Liposomes generated from proliposomes for treatment of glioma using Momordica charantia extracts

By

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DECLARATION

I declare that whilst registered as a candidate for the degree for which this submission is made, I have not been registered candidate for another award by any other awarding body. No material contained in this thesis has been used in any other submission for and academic award.

Signed

Abstract

Every year there is 2% increase in the reported cases of glioma. It is important to treat glioma from materials that are readily available in nature. One of the interesting properties of liposomes is their ability to target tumours and facilitate the cellular uptake of therapeutic agents compared to the agent alone. In this project, liposomes were prepared from SPC (Soy phosphatidylcholine) and HSPC (Hydrogenated soy phosphatidylcholine) phospholipids. Drug-free liposomes prepared by proliposomes were compared with the conventional method of producing liposomes. Conventional liposomes are biocompatible and biodegradable, however, they are physically and chemically unstable. The instability problems were avoided by formulating liposomes using the ethanol-based proliposome technology. In ethanol-based proliposome method, aqueous phase (e.g. water) was added to an ethanolic solution of phospholipid to generate liposomes. In this work, natural anticancer materials such as Paclitaxel (PTX) and *Momordica charantia* extracts (Whole fruit, Fruit alone and Seed alone) from Africa, China and India were incorporated in liposome formulations. These liposomes were analysed by investigating, the resultant size, size distribution and zeta potential of the vesicles. Either, the anticancer drug or extract of *Momordica charantia* was mixed with liposomes and checked for the efficacy of the anticancer-liposome formulations on the viability of glioma cell lines and the molecular mechanism of the cell death were also investigated. The results show that liposomes prepared by the conventional thin-film method were comparatively large in size as compared to liposomes generated from proliposomes. Liposomes generated from proliposomes (made from SPC or HSPC),

when generated by hydration with *Momordica charantia* extracts (WF – Whole fruit, FA – Fruit alone or SA – Seed alone) from Africa, China or India respectively, or when prepared using PTX in the lipid phase had significantly larger size and wider size distribution as compared to drug free liposomes. Liposomes generated from proliposomes were neutral or negatively charged and were a mixture of oligolamellar and multilamellar vesicles regardless of formulation. Particle size and size distribution of HSPC liposomes were larger than SPC liposomes on inclusion of either Paclitaxel or *Momordica charantia* extracts. Liposome generated by proliposome method using the *Momordica charantia* extracts (FA, SA and WF) exerted cytotoxic effects against glioma cells 1321N1, Gos-3 and U87-MG at the higher concentrations with or without liposomes. *Momordica charantia* extracts showed either slight or no significant effect on the normal glial cells. The liposome formulations were more effective against glioma cells as compared to drug-free liposomes. FA extract of *Momordica charantia* was very effective with and without liposomes but less than PTX liposomes. The activities of caspase 3/7, caspase 9 and cytochrome c release were elevated in cancerous glial cell line indicating apoptosis via mitochondrial cell death or intrinsic pathway.

In conclusion, the study showed that liposomes generated from proliposomes were appropriate to target cancerous glial cells by binding plant extracts *Momordica charantia* and PTX to SPC and HSPC phospholipids. The cell death was induced by apoptosis via mitochondrial cell death.

Dedicated to

My Loving Family

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List of Abbreviation

AMC	African *Momordica charantia extract*
AMPK	Adinosine Monophosphate Protein Kinase
CMC	Chinese *Momordica charantia extract*
DMEM	Dulbecco‘s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ECACC	European Collection of Cell Culture
ER	Endoplasmic Reticulum
EMEM	Eagle‘s Minimal Essential Medium
ETOH	Ethyl alcohol
FA	Fruit Alone
FBS	Fetal Bovine Serum
g	gram
GBM	Glioblastoma Multiform
HMP	Hexose Monophosphate Pathway

HSPC	Hydrogenated Soy Phosphatidylcholine
IMC	Indian *Momordica charantia extract*
LUV	Large Unilamellar Vesicle
mg	milli gram
ml	milli liter
MLV	Multilamellar Vesicle
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
MSI	Magnetic Source Imaging
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
mV	milli Volt
NADH	Nicotinamide Adenine dinucleotide Reduced
NADPH	Nicotinamide Adenine dinucleotide phosphate

OLV	Oligolamellar Vesicle
PBS	Phosphate Buffer Solution
P1	phosphatidyl-inositol
P53	Protein 53
PC	Phosphatidylcholine
PE	Phosphatidyl ethanolamine
PNA	p-nitroanilide.
PS	Phosphatidylserine
PTX	Paclitaxel
RLU	Reflective Light Unit
SA	Seed Alone
SD	Standard deviation
S.E	Standard error

Span	(90% undersize-10% undersize)/ 50% undersize
SUV	Small Unilamellar Vesicle
T_C	Phase Transition Temperature
TEM	Transmission Electron Microscopy
WF	Whole Fruit
WHO	World Health Organisation
α	Alpha
β	Beta

Chapter 1.
Introduction

1.1 Cancer

Cancer is the Latin word of crab and is an abnormal growth of cells which tend to proliferate in an uncontrolled manner and, in some cases, metastasize (i.e. spread to other organs) (Bertram, 2000). Cancer may also be referred to as malignancy or a malignant tumour or described as a neoplasm (i.e, a new growth). It is not only one disease, but also a group of more than 100 different and distinctive diseases. Cancers can involve any tissue of the body and have many different forms in each body area (Bertram, 2000). Most cancers are named after for the type of cell or organ from which they start. For example, a cancer in the breast is called breast cancer. If a cancer spreads (metastasizes), the new tumour bears the same name as the original (primary) tumour. Normal cells stop dividing at a certain point of time and cancerous cells continue to divide, as illustrated in Figure 1.1. These cancerous cells keep on growing until their food supplement and energy are depleted (Bertram, 2000).

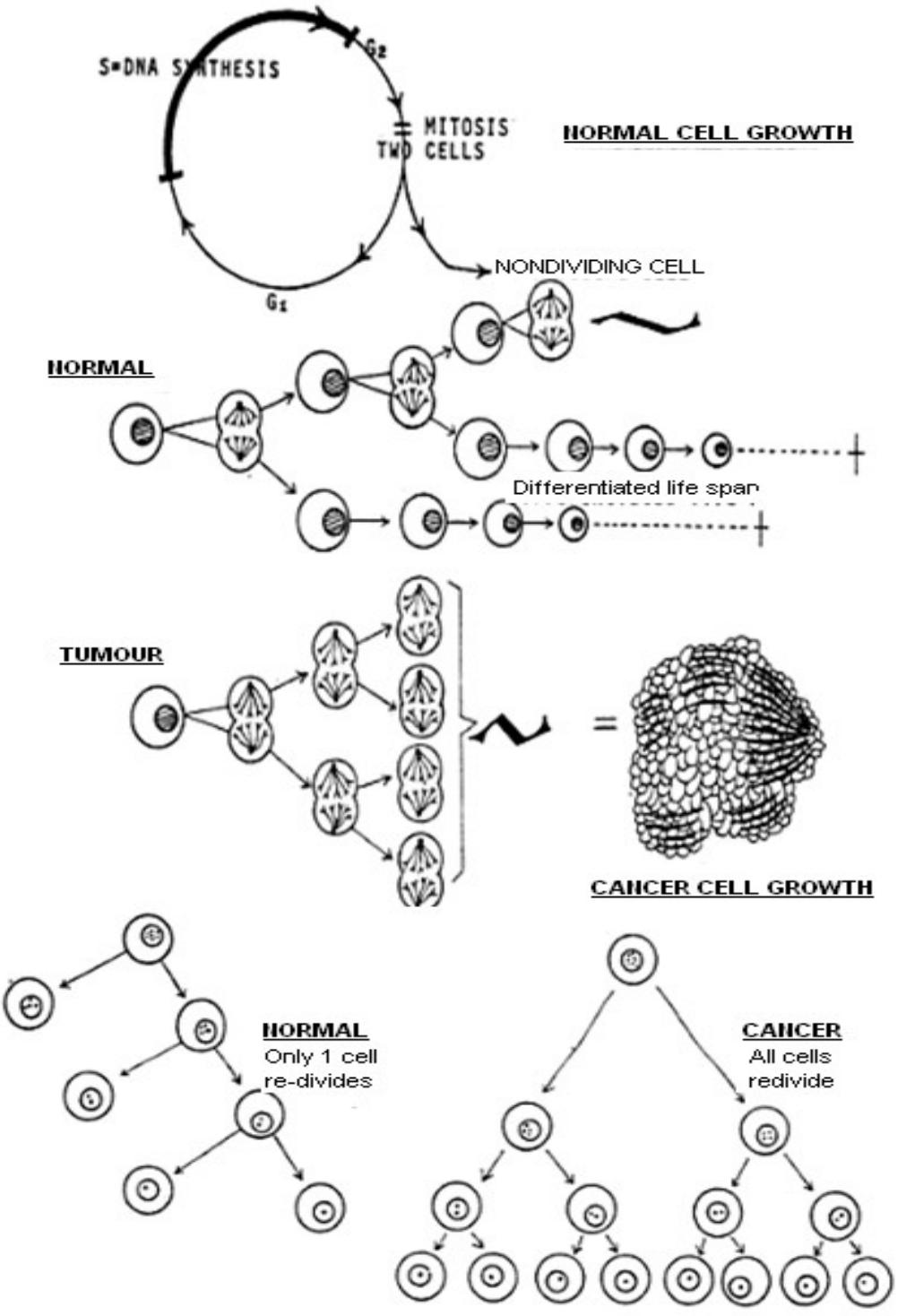


Figure 1.1: Diagrams showing above iilustrate how a normal cell can become tumours during the process of mitotic division (Modified from- Henry, 1964).

There are three phases are involved in the growth of cancer cells:

Phase 1 – Initiation

This is when either a free radical or a carcinogen changes the genetic makeup of a cell, the cell divides more often than it normally does (Troll and Wiesner, 1993; Pitot et al. 2004).

Phase 2 – Promotion

In this phase, the damaged cell multiplies uncontrollably and this phase precedes the final progression phase (Yamagiwa and Ichikawa, 1918; Troll and Wiesner, 1993).

Phase 3 – Progression

In this progression phase, cancer grows and invades the surrounding tissues, forming new blood vessels. It does so by releasing compounds that go against the natural defence of the body and penetrate into the surrounding tissues. Cancer cells also build a blood supply network via angiogenesis and invade the surrounding tissues. The growth of solid tumour cannot continue without sufficient blood supply, and the diameter of the tumour may range between 1-2 cm. Certain natural compounds have been shown to retard the spread of cancer cells in any combination of the above three phases (Pitot et al. 2004).

Table 1.1 summarises the difference between normal cells and cancer cells. These differences are extremely important because they comprise the basis for designing anticancer compounds and formulations.

Table 1.1: Comparison between cancer cells and normal cells (Adapted from: Yamazaki et al. 1995).

	Cancer cells	**Normal Cells**
1.	Cancer cells have prolonged life span by having uncontrolled manners of multiplication.	Normal cells multiply and divides normally.
2.	Cancer cells fail to perform the functions of the relevant organ.	The functions of the organ are carried out successfully.
3.	Cancer cells favour sugar as the main source of energy.	Normal cells multiply in presence of sufficient oxygen supply.
4.	Cancer cells have more insulin receptors compared to normal cells.	Normal cells have less insulin receptors when compared to cancer cells.
5.	Cancer cells cannot tolerate high temperatures (i.e. they are more heat-sensitive).	Normal cells have some tolerance with respect to high temperatures.
6.	Cancer cells form tumour or swelling due to abnormal growth and spread to the other organs through the lymphatic system.	Normal cells grow normally with the support of the lymphatic system.

1.2 Glioma

A glioma is a tumour that grows from glial cells, which are the supportive cells in the brain (McPherson, 2013) Astrocytes and oligodendrocytes are the two main types of supportive cells in the brain (Figure 1.2). Glioma is the most common type of brain tumour and it is typically categorized as primary or secondary. Primary tumours start in the brain, whereas secondary tumours spread to the brain from another site such as the breast or the lung. A glioma is graded from I to IV based on their nature and growth rate. Grade I glioma is often considered a benign tumour, while grades II, III and IV are tumours with an increasing likelihood to grow and spread quickly and hence they are considered malignant (Lassman, 2004).

1.2.1 Grading of gliomas

The World Health Organization (WHO) defines gliomas according to cell type, location and grade, and categorizes them into four classes (Lassman, 2004). These are: Grade I tumours (pilocytic astrocytomas), Grade II tumours (low-grade astrocytomas), Grade III tumours (anaplastic astrocytomas) and Grade IV tumours, which are also known as glioblastoma multiforme (GBM). Grade I tumours do not invade the surrounding brain tissue and are often curable with surgery, while tumours of grades II, III and IV are diffusive and can invade normal brain tissues. Grade III and IV tumours are the most aggressive and are referred to as ―high-grade‖ or ―malignant‖ brain tumours (Lassman, 2004). The incidence of glioma has increased since the late 1970s. Despite remarkable advances in surgical techniques and treatment options, including chemotherapy and radiotherapy, the prognosis of glioma is still very poor (Surawicz et al. 1998).

1.2.2 Classification of glioma

Gliomas are enormously heterogeneous varying from benign to malignant. Kernohan and Sayre introduced a new set and further classified glioma into astrocytomas, oligodendromas and glio-blastomas (Collins, 2004).

I. Astrocytoma

Astrocytomas (Figure 1.2) originate from astrocytes or supporting cells of the brain. Astrocytomas are divided into two groups; differentiated neoplasms which are lethal and undifferentiated neoplasms which are benign. Well differentiated neoplasms occur more in adults than in children. Astrocytic gliomas are graded pathologically depending on the most malignant stage recognized (Doolittle, 2004). Astrocytic gliomas are divided based on the malignancy into astrocytoma, anaplastic astrocytoma, and gliobastoma multiforme. The most common glial tumours are anaplastic astrocytoma and glioblastoma (Doolittle, 2004). WHO considers astrocytic glioma with nuclear atypia as grade II. Astrocytic tumours that exhibit mitotic activity are considered as grade III and tumours that demonstrate atypia, mitosis, endothelial cell proliferation and necrosis are categorized under grade IV (Hiros, et al. 1995).

II. Oligodendroglioma

The other common type of glial cells that give rise to tumour is oligodendroglioma (Figure 1.2), which is the primary myelinating cell of the central nervous system. Low grade oligodendroglioma are seen in children. WHO graded oligodendrogliomas as grade II or grade III tumours (American Brain Tumour Association, 2001). Grade II tumours are well-differentiated, which are irregular against normal cells. Grade III tumours are also known as anaplastic tumours as they are clear abnormal cells in which pleomorphism, hyperchromatism, hypercellularity,

and mitosis are increased (McDonald et al. 2005). Grade III tumours consist of numerous blood vessels and rapidly proliferative cells (Miller et al. 2006). Few anaplastic oligoastrocytomas consist of glioblastomas that are grade IV destructive cells (Reifenberger et al. 1995).

III. Glioblastoma

Glioblastoma multiforme (Figure 1.2) is the most common malignant brain tumour in adults and is uncommon in children. Glioblastoma multiforme is also the most malignant and common of all primary brain tumours. Glioblastomas are rapidly growing and destructive tumours. Often glioblastoma involves a large portion of the cerebral hemisphere. It invades the meninges and by spreading directly across the midline it causes a marked distortion of the ventricular system. Glioblastoma simulates commonly differentiated astrocytoma, piloid astrocytoma, gemistocystic astrocytoma, polar spongioblastoma, oligodendroglioma and medulloblastoma. Several tumours are diagnosed as astrocytoma subsequently reveal the appearances of glioblastoma. Metastases of glioblastoma are bones, liver, and lungs (Kleihues et al. 2002).

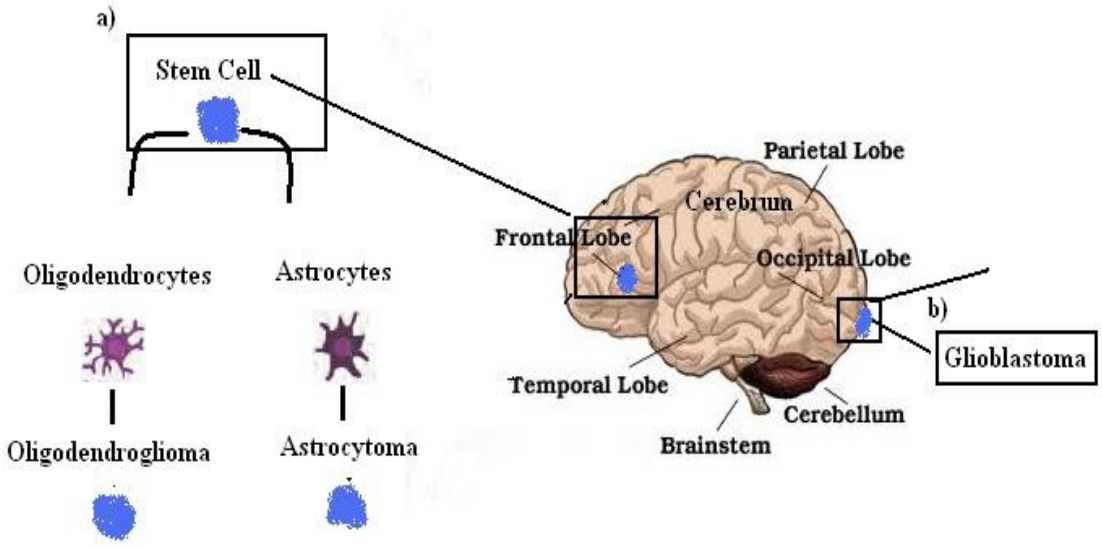


Figure 1.2: Diagram showing the origination of glioma from the brain. (a) Supportive cells- Oligodendroglioma and Astrocytoma in the cerebrum region and (b) Glioblastoma nearby to the cerebellum region.

1.2.3 Statistics of Brain tumour

The overall incidence rate of brain tumour is around 19 new cases per 100,000 people every year. The incidence rates are higher in females, and whites, and increases with age (CBTRUS, 2011). According to USA statistical report on brain tumour, in 2004 more than 612,000 persons were diagnosed with primary brain and central nervous system tumours. Also, more than 124,000 persons were suffering from malignant tumour and more than 488,000 persons were suffering from non-malignant brain tumours (Porter et al. 2010).

1.2.4 Statistics for tumour-specific glioma (CBTRUS, 2011).

1. Glioma is a term used for all tumours arising from gluey or supportive tissue of the brain, representing 31% of all brain tumours and 80% of all malignant brain tumours.

2. Glioblasatoma represents 17% of all primary tumours, and 54 % of all gliomas.
3. Astrocytoma represents 7% of all primary brain tumours.
4. Astrocytoma and glioblastoma combined represents 61% of all glioma.
5. Oligodendroglioma represents 2% of all primary brain tumours.

The majority of the primary tumours are located within the frontal, temporal, parietal and occipital lobes of the brain.

1.2.5 Treatment of glioma

The treatment of malignant gliomas is performed via surgery, chemotherapy and radiotherapy (Lonardi et al. 2005). Depending on the site of lesion and condition of the patient, surgery can include gross total excision of the tumour using image guidance or may be restricted to biopsy. The treatment remains challenging and recent advances have improved both the quality of life and survival (Liu et al. 2008). The main goal of surgery is to resect the tumour completely. However, the infiltrating nature of gliomas and their imprecise margins make this goal very difficult (Wen and kesari, 2008). Surgery usually has positive outcome in patients with low-grade and malignant gliomas (Smith et al. 2008).

A study form Eastern co-operative oncology group and radiation therapy group showed a positive correlation between survival and extent of resection. A retrospective review on three consecutive trials revealed a longer median survival for maximal resection of 11.3 months as compared to biopsy alone that led to survival for 6.6 months (Chang et al. 1983). Many recent technical advances in neuro-imaging like MRI (Magnetic Resonance Imaging), MR (Magnetic Resonance) spectroscopy and surgical technology like image guided surgery, intra-operative ultrasound and CT surgical techniques have

helped to increase the survival time of the glioma patients (Keles and Berger, 2004; Rampling et al. 2004).

1.2.5.1 Radiotherapy

Radiation therapy, also called radiotherapy, is a non-invasive technique. Also, it offers avoidance of adverse effects that are seen with chemotherapy. Radiotherapy is performed by applying radiation on glioma. The whole brain is treated with conventional external beam following surgery in fractionated doses (Lapperrier et al. 1998).

1.2.5.2 Chemotherapy to treat cancers

Approximately 45% of brain tumour patients benefit when treated with nitrosourea drugs (Kornblith et al. 1978). Despite treatment with surgery and radiotherapy, the inevitable recurrence makes high-grade gliomas the most devastating neoplasm, with eventual death. A number of randomized clinical trials have been conducted to assess the role of chemotherapy in improvement of survival for glioma patients (Lonardi et al. 2005). Various chemotherapeutic agents have been administrated before, during or after radiotherapy (Stewart et al. 2001). A randomized study undertaken by the National Cancer Institute of Canada has established the concurrent temozolomide and radiation therapy (Stupp et al. 2005). The two major classes of chemotherapeutic drugs that are being used currently in the treatment of gliomas include alkylating agents and microtubule modulators.

1.2.5.3 Alkylating agents

Alkylating agents were the first compounds identified for treatment of cancer (Espinosa et al. 2003). These compounds react directly with electron rich atoms in biological molecules to form covalent bonds. The chemotherapeutic and cytotoxic effects are directly related to the alkylation of DNA (Goeffrey et al. 2003). Temozolomide (TMZ) is an orally administrated alkylating agent and has excellent penetration across the blood brain barrier. TMZ is the current standard treatment for anaplastic astrocytomas and has also been tested in combination with other drugs in low-grade gliomas (Berger et al. 2007). Nitrosoureas are other commonly used drugs in the treatment of gliomas as they are highly lipid soluble, resulting in enhanced permeability across the blood brain barrier (Walker et al. 1978). The main nitrosureas employed in treatment of glioma are nimustine, carmustine and lomustine (Espinosa et al. 2003). However, changes in the uptake of the drug represent one form of resistance that may develop to anti-neoplastic therapy. Other forms of resistance include increases in glutathione production, which may serve as the site of drug action, reducing its effect on DNA, increasing efficiency in DNA repair, increasing metabolic degradation of the drug and failure to express the p53 gene (Jean-Pierre and Michael, 2010).

1.3 Study of Tissue culture

Tissue culture is the growth of separated tissues or cells *in vitro* in presence of nutrient medium typically facilitated via the use of liquid growth media (ref). The flask or plate containing the culture is incubated at 37°C in a sterile condition, in order to prevent contamination of the cultured cells (Carrel, 1912).

In general, there are two types of tissue culture sterile techniques (Freshney, 2000):

a. **Primary cell culture –** The growth of tissue is derived from the living organisms (e.g. biopsy material). The culture consists of mixed population of cell types. Frequently, some of the cells may survive without proliferating and will therefore be lost in the increasing population of those which are able to multiply in the conditions provided *in vitro* (Maureen, 1997).
b. **Established Cell lines-** These cells are derived from a primary culture and they can be sub-cultured in the medium indefinitely (Michael, 1998).

1.3.1 Importance of growth media in tissue culture

A liquid medium is required to support the growth of the cells in tissue culture. A minute sample of tissue is either spread on or added into the culture in biological medium (e.g. blood serum or tissue extract). Biological medium might have the appropriate nutrients, temperature and pH for the cells to be incubated. The media used for glioma cell culture are Dulbecco‘s Modified Eagle Medium (DMEM-500 ml) and Eagle‘s Minimal Essential Medium (EMEM-500 ml) (Obara, 1998). However, DMEM in comparison to EMEM contains four times higher vitamins, glucose and amino acids which are present in the original formula. Additionally, it contains iron and phenol red. DMEM is suitable for most types of cells, including human, monkey, hamster, rat, mouse, fish and chicken cell lines (Ham et al. 1979).

1.3.2 WHO grading of glioma based on genetics and histopathology characteristics

Stepwise progression from low to intermediate to high-grade glioma is denoted by number of DNA alterations.

1. **Low-grade of astrocytoma**- Mutation of the P53 and loss of heterozygosity on the large arm of chromosome 22.

Grade I: Juvenile pilocytic astrocytoma

-Low mitotic activity; little nuclear atypia.

Grade II: Astrocytoma Variant

- Fibrillary, protoplasmic and gemistocytic
- Low mitotic activity; nuclear atypia; may be infiltrative; may recur (Figure 1.3).

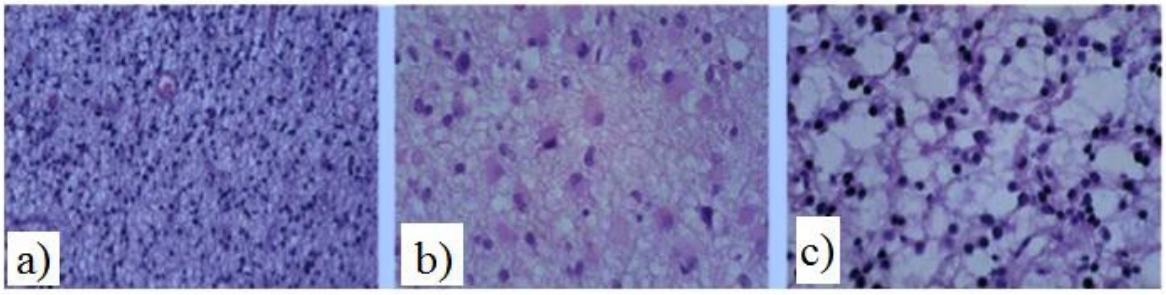


Figure 1.3: Photograph showing grade II astrocytoma: a) fibrillary, b) gemitocytic and c) protoplasmic. (Source: Brat et al. 2003).

2. **Intermediate grade of astrocytoma** – Appears to be associated with loss or inactivation of p16 tumour suppressor gene on chromosome 9 and loss of heterozygosity on the long arm of chromosome 9.

Grade III: Anaplastic oligodendroglioma

-High mitotic activity; infiltrative; cellular and nuclear atypia; often recur (Figure 1.4).

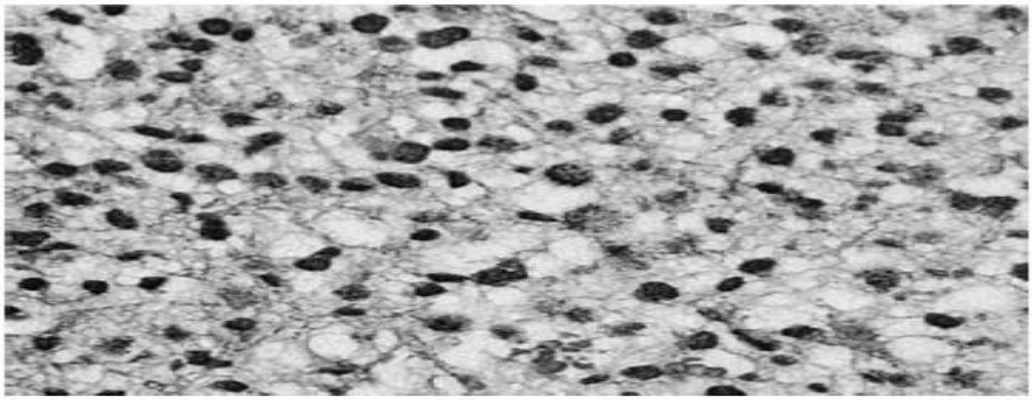


Figure 1.4: A photograph showing grade III Oligodendroglioma: Cells of an anaplastic oligodendroglioma demonstrate the classic 'fried egg' appearance: round nuclei with clear, peri- nuclear halos (Adapted from – Brat et al. 2003)

3. **High grade of astrocytoma**- Partial or complete loss of chromosome 10. Most of the high grade astrocytoma also show amplification of the c-erbB1 oncogene which encodes the epidermal growth factor

Grade IV: Glioblastoma astrocytoma

- gaint cell glioblastoma
- gliosarcoma
- High mitotic activity; high degree of infiltration; cellular and nuclear atypia; necrosis; neovascularization (Figure 1.5).

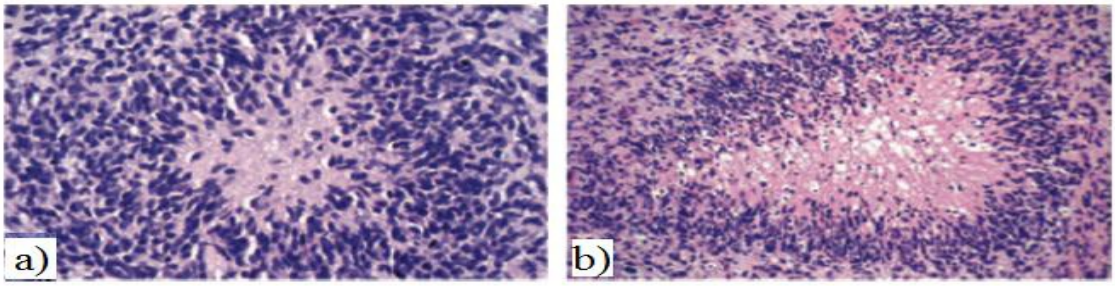


Figure 1.5: A photograph showing grade IV Glioblastoma multiforme: Pseudopalisades in glioblastoma are small (a, <100 mm) and medium (b, 200–400 mm). Note the central necrosis in the larger pseudopalisade (b) (Adapted from Brat et al. 2003).

1.4 Cell viability MTS assay to identify proliferation or inhibition of glioma cells

MTS assay is the most popular colorimetric assay in which the compound 3-(4, 5-dimethyl thiazol-2yl)-5, (3- carboxymethoxyphenyl)-2 (4 sulfophenyl)-2H (tetrazolium) is converted to a coloured insoluble formazan product by mitochondrial enzymes (Berridge and Tan, 1993). The end product is solubilised in either alcohol or detergent and the absorption is measured at 490 nm (Nikkhah et al. 1992; Lewandowicz et al. 2000). The amount of colour produced is directly proportional to the number of living cells. MTS assay has been used in several investigations for the measurement of chemo-sensitivity testing in malignant gliomas (Nikkhah et al. 1992). The enzymatic reactions (Figure 1.6**)** are based on either the oxidase or dehydrogenase reactions, which are determined either calorimetrically or fluorimetrically using different reagents including oxidative chromogenic dyes or reductive chromogenic dyes. Tetrazolium salts are one of the reductive chromogenic dyes employed in the measurement and it is a very sensitive detection system.

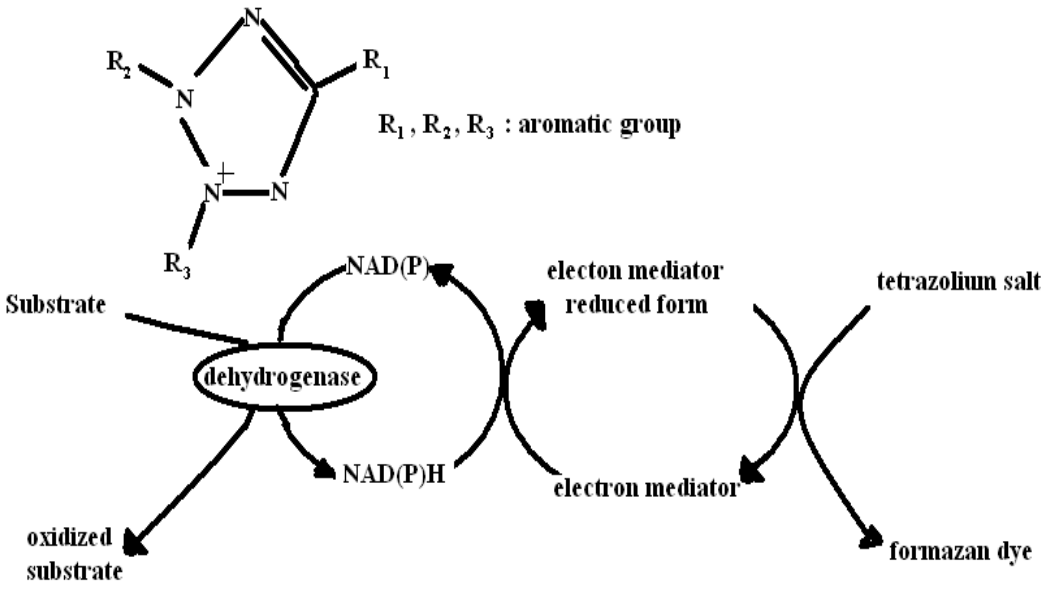


Figure 1.6: Diagram showing the structure of tetrazolium salt and electron transfer mechanism for MTS assay. (Adapted from- www.promega.com/automethods/).

1.5 Capase and cytochrome c assay used to identify pathways of cell death.

Cell death is typically discussed dichotomously as either apoptosis or necrosis (Majno and Joris, 1995). Apoptosis is described as an active programmed cell death regulated in a controlled manner. Apoptosis plays an active role in their own cell death, which is known as suicidal cells. This mechanism of cell death makes apoptosis distinct from other necrosis, which is another mechanism of cell death. Necrosis has been characterised as passive, accidental cell death resulting from environmental perturbation with uncontrolled release of inflammatory cellular contents (Kerr et al. 1972). Apoptosis is on energy dependent process, while necrosis is energy independent process (Rastogi et al. 2009).

Figure 1.7 represents the morphological features of cellular death via apoptotic and necrotic pathways. Figure 1.7A shows necrosis and in this process, the cells swell

and become leaky and finally disrupt and release their contents into the surrounding tissue, resulting in inflammation.

Apoptosis is the process where the cells undergo cellular shrinking, chromatin condensation and margination at the nuclear periphery (Van Cruchten, 2002). Cells eventually form membrane-bound apoptotic bodies that contain organelles, cytosol and nuclear fragments. These apoptotic bodies are phagocytosed by the macrophages without triggering inflammatory processes (Van Cruchten, 2002). Figure 1.7 B shows apoptotic cells, which have distinctive morphology during the apoptotic process, where the cell begins to shrink following the cleavage of lamin and actin filaments in the cytoskeleton (Figure 1.7 A). The breakdown of chromatin in the nucleus often leads to nuclear condensation and in many cases the nuclei of apoptotic cells take on a ―horse-shoe‖ like appearance (Figure 1.7 B). Cells continue to shrink (Figure 1.7 C), packaging themselves into a form that allows for their removal by macrophages. These phagocytic cells are responsible for clearing the apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death (Vermes et al.1995; Darzynkiewicz et al.1992).

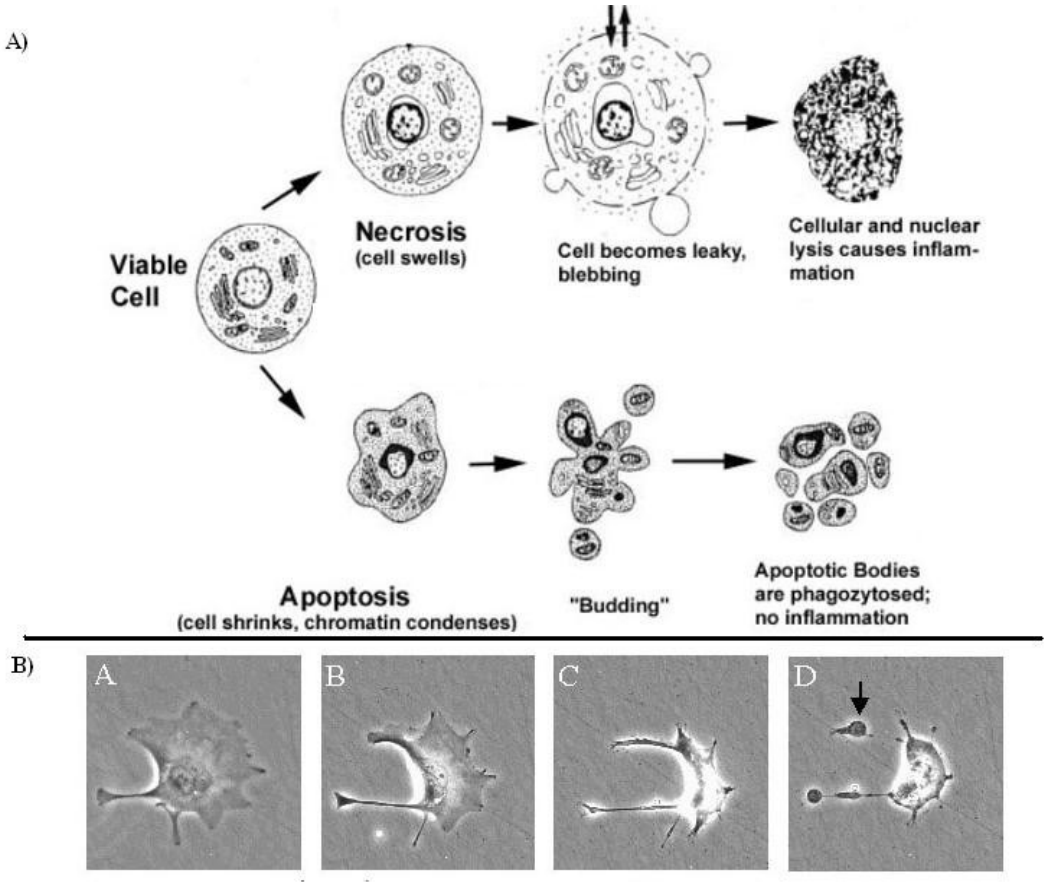


Figure 1.7: Diagrams showing the morphological representation of cell death of viable cells via A) Necrosis or Apoptosis B) Apoptosis is characterized by a variety of cellular changes including loss of membrane phospholipid symmetry, mitochondrial swelling and DNA cleavage. (Adapted form-http://www.celldeath.de/encyclo/aporev/revfigs/revfig_2.htm).

1.5.1 Mechanism of apoptosis

The apoptotic processes are highly complex and sophisticated. Figure 1.8 shows that specific signals are given to the cells to undergo apoptosis and a number of distinctive changes occur in the cells. The pathways involved in apoptosis mechanism are :

Energy Dependent pathway: i) Intrinsic Pathway ii) Extrinsic Intrinsic Pathway and iii) perforin/granzyme pathway all of which are activated by granzyme A via single

stranded DNA damage (Martinvalet et al. 2005). The Energy Independent pathway is perforin/granzyme pathway, which is activated by granzyme B.

Either extrinsic or death receptor pathway involves death ligands interaction with death receptors and inducing apoptosis. An Intrinsic or mitochondrial pathway involves activation of mitochondria macrophages to activate apoptosis and perforin/granzyme pathway. In turn, the perforin/granzyme pathway activates, cytotoxic T-cells to bind to perforin receptor which gives rise to either granzyme A (energy dependent) or granzyme B (energy independent) (Figure 5-2). The caspase family initiators (caspase-2, -8, -9, -10), effectors or executioners (caspase-3, -6,-7) and inflammatory caspases (caspase-1,-4,-5) (Cohen, 1997; Rai et al. 2005) which are responsible for regulating the extrinsic, intrinsic and perforin/granzyme pathway mediated by granzyme A. The entire three energy dependent pathways converge to enexecutive pathway for apoptosis via indication of by caspase- 3 enzymes. This enzyme breaks down or cleaves key cellular components that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other enzymes such as DNases, which cleaves the DNA in the nucleus, cross –linkage of protein formation of apoptotic bodies, expression of ligands for phagocytic receptors and finally uptake of phagocytic cells (Vermes et al. 1995; Darzynkiewicz et al. 1992).

Figure 1.8 represents apoptosis pathway via extrinsic, intrinsic and perforin/granzyme pathway. The entire pathway requires specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase (8, 9, 10), which in turn activates the executioner caspase-3. However, granzyme A works in a caspase-independent fashion. The execution pathway results in characteristic cytomorphological features including cell shrinkage,

chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally, phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages.

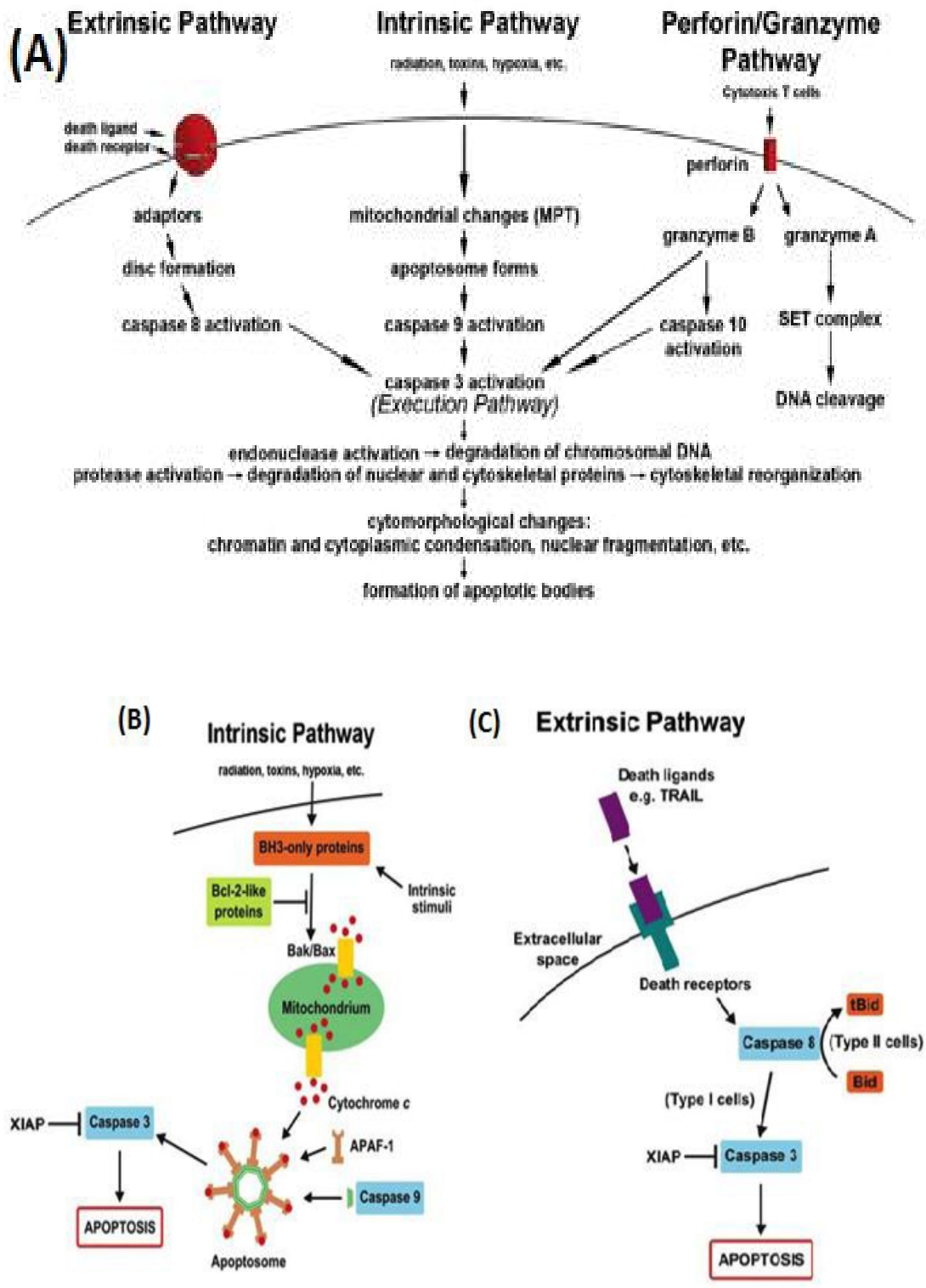


Figure 1.8: Schematic representations (A-C) of apoptotic events due to the regulation of: intrinsic, extrinsic and perforin/granzyme pathway. (Adapted from- Vermes et al. 1995; Darzynkiewicz et al. 1992).

1.5.2 Caspase activity

There are a number of caspases in mammalian cells that have been shown to be involved in the early stages of apoptosis, e.g. Caspase 2, Caspase 3, Caspase 6, caspase 7, caspase 8, caspase 9 and caspase 10. The functions of these enzymes are not yet entirely clear, but it appears that after an initial signal to the cell to undergo apoptosis, they may be responsible for the activation, amplification and execution of the apoptotic cascade such as intrinsic, extrinsic and perforin/granzyme pathway activated by granzyme A (Figure 1.8). Apoptosis probably always involves caspase-3 activated either by caspase-8 or by caspase-9. The upstream mechanisms therefore activate either caspase-8 or -9. Caspase-8 is typically activated by death receptors (Fas, TRAIL-receptors, TNFR). It appears that mitochondrial apoptosis is much more common than caspase-8-dependent apoptosis *in vivo*. Mitochondrial apoptosis is regulated through the Bcl-2-family of proteins: within this family, the BH3-only proteins (‗triggers‘) often regulate the activation of the effectors Bax and Bak; active Bax and Bak cause the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol, where it binds to Apaf-1, inducing its oligomerisation and thereby causing the activation of caspase-9.

1.5.3 Role of Bcl-2 proteins in the release of cytochrome c release

The Bcl-2 proteins are a family of proteins responsible for apoptosis. Proteins Bcl-2 and Bcl-XL are anti-apoptotic, while the proteins Bad, Bax and Bid are pro-apoptotic. Excessive concentrations of pro-apoptotic proteins in the cells cause higher susceptibility to apoptosis, whereas, excessive concentrations of anti-apoptotic proteins may increase the resistance of cells against apoptosis. The pro-apoptotic Bcl-2 proteins are often found in the cytosol acting as sensors of cellular

damage or stress. Following cellular stress, they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. An excess of pro-apoptotic Bcl-2 proteins at the surface of the mitochondria is thought to be important for the formation of the pore on the mitochondrial membrane. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic Bcl-2 proteins leading to the formation of pores in the mitochondria and subsequently the release of cytochrome c and other pro-apoptotic molecules from the intermembrane space. Cytochrome c is a small heme protein found loosely associated with the inner membrane of the mitochondrion. The release of cytochrome c from the mitochondria is a particularly important event in the induction of apoptosis. Cytochrome c is a highly soluble protein, unlike other cytochromes, with a solubility of about 100 g/L and is an essential component of the electron transport chain. Cytochrome-c is capable of undergoing oxidation and reduction (Vidal et al. 2002). Once cytochrome c has been released into the cytosol, it is able to interact with a protein called Apaf-1. These in turn leads to the formation of the apoptosome and the activation of the caspase 9 and the induction of apoptosis by activating caspase 3. There are different mechanisms via which chemotherapeutic drugs can exert their effects on cancer cells to cause cellular death.

1.5.4 Caspase Assays

Caspases consist of a group of aspartic acid-specific cysteine proteases that are activated during apoptosis. These unique proteases, which are synthesized as zymogens, are involved in the initiation and execution of apoptosis once activated by proteolytic cleavage (Nicholson et al. 1995). The activity of caspase- 3 in the cells and the tissue of the body is either a marker or indication for the cell death

mechanism (Porter et al 1999). Caspase- 3 is a crucial component and central event in many cellular apoptosis (Thornyberry et al. 1998; Wolf et al. 1999). Caspase- 9 is also known as ICE, Lap6, Mch6 and is an upstream proenzyme in the cascade of enzymatic reactions required for cellular apoptosis (Duan et al 1996). Caspase- 9 is a key regulator of apoptosis associated with apoptotic and anti-apoptotic proteins (Thornberry et al 1997; Petty et al. 1995). The Caspase-Glo® 9 assay is a homogenous luminescent assay that measures caspase-9 activity. Addition of a single Caspase-Glo®-9 reagent in and _add-mix measure' format resulting in cell lysis. Caspase cleavage of the substrate and the generation of a glow type luminescent signal produced by the luciferase reaction are obtained from cell lysis. The signal generated is proportional to the amount of caspase activity present. Numerous commercial kits and reagents are available to assess apoptosis based on caspase function.

The Caspase-Glo® Assays use the luminogenic caspase-9 tetrapeptide substrate (Z-LEHD-aminoluciferin) and caspase-3/7 substrate (Z-DEVD-aminoluciferin). The buffers are optimized for the specific caspase activity, cell lysis and luciferase activity. In the absence of active caspase, the caspase substrates do not act as substrates for luciferase and thus will produce no light. Upon cleavage of the substrates by the respective caspase, aminoluciferin is liberated and can contribute to the generation of light in a luminescence reaction (Figure 1.9). The resulting luminescent signal is directly proportional to the amount of caspase activity present in the sample (Promega, 2011).

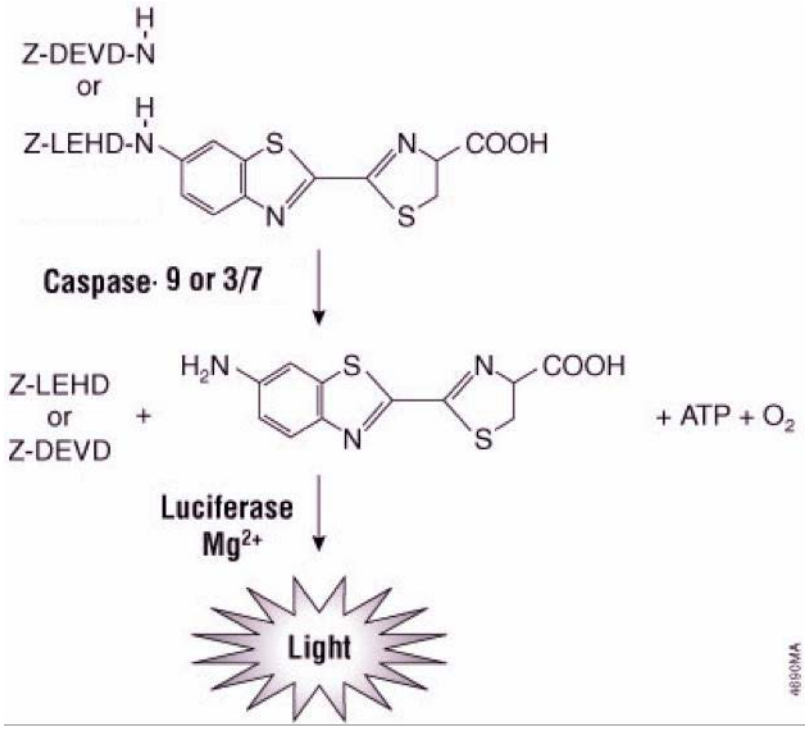


Figure 1.9: Diagram showing caspase -3/7 and caspase -9, cleavage of the proluminogenic substrates containing LEHD and DEVD. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the production of light. (Adapted from- http://www.promega.com/resources/product-guides-and-selectors/protocols-and-applications-guide/apoptosis/)

1.5.5 Mitochondrial Assays

Mitochondrial assays and cytochrome *c* release allow the detection of changes in the early phase of the intrinsic pathway. Cytochrome *c* is an intermediate compound in apoptosis, which is a controlled form of cell death in the process of development or in response to infection or the induction of DNA damage of any apoptotic programme in cell free extracts (Chandra et al. 2002; Johnstone et al. 2002). Cytochrome *c* is a small heme protein found loosely associated with the inner membrane of the mitochondrion. Cytochrome-c is capable of undergoing oxidation and reduction (Vidal et al. 2002). Eukaryotic NADPH-Cytochrome c reductase is a flavoprotein localized at the endoplasmic reticulum. It is responsible for transferring

electrons from NADPH to several oxygenases, the most important of which is the cytochrome P450 family of enzymes which are responsible for metabolism of xenobiotics (Shen et al. 1993; Plonne et al. 1999). NADPH-cytochrome c reductase is widely used as an endoplasmic reticulum marker-3 (Vidal et al. 2002; Hochgraf et al. 1997). The proapoptopic stimulus is responsible for the release of cytochrome-c and in turn it enhances the activity of caspase-9. Cytochrome-c assay kit is designed to measure the NADPH cytochrome-c reductase activity in cell and in purified microsomes of the endoplasmic reticulum. The cytochrome-c measurement is based on colorimetric assay that monitors the reduction of cytochrome-c by NADPH-cytochrome-c reductase in the presence of NADPH, resulting in formation of distinct bands in the absorption spectrum, and the increase in absorbance at 550 nm is measured with time (Vermillion et al. 1974).

1.6 Plant extracts used as traditional medicine for treatment of cancer

About 80% of the world population depend on plants for a lot of their basic health care needs (Sabir and Ahmad, 2007). Fossil records prove that even 60,000 years ago in Palaeolithic age plants were also used as medicines (Solecki et al. 1975), clearly demonstrating the historic role of nature in provision of medicines that can treat various diseases.

The use of natural medicinal agents is recognised by the WHO. Out of 250,000-500,000 plant species on the earth, only 1-10% has been studied chemically and pharmacologically for their potential medicinal value (Verpoorte, 2000). It has been estimated that about 50% of the prescribed medicinal products in Europe and USA are derived from natural materials (Newman et al. 2003). One approach to control cancer could be its prevention by diet, which inhibits one or more neoplastic events and

reduces cancer risk (Nerurkar, 2010). Dietary compounds offer a great potential in the fight against cancer by inhibiting the carcinogenesis process through the regulation of cell homeostasis and cell-death machineries (Nerurkar and Ray, 2010; Talib and Mahasneh, 2010).

There are various approaches to using medicinal plant constituents. One approach is to isolate bioactive compounds for direct use as drugs (e.g. digoxin, digitoxin, morphine, reserpine, paclitaxel, vinblastine, vincristine, etc). Alternatively, semisynthesis of novel anticancer compounds from herbal constituents can be conducted. This aims to enhance the anticancer activity and/or lower the toxicity of the extracted compounds. Examples of semisynthetic anticancer drugs include met-formin, nabilone, oxycodon, taxotere, teniposide, verapamil, and amiodarone. Also, the whole plant or one part of it might be used as herbal remedy (Daniel and Norman, 2001). One might expect any bioactive compounds obtained from plants to have low human toxicity, but there are plants that are toxic to the given endemic culture that has no reported documents (Daniel and Norman, 2001). Plants like *Momordica charantia*, Zingiber offifinale Rosc, Teucrium polium, Phagnalon rupstre, and Inula viscosa are prescribed to alleviate diseases including tumour, ulcers, diabetes, renal abnormalities, inflammations, rheumatism, and muscle spasms (Talib and Mahasneh, 2010; Sahu et al. 2011). Paclitaxel and camptothecin are plant-derived drugs that have improved the treatment of cancers (Newman et al. 2003). Numerous groups with antitumor properties are plant-derived products such as alkaloids, phenylpropanoids, and terpenoids (Kintzios 2006; Park et al. 2008).

1.6.1 *Momordica charantia*

The vegetable *Momordica charantia* L., (family: Cucurbitaceae) is a scientific name of the plant and its fruit. It is also known by other names, for instance in the USA it is known as Bitter gourd or balsam pear while its referred to as the African cucumber in many African countries. It is also called fukwa in China, kerala in India, nigai uri in Japan and ampalaya in Philippines (Behera et al. 2010). *Momordica charantia* is a flowering vine in the family of Cucurbitaceae, similar to the cucumber. The herbaceous, tendril-bearing vine grows to 5 m. It is a tropical plant vegetable and also commonly used in Indian cuisines and food shops. It has been extensively used as folk medicine for treatment of diabetes. The Latin name *Momordica* means, ―to bite‖ which refers to the jagged edges of the leaf that appear as if they have been bitten. In Ayurveda, the fruit is considered as tonic, emetic, stomachic, stimulant, antibilous and laxative. *Momordica charantia* has been used as Asian traditional medicine for long time. Its helps in enhancing the digestion like most of the bitter-tasting food. It also helps in dyspepsia, constipation, heartburn and ulcers. *Momordica charantia* is also a mild inflammation modulator, and dermeculent, and it rarely has negative effects based on the traditional reports and clinical experiences (Sathish et al. 2010).

1.6.2 Horticulture of *Momordica charantia*

Momordica charantia is adaptable to various climates (Lim, 1998). It can grow in both tropical and subtropical regions (Reyes et al. 1994). In India it is cultivated up to an altitude of 1500 meters. In the sub-tropical regions, cultivation of *Momordica charantia* can be carried out during rainy, spring and summer seasons (Lim, 1998). Temperature suitable for the growth of *Momordica charantia* is between 25 and 30°C whereas very cold climate may kill the plant or retard its growth (Larkcom, 1991;

Desai and Musmade, 1998). Furthermore, *Momordica charantia* grows well in a wide range of soil types including peat, light clay and sandy but grows best in a well-drained sandy loam soil that is rich in organic matters. It grows well in soils of shallow to medium depth (50-150 cm), and like most cucurbits, bitter gourd prefers well-drained soils. For bitter gourd, the optimum soil pH is 6.0-6.7, and may tolerate alkaline soils up to pH 8.0 (Behera et al. 2010). In India it is cultivated during the season of April to July by using 2-3 seeds in a pit. The pits are prepared at a distance of 0.5 meter and provided with manure. Only one plant is retained and seedlings are watered once or twice per week. The plant begins to flower 30-35 days after sowing and fruits are ready to harvest after 15-20 days of flowering. This plant is widely cultivated in Asia, Africa and South America (Sathish et al. 2010).

1.6.3 Origin and geographical distribution of *Momordica charantia*

Momordica charantia is cultivated in tropical Asia on hundreds to thousands of hectares. This plant was domesticated in India and southern part of China. It is found naturalized in almost all tropical and subtropical regions. It is an important market vegetable in southern and eastern Asia. Local cultivars originally from Asia are cultivated on a small scale in tropical America, and the southern part of United States (Marr et al. 2004).

1.6.4 Description of *Momordica charantia* fruit and seeds.

Seeds are flat and oval. Seeds and pith appears white in unripe fruits and ripens to red in mature ripen fruits (Sathish et al. 2010; www.rain.org, 1990).

Fruits (Figure 1.10) are 5.0-35.0 cm long, ovoid, ellipsoid, or spindle shaped, usually ridged or warty, dehiscent irregularly as a 3 valved fleshy capsule or indehiscent. The fruit has a distinct warty looking exterior and an oblong shape. It is hollow in cross-section, with a relatively thin layer of flesh surrounding a central seed cavity filled with large flat seeds and pith. The surface of the flesh is crunchy and watery in texture, similar to cucumber. The green skin is tender and edible. However, the fully ripened fruit turns orange and mushy and becomes poisonous, very bitter and non-edible (Sathish et al. 2010). Figure 1.10 shows *Momordica charantia* of various shapes and size from different location- (A) Fruit diversity (Dey et al. 2006). (B) Large fusiform fruits, pointed at both ends, numerous triangular tubercles, giving the appearance of a crocodile's back classified as *Momordica charantia* var. *Charantia* (Chakravarty 1990). (C) Chinese long fruit type, 30-60 cm, smooth ridges, light green in colour, and slightly bitter (Yang and Walters 1992). (D) Small fruits *(Momordica charantia muricata;* var. Chakravarty 1990) high in proteins, carbohydrates, iron, calcium, (Desai and Musmade, 1998) and Vitamin C (Behera et al. 2008). (E) Triangular fruit type, cone-shaped, 9-12 cm long, light to dark green with prominent tubercles, moderately to strongly bitter (Yang and Walters 1992).

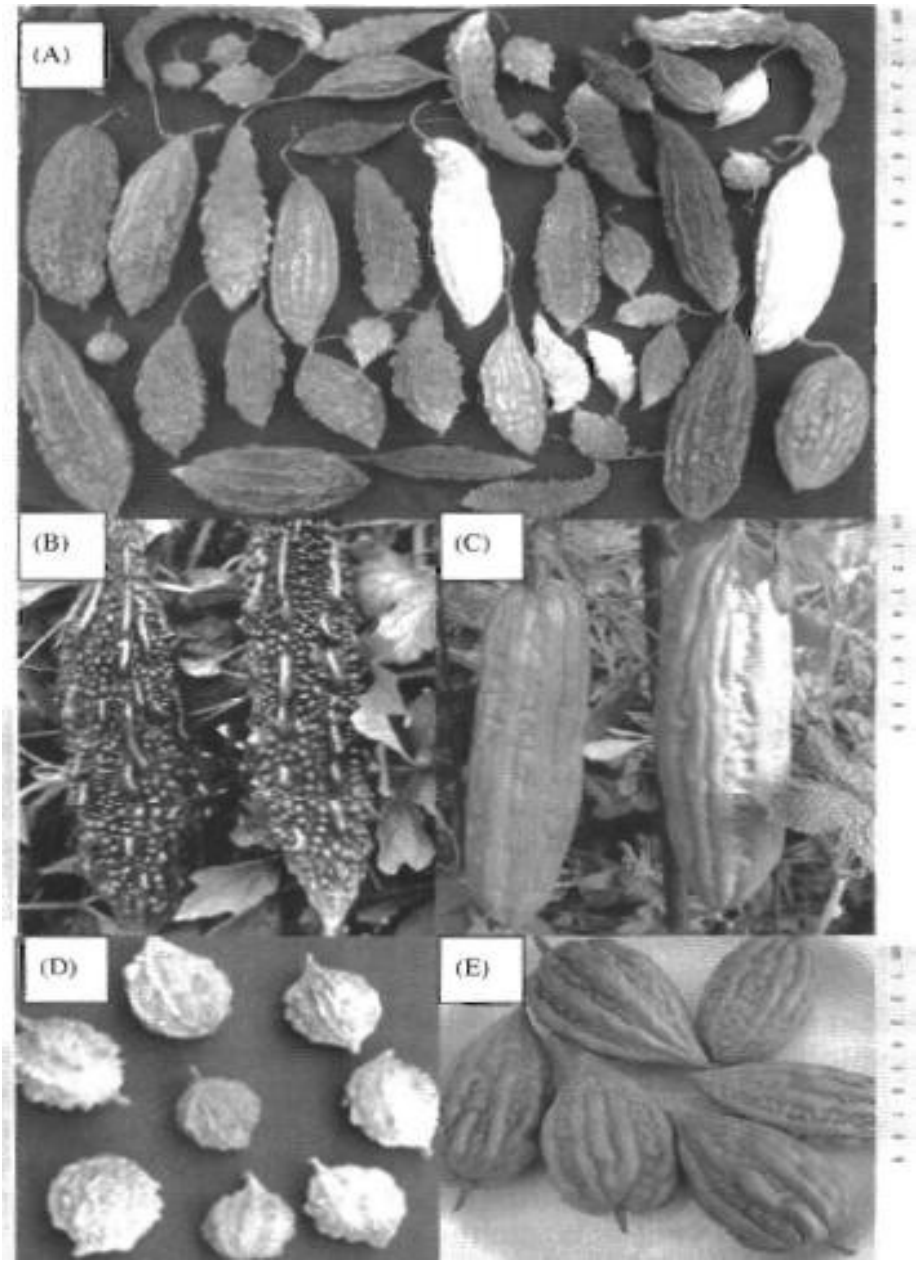


Figure 1.10: *Momordica charantia* comes in a variety of shapes and sizes: A) Fruit diversity (B) Large fusiform fruits (C) Chinese long fruit type (D) Small fruits (E) Triangular fruits. (Adapted from- http://ddr.nal.usda.gov/bitstream/10113/42264/1/IND44357776.pdf).

The three different species of *Momordica charantia* are explained, depending on the phonetype, climatic condition and soil type as stated below:

1.6.4.1 Chinese breed (Yunnan, China)

Phenotype- Chinese phenotype is 20–30 cm long, oblong with bluntly tapering round ends. It has uneven pebble like longitudinally arranged smooth ridges. It is light green in colour. *Momordica charantia* of Chinese phenotype is slight bitter (Yang and Walters, 1992) .

Climatic conditions- Yunnan has a humid monsoon climate typical of subtropical and tropical plateau zone, with distinctive dry and rainy seasons and drastic changes in climatic conditions. However the average temperature of the Yunnan is between 4 and 24 °C (Adapted from- http://www.chinatoday.com/city/yunnan.htm).

Soil type- Yunnanis covered by red soil. Red soils lack nitrogenous material, phosphoric acid and organic matter and are rich in iron. It is formed by the breakdown of the igneous rocks and metamorphic rocks. The pH of the red soil is strongly to weakly acidic (Zhenli et al. 2004),

1.6.4.2 Indian breed (Maharashtra, India)

Phenotype- Indian phenotype is 6–10 cm in length with a narrower shape and pointed ends. The surface of the plant is covered with jagged, triangular "teeth" and ridges and the colour is whitish green. *Momordica charantia* of Indian breed is strongly bitter in taste (Chakravarty, 1990).

Climatic conditions- The climate of Maharashtra is semi-dry. The temperature ranges from 10°C to 47°C. Rainfall starts in the first week of June. July is the wettest month in Maharashtra and August is also rainy. Monsoon starts its retreat with the beginning of September (Subramaniam and Roa, 1987).

Soil condition- Extensive deposits of black soil are found in the Deccan Plateau of Maharashtra. The pH of the soil is 6 - 8.5. The soil is black, thick and relatively

impermeable. The fruits of *Momordica Charantia* swell and become sticky when they are wet and they shrink when they are dried during the dry season when the soil develop wide cracks. The soil becomes rich in iron, magnesia and alumina and also contains potash and lacks phosphorus and nitrogen. Colour of the soil ranges from deep black to grey (Subramaniam and Roa, 1987).

1.6.4.3 African breeds (Kenya, Africa)

Phenotype- Fruit is ovoid or oblong-cylindric, 10 cm to 20 cm long, coarsely ridged and tapered at the end and covered with bumpy and blunt skin. It is dark-green in colour. *Momordica charantia* From Africa is moderately bitter (Chakravarty, 1990).

Climatic condition- Nairobi the capital city of Kenya has tropical climate that is hot and humid. There is plenty of sunshine throughout the year, which is appropriate for growing *Momordica charantia*. The temperature ranges from 13°C to 32°C and the long rain season occurs from April to June and a short rain season occurs from October to December. The hottest period of the year is during February and March and coldest is during July and August (UNON, 2012).

Soil condition of Kenya- Nairobi, Kenya has dry land condition. The soil is saline and sodic (Rich in sodium). It is also rich in calcium and magnesium. The pH of the soil ranges from 5.1-7.6. Nitrates may be present in appreciable quantities only rarely (Awiti et al. 2007).

1.6.5 Traditional uses of *Momordica charantia*

In the United States, bitter melon is grown for its immature fruits, which are used in Asian cooking. In other countries, the young leaves are harvested and used as a potherb (Susan, 1998). The fruit and leaves have a bitter taste because they contain the alkaloid momordicine. Alkaloid content can be reduced by parboiling or soaking the fruit and leaves in saltwater. The fruit is considered as tonic, stomachic, stimulant, emetic and laxative. The fruit may be useful in gout, rheumatoid and sub acute cases of spleen and liver diseases (Trivedi et al. 2011). *Momordica charantia* fruit is also, useful for detoxification process (Trivedi et al. 2011). Fruit also has hypoglycaemic (anti diabetic) properties as demonstrated in animal and human studies (Agarwal and Beohar, 2010).

The fruit juice and the tea made from the leaf are being used for diabetes, malaria, colic, wounds and sores. It is also being used for measles, hepatitis and fevers. Fruit pulp, leaf juice and seeds are antithelimintic, while leaves act as galactogogue and root may be used as astringent.

1.7 Phytochemistry of *Momordica charantia*

Momordica charantia fruits consist of a number of coumpounds including glycosides, saponins, alkaloids, reducing sugars, resins, phenolic constituents, fixed oil and free acids (Miniraj et al. 1993; Desai and Musmade, 1998). The leaves of this plant are nutritious sources of calcium, magnesium, potassium, phosphorus and iron. The edible fruit are great sources of the B vitamins (Sathish et al. 2010). Tables 1.2 and Table 1.3 show the main phytochemical constituents of the plant. The leaf and fruit are the good source of carbohydrate and protein, which serves as medicinal properties against some diseases, and also a good source of energy for body

metabolic activities. Other coumpounds such as α and β momorcharins and MAP30 (Momordica Anti-HIV Protein) isolated from seeds of *Momordica charantia* have been reported to exhibit antitumor and ribosome inactivating properties (Bakare et al. 2010). Charantin is the cucurbitane, which generally tastes bitter and has been found to be toxic to cancer cells. Charantin consists of stigmasterol functional group attached to glucose as shown in the Figure 1.5. Charantin is a mixture of two glucosides such as sitosteryl glucoside (Figure 1.5 G) and stigmasteryl glucoside (Figure 1.5 H). Charantin has potential activity against diabetes as well as toxic effects against tumour cells (Paul, 2010).

Table 1.2 summarises the compositions of nutrients present in *Momordica charantia* whole fruit.

Table 1.2: Nutrient composition per 100 gram of *Momordica charantia* whole fruit (Source: Gopalan et al. 1993)

Nutrients	Proximate Quantity (mg /100g)
Water	83.20
Carbohydrates	10.60
Proteins	2.10
Fibre	1.70
Calcium	0.023
Phosphorus	0.038
Potassium	0.171
Sodium	0.024
Iron	0.020
Copper	0.001
Manganese	0.080
Zinc	0.0004
β Carotene	0.126
Vitamin C	0.096

Table 1.3 summarises the active phytochemical present in the *Momordica charantia* whole fruit useful for the treatment of cancer and other diseases.

Table 1.3: Major phytochemicals in bitter gourd *(Momordica charantia)* fruit, and their health benefits.

Phytochemicals	Plant parts	Applications	References
β - momorcharin	Seeds	Glycoproteins that acts as midterm abortifacient	Leung et al. (1987)
Vicine (Figure 1.11 B)	Seeds	Hypoglycemic glycoalkaloid	Dutta et al. (1981)
Phenols	Seeds	Antioxidants that reduce blood pressure and lower incidence of cancer and cardiovascular diseases	Horax et al. (2005)
Momordicosides A and B (Figure 1.11 D)	Seeds	Triterpene glycosides that Inhibit tumour growth	Okabe et al. (1980)
MAP 30	Seeds and fruits	Basic protein that inhibits, human tumour and immunodeficiency virus (fly).	Lee et al. (1990); (1995)

Phytochemical	Plant part	Application	Reference
Polypeptide-p	Seeds and fruits	Hypoglycemic peptide (called plant insulin)	Khanna and Jain (1981)
Carotenoids	Seeds and fruits	Antiojddants that lower the incidence of cancer and cardiovascular diseases	Rodriguez at al. (1975); (1976)
Charantin (Figure 1.11 C)	Fruits	Non nitrogenous substance having hypoglycemic principle	Dutta et al. (1981)

Figure 1.11 shows the structural formulae of the active phytochemical and components present in the *Momordica charantia* whole fruit.

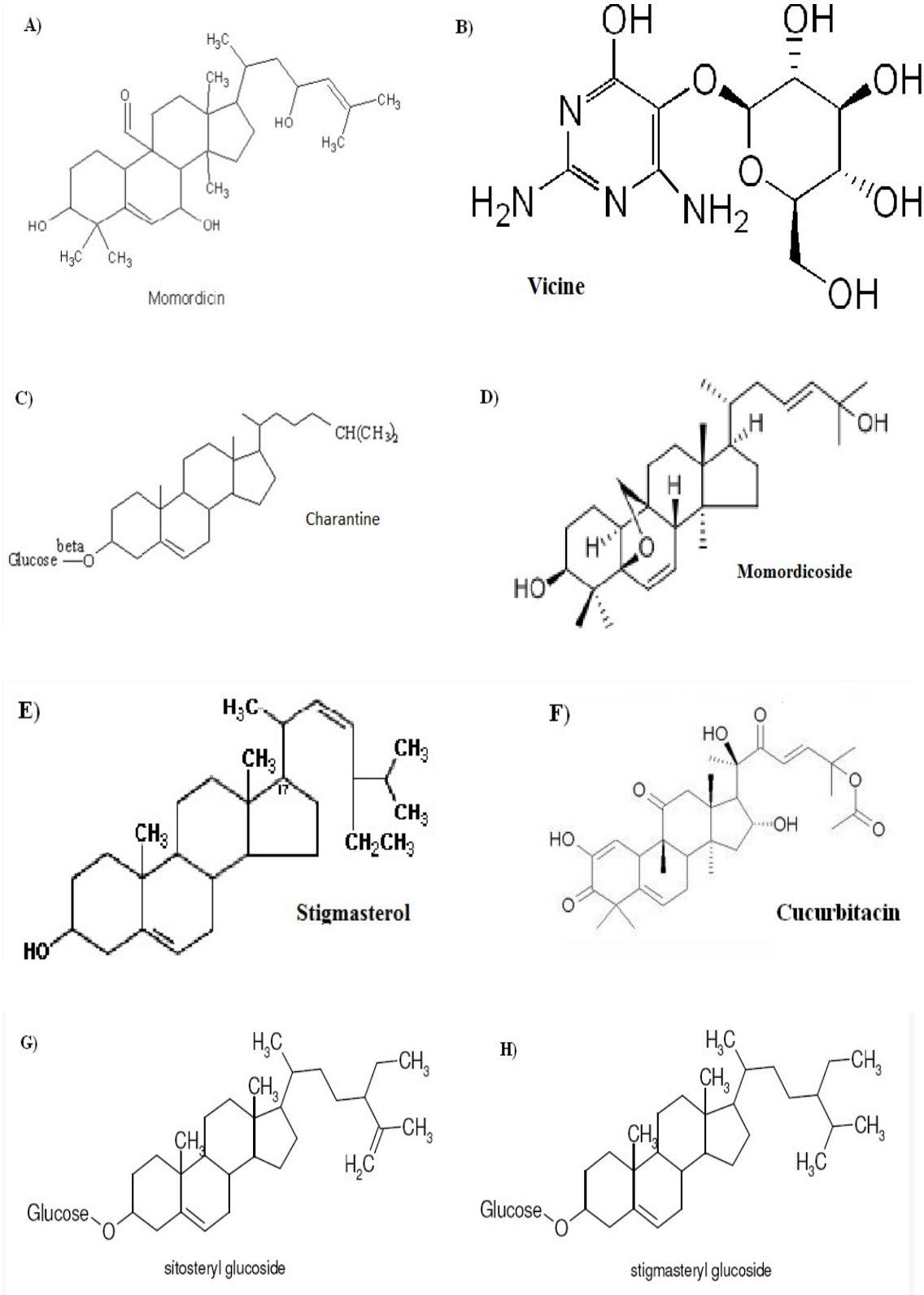


Figure 1.11: Compounds present in *Momordica charantia* (Adapted from- http://ssjournals.com/index.php/ijbr/article/view/sahu%20et%20al/sahu%20et%20al).

1.7.1 *Medical applications of Momordica charantia*

Momordica charantia has been used for centuries as medicine in India, China, Africa and Latin America. *Momordica charantia* extracts possess antioxidant, antimicrobial, antiviral, anti-cancer, antiulcerogenic and antidiabetic properties (Raman and Lau, 1996). A report also has shown that this plant has anti-tumour activity (Nagasawa, 2002). Ethno-medical reports of *Momordica charantia* indicate that it has been used in folkloric medicine for treatment of various ulcers, diabetes, and infections (Gurbuz et al. 2000; Scartezzini and Speroni, 2000; Beloin et al. 2005). The root of *Momordica charantia* has abortifacient property and the leaf and stem decoctions are also used in treatment of dysentery and gout (Subratty et al. 2005). The extracted juice from leaf, fruit and whole plant has been used for treatment of wounds and against infections, parasites (e.g., worms), measles, haepatitis, and fevers (Behera et al. 2008).

1.7.2 Anti Diabetic Activity of *Momordica charantia* in animals and humans

Enormous research studies have demonstrated the hypoglycaemic activity of *Momordica charantia* on animals including humans (Sarkar et al. 1996; Ahmed et al. 1998; Raza et al. 2000; Ahmed et al. 2001; Grover et al. 2001; Miura et al. 2001; Grover et al. 2002; Rathi et al. 2002 a, b; Kar et al. 2003; Ahmed et al. 2004; Chaturvedi et al. 2004; Miura et al. 2004; Sathishsekar and Subramanian 2005; Shetty et al. 2005) and humans (Baldwa et al. 1977; Leatherdale et al. 1981; Welihindal et al. 1986; Srivastava et al. 1993). *Momordica charantia* extracts and isolated components may exert their hypoglycaemic effects via a range of mechanisms including stimulation of insulin secretion and glucose utilization by cells and inhibition of intestinal glucose uptake (Singh et al. 2011).

1.7.3 Antioxidant Activity of *Momordica charantia*

Momordica charantia shows antioxidant properties due to the presence of carotenoids that protect plants during photosynthesis. *Momordica charantia* fruit contains as many as 14 carotenoids, depending on stage of maturity of the fruit. Other natural antioxidants present in the plant are phenolics and polyphenolic compounds. Other antioxidants present in this plant are vitamin C, vitamin B, phenolic acids, and organosulfur compounds (Simon, 1997). Bitter gourd is also a rich source of phenolic compounds, such as gallic acid, gentisic acid, catechin, chlorogenic acid and epicatechin. The concentration of phenolics varies with plant organ (fruit, leaf or root) (Jayasooriya et al. 2000; Ahmed et al. 2001).

1.8 Anti microbial activity of *Momordica charantia*

It has been demonstrated that the consumption of the fruit extract of *Momordica charantia* may increase the human and animal resistance against viral infections via the promoted production of natural killer cells (Sahu et al. 2011). Ribosome inactivating protein α-*momorcharin* stimulates the production of MAP30 (Momordica anti-HIV protein) and simultaneously suppresses HIV activity (Lee et al. 1990; Lee et al. 1995).

The fruit extracts of the *Momordica charantia* have shown antimicrobial activity against Escherichia coli, Staphylococcus, Pseudomonas, Salmonella, Streptobacillus, and Streptococcus bacteria (Omoregbe et al. 1996). Moreover, whole plant extract has shown antiprotozoal activity against Entamoeba histolytica. Also, fresh fruit extracts may exhibit antimicrobial activity against tuberculosis and helicobacteria pylori (Hussain and Deeni, 1991; Omoregbe et al. 1996; Yesilada et al. 1999).

1.8.1 Anti cancer activity of *Momordica charantia*

Chemical constituents of *Momordica charantia* include glycosides, saponins, alkaloids, fixed oils, triterpenes, proteins and steroids (Grover, 2004). Nutritional value of *Momordica charantia* is attributed to its high mineral and vitamin contents, while bitterness is attributed to the non-toxic alkaloids, momordicosides and momordicines. α- and β- momorcharins, momordins, and cucurbitacin B are a group of ribosomes inactivating proteins that are present in *Momordica charantia* and can be useful for inhibition of tumour cells (Okabe et al. 1980). Research in the last few decades has proved that *Momordica charantia* itself and its extract are useful for suppressing cancers and tumour growth. It has been reported that leukaemia lymphocyctes can be inhibited by the aqueous extract of *Momordica charantia* (Semiz and Sen, 2007). *Momordica charantia* contains charantin that is a steroidal saponin with insulin-like properties (Singh et al. 2011). Since 1928, insulin has been used to treat diabetes. Cancer cells thrive on sugar and they have more insulin receptors compared to normal cells. Depriving the cancer cells from sugar may hence be fatal to them. During insulin therapy, the blood sugar level is reduced to 50 mg/dl causing the patient to feel drowsy and weak for a short period when the cancer cells are also desperate for sugar. The cancer cells will take up sugar rapidly after being starved. Any chemotherapeutic agents, when infused with sugar, might be excessively taken up by the starving cancer cells. This is an effective approach to deliver chemotherapeutic agents to target cancer cells with less negative effects on normal cells (Jiao 2003). Among the various ribosome-inactivating proteins (RIPs) isolated from *Momordica charantia*, MAP30 (Momordica protein of 30 kDa) has displayed anti-tumour activity (Lee-Huang, 2000). The efficacy of MAP30 has been tested in estrogen-independent and highly metastatic human breast tumour MDA-

MB-231 cell lines, resulting in inhibition of cancer cell proliferation by inhibiting the expression of HER2 gene (Lee-Huang 2000). When MDA-MB-231 human breast cancer cells were transplanted into SCID mice, the mice developed extensive metastases, and all mice succumbed to tumours by day 46. Treatment of the mice with MAP30 resulted in significant increases in survival, with 20–25% of the mice remaining tumour-free for 96 days. The effect of MAP30, isolated from *Momordica charantia* seeds, was examined in premalignant and malignant human prostate cancer cell lines and caused an induction of cell cycle arrest and apoptosis (Xiong, 2009). Administration of MAP30 decreased PC3 human prostate cancer cell growth in nude mice, and this effect was primarily due to the induction of apoptosis. The *Momordica charantia* seed or fruit extracts have been shown to display anti-cancer activity in a rat colonic aberrant crypt foci model (Kohno, 2002) and a mouse mammary tumor model (Nagasawa, 2002). Akihisa et al. (2007) have isolated thirteen cucurbitane-type triterpene glycosides, including eight new compounds named charantosides I -VIII, and five known compounds, 8, 9, 14, 15, and 18, from a methanol extract of the fruits of Japanese *Momordica charantia* . Charantosides I and II exhibited marked inhibitory effects in both 7,12 dimethylbenz[a]anthracene- and peroxynitrite-induced mouse skin cancers. Seed oil of bitter melon contains more than 50–60% alpha-eleostearic acid (α-ESA) which also may suppress the growth of DLD-1 human colon cancer cells by inducing apoptosis via lipid peroxidation (Tsuzuki, 2004). α-ESA is converted to conjugated linoleic acid *in vivo*, causing a stronger suppressive effect on cancer than the non-conjugated linoleic acid. Treatment with α-ESA may also induce apoptosis in HL60 human promyelocytic leukemia cells (Kobori, 2008). Grossmann et al. (2009) have shown that that α-ESA blocks breast cancer cell proliferation and induces apoptosis. Although α-ESA

strongly induced apoptosis in HL60 cells, the acetone extract of bitter melon seed, which is probably rich in α-eleostearic acid, did not induce apoptosis in the cells. Lipophilic components of pericarp and placenta in the acetone extract may slow down the apoptosis process in HL60 cells. In fact, the acetone extract also did not suppress the colon cancer growth in xenograft model (Tsuzuki, 2004). Therefore, more work will be necessary to understand the *in vivo* activity of bitter melon and its different extracts.

1.9 Paclitaxel

Paclitaxel is a natural product with antitumor activity. Paclitaxel is a diterpene alkaloid derived from the dried bark of Pacific yew tree Taxus brevifolia (Figure 1.12 A) (Spencer, 1994). The chemical name for paclitaxel is 5β,20-Epoxy-l,2α,4,7β,10β,13α-hexahydroxytax-l l-en-9-one 4,10-diacetate 2-benzoate 13-ester with (2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine (Figure 1.12 B).

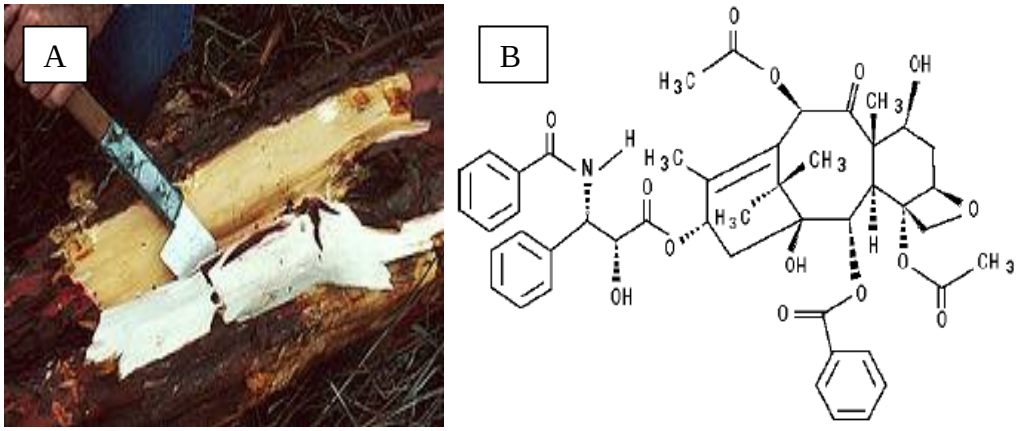


Figure 1.12: (A) The bark of the Pacific yew that is used to extract palitaxel. B) The chemical structure of Paclitaxel (Adapted from - http://www.guardian.co.uk/environment/2011/nov/10/iucn-red-list-tree-chemotherapy**).**

Paclitaxel is a white to off-white crystalline powder with the empirical formula $C_{47}H_{51}NO_{14}$ and a molecular weight of 853.9 g/mol. Paclitaxel is highly lipophilic, and melts at around 216-217°C (Singla, 2002) and is used in treatment of various advanced carcinomas such as ovarian cancer, lung cancer, breast cancer, acute leukemias, head and neck cancers and Kaposi‘s sarcoma. Paclitaxel is a mitotic inhibitor that blocks the proliferation of cancer cell (Schiff, 1979) and is commercially available as Taxol® that consists of Paclitaxel solubilised in Cremophor EL (polyethoxylated castor oil) and dehydrated alcohol (1:1 v/v) (Weiss, 1990). Unfortunately, severe adverse reactions like hypersensitivity reactions are caused by Cremophor EL (Yang, 2007). To exclude the solvent Cremophor EL and maximize the therapeutic index of the drug, different delivery systems of paclitaxel have been developed such as Abraxane® which has been approved for clinical use. Abraxne® (Abraxis Bioscience) is albumin bound paclitaxel, which is an injectable formulation used in the treatment of breast cancer (Altundag et al. 2007).

1.10 Liposomes

Liposomes have been used as a delivery system for treatment of tumour (Gregoriadis, 1973; Gregoriadis, 1977; Gregoriadis, 1978; Sofou, 2007). Many phytochemical constituents are poorly bioavailable due to their large molecular size or low water solubility. Thus, nanotechnology has infused new dimensions into the targeted drug delivery systems through self-assembling supramolecules based nanostructures (Mohanraj and Chen, 2006). Amongst many types of nanocarriers, liposomes have been investigated intensively during the last few decades to explore and exploit their intrinsic targeting potential (Mohanraj and Chen, 2006). Ajazuddin

(2010) has reported that the active ingredient of the herb curcurmin was loaded into liposomes and showed anticancer effect with prolonged circulation in the blood.

1.10.1 Architecture of Liposomes

The name liposome is derived from two Greek words: 'Lipos' meaning fat and 'soma' meaning body. It has been over 35 years since their discovery while being studied as model biological membranes (Bangham, 1961; Bangham, 1968). Liposomes are self-assembled, self-closed colloidal nanocarriers having phospholipid bilayer membranes. Phospholipids are part of all cell membranes of living organisms and play an important role in signal transduction mechanism and are arranged according to fluid mosaic pattern in biological membranes. Phospholipids are heterogeneous molecules containing phosphoric residues, polar head groups and non-polar alkyl chains (Figure 1.13) (Bangham et al. 1965).

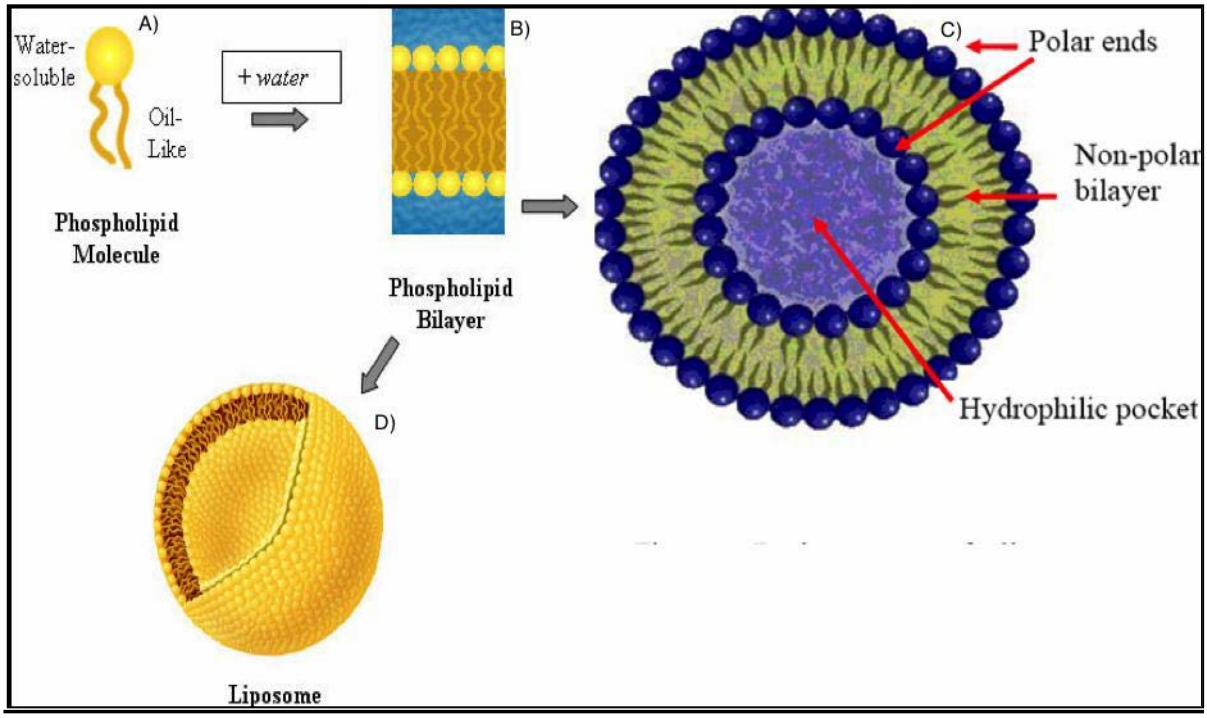


Figure 1.13: Arrangement of phospholipid bilayer in the liposomes: A) Phospholipid molecule on presence of aqueous phase forms B) phospholipid bilayers having C) polar head face outwards and non-polar tail face inwards of the liposome and forms D) colloidal and sperical shape vesicle to carry drugs. (Adapted from- http://www.uni-magdeburg.de/imos/mea_sen/img/pictures/Lipo.jpg).

Vigorous dispersion of purified phospholipid in water, by adding _process energy‘ in the form of mixing or sonication, above the phase transition temperature (T_m) of the lipid results in formation of liposomes. Phase transition temperature (T_m) of a given phospholipid is the temperature at which the bilayer transfers from the gel state to the liquid crystalline state at which the molecules are more mobile and the bilayers are more permeable. Thus, liposomes above the T_m have more flexibility and permeability. Hydrophilic molecules are entrapped in the aqueous spaces of liposomes while hydrophobic materials are entrapped in the bilayers (Ueno et al. 1991). Because of their unique size and surface properties, liposomes can enhance the performance of drugs by increasing their solubility, improving their

bioavailability, enhancing their cellular uptake and altering their pharmacokinetic properties (Mizuno et al. 2003; Haag and Kratz, 2006).

Constituent and chemistry of liposomes-

A) Phospholipids

A phospholipid molecule is amphipathic consisting of one polar head goup and two non-polar alkyl chains (Figure 1.14). When phospholipid molecules are hydrated with water, the polar heads tend to face the aqueous phase and the non-polar tails stick together and shield away from the aqueous compartments, resulting in formation of bilayers.

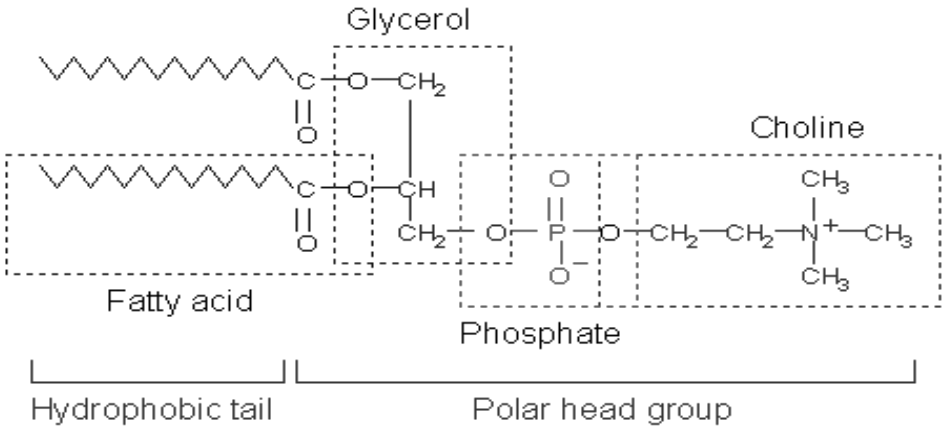


Figure 1.14: Diagram showing the chemical structure of a phospholipid (phosphatidylcholine) molecule.

i) Polar head groups of phospholipids

Most phospholipid head groups belong to the phosphoglycerides family such as phosphatidylcholine (PC) (Figure 1.14), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). The polar headgroups are respectively choline, serine, ethanolamine and inositol attached to phosphate groups and glycerol backbone (Wehrmüller, 2008).

ii) Non-polar tails of phospholipids

The non-polar tail contains two fatty-acyl chains. The fatty-acyl chains are hydrophobic in nature. The fatty-acyl chains in biological membranes usually consist of an even number of carbon atoms and are saturated or unsaturated.

B) Cholesterol

Cholestrol is a lipid that is abundant in biological membranes. The amount of cholesterol may vary depending on the type of membrane. However, membranes have nearly one cholesterol molecule per phospholipid molecule. The following figure shows the steroid structure of cholesterol with an illustration of the non-polar and polar regions of the molecule (Figure 1.15).

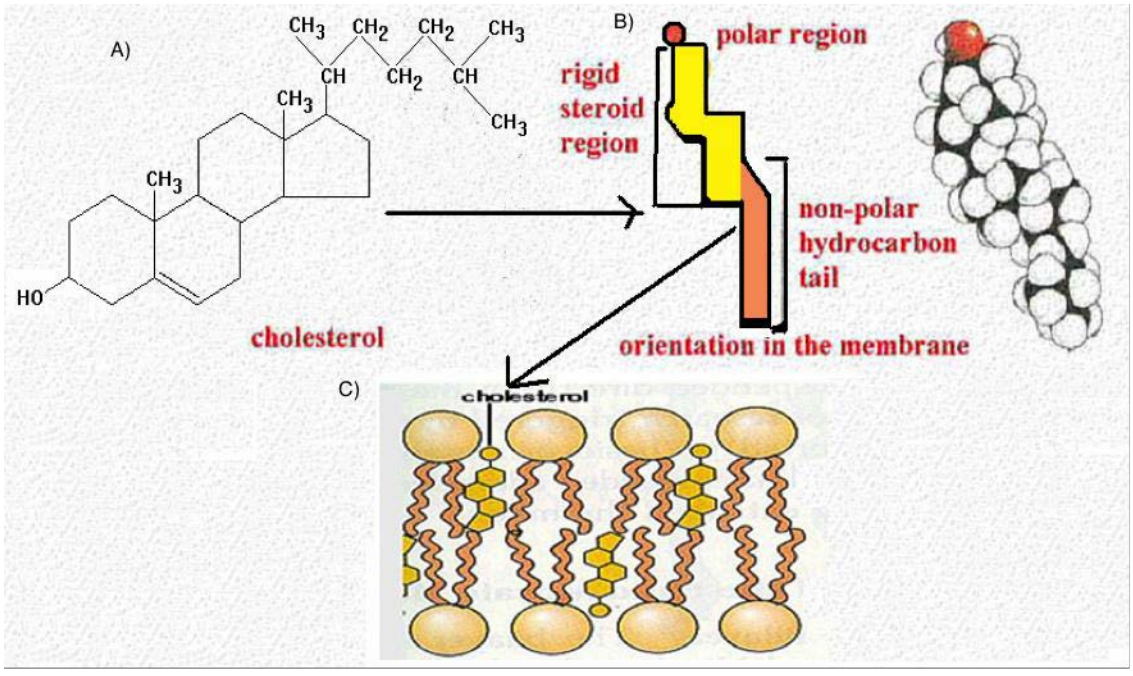


Figure 1.15: Orientation of Cholesterol in the biological membrane A) Chemical structure of cholesterol B) Polar and non-polar region in cholesterol molecule and c) Orientation of cholesterol molecule inserting itself between the membrane bilayer (Adapted from-http://www.cytochemistry.net/cell-biology/membrane_intro.htm**).**

During phase transition temperature the gel phase is converted into liquid crystalline phase and both of the hydrophobic tails of the phospholipid are freely moving as

shown in Figure 1.16. At temperatures above the T_c, the bilayer –melts‖ and the interior is fluid allowing the lipid molecules to move around, rotate and exchange places. At temperatures below the phase transition (T_c) of a given phospholipid, the bilayer is in the gel state and tightly packed. Cholesterol molecules insert themselves into the phospholipid bilayers with the same orientation of the phospholipid molecules (Samad et al. 2007).

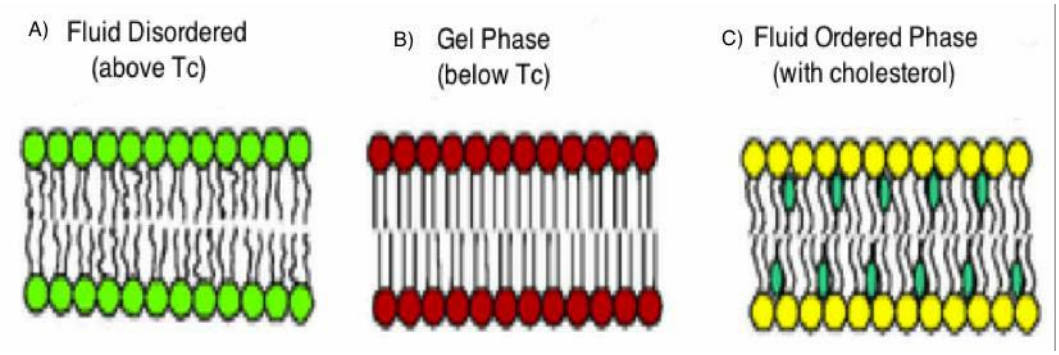


Figure 1.16: The relationship between packing style of hydrocarbon chains in bilayers and the temperature of the hydrating phase. A) shows the fluid state of hydrocarbon chain when the temperature is above phase transition (Tc) B) shows the gel phase hydrocarbon state when below phase transition C) shows rigid hydrocarbon state on insertion of cholesterol in the formulation above Tc.

(Source- http://www.cytochemistry.net/cell-biology/membrane_intro.htm**).**

<u>Functions of cholesterol functions in the membrane of liposome:</u>

1. The cholesterol embed themselves between the bilayer. The hyrophilic portion of cholesterol molecule face towards the membrane surface. Cholesterol increases the fluidity of the membrane by interfering the tight packing of the phospholipid molecule. Cholesterol prevents crystallization of hydrocarbons and phase shifts in the membrane (Yeagle, 1985).

2. Cholesterol retards the movements of the hydrocarbon alkyl chains in the phospholipid molecules. This makes the lipid bilayer less deformable and decreases its permeability to small water-soluble molecules. Without cholesterol such as in

bacterial cells cell walls are needed to maintain the function of the cells and prevent leakage of cellular components (Samad et al. 2007).

1.10.2 Soy lecithin

Soy lecithin is the most commercially important lecithin derived from plant. Soy is a subtropical plant native to South Eastern Asia and is mainly consumed in the Eastern style diet. Soyabeans are the richest vegetarian source of phospholipids. Because of the high content of phospholipid in soybeans, they are often used as raw materials for dietary supplementation as well as emulsification in many food products (Wehrmuller, 2008). Importantly, soy products contain putative protective components such as oligosaccharides and protease inhibitors, which are responsible for protection against some cancers (Davies et al 1999). Soy lecithin is a combination of naturally occurring phospholipids, which are extracted during the processing of soybean oil. The main phospholipids in soy lecithin are lysophospholipid, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine and phosphatidic acid (Figure 1.17). However, soy lecithin is sometimes used as synonym for pure soy phosphatidylcholine. There is epidemiological evidence that soy phosphatidylcholine has protective effects against colon cancer (Adlercreutz, 1998; Messina, 1994). Using soy products, anti-proliferative effects have been shown on a wide range of cell types *in vitro* including cells of the gastrointestinal tract (Yanagihara et al. 1991). Soy lecithin is an excellent source of choline, which is essential to the integrity of cellular membranes. Without choline, the cell membranes would harden, prohibiting important nutrients from entering and leaving the cell. The importance of soy lecithin is not confined to its dietary supplement of choline but also extends to include methyl metabolism, cholinergic neurotransmission,

transmembrane signalling, and lipid-cholesterol transport and metabolism (Zeisel, 2000).

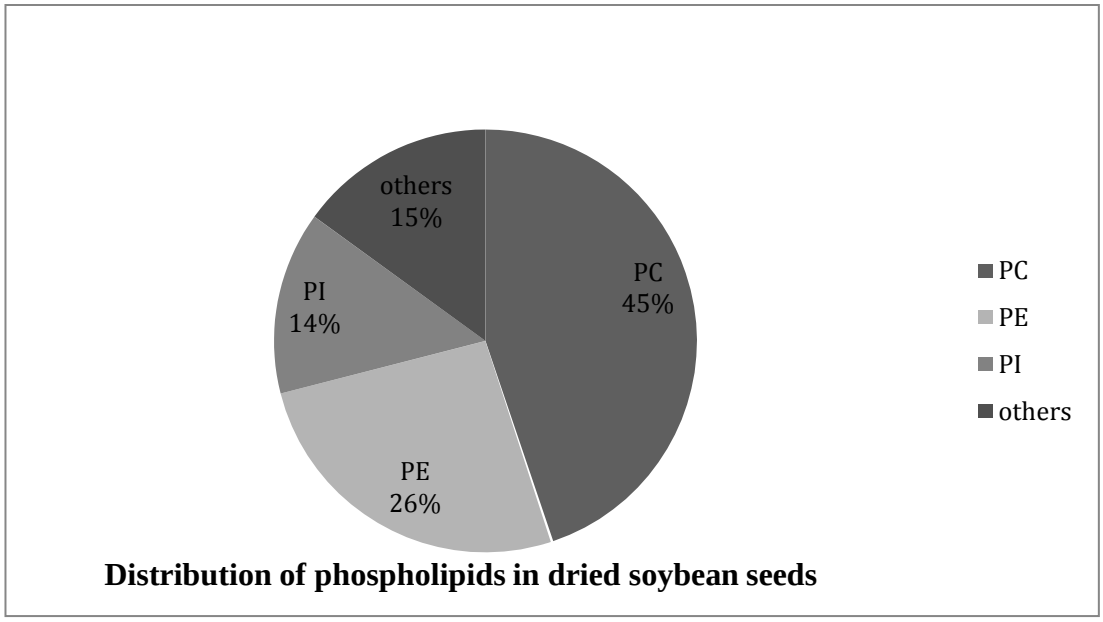


Figure 1.17: Pie chart showing the composition of soybean lecithin: Phospholipid distribution in dried soy seeds (adapted from Nyberg, 1998).

1.10.2.1 Soy Phosphatidylcholine

Lipoid S100 is a relatively pure form of soy phosphatidylcholine (SPC), which is derived from soybean (Lipoid GmbH, Switzerland). Lipoid S-100 soy lecithin is comprised mainly of soya phosphatidylcholine (94% of the dry weight) in addition to a mixture of phospholipids including N-Acyl-phosphatidylethanolamine, Phosphatidylethanolamine, Phosphatidylinositol and Lysophosphatidylcholine. The composition of Lipoid S-100 is shown in Table 1.4 and phosphatidylcholine comprises 94% of the weight of the product according to the manufacturer sheet.

Table 1.4: Composition Lipoid S-100) (Adapted from the supplier: Lipoid GmbH, Switzerland)

Composition of soy lecithin, Lipoid S-100	
Phospholipids fraction	
Phosphatidylcholine(by anhydrous weight)	94.0%
N-Acyl-phosphatidyl ethanolamine	0.5%
Phosphatidyl ethanolamine	0.1%
Phosphatidyl inositol	0.1%
Lysophosphatidyl choline	3.0%

Typical fatty acid composition in % to total fatty acids

Fatty acid	**% Of fat composition**
Palmitic acid	12-17
Stearic acid	2-5
Oleic acid	11-15
Linoleic acid	59-70
Linolenic acid	3-7

Approximate Molecular Weight*:* 790

Storage*:* -20 ºC

1.10.2.2 Hydrogenated soy phosphatidylcholine (HSPC)

Phospholipon® 90H is hydrogenated soy lecithin product and is simply referred to as hydrogenated soy phosphatidylcholine (HSPC) and was supplied by Lipoid GmbH, Switzerland). Phospholipon® 90H contains soybean-derived phospholipids with fully saturated alkyl chains such as hydrogenated phosphatidylcholine and hydrogenated lysophosphatidylcholine. As shown in Table 1.5, hydrogenated phosphatidylcholine comprises 90% of the Phospholipon®90H. The composition of saturated fatty acids (palmitic acid and stearic acid) is higher in comparison to unsaturated fatty acids (oleic acid, linoliec acid and linolenic acid) (Table 1.5).

Table 1.5: Composition of Phospholipon®90 H (Adapted from the supplier: Lipoid GmbH, Switzerland)

Phospholipon®90 H	
Phospholipids fraction	
Hydrogenated Phosphatidylcholine	min. 90%
Hydrogenated Lysophosphatidylcholine	max. 4%
Oil/ triglycerides	max. 2%
Typical fatty acid composition in % to total fatty acids	
Fatty acid	% Of fat composition
Palmitic acid	15
Stearic acid	85
Sum of stearic acid and palmitic acid	min. 98%
Sum of unsaturated fatty acids (oleic, linoleic, linolenic acids)	max 2%
Approximate Molecular Weight: 784 Storage : 0 ºC	

1.10.3 Role of phospholipids present in Soy lecithin

1.10.3.1 Phosphatidylcholine (PC)

Phosphatidylcholine (PC) is the major phospholipid constituent of cell membranes, comprising 40-50% of total phospholipids (Vance, 2008). PC is also known as 1,2-diacyl-sn-glycero-3-phosphocholine, or is simply referred to as lecithin. PC plays an important biochemical role in the maintenance and integrity of the cell membrane and is an important signalling molecule (Exton, 1994; Billah, 1990; Kiss, 1990; Kester, 1989). Recently, it has been suggested that PC by its own may have a therapeutic role in some cancers (Semalty et al. 2009). Animal studies have indicated that deficiencies in choline and PC may disrupt cell membrane signal transduction in ways that could lead to various cancers. There is evidence suggesting that liver cancer is promoted in various animals by choline-deficient diets, and it has been shown that excess choline can protect against liver cancer in a mouse model (Lois et al. 2002).

1.10.3.2 Phosphatidylethanolamine (PE)

Phosphatidylethanolamine (also sometimes called ‗cephalin') is the second most abundant phospholipid in animal and plant lipids. It comprises 20 - 50% of the total phospholipid content of the mammalian membranes. It can make up to about 20% of liver phospholipids and as much as 45% of phospholipids of the brain. Higher proportions of PE are found in the mitochondria than in other organelles of the cell (Vance, 2008).

1.10.3.3 Phosphatidylinositol (PI)

Phosphatidylinositol (PI) is an acidic (anionic) phospholipid that consists of a phosphatidic acid backbone linked via the phosphate group to inositol. Inositol is phosphorylated to form phosphatidylinositol phosphate (PIP), phosphatidylinositol bisphosphate (PIP_2) and phosphatidylinositol trisphosphate (PIP_3). PIP, PIP_2 and PIP_3, which are collectively called phosphoinositides. Two phospholipid derivatives of PI are PI phosphate and PI biphosphate have been found to be important in the signal transduction pathway located in the inner half of the plasma membrane. Inhibition of phospholipid inositol metabolism may induce cancer (Ana et al. 2001). PI may comprise up to 10% of the phospholipids of the brain. There is usually less PI than PC, PE or PS in cells.

1.10.4 Liposome morphology and preparation methods

According to their morphology, liposomes are classified into either oligolamellar, unilamellar or multilamellar. Multilamellar liposomes (MLVs) have a size range from 500 to 10,000 nm. Unilamellar liposomes can be small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs). SUVs are usually smaller than 100 nm whilst LUVs are larger than 100 nm. Liposomes of very large size are commonly referred to as giant liposomes and have a size larger than 10,000 nm and are either unilamellar or multilamellar. Some large liposomes may encapsulate smaller liposome vesicles and these are called multi-vesicular liposomes which have a size range of 2,000 - 40,000 nm. Morphology of liposomes is highly determined by the method employed to prepare them (Mohammad, 1996) (Figure 1.18).

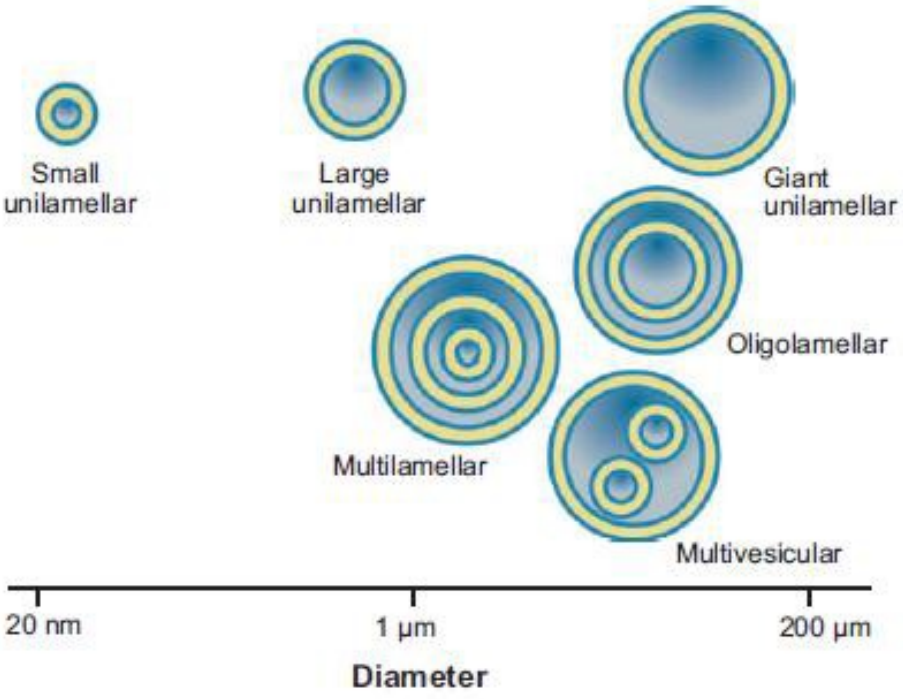


Figure 1.18: Diagrams showing the size and lamellarity of liposomes prepared using different methods (Adapted from- Jesorka, 2008)

Liposomes prepared by the conventional thin film hydration method are MLVs. This method is unsuitable for manufacturing liposomes on a large scale and because liposomes are dispersed in aqueous phase (i.e. no drying is applied) they are chemically and physically unstable during storage. Stability of liposomes depends upon the method of preparation, nature of the constituent lipids and properties and concentration of the entrapped material.

1.10.5 Usage of entrapment of material in liposomes

- Lipoosme is used to deliver hydrophilic lipohilic and amphiphatic compounds. Their location in the liposome will be dependent on physiochemical of the loaded agents. They can either be located in the liposome membrane or in the intra-liposome aqueous phase.

- Liposomes as a drug delivery system can improve the therapeutic activity and safety of drugs, mainly by delivering them to their site of action (i.e. targeting) and by maintaining therapeutic drug levels for prolonged periods of time (Abou et al. 2003; Ajazuddin and Saraf, 2010).
- Liposomes can protect a drug against degradation
- Liposomes can protect the patient against side effect of the encapsulated drug.
- Liposomes are used for the delivery of drug, protein or nucleic acid, si RNA, plasmid, DNA, peptides, proteins, viruses and bacteria (Schroeder et al. 2009) for the treatment of diseases.

1.11 Proliposomes

Proliposome methods are based on the conversion of the initial proliposome preparation into liposome dispersions by addition of an aqueous phase (Maitani et al. 2001). This method is suitable for encapsulation of a wide range of drugs with varying solubility in water and alcohol and can provide high encapsulation efficiencies when compared with other methods of manufacturing liposomes. Proliposomes are easy to scale up, potentially providing an alternative approach for the production of liposomes for clinical applications (Turanek et al. 1997; Wagner and Karola, 2011). Liposomes prepared from proliposome are safe, biodegradable and may enhance the chemical stability of the formulation since aqueous phase addition takes place immediately before administration. Proliposomes are potentially applicable for preparation of tumour targeting liposomes for anticancer drug delivery (Crommelin and Sindelar, 2002). For solid tumours, liposomes are of particular interest because of their easy manipulation and optimum size. It is known that

tumour has leaky vasculature and poor lymphatic drainage. Liposomes can be used as a targeting tool by exploiting what has been described as –Enhanced Permeation and Retention effect‖ (EPR). Proliposome formulations are either Particulate-based or Solvent-based. Solvent-based proliposomes are referred to as alcohol-based or ethanol-based when alcohol is used to dissolve the lipids.

1.11.1.1 Particulate-based proliposomes

Particulate-based proliposomes are dry, free-flowing carrier particles coated with phospholipids in order to generate liposomes upon addition of aqueous phase (Payne *et al* 1986). Particulate-based proliposomes comprise soluble carrier particles (e.g. carbohydrate) coated with phospholipids to generate MLVs by the addition of aqueous phase above the phase transition temperature (T_c) of the lipid used (Payne et al. 1986). Particulate-based proliposomes are made by attaching a flask containing the soluble carrier particles to a rotary evaporator followed by portion-wise addition of organic solution of the lipid via a feed line under reduce pressure to coat the carrier particles. Evaporation of the organic solvent results in the production of particulate-based proliposomes (Payne et al., 1986, Elhissi and Taylor, 2005). Proliposomes can then be stored below 0°C. Once the liposomes are required for administration, an aqueous phase is added to the proliposome powder. This approach offers phospholipid formulations that are free of aqueous phase during storage and hence chemical and physical instabilities of liposomes are minimised. The manufacturing procedures however is tedious and may be difficult to control, since the operation requires: (a) addition of organic solution of the lipid and subsequent solvent evaporation; (b) close monitoring to ensure that the powder is not overly wet and the unit is properly operating under vacuum; and (c) overnight drying of the

proliposomes in a desiccators under reduced pressure may also be required (Chen et al. 1987). Also, the involvement of an organic solvent is an environmental hazard.

Liposomes generated from particulate proliposomes may offer high entrapment efficiency of hydrophobic drugs as compared to hydrophilic drugs. Nicotine is amphiphilic in nature and has 45-58% entrapment efficiency in liposomes generated from particulate-based proliposomes (Chung, 1999),

The entrapment efficiency of hydrophilic drugs in proliposome-generated liposomes was much lower, for instance the entrapment efficiency of propranolol hydrochloride was approximately 4 to 10% and high phospholipid concentrations were used in order to maximise the entrapment of this drug (Ahn et al. 1995). In another study, a value of about 45 % of salbutamol sulphate was entrapped in liposomes using a micronized proliposome blend of phospholipid and carrier particles (Desai et al. 2003).

1.11.2 Solvent-based proliposomes

Solvent-based proliposoms are concentrated solutions of phospholipid, which generate liposomes upon addition of aqueous phase above the T_c of the lipid (Perrett et al. 1991). Typically, the solvent used is ethanol; hence they are commonly referred to as alcohol-based or ethanol-based proliposomes. This method offers a simple means of generating liposomes with high entrapment of hydrophilic materials (Perrett et al. 1991; Turanek et al., 1997; Elhissi et al., 2006). The spontaneous generation of liposomes from proliposomes has been demonstrated to produce large vesicles under static condition (approximately 11-13 μm), when compared to the agitated proliposome samples (approximately 1 μm) (Deo et al. 1997).

Liposomes generated by from alcohol-based proliposomes provide high entrapment of hydrophilic materials, which ranged between approximately 65 and 80% depending on phospholipid composition (Perrett et al. 1991). The ethanol-based proliposome approach may provide a means for cost-effective large scale manufacture of liposomes containing hydrophilic drugs (Perrett et al. 1991; Turanek et al. 1997).

1.12 Liposomes in anticancer therapy

Many liposome formulations of various anticancer agents have been shown to have less toxic effects than the drug itself (Gabizon, 1989). It has been reported that small liposomes are passively targeted to different tumours because they can circulate in the blood compartment for longer period of time and perfuse better into cancerous tissues. Doxil® is an intravenous liposome formulation of doxorubicin and is prepared by using the Stealth technology. Stealth liposomes are liposomes coated with polyethylene glycol, resulting in better capability to escape clearance via the reticuloendothelial system and hence they are long circulating liposomes. Caclyx® and Myocet® are liposomal formulation of doxorubicin used for treatment of ovarian cancer and recently for treatment of metastic brain cancer (Samad et al. 2007). Antibody-conjugated liposomes or immunoliposomes are liposomes bearing specific antibodies on their surfaces to target the encapsulated drug molecules to particular diseased tissues or organs. The pharmacological effect on brain cancer cells *in vivo* supports the viewpoint that macromolecules such as plasmid DNA can be delivered across biological barriers including the blood-brain barrier, the plasma membrane of the target cells within the brain tissue and the nuclear membrane (Zhang et al. 2003).

In some cases, sustained release of the liposomal formulations showed enhanced

efficacy and longer presence of therapeutic concentrations of the anticancer drug in lymphoma cells (Storm et al. 1987).

Table 1.6 shows the examples of the liposome product available in the market for the treatment of cancer.

Table 1.6: Some commercially available anticancer liposome formulations that have been investigated clinically (Adapted from: Samad et al. 2007)

Liposome product	**Drug**	**Targeted sites**
Doxil®	Doxorubicin	Kaposi‘ sarcoma
EVACT™	Doxorubicin	Refractory tumour Metastatic breast cancer
DaunoXome®	Daunosome	Advanced kaposi‘s sarcoma, breast cancer, small cell lung cancer, leukemia and solid tumours
VincaXome®	Vincristine	Solid tumour

1.12.1 Role of liposomes in anti-cancer therapy

Liposomes are biocompatible and ideal non-toxic biodegradable carriers. The phospholipid bilayer of the liposome protects the drug from degradation in the body while being targeted to the tumour site. It also prevents the exposure of active drug to the healthy tissues, while the drug is in blood circulation. The negative side effect of the drug is also reduced while in liposomes as compared to non-encapsulated drug (Brown and Khan, 2012). The principal pathway for the movement of liposomes into the tumour interstitium is via extravasation through the discontinuous endothelium of the tumour microvasculature. However, the tumour vasculature is discontinuous with gaps, allowing liposomes to extravasate and reach the tumour interstitium by transcytosis process. Non-targeted liposomes are localized in the interstitium surrounding the tumor cells and eventually they leak the drug out of the liposome. Non-targeted liposomes are not seen within tumour cells, although they are observed by the tumour macrophages, resulting in a special mode of accumulation that is different from targeted liposomes. Targeted liposomes accumulate in tumours, but on entering the tumour area are endocytosed by the tumour cells. This process results in breakdown of the liposomes by lysosomes and an increased delivery of the drug to the nucleus, resulting in cytotoxic effects in the tumour area. Large tumors are more difficult to treat than small ones, in part because of the resulting increase in interstitial pressure, which prevents access of drugs to the necrotic core (Drummond et al. 1999). The extravasation and accumulation of liposomes into tumors are depicted in Figure 1.19.

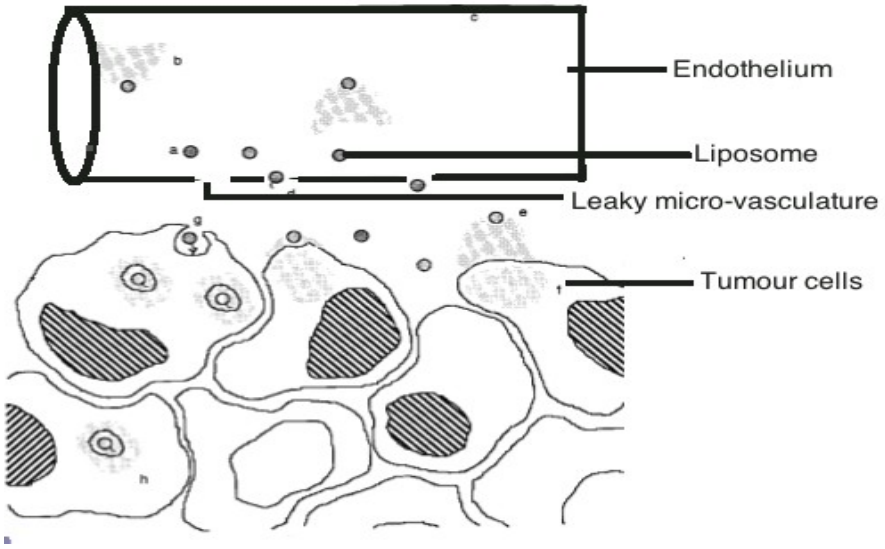


Figure 1.19: Diagram showing the gaps in the endothelial cells in tumour, allows the liposomes to accumulate in the tumour site and stop the proliferation of cancerous cells. (Adapted from – Drummond et al. 1999).

1.13 Working hypothesis

Liposomes generated from proliposomes can be used to encorporate and deliver plant-derived drugs for the treatment of gliomas.

1.14 Main Aim

The main aim of the project was to prepare liposome formulations of *Momordica charantia* extracts and to apply the extracts on glioma cell lines. Solvent-based proliposome method was used to generate liposomes. The phospholipid used in the experiment was soy phosphatidylcholine (SPC) or hydrogenated soy phosphatidylcholine (HSPC) liposomes including *Momordica charantia* extracts.

1.14.1 Specific aims

1. To undertake a thorough literature search in the area in the liposomes and *Momordica charantia.*
2. To prepare liposomes from proliposomes using solvent-based proliposome technology.
3. To study the physical and chemical properties of liposomes by size, size distribution, surface charge and surface morphology.
4. To prepare extracts of *Momordica charantia* using three different species of *Momordica charantia* from Africa, China and India.
5. To segregated *Momordica charantia* fruit into flesh of the fruit (fruit alone; FA), seeds (seeds alone; SA) or whole fruit including the seeds (WF) and produce water-soluble extracts for incorporation into liposomes generated from proliposomes.

6. Similarly, prepare Paclitaxel (PTX) liposomal formulations and compare with *Momordica charantia extracts* liposomal formulations in the experiment.
7. To deliver drug loaded liposomes on three different glioma cell lines and a normal glail cell.
8. To culture the three glioma cell lines and study their morphology before and after treatment by measuring cell viability.
9. To investigate the mechanism of extracts of Paclitaxel and *Momordica charantia* in the cell death measuring the activities of caspase and cytochrome c release.
10. To analyse the data and write the PhD thesis.

Chapter 2. General Materials and Methods

2.1 Materials

2.1.1 Momordica charantia from China, Africa and India

Three species African, Chinese and Indian of *Momordica Charantia* fruits was purchased from a local food store (Kashmir Watan Food store, Lancashire, Preston PR1 1PJ, United Kingdom).

Figure 2.1: The three different species of *Momordica charantia*. The plant source was from A) China (Yunnan), B) India (Maharashtra) and C) Africa (Kenya) and all were purchased from the local supplier Kashmir Watan food store (Kashmir Watan Food store, Lancashire, Preston *PR1 1PJ*, United Kingdom).

2.1.2 Phospholipids used in the experiment

The soy lecithin used in the experiments was Lipoid S-100 (Soy phosphatdidylcholine) and Phospholipon® 90H (Hydrogenated soy phosphatidylcholine) was purchased from Lipiod GmbH, Switzerland.

2.1.3 Other materials

HPLC grade Methanol and Ethanol was purchased from Sigma Aldrich, UK. Cholesterol (99%) was also supplied by Sigma Aldrich, UK and Paclitaxel was purchased from ChemieTek, USA. Water used in all experiments was deionised distilled water.

2.2 Methods

2.2.1 Aqueous extraction of *Momordica charantia*

The unripe fruit of *Momordica charantia* was washed thoroughly in deionised water and then was cut into small pieces. The various parts of the plant were studied for their anticancer activity. These parts were:

- Whole fruits (1000 g). These included the fruit and the associated seeds.
- The fruits alone (1000 g). These were the fruits without the seeds.
- The seeds (500 g). These were only the seeds present inside the fruits. These plant sections are shown in Figure 2.2.

Whole Fruit (WF; 1000 g) or Fruit Alone (FA; 1000 g) of *Momordica charantia* was sliced into small pieces. The sliced pieces were grinded in a blender model no-3402E19 (Thomas Scientific, UK) in presence of 900 ml of deionised water and than added 100 ml of methanol. The resultant aqueous dispersions were mixed well manually for 10 minutes and then squeezed using muslin cloth and sterile filtered using 0.45 μm Cellulose Nitrate membrane filter (Fisher Scientific, UK) in glass bottles followed by storage at 4ºC. The solid insoluble materials were discarded.

The seeds (500 g; Figure 2.1) collected from *Momordica charantia* fruits were grinded with 450 ml of distilled water and methanol (50 ml) was added to the extract, later. The dispersion was mixed well for 10 minutes and squeezed using muslin cloth, sterile filtered and stored as performed for the WF.

Figure 2.2: Diagram showing *Momordica charantia* whole fruits (A) and a fruit after being sliced into two halves with the associated seeds (B).

2.2.2 Drying of the aqueous extracts

Empty round-bottomed flasks were weighed and 20 ml of each extract (WF, FA or SA) were added and evaporated under vacuum to remove water and methanol using an R210 rotary evaporator (Buchi, Switzerland) for 2 hours. The flask was removed and kept for drying in the oven at 40 ° C for 1 hour. The flasks with the extract were weighed in order to find out the amount of the extract present.

After rotary evaporation was completed and dried in the oven for an hour at 40 ° C. The amount of WF, FA, and SA extracts from the three different species of the plant (i.e. Indian, Chinese and African) was calculated afer rotary evaporation and complete drying. The weight of the WF, FA and SA was found to be 737.6 mg, 541.2 mg and 373.4 mg, respectively for the Indian plant, 750.45 mg, 620 mg and 410.22 mg, respectively for the Chinese plant, and 720 mg, 420 mg and 340 mg, respectively for the African plant. The solid material was dissolved in deionised water (5 ml) to have the following range of concentrations: 0, 1.75, 3.50, 5.25, 7.00 and 8.75 mg.

2.3 Preparation of liposomes using thin film hydration method

Phospholipid (SPC or HSPC) was dissolved in ethanol (40 mg / ml) in 100 ml pear shaped flask and attached to the rotary evaporator. Vacuum was applied, rotation speed was set at maximum, and the water bath was set at 35C. After one hour, the vacuum pump was turned off in order to detach the flask. The pear shaped flask containing thin layer of phospholipid was immersed in water bath and hydrated with 5 ml deionised water (60 C) and shaken in order to form liposomes. The liposomes were kept immersed in the water bath at 60 C for 10 min followed by vigorous hand shaking in order to ensure the film is completely hydrated. The SPC and HSPC liposomes were allowed to cool at room temperature for 2-3 hours before performing further investigations or experiments.

2.4 Preparation of liposomes using the solvent-based proliposome method

Volumetric flasks (5 ml) were washed with methanol and dried in the oven at 80 C in order to avoid microbial contamination. SPC or HSPC (40 mg) and cholesterol (10 mg) were weighed in different volumetric flasks (Figure 2.3).

2.4.1 *Momordica charantia* Extract

Ethanol (100 µl) was added to the flask containing the SPC or HSPC (40 mg) at room temperature with gentle mixing to produce a clear solution of phospholipid. Cholesterol (10 mg) was added while keeping the flask containing the phospholipid partially immersed in a water bath (60°C) until the cholesterol was completely dissolved and a clear concentrated proliposome solution was obtained. This step was referred to as the proliposome preparation step. Increasing quantities of WF, FA or

SA of *Momordica charantia* extracts were added to a set of proliposome solutions. This was performed by dissolving the solid matter (0, 1.75, 3.50, 5.25, 7.00 and 8.75 mg) in 5ml of deionised water as previously described. The aqueous extract (2.5 ml) was added to the proliposome solutions at room temperature followed by vortex mixing for two minutes to generate liposomes. This step was referred to as the primary hydration step. The secondary hydration step involved addition of another 2.5 ml of the aqueous phase followed by vortex (A2754 - Fisons whirlimixer, Fisons scientific equipment, UK) mixing for two minutes so that the liposome solution is mixed homogenously. The preparation was allowed to settle for one hour at room temperature for SPC and 60° C for HSPC before conducting further experiments (Figure 2.2).

2.4.2 Paclitaxel

The SPC and HSPC phospholipid (40 mg) was dissolved in 500 µl ethanol solution containing paclitaxel (0, 1.75, 3.50, 5.25, 7.00 and 8.75 mg) at 60°C. The solution was transferred to a flask containing cholesterol (10 mg) at 60°C in order to obtain a clear proliposome solution of Paclitaxel. Deionised water (2.5 ml) was added at 60°C followed by gentle mixing and vortexing (A2754 - Fisons whirlimixer, Fisons scientific equipment, UK) for two minutes to perform the primary hydration. This was followed by dilution with another 2.5 ml of deionised water and vortex mixing for two minutes. Liposomes were allowed to settle for one hour at 60° C for SPC and HSPC before conducting further experiments in order to avoid re-precipitation of paclitaxel (Figure 2.2).

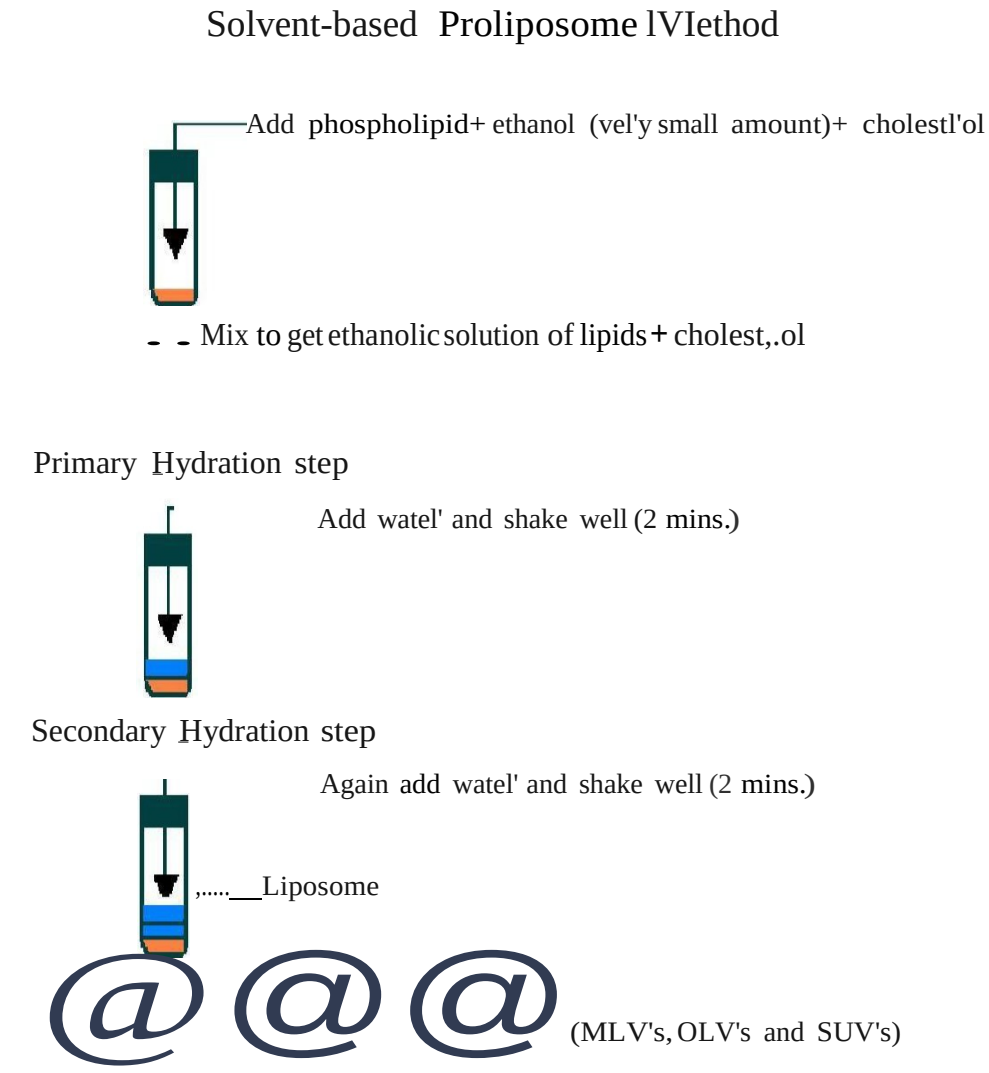


Figure 2.3: Schematic presentations for the preparation of liposomes using the solvent-based proliposome methods.

2.5 Statistical Analysis

All data generated in the study were processed using SPSS program. Control and test data were compared using student t-test and ANOVA test. Moreover, all the data were expressed as ± either Standard Error of the Mean (S.E.M) or Standard Deviation (S.D). Control (untreated) and treated data were compared and a value of $P < 0.05$ was taken as significant. All the experiments were done three times in triplicates (n=3).

Chapter 3. Characteri-sation of *Momordica charantia* prepared liposomes using ethanol-based proliposome

3.1 Introduction

The conventional thin film hydration method for preparation of liposomes is difficult to scale up, and phospholipids in liquid dispersions are liable to hydrolysis (Kensil and Dennis, 1881; Grit et al. 1989) and oxidation (Hunt and Tsang, 1981). Moreover, liposomes may aggregate or fuse during storage and leak the entrapped material. As an alternative approach to the thin film hydration method, proliposomes are stable phospholipid formulations that generate liposomes prior to administration by addition of aqueous phase (Payne et al. 1986; Perrett et al. 1991). One type of proliposomes is ethanol-based proliposomes that are concentrated alcoholic solutions of phospholipid (with or without cholesterol). The addition of aqueous phase to the proliposome formulation with shaking generates oligolamellar liposomes (Perrett et al. 1991). Liposomes generated from ethanol-based proliposomes have demonstrated different thermal profile, size and drug entrapment compared to conventional liposomes (Elhissi et al. 2006a). Recently, a study by Epstein- Barash et al. (2010) has compared the effect of liposome size and charge on the bioactivity of liposomal bisphosphonates using a wide range of cell types *in vitro* and *in vivo.* There are many potential barriers that affect the delivery of drug in its active form to the tumour site. Most of the small-molecule chemotherapeutic agents have large volume of distribution on administration. This results in narrow therapeutic index and high level of toxicity to the healthy tissues. Therefore, encapsulation of drug in macromolecules called liposomes reduces the volume of distribution of the active agent due to sustain release pattern. The drug encapsulated in macromolecules becomes very effective at the tumour site (Drummond et al. 1999). Also, the stable encapsulation of drug within the large liposome, likely prevents filtration and removal of the drug by the

kidneys (Drummond et al. 1999). Modifying and control physicochemical properties such as size and surface charge can achieve targeting of liposomes to monocytes and macrophages (Kelly et al. 2011).

Thus, many studies have shown that the biological properties of liposomes and subsequent fate of the entrapped / associated material are highly dependent on liposome characteristics including size and zeta potential. In this chapter, liposomes incorporating *Momordica charantia* extracts was prepared and characterised in terms of size, size distribution, zeta potential (i.e. surface charge) and vesicle morphology and compared to paclitaxel.

3.2 Materials

The dye used for transmission electron microscopy (TEM) was 1% Uyral Acetate dye and supplied by Sigma-aldrich, UK. Lipoid, Switzerland supplied all phospholipids used as indicated in Chapter 2.

3.3 Methods for characterisation of liposomes

3.3.1 Liposome particle size analysis using laser diffraction

The size of liposomes was analysed via laser diffraction by using the Malvern Mastersizer 2000 (Malvern Instruments Ltd., UK). Large particles scatter the light at narrow angles with high intensity whereas smaller particles scatter the light at wider angles and with low intensity (Koch et al. 2003). The instrument was switched on 30 min before starting the size measurements**.** The dispersion unit was washed several times with deionised water, by increasing the stirring speed to 3,500 rpm and subsequently reducing the speed of the dispersion unit controller to 1,360 rpm.

Deionised water (100 ml) was added in the dispersion unit for liposome size analysis. After alignment of the background, the sample was added to the dispersion unit to get the obscuration in the green range and analyse the size and size distribution of liposomes. Size and size distribution were presented by the volume median diameter (VMD, 50% undersize) and Span, respectively. Span is a term introduced by Malvern Instruments Ltd to express the size distribution of particles. Span = (90% undersize – 10% undersize) / VMD.

3.3.2 Zeta potential measurements

Zeta potential of liposomes was important because surface charge of particles could give information about formulation stability. It also specified the intensity of repulsion among similarly charged dispersed particles and attraction between oppositely charged particles. Zeta potential was measured by applying an electrical field to the suspension, which was also known as electrophoresis (Naderkhani, 2011). Using the DTS1060C Malvern‘s ZetaSizer, Liposome sample was injected through the plugs of a disposable zeta potential cell and voltage was applied through two gold plated electrodes (Figure 3.1). Particles movement under the influence of an applied electric field was exploited to determine the zeta potential of the liposome particles (Clogston and Patri, 2011).

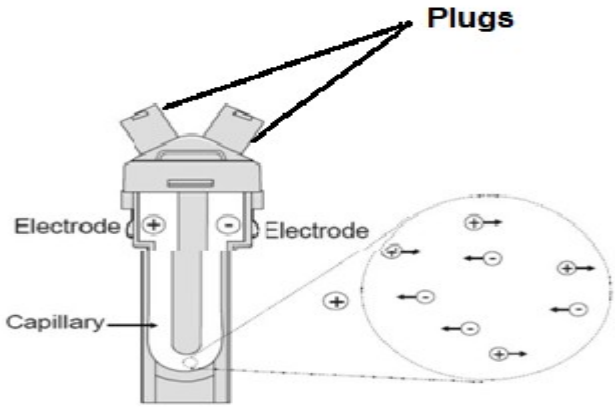


Figure 3.1: Diagram showin the zeta potential cuvette having electrode for the zeta potential analysis of liposomes.

(Adapted from- http://departments.agri.huji.ac.il/zabam/zetasizer.html).

3.3.3 Transmission electron microscopy (TEM)

Morphological and size examinations of freshly prepared blank liposomes, extract-containing liposomes were done using negative staining transmission electron microscopy. A droplet of the spontaneously formed liposomal samples was placed onto a carbon-coated copper grid (400 mesh) for about 3 minutes to permit proper absorption of the sample. The droplet was then dried using pieces of filter paper before leaving the grid for another 1 minute. A droplet of the negative stain solution (1% uranyl acetate) was added to the surface of the grid. After 1 minute, the copper mesh grid was dried using pieces of filter paper and kept in a filter paper lined petri dish. The size, morphology and lamellarity of liposomes were then viewed using a Philips CM12 transmission electron microscope and photographed at an accelerating voltage of 80 kV.

3.4 Statistical Analysis

As stated in chapter 2 in section 2.5

3.5 Results and Discussion

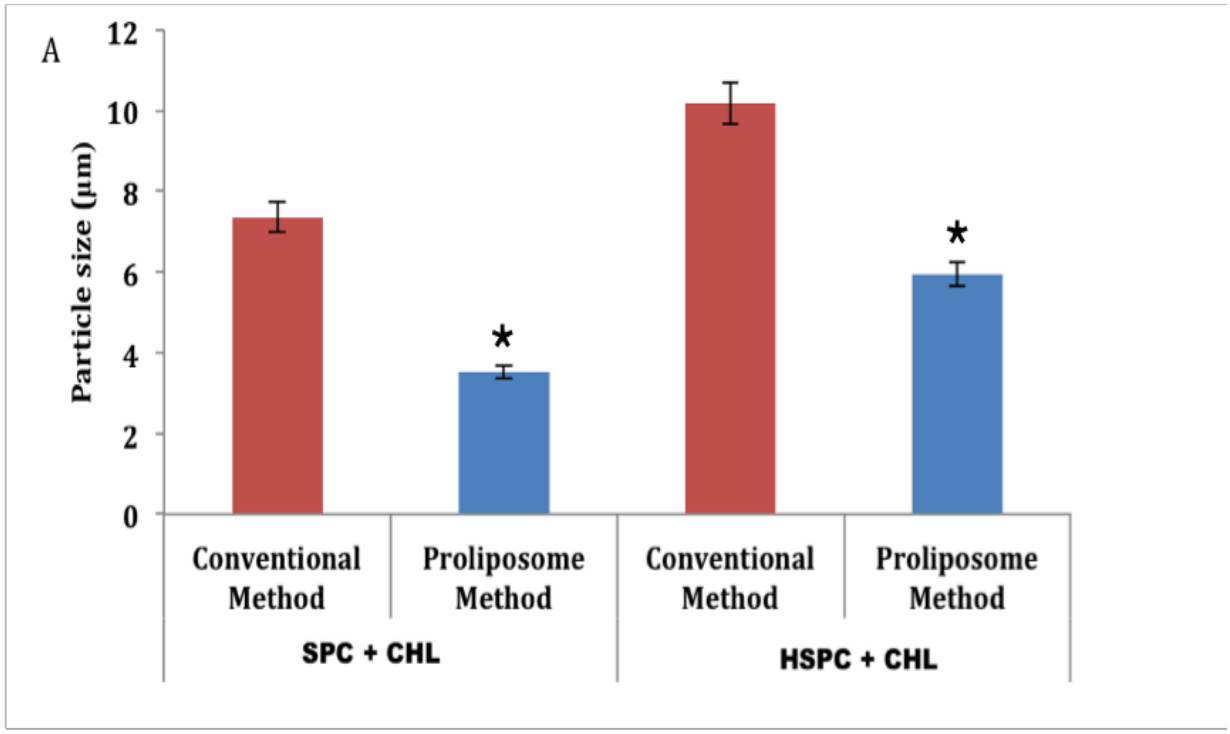
3.5.1 Size and size distribution of extract-free liposomes prepared by conventional thin film method and proliposome method

The study investigated the characteristics of extract-free liposomes prepared from SPC and cholesterol (4:1) or HSPC and cholesterol (4:1). Hydrogenated PC or partially hydrogenated PC instead of unsaturated egg PC in liposome preparation affects the stability of liposomes. Phospholipids with unsaturated acyl chains are subject to oxidation, which may affect the permeability of the bilayers and the *in vivo* performance of the liposomes (Smolen and Shohet, 1974; Konings, 1984). Thus, it is important to compare the characteristics of HSPC and SPC before performing the tissue culture studies on glioma cell lines. Moreover, liposomes generated from proliposomes were compared to liposomes generated using the thin film hydration method. The comparison was conducted in terms of size, size distribution and zeta potential. The effect of lipid composition using SPC and HSPC was also studied.

As shown in Figure 3.2 A, using blank liposomes (i.e., containing no drug or extract), vesicles generated from proliposomes had significantly ($P < 0.05$) smaller size than liposomes prepared by the conventional method for both SPC and HSPC. Moreover, student t-test analysis of particle size by conventional and proliposome method confirmed that HSPC produced significantly ($P < 0.05$) larger liposomes as compared to SPC (Figure 3.2A). Laser diffraction studies conducted by Elhissi et al. (2006a) have shown that ethanol-based proliposome method produces smaller size liposomes than thin-film conventional method (Elhissi et al. 2006a), which agrees

with the findings in the present study. The reason for the reduced size may be due to the presence of fewer bilayers as a result of ethanol inclusion in liposomes generated from proliposomes. The presence of ethanol causes interdigitation of the phospholipid bilayers by converting the gel phase into liquid crystalline phase (Elhissi et al. 2006a).

Figure 3.2B represents the span (i.e. polydispersity) of liposomes. When SPC was used to prepare liposomes, there was no significant difference in the span regardless of the method of preparation. However, liposomes made from HSPC phospholipid showed significantly ($P < 0.05$) higher span values when liposomes were prepared by the conventional method (Figure 3.2 B), indicating that when high T_m lipids are used, proliposomes could generate liposomes with lower polydispersity. Moreover, for both methods, the span was significantly higher by using HSPC phospholipid to manufacture liposomes. Higher span values indicate possible aggregation of the vesicles. This study has overall demonstrated that proliposomes-generated liposomes were superior in terms of having smaller size and narrower size distribution.



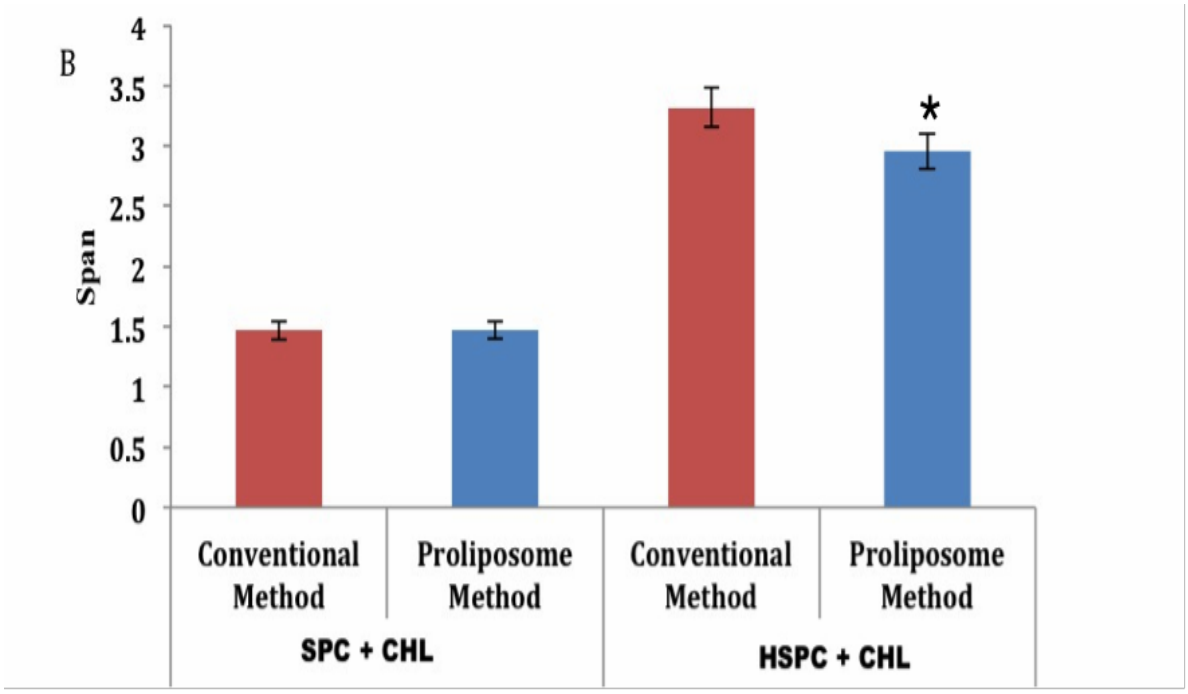


Figure 3.2: Bar charts showing A) Particle Size and B) Size distribution of extract-free liposomes prepared by thin film or ethanol-based proliposome methods using SPC or HSPC. Data are mean ± S.D and n=3. * P < 0.05 for conventional method compared to proliposome method.

3.5.2 Size and size distribution of Momordica charantia liposomes using SPC or HSPC

The median size and size distribution of SPC and HSPC liposomes including *Momordica charantia* were analysed in this study. The effect of extract type in terms of plant organ (WF, FA or SA) and source (Africa, China or India) was investigated for comparision. Similarly, the study also investigated the effect of extract concentration on liposome size and size distribution.

3.5.2.1 Particle size and size distribution of African *Momordica charantia* liposomes

When SPC was employed, the size of liposomes using the African *Momordica charantia* (FA and WF) was significantly ($P < 0.05$) increased as the concentration of the dried extract increased (Figure. 3.3 A). By contrast, for the SA extract, the particle size did not significantly change ($P > 0.05$) by increasing the extract concentration (Figure 3.3 A). Similarly, particle size using the FA component of the plant was the largest compared to the size of vesicles prepared by hydration with the SA or WF extracts (Figure 3.3 A) but this was not significant. This might be attributed to the difference in the extracted compounds from various plant organs, leading to differences in the bilayer packing and vesicle size. It has been previously shown that materials that interact with the liposome bilayers are capable of affecting the size of liposomes especially amphipathic molecules and co-solvents (Elhissi et al. 2006).

Using SPC phospholipid, liposomes containing the African *Momordica charantia* (FA, SA or WF) showed a slight, but a significant ($P < 0.05$) increase in the span value, indicating some aggregation of the liposomes upon the inclusion of higher

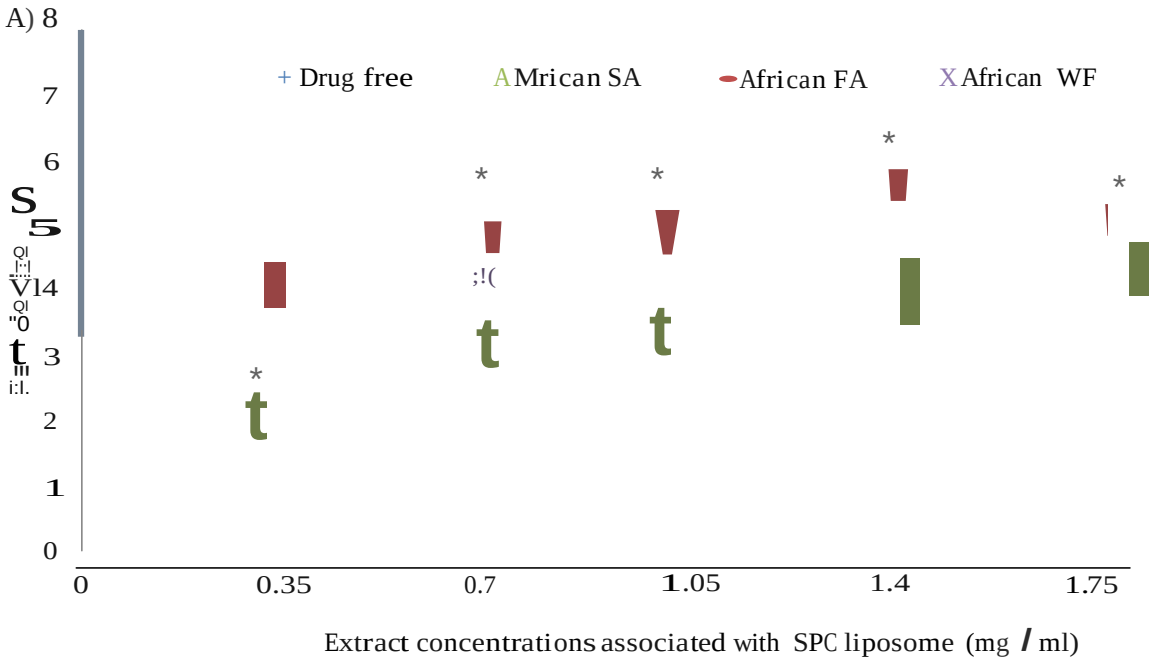
extract concentrations (1.05 - 1.75 mg / ml) as compared to the extract-free liposomes (Figure 3.3 B). This again confirms the influence of the biochemical compounds extracted from the plant on the bilayer characteristics and liposome size.

As a large number of natural product modulators have been discovered over the years, natural products such as curcumin flavonoids (natural products) can modulate ABC drug transporters (Bakare et al. 2010).

Similar to the results obtained using SPC, the size of HSPC-made liposomes with the African *Momordica charantia* (FA, SA or WF) was significantly ($P < 0.05$) increased at high extract concentrations as compared to the extract-free liposomes (Figure 3.3 C). Size measurements of the African *Momordica charantia* was the largest when using the SA extract followed by the extracts of the FA and WF. Bakare et al. (2010) have conducted a nutritional and chemical evaluation of *Momordica charantia* showing that the crude fat content in the seeds is higher than that in the fruit and leaf organs of *Momordica charantia*. This possibly indicates that the crude fat content of the seed of *Momordica charantia* may be the reason behind liposome aggregation or fusion. Previous studies have demonstrated that the increased hydrophobicity of liposome surfaces can cause fusion between adjacent vesicles (Ohki and Arnold, 1990).

When the size distribution (i.e. span) of the HSPC liposomes was studied using only the FA and WF, and when used at highest concentration (1.75 mg /ml) caused an increase in the span value compared to the extract-free liposomes. By contrast, the SA extract caused inconsistency of the span measurements and showed no clear trend upon increasing the extract concentration (Figure 3.3D).

Thus, in general, both SPC and HSPC using all African *Momordica charantia* extracts caused an increase in size and span of liposomes, suggesting that the extracted constituents have influenced the packing of the liposome bilayers with a subsequent increase in liposome size.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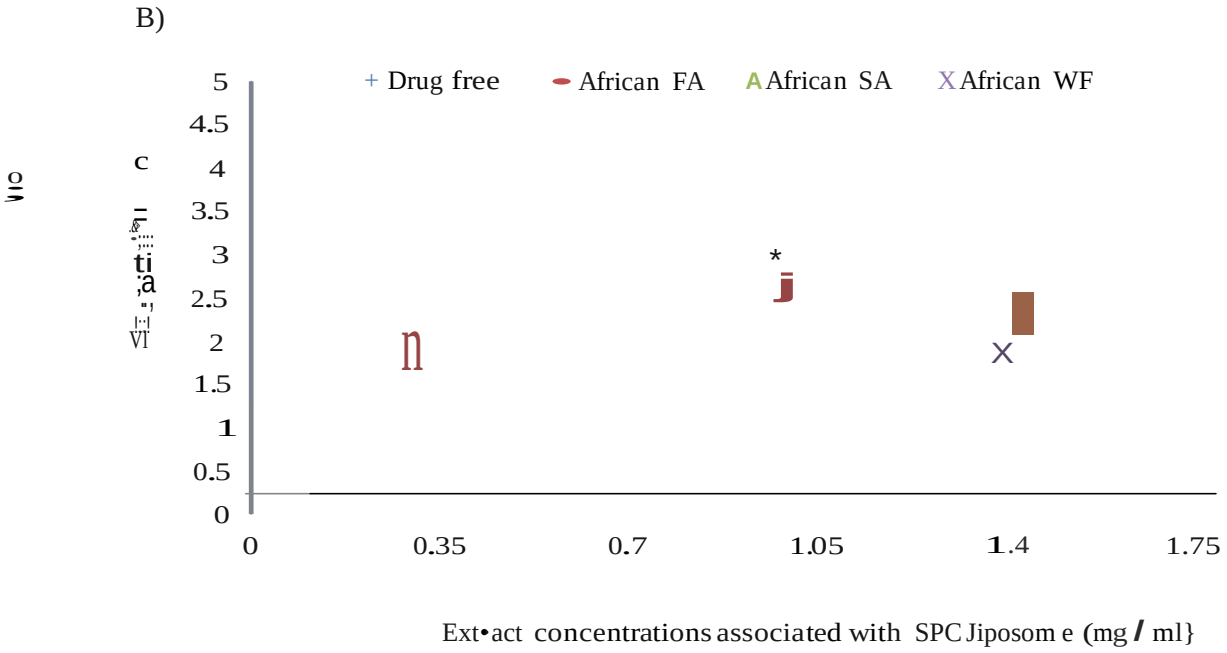
Extract concentrations associated with SPC liposome (mg / ml)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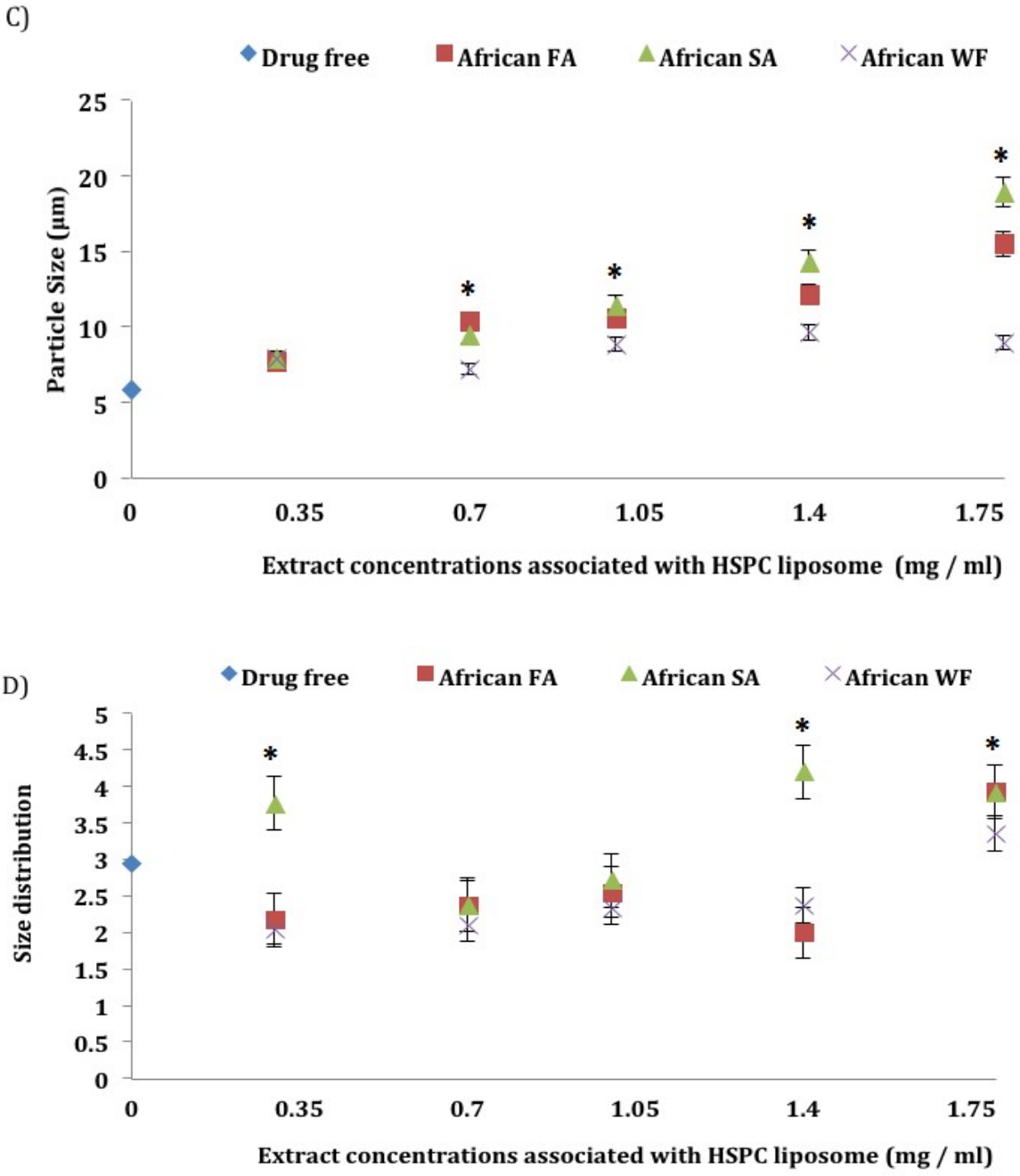


Figure 3.3: Diagrams showing the characterisation of African *Momordica charantia* extracts (FA, SA and WF) associated liposomes- A) Particle size and B) Size distribution of SPC liposomes and C) Particle size and D) Size distribution of HSPC liposomes. Data are mean ± S.D, n=3 *P < 0.05 for *Momordica charantia* liposomal formulations compared to drug free liposomal formulation.

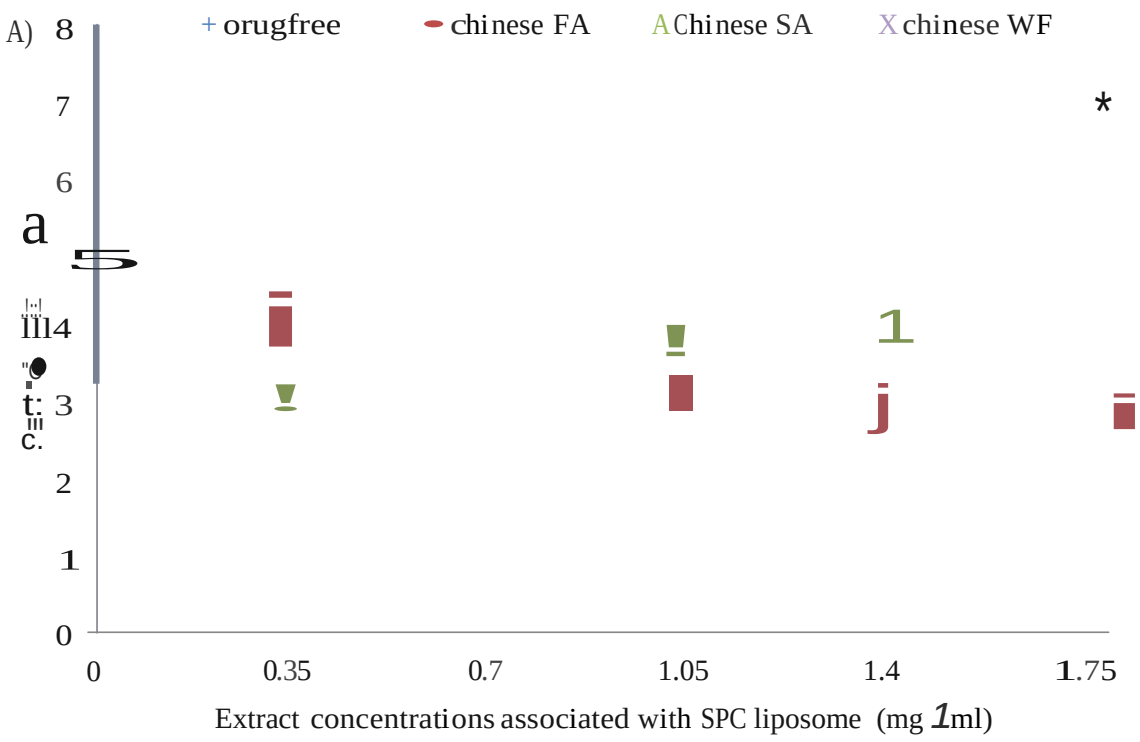
3.5.2.2 Particle size and size distribution of Chinese *Momordica charantia* liposomes

SPC liposomal formulations of Chinese *Momordica charantia* (FA or WF) showed no significant ($P > 0.05$) change in the measured particle size and size distribution at all concentrations (Figure 3.4A and Figure 3.4B). However, FA SPC formulation demonstrated a marked and significant increase ($P < 0.05$) in the span value at the concentration of 0.7 mg / ml, indicating aggregation of liposomes. This finding is difficult to justify based on the present data, suggesting that further studies are needed in the future to explore the effect of these extracts on the liposome bilayers. This investigation can be conducted using sophisticated and robust thermal techniques such as differential scanning calorimetry or solution calorimetry. However, for the SA extract formulation, although it did not show any change in the size at the low concentrations (from 0.35 mg / ml to 1.4 mg / ml), a significant ($P < 0.05$) size increase at the highest extract concentration (1.75 mg / ml) was observed when compared to the extract-free SPC liposomes (Figure 3.4 A). Seed extract of *Momordica charantia* has high contents of crude fat (Bakare et al. 2010), which might be the causative of vesicle aggregation. Thus, liposomes at the highest SA extract concentration were much larger than the extract-free liposomes for both phospholipids (Figure 3.4 A and C). When using SPC, Chinese *Momordica charantia* SA showed a significant ($P < 0.05$) and drastic increase in the span with uneven size distribution when the extract concentration was higher than 0.7 mg/ml (Figure 3.4B). These results support the finding of Hussain (2010), who demonstrated that size of liposomes increased with increasing the cucurmin concentration. Cucurmin is a hydrophobic phytochemical compound that is also present in *Momordica charantia* and has a great tendency to associate with the

hydrophobic regions of liposome bilayers. Also, there might be many other compounds present in the extract having affinity towards the hydrophobic region and making the size of liposomes larger and the size distribution wider.

HSPC liposomal formulations of Chinese *Momordica charantia* FA and WF did not show any significant ($P > 0.05$) change in the particle size at the lower concentrations (0.35 mg / ml and 0.7 mg /ml). In contrast, a significant ($P > 0.05$) increase of the particle size at the higher concentrations (1.4 - 1.75 mg / ml) was demonstrated as compared to the drug free liposomes (Figure 3.4 C). By contrast, SA of the Chinese *Momordica charantia* liposome formulation showed a significant ($P < 0.05$) increase as a result of extract inclusion (0.35 – 1.75 mg / ml) as compared to the extract-free liposomes (Figure 3.4 C). The Chinese *Momordica charantia* seed contains 90% eleostearic and 2–3% stearic acid (Chang et al. 1996). Eleostearic is unsaturated fatty acid. Unsaturated fatty acid causes oxidation of liposome at high temperature. The reason for instability of liposomes is oxidation of unsaturated fatty acid, which causes the phospholipid to aggregate and make the liposome unstable (Lee et al. 1998). *Momordica charantia* seed extract contains 90% eleostearic (unsaturated fatty acid) and this can also be the reason for increased the size and size distribution of the liposomes prepared from SPC and HSPC phospholipid containing.

HSPC liposomal formulations of Chinese *Momordica charantia (*FA, WF and SA) had a significantly ($P < 0.05$) lower span values as compared to the extract-free HSPC liposome (Figure 3.4D), again confirming the need for conducting highly sensitive thermal studies on the liposome bilayers.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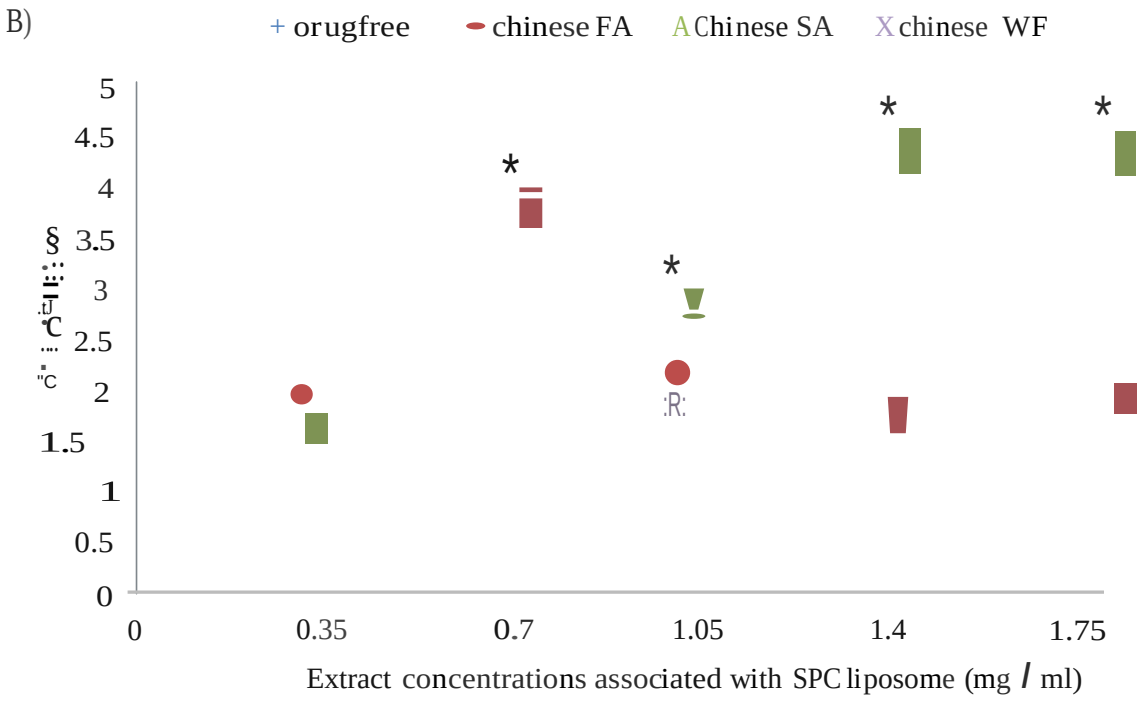
*
Extract concentrations associated with SPC liposome (mg 1ml)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1.05
1.4
1.75
*
*
*
*
Extract concentrations associated with SPC liposome (mg / ml)

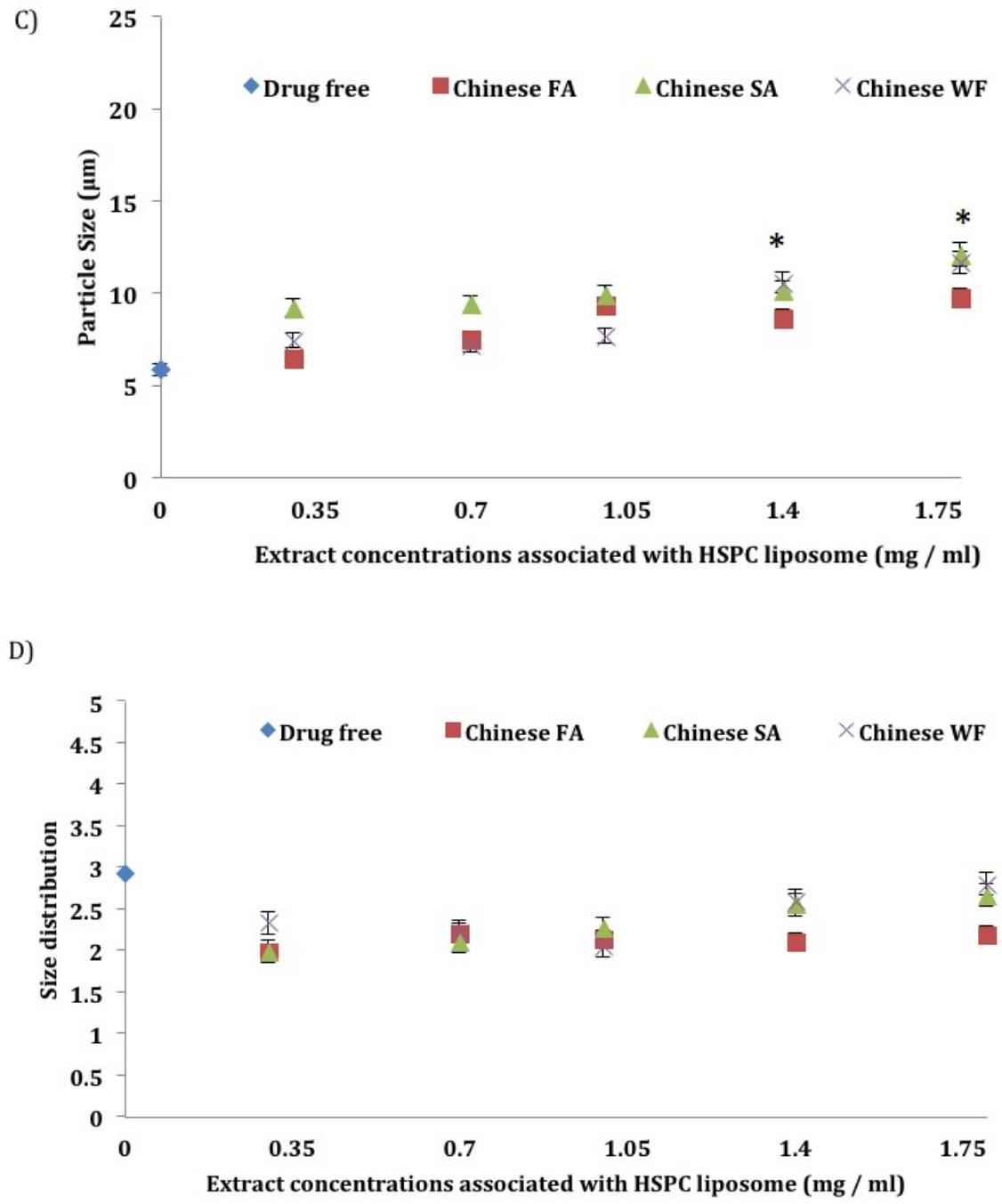


Figure 3.4: Plots showing the characterisation of Chinese *Momordica charantia* extracts (FA, SA and WF) associated liposomes- A) Particle size and B) Size distribution of SPC liposome, C) Particle size and D) Size distribution of HSPC liposomes. Data are mean ± S.D, n=3 *P < 0.05 for *Momordica charantia* liposomal formulations compared to drug free liposomal formulation.

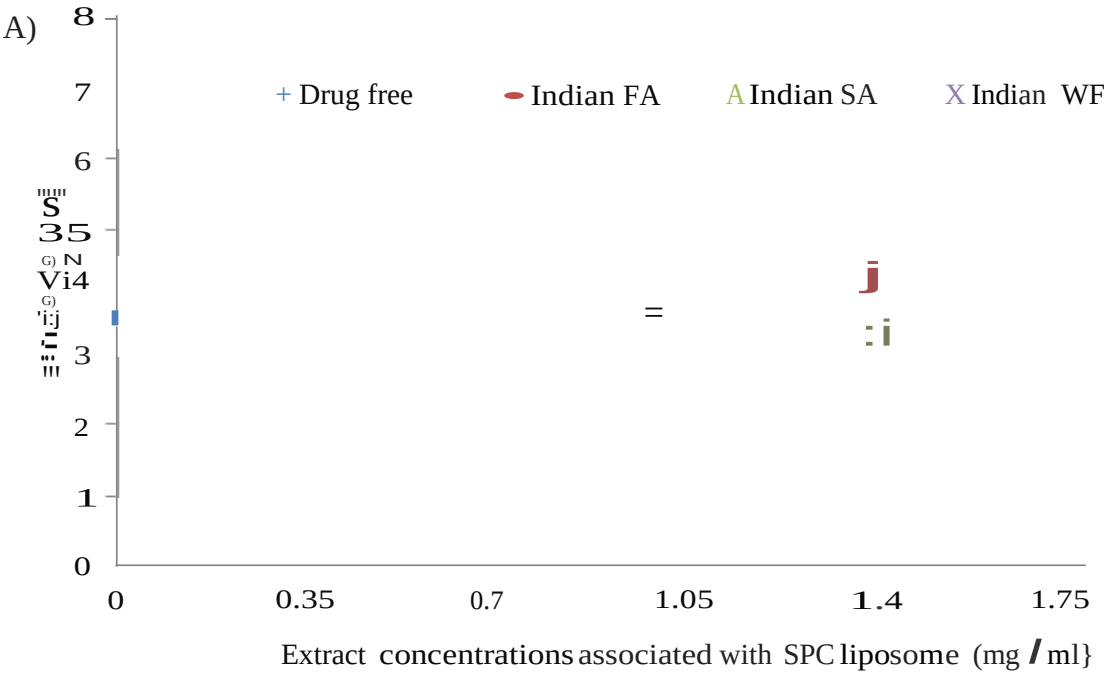
3.5.2.3 Particle size and size distribution of Indian *Momordica charantia* liposomes

The SPC liposomes of Indian *Momordica charantia* (FA, SA and WF) had either similar or slightly different particle size as compared to the extract free liposomes (Figure 3-4A). Also, the size distribution of SPC liposomes (FA or WF) and extract-free liposomes were similar. In contrast, SPC liposomes on inclusion of SA extract of Indian *Momordica charantia* had slightly larger span values and inconsistent size distribution with increasing concentrations of the extract liposomes (Figure 3-5B).

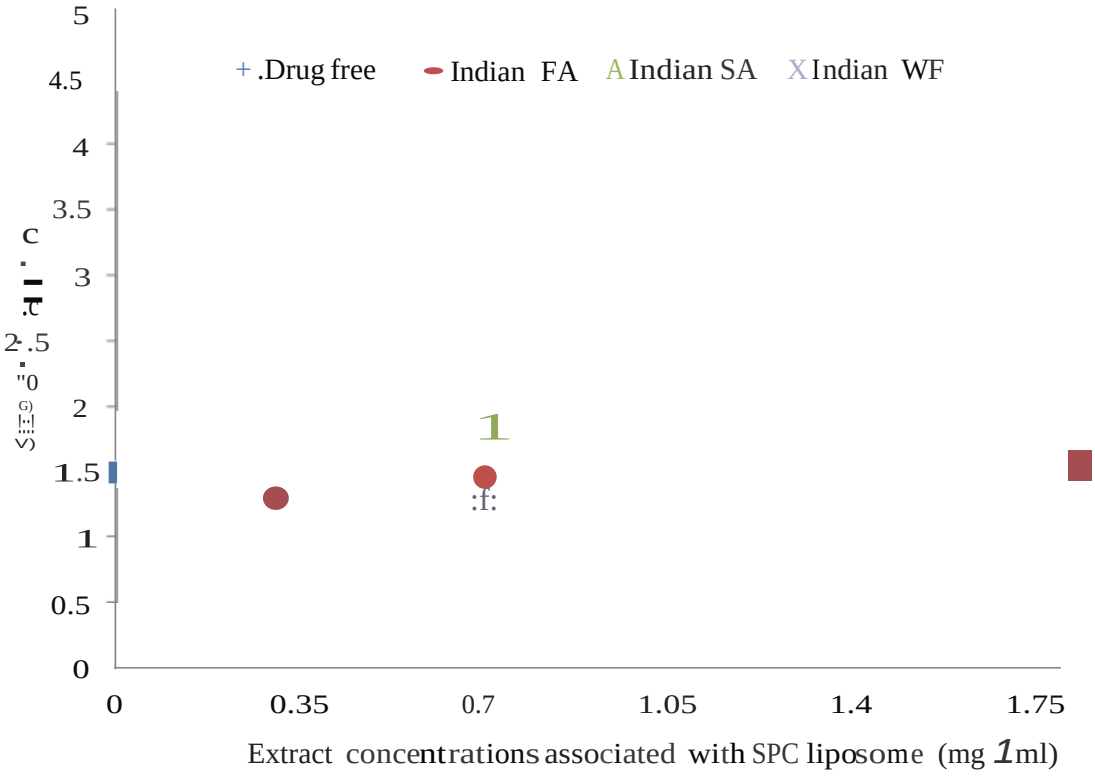
The HSPC liposomal formulations of Indian *Momordica charantia* (SA) demonstrated a significantly ($P < 0.05$) larger size with the increasing the extract concentration (0.35 – 1.75 mg / ml) (Figure 3.5 C). The same observation was seen for the FA extract but up to 1.4 mg/ml (1 -1.75 mg / ml).

When using HSPC liposomes, the range of concentrations of FA and SA extracts had only minor but not significant differences ($P > 0.05$) in the span with all values being in the range between approximately 2-3 (Figure 3.5D).

A)


+ Drug free
Indian FA
A Indian SA
X Indian WF
8
7
6
5
4
3
2
1
0
0
0.35
0.7
1.05
1.4
1.75
Extract concentrations associated with SPC liposome (mg / ml}

B)


+ .Drug free
Indian FA
A Indian SA
X Indian WF
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
0.35
0.7
1.05
1.4
1.75
Extract concentrations associated with SPC liposome (mg 1ml)

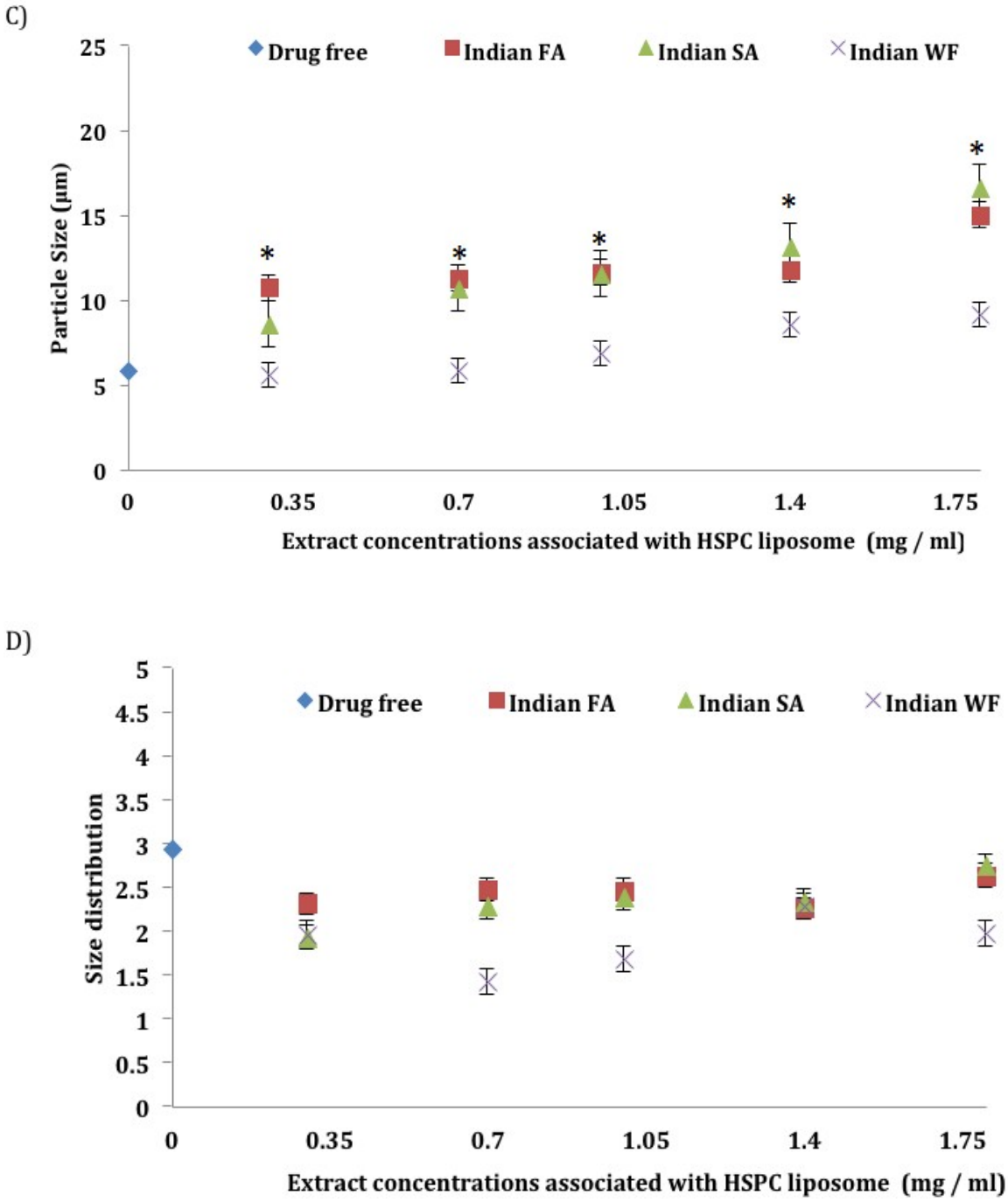


Figure 3.5: Plots showing the characterisation of Indian *Momordica charantia* extracts (FA, SA and WF) associated liposomes- A) Particle size and B) Size distribution of SPC liposome, C) Particle size and D) Size distribution of HSPC liposomes. Data are mean ± S.D, n=3 *P < 0.05 for *Momordica charantia* liposomal formulations compared to drug free liposomal formulation.

3.5.3 Particle size and size distribution of SPC and HSPC liposome formulations including PTX

Figure 3.6 (A) illustrates the relationship between paclitaxel concentration in formulation and the particle size of SPC and HSPC liposomes. The concentrations of Paclitaxel used were 0, 0.35 mg, 0.7 mg, 1.05 mg, 1.4 mg and 1.75 mg / ml. The size of Paclitaxel liposomes using SPC phospholipid was in the range of 3 – 8.9 μm, and HSPC phospholipid was in the range of 5.81 – 11.21 μm (Figure 3.6B). Moreover, the increase in paclitaxel concentration resulted in an increase in liposome size regardless of the phospholipid type. Previous study by Balasubramanian et al. (1994) also agrees with the present findings. Paclitaxel has a tendency to undergo concentration-dependent aggregation in the hydrophobic environment causing intermolecular hydrogen bonding (Balasubramanian et al. 1994; Wenck et al. 1996). Van der Waal‘s force interaction between lipid bilayer and Paclitaxel causes condensation and causes closer packing of the lipid bilayer decreasing the permeability and fluidization of the bilayers and increase aggregations of liposomes (Kan et al. 2011). For each paclitaxel concentration, the size of HSPC liposomes was significantly ($P < 0.05$) larger than that of SPC vesicles (Figure 3-6 A), possibly because HSPC has much higher phase transition temperature than SPC. It has been previously reported that saturated lipids cause rigidity of the liposome membranes (Maurer et al. 2001). It is well known that HSPC contains high proportions of saturated lipids, possibly this made PTX liposomes more rigid and the disaggregation of liposomes during hydration less efficient.

In this study, the influence of phospholipid type and paclitaxel concentration on liposome size distribution was also investigated by measuring the span of the different formulations (Figure 3.7 B). The span value of Paclitaxel formulation using

SPC was relatively and significantly ($P < 0.05$) higher than that of the drug-free formulation ($P < 0.05$). When paclitaxel was incorporated in the formulations, for each drug concentration, SPC liposomes had significantly higher span values than HSPC liposomes ($P < 0.05$) (Figure 3.7B). This finding was unexpected since the larger size measurements of HSPC liposomes (Figure 3.6 A) may initially suggest that HSPC liposomes are more liable to aggregation and subsequently may have higher span values. It seems likely that HSPC liposomes have aggregated but that was followed by fusion of the small vesicles with the larger ones. Hence this resulted in an upward shift in the size but the size distribution became narrower (i.e. span decreased). It has been reported that lipids may precipitate by cooling which might lead to larger size or heterogeneity of the final liposomes (Kumar and Spandana, 2011). It is possible that slight decreases in the temperature of HSPC liposomes during size measurement have caused some aggregation and subsequent fusion of the vesicles, which was enhanced in the presence of the hydrophobic drug Paclitaxel. This explains the increase in size and decrease in span when paclitaxel concentration is increased.

These findings also indicate that paclitaxel has interacted with the liposome bilayers causing them to become more hydrophobic with a subsequent change in both size and size distribution of the vesicles. Changes of vesicle size and size distribution due to incorporation of paclitaxel in liposome formulations has, already been reported by Bernsdroff et al. (1999). They stated that paclitaxel incorporated into saturated phospholipids causes changes in the thermotropic phase behaviour of liposomes by reducing the fluidity of the gel phase. On the contrary, incorporation of paclitaxel in liposomes made from unsaturated phospholipids causes a decrease in the fluidity of the liposome bilayers (i.e. an increase in bilayer rigidity). The aqueous core of the

unsaturated phospholipid liposomes is larger than that of saturated phospholipid vesicles. Inclusion of hydrophobic drug such as paclitaxel to SPC (unsaturated phospholipid) liposomes may cause aggregation and rigidification of liposomes and might also cause paclitaxel to precipitate out and widen the size distribution of the formulation (Feng and Huang, 2000; Bernsdorff et al. 1999). On the contrary, HSPC has larger hydrophobic domain that easily incorporates paclitaxel without causing marked aggregation and therefore the size distribution of HSPC liposomes is narrower.

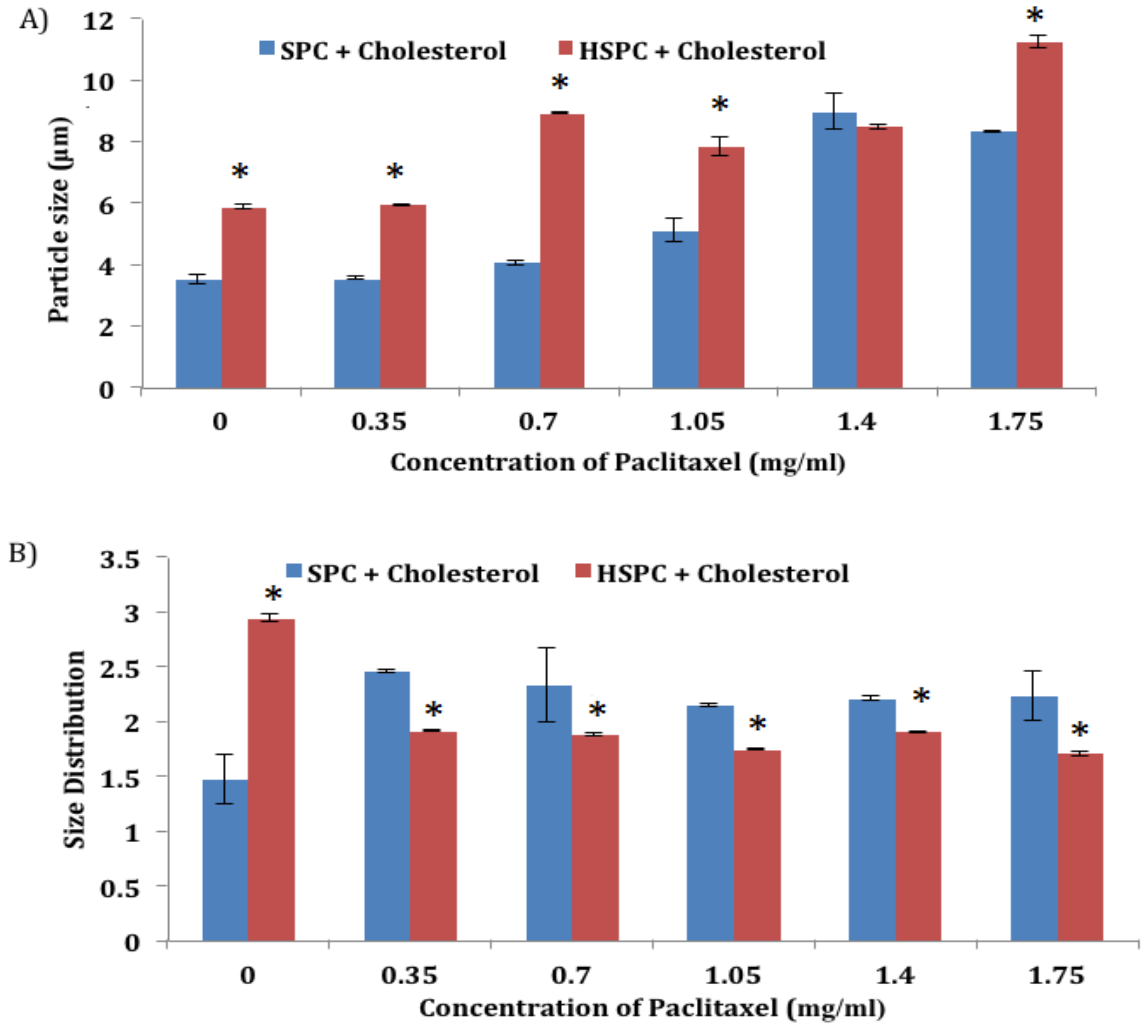


Figure 3.6: Bar chart showing characterisation of PTX associated liposomes- A) Particle size and B) Size distribution of SPC and HSPC liposomes. Data are mean ± S.D, n=3 *P < 0.05 for either SPC-PTX liposomal formulations or HSPC-PTX liposomal formulation compared to drug free liposome.

3.5.4 Zeta potential of liposomes prepared by conventional thin film method and proliposome method

In this section of the study the zeta potential of SPC and HSPC liposomes was investigated. Using no drug, the thin film hydration method was compared with the ethanol-based proliposome technology (Figure 3.7). Regardless of liposome preparation method and type of phospholipid, all formulations had negative zeta potential (Figure 3.7). However, there was a slight but significant ($P < 0.05$) difference was detected between different formulations (Figure 3.7). According to Shenoy et al. (2011), PC liposomes prepared by the conventional method generated neutrally charged liposomes since PC and cholesterol are neutral lipids. By contrast, liposomes prepared in this study (Figure 3.7) were slightly negative with zeta potential values being in the range of approximately -3.5 to – 6.5 mV. The slightly negative surface charge of liposomes (Figure 3.7) might be attributed to the presence of traces of negatively charge lipids in Lipod S-100 and Phospholipon® 90 H. When SPC was used to prepare liposomes, both methods produced vesicles having similar zeta potential values ($P > 0.05$). By contrast, when HSPC was used, significantly but slightly higher negative zeta-potential measurements ($P < 0.05$) were demonstrated for the liposomes generated from ethanol-based proliposomes (Figure 3.7). The presence of a charge on liposomes may reduce their aggregation when compared to neutral liposomes. Smaller particles with higher zeta potential may exhibit higher stability by prevention of aggregation but can also form larger and irregular shape particles (Jong and Keun, 1999). This overall indicates that HSPC liposomes prepared by using the proliposome method might be a bit more stable than thin-film hydrated vesicles in terms of stability upon storage. Further studies are required in future to investigate the validity of this assumption.

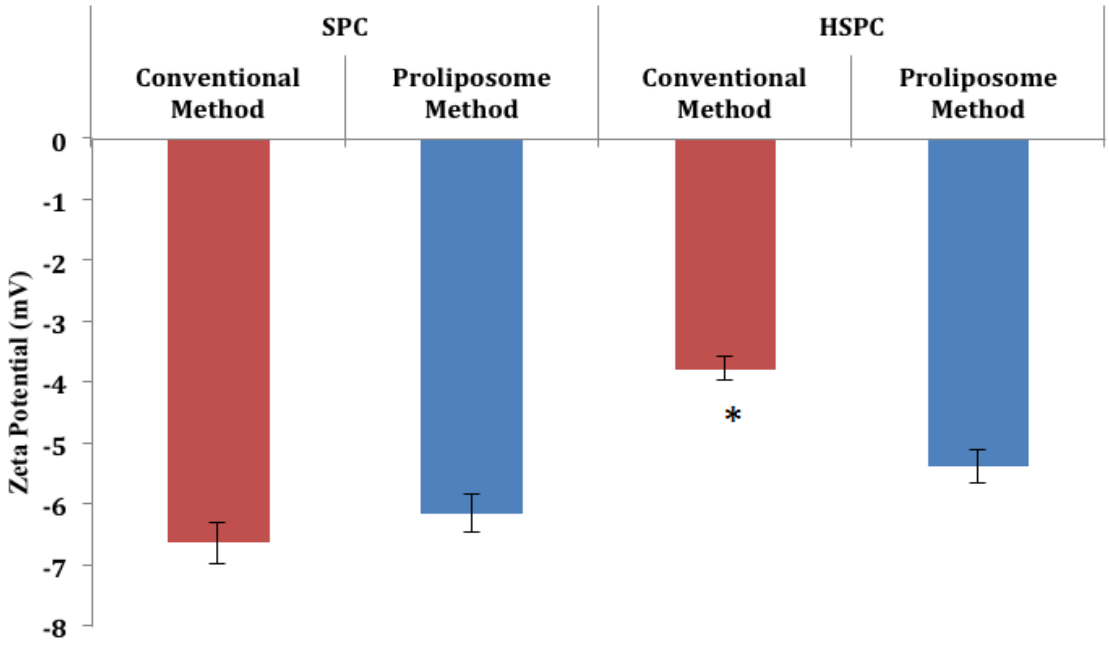


Figure 3.7: Bar charts showing a comparison of surface charges of the drug free liposomes prepared by conventional and ethanol-based proliposomes methods. Data are mean ± S.D, n=3 *P < 0.05 for zeta-potential of conventional method compared to proliposome method.

3.5.5 Zeta potential of liposomes made by hydration with the Momordica charantia extracts

3.5.5.1 African *Momordica charantia* extracts

When no extract was used for hydrating the phospholipids the zeta potential was around -6 mV and -7.5 mV for SPC and HSPC respectively (Figure 3.8). For SPC liposomes, upon using ascending extract concentrations, the negative zeta potential tended to neutralize by using the WF extract and become positive when using SA (+6 mV) or FA extracts (+4 mV) (Figure 3.8A). Regardless of plant organ, increasing extract concentrations (0.35 - 1.75 mg / ml) HSPC liposome formulations caused the negative surface charge of vesicle to convert from negative to positive. Thus, when using HSPC liposomes, positively charged liposomes with zeta potential measurements ranging between 2 mV and 7 mV were obtained as compared to the

extract-free liposomes having a negative zeta potential (-7.5 mV) (Figure 3.8B). This overall indicates that the African extracts may contain positively charged constituents that could interact with SPC and HSPC liposome bilayers, causing the surface charge to approach neutrality and revert to positive at higher extract concentrations.

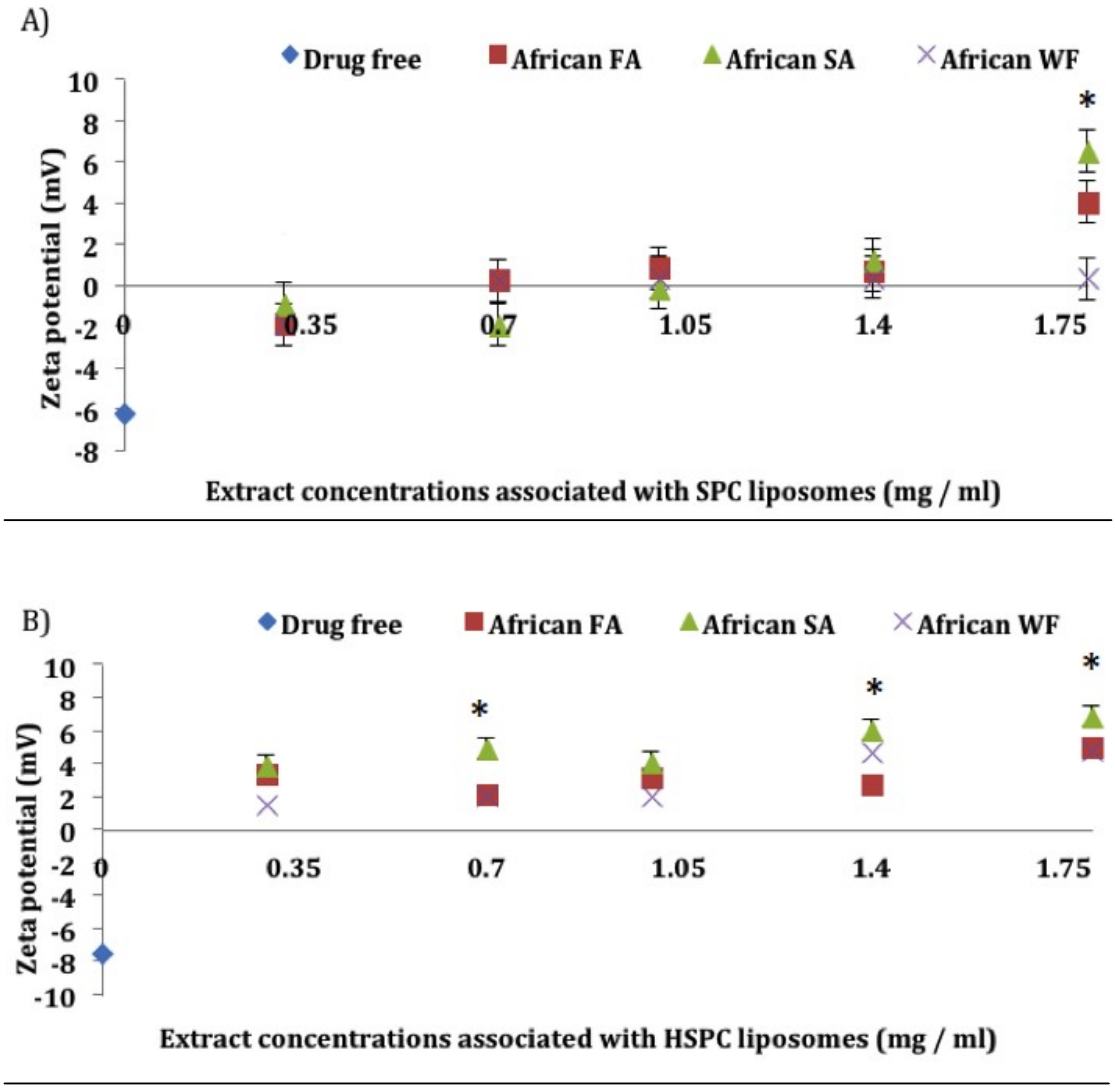


Figure 3.8: Plot showing the zeta-potential of African *Momordica charantia* extracts (FA, SA and WF) associated with liposomes A) SPC and B) HSPC. Data are mean ± S.D, n=3 *P < 0.05 for zeta-potential of African FA compared to African SA.

3.5.5.2 Chinese *Momordica charantia* extracts

The zeta potential of the increasing concentrations of Chinese extract in SPC liposomal formulations was dependent on both phospholipid type and extract concentration, (Figure 3.9). For SPC liposomes, slightly negative or neutral surface charge of liposomes ranging between -3.86 mV to +0.26 mV were measured, as compared to extract free liposomes which had a negative zeta potential (-6 mV) (Figure 3.9A). This indicates that Chinese *Momordica charantia* extracts has some positively charged constituents which is consistent with the findings for the African extract of the fruit.

However, with HSPC liposomes, inclusion of the extract resulted in higher variability in the measurements. WF extract generated neural liposomes and SA extract generated positively charged liposomes ranging between +0.36 mV and +4.5 mV. In contrast, the zeta potential measurements for the FA extract formulations were negative and in the range from -0.13 mV to -8 mV as compared to the extract-free liposomes (-7.5 mV) (Figure 3.9B). Overall, surface charge reversion from the negative range to positive range was more apparent and highly consistent for the African extracts compared with the Chinese extracts of the fruit (Figure 3.8; Figure 3.9). These findings suggest that the proportion or types of fruit constituents are different when the fruit origin is different, indicating a significant role of climatic conditions on constituents of *Momordica charantia*.

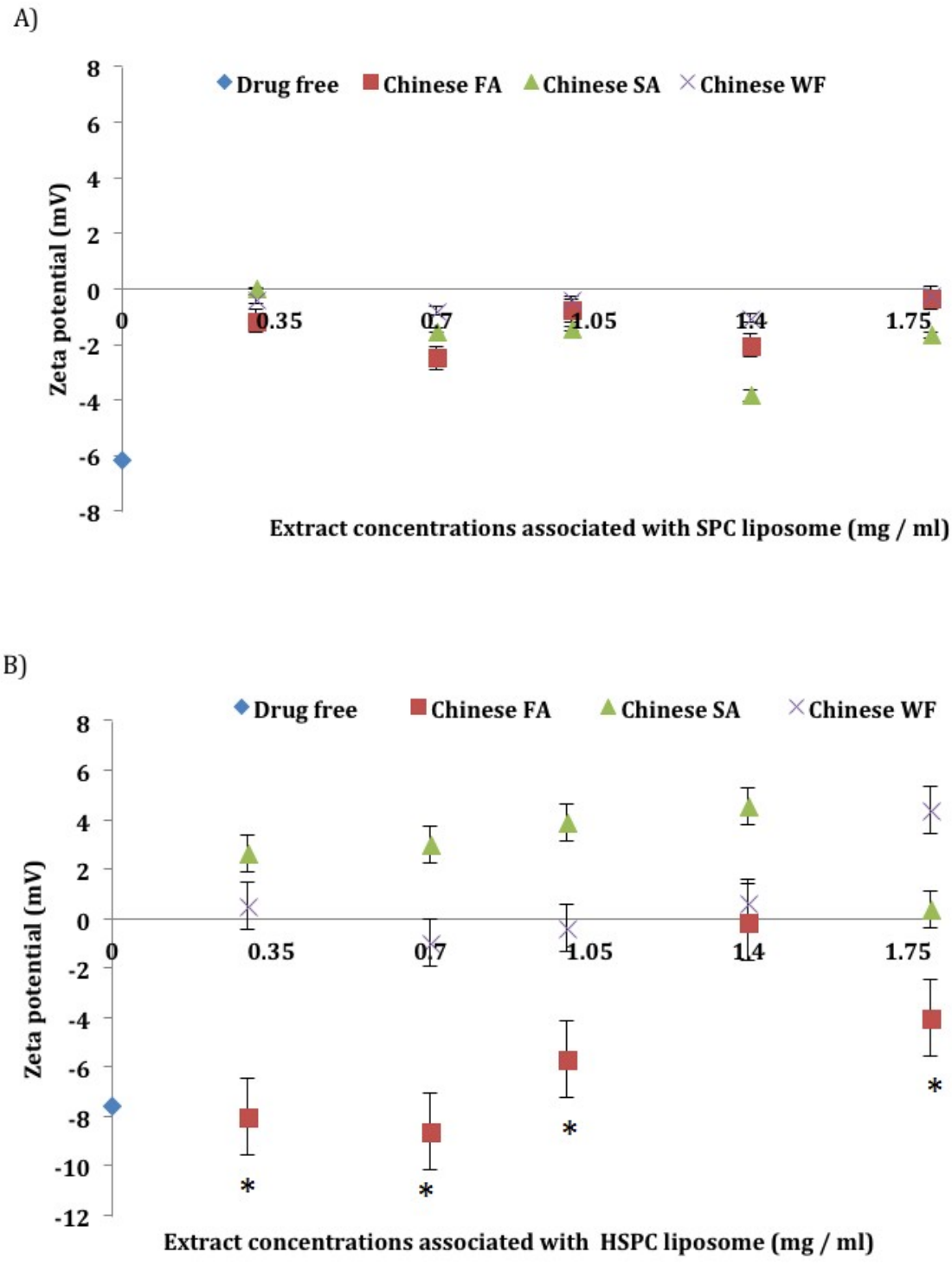


Figure 3.9: Plots showing the zeta-potential of Chinese *Momordica charantia* extracts (FA, SA and WF) associated with liposomes A) SPC and B) HSPC. Data are mean ± S.D, n=3 *P < 0.05 for Chinese FA compared to the Chinese SA.

3.5.5.3 Indian *Momordica charantia* extracts

The influence of extract concentration on the zeta potential of liposomes using Chinese *Momordica charantia* (Figure 3.10) was highly similar to that produced by the African extract (Figure 3.8) especially when the phospholipid employed was HSPC. This indicates that the trend shown by the Chinese extract was different from the African and Indian extracts of *Momordica charantia*. For SPC liposomes, the increasing concentration of Indian *Momordica charantia* extract (FA), from lower to higher concentrations (0.35- 1.4 mg /ml) showed significant ($P < 0.05$) gradual change from slightly negative towards almost neutral surface charge (-1.5 mV towards 0.5 mV) and at the highest concentration (1.75 mg/ ml) the liposomes suddenly had negative zeta potential measurement (-3 mV). The negative charge was less intense when compared to that of extract-free liposomes (-6 mV) (Figure 3.10 A). By contrast, the SA and WF extract liposomes continued towards having a slightly positive zeta potential as the extract concentration was increased (Figure 3.10A).

The trend by using HSPC liposomes was more predictable and similar for all fruit organs. The dispersion of Indian *Momordica charantia* extracts produces cationic liposomes with increasing the extract concentration (0.35 – 1.75 mg / ml). The positive surface charge of the liposomes was ranging from 2 – 6.7 mV compared to approximately -8 mV for the extract free formulation (Figure 3.10B). This may indicate that the Indian extracts of *Momordica charantia* contain positively charged constituents that can interact with HSPC liposomes, rendering vesicle surfaces positively charged. This is very interesting since the presented trend suggest that these liposomes are cationic and hence future investigations may involve these formulations in gene therapy research.

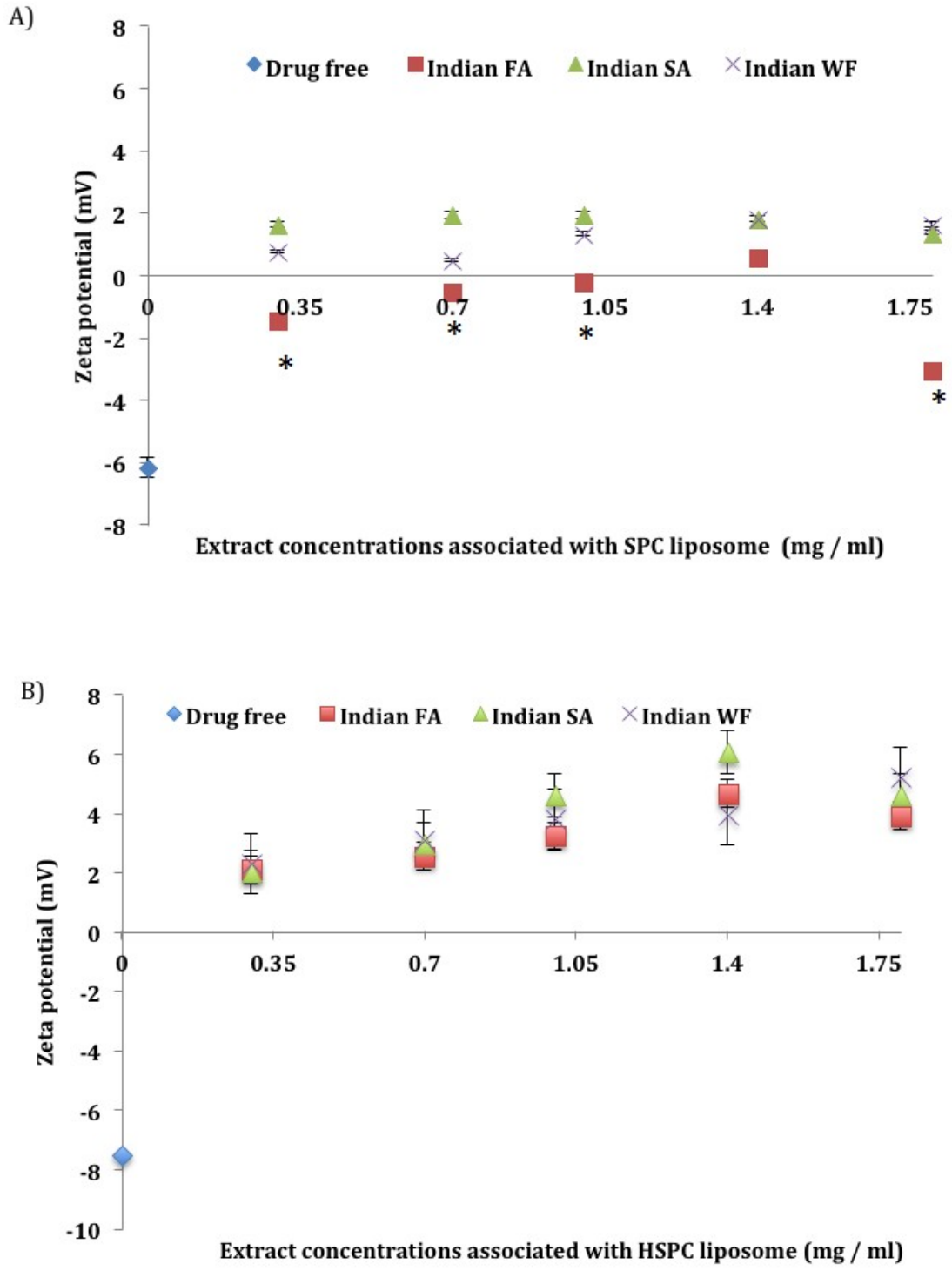


Figure 3.10: Plots showing the zeta-potential of Indian *Momordica charantia* extracts (FA, SA and WF) associated with liposomes A) SPC and B) HSPC. Data are mean ± S.D, n=3 *P < 0.05 for Indian FA compared to Indian SA.

3.5.6 Surface charge of the SPC and HSPC liposomal formulations prepared from ethanol based proliposome technology on inclusion of PTX.

In Figure 3-11 the surface charge of PTX liposomes incorporated with SPC phospholipid showed a neutral surface charge at the lower extract concentration (0.3 mg / ml) and a gradual increase in the negative charge of the particles from lower to higher concentrations (0.7- 1.75 mg / ml) ranging between -1.5 mV to 5 mV. Similarly, the surface charge of PTX liposomes encapsulated with SPC phospholipid showed significant ($P < 0.05$) neutral charged particle at the lower concentration (0.3 mg / ml). The other concentrations from 0.7-1.75 mg / ml showed slightly less negatively charged particle in the range of – 3.6 mV to -7 mV. The results showed no consistency in either increase or decrease of the surface charge and it was still less negatively charged as compare to drug free HSPC liposomes. PTX liposomes generated from SPC phospholipid produced slight less negatively charged particle as compared to PTX liposomes generated from HSPC phospholipid. Paclitaxel itself is not a pH sensitive cancer chemotherapeutic agent. The negative charge of the liposomes might be attributed due to the presence of the carboxylic group. The negatively charged liposomes are not affected by the pH difference and causes negligible zeta-potential changes (Yanga et al. 2009). Therefore, on inclusion of PTX in the negatively charged liposomes, regardless of the phospholipid does not affect the zeta-potential due to changes of the pH environment. The role of liposome membrane composition in the stability of paclitaxel-containing formulations is understood partially for neutral and anionic liposomes (Campbell et al. 2001)

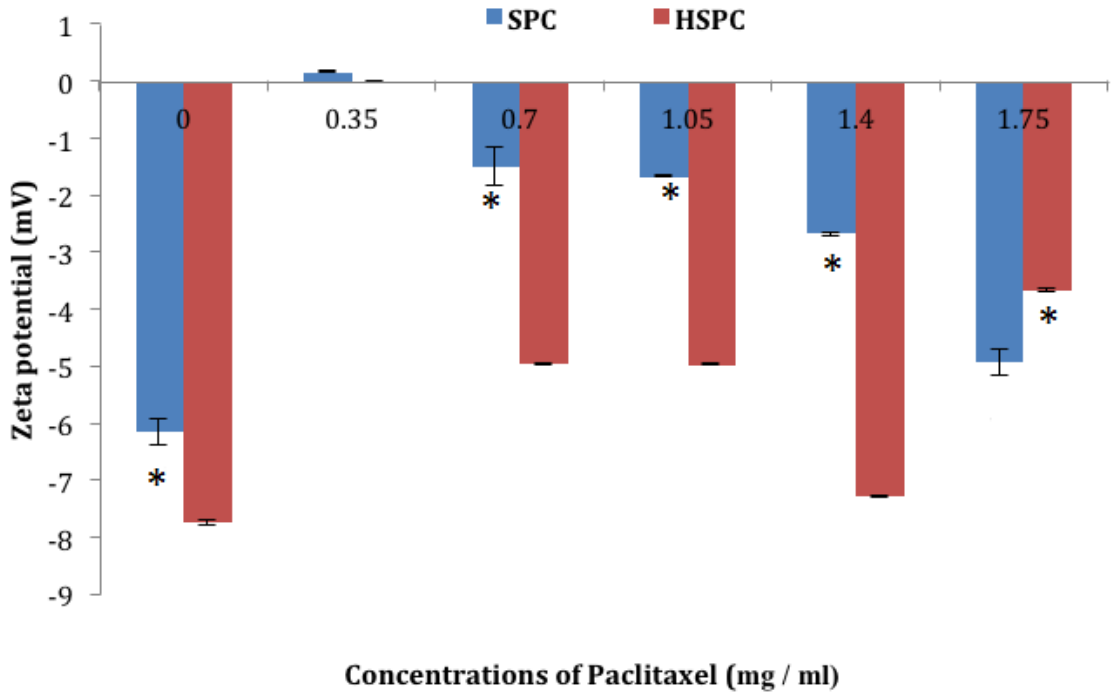


Figure 3.11: Bar charts showing zeta potential of Paclitaxel liposomes prepared from SPC and HSPC phospholipids by the ethanol-based proliposome. Data are mean ± S.D, n=3 *P < 0.05 for HSPC compared to SPC.

3.5.7 Morphology of liposomal vesicle originated by Transmission Electron Microscopy.

3.5.7.1 Drug-free liposomes

TEM as shown in Figure 3.12 represents the morphology of drug-free liposomes prepared from SPC and HSPC phospholipids (phospholipid: cholesterol; 4:1). Figure 3.12A represents SPC drug free liposome having large oligolamellar vesicles with large aqueous core. By contrast, HSPC liposomes seemed to be of smaller aqueous cores and having higher polydispersity as a group of small vesicles were seen to aggregate with one large liposomes (Figure 3.12).

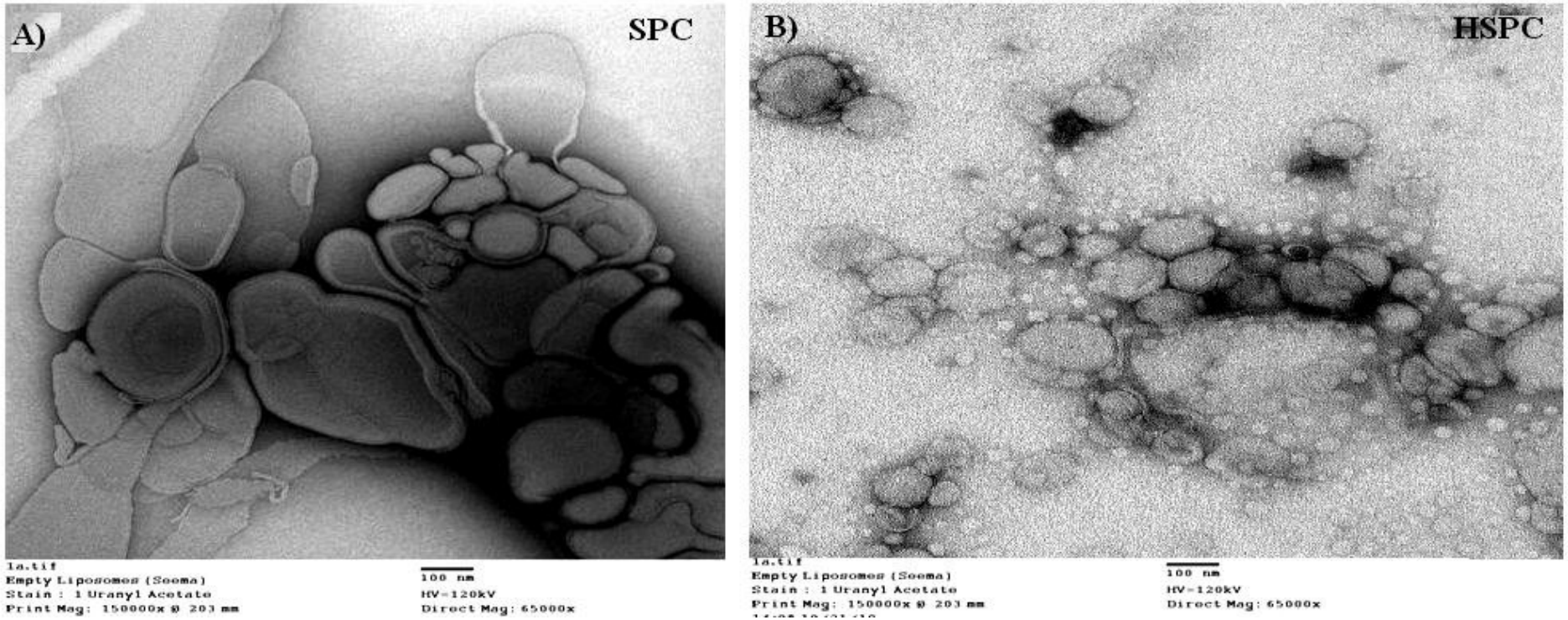


Figure 3.12: Electron micrographs of the drug free liposomes prepared from proliposome showing A) SPC liposomal formulation having oligolamellar structure with large aqueous cores b) HSPC liposomal formulation having mixture of oligolamellar and multilamellar vesicles. These micrographs are typical of three (n=3) each different experiments.

3.5.7.2 Liposomes on inclusion of *Momordica charantia* extracts

TEM as shown in Figure 3.13 represents the morphology of SPC and HSPC liposome upon using concentrations (0.35 mg / ml to 1.75 mg / ml) of *Momordica charantia* extracts (FA, SA and WF) from Africa, India and China. On the incorporation of *Momordica charantia* extracts from different countries in SPC or HSPC liposomes were oligo-lamellar and multilamellar (Figure 3.13A and Figure 3.13B). TEM also shows that HSPC vesicles have tendency to aggregate and fuse as huge vesicles were observed as compared to the SPC vesicles.

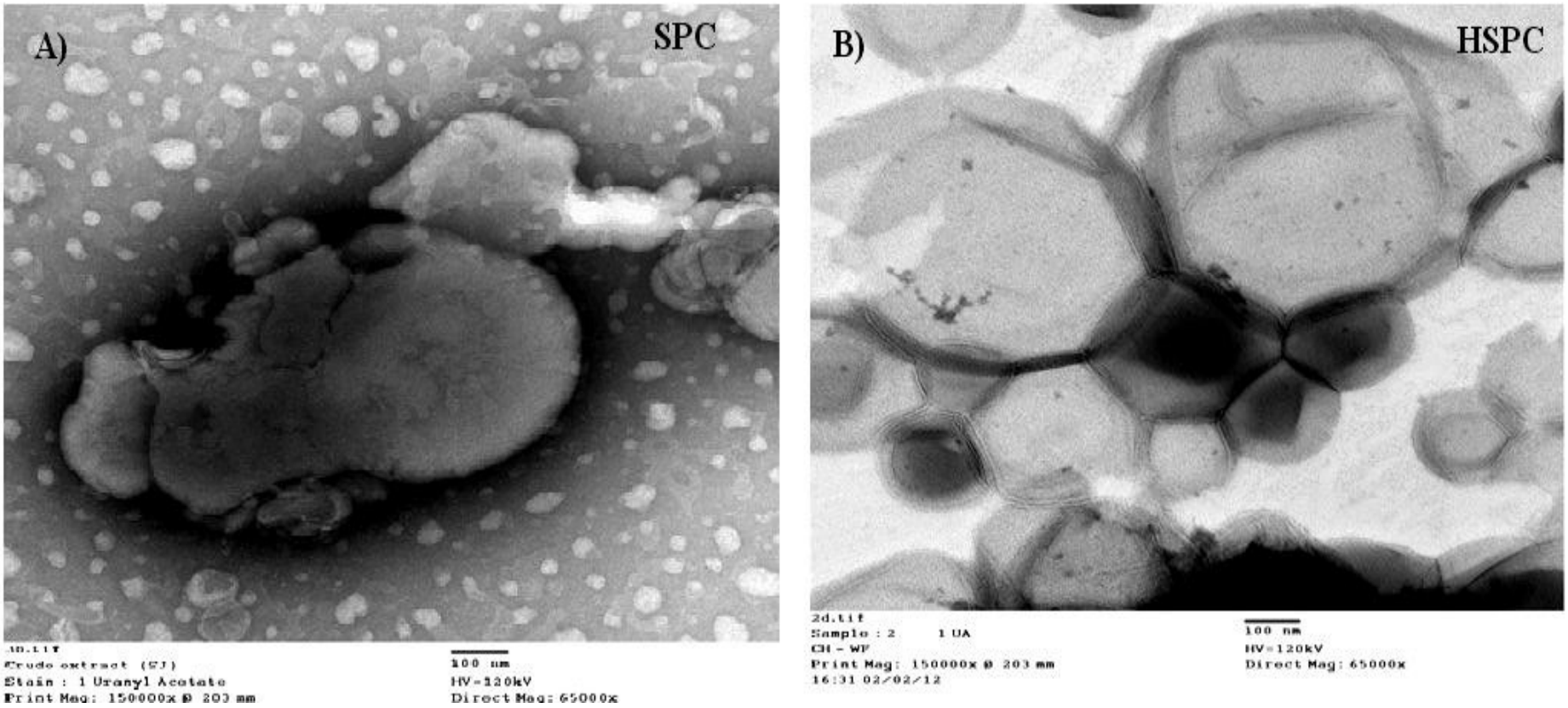


Figure 3.13: Electron micrographs of the *Momordica charantia* liposomal formulation prepared from proliposome showing A) SPC liposomal formulation and b) HSPC liposomal formulation having mixture of oligolamellar and multilamellar vesicles. Micrographs are typical of three each different experiments.

3.5.7.3 TEM of liposomes on inclusion of Paclitaxel

TEM as shown in Figure 3-14 represents the morphology of SPC and HSPC liposome upon using concentrations (0.35 mg / ml to 1.75 mg / ml) of PTX. On the incorporation of PTX extracts in SPC or HSPC liposomes were Oligo-lamellar and multilamellar (Figure 3.14A and Figure 3.14B). TEM also shows that SPC vesicles have tendency to aggregate and fuse as huge vesicles were observed as compared to the HSPC vesicles. HSPC liposome has more stable size distribution as compared to SPC liposomes.

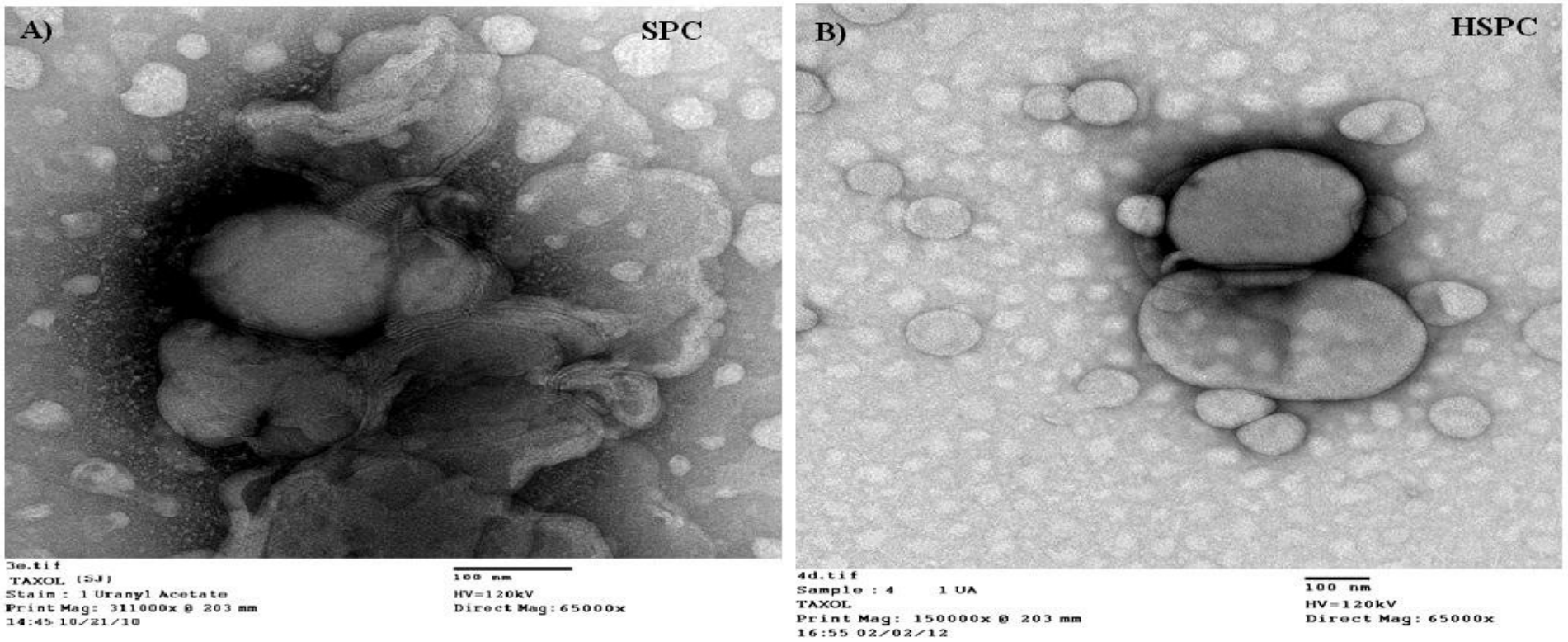


Figure 3.14: Electron micrographs of the PTX liposomal formulation prepared from proliposome showing A) SPC liposomal formulation and b) HSPC liposomal formulation having mixture of oligolamellar and multilamellar vesicles. Micrographs are typical of three each different experiments.

3.6 Conclusion

SPC and HSPC liposomes prepared by conventional method produce large size negatively charged drug free liposome as compared to liposomes prepared by proliposome method. On the inclusion of *Momordica charantia* extracts (FA, SA and WF) from Africa, China and India, HSPC phospholipid generated large size vesicles as compared to SPC. The change of size of the particle might be due to the difference in the phase transition temperature (Tm) of both the phospholipids. SPC phospholipid has low phase transition temperature, whereas, HSPC phospholipid has high phase transition temperature. Therefore, during cooling process of the liposomes formulation might be causing the phospholipid to form aggregates. SPC liposome generates large size distribution as compare to HSPC liposome may be due to larger entrapment of the hydrophilic material and formation of aggregation of SPC liposome. The surface charge of SPC and HSPC liposome were less negative, neutral or positive as compared to the drug free liposomes. The surface charge of the liposome reverts back to positive direction on the inclusion of increasing concentration of the *Momordica charantia* extract. This result indicates that the extract contains some positive constituents. On inclusion, of paclitaxel hydrophobic molecule in the HSPC phospholipid produces large size liposome as compare to SPC phospholipid. While, both the phospholipid generated large size liposome as compare to the drug free liposome. SPC produce higher span value on inclusion of PTX. As SPC has less hydrophobic domain to accommodate hydrophobic drug PTX and might be causing the drug to precipitate out. HSPC gave less span value on inclusion of PTX drug as compared to drug free HSPC liposomes. Regardless of the phospholipids, both the formulation produces anionic liposomes on the inclusion of

PTX drug. But, the surface charge of both the phospholipid was slightly less as compared to the drug-free liposome generated from SPC and HSPC by proliposome method.

Chapter 4. Tissue culture studies to investigating cytotoxicity against glioma cell

4.1 Introduction

The aim of this chapter was to use phospholipids derived from soy to manufacture solvent-based proliposomes, which could be used to generate phospholipid vesicles (liposomes) when aqueous phase (e.g. water) is added. Water-soluble materials were extracted from the fruit *Momordica charantia* and the soluble extracts were used to hydrate the proliposomes and convert them into liposomes. The efficacy of the resultant liposome formulations on the viability of glioma cells by MTS cytoxic assay was investigated.

4.2 Materials

4.2.1 Equipment and materials used in cell culture

New Brunswick Scientific ($CO_2$81R) - water jacketed CO_2 incubator, water bath, laminar flow hood, inverted phase contrast microscopes (Zeiss Axiovert, Germany), weighing balance, Tecan plate reader (Tecan Austria GmbH, 2004 model), refrigerator, freezer (- 20°C), deep freezer (-80°C), centrifuge machine, electrical aspirator, vortex mixer, 2, 20, 200 and 1000 µl pipettes, motorized pipette controller, 75 cm^2/25 cm^2 culture flasks, sterile forceps, aluminium foil, 5 and 10 ml disposable plastic pipettes, 15 ml and 50 ml centrifuge tubes (Fisher Scientific, UK), Cryovials, –Mr. Frosty‖ freezing container and liquid nitrogen, DMSO (Sigma Aldrich, UK), 0.22 µm and 0.44 µm sterile filters (Fisher Scientific, UK), syringes (Fisher Scientific, UK), pasteur pipettes (Fisher Scientific, UK), universal bottles, 5 ml volumetric flask, glass funnel, sterile spatula and clear bottom white 96 well plates (Grenier, UK).

4.2.2 Glioma cell lines

Table 4.1 represents various grades of cancerous and non-cancerous glial cells on the basis of their clinical pathological condition and origins. They are bought from different companies for the tissue culture experiments.

Table 4.1: Some types of brain cell lines used in the study with grades and companies supplying them.

Cell lines	Diagnosis	Grade	Company
1. 1321N1	Astrocytoma	II	ECACC,UK
2. U87-MG	Glioblastoma astrocytoma	IV	ECACC, UK
3. GOS-3	Mixed astro-oligodendroglioma	II/III	DSM2, Germany
4. SVGP12	Normal	-	ECACC, UK

4.2.3 Liposomes prepared from ethanol-based proliposome

The preparation of empty liposomes or liposomes including *Momordica charantia* extracts (FA, SA and WF) from different countries or by using PTX drug were performed by using the proliposome method as described in section 2.4.1 and 2.4.2

4.2.4 Media and supplements required for cell culture

DMEM (Dulbeco‘s Modified Eagle‘s Medium), MEM (Minimal Essential Medium), Foetal bovine serum (FBS), trypsin, L-Glutamine, non-Essential Amino Acid (NEAA) and sodium pyruvate were all bought from Lonza, UK.

4.2.5 Chemicals and Reagents

- Ethanol and Methanol (Fisher Scientific, UK)
- Phosphate Buffered Saline (PBS) tablets (Sigma, Aldrich, UK) –2 tablets were dissolved in 400 ml of water and stored the solution at 4 °C
- CellTiter-Glo® luminescent Cell Viability Assay kit (Promega, UK) (Figure 4-7).

4.3 Methods

4.3.1 Composition of medium for the glioma cell lines used in the study

(i) GOS-3- Dulbecco‘s Modified Eagle Medium (DMEM-500 ml), 10% Foetal bovine serum (FBS-50 ml) and 4 mM L-glutamine (10 ml).

(ii) 1321N1 - Dulbecco‘s Modified Eagle Medium (DMEM-500 ml), 10% Foetal bovine serum (FBS-50 ml) and 2 mM L-glutamine (5 ml).

(iii) U87-MG and SVGP12 – Eagle‘s Minimal Essential Medium (EMEM-500ml), 10% Foetal bovine serum (FBS-50 ml), 2 mM L-glutamine (5 ml), 1% Non-Essential Amino Acids (NEAA- 5 ml) and 1 mM Sodium Pyruvate (5 ml).

4.3.2 Cell culture and passaging of the primary glial cells (1321N1, U87-MG, Gos-3 and SVGP12)

Culture medium, PBS and trypsin (sterile) were removed from the 4°C fridge and subsequently placed in a water bath at 37°C for 30 min in order to equilibrate. The laminar flow hood was turned on for 10 min prior to commencement of the

experiment in order to purge the air inside the cabinet. The cells were incubated at 37°C in an atmosphere of 5% CO_2 in air. Thereafter, the cells were examined under an inverted contrast microscope to note confluence and their general health. The flask was passaged when the cells had reached 70-80% confluence.

The medium was aspirated from the cultured flask and washed with sterile PBS (5 ml if 75 cm^2 flask and 2 ml if 25 cm^2 flask were used) in order to remove any traces of serum from the cells, thus preventing the possible inactivation of the trypsin by the serum components. Trypsin solution (2 ml if 75 cm^2 flask and 1 ml if 25 cm^2 flask) was pipetted in the flask and incubated at 37°C in an atmosphere of 5% CO_2 in air for 3-5 min until the cells started to detach. This was confirmed by microscopic investigation at intervals. The cells were left in trypsin for an appropriate length of time to avoid damage. Complete Growth Medium (2 ml) was then added to the flask to inactivate the trypsin and the cells were pipetted up and down to break down any large cell aggregates. The cell suspension was removed from the flask and placed into a 15 ml centrifuge tube followed by centrifugation at 1000 r.p.m for 5 min. After centrifugation, the supernatant was aspirated leaving the cells pellet at the bottom of the centrifuge tube. Depending upon the cell pellet density, a volume of 1 ml to 2 ml of fresh medium was suspended in the centrifuge tube. The pellet cells were mixed properly in the medium. A volume of 20 µl of trypsinised cell suspension and 80 µl of tryphan blue (1:5) were mixed together and counted using the haemocytometer employing 1 ml of cell suspension. The total volume of the cell suspension present in the centrifuge tube was required to make 1 or 2 flasks. The cell suspension was divided in one or more flasks (depending on the cell density) and fresh growth medium (10 ml to 12 ml if 75 cm^2 flask and 5 ml if 25 cm^2 flask) was added to the flasks, which was then placed in a 5% CO_2 incubator to continue cell growth. In

addition, the same cells were seeded in 96 well plates and approximately more than 1 million cells were frozen down in liquid nitrogen depending on the number of cells present per ml.

4.3.3 Cell Counting Method

A volume of 20 µl of cell suspension and 80 µl of tryphan blue were pipetted into a micro-centrifuge tube and mixed together. A cover slip was gently placed over the chambers of the haemocytometer, and a volume of 20 µl of the cell suspension was slowly pipetted against each short side of the cover slip so that the suspension could spread into each chamber. The haemocytometer was placed onto the stage of an inverted phase contrast microscope (10X) and focused on the centre 25 squares of one chamber. The numbers of cells in these squares were then counted. These steps were repeated for the other chambers. The average number of cells in the centre grid ($1\ mm^2$) of each chamber was calculated. This number was multiplied by 10^4 to obtain the number of cells per 1 ml of suspension. The total number of cells was calculated by multiplying the number of cells per 1 ml by the total volume of the cell suspension (Figure 4.1).

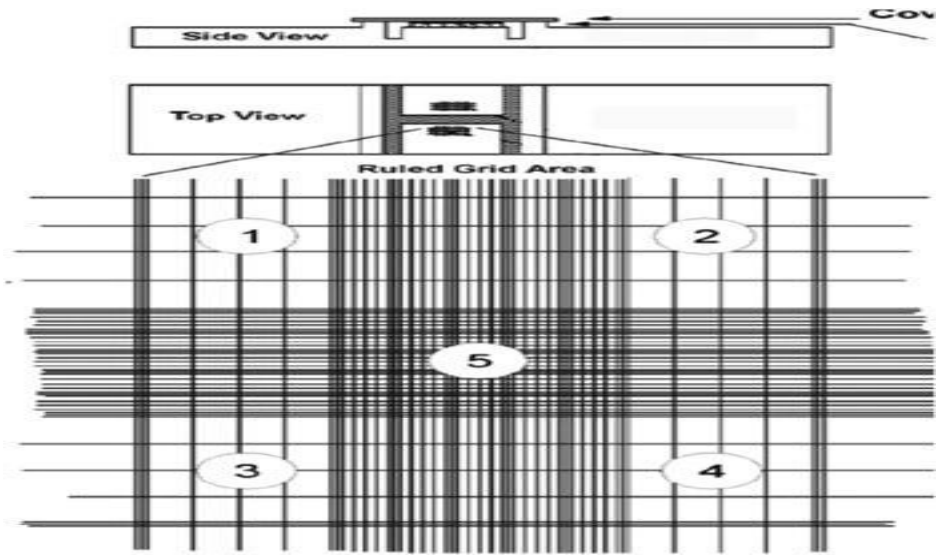


Figure 4.1: Diagram showing a hemocytometer for cell counting under objective 10X. (1,2,3,4 = Major grid line and 5 = Minor grid line of hemocytometer).

(Source: http://www.globalspec.com/reference/54403/203279/exercise-6-cell-count-by-hemocytometer-or-measuring-volume).

4.3.4 Growth curve

In this study, the standard growth curve of glioma cells was constructed. The master flask was passaged and cell count was noted. Approximately 0.23 x 10^6 (day-0) cells were taken and seeded in five sub-flasks. The sub-flasks were incubated at 37ºC in 5% of CO_2 incubator. Each sub-flask was passaged on regular basis from day-1 till day-9 and the cell count was noted down.

4.3.5 Plating of cell lines

Cell suspensions of 13121N1, Gos-3, U87-MG, and SVGP12 were acquired during passaging and were diluted in 1:10 ratio of cell suspension in the media. The required volumes of cell suspension and fresh medium volumes were calculated based on the cell count. The dilutions were made till 200 µl suspensions consisted of 2000 cells. A 96 well plate was taken and 200µl of suspension was added in each well. After the

plating was done, the plates were incubated at 37ºC for 24 hours in 5% CO_2 incubator.

4.3.6 Measurement of MTS assay

The plate was removed after 24 hrs of incubation in the 5% CO_2 incubator at 37ºC. The media in the plates were removed on sterile tissue paper. Another 150 µl of media and 50 µl of *Momordica charantia* (whole fruit, fruit or seed) extracts, Paclitaxel or liposomal dispersions of *Momordica charantia* or liposomal Paclitaxel were added to the wells and incubated in 5% CO_2 incubator at 37ºC for another 12 hours. The suspension from the plates was again removed and all the plates were washed with PBS. Again 200 µl of respective medium was added to all the plates and 20 µl of MTS reagent was added to all the plates in the absence of light. These plates were incubated at 37ºC in 5% CO_2 incubator for two hours. Absorbance was measured using the Tecan plate reader. The software used for absorbance assay was XFLUOR4GENIOSPRO Version: V 4.53 (Year, 2004). The measurements were set up by adjusting the absorbance at 492 nm, type of the plate was GRE 96ft pdf, temperature was set at 22.5ºC, and the shaking and settling time was conducted for 2 minutes. The cell viability was measured using cellTitier 96 aqueous absorbance assay which produces the result of cell viability assay (Figure 4.2).

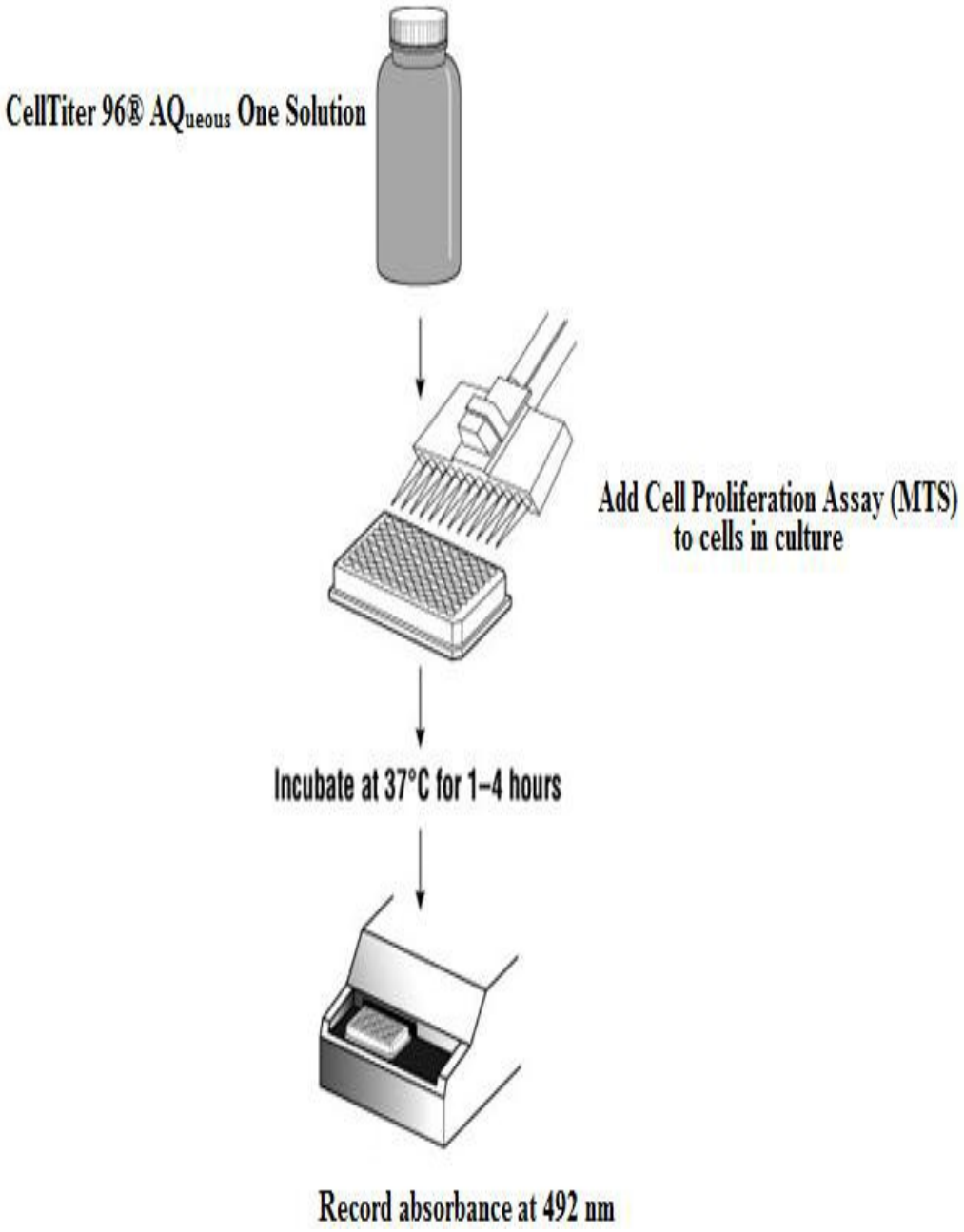


Figure 4.2: Flow diagram showing preparation and use of CellTiter 96® AQueous One Solution Cell Viability Assay Reagent (Source: http://www.promega.co.uk/resources/product-guides-and-selectors/protocols-and-applications-guide/cell-viability/?origUrl=http%3a%2f%2fwww.promega.com%2fresources%2fproduct-guides-and-selectors%2fprotocols-and-applications-guide%2fcell-viability%2f**).**

4.3.7 Statistical analysis

MTS cell viability assay experiments were performed in triplicate. Plotted the graph of cell viability absorbance in percentage measured at 492 nm and apoptosis luminescence on Y-axis and concentration of drug in percentage on X-axis. The results were expressed as mean ±Standard Deviation (SD) from three independent experiments. Statistical analysis was performed using SPSS. All the data were evaluated for unpaired variables to compare two or more groups by paired Student‘s ―t test‖ and analysis of variance (ANOVA). Values of $P < 0.05$ were considered to be statistically significant.

4.4 Results and Discussion

4.4.1 Growth curve study of the four different cell lines; 1321N1, Gos-3, U87-MG, and SVGP12

Figure 4.3 shows tryphan blue cell counting method revealing the growth pattern of the glioma cell lines 1321N1, GOS-3, U87-MG and the normal glial cells line SVGP12 in the culture for 10 days. The results observed in the graph show gradual proliferation of all the four cell lines from day 0 to day 6 and sudden decrease in the proliferation from day 7 to day 10. The proliferation of 1321N1 and Gos-3 is comparatively higher compared to SVGP12 and U87-MG cells at the initial stage. However, in a later stage, a sudden drop in the viability at a greater rate, as compared to, SVGP12 and U87-MG cells was observed. The drop in the viability of 1321N1 might be due to lack in the nutrition, in the media. Since most of the nutrients were utilized at the early stage of the growth period. Where as, U87-MG (i.e. the grade IV aggressive cells) and SVGP12 normal glial cells, initially grew slowly, so less

nutrients were utilized and the extra component used in the U87-MG and SVGP12 media provided extra nutrition. This extra nutrition helps the cells to keep proliferating for longer period of time. Low-grade glioma may appear to contain a high density of normal cells and a growth rate of less than 2%. Anaplastic glioma exhibits more atypical cells with pleomorphic nuclei, growth rate in 5-10% range but no evidence of necrosis. In contrast, U87-MG proliferated slowly at the initials days but seemed to show an increase in the proliferation rate after a few days. Gliomas with high growth rate are classified as necrosis (Alderson, 2002)

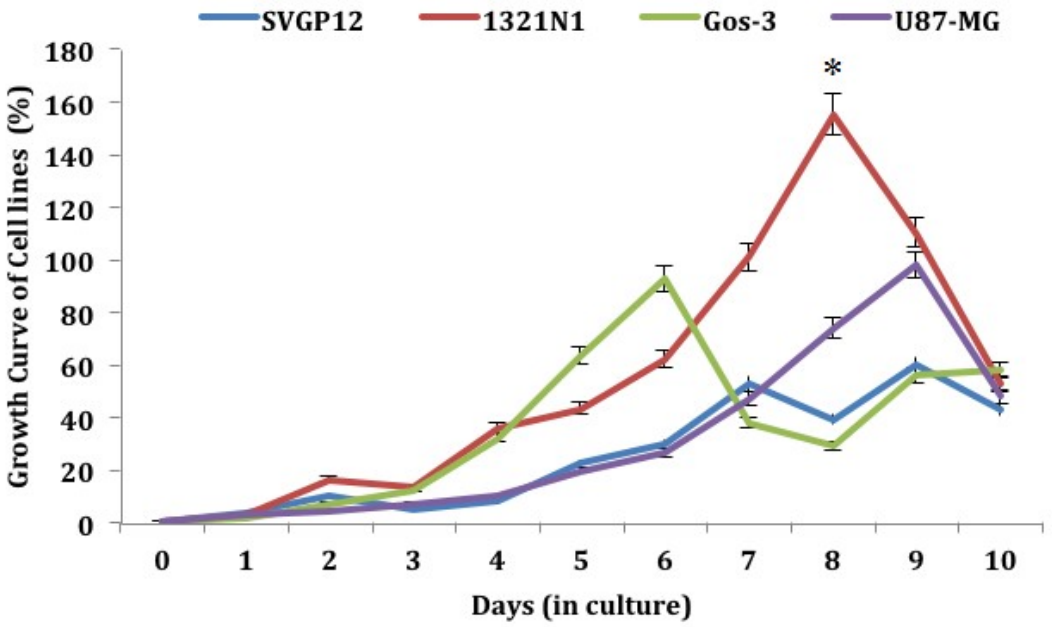


Figure 4.3: The growth curve of 1321N1, Gos,-3, U87-MG, and SVGP12 cell lines using trypan blue cell counting method under inverted microscope at magnification 10X. Data are mean ± S D; n=3; *P < 0.05.

4.4.2 Effect of water, 0.002% ETOH and extract free liposomes on the cell lines

The glioma cell lines 1321N1, Gos-3 and U87-MG and normal glial cell line SVGP12 were treated with water + 0.002% ethanol (ETOH), drug free HSPC

liposomes or drug free SPC liposomes as shown in the Figure 4.4. Water + 0.002% ETOH did not show any marked effect on the inhibition or growth of the normal or glioma cell lines. SPC phospholipid contains linoleic acid as the fatty acid. It has been reported that linoleic acid is toxic to tumour cells with little or no cytotoxic action on normal cells (Lu et al. 2010). The results shown in Figure 4.4 did not agree with the findings of Lu and co-workers (2010) since no marked effect of SPC on any of the cell lines was observed. In contrast, HSPC drug free liposome showed an increase in the viability of the normal glial cells and U87-MG high-grade glioma cells. HSPC liposomes contains high amount of palmitic and stearic acid. Saturated fatty acids such as palmitic acid and stearic acid show little or no tumour promoting effect (Tsubura, 2009), which agrees with our findings demonstrated in Figure 4.4.

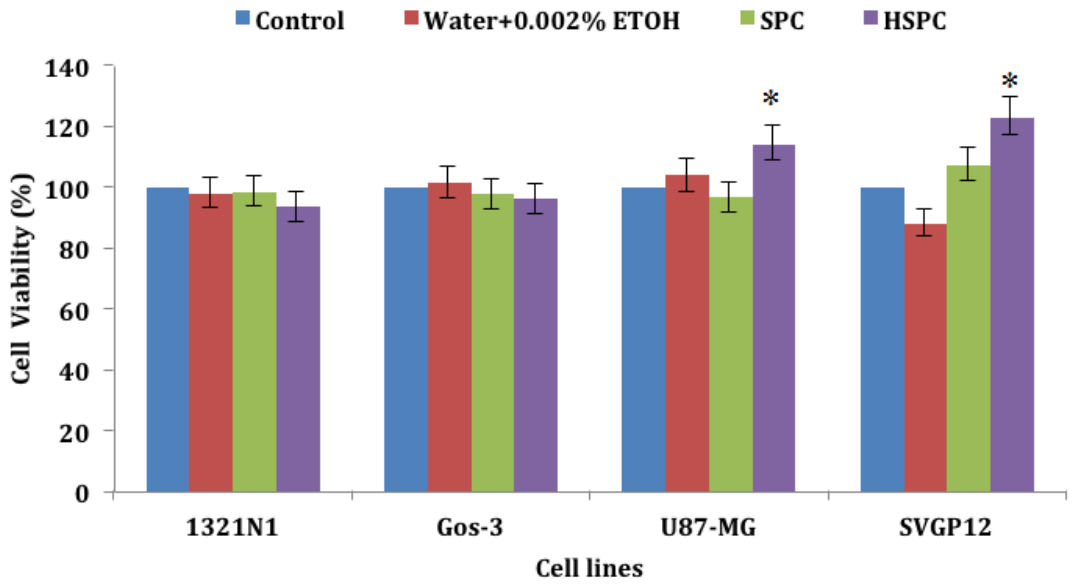


Figure 4.4: Bar chart showing treatment of glioma cell lines 1321N1, Gos-3 and U87-MG and normal glial cell SVGP12 with water +0.002% ETOH, SPC and HSPC liposomes. Data are mean ± S D; n=3; *P < 0.05 for treated cells compared to control.

4.4.3 Effect of the treatment of PTX and PTX inclusion in liposomes on the cell lines

PTX drug has a unique mechanism of action by suppressing microtubules that leads to mitotic arrest. However, PTX has a very low solubility in conventional aqueous vehicles (e.g. water). Liposomes were found to be a practicable approach for the therapeutic and effective use of PTX because they may improve its toxicological and pharmacological characteristics (Koshkina et al. 2001). Figure 4.5 shows that 0.35 mg / ml of PTX with and without liposomes, elicits inhibition of the cancerous (1321N1, Gos-3, U87-MG) and non-cancerous glial cell lines (SVGP12) as compared to the untreated cells. Low-grade glioma 1321N1, showed significant ($P < 0.05$) decrease in their growth as compared to the other cell lines investigated (Gos-3, U87-MG and SVGP12). The results have also indicated that the inclusion of PTX within either SPC or HSPC liposomes causes higher toxicity to the glioma cells as compared to PTX alone. On the other hand, either PTX with SPC or HSPC liposomes was less toxic to the glial cells as compared to PTX alone. Thus, result proved the advantage of using liposomes, which might be helpful to improve the therapeutic index of PTX (Figure 4.5). PTX was used as a positive control in the experiments. The PTX with and without liposomes showed maximum inhibition of cell as compared to *Momordica charantia* extracts from Africa, China or India as shown in Figures 4.5 to Figure 4.15.

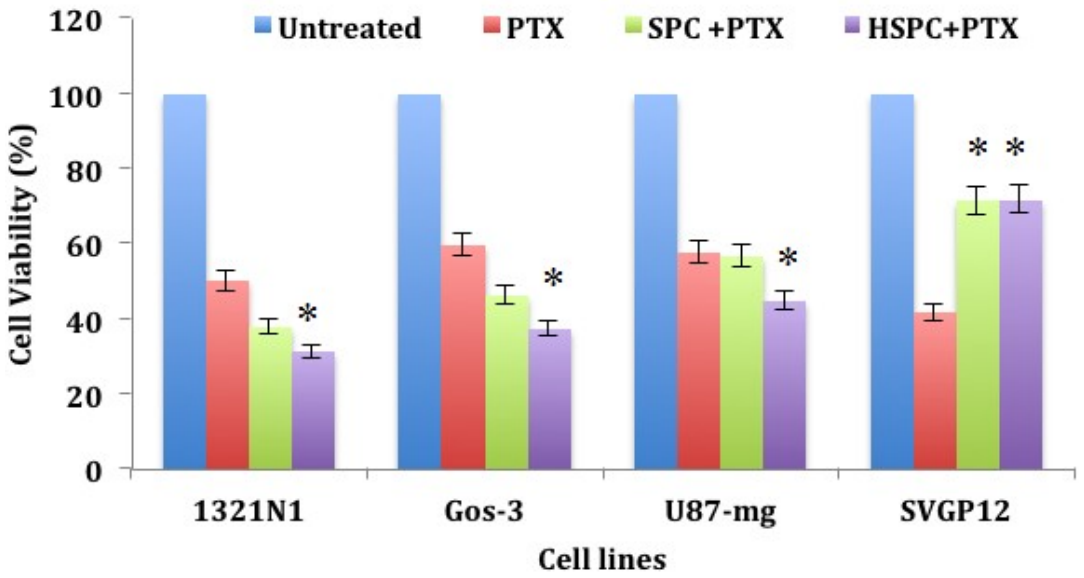


Figure 4.5: Bar charts showing treatment of glioma cell lines 1321N1, Gos-3 and U87-MG and normal glial cell SVGP12 with PTX, SPC-PTX and HSPC-PTX liposomes as compared to untreated cell lines (100%). Data are mean ± S D; n=3; *P < 0.05 for treated cells compared to untreated cells.

4.4.4 Effect of *Momordica charantia* extracts and *Momordica charantia* liposomes on the cell lines

Cancer prevention by the use of naturally occurring dietary substances is considered a practical approach to reduce the ever-increasing incidence of cancer (Baker, 2010). A vegetable commonly eaten in India, China and Africa called bitter melon (also known by the botanical name as *Momordica charantia*) has been shown in several previous studies to have a beneficial effect by reducing the levels of blood sugar and cholesterol (Anila et al. 2000; Raj et al. 2005; Islam et al. 2011). *Momordica charantia* contains glycosides such as mormordin, anti-oxidants, vitamin C, carotenoids, flavanoids, and polyphenols (Anila et al. 2000; Raj et al. 2005). It also contains terpenoids, isoflavones, anthocyanins, amino acids, minerals, and other antioxidants that might protect against cancer, cardiovascular diseases, diabetes and hypertension (Islam et al. 2011). *Momordica charantia* extract contains both chemo-preventive and suppressing agents (Wattenberg, 1992; Chiampanichayakul et al.

2001). In 1992, Wattenberg discussed chemo preventive agents as blocking agents that prevent carcinogens from either reaching or reacting with critical target sites, and as suppressing agents that prevent evolution of the neoplastic process in cells that otherwise would become malignant. Other phytochemicals that have been documented with cytotoxic activity are a group of ribosome-inactivating proteins named alpha- and beta-momorcharin, momordin, and cucurbitacin B. A chemical analog of bitter melon proteins was extracted and named MAP-30 and its discoverers reported that this compound is able to inhibit prostate tumor growth. Studies involves the antitumor activity of the entire plant of bitter melon studies reported that a water extract can block the growth of rat prostate carcinoma. Hot water extract of the entire plant can inhibit the development of mammary tumours in mice (Cunnick et al. 1990; About herb, 2007). A study by Saint Louis University Scientists has provided evidence that this plant triggers a chain of events on a cellular level that stops breast cancer cells from multiplying and also kills them (Garau, 2003). Figure 4.6 A and B shows the effect of *Momordica charantia* extract and *Momordica charantia* extract included within the liposome, on low grade 1321N1 and Gos-3 cell line, high grade glioma U87-MG and normal glial cell SVGP12. The extract was obtained from whole fruit of *Momordica charantia* of African origin and applied on the cell lines. The results illustrated the significant ($P < 0.05$) steady decrease in the proliferation of cells with increasing concentrations (0.35 -1.75 mg / ml) as compared to untreated cells. Inclusion of the extract within liposomes showed less proliferation of the glial non-cancerous cells as compared to the cancerous cells treated with the extract alone. Therefore, the results demonstrated that liposomes could be associated with large proportions of active components, facilitate their interaction with the cancerous cells and help to combat the cancer. Bisht et al. (2007) performed and experiment on

curcumin which is a yellow polyphenol extracted from turmeric. They showed that curcurmin is much less soluble in water. Thus, they increased the solubility of cucurmin by using liposomes, resulting in anti-cancer properties against pancreatic cancer (Bisht et al. 2007). Similarly, in the present study, liposomes may have improved the solubility of *Momordica charantia* components similar to cucurmin, flavonoid and large proteins etc. These active components in the liposomes have probably suppressed the proliferation of brain tumour cells.

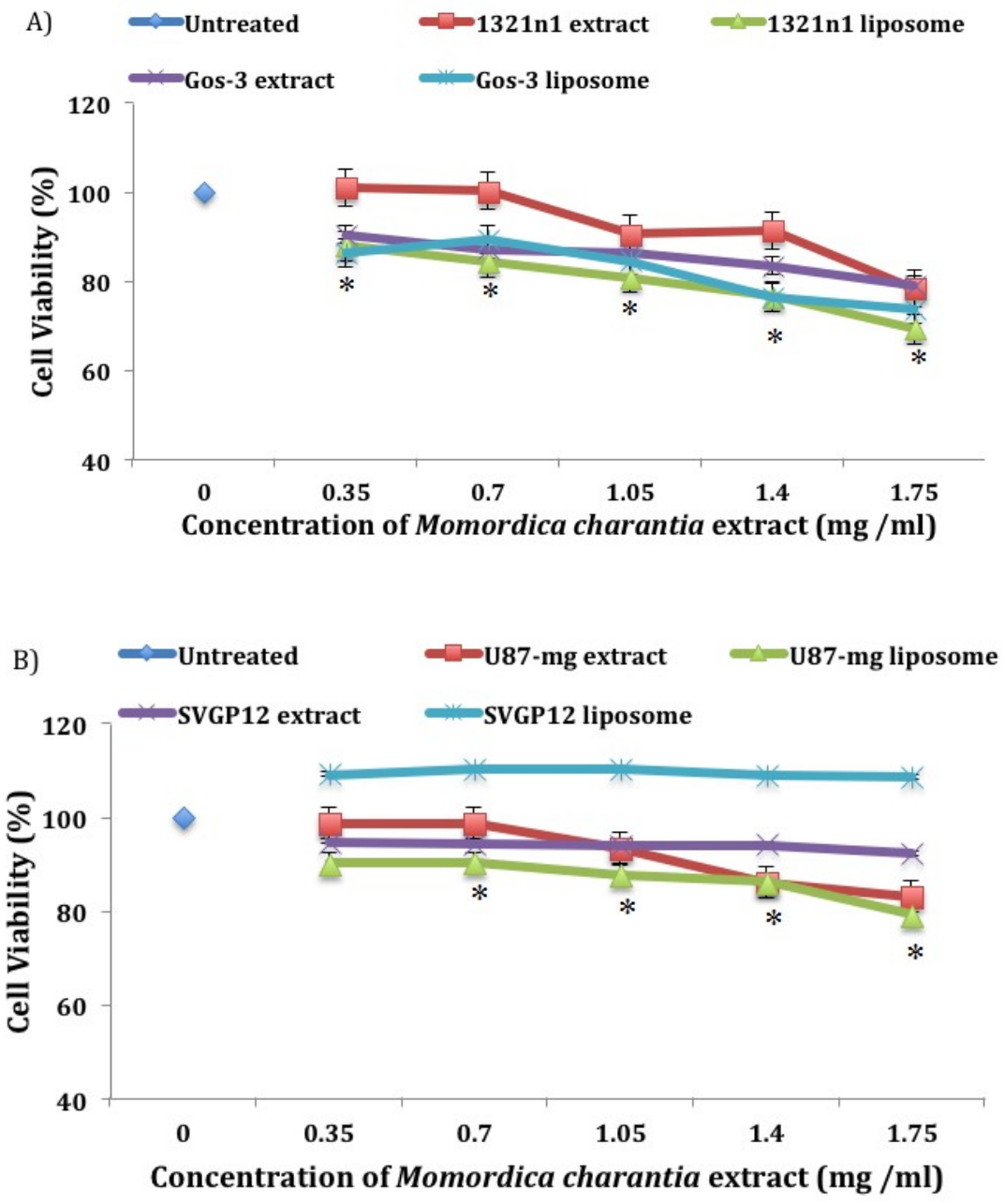


Figure 4.6: Effect of *Momordica charantia* extract with and without liposome on A) low grade tumours, 1321N1 and Gos-3 and B) high grade tumour U87-MG and also normal cell glial cell line SVGP12 as compared to untreated cell lines. Data are mean ± S D; n=3; *P < 0.05.

4.4.5 Inclusion of *Momordica charantia* extracts from different plant origins into SPC or HSPC liposomes

Anti-oxidants, like α- tocopherol in the formulation, were shown to reduce auto-oxidation of lipid components and others and prolong the shelf lives of liposomes (Hunt and Tsang 1981). Similarly, anti-oxidants from *Momordica charantia* fruit might also be assumed to prolong the shelf life of liposomes. PC represents a far more pleasant means for dietary choline repletion than choline itself. Also, PC is an excellent emulsifier that enhances the bioavailability of the co-administered nutrients. Antioxidant nutrients and especially the flavonoids are likely to be better absorbed in combination with PC (Buzzelli et al. 1993), for instance, the B vitamins, minerals, and numerous other nutrients. In The present study, *Momordica charantia* containing cancer inhibiting components were included in saturated and unsaturated soy-derived phospholipid PCs, namely HSPC and SPC, respectively. These phospholipids are known to be non-toxic. The treated and untreated cells were incubated for 24 hours with and without the African, Indian and Chinese *Momordica charantia* extracts (FA, SA and WF). The results were obtained by MTS assay absorbance at 492 nm.

Sections 4.4.6 - 4.6.14 showed the effect of different concentrations (0.3 - 1.75 mg / ml) of the African, Chinese and Indian *Momordica charantia* extracts (FA, SA and WF) on viability of three glioma cell lines 1321N1, Gos-3, U87-MG and the normal glial cell line SVGP12, as compared to the untreated cell lines. The results obtained for the four different cell lines were as follows:

4.4.6 Effect of African *Momordica charantia* extracts on cancerous and non-cancerous glial cells

Figure 4.7 A shows that cell viability depends on the concentration of the extract ($P < 0.05$) with a continuous steady decrease (cell death) in the cell viability of 1321N1 (grade II) and Gos-3 (grade II/III), upon treatment with increasing concentrations of African *Momordica charantia* (AMC) extracts, fruit alone (FA), seed alone (SA) or whole fruit (WF) (0.7 – 1.75 mg / ml) as compared to the untreated cells. At the low concentration (0.35 mg / ml) of the extracts (FA, SA and WF), 1321N1 and Gos-3 were not able to demonstrate marked decrease in the cell viability. Therefore, the viability of the cells was more or less around 100%, but the next concentrations (0.7 mg / ml to 1.75) showed a gradual decrease as compared to the untreated cells (Figure 4.7 A).

Figure 4-7 B showed no significant ($P > 0.05$) changes in the viability of U87-MG (grade IV) and SVGP12 (normal glial cells) on the treatment with AMC extracts (FA, SA and WF). Using the U87-MG cells, except for the concentration 1.75 mg / ml of the AMC- FA extracts their was a, slight but not significant ($P > 0.05$) decrease in the cell viability of U87-MG. Figure 4.7 A, B shows FA extract to exhibit maximum inhibition of 1321N1, Gos-3 and U87- MG cell line compared to other SA and WF extracts. AMC-FA extract evokes more effect on cell viability of 1321N1 cell line followed by Gos-3, U87-MG and SVGP12 (Figure 4.7 A, B). AMC extracts were more effective on low-grade glioma 1321N1 and Gos-3 than on the high-grade glioma U87-MG but there was no significant ($P > 0.05$) changes in the viability of the SVGP12 normal glial cells. Garau et al. (2003) reported that fruit extracts are found to significantly activate the liver enzymes glutathione-S-transferase, glutathione peroxidase and catalase. These enzymes showed a depression following

exposure to the anti-cancerous constituents of *Momordica charantia* fruit during carcinogenesis mediated their modulatory effect on enzymes of the biotransformation and detoxification system of the host (Garau et al. 2003).

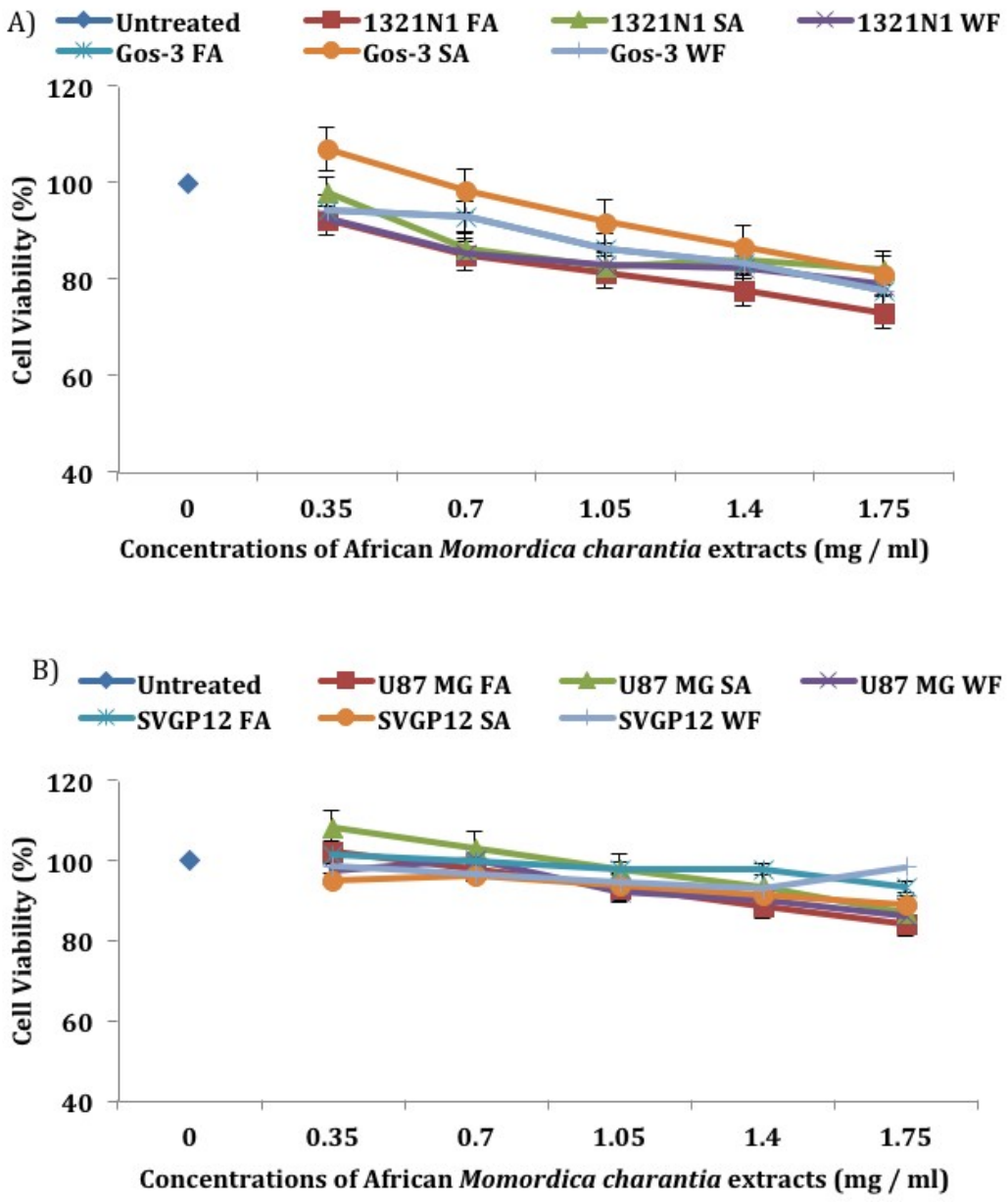


Figure 4.7: Dose response curves showing the effect of African *Momordica charantia* extracts (FA, SA and WF) on viability of A) low grade tumours, 1321N1 and Gos-3 and B) high grade tumour U87-MG and also normal cell glial cell line SVGP12 as compared to untreated cell lines. Data are mean ± S D; n=3; *P < 0.05.

4.4.7 Effect of African *Momordica charantia* extracts included in SPC liposomes to treat cancerous and non-cancerous glial cell lines.

Figure 4.8 A illustrated SPC formulation on the inclusion of AMC extracts (FA, SA and WF) showing significant ($P < 0.05$) gradual decrease in the viability of treated 1321N1 and Gos-3 with increasing concentrations (0.35 – 1.75 mg / ml). AMC – SA extract on inclusion with SPC liposomes demonstrates less inhibition of 1321N1 and GOS-3 on comparison with AMC – FA and WF extract. WF and FA extracts contains active constituents such as antioxidants, Momordicine, Map 30, Cucurmin (Raman and Lau, 1996) which might be responsible for retarding the growth of astrocytoma cells. The retardation of low-grade glioma was increased due to the incorporation of AMC extracts in the SPC phospholipid which is rapidly taken as nutrition by the astrocytomas.

Similarly, Figure 4.8 B shows U87-MG gradual decrease in cell viability as compared to untreated U87-MG on the treatment with AMC extracts (FA, SA and WF) included in SPC liposomes. By contrast, AMC SA extracts showed significant ($P < 0.05$) steady state increases in the cell viability of SVGP12, whereas, SPC liposomal formulation of AMC FA and WF showed no significant ($P > 0.05$) changes in the cell viability at concentrations (0.3 - 1.75 mg / ml) (Figure 4.8 B). AMC –FA extract was more potent than SA and WF extract to kill the cancerous cells 1321N1, GOS-3 and U87-MG (Figure 4.8 A, B). SPC phospholipid liposomes with AMC extracts (FA and WF), except for SA, showed more inhibition as compare to the individual extract alone. This result indicates that SPC liposomes enhanced the permeation of *Momordica charantia* active constituents. The inhibition of the growth of glioma cells (1321N1, GOS-3 and U87- MG) was higher upon inclusion of AMC extracts with SPC liposome as compared to the AMC extract alone. This might be

because cancer cells are more aggressive at trying to utilize the surrounding nutrition (e.g. SPC) more rapidly. The anticancer effect of the active constituents of the AMC extract was enhanced by the facilitated penetration through the cancer cells due to the presence of SPC phospholipid. SPC phospholipid liposomes have possibly entrapped appreciable proportions of the active constituents in the water-soluble AMC extracts. AMC extracts within the SPC liposome formulation served as extra nutrition and was more supportive to the growth of the normal glial cell line SVGP12 as compared to the AMC extract without liposomes.

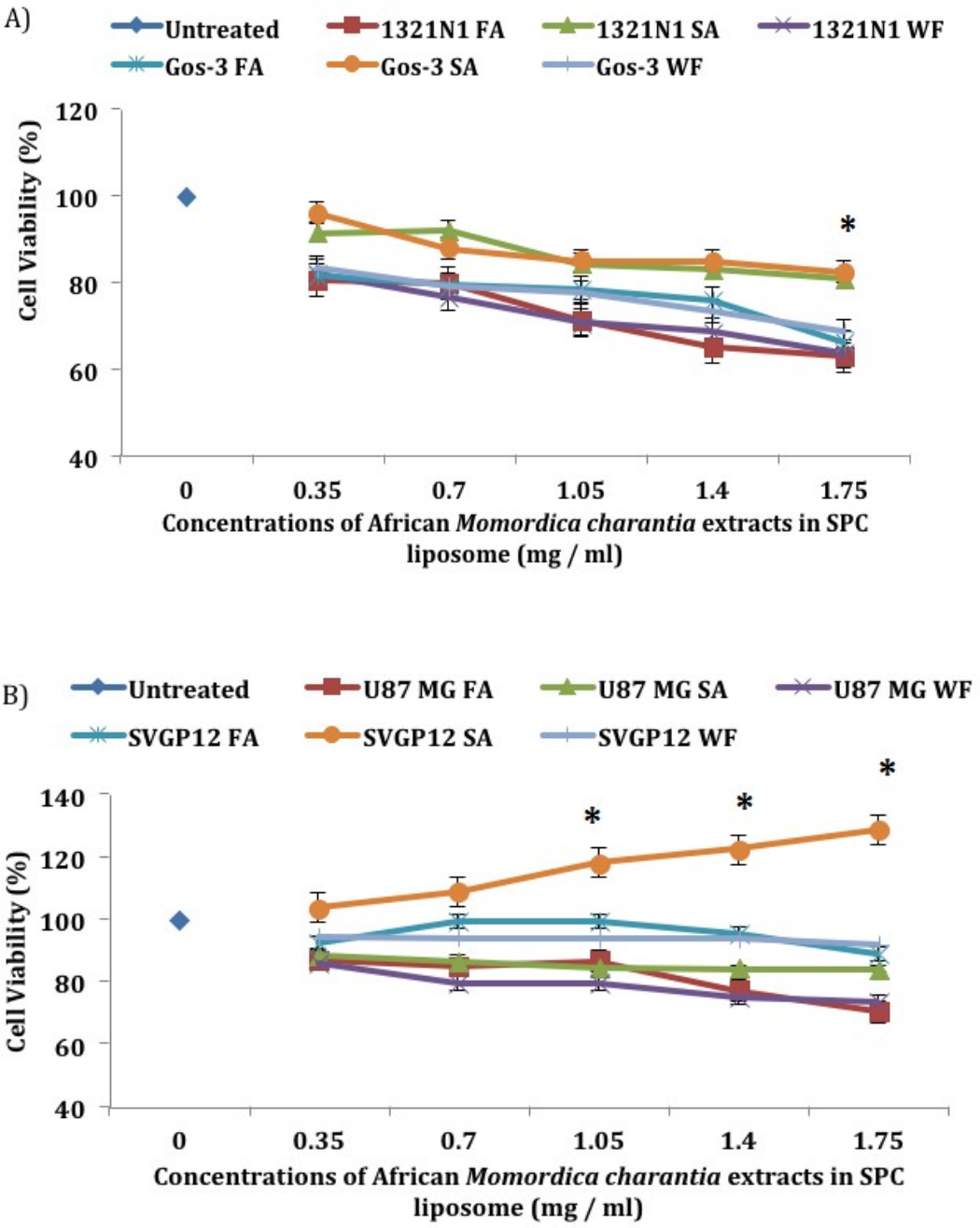


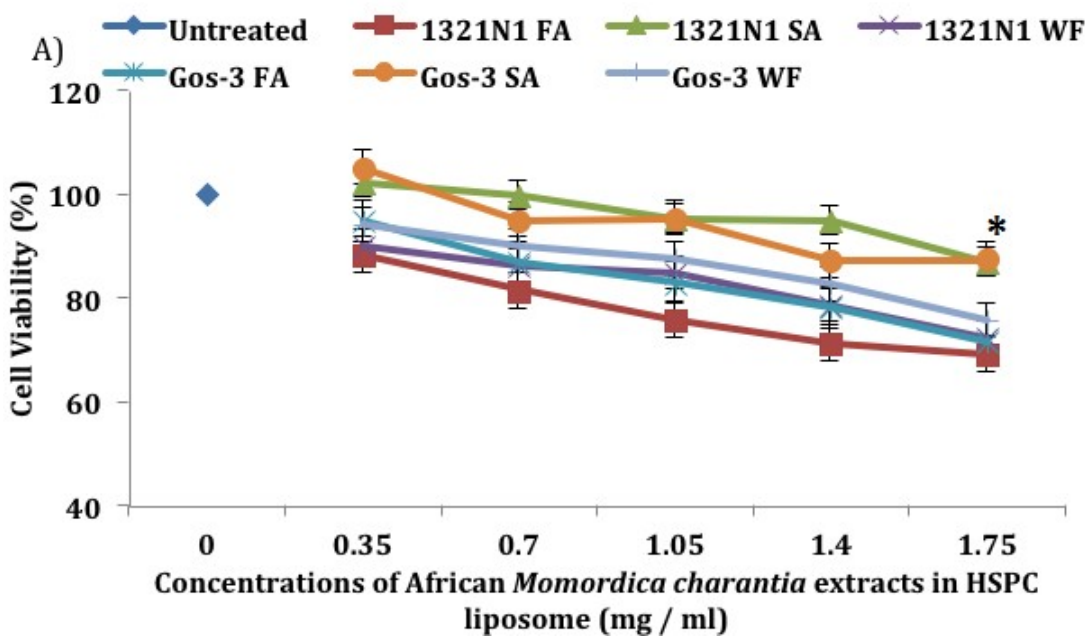
Figure 4.8: Dose response curves showing the effect of SPC liposome including African *Momordica charantia* extracts (FA, SA and WF) on the viability of A) low grade tumours, 1321N1 and Gos-3 and B) high grade tumour U87-MG and also normal cell glial cell line SVGP12 as compared to untreated cell lines. Data are mean ± S D; n=3; *P < 0.05.

4.4.8 Effect of African *Momordica charantia* extracts included in HSPC liposomes to treat cancerous and non-cancerous glial cell lines

Figure 4.9 A shows the findings when AMC extracts (FA, SA and WF) were included in liposomes prepared from HSPC phospholipid. These liposomes were used for the treatment of 1321N1 and Gos-3. Similarly, Figure 4.9 B also exhibited AMC extracts (FA, SA and WF) included in HSPC liposomes. They were used for the treatment of U87-MG and GOS-3 cell lines. Both graphs (Figure 4.9 A, B) demonstrated that AMC- FA extract on inclusion within HSPC liposomes showed ($P < 0.05$) maximum inhibition of 1321N1 and U87-MG at the concentrations 0.35 – 1.75 mg / ml. Similarly, WF extract in HSPC liposome also showed significant ($P < 0.05$) decrease in the viability of glioma cells. But, the effect of WF extract was less than that of the FA extract. By contrast, AMC-SA extract in HSPC liposomes, showed no significant ($P > 0.05$) changes in the viability of 1321N1, Gos-3 and U87-MG glioma cell line and SVGP12 normal glial cells. Similarly, AMC extracts (FA and WF) in HSPC liposomes did not show significant ($P > 0.05$) changes in the cell viability of SVGP12 (Figure 4.9 B). AMC extracts included in HSPC liposomes and liposome free AMC extract showed almost similar inhibition of glioma cancer cells, indicating that the saturated phospholipid (i.e. HSPC) has no effect or significant action in the permeation of *Momordica charantia* extract through the cancerous glial cells.

Extracts combined with the HSPC liposomes were less effective as compared to extract combined with the SPC liposomes. This might be because the HSPC liposomes were larger due to aggregation, or possibly they did not offer sufficient entrapment of the active water-soluble constituents. These materials in *Momordica Charantia* extracts might be amphipathic. This means that they might interact with

the liposome bilayers; hence their association with the liposome structures may facilitate its permeation through the target cells. Hence, it is possible that interaction of these medicinal extracts with the unsaturated phospholipid (i.e. SPC) was better than their interaction with the saturated phospholipid (i.e. HSPC). This is possibly the reason behind the enhanced anticancer effect of the extracts with the SPC liposomes compared to that using the HSPC vesicles. Unlike SPC phospholipid liposomes with AMC extracts (FA, SA and WF), HSPC liposomes with AMC extracts were slightly more effective than AMC extract alone. However, the effect of AMC extracts with SPC liposomes was higher than that of HSPC liposomes containing AMC extracts. This indicates that HSPC phospholipid also serves as nutrition for the cancer cells to thrive and shows inhibitory effect on cancer cells, when mixed with AMC extracts. HSPC phospholipid also serves as nutrition for the normal glial cell to grow and reduces the toxic effect as compared to AMC extract alone (Figure 4.7 and Figure 4.9).



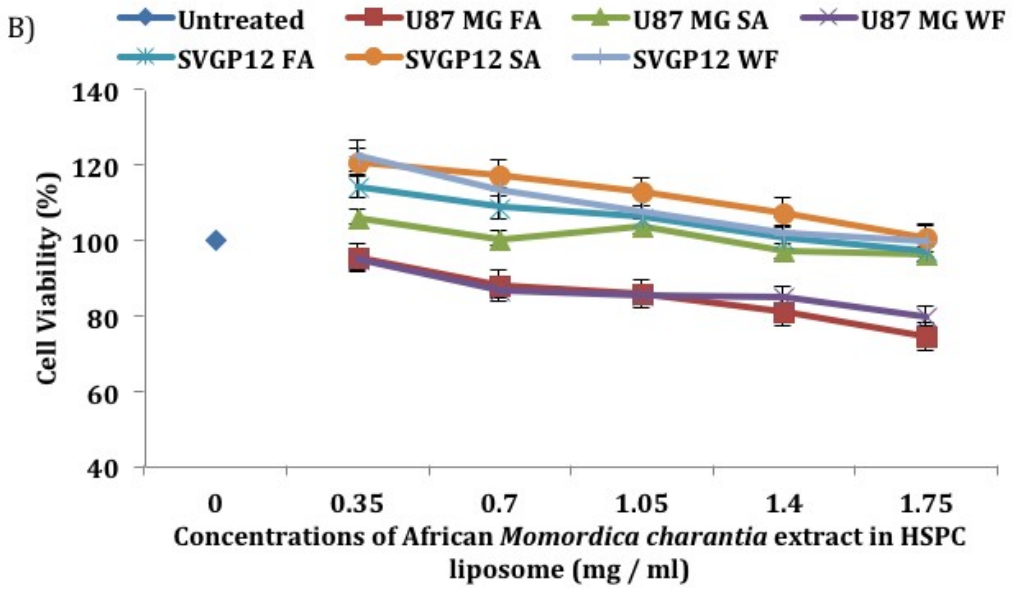


Figure 4.9: Dose response curves showing the effect of HSPC liposome including African *Momordica charantia* extracts (FA, SA and WF) on the viability of A) low grade tumours, 1321N1 and Gos-3 and B) high grade tumour U87-MG and also normal cell glial cell line SVGP12 as compared to untreated cell lines. Data are mean ± S D; n=3; *P < 0.05.

4.4.9 Effect of Chinese *Momordica charantia* extracts treatment on cancerous and non-cancerous glial cell lines.

Figure 4.10 A represents the treatment of 1321N1 and Gos-3 cell lines with Chinese *Momordica charantia* (CMC) extracts (FA, SA and WF) as compared to untreated 1321N1 and Gos-3 cell lines. Application of the extract of CMC-FA on 1321N1 and Gos-3 caused significant ($P < 0.05$) gradual decrease in cell viability. Similarly, CMC SA extract had significant ($P < 0.05$) decrease in the viability of 1321N1 and GOS-3 at the highest concentrations (1.4 -1.75 mg / ml) and no changes at the other concentrations. Similarly, CMC –WF caused a decrease of 1321N1 viability at the highest concentrations (1.4 -1.75 mg / ml). By contrast, Gos-3 cell line exhibited significant ($P < 0.05$) steady decrease at the concentration 0.7 -1.75 mg / ml of CMC-WF extract, when compared to untreated GOS-3 cell line. Thus, CMC-FA, SA and WF extract seemed to have high concentrations of potent and active antitumor ingredients like antioxidants, phenols and ribosome inactivating proteins. These compounds were possibly responsible for inhibition of the growth of the low-grade glioma.

Figure 4.10 B shows that the CMC-FA extract can exhibit significant ($P < 0.05$) and gradual decrease in the viability of the malignant cell line U87-MG. In contrast, there was no significant changes in the viability of SVGP12 as compared to untreated U87-MG and SVGP12. By contrast, CMC- SA and WF extracts also did not show any significant changes in the viability of U87-MG and SVGP12 as compared to untreated cells.

Figure 4.10 B shows the viability of 1321N1, Gos-3 and U87-MG cell lines. The results show that there was less proliferation following treatment with CMC –FA

extract alone as compared to CMC-WF and CMC-SA. A study by Zulbadli et al. in 2011 used GC-MS to analyze the structure of valuable compounds present in the *Momordica charantia* fruit extract. These researchers have identified few medicinal active compounds like vitamin E, ascorbic acid, cucurbitacin B and gentisic acid in *Momordica charantia* fruit extract. Gentisic acid and vitamin E are known for their antioxidant activity. These medicinal compounds were known for their antioxidant activity, anti-proliferative activity and hypoglycemic effect (Jayini, 2012). CMC extracts (FA, SA and WF) showed slight toxic effect on SVGP12 the normal glial cells.

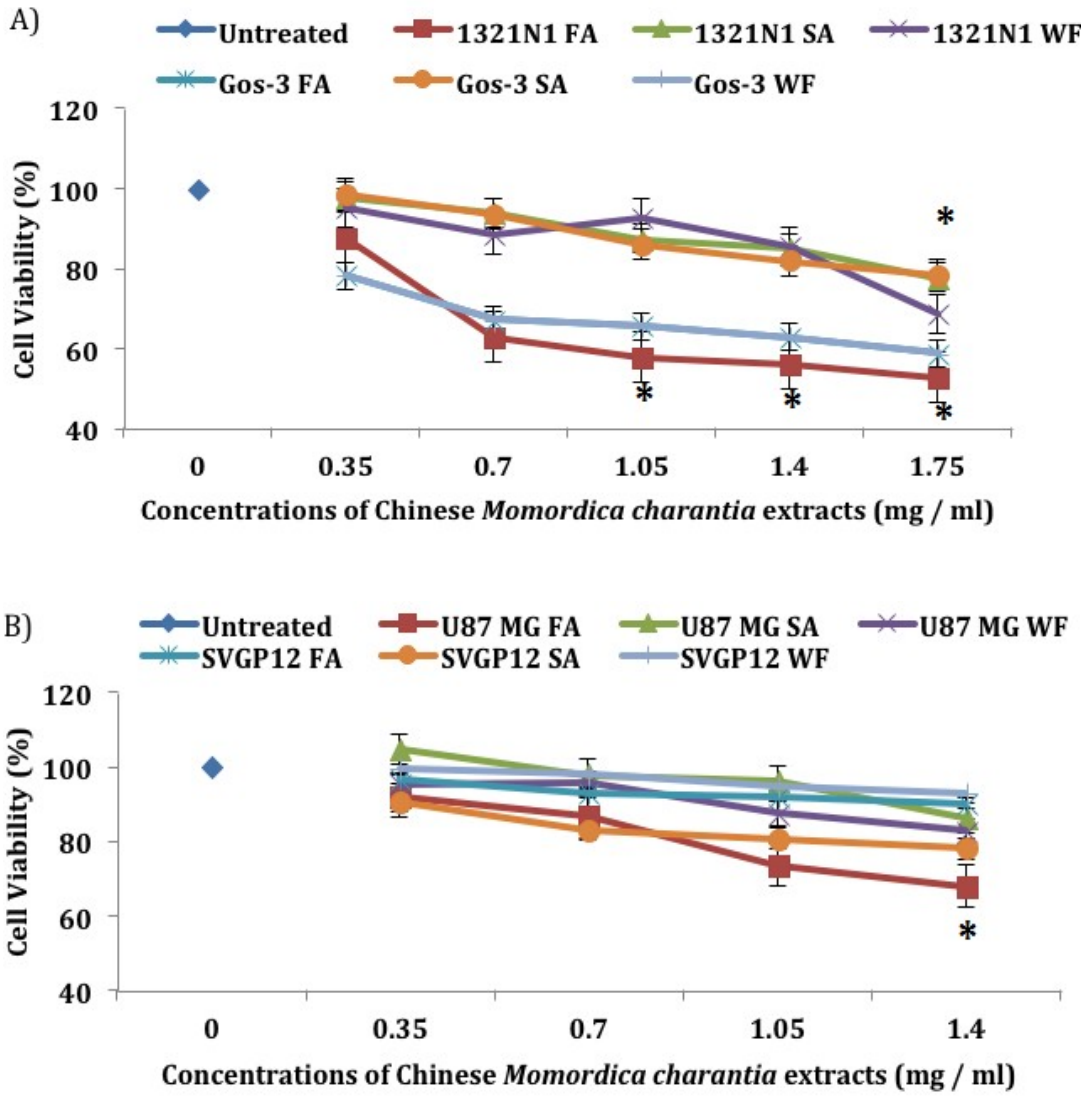


Figure 4.10: Dose response effect of Chinese *Momordica charantia* extracts (FA, SA and WF) on viability of A) low grade tumours, 1321N1 and Gos-3 and B) high grade tumour U87-MG and also normal cell glial cell line SVGP12 as compared to untreated cell lines. Data are mean ± S D; n=3; *P < 0.05.

4.4.10 Effect of Chinese *Momordica charantia* extracts included in SPC liposomes to treat cancerous and non-cancerous glial cell lines.

Figure 4.11 A illustrates the effect of Chinese *Momordica charantia* (CMC) extracts on cell viability when they were incorporated in liposome prepared from SPC phospholipids. The SPC liposomes containing CMC extracts (FA, SA and WF) were used to treat the low-grade glioma cell line 1321N1 and Gos-3 and compared to

untreated cells. SPC liposome containing CMC extracts (FA, SA and WF) demonstrated significant ($P < 0.05$) but gradual decrease in the viability of 1321N1 and GOS-3 at all concentration (0.35 – 1.75 mg / ml). In contrast,1321N1 and Gos-3 cell lines on treatment with SPC liposome containing CMC-FA extract displayed maximum inhibition in followed by CMC- WF and CMC-SA.

Figure 4.11 B shows significant ($P < 0.05$) and maximum inhibition of U87-MG as compared to untreated cells, following treatment with CMC-FA and WF extract included in SPC liposome. In contrast, U87-MG treated with CMC-SA extract on inclusion with SPC phospholipid liposome did not show any significant ($P > 0.05$) changes in the viability as compared to untreated U87-MG. This might be due to the fact that the SA extract dosage was not sufficient to kill the high-grade cancer cells. Similarly, SVGP12 treated with SPC liposomes containing FA, SA and WF extracts did not show any significant ($P > 0.05$) change in the viability as compared to untreated SVGP12. But it showed significant ($P < 0.05$) increase in the growth of SVGP12 cells when treated with extract included in SPC liposome as compared to the extract alone. This observation indicates that the cancerous cells may metabolize more extract when SPC liposomes were used to entrap the extract. Another possible explanation is that it may supports the growth or it is less toxic for the normal glial cell line.

Figure 4.11 A and B illustrates the effect of CMC extracts in SPC liposomes enhanced the penetration of active constituents in the CMC extracts. The proliferation rate of glioma cell line (1321N1, Gos-3 and U87-MG) was inhibited, when treated with SPC liposome-containing CMC extracts. CMC extract within the SPC liposomes seemed to show more promising effect for the treatment of glioma.

This result indicates that the active constituent of CMC extracts with SPC phospholipid shows additional effect to inhibit the growth of glioma.

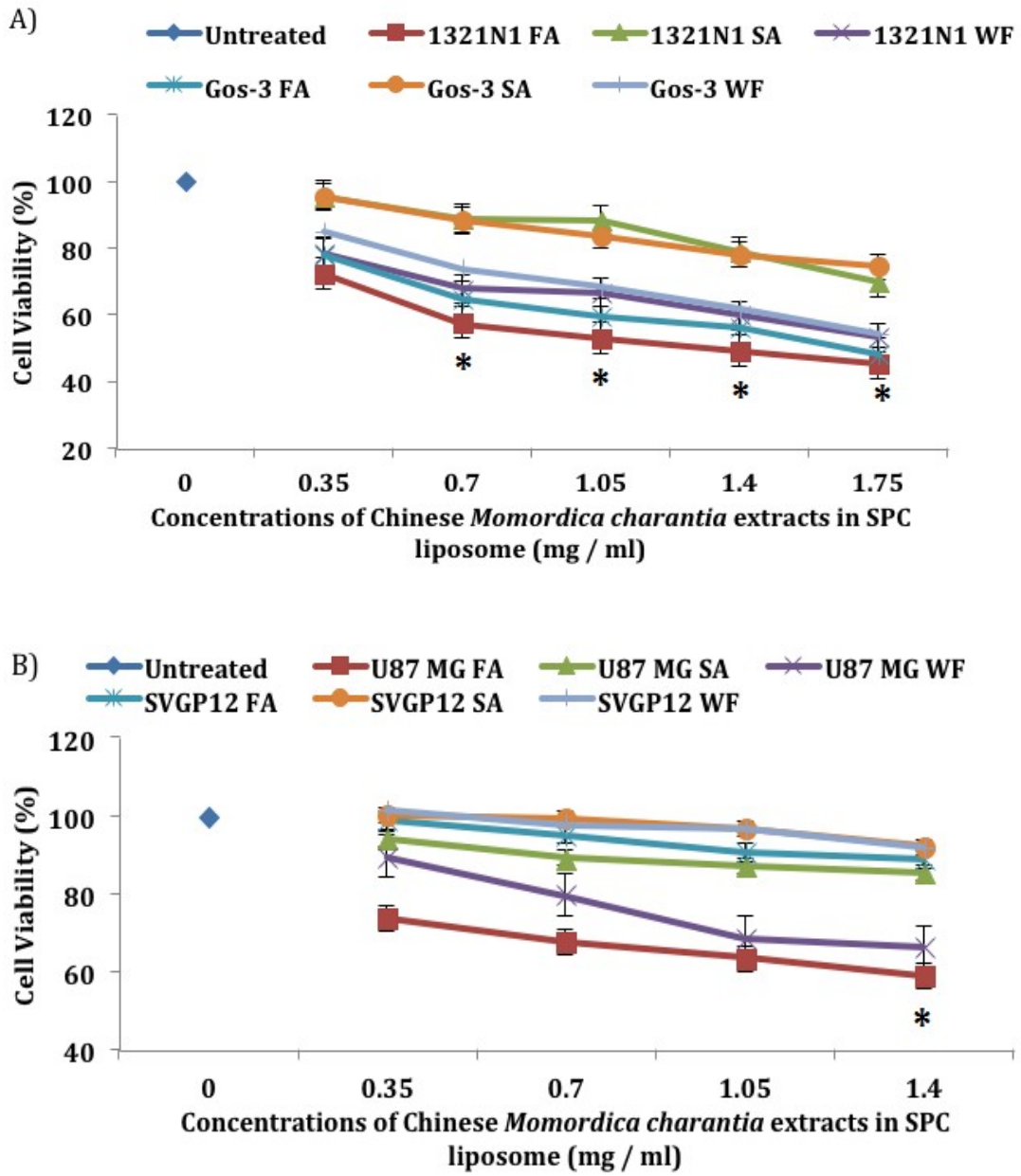


Figure 4.11: Dose response curves effect of SPC liposome including Chinese *Momordica charantia* extracts (FA, SA and WF) on the viability of A) low grade tumours, 1321N1 and Gos-3 and B) high grade tumour U87-MG and also normal cell glial cell line SVGP12 as compared to untreated cell lines. Data are mean ± S D; n=3; *P < 0.05.

4.4.11 Effect of Chinese *Momordica charantia* extracts included in HSPC liposomes upon addition to cancerous and non-cancerous glial cell lines

Figure 4.12 A demonstrates cell viability of 1321N1 and Gos-3 following treatment with increasing concentrations (0.35 - 1.75 mg / ml) of CMC extracts (FA, SA and WF) included with HSPC liposomes. On the treatment of 1321N1 and Gos-3 with CMC extracts (FA, SA and WF) entrapped in HSPC liposomes, a significant ($P < 0.05$) and gradual decrease in the viability of each cell line was observed. However, the CMC-SA extract in HSPC liposomes did not inhibit Gos-3 at the lower concentrations (0.35 – 0.7 mg /ml) as compared to untreated cells. Similarly, CMC-WF extract shows no significant ($P > 0.05$) decrease in the 1321N1 viability at 0.3 mg / ml as compared to untreated cells. Likewise, the extract free from liposome show inhibition of 1321N1 and Gos-3 cells (Figure 4.10). But, on inclusion of extract within the liposome facilitated slightly more than ±5 % inhibition of 1321N1 and Gos-3 cells at the highest concentrations 1.4 mg /ml and 1.75 mg /ml (Figure 4.12).

Figure 4.12 B shows the effect of CMC extracts (FA, SA and WF) included in HSPC liposomes on the viability of U87-MG and SVGP12 cell lines as compared to untreated cells. The normal glial cells SVGP12 showed significant ($P < 0.05$) proliferations when treated with 0.35 mg / ml and 0.7 mg / ml but with no effect when higher concentrations (1.05 – 1.75 mg / m) were used. In contrast, treatment of U87-MG with CMC-FA extract in HSPC liposomes demonstrated significant ($P < 0.05$) and gradual decrease in cell viability. SVGP12, when treated with HSPC liposomes containing CMC-SA extract showed no significant change in the viability. Whereas, CMC-WF extract in HSPC liposome displayed slightly less inhibitory effect but this was significant ($P < 0.05$) on SVGP12 cells at the higher concentrations (1.05 – 1.75 mg /ml) compared to untreated. Similarly, U87-MG

HSPC liposomes containing CMC-WF and SA extracts evoked a small but significant ($P < 0.05$) inhibition at the higher concentrations (1.05 – 1.75 mg /ml).

Figure 4.12 A, B show that the CMC extracts (FA, SA and WF) in HSPC liposomes had more potential to inhibit the glioma (1321N1, Gos-3 and U87-MG) cells. The result reveal a slight inhibition of normal glial cells (SVGP12) compared to untreated cells. The CMC extracts, when combined with HSPC liposomes showed more inhibition of glioma compared to CMC extract alone and CMC extracts along with SPC phospholipid liposomes. Except, the normal SVGP12 glial cells did not show any effect on treatment with HSPC liposomes containing Chinese *Momordica charantia*.

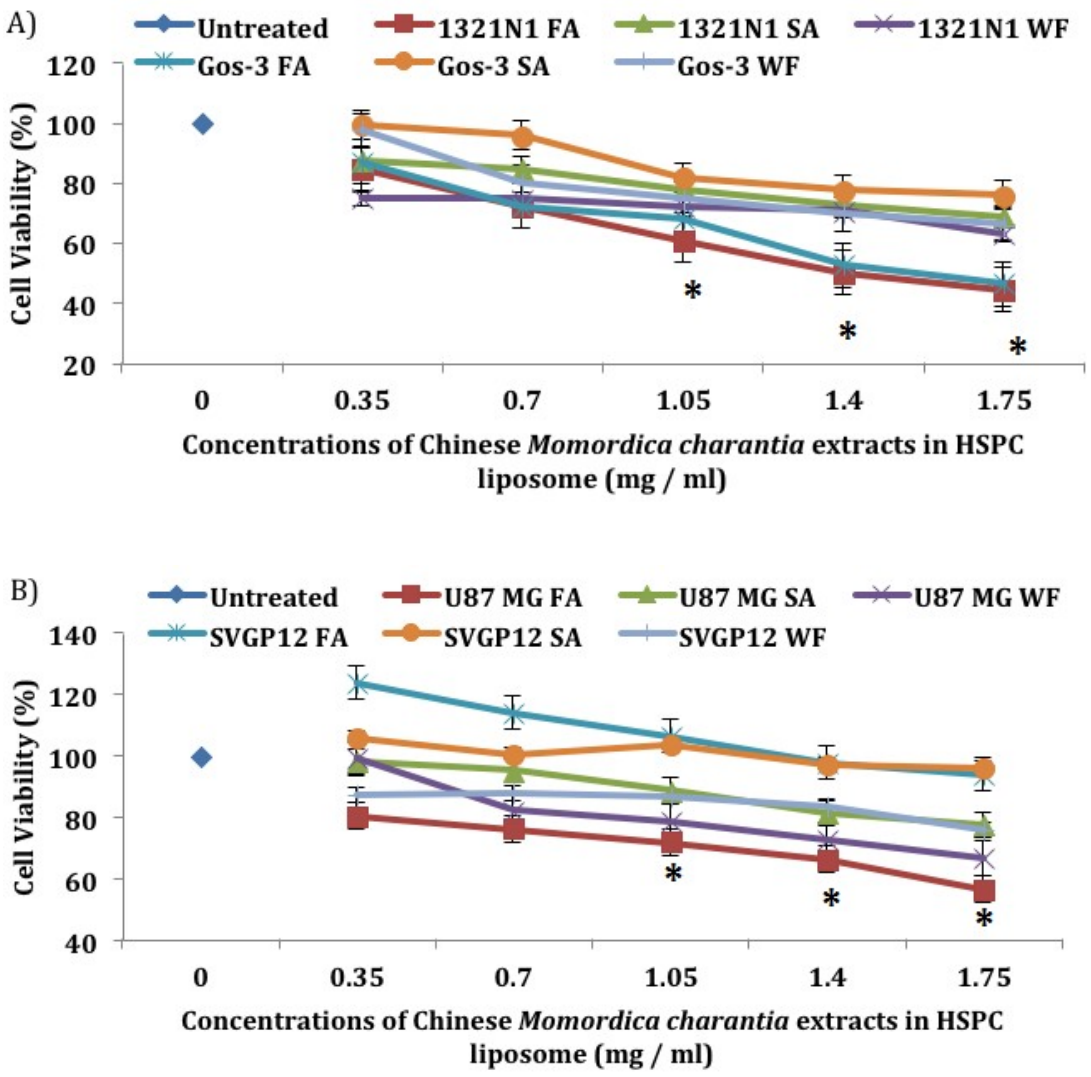


Figure 4.12: Dose dependent effect of HSPC liposome including Chinese *Momordica charantia* extracts (FA, SA and WF) on the viability of A) low grade tumours, 1321N1 and Gos-3 and B) high grade tumour U87-MG and also normal cell glial cell line SVGP12 as compared to untreated cell lines. Data are mean ± S D; n=3; *P < 0.05.

4.4.12 Effect of Indian *Momordica charantia* extracts on cancerous and non-cancerous glial cell lines

Figure 4.13 A and B shows the viability of 1321N1, Gos-3 and U87-MG glioma cell lines and SVGP12 normal glial cells when treated with Indian *Momordica charantia* (IMC) extracts FA, SA and WF. The results reveal no significant (P > 0.05) cytotoxic effect to the cells at the lower concentrations (0.35 mg / ml and 0.7 mg /

ml) of the extracts. Whereas, on the treatment with the higher concentrations of extract (1.4 mg / ml and 1.75 mg / ml) there was a significant ($P < 0.05$) decrease in the glioma cell viability. Except, Gos-3 on treatment with IMC-SA extract showed no decrease in the viability of cells at all the concentrations (0.35 -1.75 mg / ml). This clearly shows that Gos-3 cell line might need to be treated with higher concentrations of IMC-SA extract. The concentration of the SA extract should be more than 1.75 mg / ml to show a gradual decrease in the viability of Gos-3 cells. In contrast, IMC extracts WF and SA used on SVGP12 normal glial cells did not show any cytotoxic effect. In fact, these extracts supported the growth of the SVGP12 normal glial cells. FA extract derived from IMC shows a small cytotoxic effect on the normal glial cells SVGP12 at the higher concentrations 1.4 mg / ml and 1.75 mg / ml. FA extract contains active component as antioxidants, Momordicine, Map 30, Cucurmin (Raman and Lau, 1996). These components are toxic because they tend to inhibited the growth of both cancerous and non-cancerous cells.

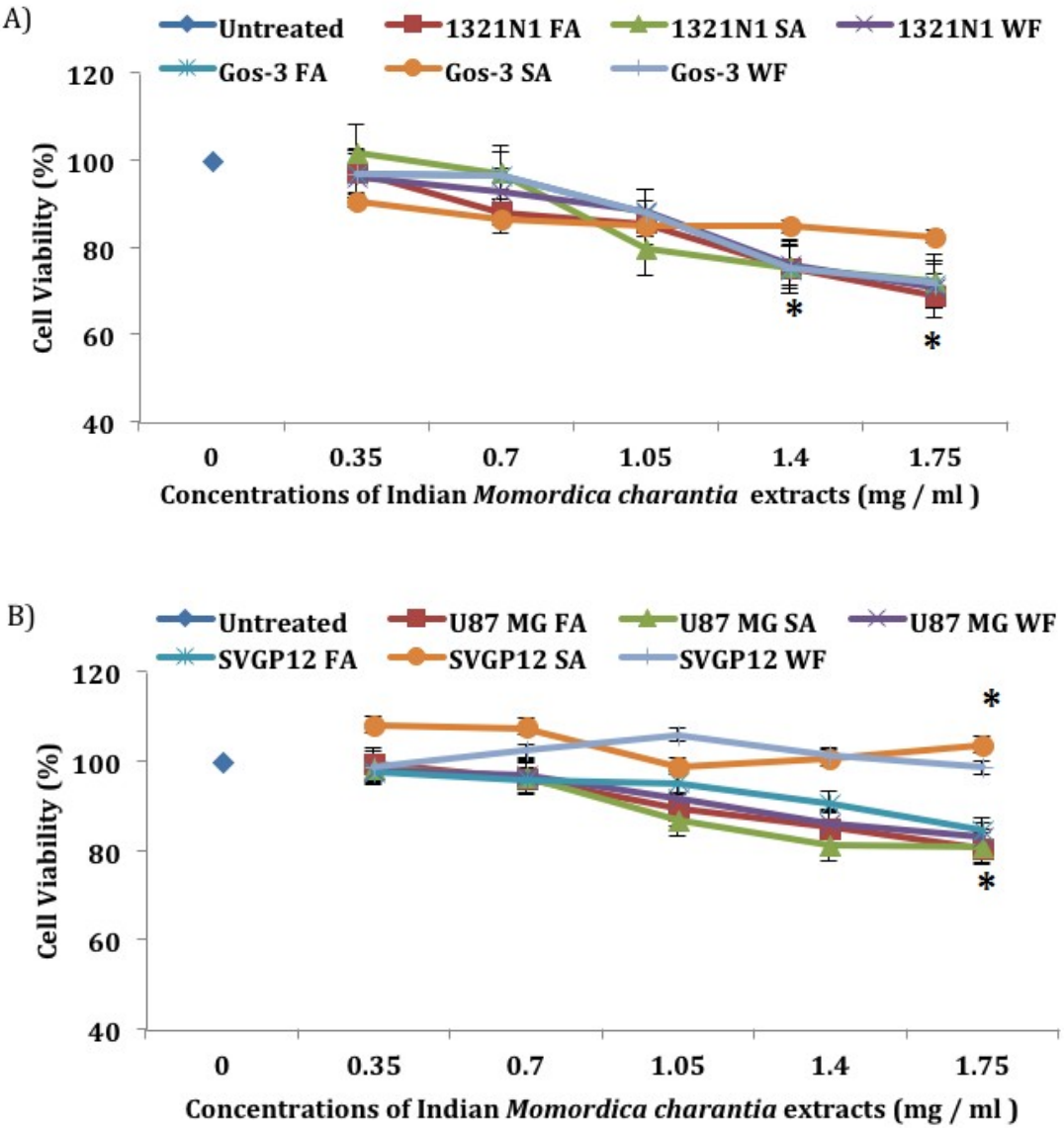


Figure 4.13 Dose dependent effect of Indian *Momordica charantia* extracts (FA, SA and WF) on viability of A) low grade tumours, 1321N1 and Gos-3 and B) high grade tumour U87-MG and also normal cell glial cell line SVGP12 as compared to untreated cell lines. Data are mean ± S D; n=3; *P < 0.05.

4.4.13 Effect of Indian *Momordica charantia* extracts included in SPC liposomes on treatment of cancerous and non-cancerous glial cell lines

Figure 4.14 A represented 1321N1 and GOS-3 treated with IMC extracts (FA, SA and WF) in SPC liposomes. The formulations had no significantly (P > 0.05) inhibitory effect on the cell growth at the lowest extract concentration (0.35 mg /ml)

of the extract. However, increasing the concentration (0.7 - 1.75 mg / m l) of IMC extracts in SPC liposomes caused a gradual decrease in the viability of low-grade glioma (1321N1 and Gos-3).

Figure 4.14 B represents the viability of U87-MG and SVGP12 cells treated with IMC extracts (FA, SA and WF) in SPC liposomes. The results show that U87-MG on the treatment with FA or WF extracts demonstrated a gradual decrease in viability, whereas the SA extract did not cause any change in viability at the low concentrations (0.35 – 1.05 mg / ml) but only a slight inhibitory effect on the cell viability at the higher concentrations (1.4 mg / ml and 1.75 mg / ml). Similarly, the viability of SVGP12 cells was not affected by treatment with the extracts formulations. FA extract with this liposome is found to be more potent in inhibiting glioma as compared to SA or WF extract with liposomes. This might be due to the fact that higher concentration of active constituents is present in the FA extract compared with the WF and SA extracts. However, Figure 4.14 B shows that IMC extract on inclusion within the liposome facilitated more cytotoxic effect on the glioma cells as compared to the cells treated with extracts without liposomes. Extracts along with liposomes are more effective on the tumour cells. Higher concentrations of FA extracts on inclusion within the liposome showed no toxic effect on SVGP12 normal glial cells. It seemed to support the growth of normal glial cells. Similarly, SA and WF extracts with liposome evoked no significant ($P > 0.05$) changes in the viability of SVGP12 cell as compared to non- treated SVGP12. Thus, seed extract might have components which act as nutrients to support the growth of healthy cells. But, component derived from seed are slight toxic to the cancerous cells.

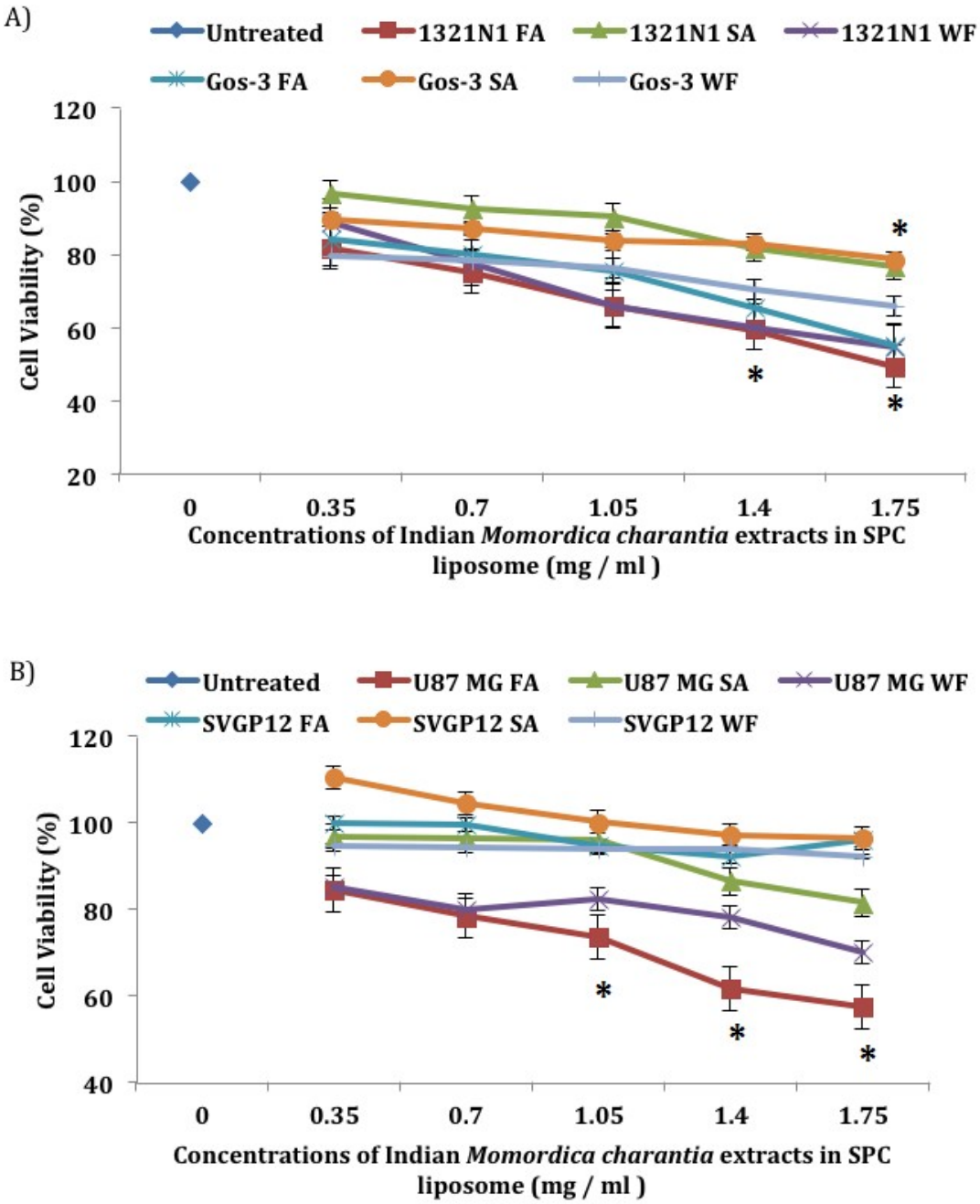


Figure 4.14: Dose dependent effect of SPC liposome including Indian *Momordica charantia* extracts (FA, SA and WF) on the viability of A) low grade tumours, 1321N1 and Gos-3 and B) high grade tumour U87-MG and also normal cell glial cell line SVGP12 as compared to untreated cell lines. Data are mean ± S D; n=3; *P < 0.05.

4.4.14 Effect of Indian *Momordica charantia* extracts included in HSPC liposomes to treat cancerous and non-cancerous glial cell lines

Figure 4.15 A shows the viability of 1321N1 and Gos-3 treated with increasing concentrations (0.35 – 1.75 mg / ml) of IMC extracts (FA, SA and WF) in HSPC liposomes. The results shows that 1321N1and Gos-3 cell lines when treated with FA and WF included in HSPC liposomes show both a significant ($P < 0.05$) and a gradual decrease in viability by increasing the extract concentration (0.35 -1.75 mg / ml). In contrast, IMC-SA included in HSPC liposomes had very slight effect on the viability of 1321N1and Gos-3 cells.

Figure 4.15 B reveal a small but a significant ($P < 0.05$) decrease in the viability of U87-MG cells upon treatment with the HSPC liposome formulations of IMC extracts (FA and WF). However, no effect ($P > 0.05$) on the U87-MG cell viability was observed upon treatment with HSPC SA extract. Similarly, the treatment of SVGP12 with IMC-SA extract in HSPC liposomes resulted in inhibition of cells, but also caused a non-consistent growth pattern. It seems that combination of both SA extract along with HSPC liposome turns to be poisonous for the healthy SVGP12 cells.

Similarly, Figure 4.15 A, B also shows that when IMC extracts included in HSPC liposomes, there was a small tendency to inhibit glioma, when compared to IMC extracts in SPC liposomes. In contrast, the extract shows more potent inhibition of cells, when compared to IMC extract without liposomes. The SA extracts in combination with HSPC liposomes were slightly toxic to both the cancerous and normal cells. The toxicity might be due to the presence of fatty acid or some other components in the *Momordica charantia* SA extract. *Momordica charantia* seeds contain around 60% eleostearic acid (α-ESA), which is a conjugated linolenic acid

(Zang et al. 2012). One of the studies published in 2012 by Zang et al. suggested that α-ESA may exhibit a potential cytotoxicity and cause apoptosis induction on human breast cancer cells, with little effect on normal cells at certain concentrations (Zang et al. 2012). This study supports the present findings using the *Momordica Charantia* extracts.

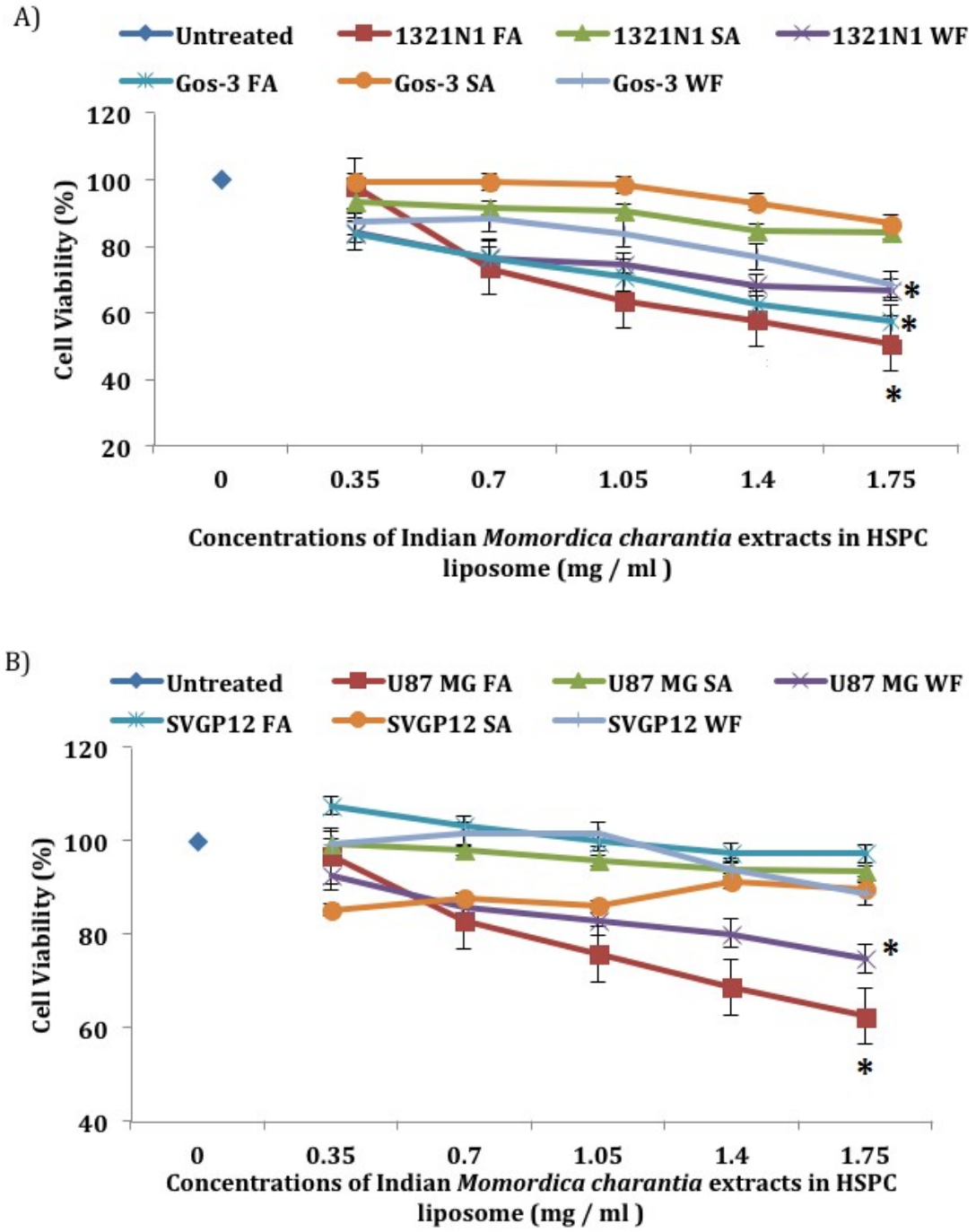


Figure 4.15: Dose response effect of HSPC liposome including Indian *Momordica charantia* extracts (FA, SA and WF) on the viability of A) low grade tumours, 1321N1 and Gos-3 and B) high grade tumour U87-MG and also normal cell glial cell line SVGP12 as compared to untreated cell line. Data are mean ± S D; n=3; *P < 0.05.

4.5 Futher Discussion and Conclusion

The growth curve of low-grade glioma 1321N1 and Gos-3 proliferates faster, and die sooner as compared to the high-grade glioma U87-MG. The low-grade glioma cells 1321N1 are not very aggressive to utilize the nutrition so they grow faster and die faster. The SVGP12 normal glail cells proliferate at normal rate utilizing sufficient nutrition to grow. Whereas, U87-MG high-grade glioma cells may utilize all the nutrition present in the media and turning the media from dark pink to light pink. U87-MG cells grow in very aggressive pattern overlapping on one another. The glioma cells treated with ETOH+water, HSPC and SPC liposomes showed no significant ($P > 0.05$) increase or decrease in the cell viability. U87-MG cells, treatment with HSPC liposome seemed to support the cellular growth, suggesting that HSPC phospholipid may serve as nutrition to the high-grade glioma. SPC and ETOH+water, did not show any effect on U87-MG. Similarly, SPC and HSPC liposome have helped the normal glial cells SVGP12 to proliferate. The normal glial cells showed a slight decrease in the cell viability on treatment with ETOH+water, indicating that ethanol is slightly toxic to the normal glial cells. On evaluation of *Momordica charantia* extract and PTX with SPC or HSPC liposomes, PTX was very toxic and killed both cancerous and non-cancerous cells. PTX therapeutic index was improved when included in liposome formulations. However, inclusion of *Momordica charantia* extract within liposomes was not as effective as PTX on cancerous cells. The really good result was exhibited by the promoted growth of the normal glial cells SVGP12 when the extract was combined with liposomes.

Momordica charantia is an excellent source of phenolic compounds, antioxidants, and antimutagens that are useful for inhibition of cancer. The results in this chapter represented the effect of *Momordica charantia* extracts (FA, SA and WF) with or without liposomes on the viability of cancerous and non-cancerous cells of the brain. *Momordica charantia* was bought from India, Africa and China and divided as FA, SA and WF. *Momordica charantia* liposomes were prepared by using SPC or HSPC phospholipids. Extract without liposomes, showed an inhibiting effect on the growth of glioma cells (1321N1, Gos-3 and U87-MG) and supported the growth of normal cells (SVGP12). On inclusion of extracts within SPC liposomes, a significant ($P < 0.05$) decrease of the glioma cells was observed. In contrast, no significant decrease in normal SVGP12 cell line was seen. Similarly, the effect of *Momordica charantia* HSPC liposomes was less effective compared to SPC liposomes. Whereas, the effect of the *Momordica charantia* HSPC liposomes was a little bit higher compared to the extract alone, but, lower compared to the SPC formulations. It was proved that FA extract and WF extract of *Momordica charantia* along with SPC or HSPC liposomes was able to show maximal inhibition effect on the glioma cell line (1321N1, Gos-3 and U87-MG) on comparison with the FA and WF extract without liposome. It also, showed that the FA and WF extract from all the countries in combination with liposome did not show any toxic effect on the normal glial cell SVGP12. Whereas, *Momordica charantia* SA extracts from Africa and China along with SPC and HSPC liposome supports the growth of the normal glial cells and did not show any changes in the growth of the glioma (1321N1, Gos-3 and U87-mg) cell line. *Momordica charantia* SA extracts from India along with HSPC showed equal amount of decrease in the viability of SVGP12. The *Momordica charantia* FA extracts from India, Africa and China, with or without liposome was more potent to inhibit the

growth of glioma (1321N1, Gos-3 and U87-Mg) cell lines as compared to *Momordica charantia* SA and WF extracts. The FA extract of *Momordica charantia* with SPC and HSPC liposome seem to be more permeable in cancer cells because it could inhibit several more cells as compared to FA extract of *Momordica charantia* without any liposome associated. The AMC and IMC FA extract showed more inhibition when in corporate within SPC liposome, rather than HSPC liposome. But, CMC FA extract showed reversed effect. The AMC and IMC FA, SA and WF extracts were able to show inhibition at the highest concentration. In contrast, there extracts were, not able to show any significant decrease of growth at the lower concentration. CMC FA extract along with HSPC phospholipid showed the maximum inhibition rate, compared to all other FA, SA and WF *Momordica charantia* extracts from India, China and Africa with or without any SPC and HSPC phospholipid liposome. This indicates that HSPC liposome could entrap maximum active constituents derived from CMC-FA extract that is useful for the inhibition of glioma (1321N1, Gos-3 and U87-MG) cell lines. It did not show any toxic effect on the normal glial cell. The AMC, CMC and IMC extracts (FA, SA and WF) either with or without liposome, killed low-grade (1321N1 and Gos-3) glioma cells and they had very little effect on high-grade (U87-MG) glioma cells but no significant effect on normal glial (SVGP12) cells. In contrast, IMC-SA on inclusion within HSPC liposome showed slight toxic effect to the normal glail cells. It is known that the seed extract contains 60% α-ESA, which at times is toxic to the normal cells at various concentrations. The combination of α-ESA with HSPC liposome enhanced the cytotoxic effect on the normal cells. The effect of AMC, CMC and IMC extracts (FA, SA and WF) either with or without liposome was not as effective as PTX with and without liposome.

In summary, *Momordica charantia* extracts inclusion in HSPC and SPC liposome prepared from solvent-based proliposome method, facilitated more permeation of the active constituents. These active constituents of *Momordica charantia* retard the growth of glioma cell lines and do not affect the growth of normal glial cells. The same part of *Momordica charantia* plant extracts obtained from different countries showed different rate of inhibition of glioma cells. This might be dur to the fruit lack the amount of active ingredient varies. The variation of active ingredient might be due to **climatic condition** including temperate, hot or humid, **soil condition**; red soil, alkaline soil, amount of nutrients and many other factors might be responsible to show the different on the glioma cell lines.

Chapter 5. Effect of *Momordica charantia* extracts and proliferation on activities of caspase 3, 7 and 9 and release of cytochrome C in glioma cell lines

5.1 Introduction

The chapter 4 of this study describes the efficacy of the resultant liposome formulations on the viability of glioma cells by MTS cytoxic assay. This chapter was specifically designed to investigate the molecular mechanism of apoptosis induced by *Momordica charantia* fruit extracts and Paclitaxel included in the liposome. Apoptosis involves permanent damage to the mitochondria leading to the release of caspase-3, caspase-9 and cytochrome-c (Kerr et al. 1972). Caspase assay and mitochondrial assay are important to identify the apoptotic-signalling pathway for death of the tumour cells.

5.2 Materials and Methods

5.3 Materials and chemical reagents

Caspase-Glo® 3/7 and Caspase-Glo® 9 activity (Promega, UK) (Figure 5-4).

Cytochrome-c (Sigma-Aldrich, UK).

Other materials used in the studies presented in this chapter were previously indicated and described in Chapter 4 (sections 4.3.1-4.3.4).

5.4 Methods

5.4.1 Plating of cell lines

Plating of the cell lines was performed as described in chapter 4 (section 4.4.5).

Measurement of Caspase-Glo® 3/7 and Caspase-Glo® 9 activity

The 1321N1, Gos -3, U87- MG and SVGP12 cell lines were incubated for 24 hrs in 5% CO_2 incubator at 37ºC. The media in the plates were removed on sterile tissue paper. Another 75 µl of media and 25 µl of liposomal solutions of *Momordica charantia* and Paclitaxel were added to the wells and incubated in 5% CO_2 incubator at 37ºC for another 12 hours. The treated and non-treated cells were incubated for 12 hrs at 37°C in 5 % CO_2. Prior to starting the assay the prepared sample of Caspase-Glo® 3/7 and Caspase-Glo® 9 reagents was equilibrated to room temperature. After 24 hr, each plate (96 well plate GRE 96 fb) was removed from the incubator for 30 min to equilibrate to room temperature. A volume of 100 µl of Caspase-Glo® 3/7 and Caspase-Glo® 9 assay reagent was added to each well of a white walled 96 well plate containing 100 µl of blank and treated cells in the culture medium (Figure 5.4). The plate was then covered with aluminium foil and mixed gently by using a plate shaker at 300-500 rpm for 2 min, followed by incubation at room temperature for 30 min. The Caspase-Glo® 3/7 and Caspase-Glo® 9 assay was performed in the absence of light. The Luminescence was measured using a Teccan Plate reader. The software used for the Luminescence assay was XFLUORHGENIOSPRO version V 4.53 (Version 2004). The plate type used from the software was specified as GRE 96 fb Pdf, and the temperature was set up at 20 – 23°C. All the values were expressed as µmol/min/ml.

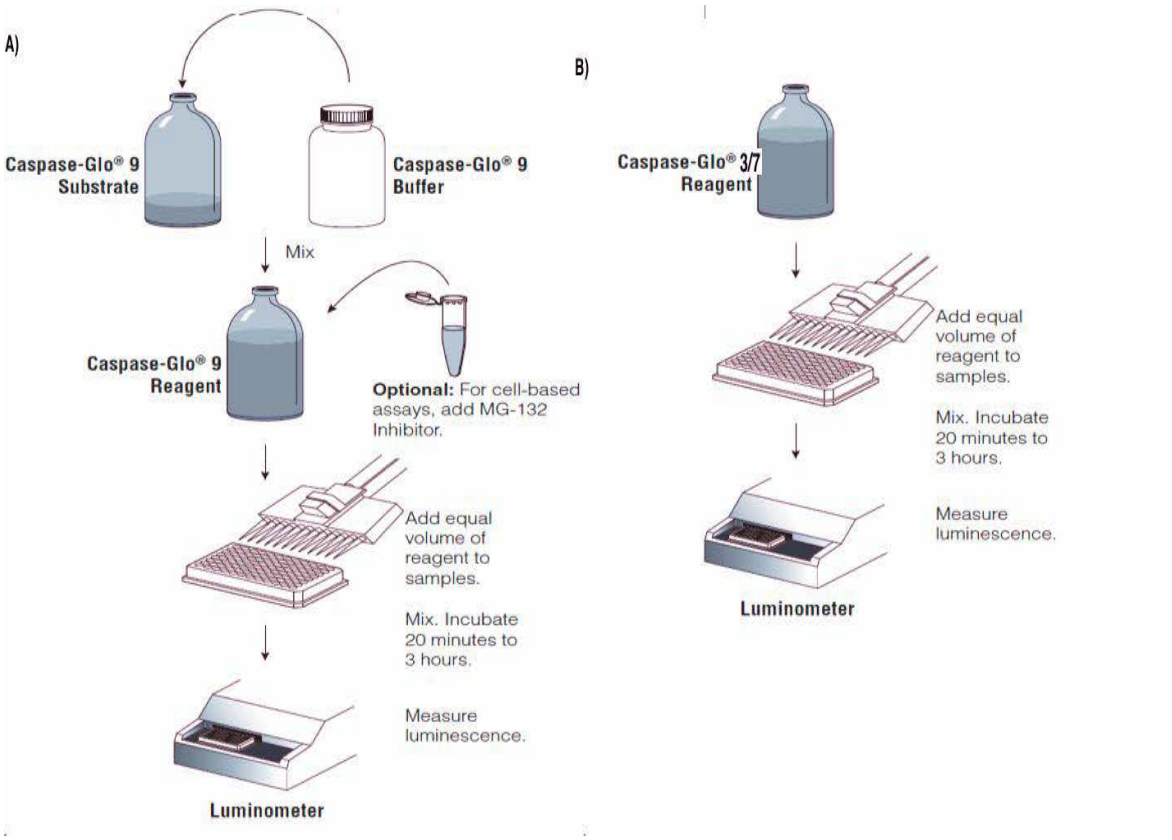


Figure 5.1: Schematic diagrams showing A) Caspase-Glo® 9 and B) Caspase-Glo® 3/7 Assay protocol (Adapted from- http://www.promega.com/~/media/files/resources/protocols/technical%20bulletins/101/caspase-glo%209%20assay%20protocol.pdf?la=en)

5.4.2 Measurement of Cytochrome-c release

The 1321N1, Gos -3, U87- MG and SVGP12 cell lines were incubated with liposomal solutions of *Momordica charantia* or Paclitaxel. The induced (treated cells) apoptosis cell suspension contained roughly 10^7 cells. A sample of non-induced (untreated cells) cells was denoted as control. For a zero-time control also contained 10^7 cells. The induced and non-induced cells were incubated for 24 hours at 37°C in 5 % CO_2. The induced cells and the non-induced cells were transferred to 15 ml centrifuge tube and centrifugation at 1000 r.p.m for 5 min at 4 °C. The supernatant was removed by gentle aspiration of both induced and non-induced cells.

The cell pellets were then washed once with 1 ml of PBS. The microsomal pellet was subsequently obtained by centrifuging at 12,000 rpm for 1 hour at and the supernatant was removed completely by gentle aspiration. The centrifuged cell pellets were then treated with 1 X lysis buffer (Sigma-Aldrich, UK) at a concentration of 100 µl per 10^7 cells, and the cells were placed on ice bath for 15-20 min. The lysed cells were centrifuged at 16,000 to 20,000 x rpm for 10 to 15 min at 4°C and the supernatants were transferred to new 1 ml tubes and subsequently frozen in liquid nitrogen and stored in aliquots at -70°C for further use. Table 5.1 shows the experimental protocol for cytochrome-c assay. A volume of 950 µl of the working solution (9 mg of cytochrome-c to 20 ml of assay buffer to obtain a concentration of 0.45 mg/ml, 36 mM) was made up by adding 9 mg of cytochrome c to 20 ml of the assay buffer in a 1 ml cuvette. A volume of 50 µl of the test sample was added to the cuvette containing the working solution. For the sample that had interference from cytochrome c oxidase activity, a volume of 20 µl of cytochrome c oxidase inhibitor solution was used for the positive control reaction. The positive control was obtained by diluting an aliquot of the cytochrome-c reductase (NADPH) to 10-fold with the enzyme dilution buffer. Each set of reactions required a total of 75 ml of the diluted positive control. A volume of 100 µl NADPH solutions was added to start the reaction. The blank reaction was measured by the value given by the reagents alone in absence of the enzyme (Table 5.1).

Table 5.1: Table below showing the reaction scheme outline to measure cytochrome–c activity (Adapted from:http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/cy0100bul.Par.0001.File.tmp/cy0100bul.pdf)

Test	Working Solution (µl)	Enzyme Dilution Buffer (µl)	Sample or Positive Control (µl)	NADPH Solution (µl)	Inhibitor solution (µl)
Blank	950	50	NIL	100	NIL
Unknown sample	950	50-X	X	100	NIL
Unknown with interference	950	30-X	X	100	20
Positive control (10-fold dilution)	950	25	25	100	NIL
Positive control (10-fold dilution)	950	NIL	50	100	NIL

* Note- NADPH solution contains 0.85 mg of NADPH per millilitre

X= Volume of unknown sample

Calculation:

Unit definition: One unit could reduce 1.0 µmole of oxidized cytochrome-c in the presence of 100 µM, NADPH per minute at pH 7.8 at 25°C

$$\text{Units/ml} = \frac{\Delta A_{550}/\text{min} \times \text{dil} \times 1.1}{21.1 \times \text{Enzvol}}$$

$\Delta A_{550}/\text{min} = \Delta A_{\text{sample}} - \Delta A_{\text{blank}}$

dil = the dilution factor of the original enzyme sample

Enzvol = volume of the enzyme sample (ml)

21.1 = extinction coefficient (EmM) for reduced cytochrome c

1.1 = Reaction volume (ml)

The reaction time was longer than 1 minute; divided the ΔA_{550} by the reaction time to obtain

ΔA_{550} / min. All the values for cytochrome -c were expressed as units/ml.

5.4.3 Statistical analysis

Caspase-Glo® 3/7, Caspase-Glo® 9 and Cytochrme C experiments were performed in triplicate. The graphs of cell apoptosis luminescence on Y-axis and drug on X-axis were plotted, and the results were expressed as the mean ±Standard Deviation (SD) from three independent experiments (n=3). A statistical analysis was performed using SPSS. All the data were evaluated for unpaired variables to compare two or more groups by paired Student‘s – t test‖ and ANOVA. Values of $P < 0.05$ is considered to be statistically significant.

5.5 Results and Discussion

5.5.1 Activity of caspase 3/7 in normal and cancerous glial cells using liposomes with or without *Momordica charantia* (FA extracts) or PTX

In chapter 4 of the study, it was discussed that liposome slightly enhances the anticancer activity, compared to the free extract solutions. The cytotoxic study, confirms that liposome merits, that the liposome enhanced the effect of *Momordica charantia* FA extracts from different countries (Africa, China and India). On the basis of the application in the chapter 4, it was necessary to investigate the molecular mechanism of cells death, adopting clinical anti-cancer techniques like gamma-irradiation, chemotherapy, suicide gene therapy or immunotherapy which can kill tumour cells. The anticancer active constituents can activates apoptosis signal

transduction pathway either by intrinsic or extrinsic pathways (Fulda and Debatin, 2006). To explore the mechanism of *Momordica charantia* extract and PTX with liposome-induced apoptosis via intrinsic or extrinsic pathway, the activation of caspase was analyzed using commercial caspase assay. Measuring caspases 3 and 7 helps in understanding apoptosis evaluation. Caspase 3 and 7 are the effecter caspases that execute some common features of the apoptotic pathway, like DNA fragmentation (Lakhani et al. 2006). This measurement was done indirectly through cleavage of a substrate by the caspases that gave a fluorescent product. Caspase-3 is one of the hallmarks of apoptosis and it is responsible for inducing apoptosis by cleaving a variety of substrates (Shu et al. 2009). Caspase 7 cleaves same substrates as caspase 3 (Fernandes-Alnemri et al. 1995).

In a recent study it was also shown that breast cancer cells MCF-7 on treatment with *Momordica charantia* extract showed PARP nuclear protein or downstream substrate activated caspase 3/7 activities (Ray et al. 2010). Figure 5.2A/B in this study show caspase 3/7 activity in the cancerous (1321N1, Gos-3, U87-MG) cells and non-cancerous glial cells (SVGP12). On treatment with drug-free liposomes, and 1.75 mg/ml of *Momordica charantia* FA (India, Africa and China) extract liposomes and 1.75 mg/ml of PTX liposomes generated from either SPC or HSPC proliposomes as compared to untreated cancerous and non-cancerous cells after 24 hours of incubation. Data are mean ± S.E.M, n=3 for different experiments in triplets (n=9). The results show a statistically significant ($P < 0.05$) increase in caspase 3/7 activity for all the samples compared to untreated cells. On treatment with *Momordica charantia* FA liposomes (SPC or HSPC) a significant ($P < 0.05$) increase in the caspase 3/7 activities was observed in all cell lines (1321N1, Gos-3, U87-MG and SVGP12), regardless of the plant origin. In contrast, no significant ($P > 0.05$)

difference was observed in the release of caspase 3/7 when cells were treated with drug-free liposomes (SPC or HSPC) as compared to the untreated cells. The release of caspase 3/7 was more pronounced in 1321N1 followed by Gos-3, U87-MG and SVGP12 on treatment with SPC liposomes including FA *Momordica charantia* extract. By contrast, on treatment with FA *Momordica charantia* extract using HSPC liposomes, the activity of capase 3/7 increased in Gos-3 followed by 1321N1, U87-MG and SVGP12. Both HSPC and SPC liposomes including Chinese *Momordica charantia* showed maximum caspase 3/7 activities followed by African *Momordica charantia* and Indian *Momordica charantia*. However, caspase 3/7 activities analysis on treatment of *Momordica charantia* extract included in the liposome (SPC and HSPC) showed less activity as compared to PTX liposomes (SPC and HSPC) in all the cell lines.

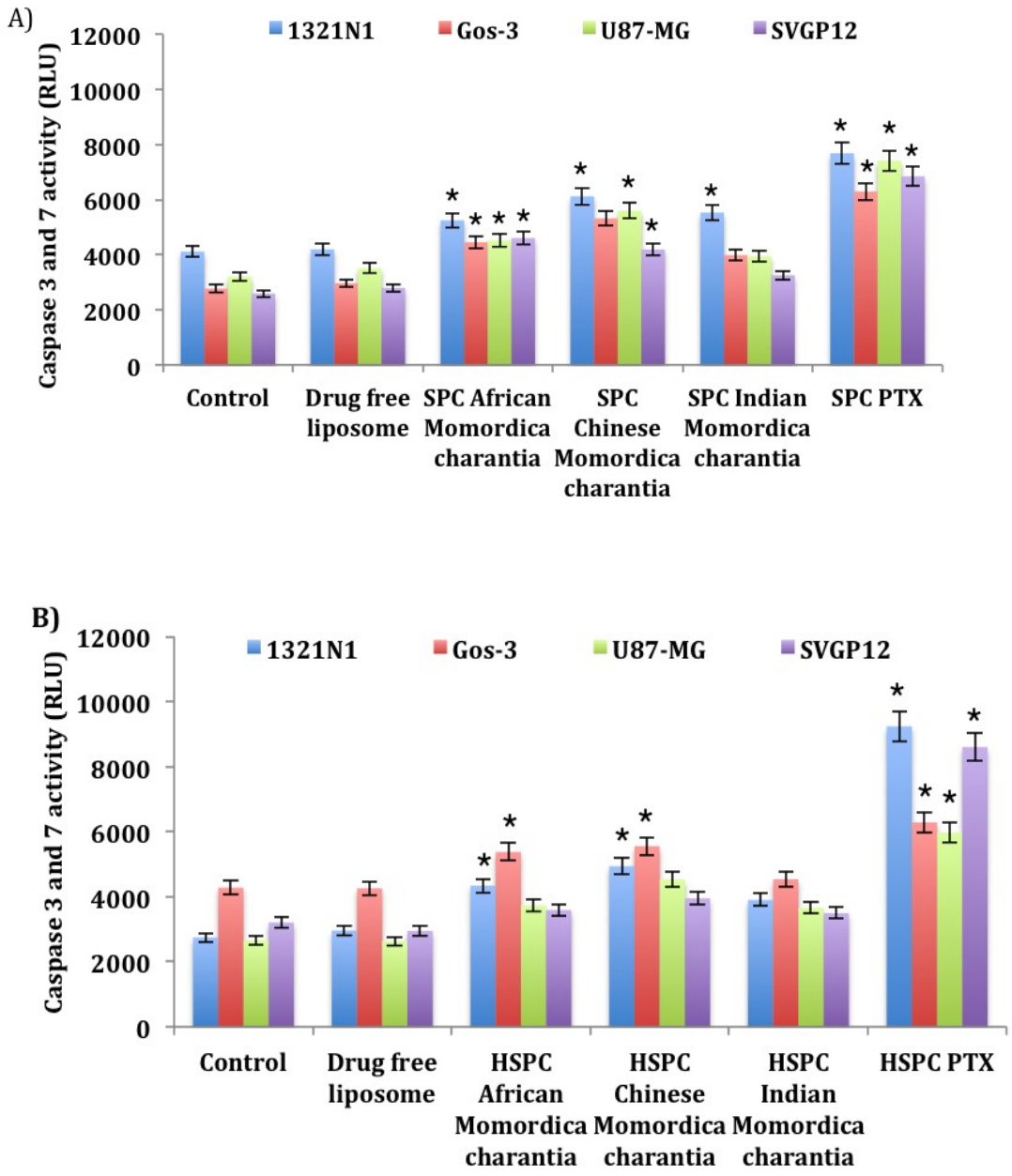


Figure 5.2: Bar charts showing A) SPC and B) HSPC liposomes, encapsulated with and without (Drug free liposome) 1.8 mg /ml of *Momordica charantia* FA from Africa, India and China and PTX involves caspase 3/7 activation on the death of 1321N1, GOS-3, U87-MG and SVGP12 as compared to untreated cells. Data are mean ± S D; n=3 in triplicates *P < 0.05 for fruit extracts liposomal formulation compared to control.

5.5.2 Activity of caspase 9 in normal and cancerous glial cells using liposomes with or without *Momordica charantia* FA extract or PTX

The caspase-9 can propagate cell death via intrinsic pathway (Bailey et al. 2005). The Apaf-1/caspase-9 apoptosome-initiated the caspase activation cascade and in turn caspase-9 cleaves and activates downstream caspases such as caspase-3 and caspase 7 (Slee et al. 1999; Srinivasula et al. 1999). The relaying an apoptotic signal from caspase-9 to cleavage of PARP is substituted by caspase-7 (Fang et al. 2012). Figure 5-3A/B shows the activity of caspase 9 in glioma cell lines (1321N1, Gos-3, U87-MG) and normal glial cells (SVGP12) measured following treatment for 24 hours with either drug free liposomes, *Momordica charantia* FA (India, Africa and China) liposomes or PTX liposomes generated from SPC or HSPC phospholipids as compared to untreated (normal and cancerous glial) cells. Data are mean ± S.E.M, n=3 different experiments in triplets. On treatment with either SPC or HSPC liposomes containing PTX or *Momordica charantia* extract from the three different origins (Africa, China and India), a significant ($P < 0.05$) increase in the caspase 9 activity was found in all cell lines investigated. Using the extracts with SPC or HSPC liposomes, 1321N1 demonstrated the maximum caspase 9 activity followed by Gos-3, U87-MG and SVGP12. However, when PTX-loaded SPC liposomes were applied, caspase 9 activity was higher in SVGP12 normal cells followed by 1321N1, Gos-3, U87-MG cells. On treatment of cells with HSPC liposomes loaded with PTX similar capsase 9 activity was obtained compared to those using *Momordica charantia* liposomes. SPC liposomes caused higher caspase 9 activities than HSPC liposomes. The use of Chinese and African extracts with SPC or HSPC liposomes resulted in similar caspase 9 activities, followed by the Indian extract. However, extract-

liposome formulations, regardless of plant origin, phospholipid type and cell line, demonstrated lower Caspase 9 activities as compared to PTX liposomes.

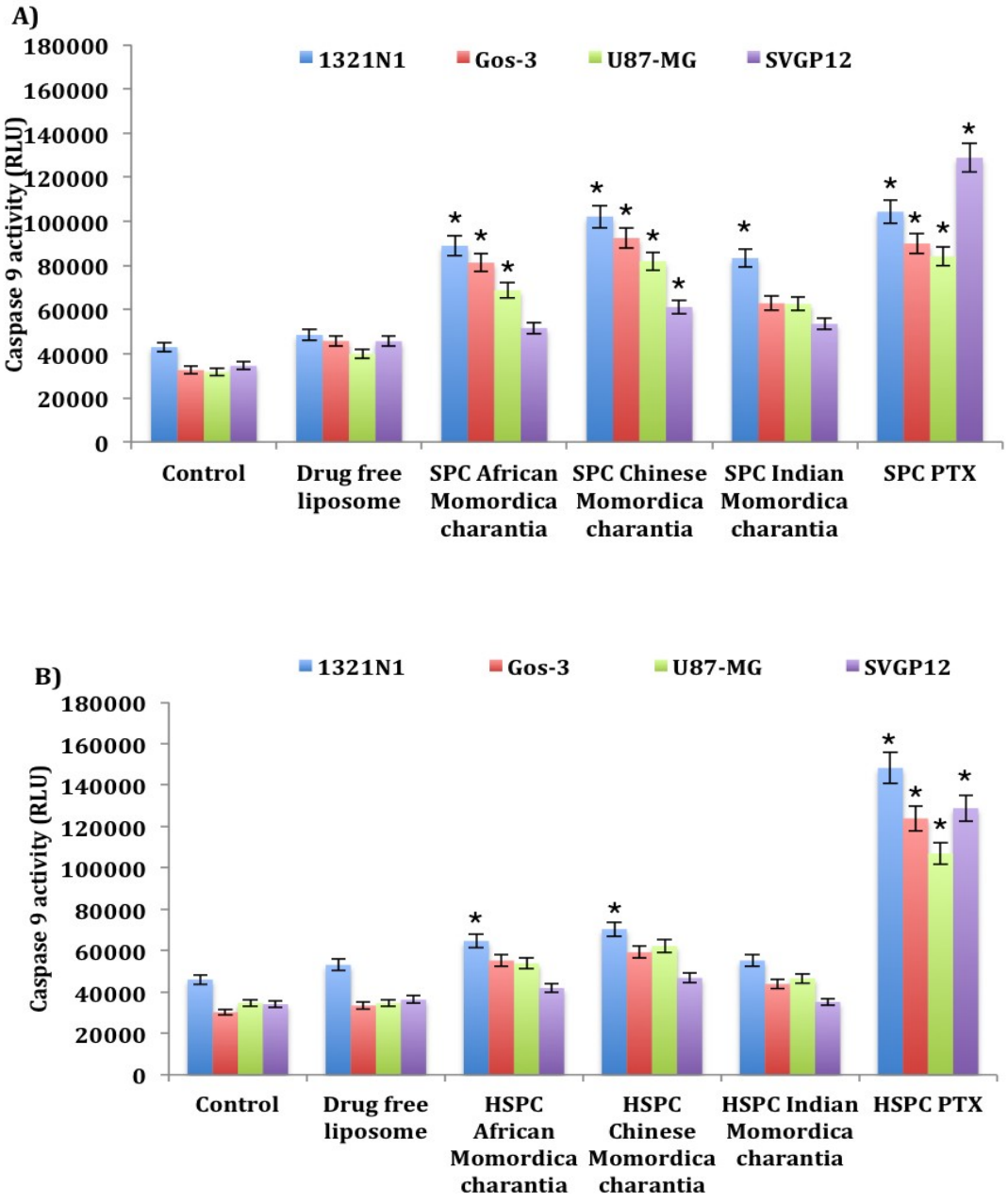


Figure 5.3: Bar charts showing A) SPC and B) HSPC liposomes, encapsulated with and without (Drug free liposome) 1.8 mg / ml of *Momordica charantia* FA from Africa, India and China involves caspase 9 activation on the death of 1321N1, GOS-3, U87-MG and SVGP12 as compared to untreated cells. Data are mean ± S D; n=3 in triplicates *P < 0.05.

5.5.3 Cytochrome C release in 1321N1, Gos-3, U87-MG and SVGP12 glial cell lines, on treatment with SPC and HSPC liposome including *Momordica charantia* extracts or PTX

Mitochondria play, a crucial and central regulatory role in apoptosis through cytochrome C pathway (reference). Mitochondria and radical species are intimately involved in the apoptosis. Increased oxidative stress from ROS and RNS changes the cellular redox potentials, depletes glutathione, and decreases reducing equivalents like NADP and NADPH. These intracellular changes are sufficient to induce the formation of mitochondrial permeability transition pores, leading to release of cytochrome c and activation of the caspases cascade (Bansal et al. 2012). The intrinsic pathway releases mitochondrial cytochrome *c* as a consequence of opening of the Bax/Bak channel. It results in the assembly of the Apaf-1/caspase-9 apoptosome and leads to activation of caspase-9 within this complex (Luo, 1998). In a previous study, it was demonstrated that apoptosis induced by *Momordica charantia* extract on human lung adenocarcinoma CL1-0 cells was through caspase- and mitochondria-dependent pathways, which change the antiproapoptotic Bcl-2 and proapoptotic Bax proteins (Li et al. 2012). In a recent investigation, Manoharan (2011) has demonstrated that cytochrome c is released using MC extracts, at an extract concentration of 800 µg. In comparison to the present findings, it is concluded that liposomes might enhance the release of cytochrome c, hence this may show the potential of liposomes. Figure 5.4 A/B shows cytochrome c release in the cancerous (1321N1, Gos-3, U87-MG) and non-cancerous glial cells (SVGP12) on treatment with either plain liposomes, *Momordica charantia* FA (India, Africa and China) with liposomes or PTX liposomes using SPC or HSPC phospholipids compared to untreated cells. *Momordica charantia* FA liposomes (SPC and HSPC)

significantly ($P < 0.05$) increased the cytochrome c release in all the cell lines (1321N1, Gos-3, U87-MG and SVGP12), regardless of fruit origin (i.e. Africa, China or India). However, no increase ($P > 0.05$) in cytochrome c release on treatment with plain liposomes (SPC and HSPC) was observed for all cell lines. Cytochrome c release was more pronounced on treatment of *Momordica charantia* Chinese FA, followed by Indian *Momordica charantia* and African FA, which showed similar increase. PTX liposomes show more increase in the release of cytochrome c, in all the four glial cells as compared to *Momordica charantia* FA liposomes. The cytochrome c release of low-grade glioma is comparatively higher than high-grade glioma and normal glial cell on the treatment with drug free liposomes or *Momordica charantia* (India, Africa and China) liposomes and PTX liposomes. In conclusion, the increase in the cytochrome c release may justify that the particular extract within the liposome is able to show maximum apoptotic activity via intrinsic pathway.

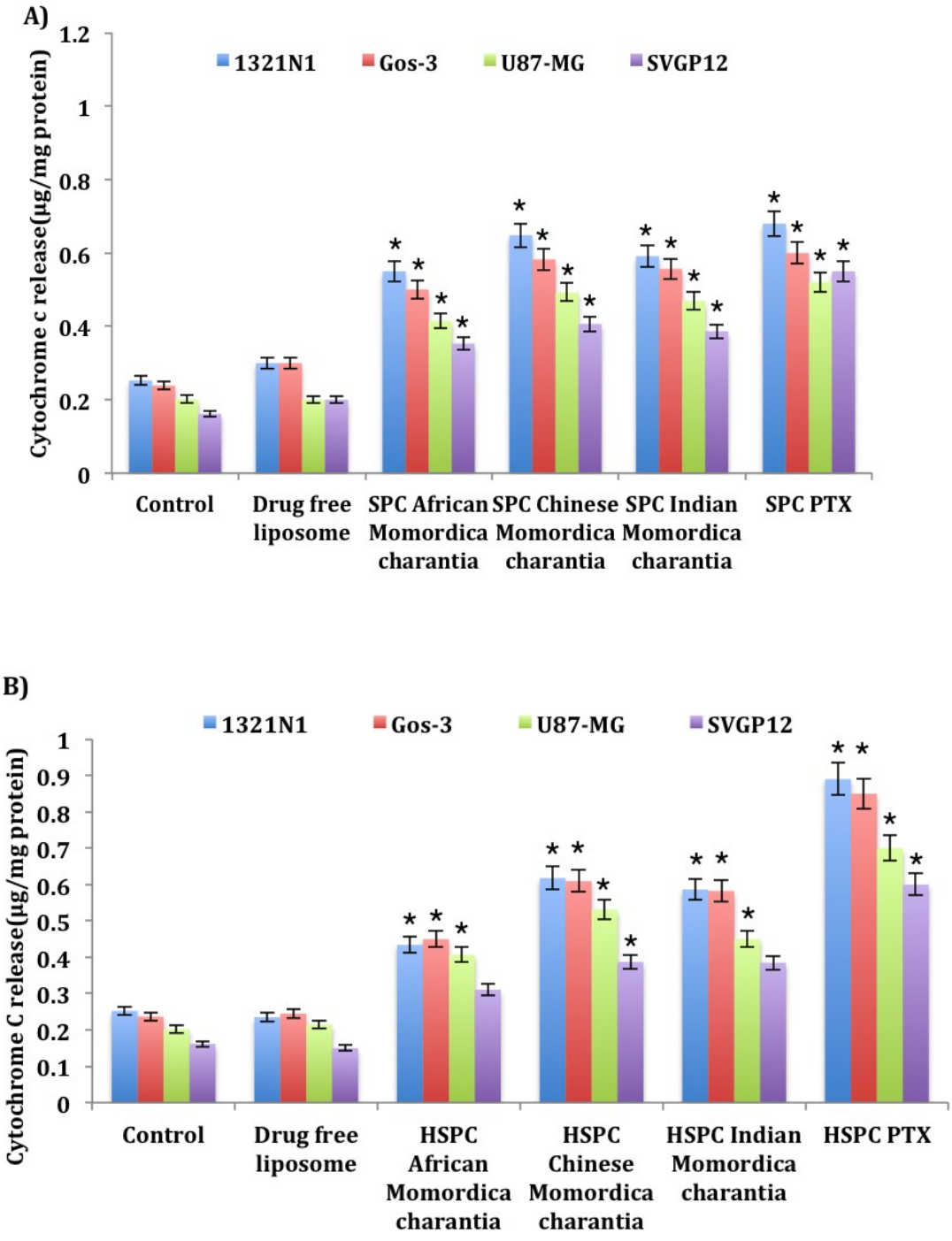
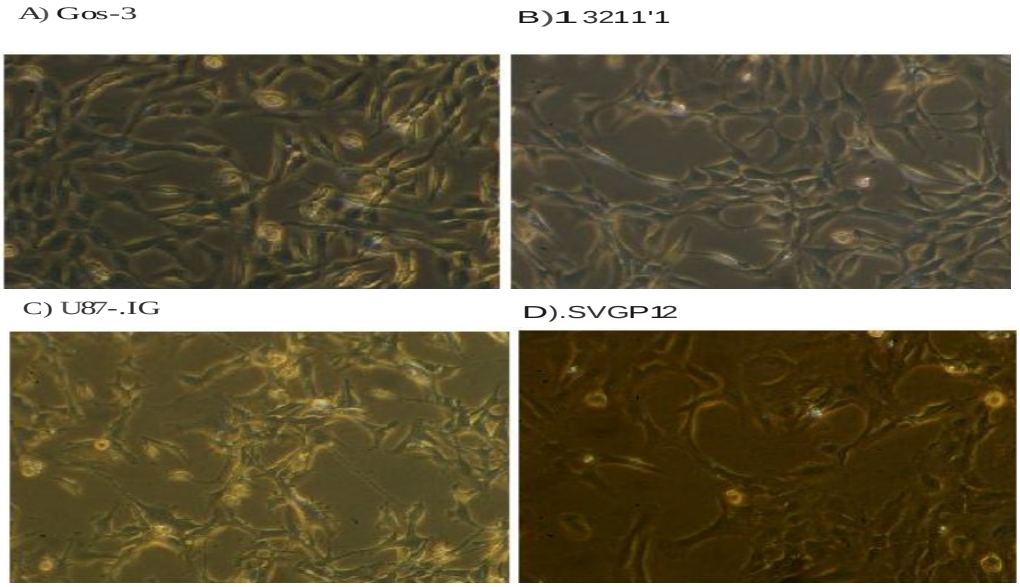


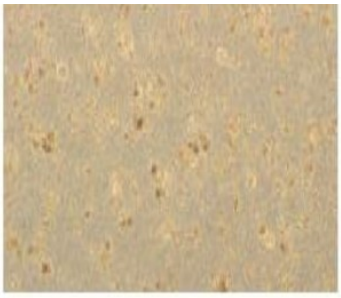
Figure 5.4: Bar chart showing cytochrome c realease of 13121N1, GOS-3, U87-MG and SVGP12 of untreated and 24hr treatment of Drug free liposome, *Momordica charantia* FA extract (African, Chinese and Indian) and PTX loaded within A) SPC ad B) HSPC liposomes. Data are mean ± S D; n=3; *P < 0.05 for treated compared to control.

5.5.4 Morphological characteristic of cell lines untreated and treated with *Momordica charantia* extract or PTX included in liposomes and drug free liposome prepared from proliposome technology

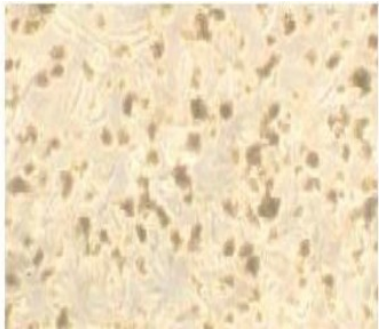
The photographs in this section give morphological description about, cancerous and non-cancerous glioma cells before and after treatment with either *Momordica charantia* extract or PTX included within SPC or HSPC liposome. The photograph was taken using an Inverted microscope under 10 X magnification. The photograph shows healthy growth of both cancerous and non-cancerous cells when they were untreated. But on treatment with liposome containing extract, they showed the shrinkage of the cell membrane. Glioma (1321N1, Gos-3 and U87-MG) cells on treatment with either *Momordica charantia* extracts or PTX within the liposomes shows less viable cells compared to the treated normal glial cells (SVGP12). The photograph in the Figure 5-8 shows healthy morphological structures of Gos-3 (Figure 5.5A) and 1321N1 (Figure 5.5B) cells in Dulbecco‘s Modified Eagle Medium (DMEM) and U87-MG (Figure 5.5C) and SVGP12 (Figure 5.5D) cells cultured in Eagle‘s Minimal Essential Medium (EMEM) media. Figure 5.5E shows shrinkage destruction and shrinkage of chromatins of U87-MG on treatment with PTX liposomes. Similarly, Figure 5.5F shows U87-MG cells on treatment with plain liposomes. Figure 5.5G is an image of GOS-3 after treatment with *Momordica charantia* liposome. Finally, figure 5.5G is an image of SVGP12 following application of *Momordica charantia* liposomes.



E) U8 -MG (LiJ)Osomal Partitaxtl *htahntnt*) F) U8 -MG (Dmg *Frtt* LiJ)Osomal hrtahntnt)



G) Gos-3 (Momonlira rharantia LiJ)Osomal hrrahntnl)



H)SVGPl2 (Momonlira charantia LiJ)Osomal hrrahntnl)

Figure 5.5: Photograph showing untreated cell line 1321Nl (A), GOS-3 (B), U87-MG (C) and SVGP12 (D) with no destruction of the chromatins or morphological features. Vice versa, treated cell lines; U87-MG with PTX liposome (E) and drug free liposome and (F), Gos-3 (G) and SVGP12 (H) treated with *Momordica charantia* liposome shows shrinking and destruction of cells. The photographs are typical of 3 such different repeats.

5.5.5 Conclusion

The results of this study have clearly demonstrated that drug free liposomes made from SPC and HSPC, PTX and crude extract of *Momordica charantia* from India, Africa and China loaded within SPC or HSPC liposomes can evoke significant decrease in cancer cell viability (cell death). Moreover, they seem to exert their anti-cancer effect on cells via apoptosis damaging the cell mitochondrial body, resulting in the elevation in cellular mediators as caspase-3/7 and caspase-9 and release of cytochrome c. Treatment of cells with drug free liposomes prepared from proliposome method employing with SPC or HSPC phospholipid had no significant ($P > 0.05$) effect on capase 3/7, caspase 9 and cytochrome c release in the cancerous and non-cancerous glial cells compared to untreated. From the results it can be conclude that drug free liposome does not kill cell via intrinsic pathway. On application of either PTX or *Momordica charantia* FA extract (Africa, China and India) with SPC or HSPC liposomes, there was more apoptotic activity in 1321N1 cell line followed by Gos-3, U87-mg and SVGP12. Chinese *Momordica charantia* FA loaded in either SPC or HSPC liposomes showed maximum caspase activation and cytochrome c release as compared to African and Indian *Momordica charantia* FA. However, the maximum apoptotic activity was observed in SPC and HSPC liposomes loaded with PTX as compared to the *Momordica charantia* extracts. Apoptotic cells show distinctive morphology during the apoptotic process. The cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton. In summary, following 24hrs of treatment with *Momordica charantia* FA within SPC and HSPC liposome can lead to elevation in the activities of caspase 3/7, caspase 9 and the release of cytochrome c. *Momordica charantia* FA inclusion within SPC and HSPC liposomes activates intrinsic mitochondrial apoptosis pathway

via activation of caspase cascade. Further experiments are required to investigate the sub-cellular and molecular mechanisms associated with the cell death including the involvement of kinase and gene expressions for apoptotic mediator.

Chapter 6. General Discussion, Conclusion and Future Work

6.1 General Discussion

Plants are invaluable source of potential new anti-cancer drugs. *Momordica charantia* is one of these plants with both edible and medical value and reported to possess anticancer activity (Fujii et al. 2000). To explore the potential cytotoxic effectiveness of the extract of *Momordica* charantia, liposomes prepared from solvent-based proliposomes were used to evaluate the cytotoxic activity on cancerous and non-cancerous glial cells. Three variates of *Momordica charantia* were obtained from Africa, India and China for this study. The rationale was to investigate whether the different phenotypic *Momordica charantia* obtained from different climatic conditions and grown in different soil condition has any variation in their anticancer effect. These fruits from different countries were segregated into FA, SA and WF extracts and were included in SPC or HSPC liposomes. The *Momordica charantia* extract liposomes prepared form solvent-based proliposomes were applied on 1321N1, Gos-3, U87-MG and SVGP12 glial cells.

To investigate the anticancer effect of *Momordica charantia* extract with liposomes, the study involved:

1. Characterization of liposomes with regard to size, size distribution and zeta-potential.
2. Cytotoxic effect of liposomes including extract by using MTS assay
3. Molecular mechanism pathway of cell death by – caspase 3/7 activity, caspase 3 activity and cytochrome c release.

6.2 Characterization of liposomes generated from proliposomes

Liposomes made from either SPC or HSPC were incorporated with either ethanol and water (1:10 v/v) extract of *Momordica charantia* (WF, FA and SA) or with the ethanol soluble anticancer drug paclitaxel. Ethanol-based proliposome technology was used for the preparation of liposomes. Ethanol-based proliposomes have been previously demonstrated to generate liposomes that offer superior entrapment efficiency of hydrophilic materials (Perrett et al. 1991; Elhissi et al. 2006). Ethanol-based proliposome method is safe, economic, convenient, time saving and self-preserved against microbial contamination and can instantly generate liposomes upon addition of aqueous phase and shaking (Perrett et al. 1991). Ethanol-based proliposome technology is also environmental friendly since no toxic solvents like chloroform or methanol are required to prepare the liposomes. Liposome interaction with biological cells is determined by a number of factors such as particle size and zeta potential.

There are many potential barriers to the effective delivery of a drug in its active form to solid tumours (e.g. brain tumour). Most small-molecular chemotherapeutic agents have a large volume of distribution following their administration (Speth et al. 1988; Chabner and Longo, 1996). Encapsulation of drugs in a macromolecular carrier, such as a liposome, may reduce the volume of distribution significantly and increase the concentration of the drug in the tumour site. The blood-brain barrier represents a formidable barrier for drug delivery to the central nervous system. Tight junctions, the lack of fenestrations, and a low transcellular pinocytic index severely limit the penetration of macromolecules to the brain (Levin et al. 1980; Seymour, 1992). Several groups have been able to show that even tumours located in the brain also have a –leaky‖ microvasculature (Siegal et al. 1995; Hobbs et al. 1998). If this is the

case, it could be assumed that the leaky microvasculature may allow the liposomes to accumulate in the cancer-affected area. The accumulation of the liposome does not allow the cancerous cells to proliferate and do not affect normal cells.

The drug free SPC and HSPC liposomes were generated from ethanol–based proliposomes or by thin-film method to target brain tumour. Size, size distribution and zeta potential of liposomes were analysed. Liposomes generated from proliposomes were smaller and their size distribution was narrower than vesicles prepared by the conventional thin film method. Also, size was smaller and size distribution was narrower for the SPC liposomes as compared to the HSPC liposomes. However, SPC liposomes were more negatively charged than HSPC liposomes. Smaller particles with higher zeta potential exhibited higher stability but formed larger and and irregular shapes (Jong and Keun, 1999).

Momordica charantia is an excellent source of phenolic compounds, antioxidants, and antimutagen useful for the treatment of cancer. Certain vegetables contain an abundance of polyphenolics, terpenoids, isoflavones, anthocyanins, amino acids, minerals, vitamins, and other antioxidants that are associated with protection from cancer (Islam et al. 2010). In this study the *Momordica charantia* was bought from the local supermarkets but obtained from different countries (Africa, China and India). They were grown, under different soil and climatic conditions. *Momordica charantia* extracts were segregated as FA, SA and WF from India, Africa and China respectively, and were included in SPC or HSPC liposomes using the proliposome technology.

Momordica charantia extracts from the three countries encapsulated in SPC phospholipids generated smaller size liposomes compared to HSPC phospholipids.

Also, the size distribution of the SPC liposomes containing *Momordica charantia* extracts (FA, SA and WF) was narrower (Span was smaller by 1- 2.5 units) compared to HSPC *Momordica charantia* extract liposomes. Except, Chinese *Momordica charantia* extracts on encapsulation with SPC phospholipid showed increased size distribution with the increasing concentrations (0.7-1.8 mg / ml) as compare to HSPC liposomes containing extract. Liposomes containing PTX using SPC were smaller than HSPC liposomes. However, the size distribution of PTX drug along with HSPC and SPC phospholipid showed vice versa results. Using PXT, the size distribution of HSPC liposomes was smaller than SPC liposomes (Figure 3-7 B).

Lipid composition can influence the liposomal surface charge. Lack of surface charge can reduce physical stability of small unilamellar liposomes by increasing their aggregation (Kaye 1981; Sharma and Sharma 1997). However, negatively charged liposomes, were believed to be more rapidly removed from circulation than neutral or positively charged liposomes (Gabizon and Papahadjopoulos 1992). It has been reported that the negatively charged liposomes are predominantly taken up by cells through coated-pit endocytosis, while cationic liposomes may deliver their contents to cells either by fusion with cell membranes or through coated pit endocytosis (Sharma and Sharma 1997). Conventional method for the preparation of liposome is difficult to scale up, and phospholipids in liquid dispersion are liable to hydrolysis (Kensil and Dennis, 1881; Grit et al. 1989), and oxidation (Hunt and Tsang, 1981), as well as liposome may aggregate and lose the entrapped material. The net charge of liposomes is also an important factor and generally anionic and neutral liposomes survive longer than cationic liposomes in the blood circulation after intravenous injection (Wang et al. 2005; Woodle and Lasic, 1992). Moreover, ethanol-based proliposome technology is suitable for preparation of liposomes on a

large scale, comprising a stable alternative to the sophisticated and time consuming rotary evaporator thin film method. SPC liposomes with *Momordica charantia* extracts produced either slightly positive slightly negative or neutrally charged liposomes. Whereas HSPC liposomes along with *Momordica charantia* extracts (FA, SA and WF) from India and Africa and WF and SA extracts from China produce positively charged liposomes. In contrast, Chinese FA extract included in HSPC phospholipid produced highly negatively charged liposome. The zeta-potential of HSPC liposomes containing PTX was more negative compared to SPC liposomes. PTX liposomes were highly negative compared to liposomes containing *Momordica charantia* extracts.

6.3 Growth curve of cancerous and non-cancerous glial cells

The growth curve experiments in this study have indicated that the glioma cell line 1321N1, Gos-3 and U87-MG proliferated faster than SVGP12, confirming that tumour cells could proliferate much faster than the normal cells (Mor et al. 2008). But sometimes even normal cells proliferate much faster (doubling time of 24 hours) than do most tumour cells (Pardee, 2002). The death of low-grade glioma 1321N1 and Gos-3 occurs at the early stage as compared to the U87-MG high-grade glioma. Glioma cell lines 1321N1, Gos-3, U87-MG on treatment with drug free liposomes did not affect the viability of cells. Patients suffering from low-grade glioma are likely to respond to the chemotherapy as compared to high-grade glioma (Engelhard et al. 2003).

6.4 Cytotoxicity of liposomes generated from proliposomes on tissue culture

The result of the study has demonstrated significant anticancer effect of *Momordica charantia* extracts from the fruit alone, seed alone and whole fruit from Africa, China and India encapsulated in liposomes and used for the treatment of cancerous and non-cancerous cells as compared to untreated glial cells.

A number of preliminary studies have previously shown the anti-cancer activity of *Momordica charantia* against lymphoid, leukemia, lymphoma, choriocarcinoma, melanoma, breast, skin and prostate cancer (Licastro et al. 1980; Ng et al. 1994; Sun et al. 2001). Maximal anti carcinogenic activity was demonstrated following chronic treatment with hot water extract of *Momordica charantia* in uterine adenomyosis and mammary tumour growth in mice (Nagasawa et al. 2002; Singh et al. 1998). The beneficial anti-cancer effects of crude water-soluble extract of *Momordica charantia* have been reported previously by several other investigators (Ganguly et al. 2000; Jilka et al. 1983; Singh et al. 1998; Raman and Lau, 1996; Takemoto et al. 1982; Yeung et al. 1988). Moreover, a number of studies have been conducted using the crude preparation of *Momordica charantia*. In all these studies, the chemical profile of the extract was not reported in the investigations. Nevertheless, some studies have demonstrated marked biological activities of several compounds including charantin, MAP 30, Momordica and alpha, beta momorcharin extracted from *Momordica charantia* (Grover et al. 2004).

All soluble compounds of either fruits or vegetables usually play important roles in preparing juices. Protein fractions obtained from the fruit and seed of *Momordica charantia* have the ability to inhibit cell growth, guanylate cyelase activity and ribosomal activity (Ram and Lau, 1996). It is hypothesised in the present project that

the distribution of active ingredients of *Momordica charantia* is different with accordance to difference in soil constituents and climatic conditions. It is also hypothesised that the concentration of plant ingredients is not the same in different parts like the skin, flesh and seeds. *Momordica charantia* fruit pulp and seed compounds possess anti-oxidant activity. *Momordica charantia* fruit pulp shows higher antioxidant activity compared with *Momordica charantia* seed, which may be ascribed to their different phenolic and flavonoid compositions. Ethanol/water extracts of both pulp and seed powders exhibited higher anti-oxidant activities compared with other solvent extracts (Padmashree, 2011).

The present study employed the extracts from either the green flesh, seed or whole fruit alone of *Momordica charantia* obtained from either Africa, India or China to investigate their anti-cancer effects. These experiments were designed to identify the anticancer effect on cancerous (1321N1, Gos-3 and U87-MG) and non-cancerous (SVGP12) cells. The results showed that the growth of the glioma cells 1321N1, Gos-3 and U87-MG was significantly inhibited at the higher concentrations of African, Chinese and Indian extracts (FA, SA and WF) of *Momordica charantia* on association with SPC or HSPC liposomes, compared to the liposome free *Momordica charantia* extracts (FA, SA and WF). Treatment of the normal glial cells SVGP12 on the treatment with African *Momordica charantia* SA associated with SPC liposomes, African *Momordica charantia* extracts (FA, SA and WF) associated with HSPC liposomes, Chinese (FA and SA) associated with HSPC liposomes showed significant increase in SVGP12 cells at the lower concentrations and no significant change at the higher concentrations was observed. The cytotoxic effect of *Momordica charantia* extracts was increased on the association with SPC liposomes as compared to *Momordica charantia* extract associated with HSPC liposomes.

Regardless of country of origin, the cytotoxic effect of *Momordica charantia* FA was higher than that of SA or WF extract. Chinese *Momordica charantia* FA extracts alone and associated with SPC or HSPC liposomes showed potent inhibition of glioma cells compared to untreated cells. Chinese phenotype extracts with or without liposomes showed more potent cytotoxic effect compared to African and Indian phenotypes.

6.5 Mechanism of cell death induced by the liposomes prepared from proliposome method

FA extract of *Momordica charantia* obtained from Africa, China or India showed maximum cytotoxic effect on the glioma cell lines. The mechanism of apoptosis for cell death of cancerous and non-cancerous glial cells is believed to occur by intrinsic or extrinsic pathway. Regardless of fruit origin, the treatment with FA extract included in SPC or HSPC liposomes resulted in different release of caspase3/7, caspase 9, calcium and cytochrome c. Apoptosis is a programmed process of cell death and it is associated with damage of cell mitochondria to elevate intracellular mediators such as caspase 3 and caspase 9 and release of cytochrome c (Bernadi et al, 1994; Chandra et al 2002). In previous studies, it was shown that anticancer drugs exert their lethality by inducing apoptosis in tumour cells targeting both mitochondrial (intrinsic pathway) and death receptors (extrinsic pathway) (Gati et al. 1991; Makin, 2002; Hajnoczy et al. 2006). Histologically, apoptotic cells are characterized by morphological alterations such as cell shrinkage, nuclear chromatin compaction, pyknosis, nuclear fragmentation, cytoplasmic condensation, and convolution of the nuclear and cell outlines. The receptor-mediated pathways is triggered by the activation of capase 8 which in turn cleaves and activates caspase 3 (Ashkenazi and Dixit, 1998). Cysteine aspartases (caspases), a protease family, are

known to be required for apoptosis induced by various stimuli (Krepela, 2001). Among mammalian caspases, comprising at least 14 known members, caspase-3 is thought to be the main effecter of caspases and it has been identified as being activated in response to cytotoxic drugs (Krepela, 2001). Activation of caspase-3 is an important step in the execution phase of apoptosis and its inhibition blocks cell apoptosis (Budihardjo et al. 1999). In mammals, caspase-3 has been identified as key executors of apoptosis and is one of the most important caspases activated downstream of apoptotic pathways (Cohen, 1997). In addition, caspase-3 which activates caspase-activated DNase, causing apoptotic DNA fragmentation. The mitochondrial pathway is initiated by cytochrome c relaease from the mitochondria, which in turn promote the activation of of caspase 9, which is responsible for the activation of cell death protease. (Ashkenazi and Dixit, 1998; Chandra et al. 2002). The present findings have demonstrated that caspase 3/7, caspase 9 and cytochrome c were released in the glioma cell lines (1321N1, GOS-3, U87-MG) when the highest concentration of *Momordica charantia* extracts was used. Maximum level of caspase 3/7, caspase 9 and cytochrome c release were observed when each glioma cell line was treated with Chinese *Momordica charantia* FA extract on association with SPC liposomes. Normal glial cells SVGP12 on treatment with *Momordica charantia* extract with SPC or HSPC liposomes showed a slight release of caspase 3/7 and caspase 9. The cytochrome c release from SVGP12 was higher than that occurred for the untreated SVGP12 cells. However, the release of cytochrome c was less in SVGP12 when compared to the treated glioma cell lines 1321N1, GOS-3 and U87-MG. The PTX liposomes generated from SPC and HSPC liposomes caused significantly more release of caspase 3/7, caspase 9 and Cytochrome c as compared to the cell lines treated with *Momordica charantia* extract FA liposomes from SPC

and HSPC phospholipids. Low grade glioma showed more cytotoxic effect compare to high grade glioma. *Momordica charantia* with liposomes (HSPC or SPC) caused cell death in brain tumour cells via mitochandrial pathway. Similarly, the release of caspase 3/7, caspase 9 and cytochrome c was higher in low grade glioma compared to high grade glioma. The incidence of cancer is increasing worldwide, in spite of substantial progress in the development of anti-cancer therapies. One approach in controlling cancer could be its prevention by diet, which may inhibit one or more neoplastic events and reduce the possibility of cancer occurrence. Dietary compounds offer great potential in the fight against cancer by inhibiting the carcinogenesis process through the regulation of cell homeostasis and cell-death machineries (Nerurka and Ray, 2010).

6.6 Conclusion

The anti-tumor activity of *Momordica charantia* has recently begun to emerge. This research focuses on recent advancements in cancer chemopreventive and anti-cancer efficacy of bitter melon and its active constituents. The main objective of this project was to develop SPC and HSPC liposomes using the proliposome method to target brain tumour. *Momordica charantia* extracts (FA, SA and WF) and PTX associated with SPC or HSPC liposomes led to aggregation of liposomes and caused enlargement of the size and broadening of the size distribution of liposomes, which also had negative zeta potential measurements. These liposomes were prepared to target the brain tumour by employing such tumour cell lines as 1321N1, GOS-3, U87-MG investigate the effect compared to normal glial cell line SVGP12. The commercial anticancer drug, PTX liposome was used to compare the anti cancer effect on the cancer cell line as compared to *Momordica charantia* extracts

liposomes. PTX liposomes (SPC or HSPC) could inhibit around 44-60 % of glioma cell lines (1321N1, Gos-3 and U87-MG), whereas, *Momordica charantia* extracts (WF, FA and SA) liposomes (SPC or HSPC) from different countries of origin could inhibit 60-80 % of glioma cells without effecting SVGP12 cells. *Momordica charantia* (FA, SA and WF) liposomes inhibited less glioma cell lines as compare to PTX liposomes. The effect of *Momordica charantia* liposomes on glioma cells was slightly more or had no effect as compare to drug without liposomes. *Momordica charantia* FA extract with or without liposomes was more effective to inhibit brain tumour as compared to *Momordica charantia* WF and SA extracts with or without liposomes. PTX liposomes generated from either SPC or HSPC liposomes caused significantly higher release of caspase 3/7, caspase 9 and cytochrome c as compared to the cell lines treated with *Momordica charantia* extract FA liposomes. Low-grade glioma shows more cytotoxic effect compared to high-grade glioma. It seems likely that high-grade glioma requires high doses of *Momordica charantia* extract to show the cytotoxic effect. Similarly, the realease of caspase 3/7, caspase 9 and cytochrome c was higher in low-grade glioma compared to high-grade glioma. In conclusion, proliposome method was developed to generate a stable and effective targeted liposome delivery system for *Momordica charantia* extracts (FA, SA and WF) or PTX to take these constituents selectively to glioma brain tumors. This targeted delivery system could potentially increase the anti-cancer activity as well as the therapeutic index of the drug compared to existing solution dosage forms.

6.7 Scope for

The study in this project opens many possibilities for future work such as-

1. Separation and isolation of active anticancer compounds from *Momordica charantia* and entrapping them individually in liposomes.
2. Reducing the particle size of the liposomes by probe sonication, or extrusion to study the effects of that on the anticancer activity.
3. To achieve easy and convenient mode of transport and handle, it is necessary to freeze dry or spray dry the liposomes for the treatment of brain tumour. The effects of drying on the anticancer activity can then be studied.
4. Usage of either sucrose or trehalose as cryoprotectants may avoid oxidation and make liposomes more stable to circulate in the body for longer period of time. For tumour cells thrive on sugar molecule for the nutrition, therefore, liposomes in combination with *Momordica charantia* and sucrose or trehalose may serve to be more toxic to the tumour cells. This hypothesis should be studied in the future.
5. Brain tumour cells expresses large amount of iron-saturated transferrin receptors. One approach is to prepare liposomes with higher specificity to bind to the tumour cells by using transferrin molecules in combination with *Momordica charantia* liposomes. A wide range of therapeutic agents into malignant sites can be delivered by transferrin-endocytosis process.
6. Using an animal model to surgically operate the brain and inject the *Momordica charantia* extracts liposomes inside the brain. This type of technology may cause sustains release of *Momordica charantia* for longer period of time without causing any toxic effect on the normal cells outside the brain.

7. Measure intracellular free Ca^{++} concentration both in cytosol and in mitochondria.

Chapter 7. Reference

7.1 References

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Chapter 8. Appendix

PROLIPOSOME TECHNOLOGY AS AN APPROACH TO GENERATING ANTICANCER LIPOSOMES.

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School of Pharmacy and Biomedical Sciences, University of central Lancashire, Preston. PR1 2HE.

AIMS

The study involves the investigation of proliposomes prepared from specific phospholipids and a drug inhibits growth of glioma.

INTRODUCTION

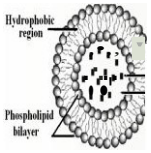
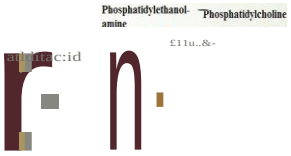


Fig 1. Basic Structure of liposomc.

The interesting properties of liposomc (Fig 1) prepared from proliposomcs is that they are safe, biodegradable, physically and chemically stable, and tumour targeting carrier for drug delivery (Katare *et al.*, 1990; Crommelin and Sindelar, 2002). They can be manufactured on a large scale from phospholipids that are similar to the lipid components present in the biological membrane (Fig 2). There arc two methods to prepare liposomes a) Particulate based proliposome and b) Solvent- based proliposome.

Particulate proliposomcs arc dry, free-flowing carrier particles coated with phospholipids that generate liposomes on addition of aqueous phase (Payne et al 1986). Secondly, a solvent based proliposome method offers a relatively simple means of generating liposomes with a high entrapment of hydroph ilic agents, by the addition of aqueous phase to a concentrated alcoholic solution of phospholipids (Perrett et al 1991). The aim of our project is to use phospholi pids to manufacture solvent-based proliposomes which can be used to generate phospholipid vesicles (liposomes) (Fig 1) when aqueous phase (e.g. water) is added. In this study, the size of the vesicles prepared from proliposomes was compared with the conventional method of producing liposomes. The model anticancer drug derived from the plant Momordica charantia was entrapped in liposomes and the efficacy of anticancer-liposome formulations on the viability of glioma cell lines 1321N1, and U87-MG was evaluated.



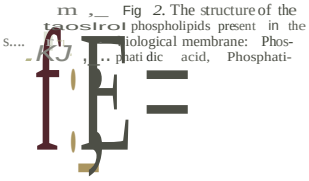


Fig 2. The structure of the phospholipids present in the biological membrane: Phosphatidic acid, Phosphati-

METHODS

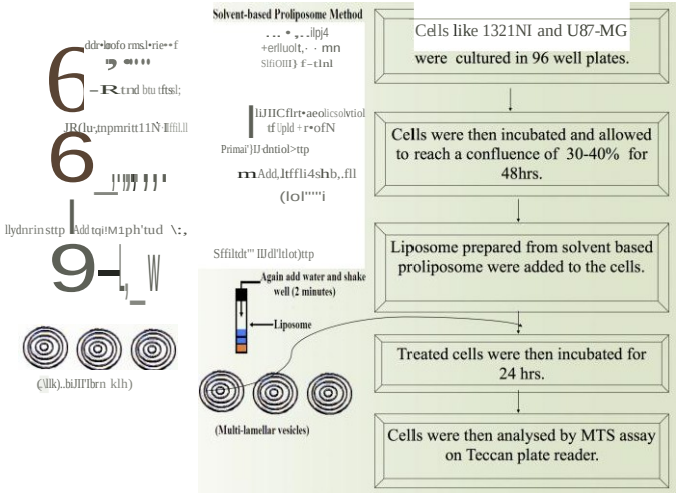


Fig 3. Liposomes preparation methods from thin film and Solvent based proliposomes methods. The liposome prepared from proliposomes are used for the treatment of glioma cell lines 1321N1 and U87MG.

A) Particle size analysis: Laser diffraction (Malvern Mastersizer) was used for particle size analysis. Sample was diluted with water and their average diameter and size distribution were determined.

B) Surface charge analysis: A Zeta-Sizer is used to measure the zeta potential (surface charge) of liposome. This was automatically calculated from the electrophoretic mobility.

RESULTS

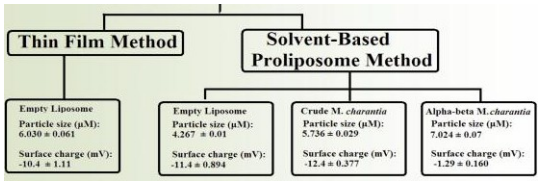
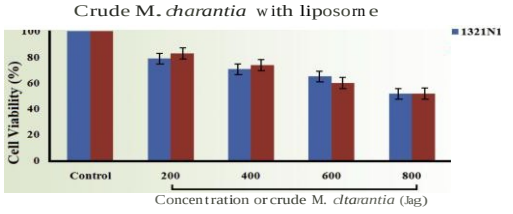


fig 4. Comparison of the particle size and charge prepared from a} Thin film method and b) Solvent–based proliposome method.

A.



B.

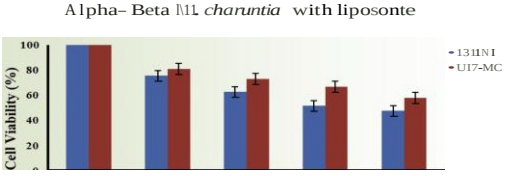


fig 5. Dose dependent effect of (A) Crude M. *charantia* with liposome and (B) Alpha-Beta M. *charantia* with liposome on the cell viability of 1321 N1 and U87-MG cell lines is measured by MTS assay on Teccan plate reader after 24 hr$ of treatment. Data for treated cells compared to untreated (control) cells are mean± S.D. n=3.

CONCULSION AND FUTURE WORK

These results have indicated that the particle size of the liposome, prepared from solvent-based proliposome method was smaller compared to the liposome prepared from thin film method. The diameter of the liposome particles prepared from solvent-based proliposome method increased on the presence of the crude M. *charantia* and Alpha-Beta M. *charantia*. The diameter of Alpha-Beta M. *charantia* was slightly larger compared to crude M. *charantia* (Fig 4). On treatment of crude and Alpha-Beta M. *charantia* entrapped liposomc on tissue culture, it was observed that Alpha-Beta M. *charantia* and crude M. *charantia* shows inhibition of cell viability of the glioma cell lines 1321N1 and U87-MG compared to the untreated (control) 1321N1 and U87-MG cells (Fig 5). Electron microscopy and High Performance Liquid Chromatography (HPLC) are required to determine the surface morphology and drug entrapment efficiency respectively.

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Treatment of Glioma Using Liposomes Generated From Alcohol-based Proliposomes

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[1] Institute of Nanotechnology and Bioengineering, [2] School of Pharmacy and Biomedical Sciences and [3] School of Computing, Engineering and Physical Sciences, University of Central Lancashire, UK.

AIMS

The study involves the *in vitro* investigation of proliposomes prepared from specific phospholipids and drugs inhibiting the growth of glioma cell lines.

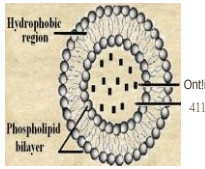


Fig 1. Basic Structure of Liposome.

INTRODUCTION

The interesting properties of liposome prepared from proliposome is, they are safe, biodegradable, physically and chemically stable, and tumour targeting carrier for drug delivery (Katare *et al.,* 1990; Crommelin and Sindelar, 2002). They can also be manufactured on large scale from phospholipids that are similar to the phospholipids present in the biological membrane. There are two methods to prepare liposomes using the proliposome approach : a) Particulate based proliposome and b) Solvent-based proliposome. Particulate proliposomes are dry, free-flowing carrier particles coated with phospholipids that generate liposomes on addition of aqueous phase (Payne ela/ 1986). Secondly, solvent based proliposomes offer a relatively simple means of generating liposomes with a high entrapment of hydrophilic agents, by the addition of aqueous phase to a concentrated alcoholic solution of phospholipids (Perrett ela/., 1991). The aim of this study is to use phospholipids to manufacture solvent-based proliposomes which can be used to generate liposomes (fig 1) when aqueous phase (e.g. water) is added. The model anti-cancer drug derived from plants Momordica charantia and taxol derived from pacific yew bark are entrapped in liposomes and the efficacy of anti-cancer liposome formulations on the viability of glioma cell lines 1321N1, Gos-3, U87-MG and normal glial cell line SVGP12 was investigated.

METHODS

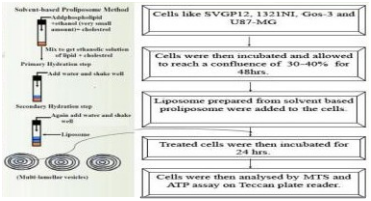
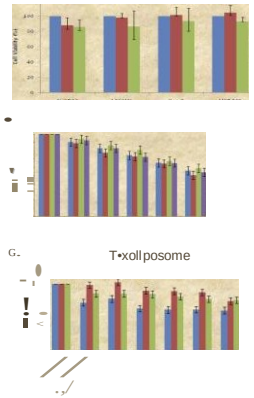


Fig 2. Glioma cell lines 1321N1, Gos-3, U87-MG and normal glial cells SVGP12 were treated with the liposomes prepared from proliposomes containing drugs such as Crude M. *charanua*, alpha-beta M. *chsrantis* and taxol.

A) **Particle size analysis:** A Malvern mastersizer 2000 is used measurement of particle size. Sample is diluted with water and their average diameter and size distribution is determined using dynamic light scattering. The technique of laser diffraction is based on the principle that particles passing through a laser beam will scatter light at an angle that is related to their size.

B) **Surface charge analysis:** A zeta-sizer is used to measure the zeta potential of liposomes. Zeta-potential will be automatically calculated from the electrophoretic mobility.



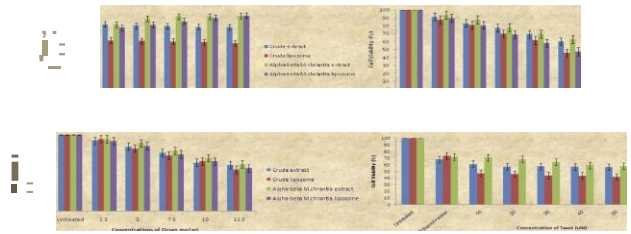


Fig 4. (A) Cells treated with water + ethanol and drug free liposome were compared with the untreated cells of SVGP12 ,1321N1, Gos-3 and U87-MG.
Dose dependent effect of Crude M.*chanrantia* and Alpha-Beta M. *charantia* extract and liposome were examined on Teccan plate reader after 24 hrs of treatment by measuring ATP release of the cells (B) SVGP12 (C) 1321N1 (D) Gos 3 and (E) U87-MG.
The cell viability of SVGP12, 1321N1 and U87-MG cell lines were measured by MTS assay on Teccan plate reader after 24 hrs of treatment with (F) Ethanol + water alone and increasing concentration of taxol dissolved in ethanol + water and (G) Taxol with drug free liposome and increasing concentration of taxol binded to liposome.
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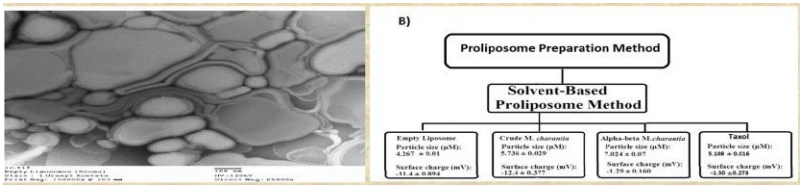

Fig 3 A) Electron microscopy of the liposomes prepared from proliposome showing oligolamellar structure with large aqueous cores B) Comparison of the particle size and charge of the formulations prepared by solvent-based proliposome method.

CONCULSION AND FUTURE WORK

On treatment with crude and alpha-beta M. *charantia* extract and entrapped with liposome on tissue culture, it is observed that Alpha-Beta and crude M. *charanlia* liposome shows slightly more inhibition of cell viability of the glioma cell lines 1321N1, Gos-3 and U87-MG and not effecting normal cell line SVGP12, when compared to the untreated cell lines (see fig 4). Taxol with liposome was observed to be more toxic to the normal cell line SVGP12 and also seem to be promoting the growth of the glioma cell line 1321N1 and U87-MG as compared to the cells treated with taxol without liposome. Further experiments are required to determine the mechanism of action of the drug inhibiting glioma. High Performance Liquid Chromatography (HPLC) is also required to determine the entrapment efficiency of the drug.

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Seema R. Jaiswal [1], Gunasekar Manoharan [1], Waqar Ahmed [2] and Abdelbary Elhissi [1]

[1]School of Pharmacy and Biomedical Sciences and [2]School of Computing, Engineering and Physical Sciences, University of Central Lancashire, UK.

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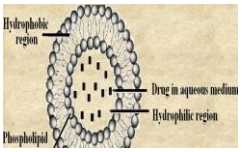
INTRODUCTION



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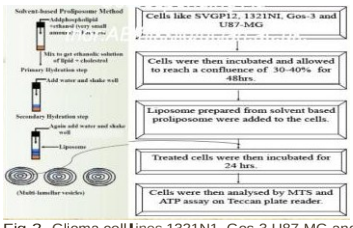
METHODS



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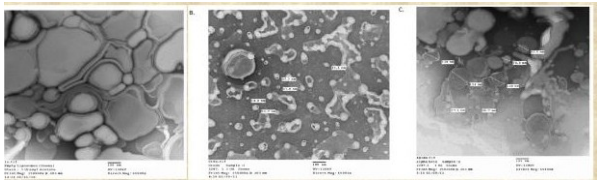
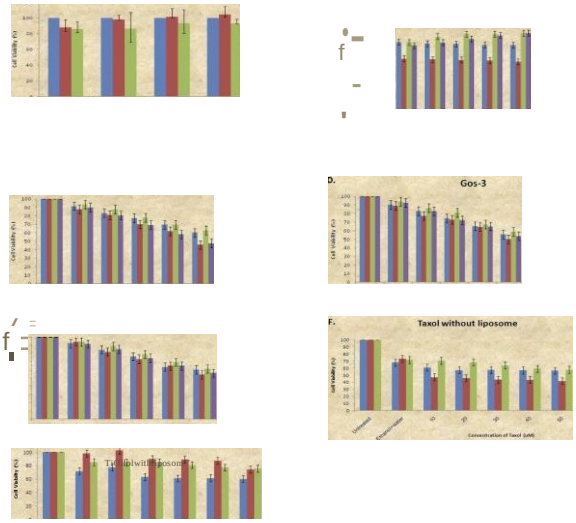


Fig 3. The electron microsoopy of the liposomes prepared from proliposome
A)Drug free liposome showing oligolamellar structure with large aqueous pores having particle size of 3.992 mm and zeta-potential charge -8.270±0.675 B) Crude M. *charantia* having oligolamellar and multi-lamellar structures having particle size of 4.730 IJffi and zeta-potential charge -o.930± 0.989 C) Alpha-beta M. *charantia* having oligo-lamellar and multi-lamellar structure having particle size of 5.320 !Jm and zeta-potential charge -2.04 ± 1.33.



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CONCULSION AND FUTURE WORK

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ACKNOWLEDGEMENTS

I would like to acknowledge my supervisors Dr Abdelbary Elhissii, Dr Waqar Ahmed and my peer Gunasekar Manoharan for their encouragement and innovative ideas. I would also like to thank School of Pharmacy and Biomedical from University of Central Lancashire for providing me all the facilities and support for my research. I am also specially thankful to the Experimental officer Mr David McCarthy from London School of Pharmacy for providing me excellent and dynamic electron microscopy pictures of my liposome prepared from Alcohol-based proliposomes.

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