DEVELOPMENT, CHARACTERIZATION AND ANTI-CANCER ACTIVITY OF LIPOSOMAL DOXORUBICIN HYDROCHLORIDE WITH PALM OIL

BAHAREH SABETI

FACULTY OF MEDICINE

UNIVERSITY OF MALAYA

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BAHAREH SABETI

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ABSTRACT

Doxorubicin hydrochloride is an anticancer medicine for treatment of various kind of cancer. Anticancer drugs are known to be highly toxic agent which may kill normal cells while inhibiting the growth of cancer cells. As drug molecules may not be able to distinguish among healthy organ's cells and cancerous cells. Similar to other anticarcinoma medicine, doxorubicin causes many side effects such as cardiotoxicity, myelosuppression, hematologic toxicity, secondary leukemia, extravasations, hepatic Impairment, alopecia and fatigue.

Several studies in field of drug delivery technology have been carried out in order to minimize side effects with enhancing therapeutic effect of doxorubicin. Liposomal drug delivery system had been proven to be one of the delivery techniques for such purpose. Encapsulating doxorubicin within liposome would decrease its toxicity and do not alter its biological activity which lead to increase its antitumor potency. Liposomal doxorubicin is FDA approved anti-cancer for treatment of ovary, lung and breast cancer. This study aimed to prepare liposomal doxorubicin using palm oil (as a part of drug delivery system) which is also known to act as a natural anticancer and antioxidant. Palm oil is rich in natural ß-carotene, γ-carotene, tocopherols and tocotrienols which may support the antioxidant and anticarcinogenic activities of anticarcenoma medicine as well. Liposome formulations were designed in this study by various porpotion of palm oil and phosphatydilcholin. Six formulations containing 0, 5%, 10%, 15%, 20% and 25% of palm oil were prepared through reverse-phase evaporation method. Formation and morphology, particle size distribution, zeta potential, entrapment efficiency and in-vitro drug release and liposome degradation of each formulation were evaluated. Liposome with 10% & 15% of palm oil showed fine formation, satisfactory zeta potential, controlled releasing pattern and less degradation compare with other formulations. Further to develop the entrapment efficiency of liposomes, liposome

formed by freeze-thaw method and pH gradient technique were carried out to active drug loading within vesicles.

Entrapment efficiency and in vitro drug release of liposome assessed using HPLC device. The HPLC results show liposome entrapment efficiency rose up to 98% by active loading with suitable and organized release pattern. Evaluation the cellular uptake and toxicity (MTT assay) of liposomal doxorubicin hydrochloride and Caelyx® (commercial form of pegilated liposomal doxorubicin) on MCF7 and MDA-MBA 231 breast cancer cells present the higher cellular uptake and effective toxicity on cancerous cells. Distribution of liposomal doxorubicin in rat organs studied using *in vivo* imaging device. Images of rat organs after intracardiac injection of liposomal doxorubicin displayed less accumulation of doxorubicin in rats' heart but more in liver, kidney and lungs.

In conclusion the results of this study proved the potential application of palm oil in preparation of liposomal. Significant proportion of palm oil utilize in formulations would improve the physical properties of liposome such as shape, stability, releasing pattern and degradation. Furthermore liposome containing palm oil showed higher uptake and IC50 which develop therapeutic index and desirable bioavailability as well.

ABSTRAK

Doxorubicin adalah ubat anti kanser yang digunakan untuk rawatan pelbagai jenis kanser. Ubat-ubatan anti kanser adalah agen yang diketahui sangat toksik yang mungkin membunuh sel-sel normal semasa menekan perkembangan sel-sel kanser olehkerana molekul ubst tersebut tidak dapat membezakan diantara sel-sel organ yang sihat dan sel-sel kanser. Sama seperti ubat-ubat anti kanser yang lain, doxorubicin menyebabkan banyak kesan sampingan seperti cardiotoxicity, myelosupresi, keracunan hematologi, leukemia menengah, extravasasi, penurunan fungsi hati, alopecia dan keletihan.

Beberapa kajian di bidang teknologi penyampaian ubat telah dilakukan untuk mengurangkan kesan sampingan tanpa mengurangkan kesan terapeutik doksorubisin. Penyampaian ubat Liposomal telah dibuktikan sebagai salah satu teknik pembawa ubut untuk tujuan yang disebutkan. Enkapsulasi doxorubicin dalam liposom akan menurun toksisitasnya disamping tidak mengubah aktiviti biologi dan menjurus kepada peningkatan potensi antitumor. Liposomal doksorubisin telah diluluskan oleh FDA untuk rawatan anticacinoma ovari, paru-paru dan kanser payudara.

Matlamat kajian ini adalah untuk menyediakan liposom doxorubicin dengan menggunakan minyak kelapa sawit yang di ketahui adalah juga agent antikanser dan anti oksidan semulajadi. Minyak kelapa sawiot adalah kaya dengan ß-carotene, γ-carotene, tocopherols and tocotrienols yang akan menyokong aktiviti antioksidan dan antikanser. Formulasi liposom-liposom telah direkabentuk dalam kajian ini dengan kandungan minyak kelapa sawit danphosphatydilcholin dalam jumlah berlainan. Enam formulasi diujudkan yang mengandungi 0%, 5%, 10%, 15%, 20% and 25% telah disediakan menggunakan kaedah “reverse-phase evaporation”. Formasi dan morfologi, size partikal, distributsi, zeta potential, “entrapment efficiency” dan perlepasan ubat secara in-vitro dan degridasi liposom untuk setiap formulatsi telah dinilaikan.

Liposom dengan 10% & 15% minyak kelapa sawit menunjukan formasi yang baik, showed fine formation, “zeta potential” yang sesuai, corak perlepasan yang terkawal dan degradasi yang lebih rendah jika dibandingkan dengan formulasi yang lain. Dengan itu kajian pelengkap telah dijalankan keatas formulasi yang mengandungi 10% & 15% minyak kelapa sawit untuk menambahbaikan “entrapment efficiency” dan indek therapeutic mereka. Liposome baru telah disediakan menggunakan kaedah “freeze-thaw”dan “pH gradient technique” dijalankan sebagai kaedah actif untuk muatkan ubat. “Entrapment efficiency” dan perlepasan ubat secar in-vitro telah dinlai menggunakan alat HPLC. Keputusan dari HPLC menunjukan “entrapment efficiency” mencapai 98% dengan pemuatan aktif. Kajian pengambilan selular dan toksisiti (esei MTT) seterusnya dijalankan keatas liposome-liposom tersebut, Doxorubicin hydrochloride dan Caelyx® (produk kormersial untuk liposom pegilated doxorubicin) telah dikaji di atas sel-sel kanser payudara MCF7 dan MDA-MBA 231. Liposom yang mengandungi 10 &15% minyak kelapa sawit mempamerkan pengambilan sel yang lebih tinggi dan toksisiti yang berkesan keatas sel-sel kanser. Kajian in-vivo telah dijalankan untuk siasatan distribusi liposom-liposom doxorubicin hydrochloride dan Caelyx® dalam organ-organ tikus. Didapati liposom-liposom yang direkabentuk menakumulasi rendah pada jantung tikus tetapi mengakumulasi lebih tinggi dalam hati, ginjaldan paru-paru.

Sebagai kesimpulan keputusan-keputusan dari penyelikan ini membuktikan potensi applikasi minyak kelapa sawit dalam sedian liposom. Kandungan sigifikan minyak kelapa sawit yang digunakan dalam formulasi-formulasi akan membaiki sifat-sifat fisikal liposom seperti rupa bentuk, kestabilan, corak perlepasan ubat dan tahap deradasi. Selain daripada itu liposom-liposom yang mengandungi minyak kelapa sawit menunjukan pengambilan oleh sel-sel yang lebih tinggi dan IC50 yang membentukan indek therapeutik kebioperolehan yang baik.

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LIST OF ABBREVAITION

TEM	Trransmision Electron Microscopy
SEM	Scaniing Electron Microscopy
ZP	Zetapotential
LUV	Large Unilamellar Vesicles
RES	Reticulo-endothelial
µg	Microgram
Ml	Mililiter
Mg	Miligram
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
PMN	Polymorphonuclear neutrophils
PEG	PolyEthylenGlycol
Min	Minute
^{o}C	degree Celsius
FDA	Food & Drug administration
UV	Ultaviolet
EE	Entrapment efficiency
DR	Drug release
RSD	% relative standard deviation
LOD	Limit of detection
LOQ	limit of quantification

CHAPTER 1: INTRODUCTION

1.1 Background of Research

Cancer is caused by abnormal growth of cells in the body and is the second major cause of death, after cardiovascular diseases, which affect people of every age, race, or gender. The type of treatments that are prescribed for cancer usually takes into account the cancerous organs and its corresponding stage, and involves surgery, chemotherapy, hormone therapy, gene therapy, and radiation therapy (Killeen, 2004; Pawaiya et al., 2011). The variety of cancers differs between races and gender; common organs afflicted with cancer are the breast, prostate, lung, kidney, bladder, and colon. Since chemotherapy is being prescribed as a form of treatment on its own or with surgery, there are many reports on drug delivery in the context of chemotherapeutic medicine.

Researchers are intensely studying the optimization of drug delivery for anticancer drug as anticancer drugs cause dangerous side effects and this renders anti-carcinoma drugs central to drug delivery studies. Anticancer medicines are highly poisonous, kill cells, and inhibit their growth, which will affect healthy tissues as well. This is a problem, as drug molecules are incapable of distinguishing healthy or infected tissues. The objective of drug delivery technology is to selectively target cancerous cells. The low therapeutic index of chemotherapeutic drugs requires higher dosages, which increases its risk. However, with the technological advance of drug delivery, smaller dosages of toxic medicine would be recommended, which leads to the reduction of adverse effects and toxicities (Killeen, 2004; Pawaiya et al., 2011).

Current efforts in the area of drug delivery include sustained drug release, development of targeted delivery, and nano-sizing. Sustained drug release extends the release time to prolong blood circulation time, reduce dosages and toxicity. Targeted drug delivery is the act of coupling a targeting antibody or ligand with drug carriers, such as liposomes. The selective system will directly target a specific site. By targeted drug delivery, the therapeutic index of medicines would increase and reduce their toxicity. Nano-sizing

reduces the size of the drug carrier, which is commonly utilized for antineoplastic drug delivery. Nano-drugs results in higher drug/carrier ratio compared to polymer conjugates, and higher degrees of protection against enzymatic degradation. Furthermore, the efficacy of nano anti-tumor drugs will improve and reduce their toxicity levels to normal healthy tissues. The nanocarriers are generally utilized for anticarcinoma medicines such as liposomes, polymeric micelles, niosomes, solid lipid nanoparticles (SLN), and nanoparticles and nanoshells (T. M. Allen & Moase, 1996; Brigger et al., 2002; Jain et al., 2007; Verma & Garg, 2001).

Liposome technology is the most studied nanocarrier, due to its purported benefits. Liposome is aqueous phospholipid vesicles formed by spherical phospholipid bilayers with an aqueous inner space. Liposome delivery systems are capable of encapsulating both hydrophilic and hydrophobic medicines inside their respective structures. They are composed of natural and nontoxic ingredient, and are naturally degraded within the body. Developing liposomal drug delivery is highly recommended for low solubility and/or toxic drugs. Encapsulating toxic drugs such as antineoplastic medicines within liposome would reduce the toxic exposure to normal organ or tissues (Akbarzadeh et al., 2013; Fang, 2006; Pinto-Alphandary et al., 2000). Liposome drug delivery and nano-sizing can amplify its therapeutic index. Nano-liposomes increase its uptake and bonding of polymers and antibody within or on the surface of liposome, increasing its selectivity and reducing its toxicities. Liposome drug delivery also enhances the circulation time and half-life of the drugs with a sustained release effect (T. M. Allen & Moase, 1996; Fang, 2006; Fenske et al., 2008; Immordino et al., 2006).

1.2 Problem statement

Doxorubicin is an antineoplastic drug that is widely administered for a variety range of cancers. It is a cytotoxic agent from anthracycline antibiotic, which inhibits the proliferation of cancerous cells and tumors. Although Doxorubicin effectively shows

results with cancer therapy, the application of this drug is far from feasible, due to its cardiotoxicity (F Arcamone et al., 2000; Hortobagyi, 1997; Zheng et al., 2006). Doxorubicin causes both acute and delayed heart failure, while the administration of liposomal is clinically proven to be less cardiotoxic. The usage of Liposomal Doxorubicin has been approved by WHO for treating breast, ovary, and lung cancers, due to better efficacy and lower cardiotoxicity (Hortobagyi, 1997; Tardi et al., 1996; Thorn et al., 2011). Liposomal doxorubicin currently available in the market is very expensive. New, cheap formulation of doxorubicin liposome using local resources is needed so as to make liposomal doxorubicin available to cancer patient in an affordable manner.

However, liposome drug delivery is also challenging, due to the lack of drug loading and the stability of vesicles. Further characterization of liposome, such as size, morphology, and the composition of lipids affected the uptake and distribution of liposomal drug carrier as well (Tardi et al., 1996).

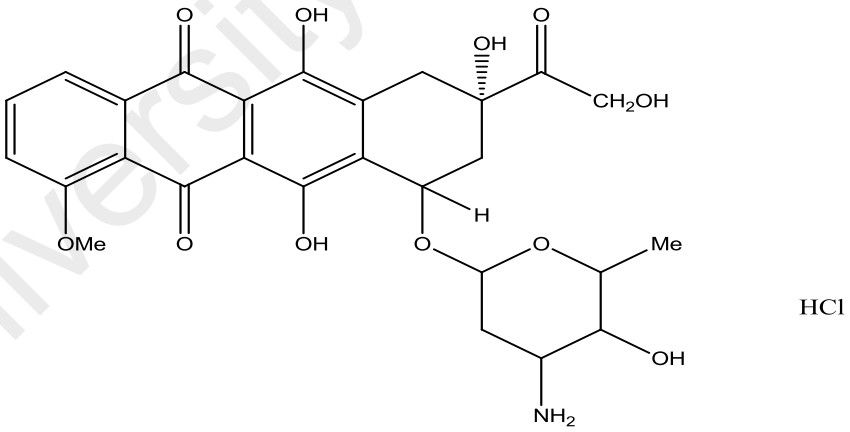


Figure 1.1: Doxorubicin hydrochloride structure

1.3 Objectives of Study

1.3.1 General Objectives

This study focuses on developing liposomal doxorubicin by utilizing the anticancer benefits of palm oil as a natural antioxidant. Palm oil has found to be rich in natural

antioxidant and anticarcinogenic chemicals, such as ß-carotene, γ-carotene, tocopherols, and tocotrienols. Palm oil consists of 50% saturated fatty acids, 39% monosaturated fatty acids, and 10.9% polysaturated fatty acids (Edem, 2002; Sundram & Chandrasekharan, 2000; Sundram et al., 2003). The main components in palm oil are palmitic acid, oleic acid, and linoleic acid. Oleic acid and linoleic acid are unsaturated fatty acids which are known to reduce cholesterol and low density lipoproteins (LDL) level of blood as well (Hassel et al., 1997; Hayes et al., 1991; O'brien, 2010).

Apart from the antioxidant and lipid lowering properties, carotenoids, tocopherols and tocotrienols also act as anti-proliferating agent that is responsible for inhibiting the growth of human breast and prostate cancer cells (Guthrie et al., 1997; Nesaretnam et al., 2004; Srivastava & Gupta, 2006). Due to anti-proliferating benefits of palm oil, this study aimed to apply some percentages of palm oil in preparing liposomal doxorubicin in order to modulate target delivery and reduce its toxicity to the normal tissues.

1.3.2 Specific Objectives

1. To prepare and characterize liposome with several percentages of palm oil
2. To make recommendation based on the best liposomal doxorubicin formulation
3. To improve drug loading and stability of liposome
4. To investigate the in vitro releasing pattern of liposomal doxorubicin
5. To determine the cellular uptake of formulated liposomal doxorubicin
6. To evaluate toxicity of liposomal doxorubicin on breast cancer cell lines
7. To assess the in vivo distribution of liposomal doxorubicin

CHAPTER 2: LITERATURE REVIEW

2.1 Liposome

2.1.1 What is Liposome?

Bangham discovered liposome in 1965. They are single or multi lipid layers that circle an aqueous core known as spheral vesicles (Bangham et al., 1965; Lautenschläger, 2006). The term Liposome is derived from two Greek words: 'Lipos', meaning fat, and 'Soma', meaning body. Taking into account the structure of liposome, there are various sizes of vesicles with bilayer lipid membrane (same as body cells) that entraps aqueous capacities within their organization (Dua et al., 2012). Liposomes are mainly composed of phospholipids, which are molecules containing hydrophilic head group with a hydrophobic tail group in the form of a long hydrocarbon chain (Akbarzadeh et al., 2013; Dua et al., 2012). Due to Bangham, liposome would form automatically, owing to self-assembly of the phospholipids. After phospholipids diffuse into the aqueous phase, vesicles will take shape, while their hydrophobic chains tend to move away from the aqueous media (Bangham et al., 1965; Y. Barenholz, 2001; Couvreur, 2013).

Since liposome was discovered, interest in liposomal-base drugs has increased, due to its pharmaceutical benefits and its ability to encapsulate a variety of drugs (Akbarzadeh et al., 2013). Vesicles generally contain hydrophilic head and a hydrophobic tail; therefore, they are capable of delivering both hydrophilic and hydrophobic drugs (Heldt et al., 2001; Mu & Zhong, 2006; Rutherford, 2011). Liposomal-base drugs can also be administered via several routes, such as oral, intramuscular, intraperitoneal, subcutaneous, inhalation, and ocular form, plus they have great advantages such as biodegradability, biocompatibility, and nontoxicity (L. V. Allen & Popovich, 2005; Y. Barenholz, 2001; Hathout et al., 2007; Lasic & Papahadjopoulos, 1998).

On the other hand, liposome have a great potential to be developed and optimized for encapsulation and delivering toxic and/or poorly soluble drugs, enzymes, and/or biologically active materials, cancer therapy, infection therapy, DNA and/or Gene

therapy and vaccine therapy to improve their respective pharmacological procedures (Hathout et al., 2007; Lasic & Papahadjopoulos, 1998). Encapsulating medicines into liposome can reduce their toxicities and adverse effects on normal tissues and targeted drug delivery, and sustain its release effect (Chen et al., 2010; Massing & Fuxius, 2000; R M Schiffelers et al., 2005). Examples of liposomal medicine that have been approved for human use are Ambisome®, Doxil®, Caelyx® and DaunoXome®. Ambisome® is Amphotericin B, and used to treat serious fungal infections, while Doxil® and Caelyx® are Doxorubicin hydrochloride, and DaunoXome® is Daunorubicin citrate, both of which are antibiotics used as anticancer drugs (L. V. Allen & Popovich, 2005; Barratt, 2000; Dua et al., 2012; Lasic & Papahadjopoulos, 1998).

2.1.2 Liposome compositions

Lipid composition plays a significant role in the formation of liposome and its physicochemical treatment properties, such as distribution, lifetime, and blood clearance (A. Gabizon et al., 1990; Grazia Calvagno et al., 2007). Phospholipids and cholesterol are generally the primary components of liposome (Akbarzadeh et al., 2013). Phospholipids are amphophilic molecules containing hydrophilic head group (charged or uncharged polar head) and lipophilic tails, which are composed of long fatty acid hydrocarbon chains (Lasch et al., 2003; Rutherford, 2011). Increasing the degree of acyl chain saturation along the chain length would enhance the maintenance of encapsulated materials in circulating liposome. Vesicles containing certain acidic phospholipids, such as phosphatidylserine, are rapidly removed from circulation by the cells of the reticuloendothelial system (RES), which reside primarily in the liver and spleen, whereas insertion of the glycolipid GM1 can dramatically increase liposome blood residence time. Increasing the lipid's dose also substantially increase the circulation lifetime of liposomes. This relationship is observed until the amount of administered lipid is sufficient to saturate the reticuloendothelial system RES, at which point further

increases in the lipid dose have little effect on the relative liposome clearance (Li & Vance, 2008).

Five main groups of phospholipids were use to prepare liposome are listed below (Doherty, 2004):

1. Phospholipid from natural sources
2. Phospholipids which was modified from natural sources
3. Semi-synthetic phospholipids
4. Fully-synthetic phospholipids
5. Phospholipids with non-natural head groups

Phosphatidylcholine is located at 50-90% of the outer part from both plants and animal cells' membrane (Rutherford, 2011). Phosphatidylcholin are derived from soy and egg yolks are the most common phospholipids used in the preparation of liposome (Eibl, 1984; Li & Vance, 2008). Since they are naturally composed of unsaturated fatty acids and have a high tendency to oxidize, adding antioxidants such as a complex of vitamin C and E during the making of liposome might improve the stability of the vesicles (Samuni et al., 2000; Schnitzer et al., 2007; Senior & Gregoriadis, 1982).

Cholesterol is another important composition of liposome, with a steroid structure (Chrai et al., 2002). It decreases the membrane fluidity of vesicles, which reduces the movement of fatty acid chains, avoiding leakage of the entrapped drugs from the liposome (Alves et al., 2013; Cócera et al., 2003; Kirby et al., 1980). Cholesterol keeps the vesicles stable and protects them from being destroyed or disintegrated by lipoproteins in the blood (Benz & Park, 2004). The absence of cholesterol can result in lipoprotein-induced vesicle destabilization and associated release of the entrapped drug (Alves et al., 2013; Cócera et al., 2003).

2.1.3 Advantages and applications of liposome

Liposom as an advance drug delivery system, were researched during the last 25 years, while some liposomal-based medicine are currently available on the market and are being clinically tested (Fang, 2006; Fenske et al., 2008). They are biocompatible, biodegradable, and nontoxic carrier, due to their natural building blocks (Hofheinz et al., 2005; Johnston et al., 2007). Since liposome was discovered, it acts as a beneficial and unique drug delivery system, which is capable of carrying both hydrophilic and hydrophobic drugs (Mu & Zhong, 2006).

Liposome can improve drug selectivity and reduce toxicities, and also increase the circulation time and half-life (Johnston et al., 2007). They are also known to facilitate drug delivery for significant objects, such as tumors, and protect it via circulation and accumulation within tumor sites, while avoiding anticancer drugs from damaging healthy tissues as well (Fenske et al., 2008). Encapsulating toxic drugs into liposome, such as antineoplastic drugs, would reduce their toxicities on healthy tissues and organs; for example, liposomal doxorubicin causes less cardiotoxicity compared with non-liposomal forms (Benz & Park, 2004; R M Schiffelers et al., 2005). Furthermore, liposome is the proper way to deliver poorly soluble medicines, such as Amphotericin B, where it is delivered systemically by encapsulating it in liposomes and improve its therapeutic index (Omri et al., 2002; R. Schiffelers et al., 2001).

On the other hand, encapsulating biologically active materials is the most recent utilization of liposome (Akbarzadeh et al., 2013). Encapsulations of amino acids, vaccines, and nucleic acids in liposome are new approaches of delivering biological principals in order to protect them from deactivation and degradation by enzymes in plasma (Immordino et al., 2006; Torchilin, 2005).

The main therapeutic and commercial aims of liposome formulation are development of oral bioavailability, decrease in viability and food dependency, development of

intravenous injection formulations, drug targeting to specific tissues (with reduction of general toxicity) and life cycle management (protection by propriety formulation techniques) (Nastruzzi, 2004).

2.1.4 Limitations of liposome

Liposome has a great potential in delivering a variety of treatments, but the market availability of liposome-based drug formulations is still scarce (Nastruzzi, 2004). The lack of sterilization methods, low stability, low efficiency or drug loading, and few reproducible methods are some of the challenges in producing liposomal-form drugs (Lasic & Papahadjopoulos, 1998; Meure et al., 2008).

Since liposome is composed of phospholipids (which are trending to oxidation and hydrolysis), it might cause physical and chemical instability, both of which are essential in their respective development and formulation (Sharma & Sharma, 1997). The addition of antioxidants may improve the stability, while the addition of cholesterol reduces leakages of drugs and stabilizes the fluidity of the membranes, thus improving the physical stability of the liposome (Chrai et al., 2002; Lautenschläger, 2006).

Despite the fact that until now, there are only a few liposome-based medicines that are available in the marketplace, it is expected that the number of liposomal formulations will increase, due to their various benefits. Furthermore, many researches are interested in optimizing and applying liposomal-base of drugs, and so far, many companies developed strategies to modify existing liposome or apply liposomal delivery technology to provide new liposomal medicine to the market. The main efforts in the development of liposome are related to optimizing: drug loading and efficiency, drug delivery and targeting, releasing manner as controlled or sustained plus physical and chemical stability of vesicles as well (M. H. kumar, 2011; Nastruzzi, 2004).

2.1.5 Classification of liposome

Liposomes are classified by several characters, such as their methods of preparation, structural properties, and compositions (such as natural, synthetic, cationic, conventional, pH sensitive & immune liposome (Sharma & Sharma, 1997). Liposome may contain one or more membranes, and their sizes vary, from 0.025 μm to 25 nm. The vesicle size is an important parameter in determining the circulation half-life of liposomes hence both size and number of bilayers affect the amount of drug encapsulation in the liposomes (Akbarzadeh et al., 2013). Table 2.1 lists the main types of liposome based on their structure and size character (Riaz, 1996).

Table 2.1: Liposome's classification based on structure and size.

Vesicle types	Diameter size	Number of lipid bilayer
Small unilamellar vesicles (SUV)	20-100nm	One
Large unilamellar vesicles (LUV)	>100nm	One
Multilamellar vesicles (MLV)	>0.5μm	Five to twenty
Oligolamellar vesicles (OLV)	0.1-1μm	Approximately five
Multivesicular vesicles(MMV)	>1μm	Multi compartmental structure

2.1.6 Methods of preparation of liposome

There are several methods of preparing liposome with a variety of lipid mixture, and phospholipids are the basic preparation constituent of any liposome (Akbarzadeh et al., 2013). Basically, there are conventional and advanced methods of synthesizing liposome (Riaz, 1996). Conventional methods that are being used include thin film hydration, reverse-phase evaporation, ethanol or ether injection, and detergent reduction (Mozafari, 2005). Also, there are advanced methods, such as freeze-drying of mono phase solutions, freeze-thaw techniques, and supercritical reverse-phase evaporation method (Meure et al., 2008). For better understanding of the formation of various vesicles, some of these methods are discussed below.

2.1.6.1 Methods of preparation MLV

Multilamellar vesicles (MLV) contain one to twenty bilayers, with a general size distribution of over 0.5 μm (Riaz, 1996; Shaheen et al., 2006). This type of liposome is prepared by several methods, such as:

A) Thin film lipid hydration Method

This is the common technique of preparing liposome, which also known as the Bangham method. It is a fast and easy way to approach liposome by dissolving lipids in an organic phase within a round-bottom flask, then removing the organic phase under pressure (Bangham et al., 1965; Meure et al., 2008). Afterwards, a thin film lipid would remain at the bottom of the flask and is rehydrated with an aqueous buffer. In addition, the mixture would shake or vortex to detach the swelling lamellae from the surface of the vessels, and organize the resulting liposomes (Bangham et al., 1965; Riaz, 1996). This method produces heterogenous-sized of MLV multi lamellar vesicles that are capable of encapsulating a variety of drugs within them (Riaz, 1996). However, the utilization of this technique is limited, because it requires a large amount of organic solvent, which is harmful to the environment and results in low entrapment efficiency of the vesicles (Meure et al., 2008; Szoka et al., 1980).

B) Solvent spherule method

In this procedure phospholipids dissolve in a light and vaporizable organic solvent and diffuse in an aqueous solution. Therefore homogeneous size MLVs will form with evaporation of light organic solvent (Kim et al., 1985).

2.1.6.2 Methods of preparation SUV

Small unilamellar vesicles (SUV) are containing one bilayer and with size destribiution of 20-100 nm. SUVs will prepare through sonication and french pressure cell methods (Shaheen et al., 2006).

A) Sonication Method

In sonication same as conventional method lipid dissolve in organic solvent then organic solvent remove under pressure and the thin lipid film layer would hydrate with buffer using sonicator (Gruner et al., 1985). SUV will form after the dispersion of phospholipids is sonicated in either a bath sonicator or probe sonicator while in this technique generally small vesicles will produce (Lopes et al., 2004). However probe sonication not recommended because of some limitations like making harmful aerosols and contamination of the liposome with the tip of metal probe respectively (Szoka et al., 1980). As bath sonicator is more safe compare with the probe sonication but still various parameters such as the position of the container in the bath or/and water temperature might cause different result during the repeatedly practices (Akbarzadeh et al., 2013; Riaz, 1996).

B) French pressure cell method

This is a quick and simple method of making SUV by extrusion of MLV through a tiny aperture at 20,000 psi at 4°C. In French pressure cell method SUVs can form reproducibly while they are larger in size compare to sonication technique. However there are some limitation for this method such as low volume of mixture and adjusting temperature (Akbarzadeh et al., 2013; M. J. Hope et al., 1993; Lasic, 1988; Lasič et al., 1987; Lawrence D Mayer et al., 1986).

2.1.6.3 Methods of preparation LUV

Large unilamellar vesicles (LUV) are containing one bilayer with size distribution of more than 100 nm (Shaheen et al., 2006). These unilamellar and oligolamellar vesicles have large aqueous volume to lipid ratios, which is four times higher than MLV and 30 times higher than SUV (Szoka et al., 1980). LUVs are preparing by solvent Injection, detergent removal, reverse phase evaporation, supercritical reverse phase evaporation, calcium-induced fusion and freeze-thaw methods.

A) Solvent Injection Methods

Ether infusion: in this method lipid dissolve in the organic phase (diethyl ether or ether/methanol mixture) and then the lipid solution is injected into aqueous phase and liposome will form under reduction of pressure at 55-65 0C (Deamer & Bangham, 1976; Schieren et al., 1978). Ethanol injection method is a simple and rapid method although there are also some limitations such as the heterogeneous size of LUVs with low entrapment efficiency, poorly solubility of some lipids in ethanol and exposure of drug or other compound with the organic solvent and some of that might remain at the end of processing which to be removed from final product (Akbarzadeh et al., 2013; M. Hope et al., 1985; Szoka et al., 1980).

Ethanol injection: through this process lipids dissolve in ethanol and injected quickly to aqueous media then LUVs will form. Same as ether method LUVs will be heterogeneous in size distribution plus removing ethanol from liposome is difficult (Batzri & Korn, 1973; Brunner et al., 1976; Stano et al., 2004).

B) Detergent removal method

Lipids are dissolved in detergents instead of organic solvent, and then LUVs will form as the detergent is removed. Detergent can be removed by several methods, such as dialysis, gel Chromatography, and adsorbing or binding to other materials (Akbarzadeh et al., 2013; Alpes et al., 1986; Enoch & Strittmatter, 1979; Gerritsen et al., 1978). While this method is reproducible and results in homogenous size of LUVs, a trace amount of detergent would remain in the final product (Riaz, 1996).

C) Reverse phase evaporation method

In the Reverse phase evaporation method, the lipid mixture are dissolved in organic solvent (diethyl ether and isopropyl ether), then added to the aqueous phase and sonicated until water forms in the oil emulsion. Afterwards, organic solvents are removed under reduced pressure, resulting in the formation of LUVs. A variety of lipids

or mixture of lipids can be used in this method, and biochemical materials and large macromolecules can be encapsulated within the LUVs (Hauser, 1982; Szoka et al., 1980; Torchilin & Weissig, 2003). However, this method is unsuitable for encapsulating fragile molecules (Meure et al., 2008)

D) Supercritical reverse phase evaporation method

Imura was the first to discover this method. It uses supercritical fluids, such as carbon dioxide, to prepare liposome. In this procedure, lipids, organic co-solvent, and compressed gas are combined in a cell, and the temperature is set above the lipid's phase transition temperature. Afterwards, aqueous solution would be slowly introduced into the cell, while the pressure is reduced to release the dense gas, forming LUVs (Imura, Gotoh, et al., 2003). Supercritical reverse phase evaporation method needs less organic solvents, and LUVs yields higher entrapment efficiency, plus carbon dioxide, which is applied in this system, is nontoxic and cheap. However this method is not without limitations, the application of high pressure and low stability or physicochemical properties of vesicles prepared by this method are rather limited (Imura, Gotoh, et al., 2003; Meure et al., 2008).

E) Calcium-induced fusion method

In the Calcium-induced fusion method, LUVs are formed by utilizing acidic phospholipids on top of the addition of calcium and EDTA, which results in the heterogeneous size range of liposome. Through this procedure, macromolecules can be encapsulated within the LUVs (Akbarzadeh et al., 2013; Mayhew et al., 1984; Riaz, 1996).

F) Freeze-thaw method

In the freeze-thaw process, LUVs will be formed by fast freezing SUVs, followed by warming or thawing them slowly (Ohsawa T, 1985; Pick, 1981). Vesicles from this

method are homogenous in size and are concentrated with phospholipid, with high abilities of encapsulation (Llu & Yonetani, 1994; Mozafari, 2005; Traïkia et al., 2000).

2.1.6.4 Methods of preparation OLV

Oligolamellar vesicles (OLV) are liposomes with 1-5 bilayers and 0.1-1 μm size distributions. OLVs with one bilayer can be formed through dialysis of sodium trichloroacetate (Oku & Macdonald, 1983). Also, OLVs with multi-bilayers can be formed using methylglucoside detergent method, plus the dialysis of phosphatidylcholine in methanol (Oku et al., 1982).

2.1.6.5 Methods of preparation MMV

(MMV) contains multi vesicles with a general size of over 1μm. The procedure is similar to the reverse phase evaporation method, where it needs additional water in oil emulsion to cross organic solvent and form MMVs. Multivesicular liposomes have very high encapsulation efficiency (up to 89%), and are capable of encapsulating glucose, EDTA, and human DNA (Kim et al., 1983).

2.1.7 Characterization of liposome

Considering the chemical and physical character of liposome, specifying their behavior, and therefore characterization of vesicles, is necessary after any preparation method of liposome (Torchilin & Weissig, 2003). Vesicles might be quantified by their chemical properties, such as analysis of the quantity of cholesterol, phosphatidylcholine, and the encapsulation efficiency or either, with physical properties, such as the formation and morphology, size distribution, thermal behavior, stability, and zeta potential values (Hathout et al., 2007; Imura, Otake, et al., 2003; Maestrelli et al., 2006; Torchilin, 2006).

2.1.7.1 Formation and morphology

Liposome can be viewed using Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Freeze-fracture electron micrographs (FEM). Electron

microscopy can determine the formation and morphology of liposome (Hauser, 1993). Lamellarity (number of lipid bilayers) and the size of the vesicles are the important properties of each liposome. Lamillarity and inner space of vesicles can be observed under Freeze-fracture electron micrographs and Transmission Electron Microscopy, whereas the outer space of the vesicles is generally studied with Scanning Electron Microscopy (Almgren et al., 2000; Gregoriadis, 2006).

2.1.7.2 Size Distribution

Particle size is one of the most important parameters of the characterization liposome, which are reported in almost all liposome studies (Gregoriadis, 2006). Particle size determinations are mostly performed to confirm that the desired liposome size range has been obtained during the preparation, and it is retained upon storage or further processing, such as sterilization (Heldt et al., 2001). Furthermore, the suitable size of particles is important for their interaction with the biological situation, for instance, through intravenous administration of loaded particles, their ability to pass or leave the vascular capillaries is effectively dependent on the size (Nastruzzi, 2004).

Size distribution can be measured using several devices and methods, such as photon correlation spectroscopy, dynamic light scattering, and gel permeation and sedimentation method (Lopes et al., 2004; Szoka et al., 1980). According to Mu and Zhong, electron microscopy can also be used to directly visualize the vesicle size of the liposomes, although using electron microscopy requires an experienced operator, and is costly (Mu & Zhong, 2006). Since photon correlation spectroscopy (PCS) is a rapid and easy method, and it only requires small amounts of samples, this method is the most common way to characterize the size of liposome (Maestrelli et al., 2006; Mu & Zhong, 2006; Nastruzzi, 2004).

2.1.7.3 Stability of liposome

The study of the stability of vesicles is another method of characterizing liposome. Both physical and chemical stability of vesicles need to be evaluated. The physical stability of vesicles is about visual changes in the liposome mixture, such as aggregation. Liposome physical stability is generally determined by measuring the zeta potential value, while chemical stability is about the degradation of liposome. As vesicles are typically composed of natural components, their structure would be depressed through the oxidation or the hydrolysis of their ingredients accordingly. Phospholipids basically involve unsaturated fatty acyl chain, which have high tendencies for oxidation. Furthermore, the presence of heat and light also accelerate the trend of hydrolysis. In order to protect liposome from oxidation, antioxidants such as butyl hydroxyl toluene and α-tocopherol can be added to the mixture. Preparing liposome under nitrogen or argon, storage at low temperature, and pH adjustment would enhance the stability of the vesicles (Akbarzadeh et al., 2013; Doherty, 2004; Gregoriadis, 2006).

A) Zeta potential

Colloidal particles are usually wrapped with surface charges, which are the result of the presence of ionized groups or ion adsorption from the dispersion medium (Hunter, 1981). These surface charges and the electrical field around the particles play an important role in the mutual repulsion of particles in their stability against aggregation (Cosgrove, 2010; Ohshima, 2007). The liposome surface charge also has an impact on drug encapsulation and in-vivo behaviors (Ohshima, 2007).

Since the surface potential of the particles can't be measured directly, the zeta potential is typically a characteristic parameter of particle charges (Hunter, 1981). Zeta potential is a measurement that assesses the stability of a colloidal system by measuring the repulsive forces between particles (Müller et al., 1996). The larger the repulsive forces, the less likely they are to aggregate, which renders the system more stable. Therefore, if

the particles have large positive or negative values, they will be kept away from each other, and acquire stability. However, if the particles have low zeta potential, these particles will tend to aggregate. Particles with zeta potentials that are more negative than -30mV or more positive than +30mV, they are normally stable (van Nieuwenhuyzen & Szuhaj, 1998).

Zeta potential can be evaluated using a zetasizer (Mu & Zhong, 2006). The Zeta potential values are generally measured by applying an electric field across the system, and the particles will migrate towards the electrode with an opposite charge. The velocity of the particle movement is proportional to the magnitude of the zeta potential (Hunter, 1981).

b) Lipid hydrolysis

Investigation of chemical stability of liposome can also be done by studying oxidation and/or hydrolysis of lipids using Proton nuclear magnetic resonance (^{1}H NMR), C-13 Nuclear magnetic resonance (^{13}C NMR), Phosphorous nuclear magnetic resonance (^{31}P NMR), and High Performance Liquid Chromatography (HPLC) devices. However, all of these devices are costly, and requires prior experiments (Gregoriadis, 2006).

Differential scanning calorimetry (DSC), Nuclear magnetic resonance (NMR), Fourier Transform Infra-Red (FT-IR), and fluorescence spectroscopy are advance techniques that can be used to study the stability of liposome, such as the measurements of phase transitions or separation, changes in parameters of chemical reactions, or physical transformation (Demetzos, 2008; Gregoriadis, 2006; Pawlikowska-Pawlęga et al., 2013).

2.1.8 Entrapment efficiency

Factors that make liposome a viable drug delivery molecule is its ability to encapsulate drug and load itself with medicine (Lasic & Martin, 1995). The quantity of drug being encapsulated in the liposome is termed the entrapment efficiency (EE). To optimize the

entrapment of liposome, some successful process, such as pH-gradients over the bilayer membrane can be utilized, which is a rapid transport of a neutral substance from the outside to the inside, where the pH is such that the substance ionizes and gets trapped (Almgren et al., 2000). Entrapment efficiency can be estimated by dialysis method, a sepharose column, and centrifugation or removing lipids using organic solvent (Nastruzzi, 2004).

2.2 Cancer

Cancer is a result of mutant genes and uninhibited growth of cancerous cells, which would stop following the normal body signals, such as increasing, growing, or cell death (Killeen, 2004; Pawaiya et al., 2011). The abnormal cancerous cells act independently from the body's normal cells, but require sustenance similar to healthy tissues, which might be detrimental to the organs or tissue in question (Burstein & Schwartz, 2008; Weinberg, 2007; H. Yu & Jove, 2004).

Tumors will form after normal cells are converted into abnormal ones. The process takes time, although it can be accelerated by external factors. Tumors are a mass of extra cells that can be cancerous or non-cancerous, which do not spread to other parts of the body, and they can be removed without incident (Hahn & Weinberg, 2002; Killeen, 2004). Malignant tumors contain cancerous cells that can attack the tissues next to them and spread throughout the body (Burstein & Schwartz, 2008; King, 2002).

Cancers are divided into primary and secondary cancers. Primary cancers are tumors that remain in the spot it grew from, while secondary tumor are those that moved away and spread to other areas of the body, typically called metastasis. There are also cancers that do not cause tumors, such as leukemia, which attacks the bone marrow and blood cells (López-Lázaro, 2010; McLaughlin, 2002).

2.2.1 Classification of Cancer

Classifications of cancers are generally based on simulating the tumor cells with the assumed function and original location. Cancers are categorized as: carcinoma, sarcoma, blastoma, germ cell tumor, lymphoma and leukemia (Louis et al., 2007; Philip, 2002).

A. Carcinoma

It is a cancer derived from reputed epithelial cells in a tissue with inner or outer surfaces of the body (Berman, 2004; Su et al., 2001). The majority of common cancers belong to this group; examples are skin, breast, lung, pancreas, prostate, and colon cancers (Boyraz et al., 2013; Travis, 2004).

B. Sarcoma

This type of cancer is derived from transformed cells of mesenchymal and connective tissue, such as fat, bone, nerve, and cartilage. Sarcoma is malignant soft tissue; hence it is more common in children, while rare in adults (Bieling et al., 1996; Borden et al., 2003; Celik et al., 1980).

C. Lymphoma

It is the cancer of the bone marrow that begins with abnormality and malignant immune system cells. Lymphoma occurs by faster division and growth of white blood cells (B or T lymphocytes) compared with normal ones, which departs from their origin to mature in the lymph nodes or blood (Clarke et al., 2004; O'Connor, 2009). It can develop in the spleen, the lymph nodes, bone marrow, blood, or other organs (Drexler, 2000; Morton et al., 2006).

D. Leukemia

Similar to lymphoma, this cancer is caused by blood cells, and they are created from bone marrow. It is the abnormal increase of young and immature white blood cells

called blasts. Leukemia is a curable cancer, with children more likely pulling through than adults (Burnett et al., 2011; Drexler & Minowada, 1998; Mathers et al., 2001).

E. Germ cell tumor

Germ cells are basically located in the ovary or testicle, where they normally develop to become sperm and eggs. Germ cell tumors are mostly oriented in the ovary or testis, but they can move to other parts and cause cancer elsewhere (Ulbright, 2005; Wilkinson & Read, 1997).

F. Blastoma

This type of cancer arises from malignancies in immature precursor cells or embryonic tissue; therefore, it more often afflicts children (Alberts et al., 2004; Koss et al., 1991).

2.2.2 Treatment of Cancer

There are several therapies for the treatment based on the type, grade, location, and stage of cancer, plus the general health of the patient as well. Main treatments of cancer consist of surgery, chemotherapy, radiotherapy and hormonal therapy. The purpose of each treatment is to decrease the occurrence or severity of any cancer. Evidence can also be collected for in vitro and in vivo researches and epidemiological and clinical studies (Alberts et al., 2004; Caprera, 2007; Carter, 2001; Cassileth & Chapman, 1996; Khan et al., 2011).

a. Surgery: it's the only therapy that is needed to cure early stage cancer via the removal of small and early stage tumors that have not expanded or spread to other organs. Furthermore, surgery is also used as diagnosis, control symptoms, or even reduce the risk of cancer. However, this treatment is performed for advanced cancers, and removes organs or tumors that are at high risk of malignant transformation in a body (Guillem et al., 2006; Wagman, 2008).

b. Radiotherapy: this method is utilized for treatment or relieving symptoms or suffering pain of metastasis of incurable cancer. Radiation therapy uses high-energy radiation to

kill cancer by damaging their DNA. It can be used by itself, such as treating cervical cancer, or combined with other treatments such as surgery to clean up an area that was formerly afflicted with tumors. Several types of radiation used in therapy are x-rays, gamma rays, and charged particles that are directed by a device via injection or surgery. However, radiation treatment is indiscriminate, and will attack healthy, as well as cancerous cells; therefore its usage should be sparse and in between in order not to damage healthy cells (Lawrence et al., 2008; Rubin & Williams, 2001; Terasawa et al., 2009).

c. Chemotherapy: this systemic therapy uses anticancer drugs that would circulate in the bloodstream, then locate and destroy cancerous cells. Anticarcinoma medicines interfere via the destruction of cancer cells, and inhibit the proliferation and their division. Chemotherapy can be the only cure for certain cancer such as certain lymphomas, and can also be applied with other therapies, such as preventing metastasis post-surgery or radiotherapy breast or colon cancers (De Flora & Ferguson, 2005; Holland, 1982; Leonard & Pwint, 2007).

Anticarcinoma medicine can be administered orally via tablets and capsules, or direct injection to the bloodstream and infusion, depending on the medicine being recommended. Moreover, "combination chemotherapy" can be advised in some cases that utilize two or more anticancer drugs simultaneously for better responses. Nevertheless, the effectiveness of applying chemotherapy agents are usually limited, due to their potential toxicity, damage, and harm caused to other normal healthy tissues in the body (Caprera, 2007; Chou, 1991).

2.2.3 Breast cancer

According to the reports, breast cancer is one of the most fatal types of cancer afflicting women, although it’s rare in men (Boyle & Levin, 2008). Breast cancer is more prominent in older women (Lacey et al., 2009). General treatments of breast cancer

include surgery and removal of the tumor, chemotherapy, and radiotherapy. Doxorubicin is one of chemotherapy agent that is commonly used for breast cancer therapy (Leonard & Pwint, 2007; Rossi, 2013). Therefore, in this study, breast cancer cell lines (MCF 7 and MDA-MBA 231) were selected to evaluate the toxicity of liposomal doxorubicin.

2.3 Doxorubicin Hydrochloride

Doxorubicin is a cytotoxic anthracycline antibiotic which is used as chemotherapy agent for a wide range of cancers (Weiss, 1992).

Chemical name of doxorubicin hydrochloride is 5,12-Naphthacenedione,10-[(3-amino-2,3,6-trideoxy-α-L-lyxohexopyranosyl)oxy] 7,8,9,10tetrahydro-6,7,11-trihydroxy-8-(hydroxylacetyl)-1-methoxy,hydrochloride (8S-cis)-; or (8S,10S)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-8-glycoloyl-7,8,s,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride, Chemical formula: C27H29NO11 (Hortobagyi, 1997). The structure of doxorubicin hydrochloride has shown in Figure 1.1.

Doxorubicin is a cytotoxic anthracycline antibiotic, which is used as a chemotherapy agent for a wide range of cancers. Anthracyclines were first revealed in 1950s by Farmitalia Research Laboratories, an Italian research company, named daunorubicin, which was a red isolated pigment from cultures of *Streptomyces peucetius* that demonstrated excellent anticancer performance on mouse tumors (Federico Arcamone et al., 1997; Kessel et al., 1968). In 1960s, clinical trials showed the successful results of treating lymphoma and acute leukemia with daunorubicin, although it is later identified as a cause of severe cardiac toxicity (Hortobagyi, 1997; Tan et al., 1967).

Later, the Italian company discovered that changing biological activity lead to some changes in the structure of the compound, resulting in a new product. Therefore, doxorubicin or adriamycin gained by mutation of a strain of *Streptomyces* using N-

nitroso-N-methyl urethane (F Arcamone et al., 2000; Di Marco et al., 1969; Tan et al., 1973). This new product demonstrates better responses than daunorobicin in treating cancers and solid tumors; however, it is still cardio-toxic (Blum & Carter, 1974; Laginha et al., 2005).

Mechanism of action: doxorubicin binds with DNA's double helix and inhibits the sequence of the topoisomerase II enzyme, which leads to the inhibition of the biosynthesis of macromolecular (Fornari et al., 1994; Gewirtz, 1999; Momparler et al., 1976). Enzyme topoisomerase II settle supercoils in DNA for transcription, since doxorubicin stabilizes the topoisomerase II complex, consequently preventing the processing duplication of DNA double helix and stop the growth of cell (Bodley et al., 1989; Frederick et al., 1990).

2.3.1 Clinical Pharmacology

Doxorubicin binds with the nucleotide and inhibits reproduction or action of DNA and RNA polymerases that resulted in the toxicity effect upon malignant cells and other normal organs in body. The significant function and mechanism of doxorubicin as a cytotoxic agent is to be incorporated with topoisomerase II to form DNA cleavable complexes. Further binding of doxorubicin to cellular membranes can also affect the function of the cells (Pang et al., 2013; Pommier et al., 2010). It also causes several changes in the characteristic morphology of cells that leads to program cellular death or apoptosis, based on its therapeutic or toxicities affects (Shen & White, 2001). Doxorubicin makes a variety enzymatic electron reduction such as reductases, oxidases, and dehydrogenases, which produces lots of hydroxyl free radical. The free radical agents reduce the level of Cu (II) and Fe (III) in cells, and causes cardiotoxicity (Shen & White, 2001; Shepherd, 2003).

2.3.2 Pharmacokinetics

Many pharmacokinetic studies of doxorubicin determined whether single or multi-agent therapy is suitable for patients with different types of tumors (Tacar et al., 2013). Due to these studies, after intravenous injection of doxorubicin, it shows multi-phasic disposition, followed by a triphasic plasma clearance. The half-life of doxorubicin distribution is 3–5 min, owing to its rapid drug uptake by the cells. The terminal half-life of doxorubicin is 24–36 h, thus its elimination from the tissue takes longer. Doxorubicin is dose-independent pharmacokinetics in the dose range of 30 to 70 mg/m2 (Gustafson et al., 2002; Reddy & Murthy, 2004).

The steady-state distribution of medicine is necessary to decrease the risk of toxicity in the body. As the steady distribution range of doxorubicin is from 500–800 l/m2, it would let body tissues get an effective amount (Reddy & Murthy, 2004). Like most drugs, doxorubicin and doxorubicinol, which is major metabolites, would bind to the plasma proteins and pass through cell membrane via passive diffusion. Entering or accumulating doxorubicin would continue till the intracellular concentration exceeds 10 – 500 time more than the extracellular concentration. Moreover, the concentration of doxorubicin in the nuclear sections is 50 times more than the other cell cytoplasm (Rook et al., 2005; Wang et al., 2000). Since the liver metabolizes drugs, doxorubicin would frequently accumulate in the liver. Further doxorubicin concentration inside white blood cells and bone marrow is 200–500 times more than the plasma (Lal et al., 2010; Tacar et al., 2013).

Doxorubicin is highly capable of entering tissues and stay within nucleated cells and intercalating with DNA, owing to its lipophilic characteristics. Due to fast distributions of doxorubicin in tissues, its blood levels fall fast correspondingly. However, despite its high ability of penetrating to the tissues, doxorubicin is unable to pass through the blood–brain barrier (Lal et al., 2010; Licata et al., 2000).

a. Distribution

According to rapid tissue uptake of doxorubicin, its main half-life distribution is about 5 minutes, whereas the terminal half-life of doxorubicin is 20 to 48 hours, due to its time-consuming elimination. Also, extensive uptake of doxorubicin by tissue is the reason for its steady distribution volume, which is around 809 to 1214 L/m2. Doxorubicin and doxorubicinol binding to proteins of plasma is about 74 to 76%, while it is independent of plasma concentration. Doxorubicin would be excreted into the milk during lactation, and it is detectable in the milk up to 72 hours after therapy (Tacar et al., 2013; Vaage et al., 1994).

b. Metabolism

Doxorubicin may experience 3 metabolic paths: one-electron reduction, two-electron reduction that yields to doxorubicinol and deglycosidation. However, around half of the administrate dosage would be eliminated in the same form from the body. The reduction of one electron is facilitated with several oxidoreductases in order to produce a doxirubicin-semiquinone radical. The primary metabolic route of doxorubicin is two-electron reduction, which yields doxorubicinol, a secondary alcohol. The minor metabolic route of doxorubicin is Deglycosidation, which is about 1-2% of the dosage, and the resultant metabolites are deoxyaglycone or hydroxyaglycone, via the reduction or hydrolysis (Gaguski & Karcheski, 2011; Richly et al., 2009).

c. Excretion

Doxorubicin metabolism in the liver is cleared through biliary excretion from Plasma, with a clearance rate of 324 to 809 ml/min/m2. Almost 40% of its applied dosage would come out into the bile in 5 days, meanwhile, just 5 to 12% of doxorubicin and its metabolites might appear in urine. Over 7 days, less than 3% of the dose in the urine was recovered as doxorubicinol. Comparing obese patients with normal ones, there is a considerable reduction in the clearance of doxorubicin without any change to the

volume of distribution, with less than 115% of ideal body weight. In obese women, systemic clearance of doxorubicin is noticeably reduced to more than 130% (Rollins & Klaassen, 1979; Tacar et al., 2013).

2.3.3 Doxorubicin Sides Effects

Cardiotoxicity, myelosuppression, hematologic toxicity, secondary leukemia, extravasations, hepatic Impairment, alopecia, fatigue, and effects at site of Injection are common side effects of doxorubicin (Thorn et al., 2011).

a. Cardiotoxicity: It is the most dangerous side effect of anthracyclines. Doxorubicin causes both acute and delayed heart failure. Acute or early cardiotoxicity is mostly from sinus tachycardia or electrocardiogram (ECG) irregularity or abnormalties like non-specific ST-T wave changes. Tachyarrhythmias (both premature ventricular contractions and ventricular tachycardia), bradycardia, atrioventricular, and bundle-branch block have also been reported. Delayed cardiotoxicity frequently occur during 2-3 months after treatment, though late event have also been reported several years after the termination of treatment. Delayed cardiomyopathy is manifested by the reduction in LVEF or signs and symptoms of congestive heart failure (CHF), such as tachycardia, dyspnea, pulmonary edema, dependent edema, cardiomegaly and hepatomegaly, oliguria, ascites and pleural effusion (Chatterjee et al., 2009; Takemura & Fujiwara, 2007). Doxorubicin cardiotoxicity is characterized as being dose-dependent, and as the cumulative dose of doxorubicin reaches 550 mg/m², the risks of developing cardiac side effects and death dramatically increase as well (Bristow et al., 1981).

b. Hematologic Toxicity: Doxorubicin may cause myelosuppression, which is decreasing the cells that is responsible for providing immunity, carrying oxygen, and those responsible for normal blood clotting (Crawford et al., 1991). Hematologic side effects have been reported in 60% to 80% of patients treated with doxorubicin, utterly depending on its dose. Patients need to be monitored carefully for red blood cell (RBC),

differential white blood cell (WBC), and assessment of the platelet counts before and during every cycle of treatment. The most common dose-limiting toxicity of doxorubicin hematologic toxicity is granulocytopenia (neutropenia) and reversible leucopenia (Perry, 2008; Stone et al., 1995). Leukopenia, with the recommended dosage schedule passing in the course reaching to its lowest point after 10-14 days of treatment, whereas recovery usually occurs on the 21st day. Thrombocytopenia and anemia might occur as well. Clinical significances of severe myelosuppression consist of fever, septicemia, infections, septic, hemorrhage, shock, tissue hypoxia, or even death (Jacobson et al., 2009; Perry, 2008).

c. Secondary Leukemia: The incidence of secondary leukemia in patients having anthracyclines in chemotherapy regiments treatment has been reported with a 1–3 year latency period. Doxorubicin and/or any DNA-damaging antineoplastic medicine commonly cause secondary AML or MDS post-therapy extensively among patients with heavily cytotoxic drugs regiments or escalated dosages of doxorubicin (Bokemeyer et al., 1995; Carvalho et al., 2009; Chabner & Longo, 2011).

d. Hepatic Impairment: As the major route of metabolism, elimination, and excretion of doxorubicin is via the hepatobiliary system, liver damage is a very common side effect of this drug (Gustafson et al., 2002). Total serum bilirubin need to be evaluated before and during therapy due to slower clearance of doxorubicin in patients with high bilirubin. The toxicity of recommended doses may be enhanced by hepatic impairment, however, lower doses are recommended in these patients. Furthermore, during therapy of patient with hepatic, conventional laboratory tests, such as SGOT, SGPT, alkaline phosphatase are also recommended, although those with severe hepatic impairment should not receive doxorubicin (Carvalho et al., 2009; Tacar et al., 2013).

e. Nephropathy and proteinuria: Kidneys have the main task of regulating chemical composition of blood and maintaining fluid balance. Doxorubicin causes toxicity in

kidneys by injuring glomerular podocytes, which occur in the form of nephropathy and proteinuria (Okuda et al., 1986). As the glomeruli are damaged, it won't be able to function normally and leads to glomerular lesions, inflammation, tubular dilation, and capillary permeability. Despite the liver being capable of regeneration, kidneys do not have the same ability. Nephropathy happens during interference of doxorubicin with the normal operation of the mitochondria and drops the activity of complexes I-IV, leading to increase in the levels of triglycerides, superoxides, and citrate synthase (Okuda et al., 1986). Furthermore, vitamin E and antioxidant compounds levels would also decrease along with lipid peroxidation. The leakage of proteins from local passages and modifying the nephron structure cause glomerulosclerosis. Proteinuria, hypertension, steroids resistance, or even renal failure, are some known glomeruli-affecting disease (Carvalho et al., 2009; Okuda et al., 1986; Tacar et al., 2013).

f. Effects at site of injection: Phlebosclerosis may occur due to the repeated injection into the same vein or into a small vessel. However, by following the recommended administration procedures, the risk of phlebitis or thrombophlebitis would be minimized at the site of the injection as well. Extravasation might also happen during the intravenous administration of doxorubicin, with or without an additional burning or stinging sensation. In the case of extravasation, or any signs or symptoms of it are observed, the infusion or injection requires immediately termination. To continue the treatment, another vein should be used (Chabner & Longo, 2011; Schrijvers, 2003).

2.3.4 Liposomal form of Doxorubicin

The anti-neoplastic drugs have nonspecific cytotoxicity on malignant and normal cells, which results in severe side effects during and after therapy. Developing novel drug delivery systems limits the drug's toxicity in order to specifically and directly affect tumors as opposed to normal cells. The number of final medicine using the new drug delivery systems is considerably increased due to efficacy, selectivity, and their

respective total effect (Carvalho et al., 2009; Tacar et al., 2013; Verma & Garg, 2001). Liposome is a current drug delivery medium that demonstrated an acceptable level of safety for therapeutic agents and anticancer drugs. They are microparticulate lipoidal vesicles, with the ability of carrying a variety of drugs for improved delivery (Verma & Garg, 2001). Investigations by many researches on the liposomal form of doxorubicin would typically reduce acute and chronic toxicities related to the administration of free drugs. Also, cardiotoxicity, which is known as clinically relevant dose-limiting side effect of doxorubicin, would be reduced by the application of liposomal form (Y. Barenholz, 2001; Poste et al., 1982). The administration of liposomal doxorubicin enhance its therapeutic index and can reduce its toxicity as well (Y. Barenholz, 2001; Elbayoumi & Torchilin, 2007; Shi et al., 2011).

2.3.5 Advantages of pegilated liposomal Doxorubicin

Encapsulating anthracycline within the liposomes reduces their toxicity, and it has been found that pegilation technology also provides longer circulation times for liposomes. Pegylation is the using of methoxypolyethylene glycols as a surface to bind itself to the vesicles (A. A. Gabizon, 2001; Moein Moghimi et al., 2006). The Pegylation liposome technology represents a favorable drug-carrier system, and renders vesicles undetected by the mononuclear phagocyte system. Therefore, the clearance of liposomal drugs would be reduced, which lead to prolonged circulation half-life and selective drug accumulation in tumor tissues (Kale & Torchilin, 2007; Koshkaryev et al., 2012; Moein Moghimi et al., 2006).

Conventional doxorubicin has a half-life of about 30 h, while pegylated liposomal doxorubicin has a half-life of around 3 – 4 days, which confirms its long circulation. The encapsulation of doxorubicin within pegylated liposome prevents its uptake by the reticulo-endothelial system, and results in a considerable prolongation in the serum (Lukyanov et al., 2004; Vail et al., 2004). Moreover, the administration of pegylated

liposomal doxorubicin results in the modified favorable tissue distribution, with consequently less cardiotoxicity, myelotoxicity, alopecia, and gastrointestinal toxicity compared to doxorubicin (Abraham et al., 2005; Y. C. Barenholz, 2012; A. A. Gabizon, 2001; Saul et al., 2003; Vail et al., 2004).

2.3.6 Doxorubicin in market

Available dosages of Doxorubicin hydrochloride and pegilated liposomal in market are as below:

1. Doxorubicin hydrochloride for Injection, a sterile red-orange lyophilized powder for intravenous use only, is available in 10, 20 and 50 mg single dose vials and a 150 mg multi dose vial.
2. Rubex®, Doxorubicin hydrochloride, available in 50 and 100 mg vials.
3. Myocet®, liposomal doxorubicin, available in 50 mg single dose vials.
4. Caelyx®, pegilated liposomal doxorubicin, available in 20 mg single dose vials.
5. Doxil®, pegilated liposomal doxorubicin, available in 10, 20 and 50 mg single dose vials.

2.4 Palm oil

The history of using palm oil as a cooking oil is back to 3000 BCE (Kiple & Ornelas, 2000). Palm oil is derivative of palm crop trees, from Elaeis guineensis, the Palmae family (Sundram et al., 2003). The oil of palm is well known as its both edible and non-edible applications and benefits. The oil of palm produces two individual oils, palm oil extracted from the mesocarp which is major edible oil in the world market and the palm kernel oil from the fruits kernel which has wide application for industry (Corley & Tinker, 2008; Gunstone, 2011). Malaysia and Indonesia are the main producer of world palm oil, while Malaysia is currently the world's largest exporter (Basiron, 2002; Pakiam, 2013).

Palm oil consists of 50% saturated fatty acids, 39% monosaturated fatty acids and 10.9% polysaturated fatty acids (Edem, 2002). The main components in palm oil are palmitic acid, stearic acid, oleic acid and linoleic acid. Furthermore it has found to be rich in vitamin E, both tocopherols and tocotrienols. Vitamin E is useful natural antioxidant and anticarcinogenic which is not found in most of other vegetable oils (Kamat et al., 1997; Sundram et al., 2003). In the crude palm oil, the vitamin E content ranges from 600-1000 parts per million with a mixture of 18-22% tocopherols and 78-82% tocotrienoles. The major tocotrienols in palm oil are alpha-tocotrienol (22%), gamma-tocotrienol (46%) and delta-tocotrienol (12%) (Sambanthamurthi et al., 2000; Sundram & Chandrasekharan, 2000).

Carotenoids are another component of interest in palm oil. The reddish coloration of palm oil is due to the presence of the carotenes in crude palm oil (Sambanthamurthi et al., 2000). Carotenoids also has natural antioxidant and anticarcinogenic properties. The major components of carotenoids are α carotene, ß-carotene and γ-carotene whereas lycopene and xanthophylls are minor parts of carotenes in palm oil (Edem, 2002; Sundram et al., 2003).

Minor component found in palm oil are Sterols such as β-sitosterol, campesterol and stigmasterol. Their presences in palm oil are negligible as compare to animal fats and only act as minor dietary source for synthesis of steroidal hormones. Other possible impurities in palm oil are tannins, flavonoids, terpenoids, hydrocarbons and ketones (Edem, 2002; Sambanthamurthi et al., 2000; Sundram et al., 2003).

2.4.1 Advantages of palm oil

Palmitic acid is the major saturated fatty acids in palm oil compare to other vegetable oils such as soy bean, corn, and coconut (Edem, 2002). Palmitic acid is assumed by people to cause hypercholesterolemic since it is a saturated fatty acid. Yet, according to Hayes study palmitic acid lowered the total cholesterol and low density lipoproteins

level (LDL) (Hayes et al., 1991). Other than that, stearic acid is another fatty acid of palm oil which also reduces cholesterol level in an animal study (Hassel et al., 1997). The unsaturated fatty acids present in palm oil are oleic acid and linoleic acid that known with the ability of reducing cholesterol level as well (O'Holohan, 1997). Although palm oil-based diets cause a higher blood cholesterol level than other vegetable oils, the consumption of palm oil will reduce endogenous cholesterol level (Edem, 2002).

Besides that palm oil include tocopherols and tocotrienoles that known as vitamin E. There are lots of benefits for vitamin E such as preventing aging, atherosclerosis, thrombosis and other cardiovascular disease. Vitamin E has antioxidant properties and removes free radical in order to protect biological membrane against oxidative damage (Sambanthamurthi et al., 2000). Kamat and Devasagayam also found that tocotrienols from palm oil reduces lipid peroxidation and protein oxidation in the brain mitochondria of rats. In addition, antioxidation properties of vitamin E also helps to resist rancidity thus improve stability of palm oil (Edem, 2002; Kamat & Devasagayam, 1995). The stability of palm oil was enhanced by antioxidant properties of vitamin E and preventing peroxidation of unsaturated fatty acids. Tocotrienoles and tocopherols have anti-proliferating properties; due to Nesaretnam study these chemicals cause inhibition of growth in human breast cancer cell (Kamal-Eldin & Appelqvist, 1996; Ling et al., 2012; Mukherjee & Mitra, 2009; Nesaretnam et al., 2004; Nesaretnam et al., 2007; F.-L. Yu et al., 2008). Guthrie also suggested that tocotrienols when used in combination with tamoxifen can effectively inhibit both estrogen –negative and –positive breast cancer cells and should be considered to be used in breast cancer therapy (Guthrie et al., 1997). Further Elson and Qureshi study on the minor constituents of palm oil such as isoprenoids, carotenoids and tocotrienol. They stated that these minor components are

potential to decrease cholesterol and LDL synthesis as well as tumor suppressive activity (Elson & Qureshi, 1995).

2.4.2 Applications of palm oil

Palm oil contains 80% edible and 20% non-edible application in a variety of product. Some food applications of palm oil are: coffee whiteners, ice cream, whipping cream, filled milk, reduced fat spread, palm-based cheese, trans fatty acid-free formulations, coconut milk powder, mayonnaise and salad dressings. Non-food products of palm oil is only 20% although its considerable and important. Palm oil utilize in non-edible productions with both direct and/or indirect application. Some direct usage of palm oil are: printing ink, soap, drilling mud, fuels in some diesel machin, engineering thermoplastics, polyacrylate coatings, polyols, epoxidized palm oil and polyuenthanes (Basiron, 2002).

Apart from palm oil edible and industrial usages, several studied offer using palm oil in pharmaceutical and medical fields due to its health benefits. The usage of natural products in pharmaceuticals has steadily seen improvements over the last decade (Corley & Tinker, 2008).

2.5 Preparation diclofenac sodium liposomes using palm oil fractions

In this study first the possibility of making liposome with palm oil fraction were investigate using diclofenac sodium. Also because doxorubicin is a toxic agent and there is a high risk of toxicity working with doxorubicin, diclofenac sodium were used to study the prospect of the formation of liposome prepared with palm oil fraction plus optimizing the formulation in order to prepare liposomal doxorubicin relatively.

On the other hand, diclofenac sodium is widely used to treat mild to moderate pain and inflammations including those associated with osteo- and rheumatoid arthritis by inhibiting cyclooxygenase enzyme. Currently available diclofenac sodium dosage forms include gel, ophthalmic solution, immediate and controlled release tablets, suppositories

and intramuscular injection (Elron-Gross et al., 2008; Taghizadeh & Bajgholi, 2011). Oral forms of diclofenac sodium are the most common in the market though they usually cause gastrointestinal problems such as abdominal cramps, nausea, constipation, diarrhea and peptic ulceration. Furthermore, 40 % of the diclofenac sodium administered orally undergoes first-pass metabolism and does not reach systemic circulation, due to its poor solubility in water and acidic medium in the stomach, it has a poor oral bioavailability and a short half-life of about two hours (Todd & Sorkin, 1988) Intramuscular injection of diclofenac sodium avoids first pass metabolism and achieves a faster therapeutic effect (Taghizadeh & Bajgholi, 2011). However, the intramuscular injection can cause cutaneous lesions at injection site. This problem can be solved by encapsulation of diclofenac sodium in liposomes the release rate of diclofenac sodium can be controlled and consequently local tissue damage can be reduced as well (Kamat et al., 1997).

CHAPTER 3: METHODS AND MATERIALS

3.1 Materials

Table 3.1 shows the materials utilized in this research project.

Table 3.1: Materials of study

Materials	Company
L-alpha-phosphatidylcholine	Sigma Aldrich (Germany)
Cholesterol	Sigma Aldrich (Germany)
Diethyl ether	Sigma Aldrich (Germany)
Methanol	Sigma Aldrich (Germany)
Chloroform	Sigma Aldrich (Germany)
Doxorubicin hydrochloride	Sigma Aldrich (Germany)
RPMI 1640	Sigma-Aldrich (Germany)
MTT powder	Sigma-Aldrich (Germany)
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich (Germany)
Phosphate Buffered Saline tablets	Sigma-Aldrich (Germany)
Diclofenac sodium	Epic Ingredients Sdn. Bhd (Malaysia)
Palmetic acid	Acidchem Int. Sdn. Bhd (Malaysia)
Stearic acid	Acidchem Int. Sdn. Bhd (Malaysia)
Oleic acid	Acidchem Int. Sdn. Bhd (Malaysia)
linoleic acid	Acidchem Int. Sdn. Bhd (Malaysia)
Sodium hydroxide	MERCK (Germany)
Sodium citrate	MERCK (Germany)
Sodium carbonate	MERCK (Germany)
Fetal bovine serum (FBS)	GIBCO (US)
Penicillin G plus Streptomycin	GIBCO (US)
Male Rats 20 days old, weighting 200-250 g	Tehran university of medical sciences

3.2 Equipment

Table 3.2 shows equipment that utilized in this research project.

Table 3.2: Equipment utilized in this study

Equipment	Company
Rotary evaporator	Rotavapour R-124, BÜCHI
Sonicator	Sonicor
TEM	ABFETEM Leo 9112
Critical point drier	Critical Point Drier, Polaron
Scanning electron microscope	JSM 6400 SEM Jeol
Water bath shaker	Memert
Particle size and zeta potential	ZEM 3600, Malvern
frige-centrifuge machine	Universal 32
UV-Visible Spectrophotometry	UV-1601 UV-Visible Spectrophotometer
RP-HPLC	Knauer
pH meter	Mettler Toledo, Delta 320A/C
Freez-Dryer Christ	Alfa 2-4 LD
Differential Scanning Calorimetry	DSC 823e (Mettler Toledo)
FT-IR spectra	Nicolet FT-IR Magna 550 spectrophotometer
^{1}H-NMR	JEOL 400 MHZ
Homogenizer	IKA
Optical microscopy	IX71, Olympus, Japan
plate reader Synergy 4	Biotek
Small animal imaging system	Kodak

3.3 Methods: Initial study

3.3.1 Liposome preparation

3.3.1.1 Liposomal diclofenac sodium

Six formulations with various proportions (%w/w) of phosphatidylcholine and palm oil fractions were designed and prepared through conventional method (Bangham et al., 1965). The components were dissolved in chloroform/methanol mixture (2:1, v/v) in a round-bottom flask then the solvent was removed under vacuum using a rotary evaporator at 50°C, 50 rpm. A thin lipid film that formed in the interior of the flask, was purged with nitrogen to remove excess organic solvent then hydrated with 10 ml solution of diclofenac sodium (20 mg/L) in phosphate-buffered saline (PBS, pH 7.4) and sonicated for 15 min in a bath-type sonicator. The liposomes were allowed to form at room temperature and kept in the refrigerator overnight. The mixture was filtered through a syringe filter (0.45 μm) 3 times. Table 3.3 shows the component of each formulation.

Table 3.3: Designed formulations of liposome with various %w/w of Palm oil fractions

Formulation	Phosphatidylcolin (mg)	Cholestrol (mg)	Palmitic acid(mg)	Oleic acid(mg)	Stearic acid(mg)	Linoleic acid(mg)
FI	66.67	33.33	-	-	-	-
FII	56.67	33.33	1.60	1.20	0.60	6.60
FIII	46.67	33.33	3.20	2.40	1.20	13.20
FIV	33.33	33.33	5.33	4.00	2.00	22.00
FV	20.00	33.33	7.47	5.60	3.40	30.80
FVI	10.00	33.33	9.07	6.80	2.80	37.40

3.3.1.2 Liposomal doxorubicin hydrochloride

Liposome formulations were designed with different proportions (%w/w) of phosphatidylcholine and palm oil (Bangham et al., 1965; Szoka et al., 1980). The palm oil provided from Sigmaaldrich contains palmetic acid 35-48%, linoleic acid 6_13%,

myristic acid 0.5-6%, Oleic acid 35-50% and stearic acid 3-7% (Sigma-Aldrich, 2017). Table 3.4 shows the component of each formulation with various weights of lipids:

Table 3.4: Designed formulations of liposome with various %w/w of Palm oil and PC

Formulations	Cholestrol (mg)	Phosphatidylcolin (mg)	Palm oil (mg)	PEG 2000 (mg)
F1	45	50	-	5
F2	45	45	5	5
F3	45	40	10	5
F4	45	35	15	5
F5	45	30	20	5
F6	45	25	25	5

Liposome prepared using reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978). First lipids were accurately weighed and added into a round-bottom flask. Then, the lipids were dissolved in chloroform: methanol mixture at (2:1, v/v). The solvent was removed under vacuum using a rotary evaporator at 40°C at approximately 50 rpm, and a thin lipid film was formed inside the flask. Then film lipid was purged with nitrogen then, and then the lipid film was dissolved in 10 mL of diethyl ether(Bangham et al., 1965; Szoka et al., 1980; Torchilin & Weissig, 2003). Then 10 ml of doxorubicin hydrochloride in distilled water solution, with a concentration of 2000 µg/ml, were added to the mixture and it was sonicated for 15 minutes. Afterward, the mixture was placed in the rotary evaporator, and the organic solvent was removed under reduced pressure at 40°C and approximately 100 rpm. The system was kept in a refrigerator overnight in order to allow the liposome to form and encapsulation of doxorubicin (Hauser, 1982; Szoka & Papahadjopoulos, 1978). Liposome were filtered with nylon syringe at 0.45 µm 5 times, and centrifuged with frige-centrifuge machine (Universal 32) at 14000 rpm for 70 minutes to separate the free drug.

3.3.2 The formation and morphology of liposome

The formation of liposome was observed with (ABFETEM Leo 9112) Transmission electron microscope (TEM). TEM Samples were prepared with applying a drop of mixture to a carbon-coated copper grid, and left for one minute to allow some of the particles to adhere onto the carbon substrate. After removing the excess dispersion with a piece of filter paper, a drop of 1% phosphotungstic acid solution was applied for one minute, and left to become air-dried before the samples were viewed (Almgren et al., 2000; Hauser, 1993; Mu & Zhong, 2006).

3.3.3 Particle size and zeta potential measurement

Particle size and zeta potential of the liposome were measured using a zetasizer. The measuring cell was washed with distilled water, followed by sample solution. Then, 50 mg of liposome weighted and dispersed in 20 ml distilled water. Afterwards, samples solution was inserted into the cell using a pipette, and sent for measurements in the zetasizer (ZEM 3600, Malvern). The measurement was done three times for each sample and the values were expressed as the mean ± SD followed by One-way analysis of variance (ANOVA.)

3.3.4 Construction of standard curve

Diclofenac sodium: dilutions range of 10, 20, 30, 40 and 50 μg/mL diclofenac sodium (in fresh Phosphate Buffer, pH=7.4) were prepared and the absorbance's were measured by UV spectrophotometry (UV-1601 Shimadzu) at a wavelength of 300 nm, afterwards a standard curve was constructed using Microsoft Excel 2007 program.

Doxorubicin hydrochloride: standard curve of the absorbance of doxorubicin hydrochloride was constructed with dilutions ranged 5, 4, 3, 2.5, 2 and 1 μg/mL were prepared, and the absorbance was measured using a UV-Visible spectrophotometry at a wavelength of 335 nm (UV-1601 UV-Visible Spectrophotometer). Then, the standard plot was constructed using MS Office Excel 2007.

3.3.5 Entrapment efficiency of liposome

Diclofenac sodium: To study entrapment efficiency of the liposome, the mixture was centrifuged (Universal 32) for 1.5h at 9000 rpm, the supernatant was collected, and the absorbance measured by UV spectrophotometer at wavelength of 300 nm. The measurement was carried out in triplicate for each sample. Entrapment efficiency (EE) of the liposomes was determined as in Eq 3.1 (Chin et al., 2002; Greene et al., 1983).

$$EE\ (\%) = [(Ci - C_f)/Ci] \times 100 \qquad \text{(Equ. 3.1)}$$

Where Ci is the initial concentration of drug used in formulating the liposomes and C_f is the concentration of drug in the supernatant.

The liposome suspension was centrifuged for 70 minutes at 14000 rpm using centrifuge Universal 32. The supernatant, which contained free doxorubicin hydrochloride that was obtained, and then 100 mg of liposome were weighted. To calculate the amount of doxorubicin that entrapped in liposome, 5ml of distilled water, Ethyl acetate, and Diethyl ether (60:40,v/v) were added to dissolve the lipids. After separation, the aqueous and organic phase of the absorbance of aqueous phase, which contains entrapped doxorubicin, was measured using UV-Visible spectrophotometry at a wavelength of 335nm. The percentage of entrapment efficiency of liposomes was determined by equation 3.2 (Mu & Zhong, 2006; Panwar et al., 2010):

$$EE\ (\%) = (Ce/Ci) \times 100 \qquad \text{(Equ. 3.2)}$$

Where Ci is the initial concentration of drug used in formulating the liposome and Ce is the concentration of entrapped drug in liposome.

3.3.6 In-vitro release study of Liposomes

Diclofenac sodium: To measure in vitro release of diclofenac sodium from the liposomes100 mg of liposome were added into a dialysis bag (Mw12000). The bag was closed at both ends and located in 50 mL of PBS buffer medium (pH 7.4) at 37oC with 60 rpm under perfect sink conditions (Saarinen-Savolainen et al., 1997; Sznitowska &

Stokrocka, 2007). At predetermined time intervals, 2 mL of the medium was taken out for while same volume of fresh media was fulfilled. The absorbance of the collected samples during 120 hours was measured using UV spectrophotometer at wavelength of 300 nm. The results recorded are the mean value of three runs carried out for each liposome concentrate then the mean ± SD followed by One-way analysis of variance (ANOVA.) Cumulative diclofenac sodium released at various time points were plotted using Microsoft Excel 2007 and computed as in Eq 3.3 (Panwar et al., 2010; Saarinen-Savolainen et al., 1997).

$$\text{Drug release (\%)} = (Ct/Ci) \times 100 \qquad \text{(Equ. 3.3)}$$

Where Ct is the concentration of drug released at time t and Ci is the initial drug concentration.

Doxorubicin hydrochloride: After the separation of the unloaded doxorubicin hydrochloride in order to estimate the in-vitro drug release, freshly phosphate buffer (pH 7.4) were prepared and added to the loaded liposome. The suspension was incubated in water bath shaker at 60 rpm at a temperature of 37ºC, while samples was taken at 30, 60, 120, 240, 360,420 and 500 minutes afterwards. The suspensions were centrifuged for 70 minutes at 14000 rpm. The supernatant was obtained to measure the amount of drug being released at certain periods, and their absorbance was measured with UV spectrophotometry at a wavelength of 335 nm. The percentage of doxorubicin released at certain times was plotted using Microsoft Office Excel 2007, and was defined by equation 3.3. Each data was stated with mean ± SD and One-way analysis of variance (ANOVA) was applied to analyze the data respectively.

3.3.7 Chemical Stability of liposomal doxorubicin hydrochloride

Liposome drug delivery is confirmed to encapsulate medicines within liposome and deliver it instantly to the site of disease. However, they failed to reach their predestinate target at a satisfactory rate. Even intravenously injection of liposome is unable to reach their intended destination (Foradada et al., 2000). As soon as liposome comes to the blood, vesicles would be recognized and removed rapidly by a mononuclear phagocyte system. PMN (polymorphonuclear neutrophils) are plasma proteins remaining from damaged cells, which may bind with phospholipids, thus destroying them (Moein Moghimi et al., 2006). Furthermore, plasma high-density lipoprotein (HDL) might spoil and destabilize vesicles by possibly removing phospholipid from the bilayer, which lead to leakages and destroyed liposomes. Therefore, the interaction of liposome with a serum component is important in the stability of vesicles (Koshkaryev et al., 2013; Patel & Ryman, 1981).

Foradada studied the interaction between serum component and liposome in the presence of heat and oxygen (Foradada et al., 2000). Foradada incubated liposome with serum component in the presence of heat and oxygen. Afterwards, he took the acidic and basic extract and studied the quantity of PC pieces after breakage using ^{1}H-NMR. It is concluded that heat cause dissipation in vesicles and oxygen concentrations, and established the interaction between serum component and liposome. He declared that the interaction between liposome and serum component is exothermic and oxygen dependent, which leads to the disruption of the vesicles.

This study also follows Foradada investigation to testify to the stability of liposome of designed formulations using ^{1}H-NMR (Hydrogen Nuclear magnetic resonance JEOL 400 MHZ). To examine the interaction between serum component and designed formulations, unloaded liposome of each formulation were prepared. After centrifugation, 100 mg of liposome were weighted and added to 10 ml RPMI 1640, with

10% FBS and 1ml penicillin G. The mixture was incubated at 37ºC in the presence of atmospheric oxygen for 2 hours, one day, and one week. After incubation, acid-base treatment was conducted in order to improve the separation of basic extract. First, NaOH 0.1 N was added until the mixture get to basic pH, and then the reaction products were extracted with Ethyl acetate and Diethyl ether (60:40, v/v). The aqueous and organic phases were separated. The organic phase, which have basic and non-ionizable substances, were washed with 10 ml HCL 0.1 N, then again, the aqueous and organic phases were separated, and the organic phase were dried over Na_2SO_4, then concentrated. The aqueous phase that contains acid ionizable substances were washed with 10 ml HCL 0.1 N, then Ethyl acetate and Diethyl ether (60:40, v/v) were added as well. The organic phase were separated and dried over Na_2SO_4, then concentrated.

3.4 Methods: Advance study

3.4.1 Preparing liposome using Freeze-Thaw Method

Due to the results of the initial study, F3 (consisting of 10% palm oil) and F4 (consisting of 15% palm oil) liposome were chosen for further analysis. F3 and F4 formulations had better stability and release pattern among other formulations. The aim of the advance study is to optimize the entrapment efficiency of liposome and increase antitumor efficacy. In this part, liposome was prepared through freeze-thaw method and loaded with doxorubicin using the pH-gradient method and studied in terms of in-vitro and in-vivo.

In this section, the entrapment efficiency and in vitro release of LUVs were measured accurately using High performance liquid chromatography (HPLC). Fourier Transform Infra Red (FT-IR) Spectrums and Differential Scanning Calorimetric (DSC) of loaded and unloaded LUVs were studied in order to detect the interaction between liposome and doxorubicin. Furthermore, ex-vivo studies were carried out using MCF7 and MDA-MBA 231breast cancer cell lines. The IC50 (half maximal inhibitory concentration) of

F3, F4 liposome and Caelyx® measured after 48h of treatment via MTT assay. In-vivo imaging studies were performed to study the distribution of F3, F4 liposome, and Doxorubicin hydrochloride in animals. The imaging of mice organs was provided right after intracardiac injection plus 24, 48 and 72 hours after injection of treatments.

3.4.2 Effect of pH-Gradient on drug loading

Many researchers have tried to optimize the entrapment efficiency across liposome (Abraham et al., 2004; Lawrence D Mayer et al., 1986; L. D. Mayer et al., 1990; Tardi et al., 1996). Several studies proved the benefits of active loading via pH-gradient, which stipulate that the interior pH of the liposome has to be acidified, while the exterior pH-value is adjusted to physiological conditions. In this part, active loading using pH-gradient method was selected to load doxorubicin within liposomes, as it is 3-10 times higher in entrapment than passive loading, which furthers its independency of entrapment from vesicle size and lipid compositions (Lawrence D Mayer et al., 1994; L. D. Mayer et al., 1990; Tardi et al., 1996; Traïkia et al., 2000).

The required active loading via pH-gradient procedure consists of three steps: preparation of the liposome, establishment of pH-gradient, and loading drugs into the liposome. To arrange pH-gradient, citrate buffer (pH=4) was applied for interior encapsulation, while sodium carbonate was used for adjusting liposome external pH (Niu et al., 2010; Tardi et al., 1996).

Step 1: lipids with the same percentage of F3 and F4 were dissolved in Chloroform/Methanol (2/1). Organic solvents were removed under vacuum rotary evaporator. The remaining thin film lipid in the flask was purged with nitrogen gas to remove the remaining solvents.

Step 2: Citrate buffer (pH= 4) were prepared by dissolving 39.35g citric acid and 33.15g sodium citrate in 1000ml deionized water, then stored at room temperature. The thin film lipid were hydrated with 10ml citrate buffer and sonicated for 15 minutes. In order

to entrap the acidic buffer, the inner liposome freeze thaw cycle was performed. Flask containing liposome was frozen under -80ºC then heated in water bath 65ºC. The freeze-thaw cycle was performed 5 times. High pressure homogenizer were used to decrease the liposome size for 15 minutes, and afterwards, the mixture is passed though nylon syringe filter 0.45µ 5 times.

Step 3: Basic buffer were used for adjusting the outer liposome to reach a physiological pH. For this reason, 53mg of sodium bicarbonate was dissolved in 100 ml, and then added dropwise to the mixture until its pH reaches 7. Afterwards, 5 ml of the concentration of 2 mg/ml doxorubicin were added to the liposome and shaken for 20 minutes for it to be loaded within the liposome (Niu et al., 2010).

3.4.3 Characterization of the liposome

Morphology of the prepared liposome was studied using scanning electron microscope (SEM-JSM 6400, Jeol). In order to study the morphology and surface of vesicles, wet samples need to be dried prior to analysis with scanning electron microscope (SEM). Critical point drying (CPD) was conducted. Direct air-drying of the samples will cause damage due to surface tension of the evaporating water, while critical point drying process would prevent damaging of liposome.

In CPD, the samples were placed in mixture of glutaraldehyde and Sorensen's phosphate buffer solution (ratio 1:1) for an hour. Glutaraldehyde is used to fix and stabilize the samples. Then, the samples were washed with Sorensen's phosphate and distilled water (ratio 1:1). The samples were placed in vials containing a few drops of osmium tetroxide and distilled water (ratio 1:1) for 14 hours. Osmium tetroxide is used to stain the lipids to provide contrast image under SEM.

Also, it fixes and stabilizes the samples and kills microorganisms in the samples. Then, the samples were washed in a series of graded ethanol for 15 minutes in each concentration as follows: distilled water, ethanol 10%, ethanol 20%, ethanol 30%,

ethanol 40%, ethanol 50%, ethanol 60%, ethanol 70%, ethanol 80%, ethanol 90%, ethanol 95% and ethanol 100% (twice). Then acetone was introduced for 20 minutes for each step in the following order: ethanol: acetone (3:1), ethanol: acetone (1:1), ethanol: acetone (1:3) and pure acetone (twice).

The samples were prepared for CPD in a critical point drier and coated with gold particles in order to provide a contrast for the image and to prevent the accumulation of static electric field on the samples during the irradiation of electrons. Then, the samples were viewed under SEM using different magnification. Images of liposome were taken and saved once captured (Cohen, 1974; Horridge & Tamm, 1969). Further the particle size and zeta potential value of liposome were measured using a zetasizer (ZEM 3600, Malvern) by dispersing 50 mg of liposome in 20 ml distilled water.

3.4.4 High Performance Liquid Chromatography

HPLC (High Performance Liquid Chromatography) is a physical separation technique, where a liquid sample is injected into a column packed with small particles. It is an accurate and significant technique for separation (into its constituent components) and quantitative analysis, and it is applicable to a large number of pharmaceutical samples (Snyder et al., 2012). High performance liquid chromatography utilizing fluorometric detection is a rapid and sensitive assay for fluorescing objects (Sepaniak & Yeung, 1980). Since doxorubicin has fluorescence excitation at 470 nm and emission at 535 nm, fluorometric detection was applied for the determination of the concentrations of entrapped and released doxorubicin. Chromatographic separation was achieved using a ChromolithTM Performance RP-8e 100 mm×4.6 mm column (protected by a ChromolithTM Guard Cartridge RP-18e 5 mm×4.6 mm, Merck, Darmastadt, Germany. Mixture of acetonitrile: heptanesulfonic acid (0.2%, pH 4) by a ratio of 25:75, applied as mobile phase with the flow rate of 1 mL/min and solution of 120 ng/ml of doxorubicin hydrochloride utilized as internal standard.

3.4.5 Construction of Calibration curve

A calibration curve of doxorubicin hydrochloride was constructed using HPLC with fluorescence detection. Dilutions of doxorubicin hydrochloride ranged 400, 200, 100, 50, 25 and 12.5 ng/mL were prepared and injected. For each injection, the graph provided the area, retention time, and height. The calibration curve was constructed using Microsoft Office Excel 2007 (Fogli et al., 1999; Mazuel et al., 2003).

3.4.6 Entrapment efficiency of liposome

To evaluate the entrapment efficiency, the centrifuge method was applied. The mixtures were centrifuged for 70 minutes at 14000 rpm toward separating of the free doxorubicin hydrochloride from the entrapped ones. Then, to approach the concentration of non-entrapped doxorubicin, 1 ml of supernatant (which obtained after centrifuge) was diluted in 99 ml distilled water. Afterwards, 1 ml of diluted medium was injected to the HPLC, and the concentration of free doxorubicin acquired using the calibration formulation (Chin et al., 2002; Greene et al., 1983). The concentration and percentage of entrapped doxorubicin were intended by equations 3.4.

$$EE\ (\%) = [(C_i - C_f)/C_i] \times 100 \qquad \text{(Equ. 3.4)}$$

where EE is percentage of entrapped drug, Ci is the initial concentration of drug used in formulating the liposome and Cf is the concentration of drug in the supernatant and EE (%) is percentage of entrapment efficiency.

3.4.7 Quantification of in-vitro release using HPLC

Dialysis method was used to estimate in *vitro* release of Doxorubicin from liposome. In order to aid the dialysis sacs (12000 M_W) were used. First, the preservative should be removed, and consequently, the dialysis sacs were soaked in distilled water for 5 hours, with the distilled water having to be refreshed every 30 minutes (Panwar et al., 2010). Later, 2 ml of liposome mixture and Caelyx® (PEG liposoma doxorubicin in market) were inserted into dialysis sacs individually. The dialysis sacs, located in 1000 ml of

fresh PBS (Phosphate Buffered Saline, pH=7), were then incubated in water bath shaker at 37ºC with 140 rpm in sink conditions (Saarinen-Savolainen et al., 1997). Samples were provided at 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 hours, and then injected into HPLC, respectively. Concentrations of doxorubicin throughout release time intend useing the calibration equation (Panwar et al., 2010; Saarinen-Savolainen et al., 1997; Sznitowska & Stokrocka, 2007). Afterwards, in-vitro drug release of doxorubicin was provided with the equation 3.5.

$$\text{Drug release (\%)} = (Ct \times 10^{-3}/Ci) \times 100 \qquad \text{(Equ. 3.5)}$$

Drug release is the percentage of doxorubicin that released at intended time, where Ct is the concentration of released doxorubicin at time t and Ci is the initial concentration of doxorubicin.

3.4.8 Fourier Transform Infra-Red

FT-IR Spectra has been an analytical instrument for decoding procedure of chemicals since the 1950s (Faix, 1992). Signals provided in FT-IR spectrum are the result of characterizing the compounds. Any sample of solid, liquids, solutions, films, fibers, pastes, powders, gases, and surfaces can all be studied using spectroscopy (Stuart, 2004).

In order to examine the FT-IR spectrum of liposome, solid samples were provided through the freeze-drying procedure using Freeze-Dryer Christ (Alfa 2-4 LD) in the direction of taking FT-IR spectrum. Potassium bromide were added to dried liposome at a ratio of 50:1, and mixed together on a thin disc (as potassium bromide disc) (Faix, 1992; Griffiths & De Haseth, 2007; Smith, 2011). FT-IR spectrums were obtained by a Nicolet FT-IR Magna 550 spectrophotometer.

3.4.9 Differential Scanning Calorimetry

DSC is a thermodynamical tool for determining the thermal properties of materials (Gill et al., 2010). This primary technique directly assesses the uptake of heat energy during

the fluctuation of temperature in order to specify any connection among temperature and physical properties of samples. Calorimetry is a suitable thermal analysis technique for qualifying the purity, the melting point, and the polymorphic forms of samples in the pharmaceutical industry. Furthermore, DSC is the only direct way for determining enthalpy (Gill et al., 2010; Höhne et al., 2003; Van Holde et al., 2006).

Calorimetry of medicinal products is considered as a tool to study the physicochemical properties and their respective stability, as well as their interactions during the formulation process (Demetzos, 2008). It is also applied to identify the thermal behavior of lipid bilayers and lipidic drug delivery systems, such as liposome, noisome, and SLN (solid lipid nanoparticles). DSC is a powerful device for quality control of liposome. Thermodynamical analysis of liposomal drug delivery system can provide and specify the temperature-dependence the heat capacity of liposome structure, due to thermal phase transitions (Ford & Mann, 2012; Gill et al., 2010). Heat capacity curves of liposome provided information of enthalpy (ΔH) and entropy (Tm), which affect the stability of the liposomal system via storage conditions (Biltonen & Lichtenberg, 1993; Demetzos, 2008). To study the calorimetry of liposome, freeze-dried liposome were provided and 5-10 mg of individual formulation packed in aluminum pan using the pierced lid. DSC instrument was calibrated by indium as a standard sample and then provided liposome samples were located in then heated from -20 to 160 °C, further doxorubicin hydrochloride and liposomal doxorubicin heated from -20 to 240 respectively, with the scanning rate of 10 °C /min (Demetzos, 2008).

3.4.10 Studies on breast cancer cell lines

Cellular uptake and cytotoxicity of F3 and F4 liposomal doxorubicin were investigated in cell culture media. MCF7 (Michigan Cancer Foundation-7) and MDA-MBA 231 human breast cancer cell lines were used to determine cellular uptake and cytotoxity of designed formulations. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium

bromide) assay was carried out to evaluate cytotoxicity of F3 and F4 liposomal doxorubicin, unloaded liposome (without drug), Doxorubicine hydrochloride and Caelyx®. Caelyx® is commercial pegilated liposomal form of doxorubicin, which is widely used as treatment for breast cancers or any other solid tumors in consequence of long circulation and less toxicity compare to Doxorubicin hydrochloride (Ranson et al., 2001).

3.4.11 Cellular uptake

Fluorescence detection methods are broadly used for studying the cellular uptake of several drugs in a cell culture media. Since doxorubicin emits red fluorescence, this technique can be utilized to monitor its uptake using either optical or fluorescence microscopy (Hu et al., 2008). Furthermore, the cellular uptake percentage of doxorubicin can be quantified by this method as well (Jiahui Yu, 2013).

To determine the cellular uptake, two breast cancer cell lines were utilized separately. MCF-7 and MDA-MBA, 231 cells were cultured in RPMI 1640 and 10% fetal bovine serum (FBS) separately. Each cell line were then seeded in 24-well plates with a density of 1 $\times 10^5$ cells/well, and incubated in 37ºC with 5% CO2 for 24h. MCF-7 and MDA-MBA 231 cells were treated with F3 and F4 liposomal doxorubicin individually. Then 50µl of Doxorubicin liposome (2000 µg/ml) were added into each well and incubated for 24h. Afterwards, the cells were washed thrice with BPS (pH=7.4), and Image analyses of cells were also performed after 24h, with confocal microscopy (IX71, Olympus, Japan) (Iwasa et al., 2006; Park & Yoo, 2010).

In order to evaluate the percentage of cellular uptake, after 2h and 24h incubation, the cells were washed thrice with BPS, then cells uptake were measured using plate reader Synergy 4, and Biotek with florescence detector. The percentages of cellular uptake are provided from the equation 3.6 (Hu et al., 2008; Jiahui Yu, 2013).

Cellular uptake (%) = (Ii/Ic) x 100 (Equ. 3.6)

Where Ii is the fluorescence intensity of treated cells, in reading time and Ic is the initial fluorescence intensity of doxorubicin hydrochloride.

3.4.12 MTT assay

The human breast cell lines (MCF 7, MDA-MBA 231) were cultured in RPMI 1640 with 10% FBS and incubated in 37ºC with 5% CO2. Afterwards, overnight cells were seeded at the density of 7000 cells per well of 96-well plates (with 100 µl of medium) and incubated in 37ºC with 5% CO2 during 24 hours. Cells were treated with various concentrations of F3 and F4 liposomal doxorubicin, caelyx®, doxorubicin hydrochloride and unloaded liposome, then incubated for 24 and 48h. After finishing incubating time, the media was removed, and 10 µl MTT were added to each well. After 4h of incubation, MTT were removed and 100 µl DMSO were added to each well. The absorbance of 96-well plates was measured with an ELISA reader at λ=595nm. The growth inhibitory concentration of cells was calculated by following equation (Akbari & Javar, 2013; Chai, 2009).

$$IC50\ (\%) = [(A_B - A_S)/ A_B] \times 100 \quad \text{(Eq 3.7)}$$

IC50 (Inhibition concentration) is the concentration of the compound, which gives the 50% growth inhibition value, where A_B is the absorbance of blank well and A_S is the absorbance of treated cells with various concentration. Each test was repeated three times then IC50 was evaluated with the mean ± SD and followed by One-way analysis of variance (ANOVA) (Hu et al., 2008; Miglietta et al., 2000; Yuan et al., 2008).

3.4.13 In vivo study

In this study, optical in-vivo imaging technique was applied for monitoring the distribution of doxorubicin hydrochloride and its liposomal encapsulated form in the normal body of rat. This technique is able to image the whole body of small animals and body cells (Weissleder, 2001). In both fluorescence in-vivo imaging and fluorescence

microscopy, a low-light camera and proper filters were used to collect fluorescence excitation and emission light from samples. In fluorescence microscopy, object of imaging are cells, slides, or culture dishes, while the whole-body of small animals is pictured with optical in-vivo imaging system. However, in-vivo imaging is technically a more challenging process, as the animal tissues are opaque or/and thick, therefore, they absorb scatters photons and generate strong auto-fluorescence. Furthermore, it is essential to apply an appropriate imaging probe, which provides biologically stable distribution and preferential accumulation at the intended target site (Ntziachristos et al., 2003). Loading near-infra red (NIR) fluorophores with drug delivery agents would be a great opportunity to follow medicine distribution with optical in-vivo imaging system without using specific conjugated antibodies. Near-infrared excitable fluorescent agents (NIR) provided deep tissue penetration and low tissue auto-fluorescence. Some of NIR lipophilic alternative carbocyanine dyes with potential auto-fluorescence are Dil, DiO, DiD, DiA, and DiR (Hilderbrand & Weissleder, 2010; Kalchenko et al., 2006; Ntziachristos et al., 2003). However, Doxorubicin has an auto-fluorescence with an excitation of 470 and emission of 535 nm, which allows us to obtain a significant signal without using NIR within liposome (Shokri et al., 2012).

The animal ethic committee approval (Appendix D) obtained from TUMS (Tehran university of medical sciences) in order to study the *in vivo* distributions of several formulation. Doxorubicin hydrochloride, F3, F4 and Caelyx® were injected the heart and after 1, 24, 48, and 72 hours the florescence intensity of organs were determined. For each treatment, 12 male rats at 20 days old and weighing 200-250 g were chosen. For each treatment, 300 µml was injected into rats' heart individually (Shokri et al., 2012). After 1, 24, 48, and 72h, the rats were dissected, and their organs were removed for quantitative analysis. Heart, lungs, kidneys, spleen, and the liver of each rat were placed in a clean dish for imaging using an *in-vivo* imaging device (Kodak). Since

doxorubicin has an autofloresence with an excitation at 470 nm and emission at 535 nm, the image of the organs were successfully taken, and the florescence intensity of doxorubicin was then determined respectively (de Chermont et al., 2007).

CHAPTER 4: RESULTS

4.1 Liposome formation and morphology

Images of designed liposome provided using Transmission Electron Microscopy (TEM). Transmission Electron microscopy shows formation and morphology of vesicles plus their physical properties. Lamellarity (number of lipid bilayers) and size of the vesicles, are the important characterization of each liposome that can be investigated with TEM images (Hauser, 1982; Mu & Zhong, 2006).

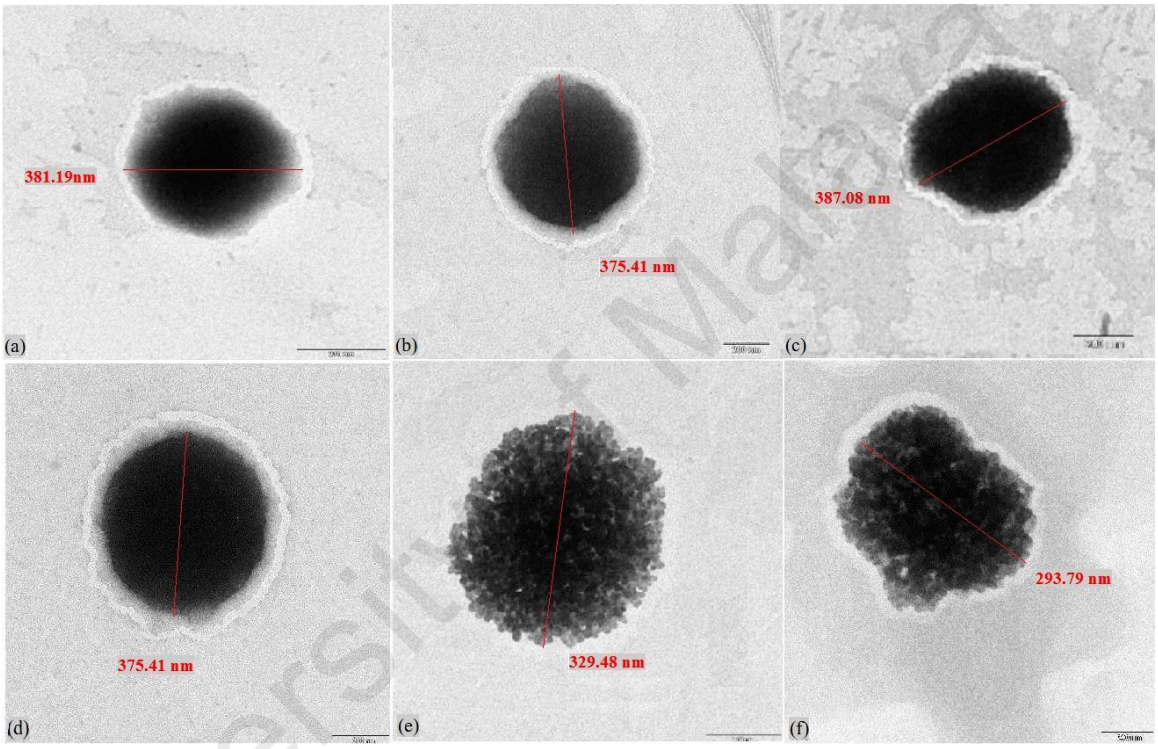


Figure 4.1: TEM images of designed liposome; (a) F1 magnification 16000x, (b) F2 magnification 16000x, (c) F3 magnification 16000x, (d) F4 magnification 16000x, (e) F5 magnification 20000x V, (f) F6 magnification 20000x

Liposomal diclofenac sodium: As TEM images show the liposomes were formed in all the formulations. FI and FII, which contained higher proportions of PC produced large and well-defined spherical vesicles; however, FIII, IV, V and VI which had palm oil fraction content of 10%, 20% , 33.33%, 46.67% and 56.67% , respectively, produced smaller, slightly deformed vesicles. These results shows however there is possibility of preparation liposomal drug delivery systems with other natural products such as palm oil fractions, but the role of PC in formation, shape and size of liposomes is not

negligible, formulation with higher percentage of PC produces more spherical vesicles in bigger size.

Liposomal doxorubicin hydrochloride: Considering TEM images in Figure 4.1, liposome formed in all formulations. As TEM images demonstrate vesicles contain one bilayer with size around 340 to 450 nm (Almgren et al., 2000; Mozafari, 2005; Riaz, 1996). F1 (Liposome formulation without Palm oil) and F2 (Liposome formulation consisting of 5% Palm oil) which composed mainly of PC have spherical fine shape. F3 (Liposome formulation consisting of 10% Palm oil) and F4 (Liposome formulation consisting of 15% Palm oil) vesicles that contain less PC, are larger and less spherical but still having acceptable and fine shape, nevertheless in F5 (Liposome formulation consisting of 20% Palm oil) and F6 (Liposome formulation consisting of 25% Palm oil) malformed and misshapen vesicles with smaller size were formed.

4.2 Particle size distribution and zeta potential measurement

Each value was expressed as the mean ± SD. One-way analysis of variance (ANOVA) was applied to analyze the data, followed by Scheffe post-hoc test using [SPSS 15 for Windows]. Differences were considered significant at $p < 0.05$.

Liposomal diclofenac sodium: Particle size distribution and zeta potential of the liposome were measured using a zetasizer device (ZEM 3600, Malvern). Table 4.1 shows the particle size of the liposomes decrease from FI to FVI and the ZP values confirm the system stability. Results demonstrated that the percentage of PC and palm oil fractions in the formulations has potential influence on zeta potential. Replacement of PC with, palm oil fractions changed the zeta potential from -31.2 mV to -50.7 mV. Further Increasing palm oil fractions to 46% and 56%, shows significant improvement on zeta potential correspondingly.

Table 4.1: Mean particle size (nm), zeta potential (mV) and entrapment efficiency (%±SD) of the liposomal diclofenac sodium (n=3)

Formulation	Mean particle size	Mean zeta potential	Mean EE
FI	439 ±15	-31.2 ±3.3	70.78 ±0.41
FII	421 ±12	-29.4 ±2.0	60.14 ±7.48
FIII	387 ±13	-29.3 ±2.4	55.63 ±2.75
FIV	392 ±21	-34.3 ±1.4	30.98 ±8.28
FV	360 ±11	-42.8 ±1.4	42. 25 ±4.43
FVI	300 ±10	-50.7 ±4.9	77.84 ±5.85

Liposomal doxorubicin hydrochloride: Particle size distribution and zeta potential of the liposome were measured using a zetasizer device (ZEM 3600, Malvern).Table 4.2 shows Mean particle size (nm±SD) of fresh prepared liposome plus Zeta potential (mV) of fresh liposome and stored in 4°C after 30 and 60 days.

Table 4.2: Mean particle size (nm) and Zeta potential (mV) of liposome (n=3)

Formulation	Mean particle size	Zeta potential fresh liposome	Zeta potential after 30 days	Zeta potential after 60 days
F1	378.84±1.3	-26.6±1.8	-24.7±3.3	-22.2±4.1
F2	407.45±2.7	-27.5±2.3	-26.3±0.9	-25.1±2.6
F3	447.21±1.7	-32.2±1.1	-31.8±2.1	-30.6±1.9
F4	438.74±2.3	-31.8±4.2	-30.3±2.8	- 29.4±4.3
F5	356.67±1.1	-29.3±2.3	-27.1±1.4	- 26.5±4.1
F6	341.45±1.8	- 28.9±2.1	-27.6±2.2	-25.7±3.8

Referring to Table 4.2, all formulation had mean particle size from 378 to 447 nm, whereas F6 had smallest and F3 showed largest mean particle size among others. However only in F3 and F4 liposome, Zeta potential values were above -30 both in fresh medium and after 30 days of storage below 4°C. Further passing 60 days of storage below 4°C, Zeta potential value of F3 remains above -30 while in F4 it was reduce to - 29.4 after.

4.3 Construction of standard curve

Diclofenac sodium:

Standard curve of diclofenac sodium in PBS, was constructed with dilutions ranged 5, 7.5, 10, 15, 20 and 30 µg/mL of diclofenac sodium in PBS (pH = 7.4). The absorbance of samples provided using UV-Visible spectrophotometry at wavelength of 300 nm. Considering Figure 4.2, equation 4.1 was obtained using Microsoft Excel 2007.

$$Y = 0.0213\ X + 0.056 \qquad \text{(Equ. 4.1)}$$

Where Y is the absorbance at the wavelength of 300nm, and X is the concentration of diclofenac sodium; the regression line was achieved $R^2 = 0.9936$

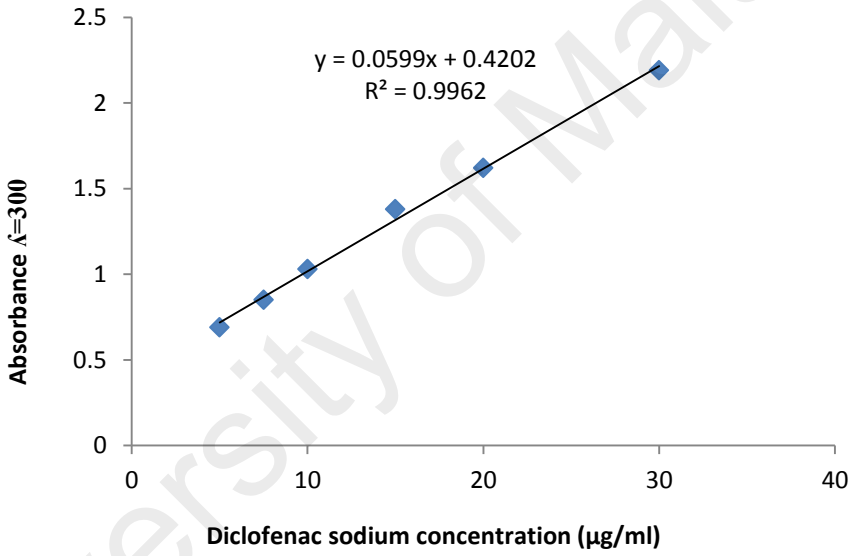


Figure 4.2: Standard curve of Diclofenac sodium in PBS (pH=7.4).

Doxorubicin hydrochloride:

Standard curve of doxorubicin hydrochloride was constructed with dilutions ranged 5, 4, 3, 2.5 and 2 µg/ml of doxorubicin hydrochloride in PBS (pH=7.4). The absorbance of samples provided using UV-Visible spectrophotometry at wavelength of 335nm. Considering Figure 4.3, equation 4.2 was obtained using Microsoft Excel 2007.

$$Y = 0.2543\,X + 0.0008 \qquad \text{(Equ. 4.2)}$$

Where Y is the absorbance at wavelength 335 nm, and X is the concentration of doxorubicin hydrochloride, the regression line was obtained $R^2 = 0.9982$ as well.

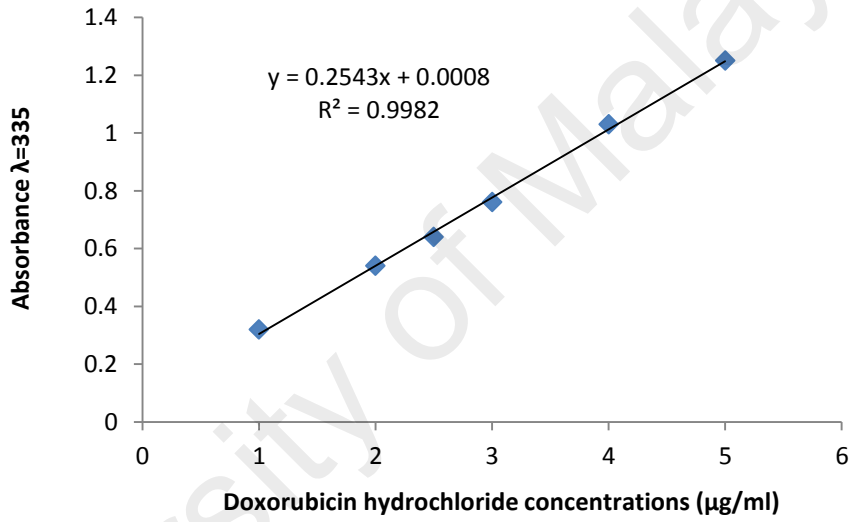


Figure 4.3: Standard curve of Doxorubicin hydrochloride in PBS (pH = 7.4).

4.4 Liposome entrapment efficiency

Diclofenac sodium: To study the quantity of diclofenac sodium that entrapped in liposomal diclofenac, the mixture was centrifuged (Universal 32) for 1.5h at 9000 rpm, the supernatant was collected, and the absorbance measured by UV spectrophotometer at wavelength of 300 nm, then entrapment efficiency (EE) of the liposomes was determined as in Eq 3.1 (Chin et al., 2002; Greene et al., 1983). Table 4.1 shows the entrapment efficiency of liposomal diclofenac sodium. According to the results, EE was variable in the formulation by increasing the percentage of palm oil fractions. The EE

was reduced from FI to FIV which had the least EE (31.0 %) but then it rose up again up to 77.8 % in FVI.

Doxorubicin hydrochloride: To determine the quantity of doxorubicin hydrochloride that entrapped within liposome, first free doxorubicin was separated from the entrapped one (using centrifuge Universal 32), then 100 mg of liposome were weighted and added to into mixture of Ethyl acetate and Diethyl ether (60:40,v/v) in order to dissolve the lipids and take out encapsulated doxorubicin. Afterwards entrapment efficiency were calculated using equation 3.2 (Mu & Zhong, 2006; Panwar et al., 2010). Table 4.2 shows the mean entrapment efficiency of prepared liposome (n=3).

Table 4.3: Mean entrapment efficiency of designed formulations (n=3)

Formulations	Entrapment Efficiency %
F1	40.02 ±0.97
F2	40.15 ±0.92
F3	40.09 ±0.81
F4	40.22 ±0.74
F5	40.15 ±1.26
F6	38.05 ±0.81

Considering Table 4.2, the entrapment efficiency of liposome in all formulations was nearly similar. Further no significant difference was observed by replacement of palm oil in formulations.

4.5 In vitro drug release study

Diclofenac sodium: Figure 4.3 demonstrates the in vitro release through 106 hours for total release of diclofenac sodium from liposomes. The absorbance of the collected samples during 120 hours, was measured using UV spectrophotometer

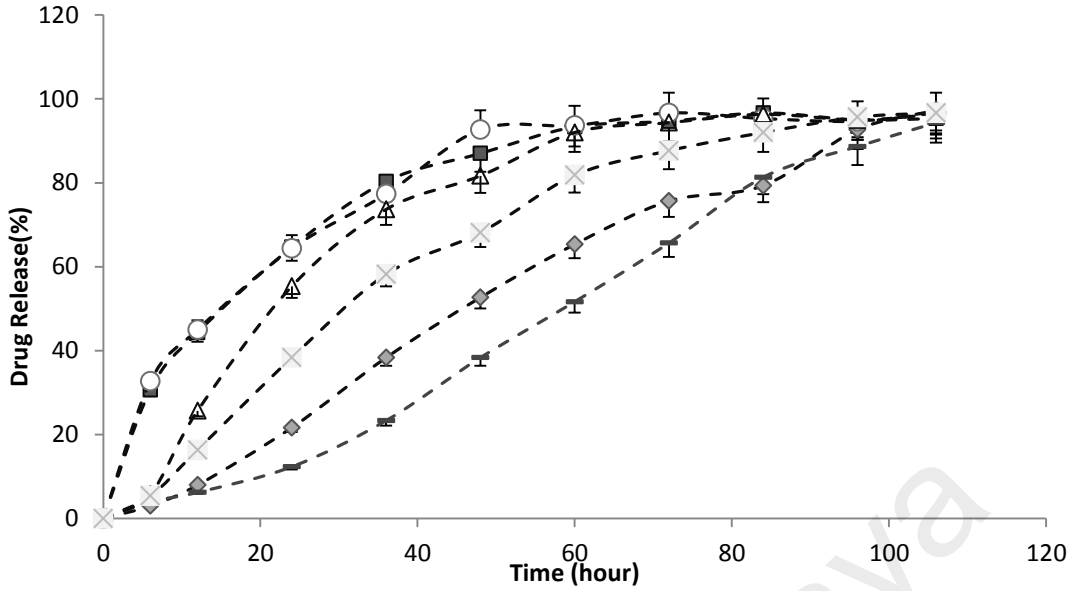


Figure 4.4: Cumulative release of Diclofenac sodium in PBS (pH 7.4). Key: FI (♦), FII (■), FIII (○), FIV (-), FV (▲), FVI (×); error bars indicate standard deviation (n=3).

Considering Figure 4.4 the drug releaseing of FI was about 10 % through first 12 hours while it slightly increased until release was complete after 106 hours. FII and FIII released drug of up to 44 and 46 %, after 12 hours respectively, and diclofenac sodium release for both was complete after 72 hours. FIV gradually released drug in 24 hours but release increased more rapidly to 85 % after 80 hours. There was a slow release of FV during the first 6 hours but it was followed by a sharp rise reaching the 91 % in 48 hour. Diclofenac sodium release from FVI release rose progressively over106 hours.

Doxorubicin hydrochloride: samples of released doxorubicin from liposome were provided at 30, 60, 120, 240, 360, 420 and 500 minutes after incubation with fresh PBS (pH=7.4). The absorbance of provided samples were measured with UV spectrophotometry at wavelength 335 nm. Table 4.3 is showing the mean absorbance, concentration and pecentage of each samples at releasing time (n=3) (Maurer et al., 1998; Panwar et al., 2010). Futher the percentage of doxorubicin released at certain times was plotted using Microsoft Office Excel 2007, and was defined by equation 3.3 respectively.

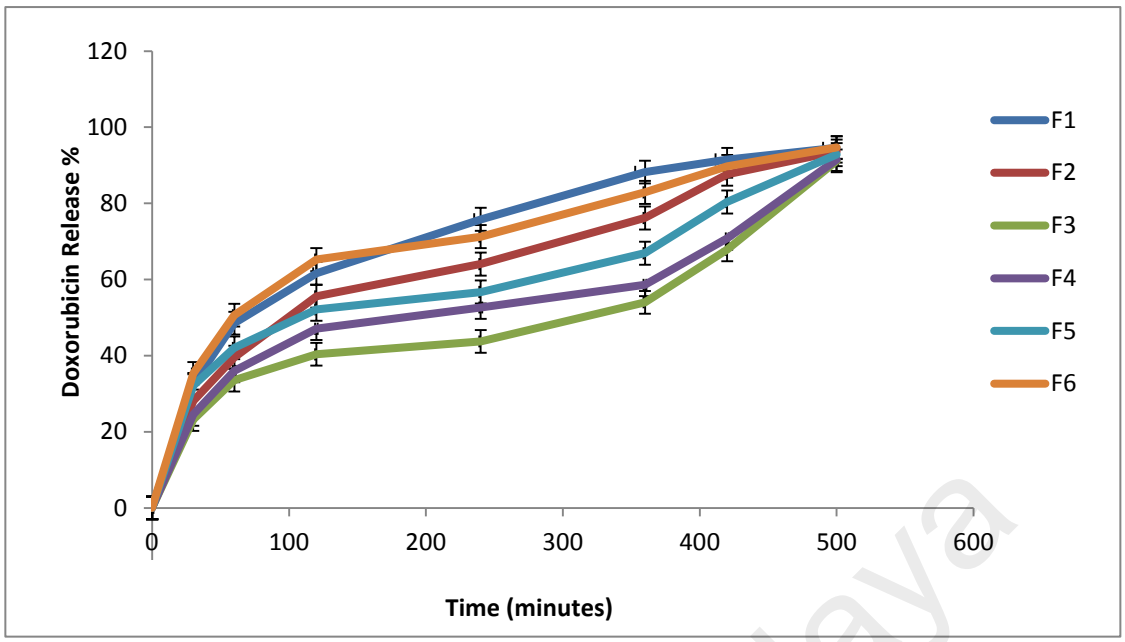


Figure 4.5: In vitro release of Doxorubicin from liposomes during 500 minutes, error bars indicate standard deviation (n=3).

Figure 4.5 and Table A.1 shows the mean percentage of doxorubicin released from liposomes during 500 minutes. Considering Figure 4.5, F1 and F6 showed the highest release of doxorubicin compare to other formulations. While F6 had more releasing rate within first 150 min, but during 150 to 500 minutes, F1 showed higher release rate than other formulations. after F1 and F6, F2 had more release although in fisrt 60 min F5 showed higher release rate. F5 had more releasing rate after F2. The lowest releasing rate observed in F3 and F4 liposomes. In first 60 minutes both F3 and F4 showed same release whereas from 60 to 360 minutes, F3 has slower and slightly release compare with F4.

4.6 Liposome Chemical Stability

Although phosphatidylcholin is essential component of forming liposomes, thay have a high tendency of oxidation due to rich amount of unsaturated fatty acids (Eibl, 1984; Li & Vance, 2008). Signals from phosphatidylcholin used as a reference for comparing degradation of vesicles after incubated with serum component in presence of heat and oxygen (Foradada et al., 2000). Prepared liposome with various percentages of palm oil

and phosphatidylcholin, were incubated with serum component at 37 ^{o}C. Afterward acidic and basic extracts of each formulation provided after 2 hours, one day and one week of incubation. ^{1}H NMR spectrums of F1-F6, acidic and basic extracts demonstrated in appendix Figures A.1 – A.36.

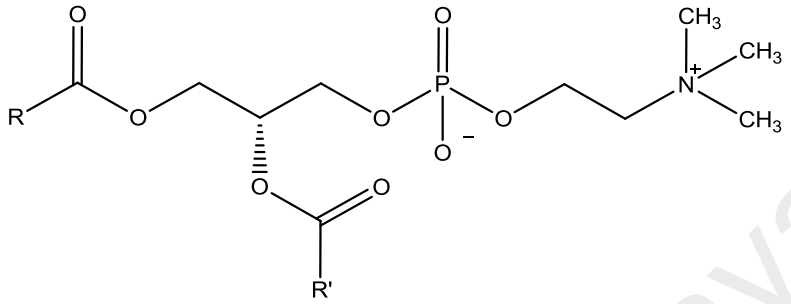


Figure 4.6: Phosphatidylcholin Structure.

4.6.1 Degradation of F1 acidic extract

Figures B.1 to B.3 show ^{1}H NMR spectrums of F1 acidic extract after 2 hours, one day and one week of incubation with serum component. Considering Figure B.1, four signals can be observed at 2.0103, 2.2757, 2.8108 and 5.2776 ppm which are matching with **-CH_2-C=C-**, **-CH_2-C=O** and **=CH-CH_2-CH=**, **–CH=CH-** (Foradada et al., 2000). Figure B.2 illustrate ^{1}H NMR spectrums of F1 acidic extract after one day incubation. Five signals detected at 2.0417, 2.2635, 3.2841, 4.301 and 5.376 ppm in ^{1}H NMR spectrum which are corresponding to **-CH_2-C=C-**, **-CH_2-C=O**, **CH_2-N^+**, **-CH_2-O-P** and **–CH=CH-** (Foradada et al., 2000). Figure B.3 shows ^{1}H NMR spectrums of F1 acidic extract after one week incubation. Same as one day incubation five signals identified in ^{1}H NMR spectrum at 2.0814, 2.2729, 3.3818, 4.2971 and 5.4015 ppm which are corresponding to **-CH_2-C=C-**, **-CH_2-C=O**, **CH_2-N^+**, **-CH_2-O-P** and **–CH=CH-** According to ^{1}H NMR spectrums of F1 acidic extract, F1 liposome showed degradation within 2h, one day and one week, which indicates the low chemical stability of formulation (Foradada et al., 2000).

4.6.2 Degradation of F1 basic extract

Figures B.4 to B.6 show the ^{1}H NMR spectrums of F1 basic extract after 2 hours, one day and one week of incubation with serum component. As it can be seen in Figure B.4, three signals at 2.0170, 2.3262, and 5.3872 ppm existed which are corresponding to **-CH_2-C=C-**, **-CH_2-C=O** and **-CH=CH-** (Foradada et al., 2000).

The ^{1}H NMR spectrum of F1 basic extract after one day incubation with serum component is shown in Figure B.5. In ^{1}H NMR spectrum four signals presented at 2.0761, 2.3190, 2.8084, 3.8228, ٤,2912 and 5.3917 ppm which are result of **-CH_2-C=C**, **-CH_2-C=O, =CH- CH_2-CH=**, **CH_2-N, CH_2-O-P** and **-CH=CH-** (Foradada et al., 2000).

Figure B.6 demonstrate ^{1}H NMR spectrum of F1 basic extract after one week incubation with serum component. Four signals distinguished in Figure B.6 at 2.1038, 2.3207, 2.8421, 3.8013, 4.2898 and 5.2983 ppm which are corresponding to **-CH_2-C=C**, **-CH_2-C=O-** , **=CH- CH_2-CH= , CH_2-N , CH_2-O-P** and **-CH=CH-** (Foradada et al., 2000). According to ^{1}H NMR spectrums of F1 basic extract, F1 liposome showed degradation within 2 h, one day and one week, which shows the low chemical stability of formulation.

4.6.3 Degradation of F2 acidic extract

The ^{1}H NMR spectrums of F2 acidic extract after 2 hours, one day and one week of incubation with serum component are shown in Figures B.7 to B.9. Considering Figure B.7, no signal in presented after 2 hours due to stability of vesicles during incubation period (Foradada et al., 2000).

Referring to Figure B.8, four signals identified in ^{1}H NMR spectrum of F2 acidic extract after one day incubation with serum component. Signals at 2.0102, 2.3685, 3.4604 and 5.4861 ppm which are result of **CH_2-C=C-, -CH_2-C=O, CH_2-N^+** and **CH=CH-** (Foradada et al., 2000).

Figure B.9 illustrate the ^{1}H NMR spectrums of F2 acidic extract after one week incubation with serum component. Six signals at 2.0184, 2.2571, 2.7544, 3.3946, 4.2552 and 5.4110 ppm are presented in ^{1}H NMR spectrum which are related to **-CH_2-C=C-, -CH_2-C=O, =CH-CH_2-CH=**, **CH_2-N^+**, **-CH_2-O-P** and **–CH=CH-** (Foradada et al., 2000). According to ^{1}H NMR spectrums of F2 acidic extract, F2 liposome didn't have any degradation within 2h, while after one day and one week, liposome showed degradation and low chemical stability.

4.6.4 Degradation of F2 basic extract

Figures B.10 to B.12 show the ^{1}H NMR spectrums of F2 basic extract after 2 hours, one day and one week of incubation with serum component. As in Figure B.10 shows there is no signals after 2 hours incubation same as acidic extract (Foradada et al., 2000).

Figure B.11 shows the ^{1}H NMR spectrum of F2 basic extract after one day incubation with serum component. Three signals are observed in ^{1}H NMR spectrum at 2.0295, 2.2990 and 5.2776 ppm that are matching with **CH_2-C=C**, **CH_2-C=O** and **-CH=CH-** (Foradada et al., 2000).

The ^{1}H NMR spectrum of F2 basic extract after one week incubation with serum component shows in Figure B.12. Four signals are distinguished in ^{1}H NMR spectrum at 2.3743, 2.8608, 3.8181 and 5.3709 ppm which are corresponding to **CH_2-C=O**, **=CH-CH_2-CH=**, **CH_2-N** and **-CH=CH-** (Foradada et al., 2000). According to ^{1}H NMR spectrums of F2 basic extract, F2 liposome didn't have any degradation within 2h, while after one day and one week, liposome showed degradation and low chemical stability.

4.6.5 Degradation of F3 acidic extract

Figures B.13 to B.15 demonstrate the ^{1}H NMR spectrums of F3 acidic extract after 2 hours, one day and one week of incubation with serum component. Considering Figure B.13, no signal observed after 2 hours because of stability of vesicles through incubation period (Foradada et al., 2000).

Referring to Figure B.14, two signals existence in ^{1}H NMR spectrum of F3 acidic extract after one day incubation with serum component at 2.0661, 2.8102 and 5.4052 ppm which are the resultant of **-CH_2-C=C**, **=CH-CH_2-CH=** and **-CH=CH-** (Foradada et al., 2000).

Figure B.15 show the ^{1}H NMR spectrums of F3 acidic extract after one week incubation with serum component. After one week five signals identified in ^{1}H NMR spectrum at 2.0177, 2.3961, 2.7814, 3.3808, 4.3013 and 5.4521 ppm which are corresponding to **-CH_2-C=C-, -CH_2-C=O, =CH-CH_2-CH=**, **CH_2-N^+** and **-CH=CH-** (Foradada et al., 2000). According to ^{1}H NMR spectrums of F3 acidic extract, F3 liposome didn't have any degradation within 2h, while after one day small signals were observed which indicate few degradation of liposome. However after one week, more signals in ^{1}H NMR spectrums confirm degradation of liposome.

4.6.6 Degradation of F3 basic extract

Figures B.16 to B.18 show the ^{1}H NMR spectrums of F3 basic extract after 2 hours, one day and one week of incubation with serum component. Considering Figure B.16 no signals exist in ^{1}H NMR spectrum after 2 hours incubation same as acidic extract (Foradada et al., 2000)**.**

The ^{1}H NMR spectrum of basic extract F3 after one week incubation with serum component shows in Figure B. 17. Four signals are detected ^{1}H NMR spectrum at 2.012, 3.7814, 4.2815, and 5.4061 ppm to that are matching to **-CH_2-C=C**, **CH_2-N^+**, **-CH_2-O-P** and **-CH=CH-** (Foradada et al., 2000)**.**

Figure B.18 illustrate the ^{1}H NMR spectrum of basic extract F3 after one day incubation with serum component. Five signals in 1H NMR spectrum are observed at 2.0421, 2.3506, 2.8693, 3.7012, 4.3685 and 5.4848 ppm which are corresponding with **-CH2-C=C-** , **-CH2-C=O**, **=CH- CH2-CH=**, **CH2-N**, **-CH2-O-P** and **-CH=CH-** (Foradada et al., 2000). According to ^{1}H NMR spectrums of F3 basic extract, F3

liposome didn't have any degradation within 2h, while after one day small signals were observed which indicate few degradation of liposome. However after one week, more signals in ^{1}H NMR spectrums confirm degradation of liposome.

4.6.7 Degradation of F4 acidic extract

The ^{1}H NMR spectrums of F4 acidic extract after 2 hours, one day and one week of incubation with serum component are shown in Figures B.19 to B.21. Considering Figure B.19, no signal is exist after 2 hours due to stability of vesicles during incubation period (Foradada et al., 2000). Figure B.20 demonstrate ^{1}H NMR spectrum of F4 acidic extract after one day incubation with serum component. Two signals observed in ^{1}H NMR spectrum at 2.0317, 3.3872 and 5.4185 ppm which are relating to **-CH_2-C=C-**, **CH_2-N^+** and **-CH=CH** (Foradada et al., 2000).

The ^{1}H NMR spectrum of F4 acidic extract after one week incubation with serum component shows in Figure B.21. Five signals at 2.1682, 2.3534, 2.7941, 3.3146, 4.3695 and 5.3279 ppm which are corresponding to **-CH_2-C=C-**, **-CH_2-C=O, =CH-CH_2-CH=**, **CH_2-N^+**, **-CH_2-O-P** and **-CH=CH-** (Foradada et al., 2000).

According to ^{1}H NMR spectrums of F4 acidic extract, F4 liposome didn't have any degradation within 2h, while after one day small signals were observed which indicate few degradation of liposome. However after one week, more signals in ^{1}H NMR spectrums confirmed the degradation of liposome as well.

4.6.8 Degradation of F4 basic extract

Figures B.22 to B.24 demonstrate the ^{1}H NMR spectrums of F4 basic extract after 2 hours, one day and one week of incubation with serum component. As in Figure B.22 shows there is no signals after 2 hours incubation same as acidic extract, (Foradada et al., 2000).

Figure B.23 shows the ^{1}H NMR spectrum of F4 basic extract after one day incubation with serum component. Four signals are identified in ^{1}H NMR spectrum at 2.0143,

2.2796, 3.7064, 4.2751 and 5.3836 ppm which are matching with **-CH_2-C=C-**, **-CH_2-C=O**, **CH_2-N**, **-CH_2-O-P** and **-CH=CH-** (Foradada et al., 2000).

The ^{1}H NMR spectrum of F4 basic extract after one week incubation with serum component shows in Figure B.24. Six signals are noticed in ^{1}H NMR spectrum at 2.0410, 2.2517, 2.7696, 3.3741, 3.7782, 4.3815 and 5.3911 ppm that are corresponding **-CH_2-C=C-** ,**-CH_2-C=O**, **=CH-CH_2-CH=**, **CH_2-N^+, CH_2-N**, **-CH_2-O-P** and **-CH=CH-** (Foradada et al., 2000)**.** According to ^{1}H NMR spectrums of F4 basic extract, F4 liposome didn't have any degradation within 2h, while after one day small signals were observed which indicate few degradation of liposome. However after one week, more signals in ^{1}H NMR spectrums showed degradation of liposome.

4.6.9 Degradation of F5 acidic extract

The ^{1}H NMR spectrums of F5 acidic extract after 2 hours, one day and one week of incubation with serum component are shown in Figures B.25 to B.27. In Figure B.25, four signals are detected in ^{1}H NMR spectrum after 2 hours incubation. Signals at 2.0173, 2.3165, 2.7619, 3.3104 and 5.4022 that are relating to **-CH_2-C=C-**, **-CH_2-C=O**, **=CH-CH_2-CH=**, **CH_2-N^+** and **-CH=CH-** (Foradada et al., 2000).

Figure B.26 demonstrate ^{1}H NMR spectrum of F5 acidic extract after one day incubation with serum component. Four signals observed in ^{1}H NMR spectrum at 2.0107, 2.2913, 3.3534 and 5.4018 ppm which are corresponding to **-CH_2-C=C-** , **-CH_2-C=O, CH_2-N^+** and **–CH=CH-** (Foradada et al., 2000).

The ^{1}H NMR spectrum of F5 acidic extract after one week incubation with serum component shows in Figure B.27. Four signals noticed in ^{1}H NMR spectrum at 2.0233, 2.3917, 3.3021 and 5.3825 ppm that are the resulting of **-CH_2-C=C-**, **-CH_2-C=O**, **CH_2-N^+** and **-CH=CH-** (Foradada et al., 2000). According to ^{1}H NMR spectrums of F5

acidic extract, F5 liposome had degradation within 2h, one day and one week, which showed low chemical stability of liposome.

4.6.10 Degradation of F5 basic extract

Figures B.28 to B.30 exhibit the ^{1}H NMR spectrums of F5 basic extract after 2 hours, one day and one week of incubation with serum component. In Figure B.28 four signals identified in ^{1}H NMR spectrum after 2 hours incubation. Signals at 2.0167, 2.3012, 2.8053, 3.7157 and 5.4023 ppm which are the resultant of **-CH_2-C=C-**, **-CH_2-C=O**, **=CH- CH_2-CH=**, **CH_2-N** and **-CH=CH-** (Foradada et al., 2000).

Figure B.29 shows the ^{1}H NMR spectrum of F5 basic extract after one day incubation with serum component. Six signals are noticed in ^{1}H NMR spectrum at 2.0312, 2.3306, 2.8144, 3.4287, 3.7586 and 5.4684 ppm that corresponding to **-CH_2-C=C-**, **-CH_2-C=O**, **=CH-CH_2.CH=**, **CH_2-N^+**, **CH_2-N** and **-CH=CH-** (Foradada et al., 2000).

The ^{1}H NMR spectrum of F5 basic extract after one week incubation with serum component shows in Figure B.30. Six signals are detected in ^{1}H NMR spectrum at 2.0210, 2.2547, 2.8261, 3.3158 3.5745, 4.3526 and 5.2764 ppm that corresponding to **-CH_2-C=C-**, **-CH_2-C=O**, **=CH-CH_2.CH=**, **CH_2-N^+**, **CH_2-N**, **-CH_2-O-P** and **-CH=CH-** (Foradada et al., 2000). According to ^{1}H NMR spectrums of F5 basic extract, F5 liposome had degradation within 2h, one day and one week, which showed low chemical stability of liposome.

4.6.11 Degradation of F6 acidic extract

The acidic extract ^{1}H NMR spectrums of F6 LUVs after 2 hours, one day and one week of incubation with serum component are shown in Figures B.31 to B.33. In Figure B.31, five signals are exist in ^{1}H NMR spectrum after 2 hours incubation. Signals at 2.0512, 3.2496, 3.6983, 4.3724 and 5.3841 that are corresponding to **CH_2-C=C-**, **-CH_2-N^+**, **-CH_2-N**, **-CH_2-O-P** and **-CH=CH** (Foradada et al., 2000).

Figure B.32 displays ^{1}H NMR spectrum of F6 acidic extract after one day incubation with serum component. Five signals are presented in ^{1}H NMR spectrum at 2.0107, 2.2986, 3.3108, 3.7751, 4.301 and 5.3771 that are corresponding to **CH_2-C=C-**, **-CH_2-C=O**, **CH_2-N^+**, **-CH_2-N**, **-CH_2-O-P** and **-CH=CH** (Foradada et al., 2000).

The ^{1}H NMR spectrum of F6 acidic extract after one week incubation with serum component shows in Figure B.33. Seven signals noticed in ^{1}H NMR spectrum at 2.1724, 2.2413, 2.8104, 3.3755, 4.3237 and 5.3870 that are matching to **CH_2-C=C-**, **-CH_2-C=O**, **=CH- CH_2-CH=**, **CH_2-N^+**, **-CH_2-O-P** and **-CH=CH-** (Foradada et al., 2000). According to ^{1}H NMR spectrums of F6 acidic extract, F6 liposome had degradation within 2h, one day and one week, which showed low chemical stability of liposome.

4.6.12 Degradation of F6 basic extract

Figures B.34 to B.36 illustrate the ^{1}H NMR spectrums of F6 basic extract after 2 hours, one day and one week of incubation with serum component. In Figure B.34 four signals identified in ^{1}H NMR spectrum after 2 hours incubation. Signals at 2.08015, 2.3475, 4.3132 and 5.3224 that are related to **-CH_2-C=C-**, **-CH_2-C=O**, **-CH_2-O-P** and **-CH=CH-** (Foradada et al., 2000).

Figure B.35 shows the ^{1}H NMR spectrum of F6 basic extract after one day incubation with serum component. Six signals are observe in ^{1}H NMR spectrum at 2.0791, 2.2394, 3.4710, 4.3015 and 5.4012 ppm that are corresponding to **-CH_2-C=C-**, **-CH_2-C=O**, **CH_2-N^+** , **-CH_2-O-P** and **-CH=CH-** (Foradada et al., 2000).

The ^{1}H NMR spectrum of F6 basic extract after one week incubation with serum component shows in Figure B.36. Five signals are detected in ^{1}H NMR spectrum at 2.0113, 2.3016, 3.4247 and 5.4149 ppm that are corresponding to **-CH_2-C=C-**, **-CH_2-C=O**, **CH_2-N^+** and **-CH=CH-** (Foradada et al., 2000). According to ^{1}H NMR

spectrums of F6 basic extract, F6 liposome had degradation within 2h, one day and one week, which showed low chemical stability of liposome.

4.7 Morphology of liposome

In advance study scanning electron microscopies (SEM) utilize to study the formation and morphology of liposome (Hauser, 1993; Mu & Zhong, 2006). SEM is generally used to study the outer space and size of the vesicles (Almgren et al., 2000; Gregoriadis, 2006). Considering Figure 4.7 the formation of liposome through freeze-thaw method will confirm. Furthermore SEM images demonstrate the spherical-shape of vesicles plus the outer feature of LUVs as well.

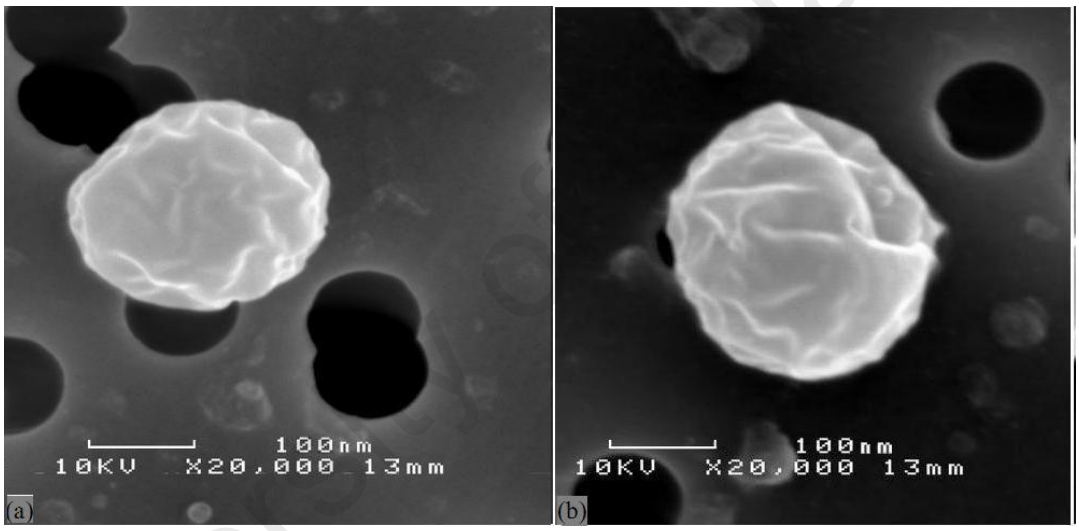


Figure 4.7: SEM images (a) F3 and (b) F4 liposome with magnification 20000X.

4.8 Particle size and zeta potential

After liposome prepared with freeze-thaw method, the particle size and zeta potential of them determined using zetasizer (ZEM 3600, Malvern).

Table 4.4: Mean liposome particle size (nm) and Zeta potential (mV) of liposome (n=3)

Formulation	Particle size (nm ± SD)	Zeta potential fresh liposome	Zeta potential after 30 days	Zeta potential after 60 days
F3	256.32 ±1.8	-32.05 ±2.7	-31.62 ±1.7	-30.09 ±2.2
F4	225.84 ±2.1	-32.36 ±1.5	-31.25 ±1.9	-30.73 ±2.6

4.9 Calibration curve of doxorubicin applying HPLC Method

Calibration curve was constructed with injection of 400, 200, 100, 50, 25 and 12.5 ng/ml doxorubicin hydrochloride using High Performance Liquid Chromatography with fluorescence detector. Afterwards calibration equation achieved by Microsoft Excel 2007 program. Figure 4.8 shows calibration curve of doxorubicin hydrochloride with the following equation, Y= 21998 X + 8938, where Y is the graph area, and X is the concentration of doxorubicin, the regression line was obtained as R^2 = 0.999 with RSD (relative standard deviation) of 8.4%, plus the limits of detection (LOD) and quantification (LOQ) were as 2.8 ng/ml and 5.3 ng/ml respectively (Alhareth et al., 2012; Carlson et al., 2014; Daeihamed et al., 2015).

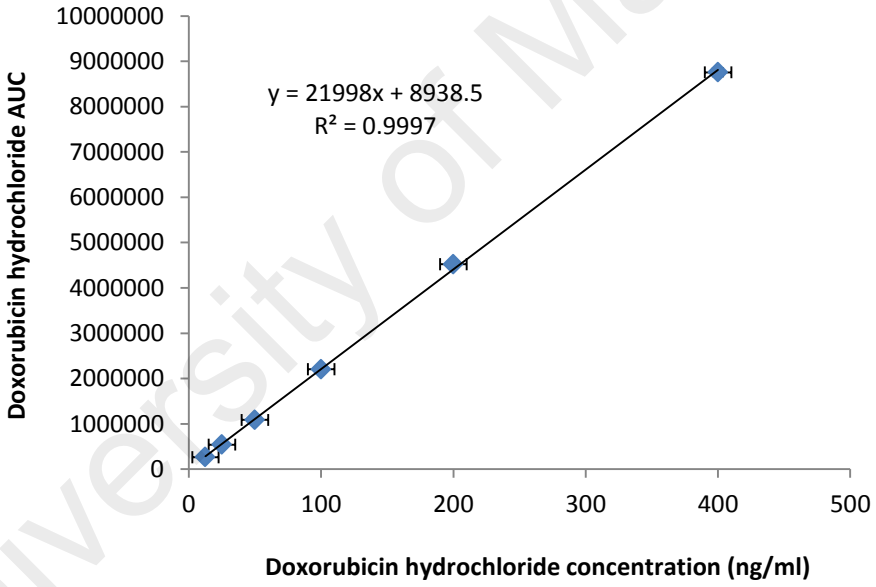


Figure 4.8: Standard curve of Doxorubicin hydrochloride in PBS (pH=7.4) using HPLC.

4.10 Quantification of liposome entrapment efficiency using HPLC

After separation of non-entrapped doxorubicin, from the entrapped one, (using centrifuged method) the concentrations of free doxorubicin measured using HPLC technique (Mu & Zhong, 2006). Table 4.7 shows area under curve (AUC), concentrations of free and entrapped Doxorubicin within liposome. Further entrapment efficiency of doxorubicin calculated using equations 3.4.

Table 4.5: AUC and Concentrations of Doxorubicin (µg/ml) in F3and F4

Formulation	F3 Liposome	F4 Liposome
AUC of non-entrapped Doxorubicin	1152295	1108483
Concentration of non-entrapped Doxorubicin (µg/ml)	0.0026	0.0025
Concentration of entrapped Doxorubicin (µg/ml)	199.94	199.99
Entrapment efficiency %	99.98 %	99.99 %

Referring to Table 4.5, the entrapment efficiency increased considerably with pH-gradient technique. As it can be seen in Table 4.7, liposome F3 and F4 entrapment efficiency increased to 99.98% & 99.99% which confirms the elevated loading of doxorubicin hydrochloride within them.

4.11 Quantification of *in vitro* release of Doxorubicin with HPLC

In order to measure the in vitro release of doxorubicin, liposome placed in dialysis bags then located in fresh PBS (pH=7.4). Samples (1 ml) were provided at 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 hours presently during incubation and then injected to HPLC.

Figure 4.9 shows dialysis bags after 60 minutes, 24, 48 and 96h of incubation and Figures C.1 to C.4 are some examples of HPLC chromatogram of *in vitro* releasing of doxorubixin hydrochloride. The consentrations of released doxorubicin from liposome provided using calibration equation further mean *in vitro* drug release calculated using equation 3.5 (Maurer et al., 1998; Panwar et al., 2010; Saarinen-Savolainen et al., 1997). Furthermore Figure 4.10 demonstrate the cumulative releasing profile of doxorubicin from F3, F4 and Caelyx® during 96 hours of incubation.

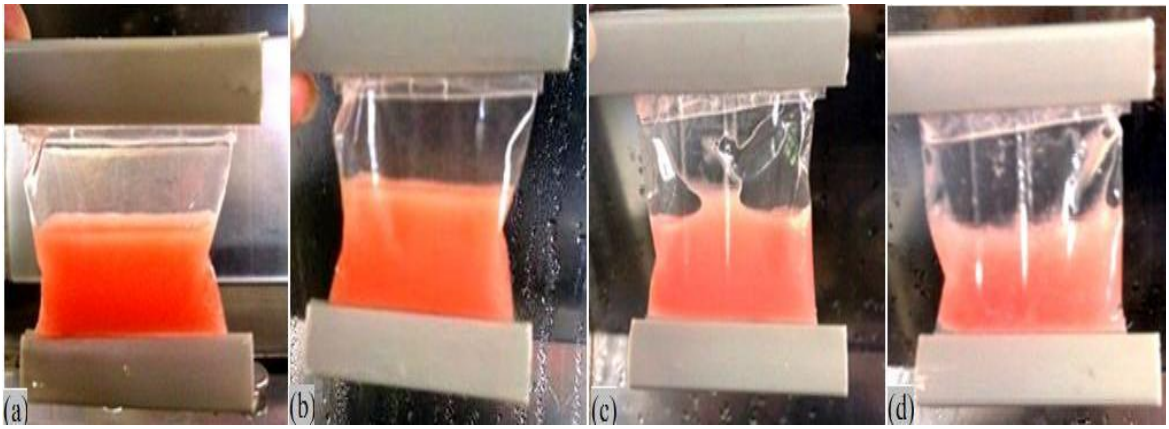


Figure 4.9: In vitro release of liposomal doxorubicin using dialyses method. Images provided from samples after 1h (a), 24h (b), 48h (c) and 96h (d) of incubation.

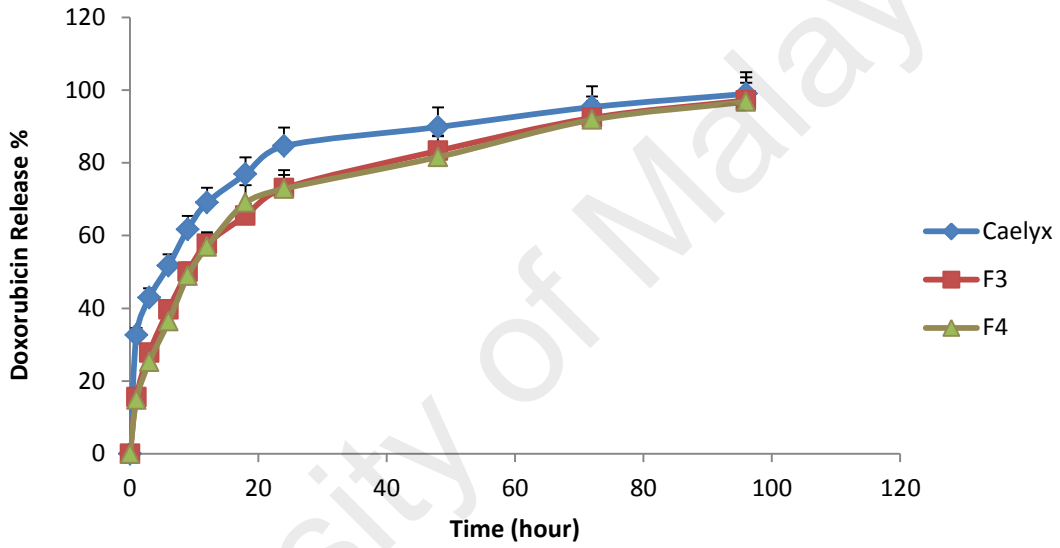


Figure 4.10: Cumulative release of doxorubicin in Phosphate Buffered Saline (pH=7.4).

Figure 4.10 demonstrate the in-vitro release of Caelyx®, F3 and F4 liposomal doxorubicin during 96 hours. According to graph, all PEG liposomal doxorubicin showed similar pattern whereas Caelyx® showed faster release compare to F3 and F4 liposome.

4.12 Fourier Transform Infra-Red (FTIR) spectrum

Potassium Bromide disc of Doxorubicin hydrochloride, F1 plus both unloaded and Doxorubicin loaded of F3 and F4 were provided then FT-IR spectrums were attained using Nicolet FTIR Magna 550 spectrophotometer (Faix, 1992; Griffiths & De Haseth,

2007; Smith, 2011). FTIR spectrums of doxorubicin hydrochloride, F1, F3 and F4 liposome are shown in Figures 4.11- 4.16.

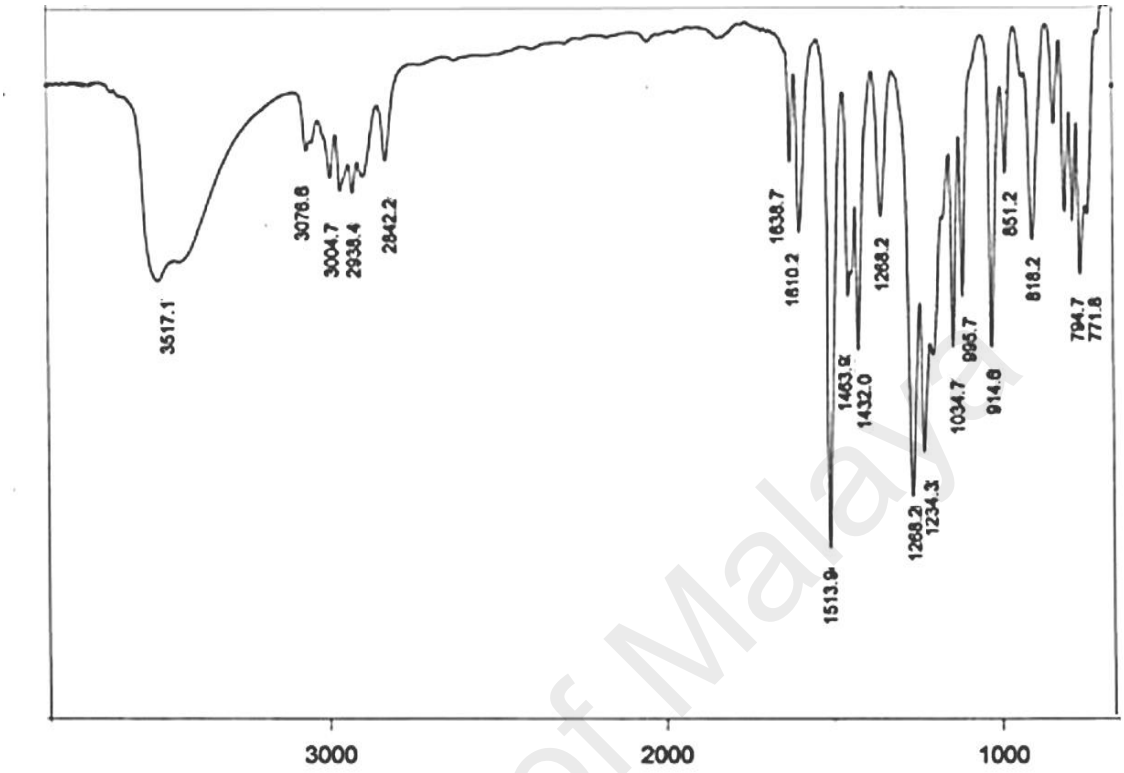


Figure 4.11: Doxorubicin hydrochloride FTIR spectra.

Figure 4.11 demonstrates the FTIR spectra of doxorubicin hydrochloride; existence of double peaks at 3076.6 is corresponding to **NH_2** while 3517.1 is singe of **O-H**. Peak at 3004.7 is match to **C-H** aromatic and peaks in 2938.4 and 2842.2 are match to **C-H** aliphatic, plus 1638.7 & 1610.2 peaks are sign of **C=O** as well. Peaks at 1513.9 and 1610 are corresponding to **C=C** while 1000 to 1300 peaks are match to **C-O**. Further peaks in 1034.7, 995.7, 851.2 & 818.22 are indicating the substitutions of aromatic rings (Griffiths & De Haseth, 2007; Smith, 2011).

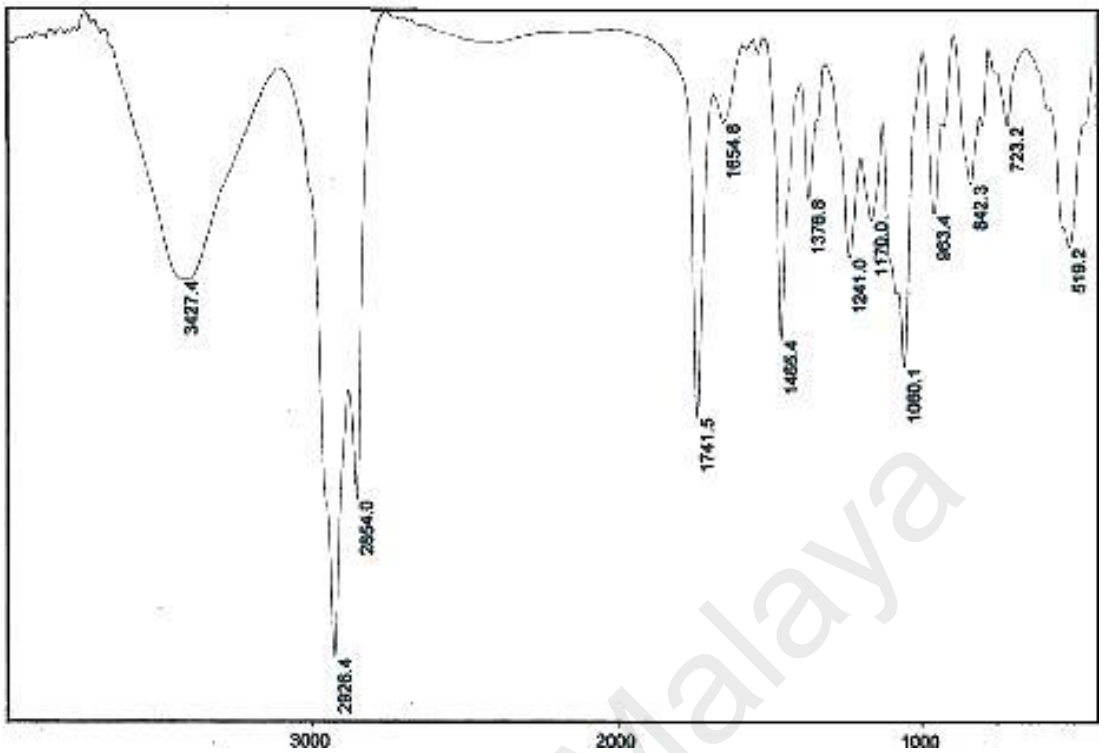


Figure 4.12: FTIR spectra of F1 Liposome.

Figure 4.12 demonstrates the FT-IR spectra of F1 liposomes (without palm oil). Looking at Figure 4.50, peak in 3427.4 is corresponding to **OH** while peaks in 2926.4 and 2854 are match with of **C-H** Aliphatic. Peak at 1741.5 indicates **C=O** plus peak 1654.8 signify **C=C** as well. Peaks at 1241.0 and 1170.0 are sign of **C-O** whereas peaks at 1465.4 and 1376.8 are point to Aromatic band. Peaks at 963.4, 842.3, 723.2 and 519.2 are also the results of substitutions on Aromatic rings (Griffiths & De Haseth, 2007; Smith, 2011).

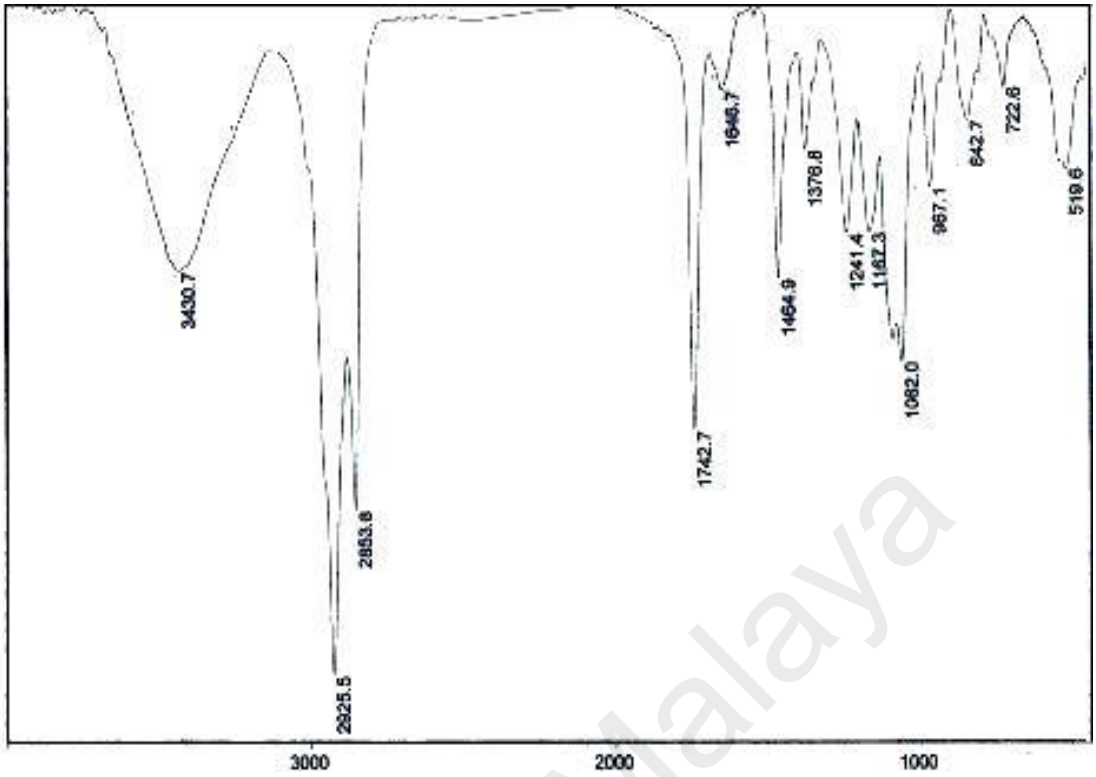


Figure 4.13: FTIR spectra of F3 liposome.

Figure 4.13 demonstrates FTIR spectra of F3 liposome. The FT-IR spectrum of F3 is similar to F1 spectrum while F3 contains 10% palm oil. Presence of peak in 3430.7 is sign of **OH** plus peaks in 2925.5 and 2853.8 are corresponding to **C-H** Aliphatic. Peak at 1742.7 is match to **C=O**, while peak at 1646.7 indicates **C=C**. Further peaks at 1241.41 & 1167.3 are match to **C-O** and peaks at 1464.9 and 1376.8 are corresponding to Aromatic band whereas 967.1, 842.7, 722.6 and 519.6 peaks are the results of substitutions on Aromatic rings (Griffiths & De Haseth, 2007; Smith, 2011).

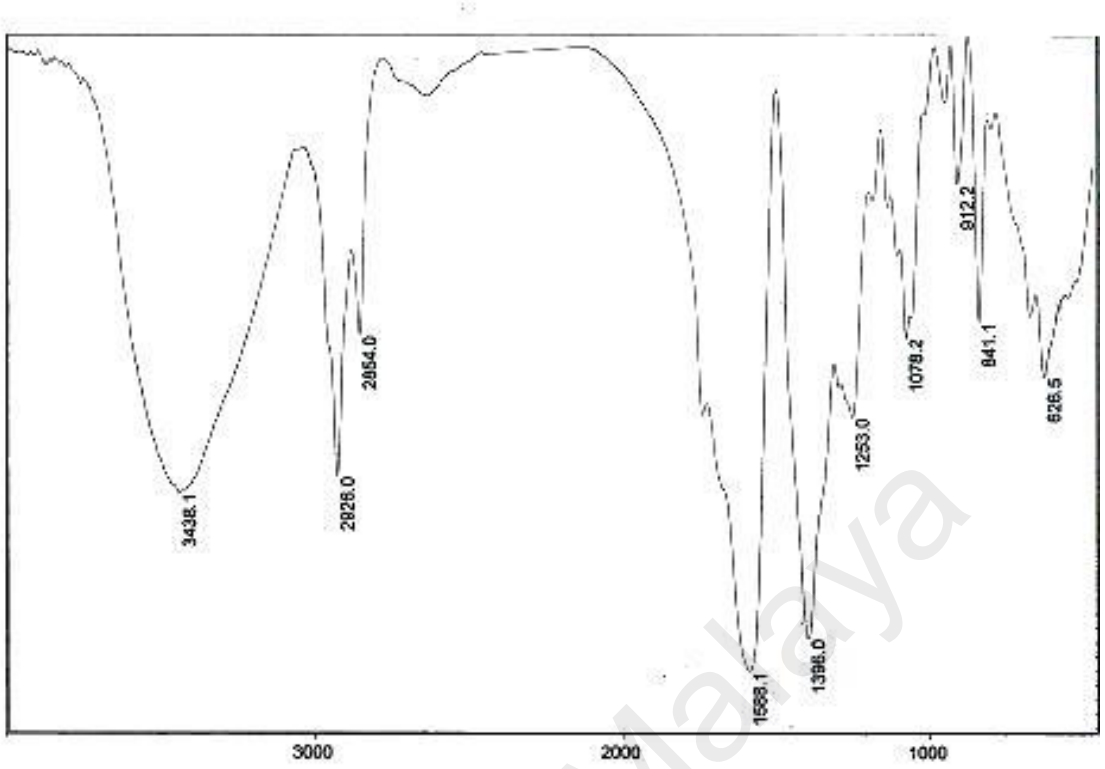


Figure 4.14: FTIR spectra of F4 liposome.

Figure 4.14 demonstrates the FTIR spectra of F4 liposome which is similar to F1 and F3 whereas it contains 15% palm oil. Peak at 3438.1 indicates the presents of **OH** and peaks at 2926.04 & 2854.0 are corresponding to **C-H** Aliphatic. Peak at 1253.0 is sign of **C-O** while peaks at 1588.1 and 1396.0 are match to Aromatic band and 1078.2, 912.2, 841.1 and 626.5 peaks are the results of substitutions on Aromatic rings (Griffiths & De Haseth, 2007; Smith, 2011).

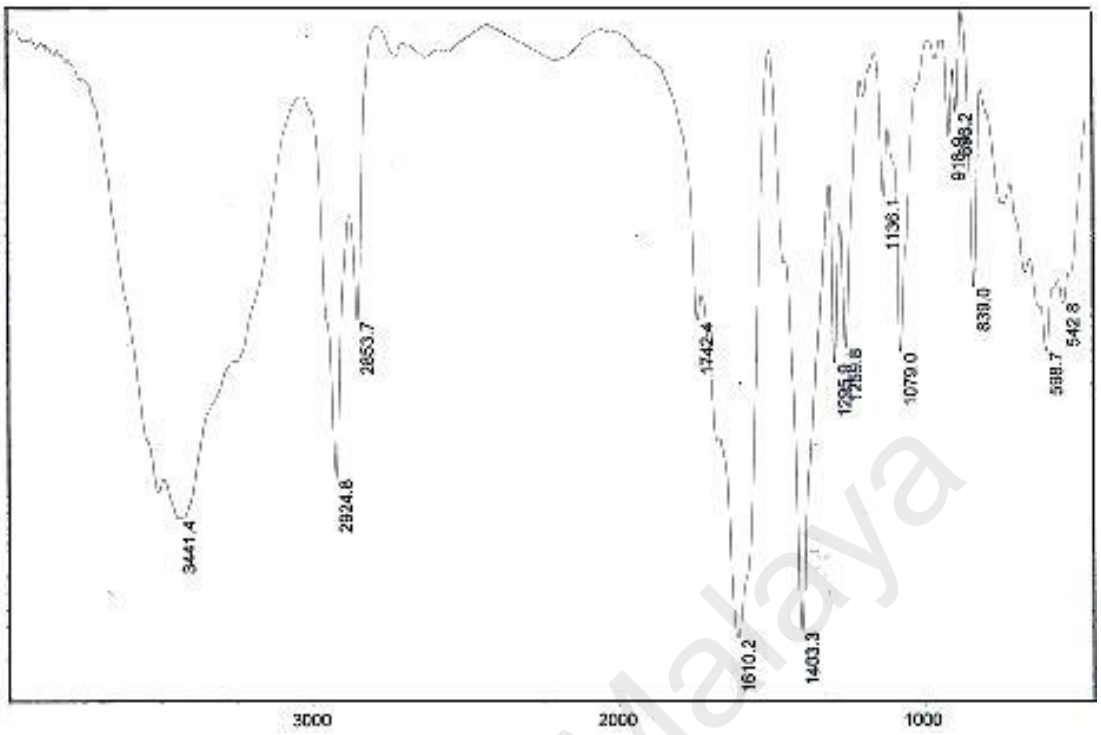


Figure 4.15: FTIR spectra of F3 liposomal doxorubicin.

Figure 4.15 demonstrates the FTIR spectra of F3 liposome which loaded with Doxorubicin hydrochloride. As it can be seen the double peaks in 3441.6 is sign of presence of **NH_2** and **O-H** of Doxorubicin. Peaks T 2924.8 & 2853.7 are corresponding to **C-H** Aliphatic and peak at 1610.2 and 1403.3 are match to **C=C**. Peaks at 1295.9, 1259.8 & 1136.1 are corresponding to **C-O** plus peaks at 1079.0, 918.9, 896.2, 839.0 and 598.7 are indicating the substitutions of Aromatic rings (Griffiths & De Haseth, 2007; Smith, 2011).

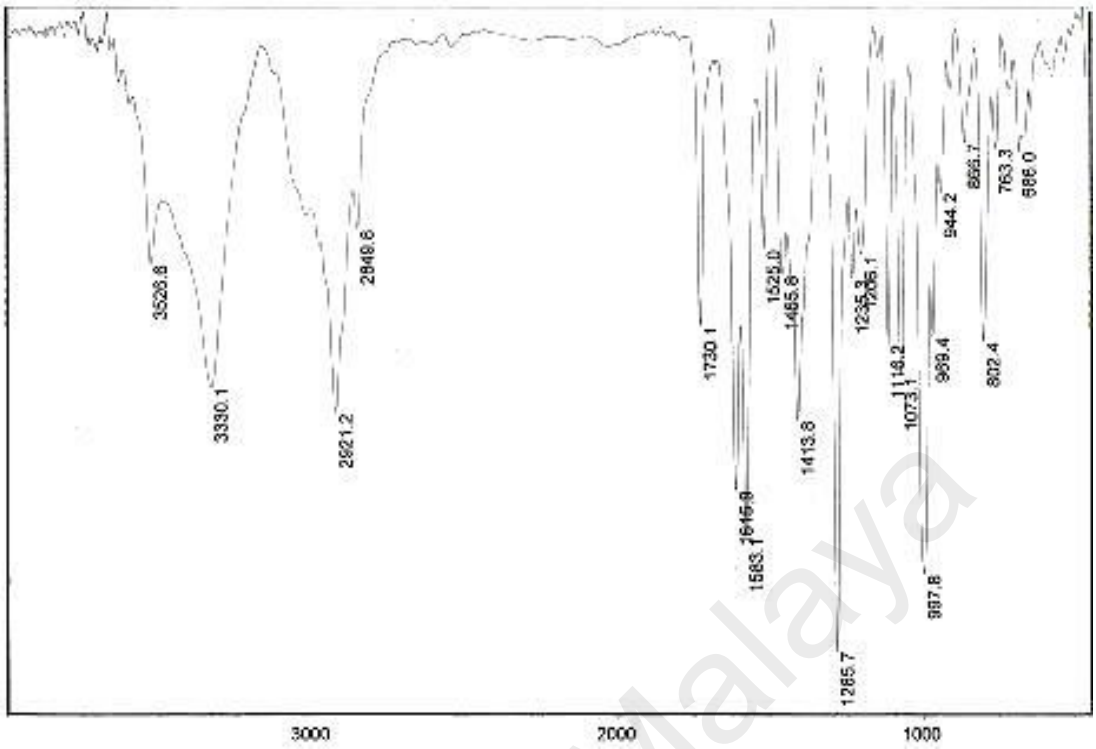


Figure 4.16: FTIR spectra of F4 liposomal doxorubicin.

Figure 4.16 demonstrates the FTIR spectra of F4 liposome which is loaded with Doxorubicin hydrochloride. Double peaks at 3526.6 and 3330.1 are indicating **NH_2** of Doxorubicin hydrochloride whereas it covers the O-H peak due to its higher proportion. Peaks at 2921.2 & 2849.8 are corresponding to **C-H** Aliphatic while 1615.9 & 1583.1 peaks are match to **C=C** plus 1235.3 & 1206.1 & 1116.2 peaks are sign of **C_2-C=O**. Peaks in 1525.0, 1465.8 and 1413.8 are match to aromatic band whereas peaks at 997.8, 969.4, 944.2, 802.4, 763.3 and 686.0 are the results of substitutions of aromatic rings (Griffiths & De Haseth, 2007; Smith, 2011).

4.13 Thermal analyzing study with DSC

The DSC test carried out to assess the influence of temperature on the liposome. Thermal analyzing study performed on doxorubicin hydrochloride, F3 and F4 liposome without loading drug and F3 and F4 liposomal doxorubicin additionally. Thermal parameters such as melting point (onset) and integral under the DSC peak were

determined respectively. The integral, provides the total enthalpy (ΔH) which is needed for changing the process.

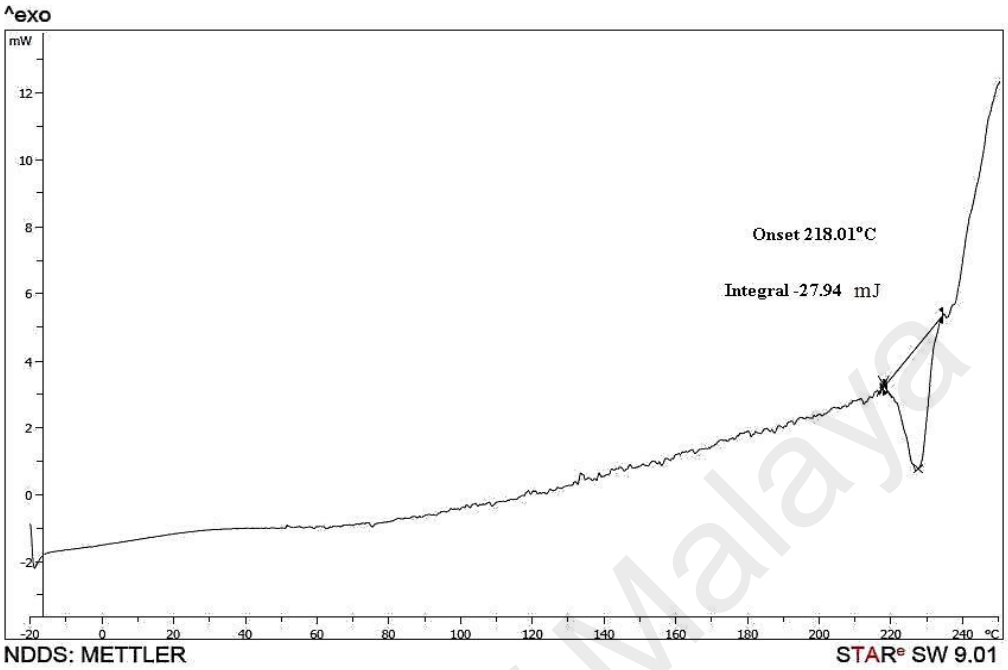


Figure 4.17: DSC curve of Doxorubicin hydrochloride.

As Figure 4.17 shows, the onset of Doxorubicin hydrochloride was 218.01^{o}C with integral -27.94 mJ.

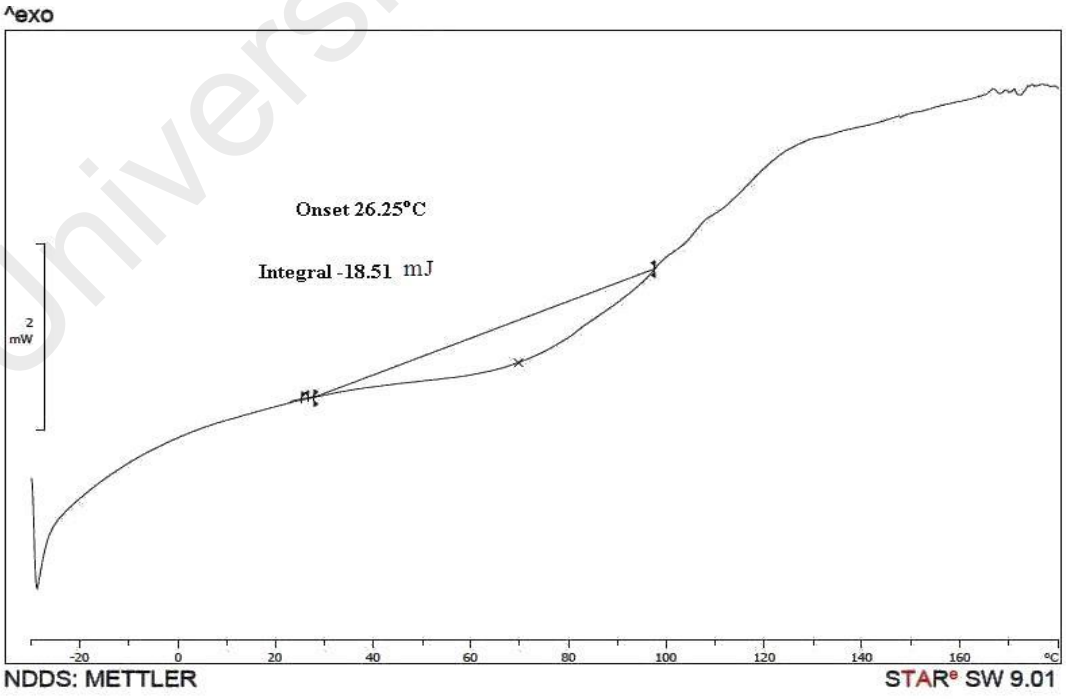


Figure 4.18: DSC curve of F3 liposome.

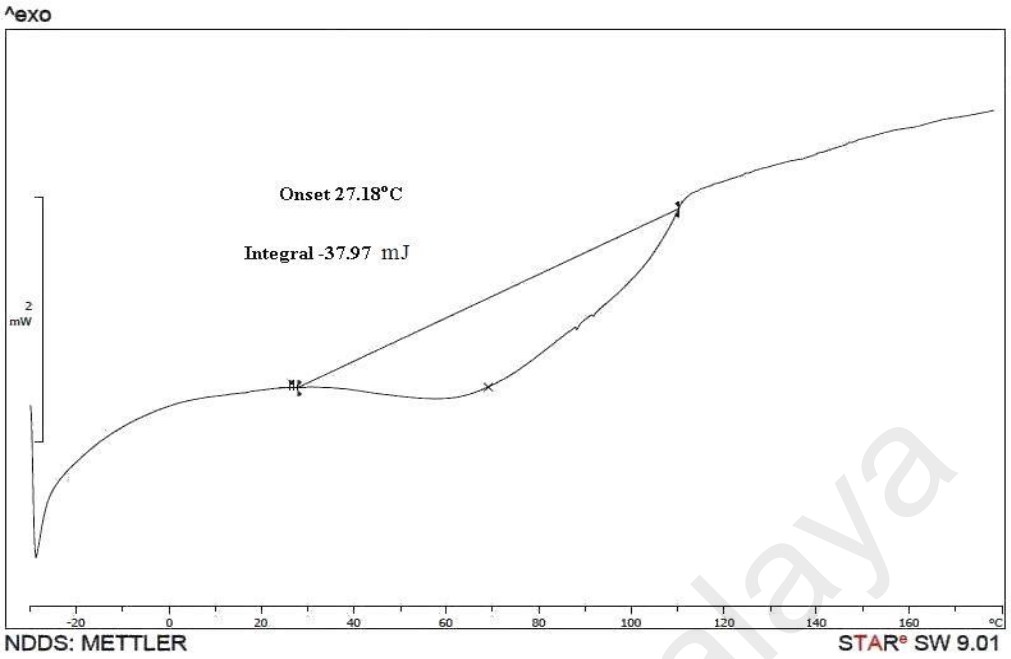


Figure 4.19: DSC curve of F4 liposome.

Figure 4.18 and Figure 4.19 show the DSC curves of unloaded F3 and F4 liposome. According to DSC curves, F3 liposome without loading doxorubicin had the onset at 26.25^{o}C with integral -18.51 mJ while F4 liposome without loading doxorubicin, showed onset at 27.18^{o}C with integral -37.97 mJ.

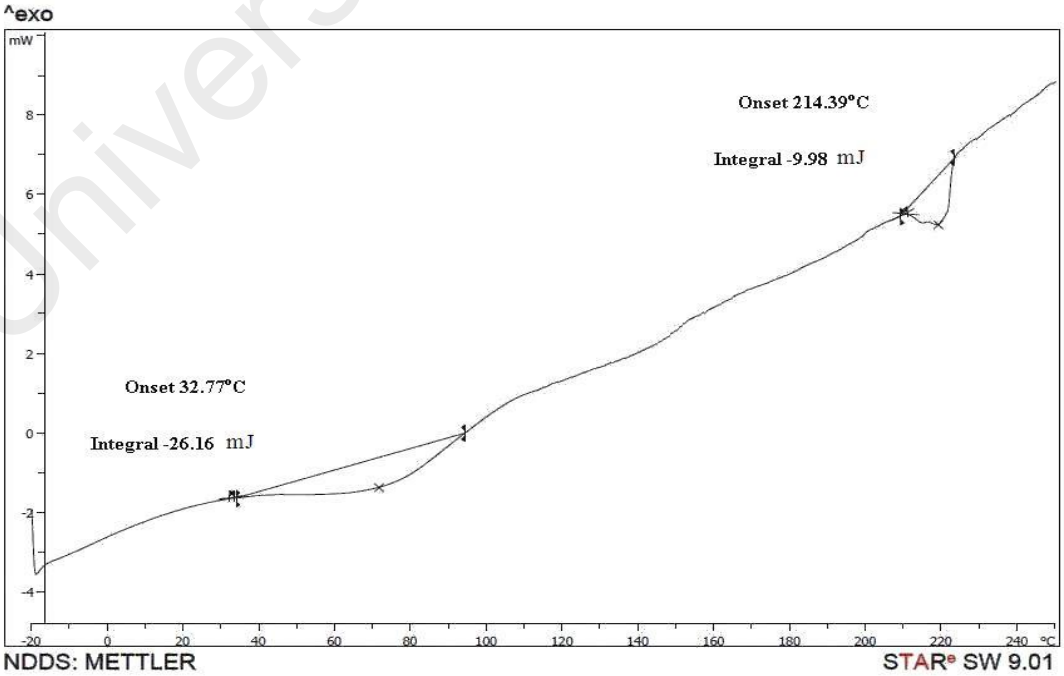


Figure 4.20: DSC curve of F3 liposomal doxorubicin.

Figure 4.20 shows the curve of F3 liposome after loading doxorubicin. Referring to Figure 4.20, two curves were observed, the onset of first curve was 32.77^{o}C with integral -26.16 mJ and the onset of second curve was 214.39^{o}C with integral -9.98 mJ. Considering the DSC curves of F3 liposome and doxorubicin hydrochloride, in Figure.20 the first curve match to the liposome while the second curve related to doxorubicin. The present of two separate curves in DSC liposomal doxorubicin confirm the well entrapment of doxorubicin within vesicles without changes or binding to the lipids.

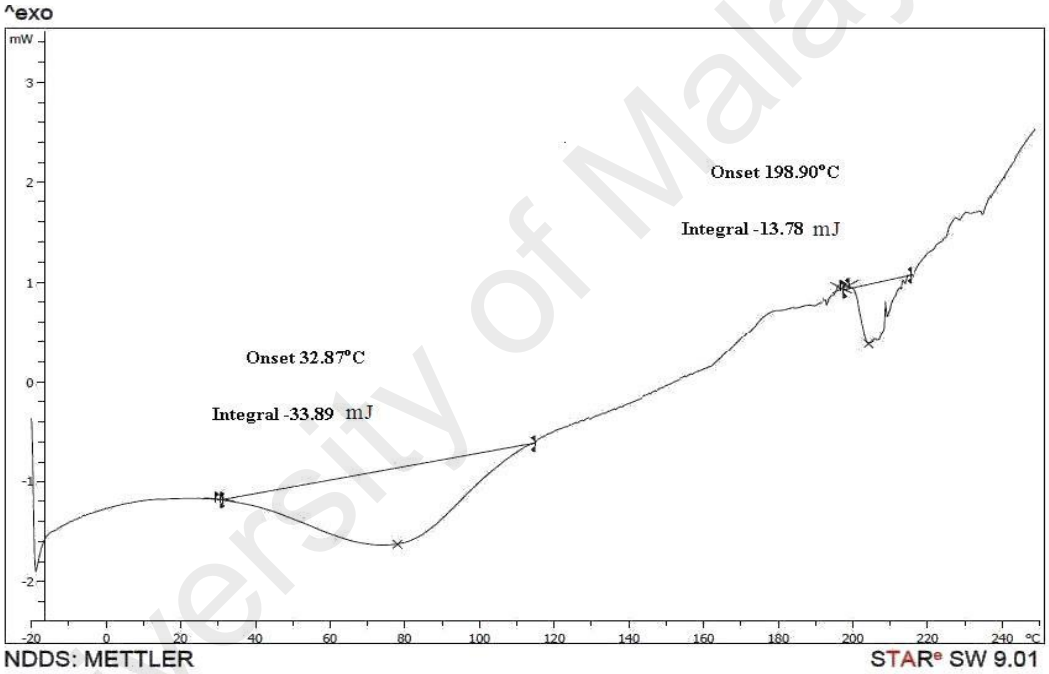


Figure 4.21: DSC curve of F4 liposomal doxorubicin.

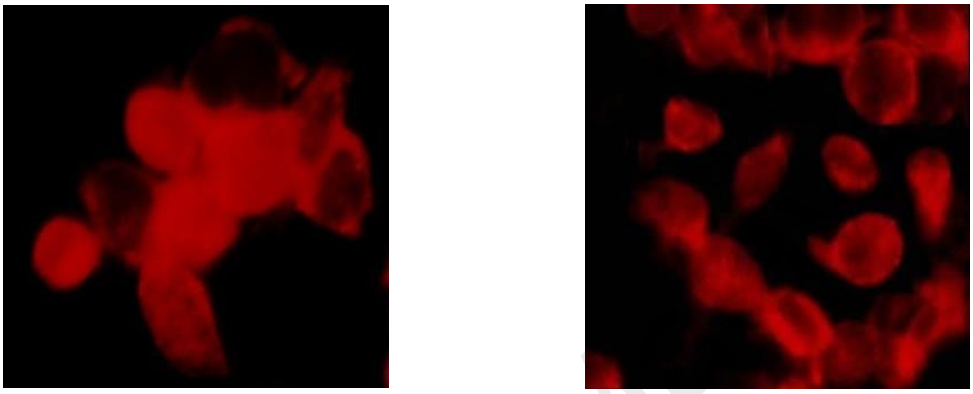
Figure 4.21 shows the curve of F4 liposome after loading doxorubicin. In Figure 4.21, two curves were identified; the onset of first curve was 23.87^{o}C with integral -33.89 mJ and the onset of second curve was 198.90^{o}C with integral -13.78 mJ.

Considering DSC curves of F4 and doxorubicin hydrochloride, the first curve belongs to the lipid while the second curve match to doxorubicin hydrochloride. The DSC curve of liposomal doxorubicin demonstrate the well entrapment of doxorubicin within liposome without changes or binding to the lipids, however the onset of lipids and doxorubicin in

liposomal doxorubicin shifted slightly compare to liposome or doxorubicin hydrochloride.

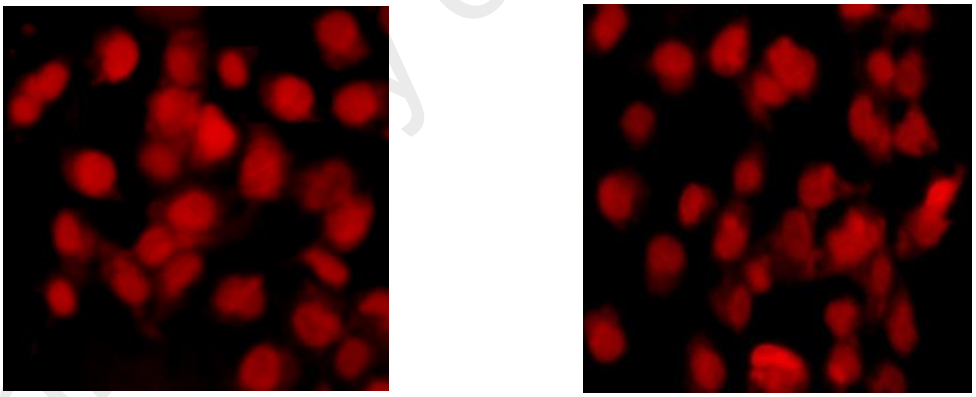
4.14 Cellular uptake

The visual cellular uptake of MCF7 and MDA-MBA 231 cell by treatment with F3 and F4 liposomal doxorubicin investigated after 24h incubation 37ºC with 5% CO2.



(a) (b)

Figure 4.22: Optical images of MCF 7 cells' cellular uptake: a (F3 liposomal doxorubicin), b (F4 liposomal doxorubicin).



(a) (b)

Figure 4.23: Optical images of MDA-MBA 231 cells' cellular uptake: a (F3 liposomal doxorubicin), b (F4 liposomal doxorubicin).

Figures 4.22 and 4.23 demonstrate the transpiration of liposomal doxorubicin to cells using confocal microscopy (IX71, Olympus, Japan). Cellular uptake of Doxorubicin hydrochloride and pegilated liposomal doxorubicin determined using plate reader Synergy 4, Biotek with florescence detector. The florescence intensity of doxorubicin obtained after cells were incubated for 2 and 24h with doxorubicin hydrochloride,

Caelyx®, F3 and F4 liposomal doxorubicin then cellular uptake was quantified respectively (Iwasa et al., 2006; Park & Yoo, 2010).

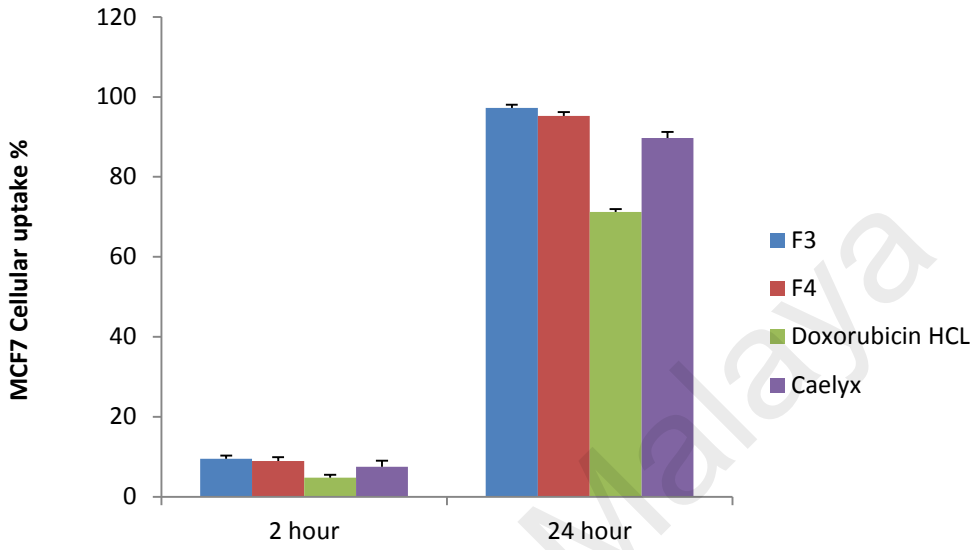


Figure 4.24: Cellular uptake of MCF7 cell line after 2 and 24 hours incubation.

Referring to Figure 4.24, after 2h incubation, F3 and F4 had 9.52% and 8.9% florescence intensity while doxorubicin hydrochloride had 4.79% and Caelyx® had 7.53% florescence intensity. After 24h incubation also F3 and F4 include higher uptake with 97.26% and 95.21% florescence intensity whereas doxorubicin hydrochloride and Caelyx® showed 71.23% and 89.73% florescence intensity. Therefore doxorubicin hydrochloride displayed the least uptake percentage at the same time as Caelyx® showed higher uptake, though F3 and F4 had the maximum uptake of others.

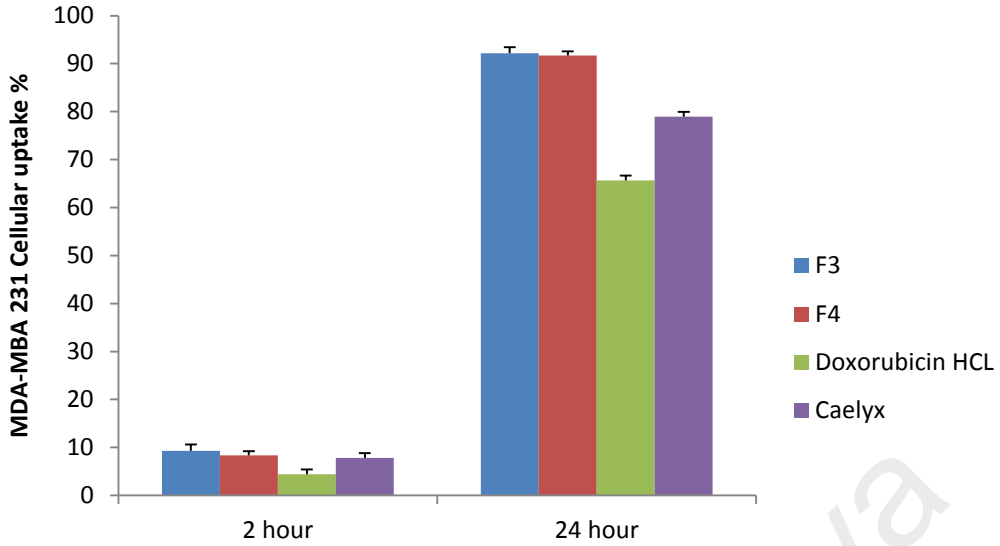


Figure 4.25: Cellular uptake of MDA-MBA 231 cell line after 2 and 24 hours incubation.

Figure 4.25 demonstrate uptake percentage of F3 and F4 liposomal doxorubicin, doxorubicin hydrochloride and Caelyx® after 2 and 24h incubation at 37ºC with 5% CO2. F3 and F4 liposomal doxorubicin showed 9.31% and 8.33% florescence intensity whereas doxorubicin hydrochloride had 4.41% and Caelyx® had 7.84% florescence intensity. After 24h incubation also F3 and F4 liposomal doxorubicin assess higher uptake to 92.16% and 91.67% florescence intensity whereas doxorubicin hydrochloride and Caelyx® showed 65.69% and 78.92% florescence intensity. As a result F3 and F4 liposomal doxorubicin had the maximum cellular uptake while Caelyx® showed less cellular uptake and doxorubicin hydrochloride had the least uptake percentage compare to other treatments.

4.15 MTT assay results

Cytotoxicity of doxorubicin hydrochloride, unloaded liposome, Caelyx®, F3 and F4 liposomal doxorubicin were evaluated on MDA-MBA 231 & MCF-7 breast cancer cell lines according to standard MTT assay and IC_{50} of treatments evaluated respectively (Chai, 2009; Hu et al., 2008; Miglietta et al., 2000; Yuan et al., 2008). The half maximal inhibitory concentration (IC50) is a quantitative evaluation of drugs that represent the

concentration of drug which cause 50% inhibition of cells (Chai, 2009; Miglietta et al., 2000). Due to MTT evaluation unloaded liposome could not reach to IC50 and didn't have any toxicity on MDA-MBA 231 and MCF-7 breast cancer cells. Figure 26-29 show the inhibition % of MDA-MBA 231 & MCF-7 after 24 and 48h treatment with liposomal doxorubicin F3 & F4, doxorubicin hydrochloride and Caelyx®.

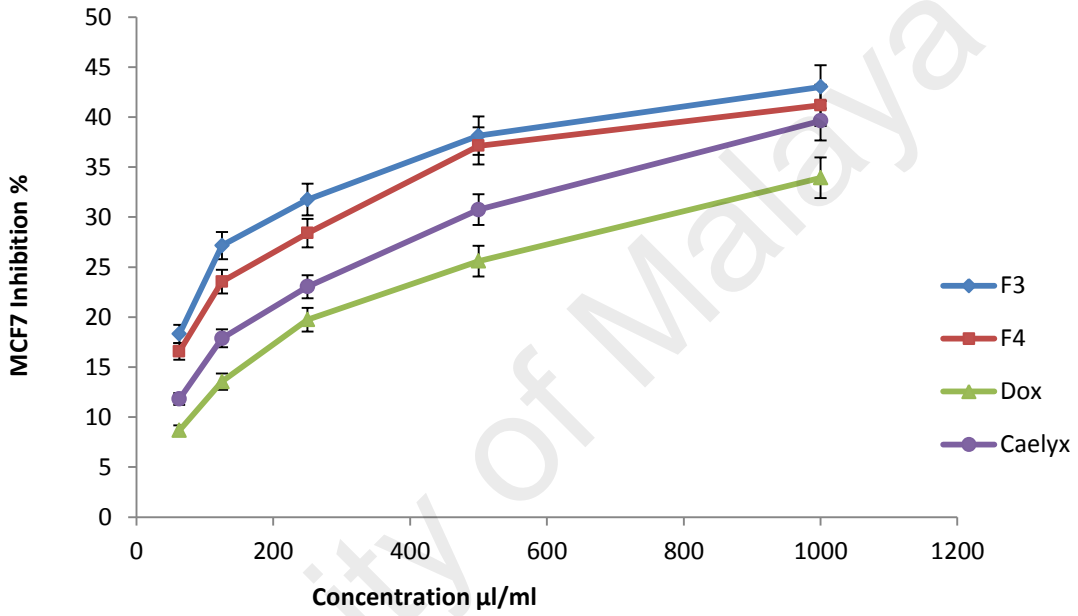


Figure 4.26: Comparison of the IC50 for F3 (), F4 (), doxorubicin hydrochloride () and Caelyx® () on MCF7 cell line, after 24 hours.

Figure 4.26 shows the IC50 of MCF7 cells, after 24h treatment with F3, F4, Doxorubicin hydrochloride and Caelyx®. Referring to Figure 4.26, MCF7 cells were not sensitive to any of treatments and could not reach IC50 in 24h incubation.

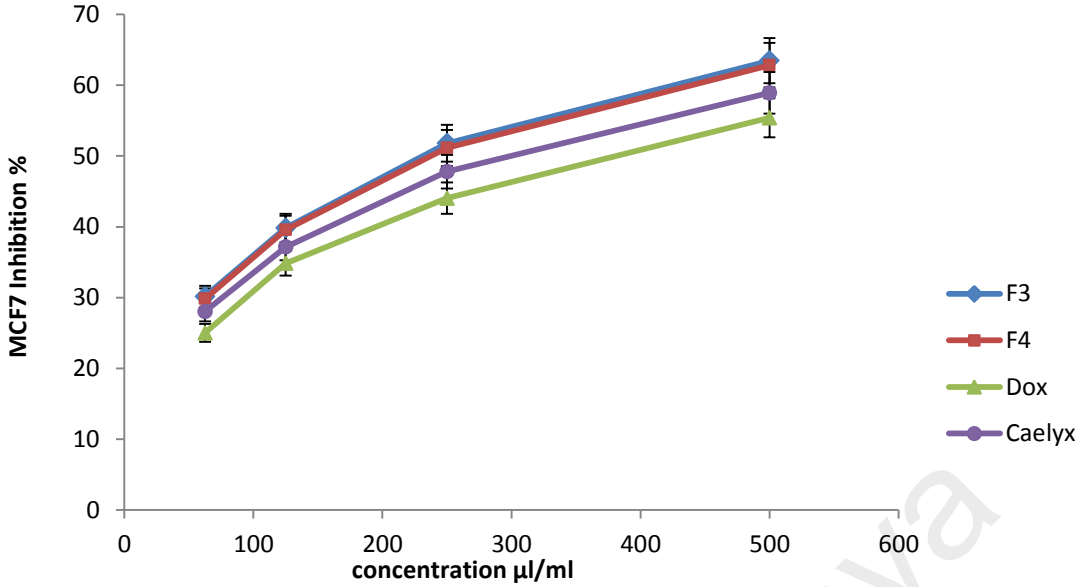


Figure 4.27: Comparison of the IC50 for F3 (—◆—), F4 (—■—), doxorubicin hydrochloride (—▲—) and Caelyx® (—●—) on MCF7 cell line, after 48 hours.

Figure 4.27 shows IC50 of MCF 7 cells after 48h treatment with F3, F4, doxorubicin hydrochloride and Caelyx®. IC50 for all treatments evaluated after 48h incubation. The IC50 values for F3 and F4 liposomal doxorubicin were determined 0.44 µM and 0.45 µM. The IC50 for doxorubicin hydrochloride and Caelyx® evaluated 0.83 µM and 0.78 µM respectively. Due to the IC50 results, MCF7 cells were more sensitive to F3 and F4 LUVs than doxorubicin hydrochloride and Caelyx®.

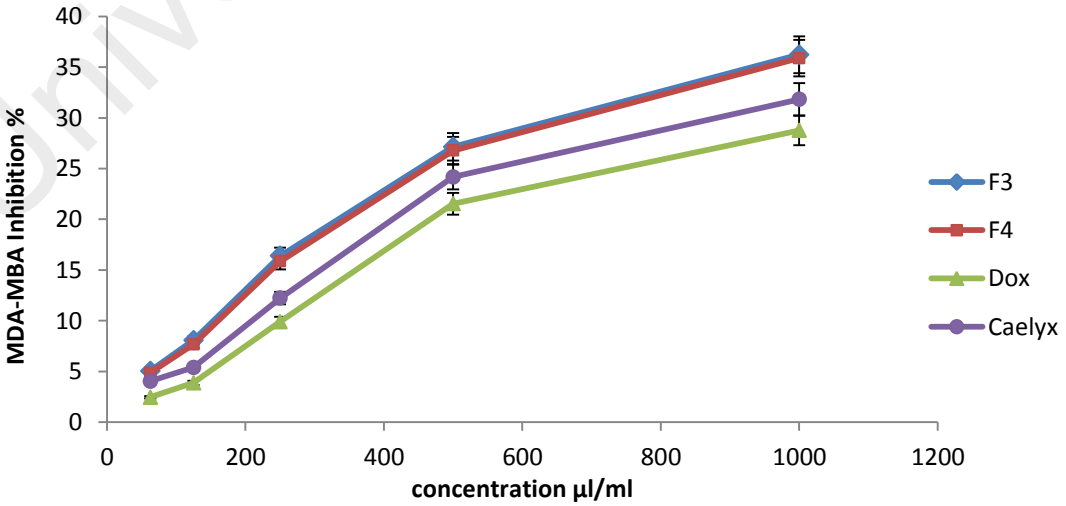


Figure 4.28: Comparison of the IC50 for F3 (—◆—), F4 (—■—), doxorubicin hydrochloride (—▲—) and Caelyx® (—●—) on MDA-MBA 23l cell line after 24 hours.

Figure 4.28 demonstrate the IC50 of MDA-MBA 231 cells, after 24h treatment with F3, F4, doxorubicin hydrochloride and Caelyx®. Referring to Figure 4.66, MDA-MBA 231 cells were not sensitive to any of treatments and could not reach IC50 in 24h incubation.

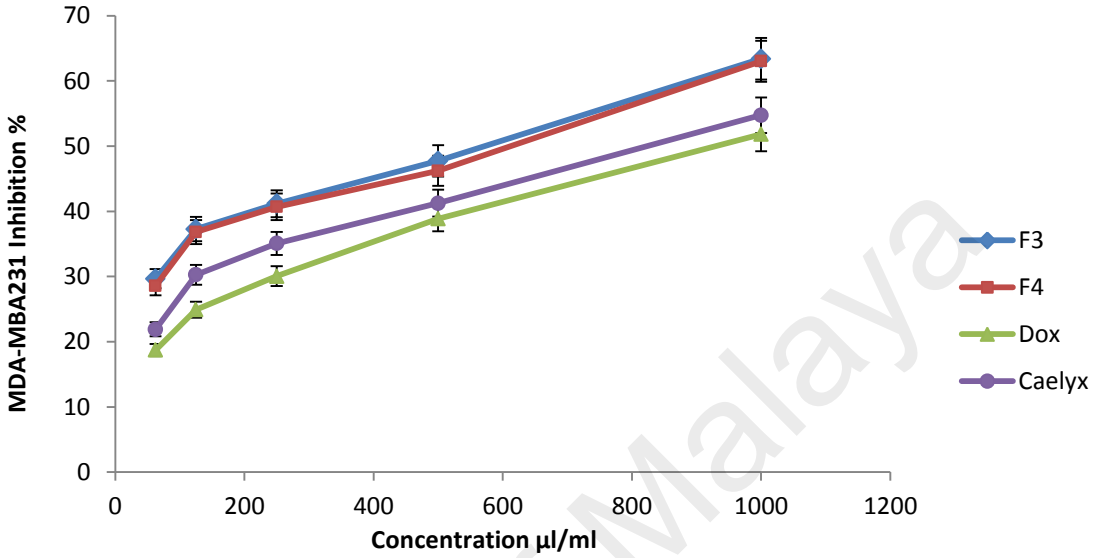


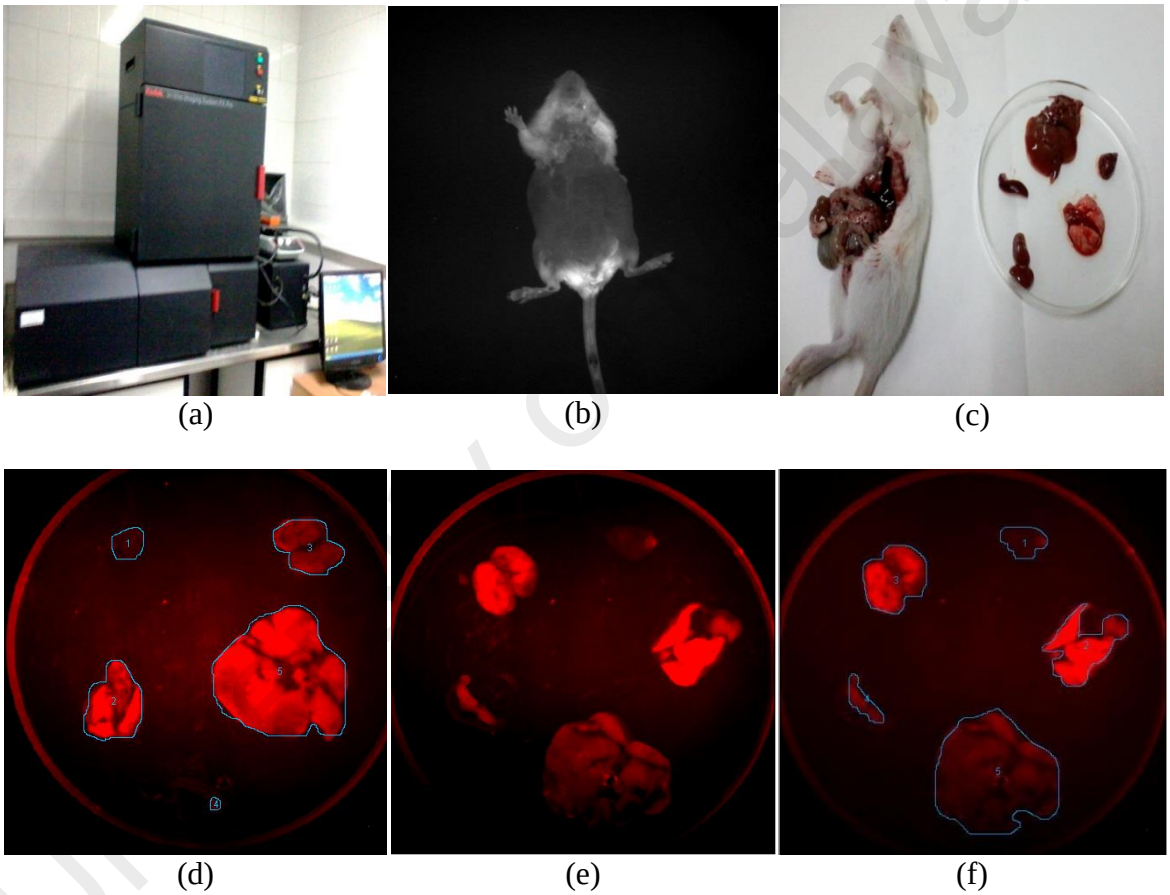
Figure 4.29: Comparison of the IC50 for F3 (—◆—), F4 (—■—), doxorubicin hydrochloride (—▲—) and Caelyx® (—●—) on MDA-MBA 23l cell line after 48 hours.

Figure 4.29 shows IC50 of MDA-MBA 231 cells after 48h treatment with F3, F4 liposomal doxorubicin, doxorubicin hydrochloride and Caelyx®. IC50 for all treatments evaluated after 48h incubation. The IC50 values for F3 and F4 were found to be 1.45 µM, and 1.46 µM respectively. Further IC50 of doxorubicin hydrochloride and Caelyx® were 1.77 µM and 1.68 µM respectively. Due to results of IC50, doxorubicin hydrochloride and Caelyx® showed more toxicity than F3 and F4 liposomal doxorubicin on MDA-MBA 231 cells.

4.16 In vivo imaging results

To obtain in vivo images of distribution of liposome formulations, rats were injected with 300 µml of each formulation individually, then images provided at the time of injection and after 24 h, 48h and 72h after injection. The distributions of each formulation in rats' organs were investigated using in vivo imaging devise (Kodak,

Japan). Organs of each rat were taken out and pictured then the florescence intensity of each organ was measured separately (Figure 30). The florescence intensity represents the accumulation and concentration of each formulation in the organs (Ntziachristos et al., 2003; Shokri et al., 2012). Figure 4.30 shows the in vivo imaging devise, provided organs and images additionally. Further, Figure 4.31 to figure 4.35 demonstrates the rats' organs distributions of doxorubicin hydrochloride, Caelyx®, F3 and F4 liposomal doxorubicin after injection.



(a) (b) (c)

(d) (e) (f)

Figure 4.30: In vivo imaging device (a), image of normal alive rat before injection (b), prepared rat organs (c) image of rat organs after 24h injection (d), image of control rat after 48h injection (e), image of rat organs after 72h injection (f).

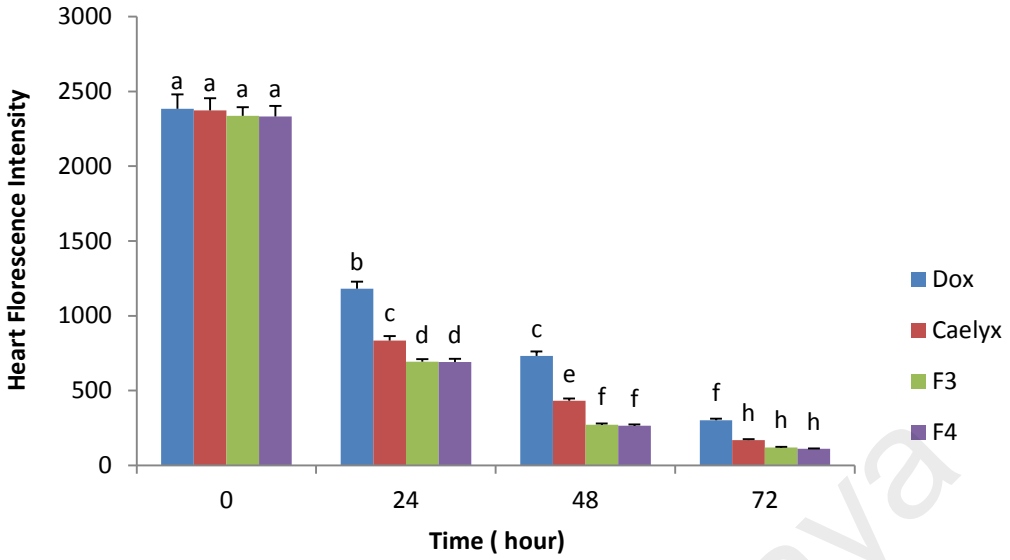


Figure 4.31: Mean florescence intensity of doxorubicin hydrochloride (■), Caelyx® (■), liposomal doxorubicin F3 (■) and F4 (■) in rat heart after 0, 24, 48 and 72h of injection.

Figure 4.31 shows the rats' heart mean intensity after 0, 24, 48 and 72h of injection doxorubicin hydrochloride, Caelyx®, F3 and F4. Referring to Figure 4.31, all treatments at the time of injection had high florescence intensity around 2300 in the heart while after one day the concentration of all treatments reduced obviously. The heart florescence intensity after 24h was reduced to: doxorubicin hydrochloride 1180, Caelyx® 835, F3 690 and F4 reduce to. After 48 h, the heart mean intensity of doxorubicin hydrochloride reduced to 731, Caelyx® to 432, F3 272 and F4 265. Further passing 72h of injection heart florescence intensity of doxorubicin hydrochloride reduced to 300, Caelyx® 169, F3 119 and F4 110 respectively.

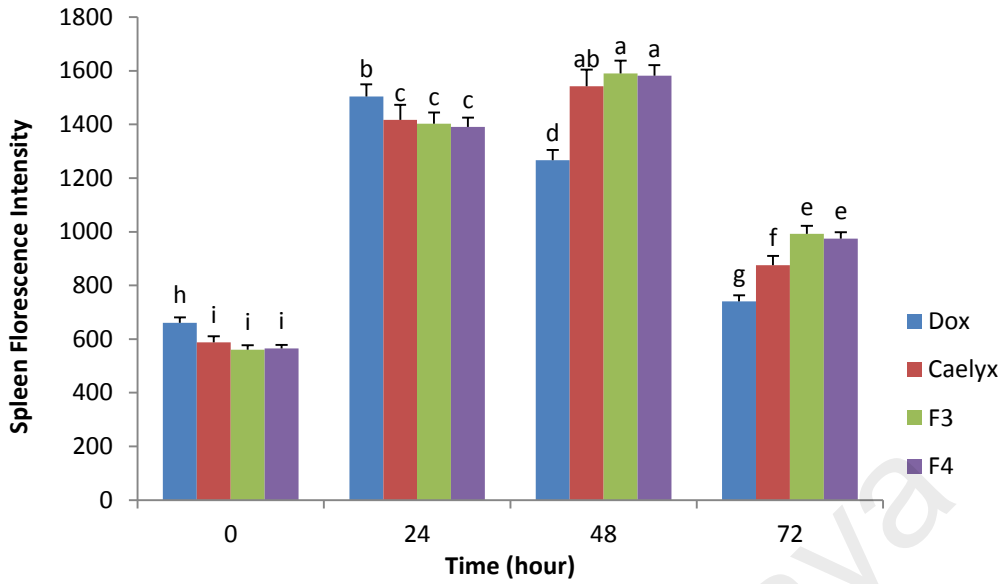


Figure 4.32: Mean florescence intensity of doxorubicin hydrochloride (■), Caelyx® (■), liposomal doxorubicin F3 (■) and F4 (■) in rat spleen after 0, 24, 48 and 72h of injection.

Figure 4.32 shows the rats' spleen florescence intensity after 0, 24, 48 and 72h of injection doxorubicin hydrochloride, Caelyx®, F3 and F4. At the time of injection the spleens' florescence intensity of were as: doxorubicin hydrochloride 661, Caelyx® 587 F3 560 and F4 564. After 24h, the florescence intensity of rats' spleen rose up to doxorubicin hydrochloride 1504, Caelyx® 1416, F3 1401and F4 1387. However passing 48 and 72h the lungs florescence intensity decreased as doxorubicin hydrochloride 740, Caelyx® 874, F3 and F4 it got to 993 and 973.

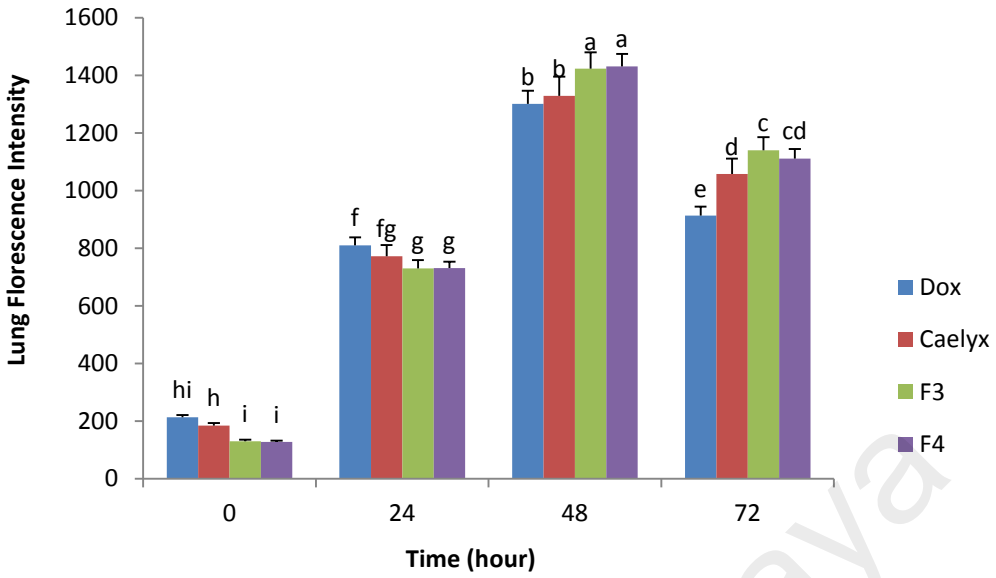


Figure 4.33: Mean florescence intensity of doxorubicin hydrochloride (■), Caelyx® (■), liposomal doxorubicin F3 (■) and F4 (■) in rat lungs after 0, 24, 48 and 72h of injection.

Figure 4.33 shows florescence intensity of doxorubicin hydrochloride, Caelyx®, F3 and F4 liposomal doxorubicin in rats' lungs after 0, 24, 48 and 72h of injection. Considering Figure 4.33 at the time of injection, rats' lungs florescence intensity was as: doxorubicin hydrochloride 213, Caelyx® 184, F3 130 and F4 128. After 24 h0ur the florescence intensity amplified as: doxorubicin hydrochloride 809, Caelyx® 772, F3 730 and F4 731. Passing 48 hours, the lungs florescence intensity has reduced as doxorubicin hydrochloride 1301, Caelyx® 1328, F3 1422 and F4 1431. Further the florescence intensity for all treatments decreased less than 1200 after 72h of injection whereas doxorubicin hydrochloride had the most reduction among others.

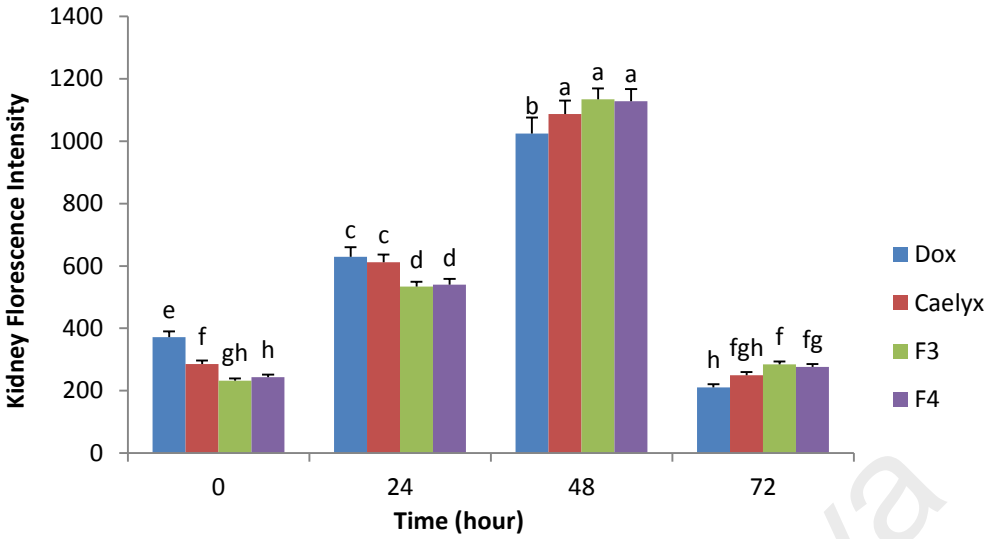


Figure 4.34: Mean florescence intensity of doxorubicin hydrochloride (■), Caelyx® (■), liposomal doxorubicin F3 (■) and F4 (■) in rat kidneys after 0, 24, 48 and 72h of injection.

Figure 4.34 demonstrates the florescence intensity of doxorubicin hydrochloride, Caelyx®, F3 and F4 liposomal doxorubicin in rats' kidneys after 0, 24, 48 and 72h of injection. As Figure 4.34 shows at the time of injection treatments had low florescence intensity in rats' kidneys as: doxorubicin hydrochloride with 371, Caelyx® 285, F3 232 and F4 243. After 24 h, florescence intensity of all treatments increased as: doxorubicin hydrochloride 628, Caelyx® 611, F3 533 and F4 539. While florescence intensity of treatment in rats' kidneys decreased significantly after 48h as: doxorubicin hydrochloride 1024, Caelyx® 1086, F3 1134 and F4 1127. Further passing 72h of injection spleen florescence intensity of all treatment condensed as doxorubicin hydrochloride 210, Caelyx® 249, F3 284 and F4 277 respectively.

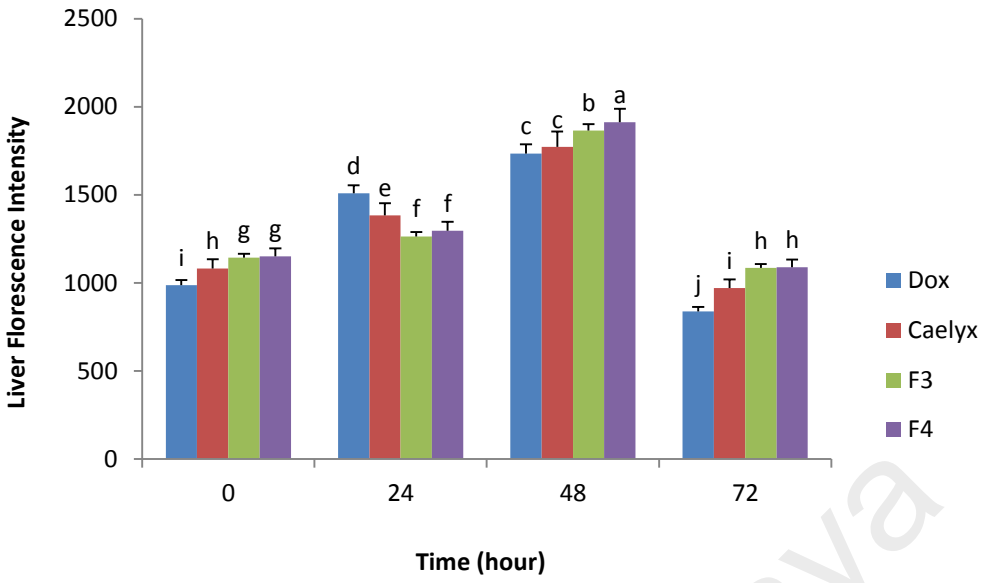


Figure 4.35: Mean florescence intensity of doxorubicin hydrochloride (■), Caelyx® (■), liposomal doxorubicin F3 (■) and F4 (■) in rat liver after 0, 24, 48 and 72h of injection.

Figure 4.53 shows the florescence intensity of doxorubicin hydrochloride, Caelyx®, F3 and F4 in rats' liver after 0, 24, 48 and 72h of injection. Referring to Figure 4.35 florescence intensity of all treatments is higher in liver, compare to other organs at the time of injection. Rats' liver florescence intensity at the time of injection were as: doxorubicin hydrochloride 987, Caelyx® 1081, F3 1143 and F4 1150. While after 48 h, florescence intensity of treatments reached to maximum as: doxorubicin hydrochloride 1733, Caelyx® 1771, F3 1864 and F4 1912. Further passing 72h, rats' liver florescence intensity were reduced as: doxorubicin hydrochloride 838, Caelyx® 971, F3 1086 and F4 1089 respectively.

According to normality test all variables were distributed normally. A two way ANOVA (factorial) was used for evaluating the effect of both Time and TRT and their interaction. The results showed significant influence of Time and their interaction on all variances at 0.01 level except the effect of TRT on kidney. R2 show a goodness of fit for the models. Following of analysis of variance, Duncan multiple range test was done for mean comparisons.

Table 4.7: Summary of ANOVA (Mean Square) for rats' organs

S.O.V	Heart	Lung	Kidney	Spleen	Liver
Time	11460391.8**	3166765**	1816703**	2247689.8**	1639918**
TRT	236091.6**	5248.0*	$594.1n^{NS}$	21545.5**	20908.3**
Time*TRT	31103.7**	16116.1**	9862.4**	35088**	31646**
CV%	6.5	4.3	4.9	3.9	2.1
R-Square	99.6	99.5	99.5	99.3	99.5

**: significant at 0.01 level, *: significant at 0.05 level, NS: non-significant

CHAPTER 5: DISCUSSION

Cancer is known as a challenging disease to treat by highly toxic anticancer medicines. Since the percentage of cancer patients has increased, medical field researches have focused on the identification of effective and selective anti-proliferative medicine with less toxicity and side effect to cure cancers(Komarasamy & Sekaran, 2012). The technology of designing the new drug delivery systems aimed to decrease the side effects and increase the therapeutic index of anticancer agent simultaneously(Jain et al., 2007)

Liposome is a form of new drug delivery system which is known for reducing the toxicity of medicines as well as optimizing their releasing time and increasing blood circulation. Some liposomal-based medicines are currently available on the market and more in clinical trials such as Caelyx® and Doxil® (Fang, 2006; Fenske et al., 2008). The physical properties and composition of liposome have a major role in their pharmacokinetics and therapeutic index (Tardi et al., 1996). Cholesterol (CH) and Phosphatidylcholine (PC) are the main component of liposome while several studies have been done on lipid composition to optimize liposome by changing the percentage of liposome ingredients (Vitas et al., 1996).

Cholesterol is a lipid-like alcohol in cell membrane with different lipid structure that found in animal tissues. It is amphipathic molecule due to its non-polar hydrocarbon body and polar hydroxyl group. Cholesterol attached ring system does not allow it to rotate around C-C bond plus provides existing rigidity and stabilize the membrane. The presence of cholesterol in construction of vesicle protects the disintegration and avoids the leakage owing to reduce the movement of fatty acid chains and decrease the membrane fluidity of vesicles respectively (Alves et al., 2013; Chrai et al., 2002; Cócera et al., 2003; de Meyer & Smit, 2009; Oldfield & Chapman, 1971).

Phosphatidylcholine is an amphihilic molecule that compose of hydrophilic head group and lipophilic tails with a long fatty acid hydrocarbon chains (Akbarzadeh et al., 2013; Lasch et al., 2003; Rutherford, 2011). However, PC has a high tendency to oxidation due to containing

large amount of unsaturated fatty acids (Samuni et al., 2000; Schnitzer et al., 2007; Senior & Gregoriadis, 1982).

Palm oil is another lipid which use in this study to prepare liposome. It composed of saturated, non-saturated and poly-saturated fatty acids. Palm oil naturally contains antioxidants chemicals such as carotenes tocopherol and tocotrienols which also known as anticancer agents (Edem, 2002). According to Sundram palm oil consist of 50-87 mg of tocoterionol which is identified as a potential antioxidant and anticancer agent (Sundram et al., 2003). Several studies have been carried out to evaluate anticancer benefit of palm oil on account of its tocopherol and tocotrienol (Alayoubi et al., 2013; Dan Postescu et al., 2010; Komarasamy & Sekaran, 2012; Wong et al., 2012).

First diclofenac sodium were applied instead doxorubicin hydrochloride due to its high toxicity. Further in pre-formulation, palm oil fractions were used to investigate the possibility of formation liposome with palm oil. Liposomal diclofenac sodium were prepared through conventional method and characterized afterwards.

Diclofenac sodium liposomes prepared in this study had size distribution ranging from 300 to 439 nm depending on the proportion of PC in the system. This might be as a result of interactions between the lipid layers of liposome with diclofenac sodium. This is according to finding of Lopes which stated due to interaction of diclofenac sodium anion with the ammonium group of PC, as the drug incorporated into the liposome the size of diclofenac sodium liposome would reduce considerably (Lopes et al., 2004). Furthermore, large standard deviations found for mean particle size may be due to the broad heterogeneous sizes of the liposome.

The Zeta potential data indicate the repulsive forces between particles in colloidal systems and thus confirm the stability of the system. Particles with lower ZP than -30 mV could result in particle aggregation and lead to caking and eventual spoilage. In this regard, FII and III with lower negative ZP and would be less stable while FI, FIV, FV and FVI higher negative ZP

would be more stable. A previous study suggests that interaction of diclofenac sodium with PC may adversely affect the structure and hence stability of the liposomes (Maestrelli et al., 2006). It was also observed that the liposomes with higher proportion of PC were consequently less stable while liposome with more proportion of palm oil fractions provided to be more stable respectively.

However the entrapment efficiency of liposome declined with increasing the palm oil lipids. Hathout obtained a maximum entrapment efficiency of approximately 40 % and this is higher than the 30 % entrapment found in the present study (Hathout et al., 2007). Imura has stated that drug entrapment increases as PC concentration rises, and hence the lower entrapment found in our study may be due to the smaller level of PC in the formulations (Imura, Otake, et al., 2003).

FII and FIII exhibited faster release among all formulations and achieved maximum 90% release within 62h. On the other hand, FIV and FI showed slower release after 6 h. Interaction of diclofenac sodium with PC might have caused deformity of the liposome besides affecting the stability and release pattern due to leakage of the drug from the unstable liposomes (Lopes et al., 2004). As the amount of PC decreased, the interaction is reduced and the stability of liposome increases. This may be the reason why FIV with a lower PC level exhibited slower release than FI which contained a higher PC content. Although FV and FVI contained higher concentrations of palm oil fractions, their liposomes were smaller.

The slower drug release obtained from FV and FVI, compared to FII and FIII, may be attributed to their higher stability (Maestrelli et al., 2006). Also according to Yamauchi drug release rate will increase as liposomal size shrinks (Yamauchi et al., 2007).

Doxorubicin hydrochloride is a hydrophilic drug which is applying as anti-carcinoma agent (Tacar et al., 2013). Encapsulation of doxorubicin hydrochloride within liposome lead to less cardiotoxicity and higher bioavailability (R. M. Schiffelers et al., 2003). Optimizing of liposome as a drug carrier with appropriate physical properties improves the therapeutic index

as well as reducing toxicity. Since LUVs have a large internal space within themselves and doxorubicin hydrochloride is a hydrophilic drug, this study aimed to prepare adjusted LUVs using palm oil. Six formulations were designed by several proportions of palm oil and PC, with the same ratio of cholesterol and PEG. Table 3.4 shows the components and the extents of designed formulation as F1 contains 50% PC and no palm oil, F2 45% PC and 5% palm oil, F3 40% PC and 10% palm oil, F4 had 35% PC and 15% palm oil, F5 had 30% PC and 20% palm oil and F6 had 25% PC and 25% palm oil. LUVs were prepared through reverse phase evaporation and freeze thaw methods and then additionally evaluated in terms of in vitro.

5.1 Formation of liposomal doxorubicin

Formations of liposomes were determined using Transmission Electron Microscopy (TEM). Szoka & Papahadjopoulos also applied reverse phase evaporation method for preparing LUV and they used electron microscopy images to confirm the formation of LUVs through this method accordingly (Szoka & Papahadjopoulos, 1978). Figure 4.1 demonstrates the formation of liposome in designed formulations. Lamellarity (number of lipid bilayers) and size of the vesicles are the important characterization of each liposome that can be investigated with TEM images (Gregoriadis, 2006; Hauser, 1993). According to transmission electron microscopy images demonstrate vesicles contain one bilayer with size around 300-400 nm which prove the well formation of LUVs with large internal space correspondingly. F1 (Liposome formulation without Palm oil) and F2 (Liposome formulation consisting of 5% Palm oil) which composed mainly of PC have spherical fine shape. F3 (Liposome formulation consisting of 10% Palm oil) and F4 (Liposome formulation consisting of 15% Palm oil) vesicles that contain less PC, are larger and less spherical but still having acceptable and fine shape, nevertheless in F5 (Liposome formulation consisting of 20% Palm oil) and F6 (Liposome formulation consisting of 25% Palm oil) by increasing the proportion of palm oil, malformed and misshapen vesicles with smaller size were formed. By increasing the ratio of Palm oil, liposome slightly becomes misshappened and defined. F1 and F2 vesicles have small and well spherical shape while F3

and F4 showed bigger and ellipsoidal formation. Liposome from F5 and F6 look misshappened with distorted appearances.

5.2 Size distribution of liposomal doxorubicin

Particle size is one of significant parameters of characterization for any vesicle. Liposome sizing is almost the same study as an evidence to approach desirable vesicle size that capable to pass through vascular capillaries efficiently (Gregoriadis, 2006; Nastruzzi, 2004). Due to Tardi study, the antitumor efficacy of liposomal doxorubicin is dependent to their vesicle size. By decreasing the size of liposome, anticarcinoma therapeutic index of liposomal doxorubicin will increase accordingly. Table 4.1 show the mean particle size of six designed LUVs provided via reverse phase evaporation method (Tardi et al., 1996). Referring to Table 4.1, F6 and F5 with the average particle size of 341.45±1.8 nm and 356.67±1.1 nm showed minimum size while F3 and F4 447.21±1.7 nm and 438.74±2.3 nm had largest average particle size. Further F1 and F2 contained particle size of 378.84±1.3 nm and 407.45±2.7 nm.

5.3 Zeta potential of liposomal doxorubicin

Value of zeta potential shows the repulsive forces between particles in colloidal systems which confirm the physical stability of the mixture. Low zeta potential cause particles to aggregate which lead to spoil the system (Gregoriadis, 2006; Ikonen et al., 2010). Table 4.1 shows the zeta potential values of fresh liposome and after 30 & 60 days of storage respectively. In fresh medium of F1 (without palm oil) and F2 (consisting of 5% palm oil), the zeta potential value were -26.6 and -27.5 mV, while after 60 days it reduced to-22.2 and -25.1 mV respectively. In F3 (consisting of 10% palm oil) and F4 (consisting of 15% palm oil) the zeta potential of fresh liposome were -32.2 and -31.1mV whereas after 60 days it reduce to -30.6 and - 29.4 mV. Further in F5 (consisting 20% palm oil) and F6 (consisting of 25% palm oil) the zeta potential of fresh medium were -29.3 and - 28.9 mV, while after 60 days it reduced to - 26.5 and -25.7 mV. One-way analysis of variance (ANOVA) applied to calculate P-value (using Microsoft

Excel 2007). Referring to Table 4.1, there is a considerable difference in zeta potential of fresh liposome and the liposome stored after 30 & 60 days due to P-value= 0.222.

Considering that particles with zeta potential more negative than -30mV or more positive than +30mV are normally stable (van Nieuwenhuyzen & Szuhaj, 1998). F3 & F4 had improved zeta potential values among other formulations. Also after 30 days of storage the zeta potential value of F3 and F4, were still below -30 however, after 60 days only F3 zeta potential was remained below -30 Mv. Consequently replacing 10% and 15% of palm oil in liposome would improve their physical stability while liposome without palm oil or lower and higher proportions of palm oil had less zeta potential value and subsequently less stability.

5.4 Entrapment efficiency and in vitro release of liposomal doxorubicin

The concentration series of 2, 2.5, 3, 4, and 5µg/mL from doxorubicin hydrochloride were prepared and the absorbance was measured using UV-Visible spectrophotometry at of 335nm. Then, the standard curve was constructed using Microsoft Excel 2007 program (Figure 4.3). The following equation was obtained, Y= 0.254 X, where Y is the absorbance at 335 nm, and X is the concentration of doxorubicin, the regression line was obtained $R^2 = 0.998$ as well.

Table 4.2 illustrates the entrapment efficiency percentages of designed liposomes. Entrapment efficiency % is the proportion of drug that entrapped within the liposomes. In order to assess EE%, first the medium was centrifuged to remove the free drug, afterwards 100 mg of liposome were added to the mixture of ethyl acetate, diethyl ether and distilled water (60:40 ml, v/v). The aqueous phase was obtained and its' absorbance measured using UV-Visible spectrophotometry at the wavelength of 335nm. Further the EE% of entrapped doxorubicin provided via equations 4.1 and 3.1 respectively (Mu & Zhong, 2006; Panwar et al., 2010). Referring to Table 4.2, all liposome formulations had approximately 40% entrapment and replacing of palm oil did not show significant effect on their drug entrapment.

Drug in *vitro* releasing of liposome was investigated under sink condition (Saarinen-Savolainen et al., 1997). After separation of free doxorubicin, freshly PBS (pH 7.4) was added

to liposome and samples were incubated for 30, 60, 120, 240, 360, 420 and 500 minutes. The absorbance of sampels were measured at 335nm and Drug release % was provided with equation 3.3.

F1 and F6 liposome had the fast release rate while F3 and F4 liposome showed slowest release. Apart from F3 and F4, F5 liposome showed slow releasing during incubation period. However F2 had lower release during the first 60 minutes but then it was increased progressively and had more release than F5.

5.5 Liposomal doxorubicin degradation study

The interaction between serum component and liposome lead to degradation of vesicles, further oxygen and heat accelerate the speed of liposome hydrolysis accordingly (Foradada et al., 2000). Since stability of liposome in presence of serum component is important to deliver its encapsulated medicine straight to the site of sickness. To determine the degradation of vesicles, 100 mg of each formulation were added to the mixture of 10 ml RPMI 1640, 10% FBS and 1ml Penicilin G. Afterwards liposome incubated at 37^{o}C in presents of atmospheric oxygen during 2 hours, one day and one week. Basic and acidic extracts were obtained from the mixture and then ^{1}H NMR spectrums of them were provided. As PC is the most important part of all liposomes to study stability of designed liposome, signals of PC piece was recognized as an indication of broken liposome. Figures B.1 and B.4 demonstrate the acidic and basic ^{1}H NMR spectrums of F1 liposome after 2h incubation with serum component. Signals of PC in ^{1}H NMR spectrums confirm the degradation of F1 liposome within 2h incubation. However, in F2 liposome no signals observed in both acidic and basic extract after 2h (Figures B.7 & B.10). Signals of F2 liposome ^{1}H NMR spectrums after one day and week incubation, also proves the degradation of liposome during the incubation period.

^{1}H NMR spectrums of acidic and basic extracts of F3 after 2h, one day and one week are shown in figures B.13 to B.18. No signal was detected in both acidic and basic extract within

2h and one day incubation with serum component. While ^{1}H NMR spectrums of one week incubation, showed signals that confirm degradation of vesicles accordingly.

Figures B.19 to B.24 demonstrate ^{1}H NMR spectrums of acidic and basic extracts of F4 liposome during 2 hours, one day and one week incubation with serum component. Same as F3 liposome, no signal was noticed in acidic and basic extracts (Figures B.19 and B.22) after 2h incubation, although passing one day, few signals appeared (Figure B.20, B.23). Further after one week, more signals observed in ^{1}H NMR spectrums (Figure B.21, B.24) due to degradation of vesicle.

^{1}H NMR spectrums of acidic and basic extracts of F5 and F6 liposome after 2 hours, one day and one week incubation with serum component, are shown in Figures B.25 to B.36. Signals of PC identified in all spectrums which confirm degradation of F5 and F6 liposome during incubation periods.

Considering the characterization results of designed liposome, F1 liposome that mainly composed of PC had fine spherical shape but low ZP value (-26.6 mV), low entrapment and fast in vitro releasing and degradation due to ^{1}H NMR spectrums findings. Further F1 liposome showed high degradation and low stability during incubation with serum component.

In F2 liposome, which contained 5% palm oil, sphere-shaped, low ZP value (-27.5mV) and drug entrapment were same as F1 vesicles. However, F2 liposome had slower in vitro release and less degradation compare to F1vesicles but since they still contain large proportion of PC, they showed quick in vitro release and low stability therefore correspondingly. F3 and F4 liposome, which contained 10 &15% palm oil, showed large ellipsoidal shape with elevated ZP value (-32.2, -31.8 mV). Also F3 and F4 liposome had deliberate drug releasing and less degradation as well.

TEM pictures of F5 and F6 liposome demonstrate malformed and misshapen formation of vesicles. While F5 and F6 contain higher ZP values (-29.59, -28.9 mV) than F1 and F2 due to less proportion of PC but, their ZP values are still less than -30 which indicate that they also

have a poor physical stability. Further F5 and F6 liposome had rapid in vitro release plus high degradation during incubation with serum component.

Since all formulations had low entrapment efficiency, active drug loading utilized to enhance the entrapment of liposome. Considering pilot study it would be concluded that the F3 and F4 liposome had fine shape, size, stability and release rate compare to other formulations. Also F3 and F4 liposome showed less degradation during incubation with serum component. Therefore F3 and F4 liposome were chosen for advance study. In this part liposome prepared using freeze thaw method and doxorubicin loaded within vesicles via pH gradient technique respectively. Afterwards liposome characterize in terms of in vitro and in vivo as well.

5.6 Morphology of liposomal doxorubicin

Figure 4.7 demonstrates the Scanning Electron Microscopy (SEM) images of F3 and F4 liposome after they prepared using freeze thaw method. SEM images show the smooth surface with fine shape and formation of F3 and F4 liposome (Almgren et al., 2000; Musumeci et al., 2006).

5.7 Calibration curve of doxorubicin hydrochloride

HPLC technique was carried out to measure the entrapment efficiency and releasing rate of doxorubicin hydrochloride after loading via pH gradient process. First calibration curve was constructed with dilutions of 12.5, 25, 50, 100, 200, and 400ng/ml of doxorubicin in PBS. Afterwards equation Y= 21998 X + 8938 obtained via Microsoft Excel 2007 program, where Y is the graph area, X is doxorubicin concentration, the regression line is $R^2 = 0.999$ (Figure 4.8).

5.8 Liposomal doxorubicin entrapment efficiency and in vitro release

Entrapment efficiency of liposome was measured after separation of free doxorubicin with centrifuge process. As Table 4.5 shows, the entrapment efficiency of liposome boosted up significantly (99.98%) using pH-gradient procedure compare with reverse-phase evaporation method. Further in vitro release of liposome measured by providing samples (1 ml) at 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 hours presently during incubation and then injected to HPLC. Figure 4.10 demonstrate the releasing of doxorubicin in Caelyx®, F3 and F4 liposome. Since F3 and F4 include same ingredient with only small difference in amount of PC and palm oil, they also have comparable releasing pattern with small variation. Caelyx® showed faster release during the incubation compare to F3 and F4. In first 6h of incubation Caelyx® had 52% drug release while F3 had 39% and F4 had 36% drug release. Further passing 12h of incubation drug release for Caelyx® was 70% and for F3 and F4 was 57%. After 24 h, drug release in F3 and F4 liposome increased to 73% while for was Caelyx® 84%. As drug releasing of Caelyx® generally was more than F3 and F4, hence during 24h higher releasing rate of doxorubicin monitored in Caelyx® than F3 and F4 as well (Hossann et al., 2007).

5.9 FTIR spectrum

FT-IR spectra utilize to analyze and characterize chemicals compounds. Figures 4.11-4.16 show FT-IR spectra's of doxorubicin hydrochloride, F1, F3 and F4 liposome loaded with doxorubicin plus pure F3 and F4 liposome without loading drug. All liposome spectra's showed comparable peak due to similar composition. F1, F3 and F4 liposome FT-IR spectra demonstrate similar peaks of OH, C-H aliphatic, C-O, and substitutions of aromatic rings. Further in doxorubicin hydrochloride, F3 and F4 liposomal doxorubicin FT-IR spectra, a double peak at 3500 corresponding to NH_2 in the formulations. While other peaks are indicating C-H Aliphatic C=O and substitutions of aromatic rings in liposomal doxorubicin as

well. The presence of double peak in doxorubicin hydrochloride, F3 and F4 liposomal doxorubicin as a result of NH_2 is the evidenced of well entrapment of doxorubicin within liposome (Griffiths & De Haseth, 2007; Smith, 2011).

5.10 Differential Scanning Calorimetry

Figures 4.17-4.21 demonstrate the Differential Scanning Calorimetry of doxorubicin hydrochloride, F3 and F4 liposomal doxorubicin plus F3 and F4 without loading drug. Thermal analyzing test gives the melting point (onset) and integral of the curves (Biltonen & Lichtenberg, 1993; Gill et al., 2010). Considering the DSC curves lipids had low onset around 25-40^{o}C while the onset of doxorubicin was over 200^{o}C. In F3 liposome the onset of lipids was 26.25^{o}C with integral -18.51 mJ whereas in F3 liposomal doxorubicin the onset of lipids was 32.77^{o}C with integral -26.16 mJ. Also in F4 liposome the onset of lipids was 27.18^{o}C with integral -37.97 mJ while in F4 liposomal doxorubicin the onset of lipids was 32.87^{o}C with integral -33.89 mJ. The lipid onset of liposomal doxorubicin was increased in both formulations, as a result of encapsulating drug within vesicles. Referring to Figure 4.17, the onset of doxorubicin hydrochloride was 218.01^{o}C with integral -27.94 mJ. Further in F3 liposomal doxorubicin the onset of doxorubicin was 214.39^{o}C with integral -9.98 mJ, while in F4 liposomal doxorubicin the onset of doxorubicin was 198.90^{o}C with integral -13.78 mJ.

Overall the doxorubicin curves in F3 and F4 liposomal doxorubicin confirms the effective encapsulation of doxorubicin hydrochloride within vesicles. Further reduction the onset of doxorubicin in liposomal doxorubicin and shifting the onset of lipids and doxorubicin in liposomal doxorubicin compare to liposome is an evidence for affecting doxorubicin and lipids on thermal analysis consequently.

5.11 Cellular uptake

MCF7 and MDA-MBA 231 cell lines were utilized to determine cellular uptake of doxorubicin hydrochloride, Caelyx®, F3 and F4 liposomal doxorubicin. The percentages of

cellular uptake of treatments determined using florescence detection method. MCF-7 and MDA- MBA, 231 cells were seeded in 24-well plates with a density of 1 ×105 cells/well, then incubated in 37ºC with 5% CO2 for 24h. MCF-7 and MDA-MBA 231 cells were treated with doxorubicin hydrochloride, Caelyx®, F3 and F4 liposomal doxorubicin individually then incubated for 2 and 24h in 37ºC with 5% CO2. Figures 4.24 and 4.25 demonstrate (florescence intensity) of doxorubicin hydrochloride, Caelyx®, F3 and F4 liposomal doxorubicin in MCF7 and MDA-MBA 231cells. Considering the cellular uptake of treatments, in both MCF7 and MDA-MBA 231 cells, liposomal treatment showed higher uptake compare with doxorubicin hydrochloride. In MCF7 after 2h F3 and F4 had 9.52% and 8.9% florescence intensity while doxorubicin hydrochloride had 4.79% and Caelyx® had 7.53% and following 24h, F3 and F4 uptake reach to 97.26% and 95.21% whereas Doxorubicin hydrochloride and Caelyx® showed 71.23% and 89.73% florescence intensity. In MDA-MBA 231 cells after 2h, F3 and F4 liposomal doxorubicin showed 9.31% and 8.33% florescence intensity whereas doxorubicin hydrochloride had 4.41% and Caelyx® had 7.84% florescence intensity. Following 24h incubation F3 and F4 liposomal doxorubicin uptake reach to 92.16% and 91.67% whereas doxorubicin hydrochloride and Caelyx® showed 65.69% and 78.92% florescence intensity. Therefore MCF7 had higher cellular uptake percentage than MDA-MBA 231 and F3 and F4 liposomal doxorubicin also showed higher uptake than Caelyx^{R} excessively.

In addition MCF7 and MDA-MBA 231 cells treated with F3 and F4 liposomal doxorubicin then incubated for 24h at 37ºC with 5% CO2. Figures 4.22 and 4.23 demonstrate the optical florescence microscopy images of MCF7 and MDA-MBA 231 cells following the treatment. After treating breast cancer cells, liposomal doxorubicin crossed over the cells and they appear with a red basis due to the red autoflorescence color of doxorubicin while the apoptosis cells exhibited brighter reds, respectively (Iwasa et al., 2006; Park & Yoo, 2010).

5.12 MTT assay

MTT assay was carried out to determine the cytotoxicity of doxorubicin hydrochloride, pure liposome without drug, and Caelyx®, F3 and F4 liposomal doxorubicin on MCF-7 and MDA-MBA 231 breast cancer cell line. Then the IC50 of treatments was evaluated after 24 and 48h incubation at 37ºC with 5% CO2 according to standard MTT assay. IC50 is a quantitative estimation of treatments that represents the 50% inhibition of cells accordingly (Akbari & Javar, 2013; Chai, 2009; Miglietta et al., 2000). Pure liposome (without drug) didn't show any cytotoxicity on breast cancer cells. Treatments which contain doxorubicin also did not reach to IC50 during 24h, although they were toxic to the cells (Park & Yoo, 2010). Mechanism of action of doxorubicin is to binds with the nucleotide (DNA's double helix) and inhibits reproduction or action of DNA and RNA polymerases that resulted in the toxicity effect upon malignant cells and/or normal cells. It also causes several changes in the characteristic morphology of cells that leads to program cellular death or apoptosis, based on its therapeutic or toxicities affects Hence, proper adequate period of incubation is important on effectiveness of doxorubicin.(Pang et al., 2013; Pommier et al., 2010). However in Postescu study cancer & normal cells first treated with palm oil liposome and then with doxorubicin hydrochloride which IC50 obtained after 24h incubation(Dan Postescu et al., 2010). Since in this study palm oil is a part of drug delivery system and pegilation technology utilized to optimize the liposome, the breast cancer cell lines were reached to IC50 after 48h of incubation accordingly. Pegilated liposomal doxorubicin were designed to prevents the uptake of liposome by the reticulo-endothelial system which result in the long circulation of liposome, accordingly (A. A. Gabizon, 2001; Moein Moghimi et al., 2006).

IC50 of treatments in MCF7 cells are as: F3 liposomal doxorubicin 0.44 µM, F4 liposomal doxorubicin 0.45 µM, doxorubicin hydrochloride 0.83 µM and Caelyx® 0. 78 µM. The IC50 of liposomal doxorubicin were more effective and potent than doxorubicin hydrochloride while F3 and F4 that include 10% & 15% of palm oil even showed higher efficacy compare to

Caelyx®.Therefore F3 and F4 liposomal doxorubicin showed approximately 2 fold higher efficacy compare with doxorubicin hydrochloride and Caelyx® on MCF7 cells due to improved cellular uptake of liposome. However in Miglietta study SLN doxorubicin had 10 fold more toxicity than doxorubicin hydrochloride (Miglietta et al., 2000). Therefore liposomal doxorubicin invented to be much more effective than doxorubicin but due to control release of doxorubicin from liposome, the IC50 of designed liposome were only double more than non-liposomal form of doxorubicin after 48h incubation.

At the same time the IC50 of treatments determined on MDA-MBA 231 cell line additionally. The IC50 of treatments are as: F3 liposomal doxorubicin 1.45 µM, F4 liposomal doxorubicin 1.46 µM, doxorubicin hydrochloride 1.77 µM and Caelyx® 1.68 µM. Considering the IC50 results, MDA-MBA 231 cells were more resistant to treatments compare to MCF7 cells whereas MDA-MBA 231 cells were more sensitive to F3 and F4 liposomal doxorubicin than doxorubicin hydrochloride and Caelyx® (Xu et al., 2009).

5.13 In vivo imaging

Distribution of doxorubicin hydrochloride, Caelyx®, F3 and F4 liposomal doxorubicin determined investigate using in vivo imaging devise (Kodak, Japan). Rats were injected with 300 µml of each formulation individually and then rats' organs taken out and images provided. As Figure 4.31 to 4.35 showed the florescence intensity of each organ was obtained at the time of injection and after 24, 48 and 72h of injection (Shokri et al., 2012). After intra cardiac injection, treatments shift to liver and spleen extensively. In vivo study were carried out to investigate the distribution of doxorubicin hydrochloride, Caelyx®, F3 and F4 liposomal doxorubicin in rats' organs using florescence imaging system. Since rats were injected into their hearts, the florescence intensity of treatment was high at the time of injection but it was decreased after 24h of injection. Liposomal formulation had less accumulation in heart than doxorubicin, whereas F3 and F4 liposomal doxorubicin showed less florescence intensity

compare with Caelyx® as well. In lungs and kidneys Florescence intensity of all treatment increase from time of injection till 48h after injection while passing 72h the florescence intensity reduced due to elimination of doxorubicin. Liver and spleen contained higher accumulation of treatment following injection due to elimination of doxorubicin in them (Campbell et al., 2002). Main metabolism and elimination of doxorubicin is through liver and major quantity of treatments accumulated in liver after injection. Passing 24h of injection the florescence intensity of liposomal doxorubicin is less than doxorubicin hydrochloride although after 48 and 72h the florescence intensity of liposomal doxorubicin was higher than doxorubicin hydrochloride due to pegilation and control releasing of the liposomal doxorubicin.

CHAPTER 6: CONCLUSION

Doxorubicin hydrochloride is a toxic chemotherapy agent from anthracycline antibiotic. Liposomal form of doxorubicin is basically promised to have a less drug-induced toxicity and more therapeutic index. In addition pegylated liposomal doxorubicin showed significant prolong circulation due to prevention of its uptake by the RES system.

This study aimed to apply palm oil in the preparation of liposome as a natural antioxidant agent, which supposed to improve the stability of liposome. Further applying natural product with inborn anticancer activity would possibility reduce side effects and improve treatment activity additionally.

Six formulations were designed with various weight percentages (%w/w) of phosphatidylcholine and palm oil then liposome formed using reverse- phase evaporation method. Liposomes were formed in all formulation although increasing the proportion of palm oil lead to formation of misshapen and malformed vesicles. Referring to TEM images, mono layer vesicles with a large internal space within them are the evidence of formation LUVs (large unilamellar vesicles) through reverse- phase evaporation method. F1and F2 liposome showed spherical shape while F3 and F4 had ellipsoidal vesicles. Formulations 5 and 6 that containing 20% & 25% of palm oil were misshapen and malformed due to large replacement of palm oil instead PC. Meanwhile adding palm oil improved Zeta potential value of vesicles where F3 and F4 liposome had acceptable ZP even after 30 and 60 days of storage at 4^{o}C. However adding palm oil to the formulations did not have any significant effect on particle size and entrapment efficiency.

Since adding 10 and 15% of palm oil improve ZP and physical stability, it also increased the chemical stability of liposome additionally. According to degradation study of liposome, F3 and F4vesicles contained less degradation during incubated with blood component in presence of heat and oxygen. No signals also identified in ^{1}H NMR

spectra of F2 liposome after 2h incubation whereas passing one day vesicles were less stable and decayed. ^{1}H NMR spectra of F1, F5 and F6 showed degradation of vesicles after 2h incubation which is evidence for low chemical stability of formulations.

Due to above results F3 and F4 which contains 10% and 15% of palm oil showed good formation, controlled release, stable ZP and low degradation. Therefore in order to develop the entrapment efficiency of the vesicles, liposome prepared using freeze-thaw method and doxorubicin encapsulated within them by active drug loading. Active drug loading using pH-gradient technique, was enhanced the entrapment efficiency of liposome up to 99.98%, which quantified by HPLC. Further both F3 and F4 formulation demonstrate controlled in vitro release during 96h of incubation in sink condition.

Fourier Transform Infra-Red spectrums were provided to investigate the structure of doxorubicin hydrochloride liposomal doxorubicin and unloaded liposome. All spectrums indicated aliphatic and aromatic band meanwhile, attendance of double pick around 3500 is evidence of NH_2 in doxorubicin hydrochloride. Referring to both F3 and F4 liposomal doxorubicin spectrums, double peaks at 3300 and 3440 are the result of qualified encapsulation of doxorubicin within vesicles.

Thermal analyzing was assessed to investigate the influence of temperature on the liposome using Differential Scanning Calorimetry. DSC curves of doxorubicin hydrochloride unloaded F3 and F4 liposome and F3 and F4 liposomal doxorubicin were provided. Considering unloaded liposome curves, F3 and F4 liposome had onset at 26 and 27^{o}C due to their lipid base, while in liposomal doxorubicin the onset increased to 32^{o}C due to encapsulating of doxorubicin within liposome. Further doxorubicin hydrochloride curve had onset at 218^{o}C whereas in both F3 and F4 liposomal doxorubicin curves the onset of doxorubicin reduced to 214 and 198.9 ^{o}C. Since encapsulating doxorubicin within liposome enhance liposome melting point, also liposome cause decreasing the onset of doxorubicin correspondingly.

Cellular uptake and toxicity of doxorubicin hydrochloride, Caelyx®, F3 and F4 liposomal doxorubicin determined on MCF7 and MDA-MBA231 breast cancer cell lines. Referring to cellular uptake results, liposome formulations showed improved uptake compare to doxorubicin hydrochloride. Further both F3 and F4 liposomal doxorubicin also had higher uptake compare to Caelyx®. In term of toxicity, MTT assay was carried out to evaluate the IC50 of treatments. Unloaded liposome was not able to inhibit cell growth and considered to be non-toxic to the cells consequently. Liposomal formulations of doxorubicin were more potent compare to doxorubicin hydrochloride due to their higher cellular uptake respectively. However MDA-MBA231 cells were more resistant to all treatment than MCF7 cells.

In vivo study were carried out to investigate the distribution of Doxorubicin hydrochloride, Caelyx®, F3 and F4 liposomal doxorubicin in rat body using florescence imaging system. Since rats were injected into their hearts, the florescence intensity of treatment was high at the time of injection but it was decreased after 24h of injection. Liposomal formulation had less accumulation in heart than doxorubicin hydrochloride, whereas F3 and F4 liposomal doxorubicin showed less florescence intensity compare with Caelyx® as well. In lungs and kidneys Florescence intensity of all treatment increase from time of injection till 48h after injection while passing 72h the florescence intensity reduced due to elimination of doxorubicin. Liver and spleen contained higher accumulation of treatment following injection due to elimination of doxorubicin in them. Main metabolism and elimination of doxorubicin is through liver and major quantity of treatments accumulated in liver after injection. Passing 24h of injection the florescence intensity of liposomal doxorubicin is less than Doxorubicin hydrochloride, although after 48 and 72h the florescence intensity of liposomal doxorubicin was higher

than doxorubicin hydrochloride due to pegilation and control releasing of liposomal doxorubicin.

According to the results of this study, it can be concluded that palm oil has potential of utilizing as a natural product in part of pharmaceutical products. Further applying palm oil in formulation of anticancer medicine can improve the antineoplastic efficacy of them consequently.

Since liposome able to delivery drug in the target tissue and improve the therapeutic index along with minimizing side effects, this study suggests utilizing natural palm oil as a part of lipid composition of other liposome formulation. Further consider palm oil as a part of other drug delivery system such as polymeric micelles, niosomes, and solid lipid nanoparticles. Considering less toxicity of liposome drug delivery, this study recommends applying palm oil in formulation of other antineoplastic medicine and investigates the toxicity behavior of liposomal antineoplastic medicines as well.

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