neonatal splice variant of $Na_v1.5$ ($nNa_v1.5$) (Fraser, 2005) and by blocking the protein, it might help a lot in preventing or reducing the cancer aggressiveness.

From a study it also has been found that the $Na_v1.5$ was expressed at approximately 1,000 fold higher levels in strongly versus weakly metastatic breast cancer cells and up-regulation of the Nav1.5 help in the metastasize process (Fraser, 2005). This suggest a VGSC blocker to be used as a tool for new anti-metastatic drug development and few study have been done. The results from the study support the notion that VGSC blockers can produce anti-metastatic effects in human breast cancer by blocking $nNa_v1.5$ at various levels which means from mRNA to functional expression. This conclusion agrees with some clinical studies reporting that systemic treatment of breast cancer patients undergoing surgery with local anesthetics, such as lidocaine, can suppress possible reoccurrence of disease and/or subsequent dependence on analgesics (Mokhtar and Djamgoz, 2012).

2.4 *Centella asiatica*

2.4.1 Background

Since ancient time, plants have been a good source of medicine and a major resource for health care. Some of the traditional herbal medicinal plants have been use for more than 2,000 years and till now still been applied by some people (Thomson, 2010). The modern pharmaceutical industry is paying more attention to plants as scientists re-

discover that plant life is an almost infinite resource for medicine development. Phytochemicals that improve human health may be consumed as fresh plant products like fruits and vegetables, or at the other extreme as highly value-added processed forms for examples in the form of extracts, powders or pills. All of these herbal and traditional plants have a common potential to enhance human health further than the simple supply of fuel for metabolic processes. Many foods consumed in Asia have been traditionally used to cure specific human ailments and one of it is *Centella asiatica.*

Centella asiatica and its preparation have been in use since ancient times especially in the Ayurvedic medical system of India and in the folk medicine of China and Madagascar. It is recommended by the World Health Organization (WHO) as one of the most important medicinal plant species to be conserved and cultivated. In Malaysia, although it has been used by our traditional healers in their herbal remedies, but its popularity are confined more as a traditional vegetable or an 'ulam' especially among the Malay communities rather than a medicinal plant.

Centella asiatica or known as pennywort or "pegaga" is a small, annual, slender, creeping herb. It has long-stalked, green leaves with rounded apices which have smooth texture with palmately netted veins. Pegaga grows wildly under a wide range of conditions, some races prefer light shade, while others do well in open sunny areas. Some even grow under more harsh conditions like on stone walls. In the wild, most of these plants are found in wet or moist surrounding like swamps, along the margins of lakes, ponds and have also been seen growing in paddy fields .

Figure 2.2: *Centella asiatica*
(Source:http://lehighacresediblegardeningexchange.blogspot.com/2011/06/dollarweed-pennywort-hydrocotyle.html)

2.4.2 *Centella asiatica* and VGSC blocker

Voltage-gated sodium channel (VGSC) blocker is a molecule or substance which can block or impair the conduction of sodium ion through the ion channel. *Centella asiatica* had been shown to have a potency to be a VGSC blocker as it have been used to treat lots of illness related to epilepsy and cognitive disorder (Veerendra Kumar and Gupta, 2002; Wattanathorn *et al.*, 2008). VGSC which can be found in the neuron cells might be the one who responsible in the action of *Centella asiatica* on those disorders.

Besides, oral administration of the *Centella asiatica* extracts have been found can retarded the development of solid and ascites tumours and increased the life span of tumour bearing mice (Babu *et al.*, 1995). Tritiated thymidine, uridine and leucine incorporation assay suggest that the fraction acts directly on DNA synthesis of the cancer cell and causing the tumor to decrease in size and increasing the life span (Babu *et al.*, 1995). It also had been found that this plant extract did not give a toxic effect on normal cells which is consider safe to be used as it only give an effect to cancer cells (Babu *et al.*, 1995).

2.5 MDA-MB-231 breast cancer cell line

The MDA-MB-231 breast cancer cell line was obtained from mammary gland of a breast which had adenocarcinoma and the cells was get from a pleural effusion on the metastatic site. With epithelial-like morphology, the MDA-MB-231 breast cancer cells appear phenotypically as spindle shaped cells and *in vitro*, the MDA-MB-231 cell line has an invasive phenotype. The MDA-MB-231 cell line is also able to grow on agarose, an indicator of transformation and tumorigenicity, and displays a relatively high colony forming efficiency. *In vivo*, the MDA-MB-231 cells form mammary fat pad tumors in nude

mice. Intravenous (IV) injection of cells into the tail vein of nude mice has been shown to produce experimental metastasis.

MDA-MB-231 also has been found to show a high expression of one of the VGSC isoform by treating it with tetrodotoxin (TTX). The outward current density was inversely related to the cells' metastatic ability, such that only very small outward currents were recorded in the MDA-MB-231cells after treated with TTX. The inward currents were blocked by TTX in a dose-dependent fashion where the IC_{50} being greater than 1 mM. Based from the high concentration of the TTX required to block the VGSC, the VGSC which is expressed in MDA-MB-231 is form TTX-resistant channel. Further molecular analysis on the MDA-MB-231 has been revealed that $Na_v1.5$ was the predominant VGSC expressed in this cell line thus, explaining its capability in metastasis and aggressiveness (Fraser *et al.*, 2003).

CHAPTER 3

MATERIALS AND METHOD

3.1 General method

3.1.1 Sterilization

All the materials used in this study need to be sterile. The apparatus were autoclaved at 121°C for 15 minutes at a pressure of 15 lbs/in^2. This technique was done in order to avoid any contamination by removing and killing all the microorganisms that might be present on the apparatus. The autoclaved apparatus need to be used in sterile condition either inside the biosafety cabinet type 2 or on the work bench which already disinfected and cleaned with 70% ethanol. All the solvent that will be used were filtered with 0.2 μm or 0.45 μm filter for sterilization process.

3.1.2 Aseptic techniques

The procedure and steps were done under aseptic technique as the condition must be sterile. Any contamination from unwanted microorganisms should be prevented from being introduced into the cell culture, sterile media, reagents and any solutions that were used throughout the project. All the basic and standard operating procedure such as wearing the laboratory coat while working with the project was done. A clean glove was also worn in order to avoid any contamination on the media and the cell culture. All the working area including the bench and laminar flow were disinfected with 70% ethanol before and after handling and work on any sample or culture. Most of the things and apparatus that were brought into the working area should also be sterile.

3.2 Cell lines

3.2.1 Type of cell lines

The cancer cell that was used in this study is MDA-MB-231 breast cancer cell line as shown in figure 3.1, which had been isolated from breast cancer and get from the chemical pathology laboratory which prepared by Prof. Dr. Nik Soriani Yaacob and team.

3.2.2 Cryopreservation of cell lines

Extra confluent monolayer cells can be cryopreserved to ensure the stock of cells is always ready. In order to cryopreserve the confluent monolayer cells, a freezing media was first prepared as this media is necessary to maintain the cells for a longer period as well as supplying the nutrients to the cells. The freezing media was prepared by mixing 20% of Fetal bovine serum (FBS) and 10% Dimethyl sulfoxide (DMSO) together with 70% Dulbecco's Modified Eagle Medium (DMEM). Then, the confluent monolayer cells were retrieved from the CO_2 humidified incubator and was placed in the biosafety cabinet. The medium was discarded and the cells were washed with phosphate buffer saline (PBS) for three times. 3 mL of 0.05% trypsin was added into the culture dish and incubate for 3 minutes in the CO_2 incubator.

After 3 minutes, the cells were observed under the microscope to ensure that all the cells already detached from the surface of the culture dish. Then, 3 or 4 mL of complete DMEM was added into the culture dish to neutralize the medium and the mixture was transfer into a sterile and clean 15 mL falcon tube. The mixture was then centrifuge for 3 minutes at 2000 rpm to get the cell pallet. The supernatant was discarded and the cell pallet was resuspend in 2 mL freezing media. The cell suspension was then transfer into a clean and sterile cryogenic vial where 1 mL of the cell suspension for 1 vial. The cryogenic vials

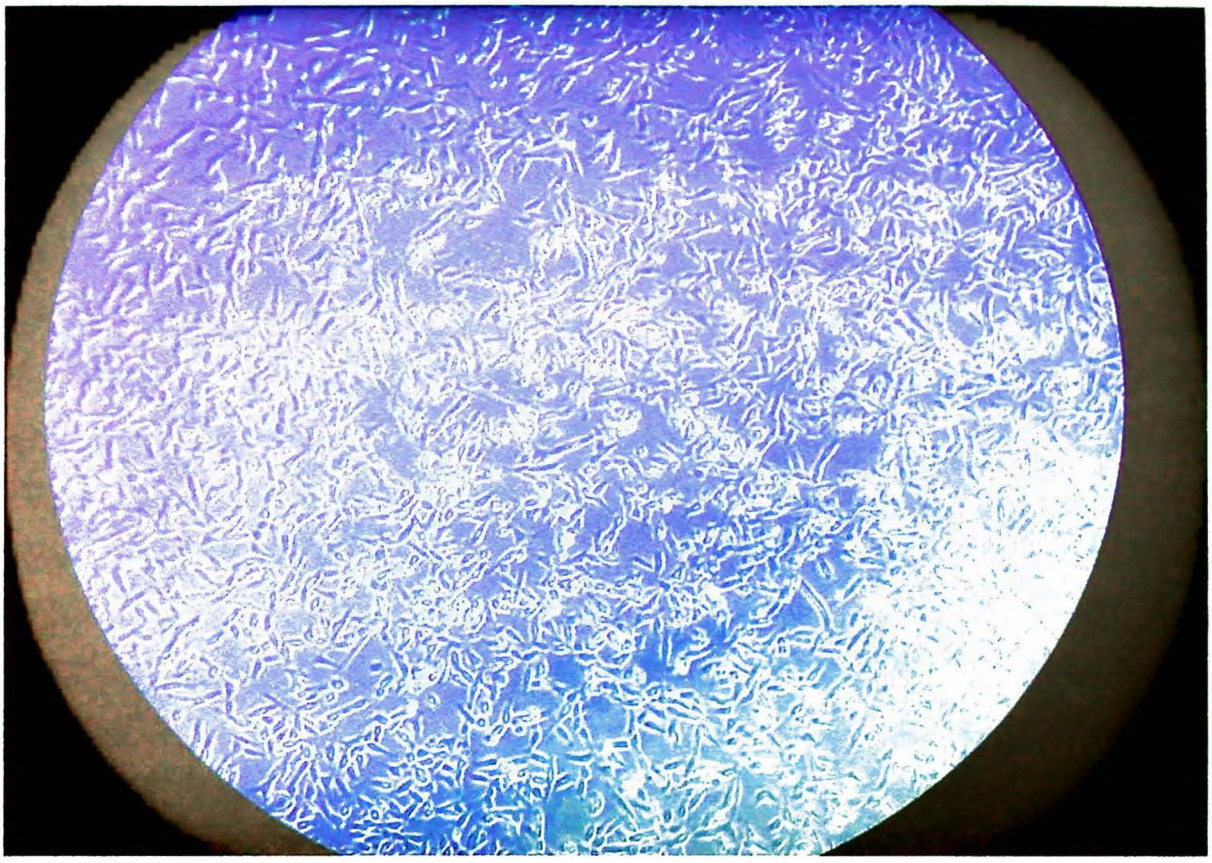

Figure 3.1 MDA-MB-231 breast cancer cell line observed under inverted microscope (10x magnification).

were stored at -20°C for 1 hour and then were transferred into -80°C for few days before transfer into liquid nitrogen tank for longer storage.

3.2.3 Thawing frozen cells

The MDA-MB-231 cell was retrieved from the cells that been cryopreserved in the liquid nitrogen tank. The cell in the cryovial was thawed by placing it in the CO_2 incubator for 5 minutes till all the cell suspensions melt down. Then, the cell suspension was transfer into two culture dish and 6 mL of complete DMEM was added into both of the dish. The cells were incubated in the humidified CO_2 incubator for overnight. Next day, the cells were observed under the microscope to check either they attached to the surface of the culture dish or not. When the cells attached, the media was change to remove the freezing media that still present in the culture dish. The cell was taken care and maintain until they are ready to be used.

3.2.4 Growth condition of cell line

The cell line was grown and maintain at 37°C with 5% carbon dioxide and enough humidity. The cell was cultured using complete DMEM in culture dish.

3.3 Medium preparation

3.3.1 Complete Dulbecco's Modified Eagle's Medium (DMEM)

The DMEM that was purchase need to be added with FBS and L-glutamine before can be used for cell culture. To prepare it, L-glutamine and FBS were thawed at room temperature. 500 mL DMEM was added with 25 mL 5% FBS and 10 mL 4 MicroMolar L-Glutamine. The preparation was done under aseptic technique inside the biosafety cabinet type-2.

3.4 Treatment or plant extracts preparation

The plant extract had been prepared by Dr. Noor Fatmawati's group and was extracted using aqueous method. As the concentration of the treatment solution that being used in this experiment is low, small number of the plant extract is weight within the range of 10 mg to prepare a stock solution. To weight the treatment plant extract, clean appendorf tube was weight and the weight was taken. Then, small amount of plant extract is added into the appendorf tube. The weight of both the appendorf tube and the plant extract is again taken and the weight of the plant extract is calculated using the formula:

$$\text{Weight of the plat extract} = \left[\begin{array}{l}\text{weight of the appendorf} \\ \text{tube with plant extract}\end{array}\right] - \left[\begin{array}{l}\text{weight of the appendorf} \\ \text{tube without plant extract}\end{array}\right]$$

The plant extract was then dissolve in 1 mL DMSO and was left to dissolve completely. The solution was again vortex to further ensure that the plant extract dissolve. From the weight of the plant extract and the volume of the stock solution, the concentration of the treatment stock solution was calculated using the formula:

$$\text{Concentration (mg/mL)} = \frac{\text{weight (mg)}}{\text{Volume (mL)}}$$

$$= \text{concentration (mg/mL)} \times 10^{-3}$$

$$= \text{concentration (ug/mL)}$$

From the treatment stock solution, the treatment solutions with different concentration were prepared and they were prepared from higher concentration to lower concentration. First, 10 ug/mL treatment solution was prepared by diluting the treatment stock solution

into complete DMEM with volume of 15 mL. The treatment solutions were prepared using the formula:

$$M1V1 = M2V2$$

Where:

M_1 = Final concentration

V_1 = Final volume

M_2 = Initial concentration

V_2 = Initial volume

The first treatment solution prepared need to be sterile by filtering it with 0.2 µL or 0.45 µL filter. Then, another two treatment solutions were prepared from the sterile treatment solution using the same formula. All the prepared treatment solutions were stored in the refrigerator at 4°C.

3.5 Agarose gel preparation

4.2 g agarose powder was weight to make up a 3% agarose gel when dissolve in 140 mL TAE buffer. The mixture was then heated in the microwave for 3 minutes to dissolve the agarose powder completely. 5 µL of EtBr was added into the agarose gel and mixed well before being poured into the tank with specific comb. The agarose gel was allowed to cool down and solidified before can be used.

3.6 Cell culture and maintenance

3.6.1 Cell splitting

All the things needed were prepared where 0.05% trypsin and complete DMEM were thaw in the 37°C water bath and other materials were prepared in the biosafety cabinet. The cell was first observed under the microscope for any unwanted microorganism and to ensure the cell was in a good condition. The percentage of the cell confluency was also observed as only the cell which confluence enough will be split. First, the media was discarded and 3 mL of 0.05% trypsin was added into the culture dish. The volume of the 0.05% trypsin and time for incubate was depending on the percentage of the cell confluency. For cell with 70% confluency, 3 mL of 0.05% trypsin was used and incubate in the CO_2 incubator for 3 minutes.

The cell was incubated at 37°C with 5% CO_2. After 3 minutes, the cell was again observed under the microscope to ensure that all the cell already detach from the culture dish and floating. If the cell still not detach, the bottom of the culture dish was tap slowly to detach it. Once the cell detach, 3 mL of complete media was added into the culture dish for neutralization. The volume of the complete media used was also depending on the volume of 0.05% trypsin used. The mixture was mixed well by pipetting it up and down and the surface of the culture dish was spray by the mixture to ensure all the cells detach.

The mixture was then transfers into 15 mL falcon tube, and was centrifuge at 2000 rpm for 3 minute to get the cell pellet. After centrifuge, the supernatant was discarded and the cell pellet was resuspend in 3 mL complete media. 1 mL of the cell suspension was pipette into new culture dish and another 6 mL of complete media was added too. The

culture dish was labeled and observe under the microscope before incubate at 37°C, 5% CO_2 until confluence.

3.6.2 Change media

The complete media was thaw in the 37°C water bath and the cell was observed under the microscope to ensure that it is in a good condition. The media was discarded and the cell was wash carefully with PBS for three times. 6 mL of complete media was then added slowly into the culture dish and again the cell was observed under the microscope. The cell was then incubated at 37°C with 5% CO_2.

3.7 Cell counting using Hemacytometer

0.05% trypsin and complete DMEM were thaw in the 37°C water bath and other materials were prepared in the biosafety cabinet. The cell was first observed under the microscope for any unwanted microorganism and to ensure the cell was in a good condition. The percentage of the cell confluency was also observed as only the cell which confluence enough will be used for experiment set up or seedling. First, the media was discarded and 3 mL of 0.05% trypsin was added into the culture dish. The cell was incubated at 37°C with 5% CO_2 for 3 minutes.

After 3 minutes, the cell was again observed under the microscope to ensure that all the cell already detach from the culture dish and floating. Once the cell detach, 3 mL of complete media was added into the culture dish for neutralization. The volume of the complete media used was also depending on the volume of 0.05% trypsin used. The mixture was mixed well by pipetting it up and down and the surface of the culture dish was spray by the mixture to ensure all the cells detach.