SINGLE AGENT AND SYNERGISTIC ACTIVITY OF LATEX C-SERUM WITH LED RED LIGHT IN CANCER CELL LINES

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AGEN TUNGGAL DAN AKTIVITI SINERGISTIK SERUM C LATEKS DENGAN CAHAYA MERAH LED TERHADAP TITISAN SEL KANSER

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LIST OF ABBREVIATIONS

µg/ml	microgram per milliliter
2-NF	2- Nitrofluorene
4-NQO	4-Nitroquinoline-1-oxide
9-AA	9-Aminoacridine
A549	Human Lung Adenocarcinoma Cells
AML	AcuteMmyeloid Leukemia
ATCC	American Type Culture Collection
ATG	Autophagy-related Genes
BRMT	Bacterial Reverse Mutation Test
Cal 27	Human Tongue Squamous Cancer Cells
CBPI	Cytokinesis-Block Proliferation Index
CCO	Cytochrome C Oxydase
CE	Cloning Efficiency
CFU	Colony-Forming Unit
CRT	Conformal Radiation Therapy
CSC	Cancer Stem Cells
cytoB	Cytochalasin B
Da	Dalton
DBP	Dialysed B-serum Precipitate sub-fraction
DBS	Dialysed B-serum Supernatant sub-fraction
DCP	Dialysed C-serum Precipitate sub-fraction
DCS	Dialysed C-serum Supernatant sub-fraction
DMEM	Dulbecco Minimum Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid

DNase	Deoxyribonuclease
DSB	Double Strand Breaks
DTBTRG	Human Brain Glioblastoma Cells
EDTA	Ethylenediamine tetra-acetic acid
EFTEM	Energy Filtering Transmission Electron Microscopes
EGFR	Epidermal Growth Factor Receptor
EMR	Electromagnetic Radiation
EMS	E;ectromagnetic Spectrum
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
ER	Endoplasmic Reticulum
EW	Empty well
FBS	Feotal Bovine Serum
FDA	The US Food and Drug Administration
GEF	Global Evaluation Factor
HDFa	Human Adult Dermal Fibroblast
HEP G2	Human Liver Adenocarcinoma Cells
HER2	Human Epidermal Growth Factor Receptor 2
his	Histidine
HS27	Human Foreskin Cells
IMRT	Intensity-modulated Radiation Therapy
IORT	Intraoperative Radiation Therapy
IR	Infrared
kDa	kiloDalton
L5178Y	Mouse Lymphoma Suspension Cell Line
IC_{50}	Median Inhibitory Concentration

LED	Light Emitting Diode
MCF-10A	Human normal breast cells
MCF-7	Human Breast Cancer Cells
MDA-MB-231	Human Breast Cancer Cells
MDS	Myelodyspalsic syndrome
MF	Mutant Frequency
mg/ml	milligram per milliliter
MGA	Minimal Glucose Agar
MI	Mutagenic Index
MKN 74	Human Stomach Cancer Cells
MLA	Mouse Lymphoma Assay
mm	milliliter
MMS	Methyl-methane Sulfonate
MN	Micronucleus
MNvit	*In vitro* micronucleus
MRC-5	Human normal lung cells
MS	Mutant Selection
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
NADP	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBCC	Nevoid Basal Cell Carcinoma
NIR	Near-infrared
nm	nanometer
NMSC	Non-melanoma Skin Cancer
OD	Optical Density
OECD	The Organization for Economic Co-operation and Development
PBM	Photobiomodulation

PBS	Phosphate-buffered Saline
PCB	Polychlorinated biphenyls
PDT	Photodynamic therapy
PE	Plating Efficiency
PPO	Polyphenol Oxydase
PR	Progesterone Receptor
PS	Photosensitizers
RI	Replicative Index
RNase	Ribonuclease
ROS	Reactive Oxygen Species
RPD	Relative Population Doubling
RPMI	Rosewell Park Memorial Institute medium
RRIM	Rubber Research Institute Malaysia
RSG	Relative Suspension Growth
RTG	Relative Total Growth
SG	Suspension Growth
SSB	Single Strand Breaks
T-25	Tissue Culture Flask 25 cm^2
T-75	Tissue Culture Flask 75 cm^2
TFT	Triflourothymidine
TG	Test Guidelines
TGF- β	Transformation Growth Factor
TGFR	Transforming Growth Factor Receptor
THMG	Thymidine, Hydrocortisone, Meth
TK	Thymidine Kinase
TNBC	Triple-negative Breast Cancer
TW	Total Well

typ	Tryptophan
UV	UltraViolet
v/v	volume over volume
VBE	Vogel-bonner Medium E

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ABSTRAK

Sub-fraksi *Hevea brasiliensis* getah lateks dialisis c-serum supenatant (DCS) telah dilaporkan mempunyai aktiviti anti-proliferasi yang berpotensi terhadap sel kanser manusia khususnya kanser payudara (MDA-MB-231) dan sel adenokarsinoma hati manusia(HepG2) melalui tapak laluan kematian sel autoschizis. Penyelidikan lebih lanjut mengenai kesan anti-proliferatif rawatan DCS (kepekatan antara 0 hingga 100 µg / mL) menunjukkan bahawa IC_{50} DCS masing-masing adalah 8.81 µg / mL dan 22.12 µg / mL untuk DBTRG (glioblastoma otak manusia) dan MKN74 (adenokarsinoma tubular gastrik manusia). Walaupun DCS menunjukkan kespesifikan dalam titisan sel kanser berdasarkan asai daya hidup sel, kesan ketoksikan gen adalah penting memandangkan penggunaannya ke atas subjek manusia jika ia dijadikan ubat anti-kanser. Ketoksikan gen sub-fraksi DCS dikaji dengan menggunakan ujian mutasi bacteria berbalik (BRMT), ujian mutasi gen sel mamalia *in vitro* (MLA) dan ujian mikronukleus *in vitro* (MN*vit*) menurut garis panduan Organisasi Kerjasama Ekonomi dan Pembangunan (OECD). Kajian-kajian tersebut diperlukan untuk memastikan keselamatan pengguna jikalau dijadikan ubat kanser pada masa nanti. Sub-fraksi DCS disimpulkan sebagai tidak mutagenik mengikut kriteria garis panduan OECD. Meskipun diod pemanca cahaya (LED) telah disarankan untuk digunakan dalam perawatan kanser, kespesifikan panjang gelombang dan kespesifikan jenis sel kanser, yang mana LED berkesan adalah penting. Oleh itu, mengenal pasti kespesifikan ini sebagai langkah awal amat

diperlukan untuk penyelidikan yang lebih lanjut dalam rawatan kanser yang menggunakan LED. Memandangkan untuk menggantikan atau / dan menyokong ubat anti-kanser konvensional yang berasaskan bahan kimia yang telah dilaporkan membawa kesan-kesan sampingan jangka pendek dan panjang, empat diod pemancar cahaya merah (LED) pada panjang gelombang 615, 630, 660 dan 730 nm, telah digunakan untuk meneroka kesan anti-proliferatif sebagai salah satu calon kaedah fizikal rawatan kanser yang berpotensi pada masa yang akan datang. Dengan penyiaran harian berulang 30 minit di bawah LED 660 nm (merah hiper), sel DBTRG menunjukkan penurunan daya hidup sebanyak 50 % 24 jam selepas penyinaran terakhir. Manakala sel MCF 7 menunjukkan penurunan 40% dalam daya hidup sel 24 jam selepas penyinaran terakhir apabila mengalami penyinaran berulang 15 minit harian bagi tiga hari yang berturut-turut. Sel kanser payudara manusia yang dirawat dengan penyinaran LED 660 nm MDA-MB-231 telah menunjukkan morfologi senesen selepas 24 jam sejak penyinaran terakhir. Perubahan morfologi senesen sel yang diperhatikan di bawah mikroskop cahaya songsang, menunjukkan bentuk sel yang diratakan dan tidak sekata, ukuran sel yang diperbesarkan dengan peningkatan kandungan sitoplasma, lalu meningkatkan jarak antara sel. Mikrograf dari mikroskopik elektron transmisi (TEM) menunjukkan perbezaan yang jelas dari segi integriti nukleus dan peningkatan bilangan autofagosom (AG) yang dapat diperhatikan dalam sitoplasma sel MDA-MB-231 yang menerima rawatan penyinaran LED 660 nm. Ini menunjukkan bahawa penyinaran LED 660 nm boleh mencetuskan tapak laluan kematian sel senesen-autophagy pada sel MDA-MB-231. Seterusnya, pelbagai kombinasi sub-fraksi DCS bersama dengan penyinaran lampu merah LED juga diselidik untuk mengekplorasi potensi terapi fotodinamik (PDT). Rawatan gabungan menunjukkan hasil yang menonjol hanya pada sel MCF-7 di

mana daya hidup sel menurun hingga 30 % dengan penyinaran 30 minit setiap hari dengan panjang gelombang 730 nm (jauh merah) pada kepekatan DCS di bawah 1 µg / mL. Berdasarkan keputusan yang diperolehi, rawatan sub-fraksi DCS dan penyinaran lampu merah LED 660 nm masing - masing sangat dinantikan untuk pengembangan lebih lanjut, sementara kombinasi sub-fraksi DCS dengan penyinaran lampu merah LED 730 nm menunjukkan potensi dalam PDT sebagai satu terapi kanser yang berlainan.

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ABSTRACT

Hevea brasiliensis rubber latex dialysed C-serum supernatant (DCS) sub-fraction has been reported to possess potential anti-proliferation activities against specific human breast (MDA-MB-231) and liver (HepG2) cancer cell lines through autoschizis cell death pathway. Further investigations on DCS treatment (concentrations ranged between 0 to 100 µg/mL) on anti-proliferative effect in this study have shown that IC_{50} of DCS was at 8.81 µg/mL and 22.12 µg/mL for DBTRG (human brain glioblastoma) and MKN74 (human gastric tubular adenocarcinoma), respectively. Although DCS showed specificity in cancer cell lines based on cell viability assays, its genotoxicity effects are important in view of its employment in human subjects if it would be developed into an anti-cancer drug. Genotoxicity of DCS sub-fraction was studied using bacterial reverse mutational test (BRMT), *in vitro* mammalian cell gene mutation test and *in vitro* micronucleus test (MN*vit*) according to the Organisation for Economic Co-operation and Development (OECD) guidelines. These tests were needed in order to ensure the user safety if it is developed into anti-cancer drug in the future. DCS sub-fraction was concluded as non-mutagenic according to the criteria of the OECD guidelines. While LED has been suggested for use in cancer treatment, the wavelength specificity and the cancer cell type specificity, against which LED is applicable, are essential. Therefore, it is crucial to identify these as an initial step for further research in cancer treatment using LED. In view of substituting and/or supporting the conventional chemical

based anti-cancer drugs that have been reported to have short- and long-term side effects, four red light emitting diodes (LED) at wavelengths of 615, 630, 660 and 730 nm, were employed to explore the anti-proliferative effect as one of the potential physical cancer treatment method in the future. With 30 minutes of repeated daily exposure under LED 660 nm (hyper red), DBTRG cells showed a 50% reduction in viability 24 hours post- last exposure. Whereas MCF 7 cells showed a 40 % of reduction in cell viability 24 hours post- last exposure when subjected to 15 minutes of repeated daily exposure. LED 660 nm treated human breast cancer cells MDA-MB-231 has shown senescent-like morphology 24 hours post last exposure. Senescent-like morphological changes were observed under inverted light microscope, such as flatten and irregular shape of enlarged cell size with increase of cytoplasmic content, which increases the cell to cell distance. Micrographs from transmission electron microscopic (TEM) showed obvious differences in terms of the integrity of nucleus and the increased number of autophagosomes (AG) observed in cytoplasm of MDA-MB-231 cells that received irradiation treatment of LED 660 nm. Hence, the result showed that LED 660 nm irradiation induced senescent - autophagy cell death in MDA-MB-231 cells. Next, various combination of DCS sub-fraction along with the LED red lights irradiation was also being investigated for potential in photodynamic therapy (PDT). Combined treatments showed prominent result only in MCF-7 cells where cell viability was decreased to 30 % with 30 minutes daily exposure of specifically 730 nm (far red) wavelength at DCS concentration below 1 μg/mL. Based on all the results obtained, both DCS sub-fraction and LED red light 660 nm irradiation individually were with great anticipation for further development, while combination of DCS sub-fraction with LED red light 730 nm irradiation has showed the potential in photodynamic therapy as another potential cancer treatment.

CHAPTER 1

INTRODUCTION

According to estimates from the World Health Organization (WHO) in 2015, cancer is the first leading cause of death before age 70 years in 91 out of 172 countries (Freddie et al., 2018). Due to its prevalence, the discovery of novel anticancer drugs is of great importance. Cancer therapeutics currently has the lowest clinical trial success rate of all major diseases partly as a result of the paucity of successful anti-cancer drugs (Cagan & Meyer, 2017). The past century brought great advances in the field of plant and microbiology research, with the development of several compounds used in cancer treatment protocols. Many successful anticancer drugs that are in clinical use and have demonstrated significant efficacy are derived from natural products as plants, marine organisms, and microorganisms (Majolo et al., 2019). Latex, as a plant based natural products that is abundant in Malaysia, has been reported to exert high specificity anti-proliferative effect on human cancer cell lines with low concentration required. The active fraction reported being the dialysed latex C serum supernatant (DCS) sub-fraction, while susceptible human cancer cell lines included human breast cancer cells (MDA-MB-231) and human liver adenocarcinoma cell line (HepG2) (Lam, 2018). With the continuous effort to explore more potential susceptible human cancer cell lines with DCS sub-fraction treatment, a panel of human cancer and non-cancer cell lines will be tested.

Historically, cancer research has been largely geared towards identifying the promoter of cancer progression or known as cancer metastasis. Some of the genetic and environmental factors and biologic mechanisms that cause or contribute to cancer development, progression, and spread have been elucidated. However,

therapeutically targeting these cancer promoters can lead to unacceptable toxicity due to their roles in overall body homeostasis (Cagan and Meyer, 2017). Basic researchers have been taking more responsibility to learn and move from bench to bedside to allow better outcomes of drug discovery projects, potential for toxicity and difficulty of compliance with complicated dosing regimens. Genotoxicity tests are designed to detect drugs which may have potential to induce damage to genetic material of an organism directly or indirectly by various mechanism of action (Francois, 2014). These tests are useful in selecting potential drug candidates in early drug development as required by the regulatory.

Pharmaceutical companies too employed genotoxicity tests to eliminate potential genotoxic chemical series during pre-screening, in order to ensure that development candidates have insignificant genotoxic liability, and to shed light on the risk to human health after a positive finding in one or more assay. So far, no single test able to identify all types of DNA damage, while a battery of tests is required prior to clinical trials. In fact, an increase in both spontaneous chromosome breakage and sister chromatid exchange after chemotherapy that were reported by several authors (Gebhart et al., 1980; Aronson et al., 1982; Carbonell et al., 1996; Tekcan et al., 2012) has indicated genotoxicity test(s) are important for preclinical drugs. Furthermore, it was also reported that cancer patients treated with cytostatic drugs which carries mutagenic effects targeting the DNA of the cells developed second cancer/ malignancy after treatment. It was suggested in the same report that it is very important to have a simple method to measure and evaluate the persistence of the genetic damage induced by such treatments (de Mesa et al., 2002).

Development of second cancer/malignancy is one of the rare complications due to cancer therapy and often occurs at the later stage of treatment regardless on the same site or different site is one of the most serious (Fung et al., 2019). Despite it is difficult to extract out the literal cause of another type of cancer development on the same person, the risk of causing a different type of cancer that may related to the previous cancer therapy is important to be identify before any new development of treatment (Fung et al., 2019). For numerous new treatment methods for cancer that were developed, the enduring effects that may lead to second cancer are still unknown during the cancer treatment development process. The need for risk assessment of the treatment which possibly leads to developing rare, therapy-related second cancer after cancer treatment of the potential anti-cancer compounds is highly recommended (Morton et al., 2018). Anti-cancer effect exerted from latex C-serum has been an unexpected outcome when the *in vitro* studies conducted were made on an unprecedented scale based on the findings reported by Lam (2018). In the sequel after promising results were obtained, the risks of DCS sub-fraction needed to be accessed by conducting the genotoxicity tests. Thus, DCS sub-fraction is needed to subject to a series of standard investigation of potential genotoxicity/ mutagenic potential using bacterial reverse mutation test (also known as Ames test), *in vitro* cell gene mutation test (MLA) and *in vitro* micronucleus test.

Conventional radiotherapy is the use of high-energy beam such as X-ray and Gamma ray to kill cancer cells either by external beam radiation (outside the body) or placed internally as localized therapy (Morgan et al., 2018). Despite radiation therapy was one of the frequently used and effective methods in cancer treatment, it is also known to potentially causing cancer in the past. The likelihood of getting second cancer after received radiation therapy was found more common in

young patients compared to adult as they are likely to survive for a longer duration after anti-cancer therapy at 25 years after first cancer diagnosis. Mortality due to radiation-induced second malignancies has increased compared to that of first cancer diagnosis (Chinna et al., 2018). While LED was designed to emit low intensity infrared radiation, it is commonly used in various commercial electronic products (Okon and Biard, 2015) and then the biological significance of LED was also discovered (Opel et al., 2015). Several FDA approved LED devices especially red and infrared (IR) was rapidly rising in the market for biological applications such as wound healing, anti-wrinkle, photodynamic therapy with or without photosensitizers, anti-inflammatory were reported (Matsumoto et al., 2014; Opel et al., 2015).

In addition, LED red light was reported appears to be a promising treatment option for premalignant and malignant lesions, as well as photodynamic therapy with injection of sensitizer (Opel et al., 2015). The invention of light emitting diode has an advantage over the ordinary incandescent light bulbs as no filament that often burn out was used in LED design, which makes it more efficient, durable, versatile, cost-saving and last longer than the commonly use light bulb (Okon and Biard, 2015). With the advantages of the LED properties found, it is therefore with great interest to explore LED red light specifying at the wavelength range of 615 – 730 nm for anti-proliferating effect on human cancer cell lines. In recent years, scientists have extended approaches with a new cancer treatment method called photodynamic therapy (PDT), which would benefit from uniting two existing cancer treatment methods to achieve better therapeutic index in cancer patients (Juarranz et al., 2008; Kwiatkowski et al., 2018). Hence, due to the non-invasiveness of LED-based radiation advantage has enabled the possibility to further explore the possible photodynamic therapy (PDT) effect by combining the dialysed

C-serum supernatant (DCS) treatment along with the LED red light irradiation in this study. A flowchart summarizing the researches for this study is illustrated in Figure 1.1.

1.1 Objectives of research

The objectives of this study are:

i. To determine the anti-proliferative effects on various human cancer and non-cancer cell lines treated with latex C-serum (DCS) and LED red light, respectively,

ii. To determine the anti-proliferative effects on various human cancer and non-cancer cell lines treated with combination of both latex C-serum (DCS) and LED red light,

iii. To elucidate the type of cell death involved in LED red light treated human cancer origin cell line,

iv. To determine the genotoxicity effects of DCS sub-fraction treatment at both gene and chromosome level.

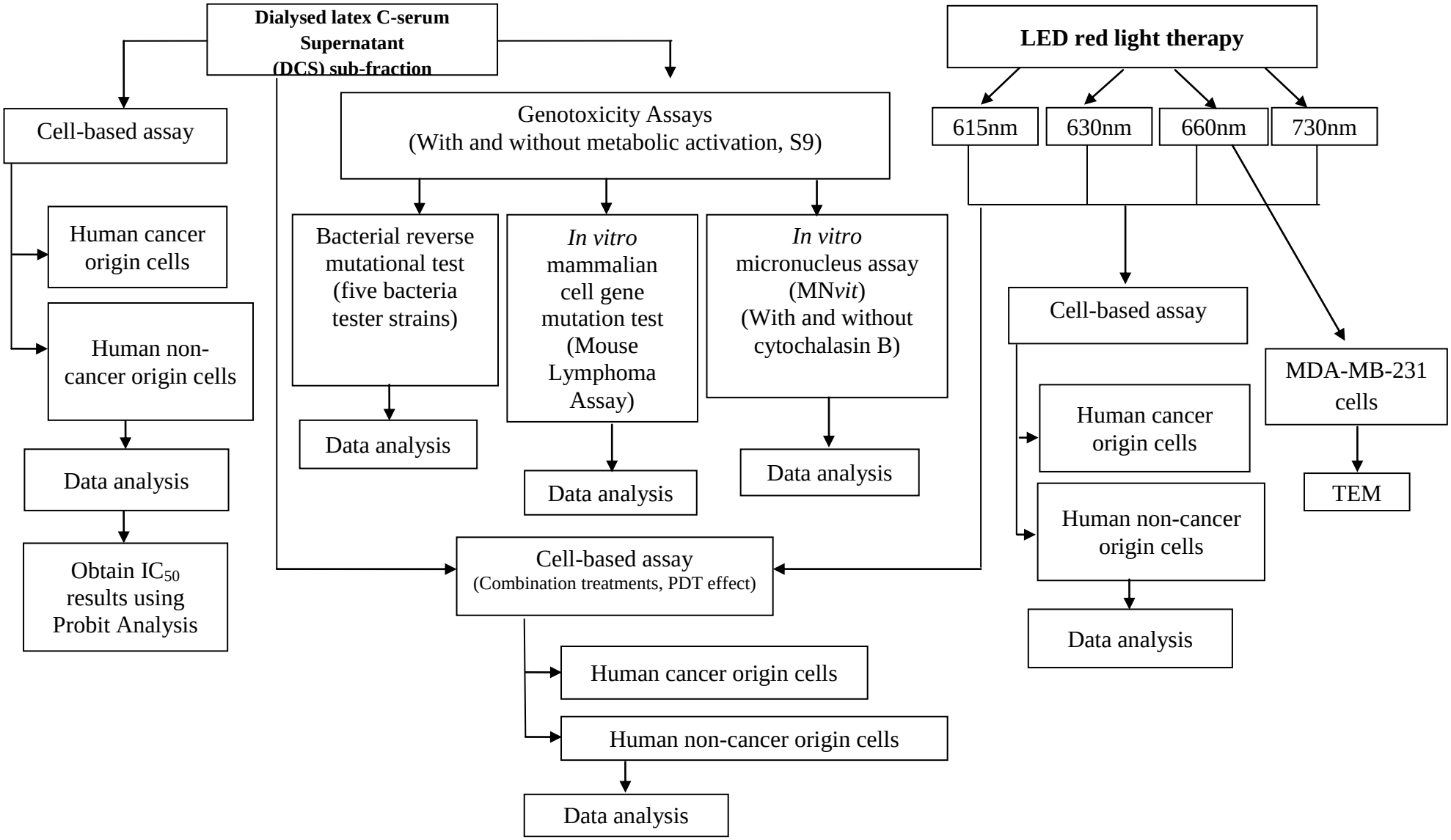

Figure 1.1 Flow chart of research

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer therapies

Conventional cancer treatment methods such as chemotherapy, surgery, and radiotherapy are among the three most common practiced methods (Morz et al., 2011). Each method has it own limitations and effectiveness and often needed the combination of therapies. With the technology advancement, more cancer treatment methods were being developed such as immunotherapy, targeted therapy, radiofrequency ablation and hormone therapy. These methods were developed in order to gain higher therapeutic index to cure cancer more specific and prefereably without side effects fot the patients (Peidaee, 2014).

2.1.1 Surgery

Surgery is one of the oldest, most effective and common method to remove solid cancerous tumour. Misconceptions about cancer that it is an incurable dieases with reported recurrence of cancerous tumours, and other complications along with primitive cancer surgery procedures lead to slow progress in the development of cancer treatment (Peidaee, 2014). Till date, technology advancement has created useful instruments such as fiber optic, laparoscopic and thorascopic technology to aid in surgery procedure to achieve low level of invasiveness in cancer removal surgery. There are some new methods such as cryosurgery or cryotherapy uses liquid nitrogen spray to cryopreserve cells that behaved abnormally, or lasers to replace scalpel to seize cancer growth tissue or even through cautery excision of cancer. Despite these

new methods for cancer surgery reduces side effects but some issues were still remained after the surgery, for example damaging or deforming organs or part of the body, infections from surgical procedure, blood loss, and other illness such as pneumonia (Peidaee, 2014).

2.1.2 Chemotherapy

The term “chemotherapy” was coined by the German scientist Paul Ehrlich, who has a particular interest in alkylating agents, and later described the chemical treatment of disease (Manuel et al., 2011). Chemotherapy as one of the widely used conventional methods of cancer treatment often utilizes western medicine, of which these chemotherapy drugs can be categorized in a few categories according to their mechanism of actions such as alkylating - agents, antimetabolites, antitumour antibiotics, inhibitors for topoisomerase, mitotic, protease and tyrosine kinase, hormonal drugs, and plant alkaloids (Le et al., 2017).

Generally, chemotherapy drugs work on attacking the genome at different stages of the cell cycle as they grow or divide. For instance, alkylating agents bind to DNA in the nucleus to prevent cell division; antimetabolites agent were used to replace the normal building blocks needed for RNA and DNA replication with an in active substances, antitumour antibiotics would interfere with DNA structure/ enzyme and prevent DNA from uncoiling or replication, and the others such as topoisomerase inhibitors, mitotic inhibitors and cortisteroids, which are used for cancer treatment (Huang et al., 2017).

Based on the mechanisms of action of the chemotherapy drugs, it can be deducted that chemotherapy could generally work in both normal and abnormal cells by interrupting their chemical processes in cell cycle (Huang et al., 2017). These chemotherapy drugs would also interfere with those non-targeted normal cells that are consistently growing and dividing, such as skin, hair follicle, lining of digestive system, and bone marrow which continuously producing new blood cells. Hence, a variety of mild to severe, short-term and long-term side effects from chemotherapy on cancer patients are inevitable (Ramirez et al., 2009**;** Peidaee, 2014). Some of the side effects were summarized in Table 2.1.

Table 2.1 Side effects from chemotherapy on cancer patients (Ramirez et al., 2009**;** Peidaee, 2014)**.**

Early side effects		**Late side effects**
1. Allergic reaction 2. Anemia 3. Appetite loss 4. Bleeding and Bruising (thrombocytopedia) 5. Uncontrollable bowel system 6. Edema (swelling) 7. Flu-like symptoms 8. Pain in joint/ bone	9. Ulcers at mouth/ throat area 10. Nausea/vomiting, Peripheral neuropathy (nerve problems) 11. Skin and nail changes, 12. Insonia 13. Uninary-bladder problems	1. Hair loss 2. Fatigue 3. Fertility issues 4. Organ-related inflammatory/ immunotherapy 5. Sexual health problems (early menopause symptoms) 6. Seizures 7. Rare secondary malignancy

Combination chemotherapy was then discovered with better therapeutic effect towards certain fast-growing cancer such as leukemia and lymphoma. With appropriate combination of chemotherapy with radiotherapy or surgery, it has been proven successfully to increase the cancer curing rate. Using chemotherapy to shrink the tumour before surgery is called neo-adjuvant chemotheraphy. On the other hand, chemotheraphy given post-operative to eliminate undetected cancer cells and to

reduce the chances of reccurrance is known as adjuvant chemotherapy (Peidaee, 2014). Pre-treatment with chemotherapy causes cancer cells to become sensitive to the following radiation therapy is called chemoradiotheraphy (Huang et al., 2017).

2.1.3 Radiotherapy

Radiation oncology being a discipline in which various health and science professionals from numerous disciplines works together. Generally, radiotherapy uses higher energy radiation such as X-ray or gamma rays (different forms of ionizing radiation) targeted to destroy cancer cells by inhibiting their growth and division while some are targeted to shrink localised tumors. These radiations would cause damage of the DNA of the radiated cancer cells which immediately lead to cell death or weaken them to the degree that unable to proliferate (Peidaee, 2014).

"X-ray" was the first kind of ray that was utilised as a thareapeutic radiation for cancer treatment (radiotherapy) in the late 19^{th} century, discovered by a german physicist, named Wilhelm Conrad Roentgen. Later it was discovered that radiation could cause second cancer/ malignancy development when many of the radiologists developed leukemia as a result of them used their arm skin to obtain proper daily dose of radiation for patients. There is no surprise that many of them developed leukemia later on (Peidaee, 2014). The existence of high-energy radiation may overpower the ability of damaged cells to repair, resulting in a further risk of second cancer. Normal cells surrounding the area facing the risk to be affected by the radiation and this has significantly restricted the treatment results (Damyanov et al., 2018). Maximum radiation dose by which death of localized cancer cells has low acute and late morbidity relative to the average radiation dose of non-cancer cells

may contribute to the therapeutic ratio for cancer treatment in radiotherapy (Beasley et al., 2005). With advancements in technology, more complex and precise radiation machines were developed. Nowadays, advanced radiotherapy machines expose cancer affected areas with a very high precision beam, which lead to reduction of side-effects of radiatherapy on normal surrounding tissues (Peidaee, 2014).

Among the two types of radiations used to threa cancer, photons radiation (X- rays and gamma rays) are more widely used compared to particle radiations (electron, proton and neutron beams) (Baskar et al., 2012). Photon beams carry a low radiation charge and have a much lower mass. X-rays and gamma rays are regularly used photons in radiation therapy to treat various cancers. X- rays and gamma rays are sparsely ionizing radiations, considered low LET (linear energy transfer) electromagnetic rays and further composed of massless particles of energy are called photons. X-rays are generated by a device that excite electrons (for example cathode ray tubes and linear accelerators), while gamma rays initiate from the decay of radioactive substances (for example cobalt-60, radium and cesium) (Baskar et al., 2012).

The use of radiotheraphy cancer treatment was not limited by using radiation alone, but it was also incorporated with surgery and chemotheraphy a combination treatment to achieve a more effective healing response. Along with surgery and chemotherapy, radiation therapy or radiotherapy remains an important modality used in cancer treatment being a highly cost-effective single modality treatment accounting about only 5 % of the total cost of cancer care (Ringborg et al., 2003; Baskar et al., 2012). Furthermore, approximately 50 % of all cancer patients received radiation therapy during their course of illness (Begg et al., 2011) with an

estimation that radiation therapy contributes to around 40 % towards curative treatment (Barnett et al., 2009).

Radiothrapy works through in various ways to remove the cancer cells: direct radiation and indirect radiation. Both ways were able to cause changes in DNA such as mutation or rearrangement of chromosome during its DNA repair phase (Baskar et al., 2012; Maier et al., 2016). Direct radiation on DNA molecule does not need any intermediate medium. Radiation energy will be deposited directly on the DNA molecule to create DNA lesions through induction of chromosome aberration and gene mutation. Indirect radiation occurs when the radiation interact with dilute aqueous solution (particularly with water component of the cells) lead to hydrolysis reaction (ionization), rapidly producing oxidizing and reducing free hydroxyl radicals (OH^-) (Han and Yu, 2009; Baskar et al., 2012). These free radicals are highly reactive due to an extra unpaired electron and able to diffuse far enough to interact with DNA molecules to cause a molecular structural damage (Desouky et al., 2015).

Radiation can be delieverd to the location of the cancer either by externally or internally. External beam radiation is delivered from outside the body by aiming high-energy rays (photons, protons or particle radiation) to the location of the tumor. This is the most common approach in the clinical setting. Internal radiation or brachytherapy is delivered from inside the body by radioactive sources, sealed in catheters or seeds directly into the tumor site. This is used particularly in the routine treatment of gynecological and prostate malignancies as well as in situations where retreatment is indicated, based on its short range effects (Baskar et al., 2012).

2.1.4 Photodynamic Therapy (PDT)

In addition to the conventional cancer treatments such as surgery, chemotherapy and radiotherapy, photodynamic therapy (PDT) arose as an interventional cancer treatment that combined two conventional methods which yield more promising results in cancer treatment. PDT consists of three essential components: photosensitizer (PS), a particular harmless non-thermal visible light to produce reactive oxygen species (ROS) to destroy cancer cells (Morz et al., 2011). None of these is individually toxic, but together they initiate a photochemical reaction that culminates in the generation of a highly reactive product termed singlet oxygen ($^1O_2$).

There are two forms of reactions may be involved in PDT. Type I (electron transfer) involves the transfer of either hydrogen atom or an electron between the excited PS and the substrates, leading to the generation of free radicals. These radicals then react with oxygen, resulting in the production of ROS such as superoxide and hydroxyl radicals. Type II (energy transfer) involves the energy transfer between the excited PS and the molecular oxygen in the ground state ($^3O_2$), resulting in the formation of highly reactive state of oxygen as singlet oxygen ($^1O_2$) (Figure 2.1). The resulting ROS can cause irreversible damage to target tissues/cells. Singlet oxygen can rapidly cause significant toxicity leading to cell death via apoptosis or necrosis (Morz et al., 2011). Photosensitizer (PS) is typically injected systemically (from low to moderate dose) and makes its aggregation at the desired site for malignant cells uptake, then light is transmitted to create ROS molecules cytotoxicity and serious vascular damage that impairs blood supply to the specific area being treated (Engbreht et al., 1999). PDT was a promising therapeutic method

for malignant and pre-malignant tumors (Pass, 1993) and other diseases (Oleinik and Evans, 1998).

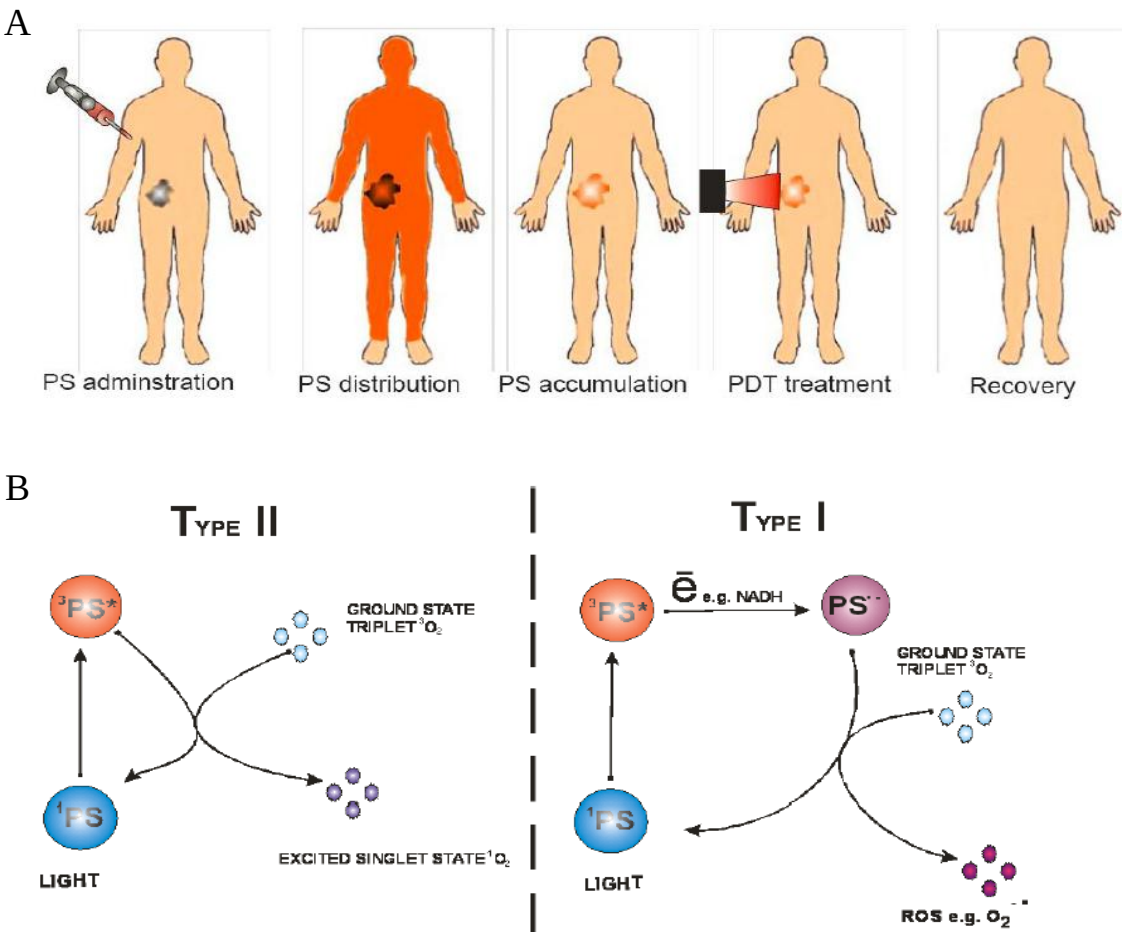

Figure 2.1 **Concept of photodynamic therapy (PDT) effect**. (A) Illustration of the mechanism of PDT; (B) Types of reaction (Type I and Type II) involved in PDT. Figures adapted from Morz et al. (2011).

The biological effects of PDT are still limited to a few limitations such as the exposed area of light, the photosensitizer's compatibility with the activation of the specific wavelength and the light intensity to assess the degree of penetration into the target site (Wilson, 2002). Furthermore, PDT was reported leads to activation of tumor directed and systemic immune responses. PDT effects occurs only if the photosensitized cells are irradiated, sufficient intracellular concentration of PS agent of the cells, and formation of reactive oxygen species (ROS) begin only when irradiation takes place (Castano et al., 2006). On the other hand, manipulating the temperature of irradiation could possibly alter the immediate consequences (Pryde et al., 2000).

Phtodynamic therapy (PDT) is often designed based on the PS agents chemical properties to localize the malignant cells and tissues, and the optimum absorbance light wavelength of the PS, to convert oxygen molecules in cells into reactive oxygen species (ROS). These cellular molecules would then take effect by typically oxidize surrounding lipids and proteins (Kessel and Oleinick, 2009). The effect can result in severe oxidative stress in the cells and is very specific for tumor eradication (Dougherty et al., 1998). PDT can cause different types of cell death such as apoptosis, necrosis and autophagy depending on the types of photosensitizers used, location of light irradiation, cells and photodynamic dosage (Liang et al., 2015). If apoptosis is not activated, autophagy as the type II programme cell death would then activated in the mode of cell death or predominant cell death after PDT (Kessel et al., 2006; Xue et al., 2007; Buytaert et al., 2008).

Several photosensitizing agents have been documented to be licensed for clinical use since 2009, and there are many others in clinical andpre-clinical studies.

Some agents were porphyrins compounds, derivatives such as benzoporphyrins or pheophorbides, or associated macrocycle structure forms such as phthalacyanins. They have been reported to require additional high level of purity from commercial sources. Nevertheless, some PS agents prepared for clinical trials or those obtained directly from synthetic chemist are usually more refined (Kessel and Oleinick, 2009).

An ideal PS agent should be a single pure compound to allow quality control analysis with low manufacturing costs and good stability in storage. It should have a high absorption peak between 600 and 800 nanometers (nm) (red to deep red), because absorption of photons with wavelengths longer than 800 nm does not provide enough energy to excite oxygen to its singlet state and to form a substantial yield of reactive oxygen species (Agostinis et al., 2011). Photosensitizer (PS) agents with the structure of the hydrophobic ring system, including non-porphyrin photosensitizers like hypericin is critical for controlling PS concentration as well as cell entry through the hydrophobic intracellular sites and incubation time. In general, PS agents are dissolved for the delivery of PS to cells in biocompatible organic solvent. It is important to test that the vehicle is non- toxic to cells, and the organic solvent level should always remain below 0.1 % of the medium volume (Kessel and Oleinick, 2009).

LED or laser appeared as the two main light sources used in PDT (Dougherty et al., 1998; Wilson, 2002). Due to high energy of radiation used in laser light, it can be directed through fiber optic cables to the tumor site. Argon laser was used in PDT which can penetrate for approximately an inch through the tissues without damaging them, which mostly used for internal organ cancers (Peidaee, 2014). In dermatology, PDT is commonly used and it was approved for the treatment

of oncological conditions such as actinic keratosis, Bowen disease and superficial basal cell carcinoma. In the last two decades however, PDT has also been used for the treatment of several nonneoplastic dermatological diseases (Megna et al., 2016). Due to the minimal invasiveness advantage of PDT, a number of research and clinical studies on the potential role of photosensitizing agents are still conducting underway. For instance, various light wavelengths that have been used in PDT clinical studies for skin cancer treatment, included blue light (approximately 475 nm) with 20 % aminolevunic acid (ALA) for treatment of nevoid basal cell carcinoma (NBCC) (Kwasniak et al., 2010). Another study has reported to use five wavelengths (460, 525, 630, 730 and 850 nm) ranging from visible light to near-infrared light range with difference concentration of oxygen of the hemoglobin on non-melanoma skin cancer cell lines (Saager et al., 2013). PDT was also being reported to have a potential by further optimize parameters in the execution of PDT method for its various intensities and duration, which in turn potentially to improve the treatment output (Saager et al., 2013).

In a nutshell, PDT is still considered to be one of the new and promising cancer therapy strategies. Its full potential has yet to be discovered, and its range of applications alone or in combination with other approved or experimental therapeutic approaches is definitely not exhausted. The advantages of PDT compared with conventional cancer therapy methods such as surgery, chemotherapy, or radiotherapy are reduced long-term morbidity (Agostinis et al., 2011). Furthermore, it is with the fact that PDT does not compromise future treatment options for patients with residual or recurrent disease. Due to a lack of natural mechanisms of singlet oxygen radical ($^1O_2$) elimination and a unique mechanism of cytotoxicity, mutations that confer resistance to radiotherapy or chemotherapy do not compromise anti-tumour

efficacy. Many conventional anti-tumor treatments carry a biological risk of inducing immunosuppression. PDT-induced immunogenic cell death associated with induction of a potent local inflammatory reaction offers the possibility to flourish into a therapeutic procedure with excellent local anti-tumor activity as well as the capability of boosting the immune response for effective destruction of metastases (Agostinis et al., 2011).

2.2 *Hevea brasiliensis* latex

2.2.1 Background

Hevea brasiliensis, also known as rubber tree, was grown for timber production which mainly used in manufacture of furniture, and being the main source for natural rubber (NR). This particular species of rubber tree possesses rapid growth rate and with high quality latex production. Latex of the *Hevea brasiliensis* was being exploited extensively many decades ago. It is the milky white fluid comprises highly specialized articulated cells, which also known as laticifers (Moir, 1959, d'Auzac and Jacob, 1989). In addition to water content, polymeric hydrocarbon cis-polyisopryene tended to be the next major component in latex while proteins make up around 1-2 percent of the latex's fresh weight (de Fay¨ and Jacob, 1989).

Upon ultracentrifugation, freshly collected latex divided into three main fractions, where the uppermost layer known as rubber particle phase, a transparent clear aqueous layer known as C-serum and a lower bottom fraction (pellet) known as B-serum (Figure 2.2). Rubber phase comprises rubber particles and two insoluble proteins, while the C-serum and B-serum consisted of mainly water-soluble proteins.

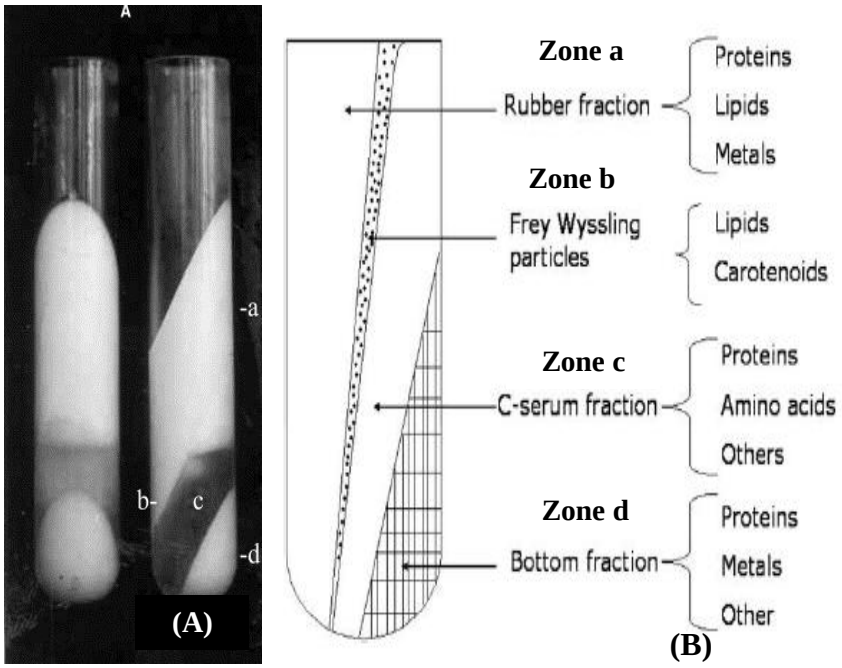

Figure 2.2 **Separation of *Hevea brasiliensis* latex upon ultracentrifugation and protein contents distribution. (A)** Front and side perspective view of the centrifuged tube. Zone a - rubber particle phase, Zone b as the presence of Frey wyssling particles, Zone c - C-serum fraction and Zone d- bottom fraction (pellet). Figure adapted from Yeang et al., (2002); **(B)** Distribution of protein contents in separated fractions Figure adapted from Ferreira et al., (2009).

Latex B-serum can be obtained by repeated freeze-thaw process of the solid bottom fraction of centrifuged (Figure 2.3).

B-serum comprises mainly lutoids and some other minor organelles such as ribosomes and endoplasmic reticulum sedimented by centrifugation. Lutoids are the lysosome-like organelles that make up 10-20 % of w/w of latex (Thepchalerm et al., 2015). Heveins, a lectin-like protein which also known as latex allergens, were those major components in the B-serum (Thepchalerm et al., 2015). Many of the heveins called Hev b 1, 2, 3, 4, 6, 7, and 10 are also plant protection or stress-related proteins (Sussman et al., 2002), while other latex allergens such as Hev b 5, 7, 8, 9, and 10 have varying degrees of homology with recognized food, pollen, and mold allergens (Yagami, 2002; Yeang et al., 2002). It was reported that latex B serum has shown anti-proliferative effects on human breast epithelial cells, but lack of specificity in killing human B lymphocyte cell line (BDCM) (Lam, 2018). The anti-proliferation agent(s) in latex B serum was proved heat sensitive as the anti-proliferative activity was diminished in pre-heated B serum fractions (Lee et al., 2012).

Using SnakeSkinTM tubing with molecular weight cut off at three kDa against distilled water for 48 hours at about 5 °C, latex B-serums and C-serum were then subjected to dialysis. Following of that, both dialysed latex serum were centrifuged to prepare the two separate supernatants known as dialysed B-serum supernatant (DBS) and dialysed C-serum supernatant (DCS) fractions respectively (Sunderasan et al., 2015).

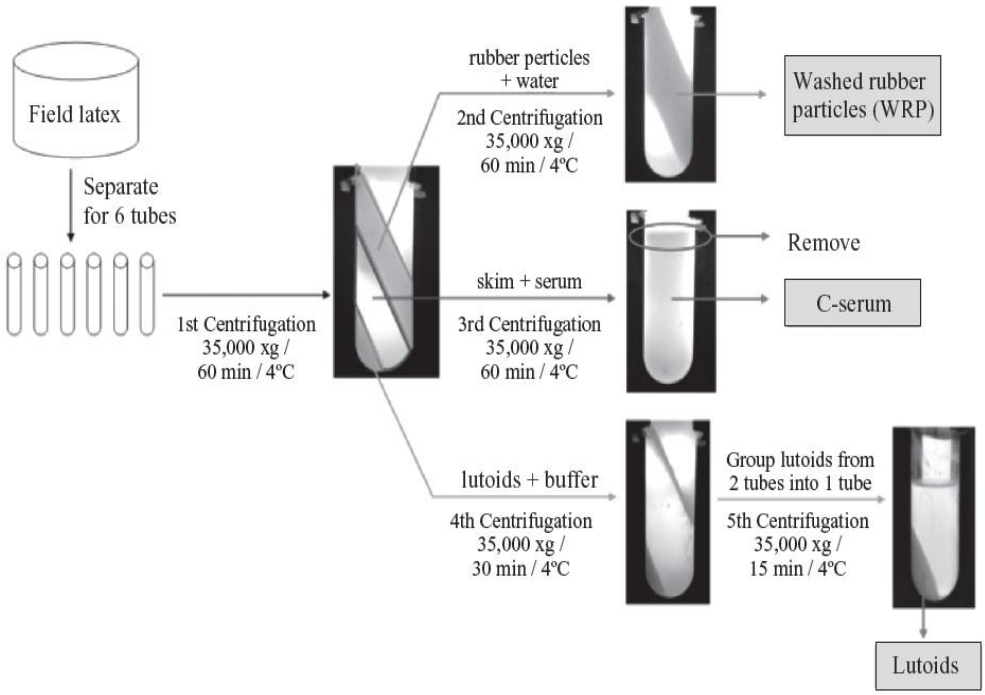

Figure 2.3 **Methods used to fractionise the latex collected into three individual fractions.** Field latex collected was subjected to first centrifugation into three separate fractions. Each fraction was removed carefully after centrifugation into different tubes. Bottom fraction was pulled into a tube and subjected to centrifuge again with buffer. Figure adapted from Thepchalerm et al. (2015).

2.2.2 Anti-fungal effects

Plant diseases caused by fungal infection often lead to considerable damage to the economically important crops worldwide. Due to the absence of an immune system such as in humans, plants have developed an amazing array of structural, chemical and protein-based defenses specifically designed to defend against invasive harm before any major damage.

Hevea brasiliensis latex B-serum and C-serum were found to have positive results in anti-fungal activities towards *Candida albicans* and *Apergillus niger*, respectively (Daruliza et al., 2011 a, b). The presence of *Hevea brasiliensis* allergens in latex B-serum, particularly Hev b2 has been reported to have β-1,3-glucanase activity observed contained in the lower fraction of *Hevea* latex where two acidic isozymes and three basic isozymes were also being detected (Sussmann et al., 2002). A recent study has been conducted to investigate the involvement of polyphenol and polyphenol oxidase (PPO) in latex C-serum was responsible to the anti-fungal effect. Type of polyphenols that were successfully identified included gallic acid, quercetin and napthoic acid (Mubarak et al., 2018). It was suggested that these species-specific anti-fungal effects from both latex B- and C- serums can be used as a value-added property to serum-specific anti-proliferative properties of the current cancer cell line to fight fungal infection in patients with immune-compromised cancer (Daruliza et al., 2011a).

2.2.3 Cytotoxic effects on cancer cells

Latex from *Hevea brasiliensis* has been studied extensively for its antimicrobial and antifungal properties (Daruliza et al., 2011a). However, a group of scientists has made a valiant attempt to explore the medicinal value of the latex although it is well known for its allergenic nature. The finding has shown promising results were obtained from anticancer studies that were conducted using latex serums. Latex B-serum sub-fraction was first reported to elicit anticancer effect on a human cervical cancer cells, HeLa cells (Ong et al., 2009).

Further studies were conducted using human breast cancer cell lines (MDA-MB-231 and MCF-7) as well as human liver cancer cells (HepG2). The result has showed HepG2 and MDA-MB-231 cells were more susceptible towards latex B-serum DBP (dialysed B-serum precipitate) sub-fraction than MCF-7 cells (Yang et al., 2012). In line with these results, latex C-serum sub-fractions were also being tested concurrently. It is concluded that DCS (dialysed C serum supernatant) sub-fraction is the most potent among the tested cancer cell lines, while the non-cancer origin cell line HS27 was not susceptible (Lam, 2018). According to Lam (2018), the Dimethylthiazol-diphenyl tetrazolium bromide (MTT) viability results showed that the anti-proliferative agent in DCS treatment with concentrations 0 to 1μg/mL, showed cell line specific to cancer origin cells with cell susceptibility towards DCS in a descending order: HepG2 > MDA-MB-231 > HeLa > CaOV-3 > MCF-7 > CAL27 > HT29 > HTC116 > HS27. However, the anti-proliferative agents in C serum were found sensitive to heat treatment when heat-treated latex C serum showed no significant decreased of cell viability of HS27, MCF-7 and MDA-MB-231 (Lam, 2018).

2.2.4 Latex C-serum

In the laticiferous cytoplasm, latex C-serum was found metabolically active with all the glycolytic and cytosolic enzymes for isoprenoid pathway. Protein content in latex C-serum has covered up to 60 % of the latex protein (approximately 200 polypeptides) which makes it rich in organic compounds. Malic acid and citric acid comprised 90 % of the organic acid in latex C-serum. Gluthanione, cysteine and ascorbic acid as the reducing agents, and several major groups of amino acids such as alanine, aspartic acid, glutamic acid and its amide are also found present in C-serum. (D'Auzac and Jacob, 1989; Wititsuwannakul and Wititsuwannakul, 2001). Among these proteins, there were nineteen anionic proteins and five cationic proteins, alpha-globulin, calmodulin (calcium-modulated protein) and *Hevea* latex lectin-binding protein (HLL). Alpha-globulin which has high binding affinity for adsorption into rubber particles has made it important in latex and rubber particles' colloidal stability (Archer et al., 1969). Calmodulin appeared as a heat-stable calcium-binding protein which activates the hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase enzymes found in B-serum. HLL protein was reported for its role in anti-coagulating latex to maintain colloidal stability (Wititsuwannakul et al., 2008). Bealing (1981) reported that 75-95 % of the total carbohydrates content in latex C-serum is made up of sucrose, glucose, fructose, raffinose, and quebrachitol (chemically known as 1-methyl inositol).

Recently, protein profiling on the most abundant *Hevea* latex serum proteins were conducted by Norazreen and Mohd Fazri (2018). The protein idenfication analysis with the protein score has successfully identified several protein clusters in the latex, which were small rubber particles protein (SRPP), hevamine, elicitor-