CYTOTOXIC EFFECT, ANTI-PROLIFERATIVE AND CELL DEATH MECHANISM OF LATEX C-SERUM AND SUB-FRACTIONS USING CELL-BASED ASSAY

by

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dATP	2'-deoxyadenosine triphosphate
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
BrdU	5-bromo-2'-deoxyuridine
7-AAD	7-Aminoactinomycin D
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine-5'-triphosphate
ATCC	American Type Culture Collection
APS	Ammonium Persulphate Solution
AIF	Apoptosis-inducing factor
BAK	BCL-2-antagonist/killer-1
BAX	BCL-2-associated X protein
BID	BH3 interacting-domain death agonist
BBP	Boiled B-serum Precipitate
BBS	Boiled B-serum Supernatant
BCP	Boiled C-serum Precipitate
BCS	Boiled C-serum Supernatant
CO_2	Carbon Dioxide
CLL	Chronic lymphocytic leukaemia
CML	Chronic myelogenous leukemia
Cdk	Cyclin-dependent kinase
Da	Dalton
DNase	Deoxyribonuclease
DNA	Deoxyribonucleic Acid

DBP	Dialysed B-serum Precipitate
DBS	Dialysed B-serum Supernatant
DCP	Dialysed C-serum Precipitate
DCS	Dialysed C-serum Supernatant
DMSO	Dimethyl sulfoxide
DIABLO	Direct IAP-binding protein with low pI
DMEM	Dulbecco Minimum Eagle Medium
endoG	Endouclease G
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
EDTA	Ethylenediamine tetraacetic acid
FLIP	FLICE inhibitory protein
FBS	Foetal Bovine Serum
g	Gravitational force
HTRA2	High temperature requirement protein A2
H_2O_2	Hydrogen peroxide
IC_{80}	Inhibition Concentration 80
IAP	Inhibitor of apoptosis protein
pI	Isoelectric point
kDa	KiloDalton
L	Liter
IC_{50}	Median Inhibitory Concentration
µg/ml	Microgram per milliliter
mg/ml	Milligram per milliliter
mM	MilliMolar

MAC	Mitochondrial apoptosis-induced channel
MMP	Mitochondrial membrane potential
MDM2	Mouse double minute 2 homolog
ng/ml	Nanogram per milliliter
nm	Nanometer
NADP	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NHL	Non-Hodgkin lymphoma
OD	Optical Density
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PCD	Programmed cell death
PI	Propidium iodide
ROS	Reactive oxygen species
RNase	Ribonuclease
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RRIM	Rubber Research Institute Malaysia
SEM	Scanning electron microscopy
SDS-PAGE	SDS Polyamide Agarose Gel Electrophoresis
SMAC	Second mitochondria-derived activator of caspases
SDS	Sodium Dodecyl Sulphate Solution
SRP	Staurosporine
TEMED	Tetramethylethylenediamine
T-25	Tissue Culture Flask 25 cm^2

T-75	Tissue Culture Flask 75 cm^2
TRADD	TNFR type 1-associated death domain protein
TEM	Transmission electron microscopy
TNF	Tumour necrosis factor
TNFα	Tumour necrosis factor alpha
TNFβ	Tumour necrosis factor beta
TNFR	Tumour necrosis factor receptor
TRAIL	Tumour necrosis related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
VC	Vitamin C
VK_3	Vitamin K
v/v	Volume over volume
w/v	Weight over volume
WB	Whole B-serum fraction
WC	Whole C-serum fraction

LIST OF APPENDICES

KESAN TOKSIK, ANTI-PROLIFERATIF DAN MEKANISME KEMATIAN SEL OLEH LATEKS SERUM-C DAN SUB-FRAKSINYA MENGGUNAKAN PENGASAIAN BERPANDU SEL

ABSTRAK

Lateks daripada *Hevea brasiliensis*, pokok getah asli, tidak pernah dilaporkan sebagai ubat-ubatan tradisional walaupun kepentingan ekonominya dipandang tinggi. Lateks memainkan peranan biologi dalam sistem pertahanan tumbuhan terhadap mikroorganisma dan serangga. Oleh kerana maklumat yang terhad dalam kajian kesan biologi pada sistem mamalia, penilaian daya tahan sel dengan menggunakan MTT, penilaian PCR masa nyata (real-time PCR), penilaian kitar sel (cell cycle) dan penilaian profil protein telah digunakan untuk menilai kesan anti-kanser dan laluan kematian sel yang disebabkan oleh serum-C (WC) dan pecah-pecahannya terhadap warisan sel kanser asal. Pecahan serum yang selanjutnya yang digunakan dalam kajian ini telah disediakan melalui rawatan dialisis dan pra-haba. Ini menghasilkan empat pecahan daripada lateks serum-C: DCP iaitu mendakan serum-C hasil daripada dialisis; DCS iaitu supernatan serum-C hasil daripada dialisis; BCP iaitu mendakan serum-C hasil daripada pra-dipanaskan dan BCS iaitu supernatan serum-C hasil daripada pra-dipanaskan. Penilaian MTT mendedahkan bahawa rawatan DCS dan DCP adalah khusus dan menyasarkan sel-sel HepG2 dengan nilai IC_{50} = 0.17 µg/mL ± 0.02 dan 1.81 µg/mL ± 0.08 masing-masing. Sel-sel MDA-MB231 didapati mudah terdedah kepada rawatan DCS dan DCP, dengan nilai-nilai IC_{50} yang terletak di 2.00 µg/mL ± 0.04 dan 5.49 µg/mL ± 0.01 masing-masing. DCS telah terbukti

lebih berkesan berbanding dengan DCP dalam kesan anti-proliferasi tertentu terhadap garisan sel kanser asal yang diuji. Aktiviti anti-proliferasi daripada BCP dan BCS yang pra-dipanaskan, dikurangkan dengan ketara dan keputusannya mencadangkan bahawa komponen aktif tidak dapat menahan haba. Analisa pengekspresian gen menggunakan PCR masa nyata, profil protein, pewarnaan sel dan frakmentasi DNA menunjukkan bahawa kematian sel yang disebabkan oleh DCS dan DCP tidak melalui kematian sel yang bergantung kepada caspase. Analisa kitaran sel menggunakan integrasi BrdU menunjukkan bahawa kitaran sel dirawat dengan DCS dan DCP terbantut dalam fasa G_0/G_1 kerana regulasi p21 yang tinggi (keputusan yang didapati daripada analisa profil protein). Tambahan pula, perubahan ultrastruktur sel yang dirawat dengan DCS dan DCP yang diperhatikan dengan menggunakan SEM dan TEM mengesahkan mekanisme kematian sel adalah dicetuskan oleh autoschizis. Oleh itu, selain untuk kegunaan perubatan yang berpotensi dalam rawatan kanser, fraksi DCS juga boleh digunakan untuk mengkaji laluan isyarat molekul yang terlibat dalam autoschizis. Dengan penemuan baru-baru ini, lateks *Hevea brasiliensis* akan menyumbang bukan sahaja dalam industri polimer, tetapi juga, berpotensi tinggi, dalam sektor farmaseutikal dan penjagaan kesihatan.

CYTOTOXIC EFFECT, ANTI-PROLIFERATIVE AND CELL DEATH MECHANISM OF LATEX C-SERUM AND SUB-FRACTIONS USING CELL-BASED ASSAY

ABSTRACT

Latex from *Hevea brasiliensis*, the natural rubber tree, has never been reported for folk medicinal use although highly regarded as an economic importance. Latex was thought to play a biological role in the plant defense system against microorganisms and insects. Due to scarce information available on the studies of its biological effects on mammalian system, cell viability assay using MTT, real-time PCR, cell cycle analysis and protein profiler analysis were used to evaluate the anticancer effects of and cell death pathway induced by whole C-serum (WC) and its fractions against the cancer-origin cell lines. Further fractions of the sera used in this study were prepared via dialysis and pre-heat treatment. The resulted four fractions from the whole C-serum were as follows: DCP = dialyzed C-serum precipitant; DCS = dialyzed C-serum supernatant; BCP = boiled C-serum precipitant and BCS = boiled C-serum supernatant. MTT assay revealed that DCS and DCP treatments specifically target HepG2 cells with IC_{50} values of 0.17 µg/mL ± 0.02 and 1.81 µg/mL ± 0.08 respectively. MDA-MB231 cells were found to be susceptible to DCS and DCP treatments, with IC_{50} values at 2.00 µg/mL ± 0.04 and 5.49 µg/mL ± 0.01 respectively. DCS was proven to be more effective compared to DCP in conferring specific antiproliferation effects against the tested cancer-origin cell lines. The antiproliferation activity of the pre-heated BCP and BCS was significantly reduced

and the results suggested that the active component(s) were heat-labile. Gene expression analysis using real-time PCR, protein profile array, cell staining and DNA fragmentation assay indicated that the cell death induced by DCS and DCP was not promoted via caspase-dependent cell death. Cell cycle analysis using BrdU incorporation showed that the DCS- and DCP-treated cells were arrested in G_0/G_1 phase. Furthermore, ultrastructural changes of DCS- and DCP-treated cells observed using SEM and TEM confirmed the cell death mechanism triggered by autoschizis. Hence, in addition to its potential medical use in cancer treatment, DCS fraction could also be used to study the molecular signaling pathway(s) involved in autoschizis. With these recent findings, latex from *Hevea brasiliensis* would contribute not merely to the polymer industry, but also, with high potential, in the pharmaceutical and healthcare sectors.

CHAPTER 1 INTRODUCTION

1.1 Rubber in Malaysia

The agricultural land in Malaysia is suitable for a diverse variety of tropical crops. Due to the availability of large agricultural land area, a number of crop plants were introduced by the British in the late 19th century. One of the important crops introduced was the rubber tree (*Hevea brasiliensis*) that originates from the Amazon basin in South America. Malaysia became an important producer when Sir Henry Nicholas Ridley heavily promoted the commercial production of rubber in 1881-1911 during his tenure as the first Scientific Director of the Singapore Botanic Gardens. Rubber seeds were distributed to many plantations. With concerted research and development, it did not take long to develop the first technique for tapping trees for latex without causing serious harm to the tree. Rubber tapping technique was considered a breakthrough as the latex can be harvested every 2-3 days with almost the equivalent amount of latex as the very first day it was cut.

Of all the latex-producing plants, *H. brasiliensis* is the only species successfully established as a continuous commercial supply for natural rubber. Its capability of producing high yield of rubber with properties suitable for the manufacture of rubber products proved that *H. brasiliensis* is a remarkable sustainable resource for downstream industries. Even after 20 years of tapping rubber, the timber of the felled rubber tree can be used to manufacture furniture and materials for flooring. Although the natural rubber tree may suffer from stiff competition from production of synthetic rubber, there is still considerable difference in physical and mechanical properties. Constant research is carried out in

order to study and improve the production of natural rubber. In Sungai Buloh Reserve, an experimental research station was set up during the 1930s and since then it has grown into an operational unit spearheading research on crop management, improvement and protection, besides efforts in biotechnology that includes tissue culture and genetic transformation of the rubber tree.

Genetically transformed *H. brasiliensis* is an ideal candidate as natural "factories" for the production of the foreign proteins for pharmaceuticals. Recombinant proteins synthesised in the latex could be extracted continually and non-destructively by tapping the tree. Transgenic rubber trees require low maintenance, are environment-friendly and cost efficient as compared to bioreactor systems by which many pharmaceuticals are currently manufactured. Experimental transgenic rubber trees at the Rubber Research Institute (RRI) of Malaysia have successfully produced bacterial enzyme, β-glucuronidase (GUS) enzyme, mouse antibody and human serum albumin (retrieved from http://www.lgm.gov.my/RnD/biotech.aspx on 14th March 2013).

1.2 Research Rationale

Latex is often processed to be used in the tire, electrical and medical devices industry. Much research has been conducted on latex's phytochemical, biochemical and physiochemical properties in order to exploit its many industrial-related uses. However, there is a lack of knowledge in the bioactivities of this hydrocarbon polymer and with increased competition since the discovery of chemically-synthesized isoprenes (synthetic rubber), the rubber industry is losing its luster.

Therefore, it is upmost important to increase the latex value as a potential medicinal source to sustain its position in the global market. Extensive research has

been conducted over the years in RRI and has successfully produced stable and reproducible clones. Thus rubber is a more consistent counterpart when compared to the traditional herbs, where a full-scale research would have to be launched in terms of plantation, harvesting and processing, product formulation, manufacturing, safety, quality and standard efficacy assessment. Rubber has the advantage to be manufactured as a pharmaceutical therapeutic due to the years of experience at RRI. Not only that, the *H. brasiliensis* tree can be its own natural "factory" for pharmaceutical therapeutics production for the next 20 years.

Research work on latex serum to explore its potential use as pharmaceutical therapeutics for cancer treatment began in 2006 by a group of Malaysian researchers. Cytotoxicity assay via spectrophotometric measurement of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole reduction to purple formazan in living cells, was adopted to determine the viability of Vero (non-cancer origin, African Green Monkey kidney epithelial cells) and human cervical cancer cells, HeLa, when treated with bark extract, latex B- and C-sera. The group managed to identify that the latex B- and C-sera could potentially possess active anticancer properties.

Further fractionization according to the size of sera contents resulted in four fractions from latex B- and C-sera: BHM = B-serum high molecular weight; BLM = B-serum low molecular weight; CHM = C-serum high molecular weight and CLM = C-serum low molecular weight. In this preliminary work, BHM was found to be the active fraction with specific killing effects on HeLa cells while BLM, CHM and CLM displayed the effects with non-specificity towards the cancer cells (Ong *et al.*, 2009).

Further research work on latex B-sera, C-sera and their fractions' (Fig 1.3) is scheduled to investigate the potential of killing and the specificity of these against an array of human cancer cell lines. Current research work is to focus on the specificity and mechanism of action which the latex C-serum acts upon on specific human cancer cell lines. Once cytotoxicity or cell proliferation inhibition has been confirmed, the cell death signaling pathway will be studied to better understand the mechanism of action.

Preliminary screening of *Hevea* latex fractions (B- and C-sera) and bark extract using potato tuber disc bioassay had shown that latex B-serum could inhibit growth of crown gall tumour (Sunderasan & Yeang, personal communication). With studies indicating that latex serum is potent not only to specific cancer cells but also against other opportunistic microbial infections (Daruliza *et al.*, 2011), investigations of the latex serum as a possible therapeutic for cancer will be able to provide cancer patients an alternative route to combat the disease without further deteriorating the patient's health, hence allowing the body a higher chance of recovery.

Latex C-serum was subjected to dialysis using SnakeSkin™ (Pierce, IL, USA) tubing with molecular weight cut-off 3 kDa and pre-heat treatment by immersing in a boiling water bath to obtain the dialyzed and pre-heated (boiled) fractions as specified in Figure **1.1**. Research was conducted according to Figure 1.2.

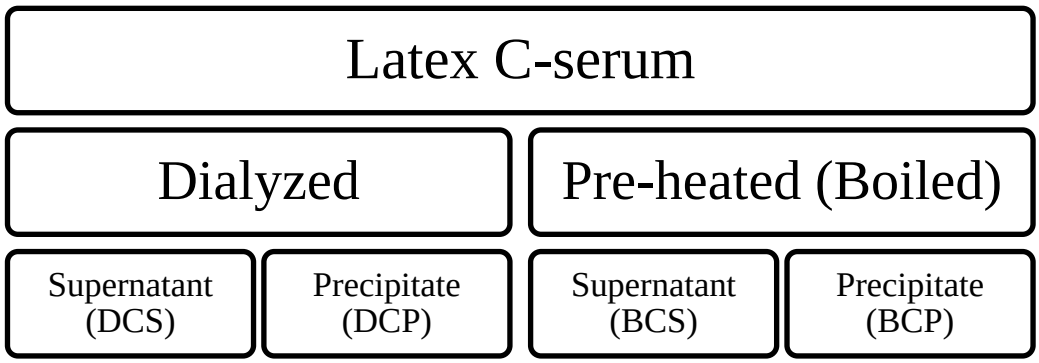

Figure 1.1 Latex C-serum and its fractions.

1.3 Research Objectives

- ✓ To carry out cell-based cytotoxicity and anti-proliferation assay using whole C-serum (WC) and its further sub-fractions against an array of human cancer cell lines.
- ✓ To determine the fraction that possesses the best anti-proliferation effect against the nine adherent human cell lines and two suspension human cell lines.
- ✓ To visualize the cell death morphology of specific susceptible cell lines using Field Emission Scanning electron microscopy (FESEM) and Energy Filtered Transmission electron microscopy (EFTEM)
- ✓ To study the downstream mechanism that trigger anti-proliferation and cytotoxicity effects on the cell death signaling pathway of specific susceptible cell lines using protein profiler analysis using RayBio® Human Apoptosis Antibody Array kit, gene expression analysis using real-time PCR and cell cycle analysis using flow cytometry with BrdU incorporation

1.4 Experimental flow chart

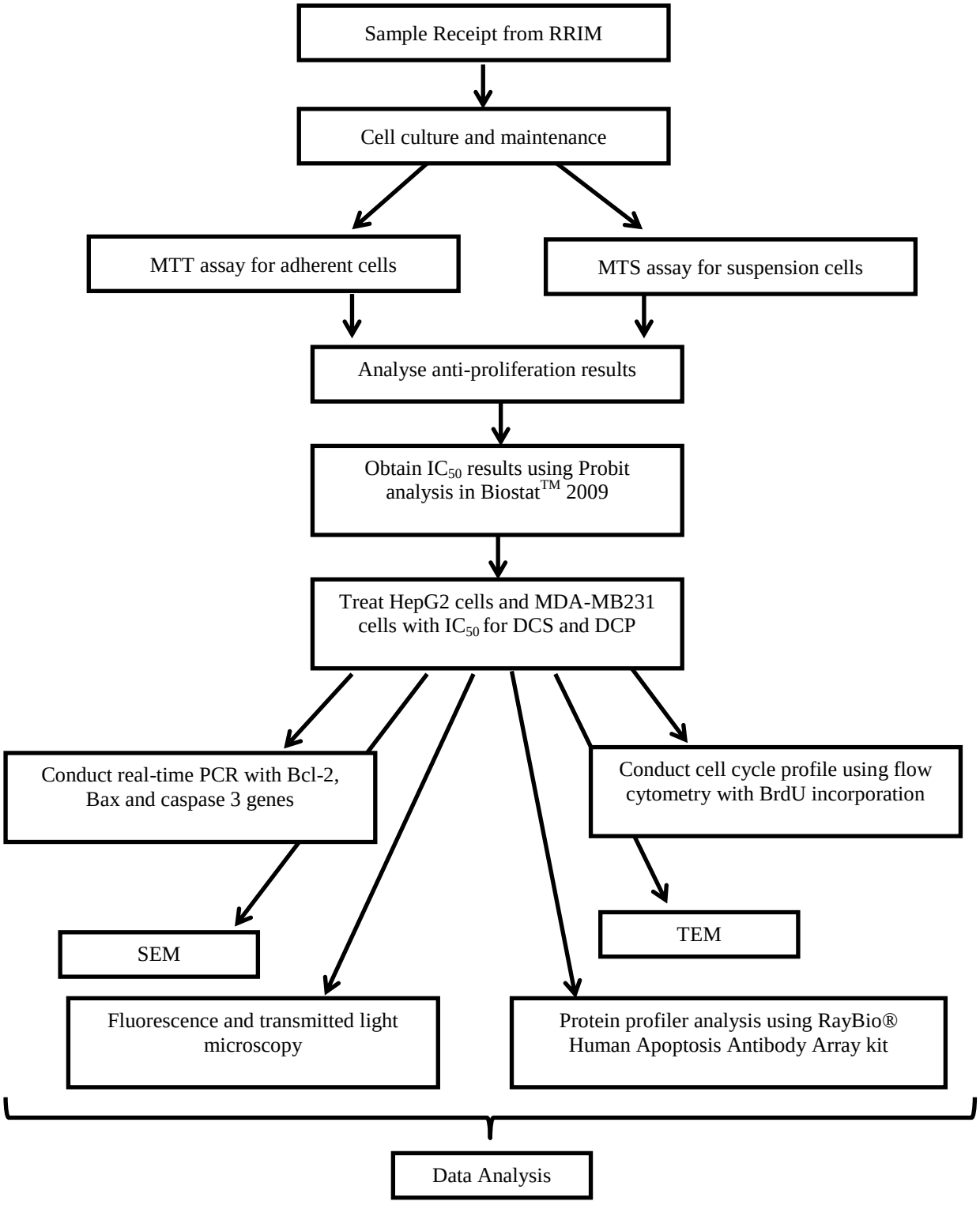

Figure 1.2 Flow chart of research. Refer to section 3.0 for further explanation.

CHAPTER 2 LITERATURE REVIEW

2.1 Terpenoids

Terpenoids constitute the most abundant and structurally diverse group of plant secondary metabolites that play an important role in plant-insect, plant-pathogen, and plant-plant interactions (Cheng *et al.*, 2007; Zwenger & Basu, 2008). Based on its structures, terpenoids consist of five subclasses, including monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids and tetraterpenoids. Many of these terpenoids are identified as major bioactive constituents in traditional Chinese medicine (Huang *et al.*, 2012), used for thousands of years in its clinical practices. Paclitaxel (Taxol®) and docetaxel (Taxotere®) are popular chemotherapeutic agents, derived from a class of diterpenoids called taxanes, to treat many different types of cancer including breast, lung, myelomas, lymphomas, and leukemias (Gligorov & Lotz, 2004).

Thoppil and Bishayee conducted an extensive literature review in 2011 and listed terpenoid compounds such as d-limonene, cucurbitacin B, ursolic acid, β-carotene and lycopene to have the greatest therapeutic potential for liver cancer prevention and treatment. Preclinical studies indicated that d-limonene not only inhibits tumor cell proliferation and increase acceleration of the rate of tumor cell death, d-limonene also induces phase I and phase II carcinogen-metabolizing enzymes (cytochrome p450), which metabolize carcinogens to less toxic forms and prevent the interaction of chemical carcinogens with DNA (Sun, 2007). Not only terpenoids have shown to exert biological activities but have also exhibited little or no toxicity to non-cancerous cells in *in vitro* studies. In the review, Thoppil and Bishayee suggested that terpenoid compounds can be used in combination with

other chemotherapeutic drugs and radiation therapy to enhance their therapeutic efficacy while limiting chemo- and radio-therapy-associated unwanted side effects.

2.2 Latex from *Hevea brasiliensis*

The rubber tree is primarily cultivated for the rubber particles contained in its latex, a milky white sticky emulsion that exudes upon damage from specialized canals called articulating laticiferous cells which are found on all parts of the tree – leaves, flowers, fruits and even the root (Kekwick, 2001; Agrawal & Konno, 2009). Latex from *H. brasiliensis* consists of many isoprene units in a long *cis* double bonds chain called polyisoprene. Latex is a terpenoid, chemically known as cis-1,4-polyisoprene and possesses many valuable constituents such as proteins, lipids, quebrachitol, ribonucleic acids and organic salts in relatively small amounts; consistent with its cytoplasmic nature (Chow & Draper, 1970; Bealing, 1981; Burton *et al.*, 1985; Wajant & Foster, 1996; Effenberger *et al.*, 2000). Therefore the latex from *Hevea brasiliensis* has a wide range of high molecular weight distribution.

Latex can be separated into three fractions consisting of the rubber cream on the top layer, clear centrifuged serum (C-serum) in the middle portion and a thick pale yellowish bottom fraction after high speed centrifugation. The bottom fraction mainly contains vacuole-like organelle known as lutoid body. The fluid released from a continuous freeze-thaw cycle to rupture the lutoid bodies is known as B-serum (Figure 2.1). Lutoid particles have long interested biochemists because they resemble lysosomes in the cells and are considered the site with the most hydrolytic activity and pathogenesis-related proteins (Wititsuwannakul & Wititsuwannakul, 2001).

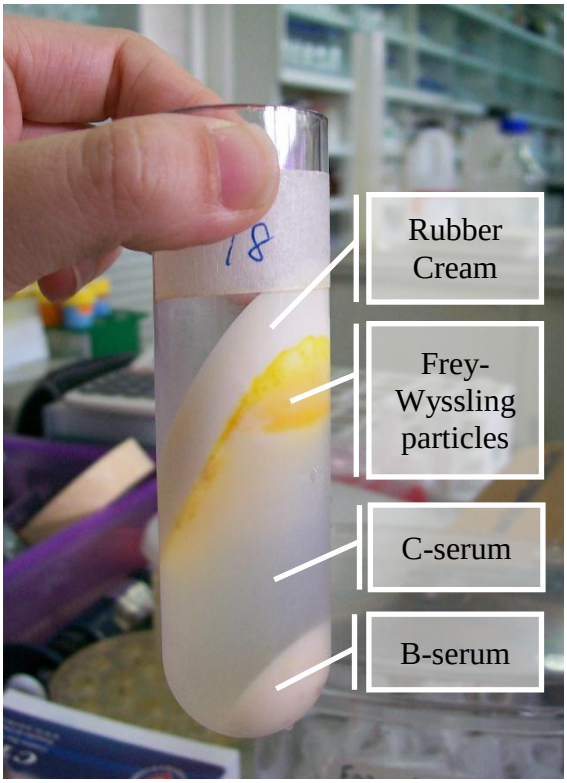

Figure 2.1 The separated zones of *Hevea brasiliensis* natural latex can be observed after centrifugation. The various zones are distinguishable by colour or texture or both. (White rubber layer, clear solution and yellowish pellet). Clear solution is the latex C-serum solution.

2.2.1 Latex B-serum

B-serum is the fluid released from ruptured lutoid bodies with seven anionic proteins and six cationic proteins (Subramaniam, 1995). Lutoid bodies are osmotically active spheres of a single layer membrane measured about 1-3 µm in diameter (Wititsuwannakul & Wititsuwannakul, 2001). The lipids of lutoid membrane are rich in phosphotidic acids and saturated fatty acyl residues that play an important role in colloidal stability in latex (Dupont *et al.*, 1976). The fluid inside these lutoid bodies contains high acid hydrolase content similar to lysosomes and is rich with microfibrils that act as nitrogen reserves. B-serum contains approximately 50% of Hevein, a chitin-binding protein and 30% of Hevamine, a plant defense protein (Wititsuwannakul & Wititsuwannakul, 2001). Although not much is known about

the enzymes and proteins found in B-serum, it is thought that the contents of the lutoid bodies play a role in the metabolic processses and homeostasis in latex, as well as metabolic interactions with latex C-serum. Chitinase and β-1,3-gluconase found in B-serum are also implicated in wound response induced by the multiple tappings that serve as a protection against possible attacks from pathogenic microorganism (Wititsuwannakul & Wititsuwannakul, 2001).

2.2.2 Latex C-serum

Latex C-serum is the metabolically active fraction where all the glycolytic enzymes and cytosolic enzymes for isoprenoid pathway are found in the laticiferous cytoplasm. Rich with organic compounds, about 200 polypeptides (60% of the latex protein) can be found in the C-serum. Comprised of nineteen anionic proteins and five cationic proteins, its major component is α-globulin (pI =4.55) that has been reported to act as protein storage in the seeds of plants. However α-globulin was thought to play an important role in latex and rubber particles' colloidal stability due to its high binding affinity for adsorption into rubber particles (Archer *et al.*, 1963). C-serum also contains a heat-stable calcium-binding protein called Calmodulin which activates HMG-CoA reductase in the lutoids found in B-serum (Wititsuwannakul *et al.*, 1990). Wititsuwannakul *et al.* (2008) recently identified another protein in the C-serum that plays an anti-coagulating role in maintaining colloidal stability in the latex called Hevea latex lectin-binding protein (HLL).

Other than sucrose, glucose, fructose and raffinose, quebrachitol or chemically known as 1-methyl inositol makes up about 75% to 95% of the total carbohydrates present in the C-serum (Bealing, 1981). Major amino acids found in C-serum are alanine, aspartic acid, glutamic acid and its amide. Malic and citric acid makes up

for 90% of its organic acids, while glutathione, cysteine and ascorbic acid are the reducing agents found in C-serum (d'Auzac and Jacob, 1989; Wititsuwannakul and Wititsuwannakul, 2001). However, the primary metabolic and physiological functions of these components are still poorly understood.

2.2.3 Latex allergens

Latex allergy was first reported in 1979, even though irritant and delayed-contact reactions to rubber products were increasingly recognized since 1927 (Ownby 2002). Both Latex B- and C-sera are known to contain proteins, and a multitude of organic compounds as shown in Table 2.1 (Archer *et al.,* 1969). According to Archer *et al.* (1969) and Subramaniam (1995), latex B-serum mainly consists of a protein rich in sulfur called Hevein (50%), a chitin-binding protein and Hevamine (30%), a plant defense protein, which has also been identified as major allergens to humans.

According to Yeang *et al.* (2002), latex C-serum contains four of the fourteen International Union of Immunological Societies (IUIS)-recognized latex allergens, which are *Hev b 5, 7, 8,* and *9.* The most important allergen of this subgroup is *Hev b 5,* a heat-stable protein (Rihs and Raulf-Heimsoth, 2003; Beezhold *et al.,* 2004). However, all of the recognized allergenic proteins located in the latex bottom fraction—*Hev b 2, 4, 6,* and *10*. The molecular weight of identified latex allergens are as in Table 2.2.

Table 2.1 Composition of fresh natural latex (reproduced from Subramaniam, 1995. The chemistry of natural rubber latex. Immunology and Allergy Clinics of North America 15, p. 2, Table 1.)

Component	Content * (%, weight/ volume)
Rubber hydrocarbon	25-45
Protein	1.0-1.8
Carbohydrate	1.0-2.0
Neutral lipids	0.4-1.1
Polar lipids	0.5-0.6
Inorganic constituents	0.4-0.6
Amino acids, amines etc.	0.4
water	-

*The percentage content of the components varies according to clonal variations of the rubber clones

Table 2.2 Natural rubber latex allergens recognized by the International Union of Immunological Societies (IUIS) (reproduced from Yeang *et. al*., 2002 Allergenic proteins of natural rubber latex. Methods 27, p.34, Table 2 with updates)

IUIS code	Identity	Molecular mass (kDa)	Location in latex
Hev b 1	Rubber elongation factor (REF)	{14.6}[28], 58[b]	Rubber particles
Hev b 2	β-1,3-Glucanase	[36][d], 36[76], 34–36[75;79,] (35)[44]	B-serum
Hev b 3	Small rubber particle protein (SRPP)	[22–23][27], (22)[25;43]	Rubber particles
Hev b 4	Microhelix, cyanogenicglucosidase	50–57[e]	B-serum
Hev b 5	Acidic protein	[16][47] (16.0)[47]	C-serum
Hev b 6.01 *Hev b 6.02* *Hev b 6.03*	Prohevein Hevein Prohevein C terminus	20[90] (19c)[24] {4.7}[84] (4.7)[24] 14[90] (13.3c)[24]	B-serum
Hev b 7.01 *Hev b 7.02*	patatin homologue from B-serum patatin homologue from C-serum	42.9 [44][56] (43)[57;58]	B-serum C-serum
Hev b 8	Prolin	10.2, 14.2, 15.7[64] (14.0)[66]	C-serum
Hev b 9	Enolase	51[36], 48[71] (48)[71]	C-serum
Hev b 10	Mn-superoxidedismutase	45[101], 25[36] (23)[102;103]	B-serum
Hev b 11	class I endochitinase	33	B-serum
Hev b 12	lipid transfer protein	9	B-serum
Hev b 13 (= Hev b 7.01)	latex esterase Early nodule specific protein (ENSP)	42.9	B-serum
Hev b 14	Hevamine	30	B-serum

2.2.4 Latex as a medicinal source for cancer

Due to its allergenic nature, latex has never been thought to possess any medicinal properties even though latex had strongly been implicated in plant's defense against herbivorous insects (Agrawal and Konno, 2009) and microorganisms (Van Parijs *et al.*, 1991). Latex B- and C-sera possess plant defense proteins but identified as allergenic proteins to humans (Yeang *et al.*, 2002). Latex was thought to have a primary role in entrapping whole insects in its sticky emulsion (Agrawal and Konno, 2009). The elasticity of its cis-polyisoprene structure, coagulation of its rubber particles and the adhesiveness of rubber particles to the surfaces of the insects play important roles in this clever entrapment.

Latex from *H. brasiliensis* has also been reported to possess specific antimicrobial properties (Daruliza *et al.*, 2011). *Hevein* was found to possess strong antifungal properties against human pathogenic fungi including *Candida albicans*, *Candida tropicalis*, *Candida krusei* and *Candida glabrata*. *Hevein* is a small protein with a molecular size of 4.7 kDa that could penetrate and bind to the pathogenic fungal cell wall matrix and caused a Ca^{2+}-dependent aggregation in fungal cell wall (Kanokwiroon *et al.*, 2008). This could prove a valuable finding as synthetic antifungal drugs available for the treatment of *Candida* infections have very serious side effects to immunocompromised and AIDS patients who suffer from immune dysfunction. For instance, Fluconazole and Itraconazole, two very popular antifungal drugs, have significant negative interactions with chemotherapeutic drugs and AIDS-related drugs (Albengras et al, 1998). Besides that, multidrug resistance has emerged in recent years due to the widespread use of antibiotics and immunosuppressive agents (Vandeputte *et al.*, 2012). However, further work may

need to be conducted in order to remove or mask the allergenicity without losing its antifungal property.

2.3 Cancer in Malaysia

Cancer remains a rapid growing problem that seeks a global attention. In Malaysia alone, the overall age-standardized incidence (ASR) for males was 85.1 per 100,000 population and 94.4 per 100,000 population for females (Malaysia National Cancer Registry Report 2007 by Malaysia Ministry of Health, 2011) with breast cancer being in the lead rank followed by colorectal cancer. Oncologists have predicted from the increased number of cases (year-to-year) that cancer would surpass cardiovascular disease taking its leading rank as the main cause of death as early as 2030 with more than 13.2 million deaths (Boyle and Levin, 2010). Thus, researchers are relentlessly learning about cancer in order to discover any forms of potential remedy or useful natural substances that may fight against cancer specifically. It is thought that these would cause less side-effects compared to those synthetic drugs that chemically induce cell death in cancer cells (Hamblin, 2006).

2.3.1 Cancer progression

Cancer is a disease where cell proliferation occurs without any cell regulation and continues to divide abnormally (Cancer Facts & Figures 2016 by American Cancer Society, 2016). These cancer cells have the potential to break free from its original cells and travel in the blood or lymphatic system in order to invade and spread to other parts of the human body. These type of cancer cells are considered malignant with invasive characteristics, whereas cancer cells which grow into a solid tumor mass without any invasive characteristics are known as benign tumors, which can be

surgically excised. Cancer cells do not mature into distinct cell types with specific functions, unlike normal cells (National Cancer Institute, 2016). This is one of the reasons cancer cells continue to grow uncontrollably.

2.3.1(a) Cell cycle regulation

This uncontrolled growth is influenced by genetic factors such as DNA mutation, hereditary oncogenes and "faulty" tumor suppressor genes that affect the normal cell cycle. The cell cycle governs the cell replication and division process of the cell into two daughter cells. The cell cycle is divided into four sequential phases, namely G_1 phase, S phase where DNA replication occurs, G_2 phase and mitosis also known as M phase where the cell divides into two daughter cells. The sequential phases follow a strict regimen and this regimen does not allow mitosis to occur unless DNA synthesis has completed and vice versa (Garrett, 2001). The cell cycle has strict checkpoints at each G_1 and G_2 phases in order to check and ensure DNA replication and mitosis has been conducted before the cell continues to the next phase of the cell cycle (Figure **2.2**).

The first checkpoint occurs at the G_1/S phase transition and is a major sensor of DNA damage. Cells may be arrested in S phase due to incomplete DNA replication or any DNA damage. Next checkpoint occurs at G_2/M checkpoint, which monitors the fidelity of DNA replication and like the G_1/S checkpoint is an important sensor of DNA damage. Another checkpoint occurs at the mitosis phase known as the spindle checkpoint where if a functional mitotic spindle has not been formed correctly, cell cycle arrest occurs (Garrett, 2001). For cells that did not receive enough cues and signals for proliferation will not be able to go through the restriction point (R) and remain in G_0 phase. These cues and signals depend on

cyclin dependent kinase (Cdk) family that governs the cell cycle progression through specific protein phosphorylation at each phase (Garrett, 2001; Malumbres and Barbacid, 2007).

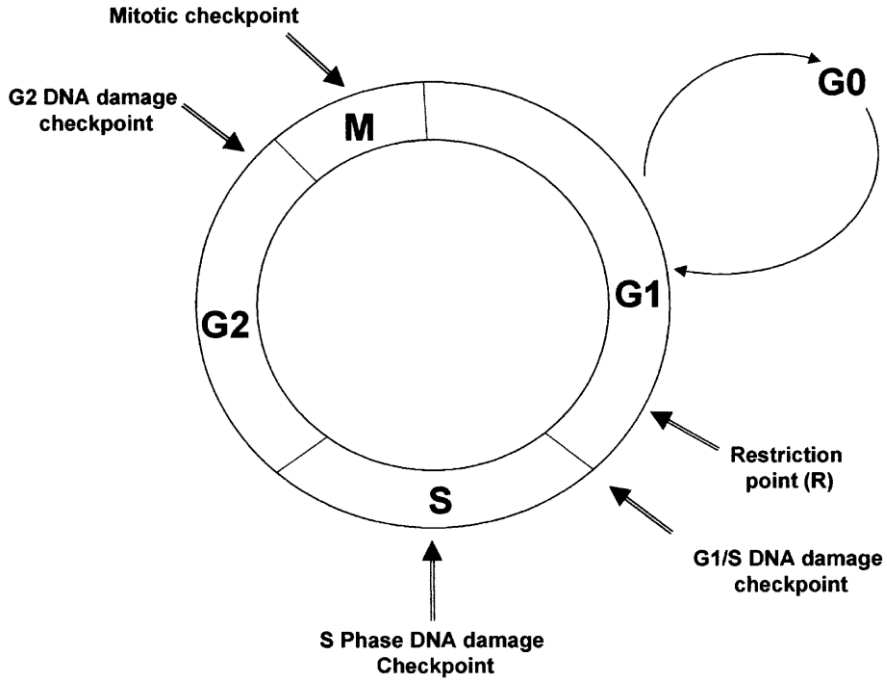

Figure 2.2 Schematic representation of the cell cycle and its checkpoints. (Figure reproduced from Garrett, 2001. Cell cycle control and cancer. Current Science 81:5 p.515, Figure 1).

2.3.1(b) Environmental factors

Environmental factors such as lifestyle, stress, diet, and even exposure to cancer-causing substances, chronic inflammation and infectious agents plays a role in cancer progression (Cancer Facts & Figures 2016 by American Cancer Society, 2016). One example of infectious agent is *Helicobactor pylori*, a spiral-shaped, Gram-negative bacterium, that grow in the digestive tract and have a tendency to attack the stomach lining that cause ulcers in the stomach and small intestine (Wroblewski *et al.*, 2010). Infection with *H. pylori* is the strongest known risk factor for gastric cancer and was declared as type I carcinogen in 1994 (Parkin *et al.*, 2005).

In 2010, the Malaysian Ministry of Health (MOH) started human papillomavirus (HPV) vaccination programme for preteen girls aged 13 (MaHTAS by MOH Malaysia, 2011). HPV infections are a common, sexually transmitted disease with more than 130 subtypes and about 70 subtypes infect human (Kawana *et al.*, 2009). High-risk HPV genotypes have been associated to cervical cancer (Burd, 2003). Although many developing and developed countries have initiated this vaccination programme, it is still highly recommended for women 30 years above to conduct regular screenings (Burd, 2003; WHO Factsheet 2016). This is because the two HPV vaccines developed and approved, Gardasil®, a quadrivalent vaccine which targets HPV-6, -11, -16 and -18 and Cervarix®, a bivalent vaccine which targets HPV-16 and -18, contains only L1 virus-like particles (VLPs) derived from HPV-16 and -18 which are most frequently associated with cervical cancer but not the other possible subtypes associated with cervical cancer (Kawana *et al.* 2009).

2.3.2 Cancer description and stage

Clinicians and doctors determine the severity of cancer through a method called staging. Staging describes the extent or spread of cancer at the time of diagnosis. Proper staging is essential for the doctors and clinicians to assess the prognosis and to optimize therapy options available (Cancer Facts & Figures 2016 by American Cancer Society, 2016). Generally, a cancer stage is based on the size or extent of the primary tumor (T), whether it has spread to nearby lymph nodes, therefore absence or presence of regional lymph node involvement (N) or it has spread to other areas of the body, the absence or presence of distant metastases (M). Once the T, N, and M categories are determined, a stage of 0, I, II, III, or IV is assigned, with stage 0 being *in situ*, stage I being early, and stage IV being the most advanced disease

(Cancer Facts & Figures 2016 by American Cancer Society, 2016). Different cancer types require different staging methods that involve descriptive and statistical analysis, such as leukemia, brain and spinal cord tumors (National Cancer Institute, 2016).

2.3.3 Cancer treatment

Cancer treatments available in Malaysia are quite extensive, ranging from surgery to chemotherapy comprising of one or more chemotherapeutic drugs, to modernized radiotherapy referred as stereotactic radiosurgery (SRS) and stereotactic radiation therapy (SRT). SRT uses high energy x-ray beam to shrink or control the growth of a tumor or abnormal cells by either killing the cells directly or by disrupting the ability of the cells to grow. Both the SRS and SRT are similar but different in dosage quantities during radiation. SRS delivers a large dose of radiation at a single time, whereas SRT delivers lower dose of radiation over a period of fractionated treatment schedule (Halasz and Rockhill, 2013).

Chemotherapeutic drugs or agents consist of a variety of molecules that act upon actively proliferating cells, without differentiating cancer cells from normal cells. Therefore normal cells are destroyed as well and this causes unwanted side-effects to the patients. One example is alkylating agents such as mechlorethamine (nitrogen mustard) that directly form covalent bonds at two nucleophilic sites on different DNA bases to induce interstrand (between two opposite strands) and/or intrastrand (on same strand) cross-links to keep the cell from proliferating (Siddik, 2002). Alkylating agents act on all phases of the cell cycle and are used to treat many different cancers, including leukemia, lymphoma, Hodgkin disease, multiple myeloma, and sarcoma, as well as cancers of the lung, breast, and ovary.

Antimetabolite drugs were among the first effective chemotherapeutic agents discovered and are folic acid, pyrimidine or purine analogues that interfere with DNA and RNA replication by competing and substituting essential pyrimidine or purine required for normal physiological functions. Generally, antimetabolites such as 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), Fludarabine and Methotrexate induce cell death during the S phase of cell growth when incorporated into RNA and DNA or inhibit enzymes needed for nucleic acid production, causing DNA damage and apoptosis induction (Sampath *et al.*, 2003; Parker, 2009).

Doxorubicin is commonly used to treat leukemia, Hodgkin's lymphoma and a wide variety of cancers. Doxorubicin is an anthracycline, an anti-tumor antibiotics that interfere with enzymes involved in DNA replication in the cell cycle. It also causes buildup of free oxygen radicals that induce DNA and cell membrane damage (Sangeeta, 2014). However, anthracyclines can permanently damage the heart and weaken the heart muscles if given long term (Cardinale *et al.*, 2010).

Topoisomerase inhibitors are another group of chemotherapeutic drugs, which act upon the topoisomerase enzyme responsible for the uncoiling and coiling of the double helix strands during DNA replication in the S phase. Topoisomerase inhibitors will bind to the topoisomerase molecule, rendering it nonfunctional and unable to bind DNA back together after it has been cut. Therefore cuts are made to either one or both strands of the DNA molecule which are never repaired, ultimately leading to death of the cell (Ewesuedo and Ratain, 1997; Binashi *et al.*, 1995).

Chemotherapeutic drugs also consist of mitotic inhibitors, which are often plant alkaloids and other compounds derived from natural products (Zulkipli *et al.*, 2015). Mitotic inhibitors function by inhibiting cell division or mitosis in the M phase, where a single cell divides into two genetically identical daughter cells.

Mitotic inhibitors bind to tubulin and inhibit its polymerization into microtubules (Sudakin and Yen, 2007). Taxanes such as paclitaxel (Taxol®) and docetaxel (Taxotere®) and vinca alkaloids such as vinblastine (Velban®), vincristine (Oncovin®), and vinorelbine (Navelbine®) are some of the examples of mitotic inhibitors (Zulkipli *et al.*, 2015). Nerve damage may occur if administrated in high doses (Bass and Ahmad, 2013).

Other forms of chemotherapeutic drugs require more targeted therapies in order to reduce the side effects cancer patients suffer from chemotherapy. Such example is Imatinib, a tyrosine-kinase inhibitor that kills cancer cells by blocking the action of tyrosine kinases. Imatinib is particularly useful in Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia (CML) cells where one tyrosine kinase enzyme, BCR-Abl, is constantly actively adding phosphate groups. Fortunately, this BCR-Abl tyrosine kinase enzyme exists only in cancer cells and not in healthy cells, thus Imatinib works as a form of targeted therapy, where only cancer cells are killed through the drug's mechanism (Goldman & Melo, 2003).

Rituximab (Mabthera®) is another form of targeted therapy which uses monoclonal antibodies also known as immunotherapy that targets specific protein receptors on the surface of cells. Rituximab is used to treat non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukaemia (CLL), as it specifically targets CD20 antigens on B lymphocytes. Even though CD20 antigens are available on the surface of normal B-cells and Rituximab will sensitize both malignant and normal B-cells to be killed, full recovery of normal B-cells in the peripheral blood is usually seen 9-12 months after therapy (Plosker and Figgitt, 2003).

2.4 Programmed cell death (PCD) – Apoptosis

Programmed cell death (PCD) has long been studied in the structural and developmental systems biology. It is an important biological process that regulates and eliminates unwanted cells such as those with potentially harmful genomic mutations, autoimmune reactive lymphocytes and virally-infected cells (Fesik, 2000). Kerr *et al.* first coined the word "apoptosis" in 1972 after observing a well-organized and conserved mechanism with unique morphological features that often occurs during cell death. During apoptosis, the cell receives a "death" signal and begins to shrink and loses its viability with chromatin condensation, plasma membrane blebbing and nuclear DNA fragmentation within its cellular compartment (Wyllie 1980). The apoptotic bodies are then engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response.

This highly controlled sequence of events is very important in embryogenesis, development of reproductive system, homeostasis of the immune system and only occurs in the cells which have been internally predetermined to die (Gewies, 2003). This programmed cell death is so precisely executed that in the development of the nematode *Caenorhabditis elegans*, exactly 131 cells die according to a well-regulated genetic programme (Hengartner and Horvitz, 1994). Apoptosis plays a protective mechanism, directing lysis of virus-infected cells, foreign cells or incipient neoplasm. Over-regulated PCD can contribute to the acquired immune deficiency syndrome (AIDS) and neurodegenerative diseases like Alzheimer and Parkinson syndromes, and ischaemic injury such as myocardial infarction. Under-regulated PCD could lead to cancer, persistent viral infection, or autoimmune disorders. In short, apoptosis is an active, metabolic, genetically encoded and evolutionarily selected death pathway.

Apoptosis can be triggered by various stimuli from outside or inside the cell, dividing its signaling pathway to extrinsic and intrinsic pathways. The extrinsic pathway begins outside a cell, when conditions in the extracellular environment determine that a cell must die. Based on the triggering stimulus and nature of the components involved, at least two apoptotic pathways can be differentiated; one involving receptor systems and the other triggered by cytotoxic stress. Apoptosis has been widely studied by scientists in order to determine whether cell death occurrence by an active molecule is triggered by receptor systems or cytotoxic stress to improve drug deliverance to its actual active site and reduce the risks for drug suppression.

2.4.1 Extrinsic pathway

The extrinsic pathway involves the activation of "death receptors" which are cell surface receptors that transmit apoptotic signals upon binding with specific ligands such as tumour necrosis factor receptor (TNFR) gene superfamily. The TNFR family is a large family consisting of 29 transmembrane receptor proteins, organized in homotrimers and activated through binding of respective ligand(s) (Figure **2.3**). There are at least 19 members of the TNF ligand family and each binding may result in a number of different responses including proliferation, inflammation and apoptosis, depending on the adaptor proteins associated with the activated receptor (Aggarwal, 2003). For example, TNFR may also stimulate pro inflammatory pathways leading to activation of NFκB (Nuclear factor kappa-light-chain-enhancer of activated B cells), through the binding of receptor interacting protein, RIP to TNFR type 1-associated death domain protein (TRADD). Popular death receptors that mediate apoptosis are the tumour necrosis factor receptor (TNFR-1), Fas/CD95,

and the tumour necrosis related apoptosis-inducing ligand (TRAIL) receptors DR-4 and DR-5, which binds to TNFα, CD95 and TRAIL, respectively (Figure **2.3**).

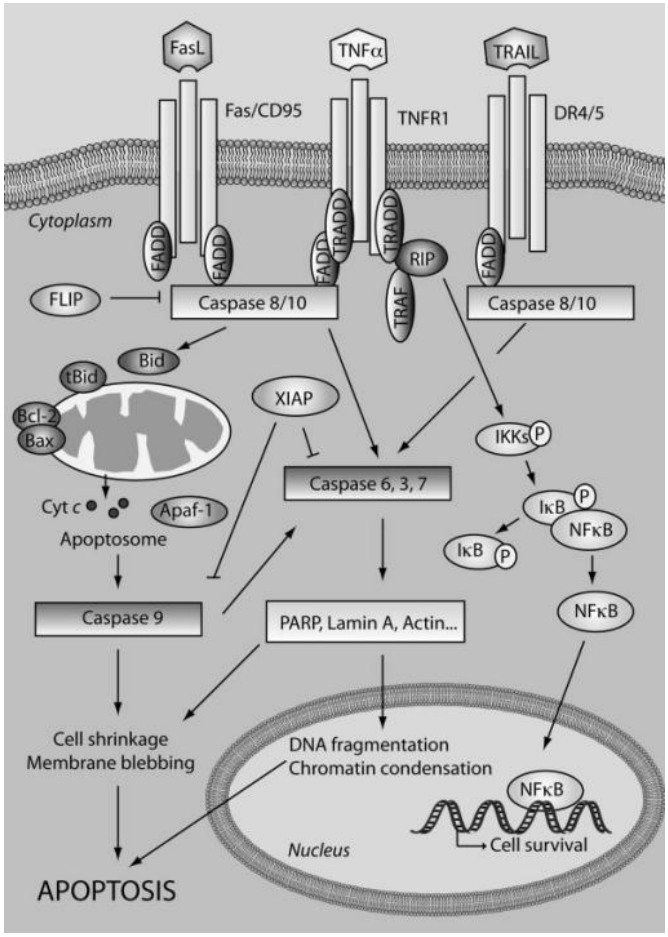

Figure 2.3 Schematic representation of the extrinsic apoptosis pathway. The extrinsic apoptosis pathway is activated upon ligand binding to death receptors (TNFR1, Fas/CD95, DR4/5) which results activation of a caspase cascade and eventually cleavage of both cytoplasmic and nuclear substrates. The extrinsic apoptosis pathway indirectly triggers and often converges with the intrinsic pathway in the caspase cascade causing cellular shrinkage, membrane blebbing and DNA fragmentation, hallmarks of apoptosis cell death (Figure reproduced from Krakstad and Chekenya, 2010. Survival signalling and apoptosis resistance in glioblastomas: opportunities for targeted therapeutics. Molecular Cancer, 9:135 p.7 of 14, Figure 3).

Subsequent signaling is mediated by the cytoplasmic part of the death receptor, which contains a conserved sequence termed the death domain (DD) and forms a death inducing signaling complex (DISC) to activate the initiator caspase-8 and caspase-10. Activated caspase-8 or caspase-10 then initiates a caspase cascade by processing the effector caspases-3, -6, and -7 which in turn cleave a number of

protein substrates. Cleavage of caspase substrates eventually leads to the characteristic morphological and biochemical features of apoptosis (Gewies, 2003; Elmore, 2007). The activated caspase-8 also triggers BH3 interacting-domain death agonist, known as BID in activating pro-apoptotic BAX protein (BCL-2-associated X protein) and/or BAK protein (BCL-2-antagonist/killer-1) to form an oligomeric pore known as mitochondrial apoptosis-induced channel (MAC) that governs the release of cytochrome c. This is where the extrinsic and intrinsic apoptosis pathway meets and converges as one leading up to a series of events in apoptosis cell death. Kruger *et al.* (2001) summarized that the activation of caspase-8 could be prevented by FLICE inhibitory protein (FLIP) as a potential competitive inhibitor.

2.4.2 Intrinsic pathway

The intrinsic pathway involves the mitochondria as a central role in the integration and propagation of death signals originating from inside the cell such as DNA damage, oxidative stress, starvation, hypoxia as well as those induced by chemotherapeutic drugs. These stress-inducing signals then disrupt the mitochondrial inner transmembrane potential ($\Delta\psi$) and cause the sudden increase of the inner mitochondrial membrane permeability to solutes with a molecular mass below approximately 1.5 kDa. Due to this, osmotic swelling occurs in the mitochondria and ruptures the outer mitochondrial membrane, resulting in the release of pro-apoptotic proteins from the mitochondrial intermembrane space into the cytoplasm. Released proteins include cytochrome c and dATP binds to the Apaf-1 protein and forms the apoptosome, a cytosolic death signalling protein complex that triggers the activation of procaspase-9 (Gewies, 2003; MacFarlane and Williams, 2004; Elmore, 2007; Taylor *et al.*, 2008). The activated caspase-9