ELUCIDATION OF ANTIPROLIFERATIVE AND ANTI-METASTATIC PROPERTIES OF *CAULERPA LENTILLIFERA* IN THE HEPG2 CELL LINE

by

RAMMIYA A/P RAJASEGARAN

DISSERTATION IS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

ADVANCED MEDICAL AND DENTAL INSTITUTE
UNIVERSITI SAINS MALAYSIA

2023

ACKNOWLEDGEMENT

First and foremost, I would like to thank God, the Giver of great protection, and blessings as well as to the universe for supporting me with great positive energy. I am very thankful for giving myself strength in both mentally and physically throughout my life and during conducting my dissertation.

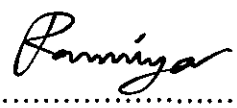
Furthermore, I would like to express my sincere gratitude and appreciation to my supervisor, Associate Professor Datin Dr. Shahrul Bariyah Binti Sahul Hamid, for her guidance, support, encouragement, and for her unconditional care towards me. I am greatly honoured to work under her supervision and I truly treasure all her commitments despite the time and suggestions that she had provided to me.

I am honoured to further my studies in Universiti Sains Malaysia as a postgraduate student in Medical Research program which has given me an opportunity to widen my range of knowledge and skills for my future career. I would also like to express my gratitude to my seniors, Ms. Auni Fatin Bt Abdul Hamid, and Ms. Nurnabila Bt Md Zailani for assisting me, providing care and motivational support throughout my research in laboratory.

I would like to thank my best friend, Soonali Wasdewa, for the support and unconditional love that she has provided me not only for my dissertation, but also throughout my journey. Not forgetting the pillar of my life, Mr. Rajasegaran Ramasamy and Mrs. Rathika Ramanaidu. The reason why I chose to further my studies in master degree of Medical Research, being motivated and actively participating in my research is because of their support, motivation, continuous love, and care.

DECLARATION

I hereby declare that this research was sent to Universiti Sains Malaysia (USM) under the Masters of Science in Medical Research program. It has not been sent to other public or private universities. With that said, this research can be used for the purpose of consultation and can be photocopied for reference purposes.



.....

RAMMIYA A/P RAJASEGARAN

Matric No.: P-IPM0024/22

Date: 29 August 2023

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

α	alpha
β	beta
eV	electron volt
g	grams
κ	Kappa
m	metre
m/z	mass-to-charge ratio
mbar	millibars
mg	milligram
mg/mL	milligram per millilitre
mL	millilitre
nm	nanometre
rpm	revolutions per minute
µg/mL	microgram per millilitre
µL	microlitre
µm	micrometre

LIST OF ABBREVIATIONS

A172	Human brain glioblastoma cell line
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
Akt/ PKB	Protein kinase B
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl-6	B-cell lymphoma 6
C57BL/KsJ	Leptin receptor deficient
c-MET	Mesenchymal-epithelial transition factor
Cdc25C	Cell division cycle 25C
Cdk1	Cyclin-dependent kinase 1
CEMACS	Centre for Marine and Coastal Studies, USM
CFSE	Carboxyfluorescein succinimidyl ester
CHO	Chinese Hamster Ovary
CLE	*Caulerpa lentillifera* extract
CLHE	*Caulerpa lentillifera* hexane extract
C. lentillifera	*Caulerpa lentillifera*
CO2	Carbon dioxide
COX-2	Cyclooxygenase
CTNNB1	Catenin beta 1
db/db mice	Diabetic mice
ddH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunoassay
EMEM	Eagle's minimum essential medium
ERK	Extracellular regulated kinase
FAMEs	Fatty acid methyl esters

FASN	Fatty acid synthase
FBS	Fetal Bovine Serum
Fox0	Forkhead box 0
GC-MS	Gas chromatography- mass spectrometry
GLUT4	Glucose transporter protein type-4
GSK3β	Glycogen synthase kinase-3 beta
H2O2	Hydrogen peroxide
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCs	Hepatocytes
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HepG2	Human hepatoma cancer cell line
HIF1 α	Hypoxia-angiogenesis
HL-7702	Normal human liver cell line
HPLC	High performance liquid chromatography
HSCs	Hepatic stellate cells
HuH7	Human hepatoma-derived cell line
IC	Half-maximal inhibitory concentration
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IRS	Insulin receptor substrate
ISO	Isoorientin
JC-1	5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimi-dazoylcarbocyanine iodide fluorescence dye
Jnk	c-Jun N-terminal kinase
KCs	Kupffer cells
LA	Linoleic acid
LPS	Lipopolysaccharide
LSECs	Liver sinusoidal endothelial cells
miR-211	MicroRNA-211
MAPK	Mitogen activated protein kinases
MCF-7	Breast cancer cell line
MDA-MB-231	Human breast adenocarcinoma epithelial cells
MMP	Mitochondrial membrane potential

MS	Mass spectrometry
MTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NAFLD	Non-alcoholic fatty liver disease
NF-κB	Nuclear factor kappa B
NIST	National Institute of Standards and Technology
NO	Nitric oxide
OD	Optical density
OPE	Ophiorrhiza pumila ethanolic extract
P13K	Phosphatidylinositol 3-kinase
PA	Palmitic acid
PARP	Poly-ADP ribose polymerase
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
pH	Potential of Hydrogen
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
R.T.	Retention time
RAW 264.7	Macrophage cell line from tumour in male mice
RB	Retinoblastoma gene
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RTKs	Receptor-tyrosine kinases
SC	Stromal cells
SOAT1	Sterol O-acyletransfrase1
SMMC-7721	Liver cancer cell line
SPSS	Statistical Package for the Social Sciences
SREBP2	Sterol regulatory element binding protein 2
STING	Stimulator of interferon genes
TAM	TrueAlgaeMax
TAZ	Transcriptional co-activator with PDZ-binding motif
TDA	Tridecanoic acid/ tridecylic acid
TERT	Telomerase reverse transcriptase
TNF- α	Tumour necrosis factor alpha

TP53	Tumour protein 53 gene
TSG	Ethanolic extract of Prunus persica (L.)
ULK1	Autophagy activating kinase
UMMC	Universiti Malaya Medical Centre
USM	Universiti Sains Malaysia
WHO	World Health Organisation
WNT	Wingless-related integration site
XYL5	β -1,3-xylooligosaccharides with average degree of polymerisation of 5
YAP	Yes-Associated protein mediator
YLE	Smallanthus sonchifolius leaf (Yacon)

PENJELASAN MENGENAI SIFAT ANTIPROLIFERATIF DAN ANTI-METASTATIK OLEH CAULERPA LENTILLIFERA PADA TITISAN SEL HEPG2

ABSTRAK

Karsinoma hepatoselular (HCC) adalah salah satu kanser hati yang biasanya disebabkan oleh sirosis. Walau bagaimanapun, terapi yang biasa digunakan masih tidak dapat mengekang penyebaran penyakit kanser kerana ia menjadi rintangan terhadap terapi. Oleh itu, penerokaan ke dalam sebatian terapi baru terjamin untuk mengatasi penyakit ini. Caulerpa lentillifera adalah rumpai laut hijau yang tumbuh di kawasan pesisir dan dilaporkan mengandungi peranan anti-radang. Tujuan kajian ini adalah untuk mengenal pasti sebatian mudah meruap yang terdapat dalam ekstrak metanolik C. lentillifera dan mekanisma tindakan dalam sel barah hati HepG2 dengan menggunakan ujian MTT dan ujian klonogenik. Sampel C. lentillifera telah dikeringkan dan digiling menjadi bentuk serbuk sebelum pengekstrakan metanolik. Ekstrak metanolik C. lentillifera telah dianalisis untuk mengenal pasti kehadiran sebatian mudah meruap dengan menggunakan instrumen GC-MS. Asid heksadekanoik dan asid tridekanoik dikesan sebagai sebatian yang paling banyak dalam sampel C. lentillifera. Sel kanser hati HepG2 dikultur untuk diuji dengan ekstrak metanolik C. lentillifera. Ujian MTT dan ujian klonogenik dilaksanakan untuk mengenal pasti mekanisma tindakan pada sel kanser hati HepG2. Kajian menunjukkan bahawa sebatian aktif yang terdapat dalam ekstrak metanolik C. lentillifera mempunyai kesan sitotoksik terhadap daya maju sel HepG2. Kepekatan separuh maksimum (IC_{50}) yang diperoleh telah diuji secara lebih lanjut dalam ujian klonogenik. Selain itu, ekstrak metanolik menunjukkan penindasan pembentukan koloni HepG2 yang ketara

($p<0.05$), namun, pengurangan diameter koloni adalah tidak signifikan. Secara keseluruhan, C. lentillifera mempunyai kemampuan untuk menghalang percambahan sel kanser hati HepG2 yang berkemungkinan melalui apoptosis. Walau bagaimanapun, kajian yang lebih lanjut diperlukan untuk mengenal pasti sasaran molekul tepat yang terdapat dalam C. lentillifera.

ELUCIDATION OF ANTIPROLIFERATIVE AND ANTI-METASTATIC PROPERTIES OF *CAULERPA LENTILLIFERA* IN THE HEPG2 CELL LINE

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common primary liver cancers which is usually caused by cirrhosis. However, standard therapies are still unable to curb the spread of the disease as it becomes resistant. Hence, exploration into new therapeutic compound is warranted to address this disease. *Caulerpa lentillifera* is a green seaweed that grows in the coastal regions and reported to contain anti-inflammatory role. This research aims to identify volatile compounds present in *C. lentillifera* methanolic extract and the mechanism of action in HepG2 liver cancer cells using MTT assay and clonogenic assay. The *C. lentillifera* sample was dried and grinded into powder form prior to methanolic extraction. *C. lentillifera* methanolic extract was analysed to identify the presence of volatile compounds using GC-MS instrument. Hexadecanoic acid and tridecanoic acid were detected as the most abundant compounds in *C. lentillifera* extract. HepG2 liver cancer cells were cultured to be tested with *C. lentillifera* methanolic extract. MTT assay and clonogenic assay were performed to identify the mechanism of action in the HepG2 liver cancer cells. Findings suggest that the active compounds present in *C. lentillifera* methanolic extract have cytotoxic effect on the viability of HepG2 cell. The half maximal concentration (IC_{50}) obtained was further tested in the clonogenic assay. Similarly, the methanolic extract demonstrated significant suppression ($p<0.05$) of HepG2 colony formation and showed insignificant reduction in the diameter of colonies. Taken together, *C. lentillifera* has the properties that able to inhibit the proliferation of HepG2 liver cancer

cells probably via apoptosis. However, further studies are needed to identify the exact molecular targets of the *C. lentillifera*.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Liver is the second largest organ in a human body with two main lobes which comprises of eight segments of functional basis with many smaller lobules. (Mahadevan, 2014). This organ consists of numerous cells that play important roles in cellular level such as detoxification, metabolism, coagulation, and immune responses (Trefts *et al.*, 2017). The hepatocytes (HCs), Kupffer cells (KCs), hepatic stellate cells (HSCs), and the liver sinusoidal endothelial cells (LSECs) are the main four liver cells that works together to maintain the shape and function of the organ. Approximately 70% of the total liver cell population consist of HCs which are parenchymal liver cells. HCs plays crucial role in the basic functions of the liver such as drug metabolism, as well as the secretion of coagulation and complement factors like fibrinogen, and factor V. LSECs represents 50% of the nonparenchymal cells which functions as a permeable barrier to maintain the immune homeostasis and the metabolism between the blood cells with both HCs and HSCs. One-third of the nonparenchymal cells are the KCs that functions to eliminate the pathogens or foreign substance by secreting cytokines like tumour necrosis factor- α (TNF- α), interleukin 6 (IL-6), and several chemokines whereas the remaining 5% of the liver cells play essential role in vitamin A and lipid storage (Ding *et al.*, 2016).

Liver cancer cells occurs when the DNA of the liver cell is mutated in such a way the cell grows without the presence of proliferative signaling. This leads to continuous growth of cells that subsequently forms a tumour. Liver cancer can be classified into two types which are primary liver cancer and secondary liver cancer. Cancer that begins in the liver are known as primary liver cancer and the most common

form of liver cancer are hepatocellular carcinoma (HCC) which grows in two different patterns that are either beginning with a single tumour in larger size or beginning with many smaller nodules throughout the liver. Besides that, intrahepatic cholangiocarcinoma also known as bile duct cancer occurs in the cells that forms the small bile ducts outside the liver. Angiosarcoma and hemangiosarcoma are rare cancer conditions that occurs in the cells lining to the blood vessels of the liver whereas for hepatoblastoma, this kind of cancer is also a rare condition that develops in children that are younger than four years old. Furthermore, cancer cells that have metastasised from its original site such as from stomach, pancreas or lung are known as secondary liver cancer. Example of secondary liver cancer are benign liver tumour, hemangioma, hepatic adenoma, and focal nodular hyperplasia (Tunissiolli *et al.*, 2017).

The possible risk factors that cause primary liver cancer are due to chronic infection with HBV or HCV, cirrhosis which causes scar tissue formation in liver, several inherited liver diseases that includes hemochromatosis and Wilson's disease, diabetes, non-alcoholic fatty liver disease (NAFLD), exposure to aflatoxins and excessive alcohol consumption. Signs and symptoms may appear in the beginning stage of primary liver cancer such as loss of appetite, frequent tiredness, swelling of abdomen, jaundice, weight loss, nausea, and vomiting (Janevska *et al.*, 2015).

According to Mohamed *et al.* (2018), liver cancer is the eighth most common cancer in this country. The death caused by liver cancer had risen by 31.5% from 1990 to 2020. According to Malfettone *et al.* (2020), liver cancer deaths in Malaysia was 1,984, accounting 1.18% of total deaths. However, recently WHO reported liver cancer deaths in Malaysia has increased to 5,647 of total deaths. The annual mortality rate per

100,000 people from liver cancer in Malaysia is 6.96% which is ranked as the 57th in world liver cancer ranking (Mohamed *et al.*, 2018).

The liver cancer is the fifth most common cause of cancer among men in Malaysia but it is the eighth most common cause of cancer in this country. According to the Mohamed *et al.* (2018), there were 2149 liver cancer cases in 2020. Out of these cases, 1553 cases or 72.27% involve men and 596 cases or 27.73% involve women. Individuals with high risk to develop liver cancer are those more than 70 - 74 years for men with 30% per 100,000 of population compared to women is 75 - 79 years with 12.5% per 100,000 of population (Mohamed *et al.*, 2018).

Malaysia National Cancer Registry Report from 2012 to 2016 indicated that most of the patients with liver cancer are Chinese with 1284 cases (47.66%), followed by Malays with 1263 cases (46.88%) and Indian with 147 cases (5.46%) (Azizah *et al.*, 2019). Similarly, another study by Mohamed *et al.* (2018) that was conducted at the Universiti Malaya Medical Center (UMMC) found out that out of 115 patients, Chinese ethnicity was most affected with liver cancer, which is 69 patients (60%), followed by Malays with 33 patients (28.7%) and Indians with 13 patients (11.3%).

From the aspect of causes, Malays and Chinese patients with liver cancer most likely caused by HBV and for Indians it was cryptogenic (29.0%) and alcohol (26.3%) induced the liver cancer. Hepatitis C was more in Malays (18.3%) but less in Chinese and Indians. Overall etiology shows hepatitis B (59.2%) being the major cause followed by cryptogenic (16.4%) and hepatitis C (10.6%). Moreover, cryptogenic can be correlated with the increasing incidence of diabetes and obesity in Malaysia. According to obesity statistics in the Asia-Pacific region, it showed that Malaysia is the most obese country by which the obesity prevalence has increased tremendously

from 43.1% in 2006 to 60% in 2014, and the diabetes mellitus prevalence is exhibiting an increasing trend from 14.9% in 2006 to 20% in 2011 (Mohamed *et al.*, 2018). In year 2022, the percentage of obesity in Malaysia is 15.60% per population while the percentage of diabetes is 19% per population in Malaysia. Due to the rising epidemic of obesity and diabetes in Malaysia, these being the underlying cause of NAFLD which contribute to aggressive form of liver cancer (Mohamed *et al.*, 2018).

1.2 Problem Statement

There are possible treatments available for HCC in Malaysia which are ablation techniques, surgery, chemotherapy, and radiotherapy, targeted therapy, and immunotherapy. Radiofrequency ablation also known as the tumour removal treatment is most common method used to destroy the affected tissue. Furthermore, surgery or liver transplant methods aim to remove the affected parts of liver or the whole liver and replacing the damaged liver with a healthier liver. As for chemotherapy and radiotherapy, chemoembolisation and radio embolisation are performed to destroy the cancer cells and preventing the remaining cancer cells from proliferating. Targeted therapy is done using regorafenib (Stivarga) and cabozantinib (cabometyx) drugs that specifically targets either genes, proteins or the tissue environment that supports the cancer cell growth and survival. Immunotherapy is also referred as biologic therapy that functions to boost the immune response against cancer cells (Mansoori *et al.*, 2017).

The report on patients who have been treated with these possible treatments in five-years survival rate of liver cancer from 2012 to 2016 has shown that the survival rate was only 12.8%. In some liver cancer patients, the targeted therapies fail due to the gain of resistance of drugs used for treatments. The resistance to drugs is caused

by the alteration of a single genetic in liver cancer cells known as an intrinsic factor which can occur either before or during the treatment. The pH, hypoxia, and paracrine signalling interactions with stromal as an extrinsic factor also contribute to tumour heterogeneity. Moreover, tumour microenvironments such as stromal cells (SC), extracellular matrix (ECM), and soluble factors including cytokines and growth factors provide direct cell interaction mediated by drug resistance and cause cancer cell survival. It has been analysed that overexpression of the ATP-binding cassette (ABC), drug transporters such as ABCB1, which encodes P-glycoprotein, and ABCG2 help to prevent cancer stem cells to interact with chemotherapeutic agents (Mansoori *et al.*, 2017).

Furthermore, these treatments come with side effects towards the liver cancer patients. Side effects after the radiofrequency ablation experienced by patients is mild headache, soreness at the injection area, external burning sensation and numbness. Besides that, the other side effects of chemotherapy include hair loss, diarrhoea, loss of appetite, nausea, and vomiting. Skin problems like acneiform rash and skin redness are some of the common side effects that may occur to patients treated with targeted therapy. For immunotherapy, side effects including skin irritation, muscle cramp, diarrhoea, and anaemia that may occur due to immune checkpoint inhibitors (Mansoori *et al.*, 2017).

To overcome poor response to existing therapies, new therapy is imperative to address this challenge. Over the last decade, intense research has diverted towards natural sources with less toxicity as therapy for liver cancer. One of the natural sources that has not been tapped into its anticancer property is the *Caulerpa lentillifera*. It is a macroalgae that grows in the coastal areas in Malaysia.

Caulerpa lentillifera is a type of green seaweed that can be found in the Asia-Pacific regions. It is a green alga known as sea grapes or green caviar from ulvophyte species. It grows on rocks and coral rubble in small clumps. *C. lentillifera* is widely consumed as a fresh vegetable and have been traditionally consumed by Southeast Asians. It can be eaten as raw on their own or in salads. *C. lentillifera* is rich in iodine, polyunsaturated fatty acids, minerals, vitamins, and bioactive compounds like caulerpin that contributes to many human health benefits. *C. lentillifera* also has diabetes lowering properties which help to enhance insulin secretion in pancreatic β-cells and increase insulin sensitivity. Furthermore, anti-cancer properties contained in *C. lentillifera* has showed that the extract can inhibit the cell proliferation, reduce the cell viability, and induces apoptosis of cancer cell.

Studies by Maeda *et al.* (2012), Varitta Tanawoot *et al.* (2021), Yoojam *et al.* (2021), as well as Sharma, *et al.* (2015) have showed that *C. lentillifera* has anti-cancer properties which can inhibit the cancer cell proliferation, cell cycle arrest, suppress the ROS formation and inhibit the signaling pathway of cancer cell such as MCF-7 cells, A172 human glioblastoma cells, and RAW264.7 macrophage cells, however, these studies have not been tested on HepG2 liver cancer cell line.

Therefore, the present study aims to identify the volatile compounds present in *C. lentillifera* sample and to determine the phenotypic characterisation associated with proliferative and metastasis in HepG2 liver cancer cells using MTT assay and clonogenic assay. These methods were used to allow better research on the identification of specific compound or biomarker that can be used to treat liver cancer patients via targeted drug therapy treatment.

1.3 Objectives

The objectives of this study were as below;

1.3.1 Objective 1

To perform methanol extraction of *C. lentillifera* sample.

1.3.2 Objective 2

To identify volatile compounds present in *C. lentillifera* sample.

1.3.3 Objective 3

To determine the phenotypic characterisation associated with proliferative and metastasis in HepG2 liver cancer cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Characterisation of liver cancer cell

Cell lines are immortal cells that are very priceless due to consistent natural population of cells with reproducible results. The cell lines have scientific revolution as well as largely applied in production of vaccine, in drug metabolism testing and cytotoxicity testing, antibody production, to study the gene function, to generate artificial tissues like artificial skin, and last but not the least, biological compounds synthesis such as therapeutic proteins (Kaur and Dufour, 2012).

The immortalised liver cancer cell lines have been used as worldwide practice for the cancer research, and have been applied to study the hepatitis B (HBV) and hepatitis D (HDV) viral infections. According to Verrier *et al.* (2016), the replication and expression of chronic HDV in full cell cycle as well as the chronic HBV replication were found in HuH7 and HepG2 cell lines. Among 40 various types of liver cancer cell lines, the cell line that has been widely used in scientific research is HepG2 cell line as shown in Figure 2.1 (Kaur and Dufour, 2012).

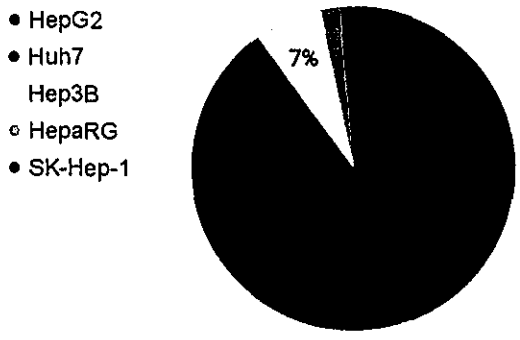


Figure 2.1 Percentage of human hepatic cell lines manuscripts that is searched on PubMed until 2021 (Arzumanian *et al.*, 2021).

The HepG2 cells has eight hallmark capabilities that provides specific roles in supporting the progression, development, endurance of tumours as well as their respective cells, and metastatic dissemination to survive. The ten biological hallmarks of cancer are as shown in Figure 2.2 (Hanahan, and Weinberg, 2011).

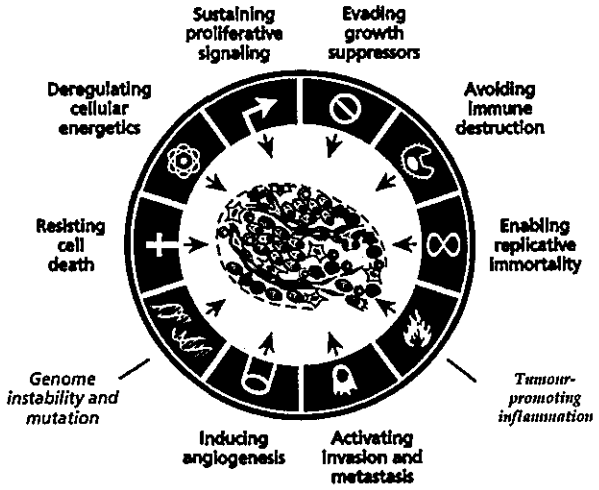


Figure 2.2 The 8 biological hallmarks of cancer with 2 additional hallmark representing as genome instability and mutation as well as tumour-promoting inflammation (Arzumanian *et al.*, 2021).

2.1.1 Sustaining proliferative signaling

HepG2 cells stimulates their own growth due to the cells do not require extracellular signals like epidermal growth factor receptor (EGFR). This is because the cells have their own and sufficient growth signals. The proliferation of cancer cells is mainly depending on Akt, MAPK/ERK and MTOR pathways. Akt also known as the protein kinase B (PKB) was discovered as proto-oncogene which functions in the regulation metabolism, survival, protein synthesis, proliferation, and the transcription of cells. This proto-oncogene is activated mostly by involving in the lipid kinase phosphatidylinositol 3-kinase (P13K) that is an important cell survival regulator which inhibits the programmed cell death effects of signaling pathways and proteins such as

Fox01. Mitogen activated protein kinases (MAPKs) are main element in many signaling cascades that disseminate proliferation, and viability signals from cell surface to nucleus. Growth factors that stimulate receptor-tyrosine kinases (RTKs) or alteration in the cellular homeostasis can aid in the signaling pathway activation which involves extracellular regulated kinase (ERK), p38, MAPK or c-Jun N-terminal kinase (Jnk). Respective kinases will then promote the stimulation of transcription factors that result in cell growth, survival, proliferation, and repair. Last but not the least, MTOR is a protein kinase that plays a role as ATP and as amino acid sensor to stabilise the availability of energy and nutrient including the cell growth. MTOR can also activate or inhibit other signaling pathways like Akt, MAPK and ERK (Hanahan, and Weinberg, 2011).

2.1.2 Evading growth suppressors

HepG2 cells avoid inhibitory signals or factors that stops the cellular growth and allow continuous proliferation. TP53, PTEN and RB genes are the suppressive genes that cause inhibition of cell proliferation and growth. When the genes are oppressed, the gene would not be activated, thus, cell growth would not be restrained. HepG2 cells can lose the suppressive gene entirely or cause mutation to the gene so that the genes do not express or activated (Hanahan, and Weinberg, 2011).

2.1.3 Resisting cell death

Generally, apoptosis mechanism prevents infected or damage cells on the organism by maintaining the body tissue healthy via growth and development. For cancer cells to survive and proliferate, the cells must evade apoptosis by modifying the mechanisms that detects the damage or irregular cells. Therefore, the cancer cells can prevent the normal signaling of cells and activation of apoptosis. HepG2 cells can also

involve themselves to defect the downstream signaling or involve the signaling proteins in apoptosis such as increasing or decreasing the anti-apoptotic or pro-apoptotic gene expression to prevent a proper apoptosis (Hanahan, and Weinberg, 2011).

2.1.4 Enabling replicative immortality

HepG2 cells can degenerate to pre-differentiated stem cell-like phenotype which enables the uninhibited cellular division and other metabolic adaptations to survive in adverse conditions. Hippo signaling pathway and WNT signaling pathway are the two main pathways that enable the replicative immortality to occur in this cell. Dysregulation of Hippo signaling that functions to regulate the cell proliferation can contribute to the development of cancer by triggering the YAP and TAZ mediators. WNT/β-Catenin pathway that regulates the stem cell pluripotency and cell development also can contribute to cancer cell's ability to replicate continuously (Hanahan, and Weinberg, 2011).

2.1.5 Inducing angiogenesis

Cancer cells requires oxygen and nutrients to grow as well as excrete metabolic wastes and carbon dioxide like other cells. Hence, formation of new blood vessels also known as angiogenesis is created to satisfy the needs of the cancer cells. When angiogenesis is activated, the tumour cells will exhibit various patterns of neovascularization to various locations and the angiogenesis induced by these oncogenes can drive the proliferative signaling which then allows the enlargement of tumour cells and cause invasion of cells to nearby tissue as well as metastases (Hanahan, and Weinberg, 2011).

2.1.6 Activating invasion and metastasis

The HepG2 cells actively invades the local tissues and spreads to various sites via invasion and metastasis. Tissue invasion mechanism is when the tumour cells spread to neighbouring cells or tissues and metastasis is a mechanism of tumour cells been break away from the primary tumour cell and migrates to other location to establish a new secondary tumour. Adherens junction signaling pathway is the pathway used by tumour cells to invade and undergo metastasis. Adherens junction has multiple function namely initiation and stabilisation of the adhesion of cells, regulates the actin cytoskeleton, intracellular signalling as well as regulation of transcription. When the adherens junction is defective, the cell-cell junction causes extensive variety of tissue abnormalities that will interrupt the homeostasis of cell (Hanahan, and Weinberg, 2011).

2.1.7 Deregulating cellular energetics and metabolism

Cancer cells requires abundance of energy to proliferate and the energy these cells consume are from signaling pathways that leads to Warburg effect also known as aerobic glycolysis. In this pathway, P13K/Akt, ERK1/2, MAPK, ULK1 (autophagy), p53, MTOR, AMPK, and hypoxia-angiogenesis (HIF1 α) are represented. HepG2 cells uses glucose, glutamine, lysophospholipids, acetate, amino acids, and extracellular proteins as their fuel source to supply macromolecular precursors for cell division. One of the fuel sources that cancer cell commonly use is glutamine because glutamine can enter mitochondria and activate the Krebs cycle through α-ketoglutarate to enable further oxidation. This increases oxygen and ATP production and hence promotes the cancer cell proliferation and growth (Hanahan, and Weinberg, 2011).

2.1.8 Avoiding immune destruction

Immune checkpoints are the integrated control mechanism of immune system that regulates the self-recognition and prevent security damage during the physiological immune response. HepG2 cells hijacks the normal mechanism of immune checkpoint control and regulation of innate immune response through stimulator of interferon genes (STING). These cells evade the immune elimination via the loss of antigenicity or the loss of immunogenicity as well as by synchronising with the immunosuppressive microenvironment. Hence, the cancer cells will be recognised as normal cells in that specific host and will not be attacked by the immune system (Hanahan, and Weinberg, 2011).

2.2 Inhibition of P13K/Akt signaling pathway in mitochondria of HepG2 cells

According to Yuan *et al.* (2012), the use of isoorientin (ISO) compound extracted from few plant species namely *Phyllostachys pubescens*, *Patrinia*, and *Drosophyllum lusitanicum* towards HepG2 cells has caused reduction in cell proliferation and induction of cell death in dose-dependent manner without releasing toxicity to human liver cells (HL-7702). The ISO compound triggered several apoptotic characteristics such as the cellular shrinkage, and the cleavage of poly-ADP ribose polymerase (PARP) and DNA fragmentation. The mechanism that underlies with the ISO-induced apoptosis in HepG2 cells are examined through the activation of caspase-3. Presence of caspase-3 in cytosol as zymogen occurred because of induction of caspase-dependent pathway that corresponds to mitochondria-mediated apoptosis pathway (Lin *et al.*, 2016). The apoptosis stimuli cause loss of mitochondrial membrane potential (MMP) and cytochrome c ease out of mitochondria and into cytosol. The release of cytochrome c stimulates caspase-3 subsequently cleaving the specific substrates of caspase-3 like PARP to apoptosis. Furthermore, ISO induces the down-

regulation of P13K/Akt pathway by increasing Fox04 expression in HepG2 cells. Fox04 also known as AFX is a member of forkhead transcription factor family that can induce the programmed cell death by reducing the Bim and Bcl-6 activities (Jiang *et al.*, 2018).

2.3 Inhibition of cell proliferation, cell cycle arrest, necrosis, and suppression of ROS formation in HepG2 cells

According to Myint *et al.* (2019), methanol extract of *Smallanthus sonchifolius* leaf (YLE) in dose-dependent manner has showed suppressive effect on HepG2 cell growth, cell migration and suppression on ROS formation. YLE has few pharmacological properties that includes anti-oxidant, anti-cancer effects, anti-allergic, and anti-inflammatory effect (Lee *et al.*, 2015). Higher concentration of YLE of about 90% at 100 μg/mL has effectively suppressed HepG2 cell proliferation due to the contribution of YLE in hindering the metastasis progression of cancer cell (Seyfried, and Huysentruyt, 2013). Besides that, YLE extract dose-dependent manner showed increase in percentage of cells at G0/G1 phase of HepG2 cell cycle through flow cytometry analysis (Grzmil, and Hemmings, 2012). This indicated that YLE extract has influenced the HepG2 cell cycle by causing arrest at G0/G1 phase of cell cycle. YLE also has induced necrosis in liver cancer cells by avoiding the caspase-3 or caspase-8 activation which regulates for apoptosis in cells. Furthermore, cancer cells have high levels of ROS because of its high metabolomic activity that has specific functions in the cell development (Yang *et al.*, 2018). YLE supresses the formation of ROS in HepG2 cells. This is because YLE contains abundance of polyphenols that supports the inhibition of HepG2 cell proliferation (Kumari *et al.*, 2018).

2.4 Suppression effect on cell proliferation, cell migration, and NF-κB signaling pathway

Ophiorrhiza pumila ethanolic extract (OPE) was tested on two human cell lines which were HepG2 and SMMC-7721 cells and both cells have showed cytotoxic effects in a time and concentration-dependent manner. The OPE has cause inhibition to cancer cell proliferation through arrest of cell cycle progression. From the analysis of previous studies on the MS fragmentation behavior, the main components contained in OPE were β-stigmasterol, deoxypumiloside, aknadinine, camptothecin, and pumiloside as shown in Table 2.1 (Liu *et al.*, 2020).

Peak	Retention time (min)	MS (m/z)	MS^2 (m/z)	Tentative compounds	Relative peak area (%)
1	7.2	513.26	351.16	Pumiloside	4.37
2	12.6	497.25	335.19	Deoxypumiloside	3.39
3	14.5	349.11	337.19	Camptothecin	8.48
4	32.5	383.26	327.15	Aknadinine	16.0
5	46.1	413.31	301.11	β-Stigmasterol	2.42

Table 2.1 Molecular compounds of OPE that are identified from chromatogram by HPLC-MS^2 (Liu *et al.*, 2020).

According to Gao *et al.* (2019), β-stigmasterol was been recorded to inhibit the human gastric carcinoma cell (SGC-7901) proliferation by inducing at G0/G1 arrest in carcinoma cells. However, OPE has caused G2/M phase arrest in both HepG2 and SMMC-7721 cell cycles. Thus, suppressing the cancer cell proliferation. Besides that, OPE has enhanced the mechanism of programmed cell death in both cancer cells by increasing the caspase-3 and Bax cleave expression and decreasing the expression of Bcl-2 in both HepG2 and SMMC-7721 cells (Renault *et al.*, 2011). Furthermore, OPE has increase the intracellular ROS level causing the suppression of HepG2 and SMMC-7721 cell viability that leads to damage of several macromolecules such as DNA damage, and protein denaturation (Liu *et al.*, 2020). OPE in dose-dependent manner has

inhibited the migratory and invasive ability of HepG2 and SMMC-7721 cells which caused in reduction of matrix metalloprotease, MMP-2, and MMP-9 expressions. OPE has also oppressed the p65 phosphorylation implying in the downregulation of NF-κB signaling pathway via cytotoxic effect (Liu *et al.*, 2020).

2.5 Induction of cell cycle arrest and suppression of HepG2 cell migration

According to Shen *et al.* (2017), ethanolic extract of *Prunus persica* (L.) root also called as TSG has showed that TSG extract can inhibit the HepG2 cell growth in dose and time-dependent manner. The apoptosis of HepG2 cells occurred when handled with higher-dosage of TSG extract causing the cell cycle arrest in G2/M phase. The protein expression levels of the Cdc25C and Cdk1 protein showed decreasing in amount and was detected using western blotting analysis. This is because Cdc25C and the Cdk1 proteins are the key proteins for modulation of mitosis-phase entry that sets the cell for division phase and the regulation of G2/M phase progression to ensure the DNA information are transmitted to daughter cells without damage as shown in Figure 2.3 (Liu *et al.*, 2020). The movement of HepG2 cells was hindered by TSG extract along with the decreased in extracellular MMP-3 and MMP-9 expressions (Shen *et al.*, 2017).

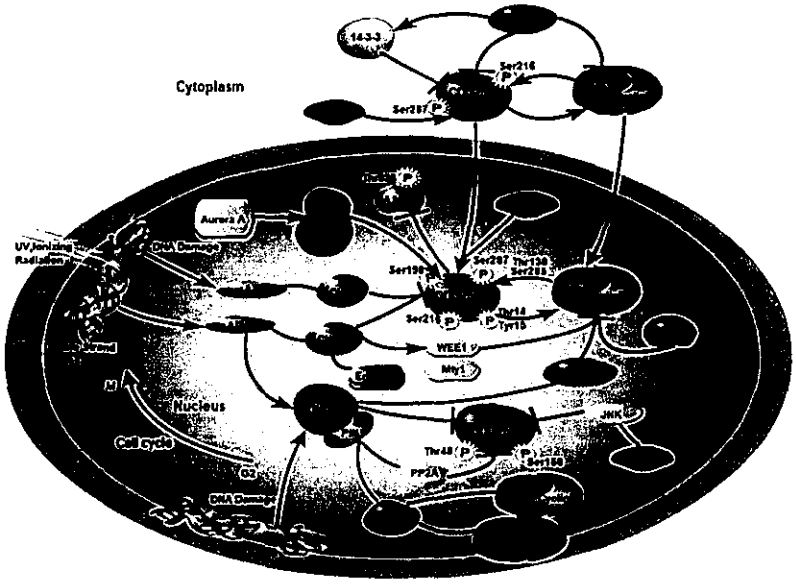


Figure 2.3 The role of CDc25C in regulation of the G2/M cell cycle (Liu *et al.*, 2020).

2.6 Properties of *Caulerpa* species

2.6.1 Physical properties

Caulerpa genus does not have a common name, but the species with vesiculate branchlets were commonly called as the sea grapes or grape algae such as *C. racemosa* or for the vesiculate branchlets that are in small and green caviar structure such as *C. lentillifera*. *Caulerpa* is a polynuclear siphonous green macroalga that are differentiated from other coenocytic green algae by internal elaborations (trabeculae) that is known to provide mechanical support to the algae via thallus. The growth of *Caulerpa* is regulated by environmental factors that includes water current, the light intensity, wave exposure, the seasons, depth of sea, and minimal pressure (Zubia *et al.*, 2019).

Caulerpa species have haplontic life-cycle in which the species has haploid gametophyte with microscopic zygote stage. The reproduction of this species can be sexually like biflagellate gametes that are liberated through the branch papillae as shown in Figure 2.4 or asexually that is anisogamous. Anisogametes are developed inside the coenocytic thalli whereas gametogenesis process involves migration of cytoplasm into lattice of unspecialised gametangia that are concentrated at terminal ends of fronds.

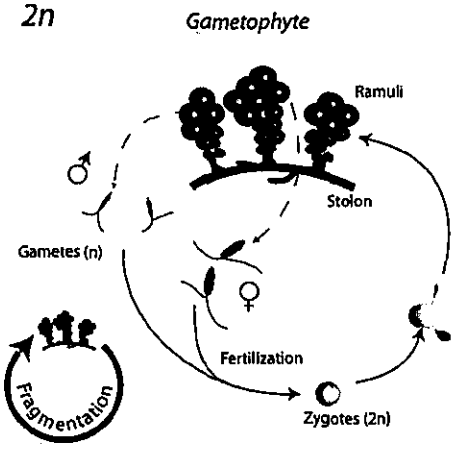


Figure 2.4 Life cycle of *Caulerpa* species (Zubia, 2019).

2.6.2 Chemical properties

According to Zubia *et al.* (2019), *Caulerpa* species has high concentration of minerals that can contain up to 55% of dry matter. This is evidence of dietary benefits for humans because seaweeds are used as mineral and iron nutritional supplements. Besides that, carbohydrates are abundantly found in *Caulerpa* species which varies from 3.6% to 83.2% of dry matter in the edible species of *Caulerpa*. The polysaccharides in the seaweed are taken as an alternative source of dietary fibre in multiple nutraceutical products because this fibre helps in human digestive tract. Furthermore, *Caulerpa* species has rich amount of chlorophyll a and chlorophyll b that contained 20 times more of β-carotene which contributes to anti-cancer properties. Siphonaxanthin is an oxidative metabolite of lutein that are found in *C. lentillifera* species. This compound plays a role in preventing cancer activity and inhibiting lipogenesis effects (Sharma *et al.*, 2017).

The secondary metabolite that contained the highest in *Caulerpa lentillifera* is caulerpin as shown in Figure 2.5 (Kase *et al.*, 2020). Caulerpin is a cyclooctatetraene ring that comprises of orange-red pigment that functions as growth promoter. The presents of caulerpin in dimer of indole-3-arcylic acid form acts like indole auxin

performs antimicrobial effect against food pathogen as well as inhibits the pro-inflammatory cytokines TNF-α and IL-6 production in a dose-dependent manner when tested on RAW 264.7 cells using MTT assay (Nagappan and Vairappan, 2013).

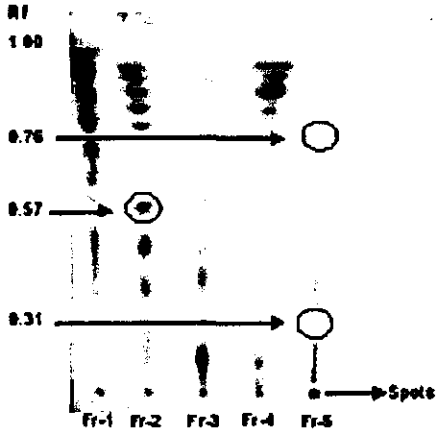


Figure 2.5 Chemical separation of compound contents in *Caulerpa* species using thin layer chromatography and the spot in red pigment is indicated as caulerpin (Kase *et al.*, 2020).

2.7 Apoptosis inducing activity

According to Varitta Tanawoot *et al.* (2021), *C. lentillifera* hexane extract (CLHE) has performed inhibitory effect against A172 human glioblastoma cells by inhibiting the cell proliferation and induced apoptosis of cancer cell. Using carboxyfluorescein succinimidyl ester (CFSE) assay, the drastic decrease in viability of A172 cells were analysed when CLHE concentration increased. Inhibition of cell growth was detected from the collection of CFSE fluorescence dye in daughter cell. Increased in fluorescent dye level indicated the increasing in level of cell division disruption. Besides that, CLHE promotes cell cycle arrest at G0/G1 phase of A172 cells, thus, inhibits cell proliferation. Furthermore, CLHE treatment towards A172 cells has also showed a rise in the apoptotic cell population. The loss of mitochondria membrane integrity that activates the mitochondrial-mediated pathway of apoptosis was analysed

using JC-1 fluorescence staining assays and the result as shown in Figure 2.6 illustrated that at concentration of 250 μg/ml, CLHE extract has promoted the loss of MMP. Additionally, the detection of intracellular ROS levels in A172 cells were analysed using dichlorodihydrofluorescein diacetate fluorescence labelling and the result showed that CLHE extract increases the ROS level in A172 cells. This concludes that the CLHE extract can hinder the growth and induce apoptosis of A172 glioblastoma cells (Varitta Tanawoot *et al.*, 2021).

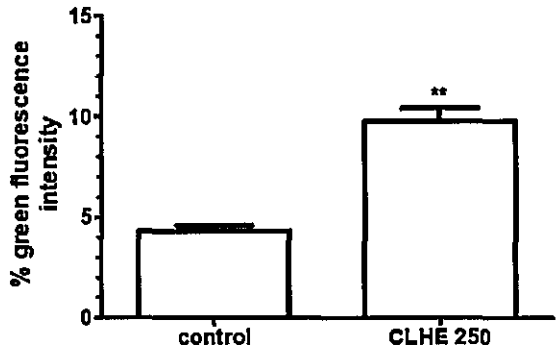


Figure 2.6 Bar chart graph of percentage of green fluorescence intensity with control and CLHE at concentration of 250 μg/ml for analysis of the loss of mitochondria membrane integrity in A172 cells (Varitta Tanawoot *et al.*, 2021).

According to Maeda *et al.* (2012), the oligosaccharides from *Caulerpa lentillifera* has showed decrease in viable human breast cancer MCF-7 cells in dose-dependent manner and this has indicated that the extract can influence the cell apoptosis. The oligosaccharide was prepared by mild acid hydrolysis of β-1,3-xylan in order to obtain in water-soluble samples as β-1,3-xylan is insoluble in water. The effect of β-1,3-xylooligosaccharides with average degree of polymerisation of 5 (XYL5) treatment was first analysed on MCF-7 cells by MTT assay and the results manifested that there were reduce in number of viable cells based on dose-dependent manner. Then, the

treatments with XYL5 and H_2O_2 was analysed on MCF-7 cells by staining with Hoechst 33258 dye (Dojindo) to visualise the condensation and fragmentation of chromatin. There was presence of condensed chromatin when observed in XF Imaging. The results based on analysis of XYL5 treatment using western blotting showed that the degradation of PARP by caspase has triggered the apoptosis of MCF-7 cells (Maeda *et al.*, 2012).

2.8 Anti-inflammatory effect

According to Yoojam *et al.* (2021), *Caulerpa lentillifera* extract (CLE) has showed effects on the inhibition of inflammatory responses in LPS-activated RAW264.7 macrophage cells by suppressing the inflammatory cytokines and mediators. CLE had shown anti-inflammatory effects in the LPS-stimulated RAW264.7 cells by decreasing the production of IL6 and TNF-α cytokines, as well as the related gene expression. In this study, phenolic compound called catechin was found in CLE had exhibited the suppression of inflammatory response of RAW624.7 cells by hindering the pro-inflammatory cytokine genes expression which were comprised of IL-1α, IL-1β, IL-6, and IL-12p35, as well as increasing the anti-inflammatory cytokine genes expression which comprised of IL-4 and IL-10 in 3T3-L1 adipocytes (Cheng *et al.*, 2019). Tannic acid presented in CLE also suppressed the IL-6 and IL-1β production and reduced the expression of TNF-α protein (Wu *et al.*, 2019). Besides that, CLE has showed results on the decreasing of NO, reduction of PGE2 levels and COX-2 enzyme due to reduce in expression of pro-inflammatory cytokines that are essential mediators for iNOS and COX-2 gene transcription. The chemicals that were produced from reactive oxygen and nitrogen species (RONS) and presence of LPS can influence in DNA damage. In this study, the presence of catechin in CLE extract caused reverse in

DNA damage whereby the level of prompt DNA strand to break decreases as shown in Figure 2.7 (Yoojam *et al.*, 2021).

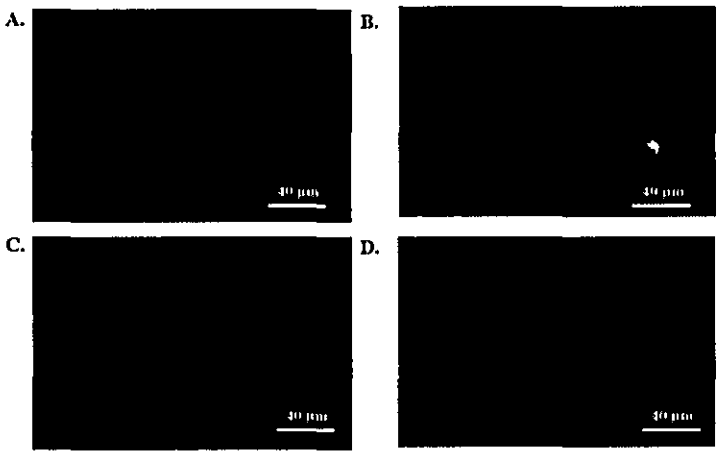


Figure 2.7 The CLE effect towards DNA damage in presence and absence of LPS-stimulated RAW264.7 macrophage cells using Hoechst 33342 fluorescent stain for DNA labeling and viewed under an inverted fluorescence microscope. A indicated as control, B indicated as changes in morphology of DNA with presence of LPS, and for C and D, both indicated as no changes in parameters for CLE-treated cells and CX-treated cells (Yoojam *et al.*, 2021).

2.9 Regulation of glucose metabolism

CLE has showed enhancement to insulin resistance and has regulated the glucose metabolism in myocyte of C57BL/KsJ-db/db mice through the PI3K/Akt signaling pathway (Sharma *et al.*, 2015). When the glucose is accumulated in the blood, the homeostasis of glucose will be distorted by the defects of PI3K/Akt signaling pathway in insulin-sensitive tissues. Therefore, in this study, the CLE has caused a decrease in blood glucose by increasing the glycogen content in the liver and reducing the inflammatory mediators that are linked to the insulin resistance in the plasma of mice. The increase in glycogen also has cause significant increase in muscle weight. This is because CLE has enhanced the insulin-signaling molecules activation which

includes IRS, P13K, Akt, and GLUT4 in the muscle of mice and in L6 myocytes but inhibits the AMPK phosphorylation as shown in Figure 2.8. Thus, the CLE in this study decreases production of inflammatory mediators like IL-6, TNF- α and FFA in db/db mice because of the decrease in HOMA-IR and insulin which improves the insulin resistance in target tissue (Sharma *et al.*, 2015).

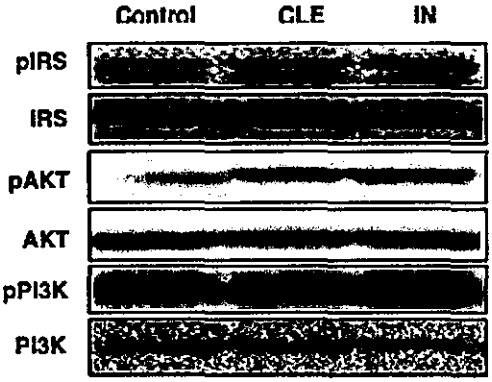


Figure 2.8 The result of CLE effect on the insulin-signaling proteins' expression in L6 myocytes (Sharma *et al.*, 2015).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemicals and reagents

The chemicals and reagents that were used in this study are listed in Table 3.1.

Table 3.1 List of general chemicals and reagents

Chemicals and reagents	Brand/Manufacturer
• Distilled water (ddH_2O)	Barnstead
• RPMI medium	ATCC
• Fetal Bovine Serum (FBS)	Gibco
• Methanol solvent	Fisher Chemical
• Antibiotics containing streptomycin and penicillin	Nacalai tesque
• EMEM complete media	ATCC
• Crystal violet	Sigma® Life Science
• DMSO	COCONANA
• Phosphate-buffered saline (PBS)	AMRESCO

3.1.2 Laboratory apparatus and equipments

The laboratory and equipments that were used in this study are listed in Table 3.2.

Table 3.2 List of general laboratory apparatus and equipments

Laboratory apparatus and equipments	Brand/Manufacturer
• Micropipette	Eppendorf Research
• Round bottomed flask	SCHOTT Duran
• Water bath	Memmert
• Whatman No. 1 filter paper	Cytiva
• 96-well plate	Corning Incorporated
• 6-well plate	Sorfa Life Science Research Co., Ltd.
• 15 mL Falcon tubes	TARSONS