STUDIES OF THE ANTIFUNGAL AND ANTICANCER PROPERTIES OF *Hevea brasiliensis* LATEX B-SERUM AND THEIR SUB-FRACTIONS

by

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LIST OF ABBREVIATION

APS	Ammonium Persulphate Solution
ATCC	American Type Culture Collection
BBP	Boiled B-serum Precipitate sub-fraction
BBS	Boiled B-serum Supernatant sub-fraction
BSLT	Brine Shrimp Lethality Test
CaOV-3	Human Ovarian Cancer Cells
CFU	Colony-Forming Unit
CO_2	Carbon Dioxide
Da	Dalton
kDa	kiloDalton
DBP	Dialysed B-serum Precipitate sub-fraction
DBS	Dialysed B-serum Supernatant sub-fraction
DMEM	Dulbecco Minimum Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
EDTA	Ethylenediamine tetra-acetic acid
ER	Estrogen Receptor
EGFR	Epidermal Growth Factor Receptor
FBS	Feotal Bovine Serum
HCT 116	Human Colon Carcinoma Cells
HeLa	Human Cervical Cancer Cells
HepG2	Human Liver Cancer Cells

Hs27	Human Foreskin Cells
LC_{50}	Median Lethality Concentration
LC_{80}	Lethality Concentration at 80%
OD	Optical Density
MDA-MB231	Human Breast Cancer Cells
MCF	Human Breast Cancer Cells
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
mg/ml	milligram per milliliter
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
NADP	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
nm	nanometer
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PS	Physiologic saline
RNase	Ribonuclease
RPMI	Rosewell Park Memorial Institute medium
RRIM	Rubber Research Institute Malaysia
SDS	Sodium Dodecyl Sulphate Solution
SDS-PAGE	SDS Polyamide Agarose Gel Electrophoresis
T-25	Tissue Culture Flask 25 cm^2
T-75	Tissue Culture Flask 75 cm^2

TGFR	Transforming Growth Factor Receptor
v/v	volume over volume
WB	Whole B-serum fraction
µg/ml	microgram per milliliter

LIST OF APPENDICES

LIST OF PUBLICATIONS

1 K. M. A. Daruliza, K.L. Yang, K.L. Lam, J. T. Priscilla, E. Sunderasan & M. T. Ong. (2011). Anti-Candida albicans activity and brine shrimp lethality test of *Hevea brasiliensis* latex B-serum. *European Review for Medical and Pharmacological Sciences*, 15: 1163-1171. (Impact factor: 1.04)

2 K. M. A. Daruliza, K.L. Lam, K.L. Yang, J. T. Priscilla, E. Sunderasan & M. T. Ong. (2011). Antifungal effect of *Hevea brasiliensis* latex C-serum on Aspergillus niger. *European Review for Medical and Pharmacological Sciences*, 15: 1027-1033. (Impact factor: 1.04)

3 K.L. Yang, K.L. Lam, S. M. Mansor, S. G. Teoh, E. Sunderasan & M. T. Ong. (2012) Anti-proliferation effect of *Hevea brasiliensis* latex B-serum on human breast epithelial cells. *Pakistan Journal of Pharmaceutical Sciences*. 25 (3), 645-650. (Impact factor: 1.103)

KAJIAN KE ATAS SIFAT-SIFAT ANTIKULAT DAN ANTIKANSER LATEKS B-SERUM DAN SUB-FRAKSI *Hevea brasiliensis*

ABSTRAK

Kajian terdahulu menggunakan lateks B-serum yang diekstrak daripada *Hevea brasiliensis* untuk menguji sifat antikanser pada sel-sel HeLa telah merintis jalan untuk menjalankan analisis lanjutan dengan menggunakan pelbagai jenis kumpulan sel-sel kanser manusia dengan indeks terapeutik yang lebih tinggi. Dalam kajian ini, toksisiti lateks B-serum (WB) dan sub-fraksinya (DBP, DBS, BBP dan BBS) telah dinilai dengan Ujian Maut Udang Air Masin (BSLT). Ciri-ciri antikanser dinilai dengan menggunakan ujikaji MTT di mana panel sel-sel kanser yang berasal dari manusia telah digunakan. Ciri-ciri antikulat *Hevea brasiliensis* latex B-serum kemudian diuji menggunakan kaedah peresapan cakera , kepekatan perencatan minimum (MIC) dan kepekatan antikulat minimum (MFC). Mekanisme kematian sel yang berkemungkinan diinduksi oleh WB dan sub-fraksinya telah dikaji dengan menggunakan kaedah fragmentasi DNA, diikuti oleh kaedah "real-time reverse transcriptase- qualitative polymerase chain reaction" (RT-qPCR). Keputusan daripada BSLT menunjukkan bahawa WB dan sub-fraksinya mempunyai tahap ketoksikan yang rendah (461.0 mg/ml) menurut klasifikasi daripada Meyer et al., (1982). Keputusan ini turut disokongkan oleh ujikaji MTT bahawa keupayaan sel Hs27 (bukan berasal daripada kanser) tidak terpengaruh oleh rawatan WB dan sub-fraksi di mana keupayaan sel itu dikekalkan melebihi 90% walaupun selepas 72 jam rawatan. Sementara itu, di kalangan keenam-enam barisan sel kanser manusia, hanya barisan HepG2 dan MDA-MB231 menunjukkan kencenderungan yang tinggi ke arah rawatan WB dengan nilai LC_{50} masing-masing pada 8.657 μg/ml dan 85.86 μg/ml,

dan rawatan DBP dengan nilai LC_{50} pada 1.172 µg/ml and 5.364 µg/ml masing-masing. Walaubagaimanapun, ujikaji yang dijalankan dengan menggunakan sub-fraksi BBS dan BBP tidak memaparkan keputusan yang sama. Menariknya, MCF-7 yang merupakan satu lagi barisan sel kanser payudara manusia tidak menunjukkan sebarang kecenderungan seperti yang ditunjuk oleh MDA-MB231. Ini telah mencadangkan bahawa lintasan kematian sel apoptosis yang bersandar kaspase-3 berkemungkinan teraruh semasa rawatan. Dalam kaedah fragmentasi DNA, tiada penanggan DNA dapat diperhatikan dan keputusan RT-qPCR yang dijalankan menunjukkan ekspresi yang rendah oleh gen bax dan kaspase-3 setelah rawatan dengan WB dan DBP. Keputusan ini menunjukkan tiada lintasan apoptosis dapat diaruh pada kedua-dua barisan sel HepG2 dan MDA-MB231 yang rentan pada sub-fraksi WB dan DBP. Dalam ujian keterentanan antikulat, WB menunjukkan zon perencatan terhadap *Candida albicans* manakala tiada zon perencatan yang diperhati terhadap *Aspergillus niger*. Nilai MIC yang diperolehi daripada *C.albicans* yang dirawat dengan WB ialah 2.5 mg/ml. Sabagai kesimpulan, kesemua keputusan yang diperolehi menyarankan bahawa sub-fraksi DBP, harus diambilkira untuk kajian lanjutan supaya sifat antikanser yang berpotensi dapat diterokai memandangkan ia dapat meminimakan jangkitan yang disebabkan oleh *C. albicans*, sejenis agen jangkitan oportunis dalam pesakit-pesakit kompromi-imun. Sebagai tambahan, mekanisme untuk menentukan lintasan kematian sel yang mana diaruh setelah dirawat dengan B-serum juga memerlukan penjelasan.

STUDIES OF THE ANTIFUNGAL AND ANTICANCER PROPERTIES OF *Hevea brasiliensis* LATEX B-SERUM AND THEIR SUB-FRACTIONS

ABSTRACT

Previous studies using latex B-serum, extracted from the *Hevea brasiliensis* latex has exhibited its anticancer properties on HeLa cells. This has paved the way further analysis of the anticancer properties on different human cancer cell lines with higher therapeutic index. In this study, toxicity of latex whole B-serum (WB) and its sub-fractions (DBP, DBS, BBP and BBS) was first assessed using brine shrimp lethality test (BSLT). Anticancer properties were assessed using MTT assay where a panel of human cancer-origin cells was applied. Antifungal properties of *Hevea brasiliensis* latex B-serum was then tested using disc diffusion, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) assay. Possible cell death mechanism induced by WB and its sub-fractions was studied using DNA fragmentation assay, followed by real-time reverse transcriptase- quantitative polymerase chain reaction (RT-qPCR). Results from BSLT indicated that WB and its sub-fractions have low toxicity level (461.0 mg/ml) according to the classification by Meyer et al., (1982). This result was further confirmed by MTT assay that the Hs27 (non-cancer origin) cell viability was not affected by the treatment of WB and its sub-fractions where the cell viability was remained above 90% at treatment up to 72 hours. Meanwhile, among the six human cancer-origin cell lines tested, HepG2 and MDA-MB231 cell lines showed high susceptibility towards the treatment of WB with the LC_{50} value 8.657 and 85.86 µg/ml, respectively and DBP with the LC_{50} value 1.172 and 5.364 µg/ml, respectively. However, no similar effect was observed in BBP and BBS sub-fractions treated cells. Interestingly, similar effects were not

observed in MCF-7, another tested human breast cancer cell line, suggesting that caspase-3 dependent apoptosis cell death pathway might be induced during the treatment. In DNA fragmentation assay, no DNA laddering was observed and RT-qPCR results showed low expression of Bax and caspase-3 genes when the related cells were treated with WB and DBP confirming the fact that no apoptosis pathway was induced in both HepG2 and MDA-MB231 cell lines that were susceptible to WB and DBP sub-fractions. In the disc diffusion antifungal test, WB showed an inhibition zone against *Candida albicans* while this was not observed with *Aspergillus niger*. The MIC value obtained for WB-treated *C. albicans* was 2.5 mg/ml. It is highly recommended that DBP sub-fraction should be subjected to further studies to explore the potential anticancer properties in view of developing a drug with additional ability to minimize infection caused by *C. albicans*, an opportunistic infectious agent in immune-compromised patients. In addition, the mechanism(s) of cell death induced by the sub-fraction also needs to be elucidated.

CHAPTER 1

1.0 INTRODUCTION

Candidiasis is a common infection of the skin, nails, oral cavity, esophagus and vagina and in critically ill non-neutropenic patients (Eggimann et al., 2003), caused by the *Candida* genus accounting for more than 90% of candidiasis cases (Douglas, 2003; Edward, 1995). Systemic yeast infections are a common consequence of immuno-suppression, long-term indwelling catheters, and endocrinopathies. *Candida albicans* is the most common pathogen causing the fungal opportunistic infection. Additionally, it has the ability to adhere to host surfaces or to prosthesis leading to the formation of biofilms which further facilitate their adhesion, infection and resistance to the antifungals (Parahitiyawa et al., 2006). *Aspergillus* spp is another documented causative agents of human disease while *Aspergillus niger* being the first most common causative species (Kwon-Chung & Bennett, 1992). With pathogenic fungi, the situation is not so bright, where amphotericin B was for many years the only treatment available for fungal infections. Due to its severe toxicity level that would lead to impairment of renal function, the use of amphotericin B has been limited (Hossain & Ghannoum, 2000) for treatment of human diseases caused by fungal infection. Hence, increasing incidence of drug-resistant pathogens has drawn the attention of the pharmaceutical and scientific communities towards studies on the potential antimicrobial activity of plant-derived substances, an untapped source of antimicrobial chemotypes, which are used on traditional medicine in different countries (Savoia, 2012). In recent years, the antifungal drug discovery is

accelerated due to various challenges such as limited number of effective anti-fungal drugs, toxicity of the available anti-fungal drugs, resistance of fungi to commonly use anti-fungal drugs, relapse of fungal infections and the high cost of anti-fungal drugs (Mustafa et al., 1999; Klepser, 2001; Khan et al., 2003; Runyoro et al., 2006).

Compared with chemical synthesis, plant-derived natural products represent an attractive source of biologically active agents since they are natural and available at affordable price (Ghosh et al., 2008). Medicinal plants represent a rich source of antimicrobial and antifungal agents (Mahesh & Sastish, 2008; Ali et al., 2001; Ho et al., 2001). However, many plants that were used as the resources of natural products, have not much been thoroughly explored so far. Latex serum had been reported to be anti-microbial due to the presence of hevein, a chitin binding protein, and a number of hydrolytic enzymes (Martin, 1991; Parijs et al., 1991), which contained mainly latex B-serum. Nevertheless, it has been suggested that latex B-serum alone was not sufficient to cause an inhibition of yeast growth (Giordani et al., 1999). Other reports tried to attribute the antifungal property of latex B-serum to the enzymatic activities (Jacob et al., 1993). If properly studied, *Hevea* extract could potentially be employed as a relatively low cost resource for various antimicrobial activities due to the simplicity of latex preparation and the abundance of latex that can be obtained in rubber producing regions.

Screening by using *in-vitro* evaluation is a useful tool for the discovery of new potential antifungal agents from natural products such as essential oil and extracts derived from plants (Veronica et al., 2011). The present study was also aimed at examining the antifungal property of latex B-serum based on the difference between *C. albicans* (single-cell fungus) and *Aspergillus niger* (a filamentous fungus). In addition, this antifungal study would serve as a preliminary test in

characterizing latex B-serum activities in biological system before embarking on mammalian cell-based cytotoxicity assay.

Toxicity test is as of most basic parameter to be conducted to identify the toxicity level of a substance and decipher if it could be further developed into next step. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose, and toxicology is simply pharmacology at a higher dose. Thus, in vivo lethality in a simple zoologic organism can be used as a convenient monitor for screening and fractionation in the discovery and monitoring of bioactive natural products (McLaughlin et al., 1991). Brine shrimp lethality test (BSLT) is one of the common and easy handled method to study toxicity level of a test substance (McLaughlin et al., 1998).

An integrated part of cancer cell development is the resistance to programmed cell death (apoptosis). Re-establishment of apoptosis in cancer cells is a target mechanism for anticancer agents (Joshi et al., 1999). In fact, many of the cancer research studies have been carried out to study the mechanism of cell death induced by natural products. Many conventional anticancer drugs have an associated lack of safety by their toxicity. Relatively faster mutations in tumor cells, compared to normal cells, pose a significant obstacle in treatment of cancer (Vandana et al., 2011). In spite of the several successes of previously studied anticancer plant products, the development of multi-drug resistance in cancer chemotherapy is still one of the major problems (Xu et al., 2009).

Preliminary studies on latex whole B-serum (WB) has shown the potential in anticancer properties (Ong et al., 2009). Further detailed studies on the anticancer properties of latex B-serum and it's sub-fractions were conducted in this present study by using a panel of human cancer-origin cell lines from different sites of the

body. In line of this, a human non-cancer-origin cell line was employed in the same experiment to investigate if the anticancer effects would be detrimental to other non-cancer-origin cells if it is to be developed into anticancer drugs in the future. It would be an advantage if the latex B-serum could be tested on its antifungal effects, especially on *Candica albicans* which is an opportunistic fungus that caused infection in immune-compromised cancer patients. As of to date, there was no work being carried out to investigate on the toxicity level of the latex B-serum. Therefore in this thesis, further experiments were undertaken to explore the toxicity level, antifungal properties onto *Candida albicans*, and to identify the specificity of the latex B-serum towards human cancer-origin cell lines using appropriate and reliable bioassays.

1.1 OBJECTIVES

The objectives of the work carried out in this thesis are as follows:

- To determine the level of toxicity of latex whole B-serum (WB)
- To investigate the antifungal properties of latex whole B-serum (WB)
- To investigate the anticancer properties of latex whole B-serum (WB) and its sub-fractions through cell-based assays on various human cancer-origin and non-cancer-origin cell lines.
- To develop the median lethal concentration (LC_{50}) values of the latex whole B-serum and its sub-fractions in anticancer studies using probit analysis method.
- To determine the anticancer activities of latex whole B-serum (WB) and its sub-fraction (Dialyzed B-serum Precipitate - DBP) in inducing apoptosis of cancer cells through DNA fragmentation assay and real time reverse transcriptase - quantitative polymerase chain reaction (RT-qPCR).

CHAPTER 2

2.0 LITERATURE REVIEW

2.0.1 Current trends of cancer

Since 1990 there has been a 22% increase in cancer incidence and mortality with the four most frequent cancers being lung, breast, colorectal, and stomach cancers and the four most deadly cancers being lung, stomach, liver, and colorectal cancers (Parkin et al., 2001). Over ten million new cases of cancer (all sites excluding non-melanoma skin), with over six million deaths, were estimated in the year 2000 (Parkin, 2001; Parkin et al., 2001). Cancer is the second leading cause of death in the United States (U.S.), surpassed only by cardiovascular disease as reported by Jemal et al., (2005). Breast cancer appeared to be the most frequent cancer, all residence in Malaysia, according to the National Cancer Registry Report year 2007 and Globocan (2008). While the most common cancer occurred among women in Malaysia is breast cancer which is 26.5 percent, it is the highest number of statistics compared to another cancer such as cervix uterine, 12.6 percent, Colorectal 9.9 percent, lung, 5.8 percent and ovary 5.4 percent (Globocan, 2008). Experts project that by the year of 2010 about 1.35 million new cases of the disease will be diagnosed per year. The proportion of these people who will die from breast cancer is unknown (Shadiya et al., 2012). Nonetheless, in US, an estimation of 230,480 new cases of invasive breast cancer will be diagnosed among women in the year of 2011 based on 1995-2007 incidence rates in US, by the American Cancer Society. However approximately 39,520 women are expected to die from breast cancer. Only lung cancer accounts for more cancer deaths in women (Rick et al., 2011). Lung cancer being the most

common cancer covering 16.5% of all new cases in men while breast cancer being the most common cancer diagnosed in women as high as 23% of all new cases in women (IARC, 2012).

This could be possibly being a result of world cancer westernization trends. Less developed countries have been becoming more "westernized" due to rapid increase changes in terms of social and economy. As such, many of those infection-related cancers such as cervix and stomach cancer cases have been reduced, alongside increasing incidence rates of cancers more associated with reproductive, dietary and hormonal factors such as female breast, prostate and colorectal cancers (IARC, 2012).

Viewing at the current trends in major cancers globally, it is projected in the future that there will be increasing to 22 million new cases each year by 2030, representing a 75% increment compared with 2008 (IARC, 2012). In line with this, more efforts to develop effective cancer controls as well as cancer treatments are highly required with this knowledge about cancer burden as reported.

2.0.1(a) Plant-based anticancer agents

Traditionally, it is believed that plant-based drugs have fewer side effects when consumed, unlike those synthetically produced in laboratory. There were at least 120 distinct chemical substances derived from plants used to treat various sicknesses, and to name a few such as anti-inflammatory, pain killer, analgesic, laxative, local anaesthetic, antitumor agent, etc. (Leslie, 2000).

In particular, researches involved in searching anticancer agents from plant sources began in earliest in the 1950s. Various anticancer agents were discovered and developed such as vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins (Cragg & Newman, 2005). Among all, vinca alkaloids, vinblastine and vincristine were the first agents that advanced into clinical use for the treatment of cancer (Cragg & Newman, 2005). Combination of vinblastine, vincristine and other chemotherapeutic drugs are able to treat cancers such as leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma effectively (Cragg & Newman, 2005). Paclitaxel, extracted from the bark of Pacific Yew trees, was clinically introduced to the US market in the early 1990s (Wani et al., 1971; Rowinsky et al., 1992) due to its activity against human ovarian cancer, advanced breast cancer, small and non-small cell lung cancer (Rowinsky et al., 1992). The natural alkaloid camptothecin isolated from the Chinese tree *Camptotheca acuminate* is another useful chemotherapeutic agents used in treatment of gastric, rectal, colon and bladder cancers (Cragg & Newman, 1999; Wall et al., 1966).

Above mentioned were among the first few anticancer drugs that were discovered from plants and developed into useful chemotherapeutic drugs. This has evidenced that plant-based anticancer drugs is no more a new era. Despite existing anticancer drugs found in the market, demand in searching for potential anticancer drug, with equivalent or higher therapeutic index with little side effects, is getting higher. In addition, various factors that lead to anticancer drugs resistance have prompted scientists to continue searching for more effective anticancer drugs. Eventually, increasing amount of cancer research is being directed towards the

investigation of plant-derived anticancer compounds, many of which have been used in traditional herbal treatments for centuries.

2.1 Introduction to *Hevea brasiliensis*

2.1.1 *Hevea brasiliensis* and its origin

Rubber is a well-known tropical tree crop that mainly grown for industrial production of latex, natural rubber (NR). It is scientifically known as *Hevea brasiliensis (*Mueller (Arg.). Latex is obtained by tapping the rubber tree bark and excreted from the latex vessels (Figure 1.0). Production of good quality of latex required stable rainfall and high temperature throughout the year. Richness of soil is less important in comparison to its well drainage.

The genus *Hevea* is native to South America, where it grows wild in the Amazon and Orinoco valleys. Before the discovery of the New World, native Indians used the latex of various plants for making balls, bottles, crude footwear and waterproofing fabric. Only one of these plants, *Hevea brasiliensis* later developed as the major latex-producing crop. The first rubber plantations in Malaysia were established as early as 1890. *Hevea* was introduced in Africa early in the 20th century: in Uganda and Nigeria (1903), Congo (1904), and Liberia (1924) by the Firestone Tyre and Rubber Company. Today, most latex production is concentrated in industrial estates in tropical Africa and the Far East (Verheye, 2010).

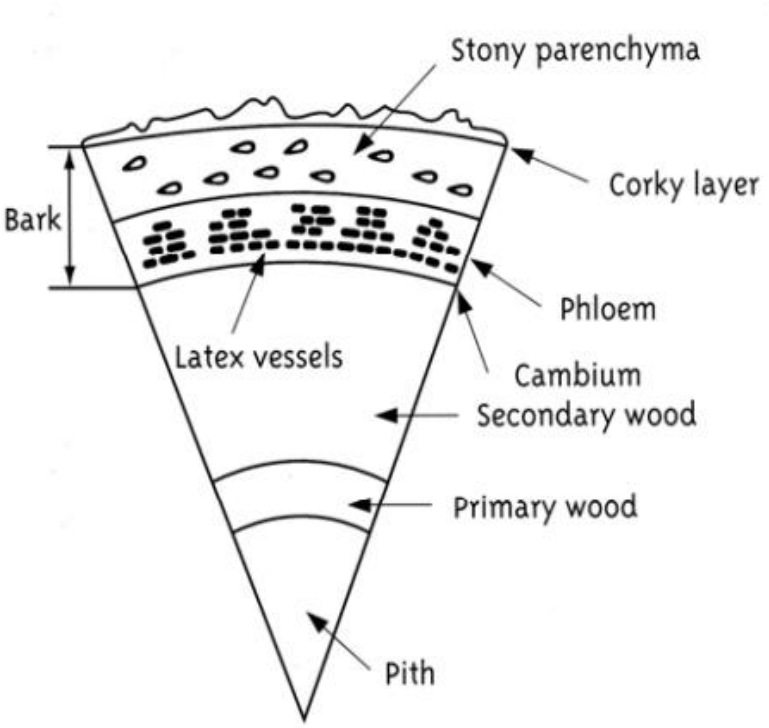

Figure 1.0: Cross section of an adult tree showing the composition of the bark and the position of the latex vessels in the soft bark tissue (Courtesy Boedt, 2001).

2. 1. 2 *Hevea brasiliensis* latex and its composition

Natural rubber exists as latex, a milky white sap, which is colloidal suspension of rubber particles in liquid form. The latex is extracted from the rubber tree and collected for processing into a either dry rubber or latex concentrate. The economic life of latex-yielding tress ranges from 20 to 30 years.

Natural latex of *Hevea brasiliensis* is the cytoplasm of specialized cells known as laticifers. Upon high speed centrifugation (Figure 1.1), latex is separated into the rubber cream, the latex serum (C-serum) and the 'bottom fraction' which consists mainly of vacuole-like organelles called lutoids. The lutoids contain a fluid, B-serum, which has long been of interest to biochemists and physiologists as it contains several hydrolases and some pathogenesis-related proteins. B-serum was obtained by repeatedly freeze-thaw of the ‘bottom fraction’ followed by high speed centrifugation. Based on the research done by Jacob et al. (1993), it has been shown that there were many low molecular weight organic, mineral solutes as well as large quantities of anionic and cationic proteins which could exert chitinase and/or lysozyme enzymatic properties in lutoids.

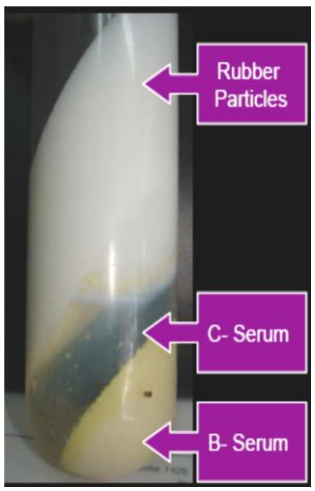

Figure 1.1

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

The bottom fraction of fresh latex consists of many organic non-rubber constituents such as proteins, phospholipids, ubiquinol, sterols, trigonelline and ergothioneine (Archer et al., 1969).

The proteins of *H. brasiliensis* latex were being studied by Archer et al. (1955). Several experiments were conducted such as elementary analyses, protein nitrogen analyses and paper electrophoresis, in order to understand the protein contents in natural rubber latex (Archer et al., 1955).

Following that, the chemistry of lutoid was also being studied by other researchers. Each composition of lutoids and their functions are summarized in Table 1.1.

Table 1.1: Lutoid compositions in latex B-serum and its function. (Source: Jacob et al., 1993)

Structure	**Constituents**	**Functions**
Membrane	**Phosphatidyic acid** **ATPase** **Pyrophosphatase** **NADH-cytochrome c-reductase**	Influx proton pump Outgoing pump of protons Outgoing pump of protons
Vacuoles Contents (pH 5.5)	**Anionic proteins** (hevein) **Cationic proteins** (heveamines A & B) **Hydrolases acids** (acid phosphatase, mannosidase, RNase, DNase, etc.) **Peroxidase**	Strong antifungal activities; allergens Chitinase activities (Martin, 1991.)

Latex B-serum extracted from *H. brasiliensis* rubber tree has long ago been well researched for its antifungal properties and vase industries for rubber products. In recent year, researches involved using this latex B-serum and its sub-fractions to treat human cancers were initiated by Rubber Research Institute Malaysia (RRIM) though it is still in infant stage.

2. 1. 3 Pharmacological activities of *Hevea brasiliensis* latex

Malaysia is a country that hosts thousands of endemic plants and animals with limitless potential for natural products discovery and development. It is often said that the myriad species found in tropical rain forests may harbour cures to many diseases such as cancer, diabetes, hypertension and so on, that afflict the world, and that the development of drugs based on these species could help pay for the forests' conservation. Whilst being the world third largest rubber producers for natural rubber in the world, the potential use of natural rubber and its properties shall be exploited for more industries and not only limited to rubber industry.

Since 1991, it was founded that hevein, as a small cysteine-rich protein in the latex of *H. brasiliensis*, displayed antifungal properties against eight fungi tested (*Botrytis cinerea, Fusarium culmorum, Fusarium oxysporum* f. sp. pisi, *Phycomyces blakesleeanus, Pyrenophora triticirepentis, Pyriculariaoryzae, Septoria nodorum,* and *Trichoderma hamatum*) (Parijs et al., 1991). It was suggested in the same study that the antifungal properties of the hevein might be due to the chitin-binding properties of hevein.

Later in the year of 1999, it was reported that both lutoidic and cytoplasmic (L-serum and C-serum, respectively) did not show yeast cell growth inhibition (Giordani et al., 1999). However, determination of glycosidic activities in latex L-serum from *H. brasiliensis* showed strong activity of N-acetyl-β-D-glucosaminidase and, in a lesser level, α-D-mannosidase activity, two enzymatic activities which are potentially able to degrade the cell wall of *Candida albicans* (Giordani et al., 1999). Also, in the same article, it was reported that synergy occurs when the antifungal effect resulting from a combination of low concentration of latex serum and fluconazole is greater than the sum of separate activities of each member of that combination (Giordani et al., 1999). Subsequently, it was also evidenced that there is a synergistic effect between *H. brasiliensis* latex serum and amphotericin B against *C. albicans* (Giordani et al., 2002). As the technology advanced, a much simpler method to obtain a pure hevein sample from *H. brasiliensis* was developed by Kanokwiroon et al., (2008). The results showed a strong inhibitory effect of that pure hevein sample against *Candida spp* (Kanokwiroon et al., 2008). It was predicted that proteins of not larger than 15-20 kDa will pass through the fungal cell wall, while hevein with a molecular size of 4.7 kDa could penetrate the fungal cell wall matrix and exhibited the antifungal property (Kanokwiroon et al., 2008).

Recently, preliminary experiments carried out using the rubber extract from *H. brasiliensis* sub-fractions on various cancer-origin and non-cancer-origin cell lines have shown that specific sub-fraction (Latex B-serum with higher molecular weight proteins – BHM) exerted interesting anticancer properties (Ong et al., 2009). The sub-fractions were able to specifically reduce high number of cancer-origin cells, Hela cells as used in this particular study, with a therapeutic index equivalent or

higher than the existing anticancer drugs found in the market. Meanwhile no anti-proliferation effects were observed in treated non-cancer-origin cells.

2. 1. 4 Traditional uses of *Hevea brasiliensis* latex

The primary product of rubber in the manufacture of a variety of rubber products are for the automotive, mining, agriculture, shipping, chemicals, pharmaceutical, and consumer industries. A secondary product is lumber. Other rubber products is manufactured for medical and consumers uses are inflatable, footwear, sporting goods, toys, bicycles and motorcycles tires, and latex products such as globes, prophylactics, medical tubings, and feeding bottle nipples.

2.2 Toxicity Evaluation

Toxicity, also known as the adverse effects can be tested using various biological assays. The development of methods of biological assay which estimate the potency of solutions from observations of their effects on living tissues is one of the fundamental achievements of pharmacology. During the past decade, traditional systems of medicine have become increasingly important in view of their safety. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs (Krishnaraju et al., 2006). Knowledge of the potential toxicity on new synthetic chemicals was absent prior to exposures of the general public has caused a in the mid-twentieth century (Bhardwaj & Gupta, 2012). Hence, the toxicity remains the first issue to be solved before any further steps taken for anticancer drug development for human consumption.

2.2.1 Acute, sub-acute and chronic toxicity test

These types of toxicity test usually involved at least a rodent and a non-rodent animal for potential toxicity evaluation due to the different responses of the species towards a toxic agent.

Acute toxicity test was generally the first test conducted with single or a brief exposure, either by route of oral, dermal or inhalation exposure. Young adults of rats or rabbits are in preference to be the basic parameters for acute toxicity tests. It is often used to determine the therapeutic index, i.e. ratio between the lethal dose and the pharmacologically effective dose in the same strain and species (LD_{50}/ ED_{50}). The greater the index, the safer is the compound (Bhardwaj & Gupta, 2012).

In chronic toxicity test, repeated-dosing toxicity studies are essential to determine the side effect/ toxicity from the exposure for a drug at lower dosage than those used in acute toxicity studies. It also determines safe dosages to be used in the initial human clinical trials. The duration for chronic toxicity test is set from 1 to 2 weeks to 1 to 2 years; depended mainly upon the requirement of treatment and the 118 Animals in Drug Development intended clinical dosing regimen. Chronic toxicity test is encouraged to be conducted in stages so that the results of one study can be used to design the subsequent study of a longer duration. For instance, the first stage is set at 2 weeks, followed by 1-month, 3-month, 6-month and then 1-year studies. In selecting the animals for chronic toxicity test, young adults of at least two species, rodent and non-rodent as in rat and dog are the basic parameters. The number of test animals should also be considered for their gender of each species (Bhardwaj & Gupta, 2012).

Sub-acute toxicity test lies in between acute and chronic toxicity test where it is employed to determine toxicity which is likely to arise from repeated exposures of several weeks to several months. Various standardized tests for sub-acute test that are available such as oral, dermal and inhalation exposure have been developed. Depending on the exposure route to be tested, rodents such as rats and rabbits, while non-rodent such as dogs will be used as the basic parameter for short term assessment (Bhardwaj & Gupta, 2012).

2.2.2 Brine Shrimp Lethality Test (BSLT)

Considering the animal welfare issues, cost and time consumption issues, brine shrimp lethality test (BSLT) is another option used for natural products extracts, fractions or pure compounds toxicity test. It is indeed easily mastered, costs effective, and utilizes a small amount of test materials (Pisuthanan et al., 2004) compared to previously described methods. For instance, the brine shrimp lethality test has advantages of being rapid (24 hours), inexpensive (readily available at pet shop in dry from), and simple (e.g. no aseptic techniques are required). It easily utilizes a large number of organisms for statistical validation using Probit Analysis, and requires no special equipment and a relatively small amount of sample (2 – 20 mg or less). In addition, it does not require animal serum as is needed for cytotoxicities (McLaughlin et al., 1998). It has been suggested that, animal models would not give more information than *in vitro* screening using a large panel of human tumour cell lines. Hence, major efforts were dedicated to the development of *in vitro* assay based on a large panel of human cell lines representing various tumour types (Teicher, 1997). It was reported that *in vivo* lethality test has been successively employed for bioassay-guided fractionation of active cytotoxic and antitumor agents such as

trilobacin from the bark of *Asimina triloba* (Zhao et el., 1992), *cis*-annonacin from *Annona muricata* (Rieser et al., 1996) and ent-kaur-16-en-19-oic acid from *Elaeoselinum foetidum* (Mongelli et al., 2002). According to the classification by Meyer et al. (1982), crude extracts and pure substances are classified as toxic when LC_{50} value <1000 µg/ml or non-toxic when LC_{50} >1000 µg/ml.

2.3 Anticancer properties

2.3.1 *In-vitro* cytotoxicity assay

The evaluation of compounds from plant extract, such as cytotoxic agents, necessitates screening of a great number of chemicals. Animal models have always played an important role in drug evaluation, but with the development of a large number of cytotoxic drugs, animal models are too costly and the delay is too long for the models to be used for high-throughput screening. Furthermore, experiments involving animals are often facing more hindrance due to ethical issues; cell-based assay is therefore a good approach to gather enough experimental data before moving to animal models.

Cell-based toxicity test is also known as *in-vitro* cytotoxicity test. *In vitro* (literally 'in glass') testing methods are employed primarily to identify potentially hazardous chemicals and/or to confirm the lack of certain toxic properties in the early stages of the development of potentially useful new substances such as therapeutic drugs, agricultural chemicals and food additives. Most toxicologists believe that *in vitro* toxicity testing methods can be more useful, more time effective and cost effective than toxicological studies in living animals (which are termed *in vivo* or "in life" methods).

There are various types of endpoint assay can be conducted for cytotoxicity evaluation. Several endpoint assays are briefly discuss in the following:

Cell viability endpoint assay using (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) salt or MTT, is one of the simple, accurate and yields reproducible results, yet common and reliable cell-based cytotoxicity endpoint assay. This method measures on the mitochondrial activity of the live and viable cells after treatment. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, leading to the formation of purple crystals which are insoluble in aqueous solutions. The crystals are re-dissolved in acidified isopropanol and the resulting purple solution is measured spectrophotometrically. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material. The more is the amount of formed formazan, the safer is the substance. This treatment destroys the cells under investigation allowing only a single time point measurement and it is generally cytotoxic (Mossman, 1983). Currently there is an updated version of the validated MTT method, called MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 tetrazolium) assay has the advantage of being soluble and hence no solubilisation step is required. MTT assay is employed in this study.

Another cell viability endpoint can be Neutral Red uptake. Neutral Red (NR), a weak cationic dye penetrates cellular membranes by non-diffusion and accumulates intercelluarly in lysosomes. Viable cells take up the NR dye, damaged or dead cells do not. In fact, Neutral red (3-amino-m-dimethylamino-2-methyl- phenazine hydrochloride) has been used previously for the identification of vital cells in cultures. This assay quantifies the number of viable, uninjured cells after their

exposure to toxicants; it is based on the uptake and subsequent lysosomal accumulation of the supravital dye, neutral red. Quantification of the dye extracted from the cells has been shown to be linear with cell numbers, both by direct cell counts and by protein determinations of cell populations (Borenfreund & Puerner, 1985, 1986).

Besides measuring the cell viability after exposure to the toxicants, there are also assay that measured on the cell death. For instance, one parameter for cell death is the integrity of the cell membrane, which can be measured by the cytoplasmic enzyme activity released by damaged cells. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane (Korzeniewski & Callewaert, 1983). The LDH activity is determined in an enzymatic test. The first step is the reduction of NAD+ to NADH/H+ by the LDH-catalyzed conversion of lactate to pyruvate. Following of that, the catalyst (diaphorase) transfers H/H+ from NADH/H+ to the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazoliumchloride (INT), which is reduced to a red formazan (Decker and Lohmann-Matthes, 1988; Lappalainen et al., 1994; Nachlas et al., 1960). The greater the red formazan formed, more toxic is the substance.

Another possible endpoint assay is measured on the total protein content of the treated cells. This Sulforhodamine B (SRB) assay is based on binding of the dye to basic amino acids of cellular proteins, and colorimetric evaluation provides an estimate of total protein mass, which is related to cell number. This assay has been widely used for the in vitro measurement of cellular protein content of both adherent and suspension cultures. The Sulforhodamine B method, is simple, accurate and yields reproducible results. The cells are briefly washed, fixed,

and stained with the dye. The incorporated dye is then liberated from the cells with a Tris base solution. An increase or decrease in the number of cells (total biomass) results in a concomitant change in the amount of dye incorporated by the cells in the culture. This indicates the degree of cytotoxicity caused by the test material. The advantages of this test as compared to other tests include better linearity, higher sensitivity, a stable endpoint that does not require time-sensitive measurement, and lower cost. The disadvantage lies in the need for the addition of trichloroacetic acid (TCA) for cell fixation. This step is critical because, if not added gently, TCA could dislodge cells before they become fixed, generating possible artifacts that will affect the results (Giuseppe et al., 2006).

Cell lines in culture, which are commonly used in many *in vitro* studies, are usually *in vitro* derived from tumours and are thus transformed. Human cells derived from normal tissues need to be immortalized. In other *in vitro* approaches primary cells are used. These cells can only be kept in culture for a certain time (hours to days) and in many cases will lose their differentiated properties during this culture time. These intrinsic weaknesses result from the fact that, *in vivo*, the cells are isolated from their natural environment and are no longer integrated into an ordered tissue and organ topology. This entails intrinsic limitations concerning reduced survival, imbalance of metabolic competence, and lack of cell to cell interaction, destroyed organ topology and absence of tissue communication. Furthermore, the balance of metabolic activation and inactivation requires a highly ordered interplay of many enzymes and cofactors in most cases (Eisenbrand et al., 2002).

2.4 *In vitro* Antifungal Susceptibility Testing (AST)

Plants have their intrinsic ability to resist pathogenic microorganisms and this has led the researchers to investigate their mechanisms of action and isolation of active compounds. In view of the large number of the plant species, potentially available for the study, it is essential to have efficient systems of the methods to evaluate efficacy of medicinal plants as antimicrobial agent. The evaluation for antimicrobial agent of plant origin begins with thorough biological evaluation of plant extracts to ensure efficacy and safety followed by identification of active principles, dosage formulations, efficacy and pharmaco-kinetic profile of the new drug. This novel search entails extensive research and it is therefore imperative to follow standard methods to authenticate claims of antimicrobial action (Das et al., 2009). There are three main methods to test on antifungal susceptibility, i. e. diffusion, dilution and bioautography (Cos et al., 2006).

2.4.1 Agar disc diffusion method

Agar disc diffusion method of antimicrobial test was first developed in 1940 (Heatley, 1944), as a modification of that described by Bauer, Kirby, Sherris and Truck (commonly known as Kirby-Bauer test) (Bauer et al., 1959, 1966). It was accepted by NCCLS (which now named as Clinical and Laboratory Standard Institute, CLSI) and has been widely used nowadays to assay plant extract for antimicrobial activity (Freixa et al., 1996; Salie et al., 1996; Ergene et al., 2006).

The disc diffusion method allows for the simultaneous testing of a large number of antimicrobials in a relatively easy and flexible manner. There are three

categories of antimicrobial level to be assigned based on the result of the diameter of the inhibition zone and the Clinical and Laboratory Standard Institute (CLSI) interpretative criteria, namely susceptible, intermediate, or resistant. Of course, the bigger the diameter of the inhibition zone, the more susceptible is the microorganism to the antimicrobial. However, one of the major disadvantages of this method is unable to generate a quantitative value such Minimum Inhibitory Concentration (MIC) value and it is difficult to examine the susceptibility of fastidious and slow-growing bacteria (Wilkins & Thiel, 1973; Dickert et al., 1981). Furthermore, different from antimicrobial agents used in clinical settings, there are currently no standard CLSI interpretive criteria of disc diffusion results to support natural antimicrobials susceptibility testing; thus, it is unable to explain the zone diameter generated by disc diffusion for natural antimicrobials. Besides, disc diffusion is labor-intensive and time-consuming (Klancnik et al., 2010).

It has been reported (Klancnik et al., 2010) that this method is not always reliable for determining the antimicrobial activity of natural antimicrobials, i.e., plant extract, because the polarity of the natural compounds can affect the diffusion of compounds onto the culture medium. Compounds with less polarity diffused slower than more polar ones (Moreno et al., 2006). Due to these concerns, disc diffusion may not be a suitable one to determine the antimicrobial activity of natural compounds.

2.4.2 Dilution methods

Broth dilution method has been categorized into macrodilution and microdilution methods. Both the dilution methods have been widely used for many years to

accurately measure antibacterial and antifungal activity as well as susceptibility testing. It is important to note that the substances normally tested by these methods are generally hydrophillic in nature (Janssen et al., 1987).

The earliest antimicrobial susceptibility testing methods was the macrodilution method. This procedure involved preparing two-fold dilutions of samples in a liquid growth medium, also called broth, dispensed in test tubes (Ericson & Sherris, 1971). After certain period of incubation of the samples with the microbes, the tubes were examined for visible bacterial or fungal growth as evidenced by turbidity. The lowest concentration of substances that prevented growth represented the minimal inhibitory concentration (MIC). The precision of this method was considered to be plus or minus 1 two-fold concentration due in large part to the practice of manually preparing serial dilutions of the substance. The advantage of this technique was the generation of a quantitative result (i.e., the MIC). The principal disadvantages of the macrodilution method were tedious, manual task of preparing the sample solutions for each test, the possibility of errors in preparation of the sample solutions, and the relatively large amount of reagents and space required for each test (Jorgensen & Ferraro, 2009).

The miniaturization and mechanization of the test by use of small, disposable, plastic “microdilution” trays has made broth microdilution testing practical and popular. It is a widely utilized method, allowing for the simultaneous testing of multiple antimicrobials with ease particularly when commercially prepared microtiter trays are used. Hundreds of identical trays can also be prepared from a single master set of dilutions in a relatively brief period (Jorgensen & Ferraro, 2009). Compared with agar-based method, broth microdilution can decrease much labor and time. In this study, microdilution method is employed. The advantages of the