Nanoparticles Mediated Targeted Drug Delivery System of Some Antineoplastic Agents for the Treatment of Breast Cancer.

Thesis submitted to

The Tamilnadu Dr. M.G.R. Medical University, Chennai,

for the Partial Fulfillment of the Requirements for the Award of Degree of

Doctor of Philosophy

In

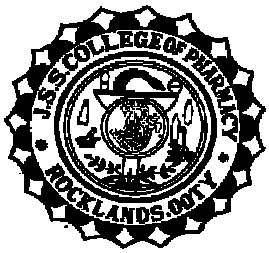
Pharmacy

By

Manjunatha N, M.Pharm.

Under the guidance of

Dr. Malay K. Samanta, M.Pharm., Ph.D., AIC.



January 2011

Department of Pharmaceutics

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Dedicated to My Beloved Parents,

the Guru

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JSS COLLEGE OF PHARMACY

(Off Campus College of JSS University, Mysore)

Accrediated by : National Board of Accreditation of AICTE - New Delhi

CERTIFICATE

This is to certify that this dissertation work entitled **"Nanoparticles Mediated Targeted Drug Delivery System of Some Antineoplastic Agents for the Treatment of Breast Cancer"** is a record of research work, independently carried out by the candidate under my direct supervision. **Mr. Manjunatha N**, M.Pharm., has conducted the study very sincerely, meticulously and methodically. I am satisfied with the work of **Mr. Manjunatha N** which was done at JSS College of Pharmacy, Ootacamund, India, during the year 2008-2010, being submitted by him for the partial fulfilment of the requirements for the award of Degree of Doctor of Philosophy in Pharmacy, to **The Tamilnadu Dr. M.G.R. Medical University**, Chennai, India. I also certify that this thesis or any part thereof has not formed the basis for the award of any other research degree, of this or any other university, previously.

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DECLARATION

I hereby declare that the thesis work entitled **"Nanoparticles Mediated Targeted Drug Delivery System of Some Antineoplastic Agents for the Treatment of Breast Cancer"** submitted by me to **The Tamilnadu Dr. M.G.R. Medical University,** Chennai, India, for the partial fulfilment of requirements for the award of Degree of **Doctor of Philosophy in Pharmacy**, is the result of my original and independent work carried out at Department of Pharmaceutics, J.S.S. College of Pharmacy, Ootacamund, India during the year 2008-2010, under the direct supervision of **Dr. Malay K. Samanta,** M. Pharm, Ph.D., AIC. The work has not formed the basis for the award of any degree, diploma, associateship, fellowship, or any other similar title previously.

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CONTENTS

Introduction

INTRODUCTION

The section acts as a bridge to "place" the need of this investigation with the merits and demerits of the past and present outcomes.

Cancer is the term derived from the greek word *Karkinos* which mean CRAB. Hippocrates used the term for the first time because of the fact that the large tumours have a figure of the crab with its mass and vessels resembling to the body and legs of it [1].

Cancer literally meaning tumour, in medical usage refers neoplasm "new growth". Tumour is said to be "benign" when its cytology and gross characteristics are considered to be localized and "malignant" when it can destroy the adjacent structure to invade distinct sites. Being a multi-process disorder the cancer is defined as an "abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same manner after the cessation of the stimuli which evoked the change. The Fig 1 provides the basic molecular pathogenesis of the cancer [2].

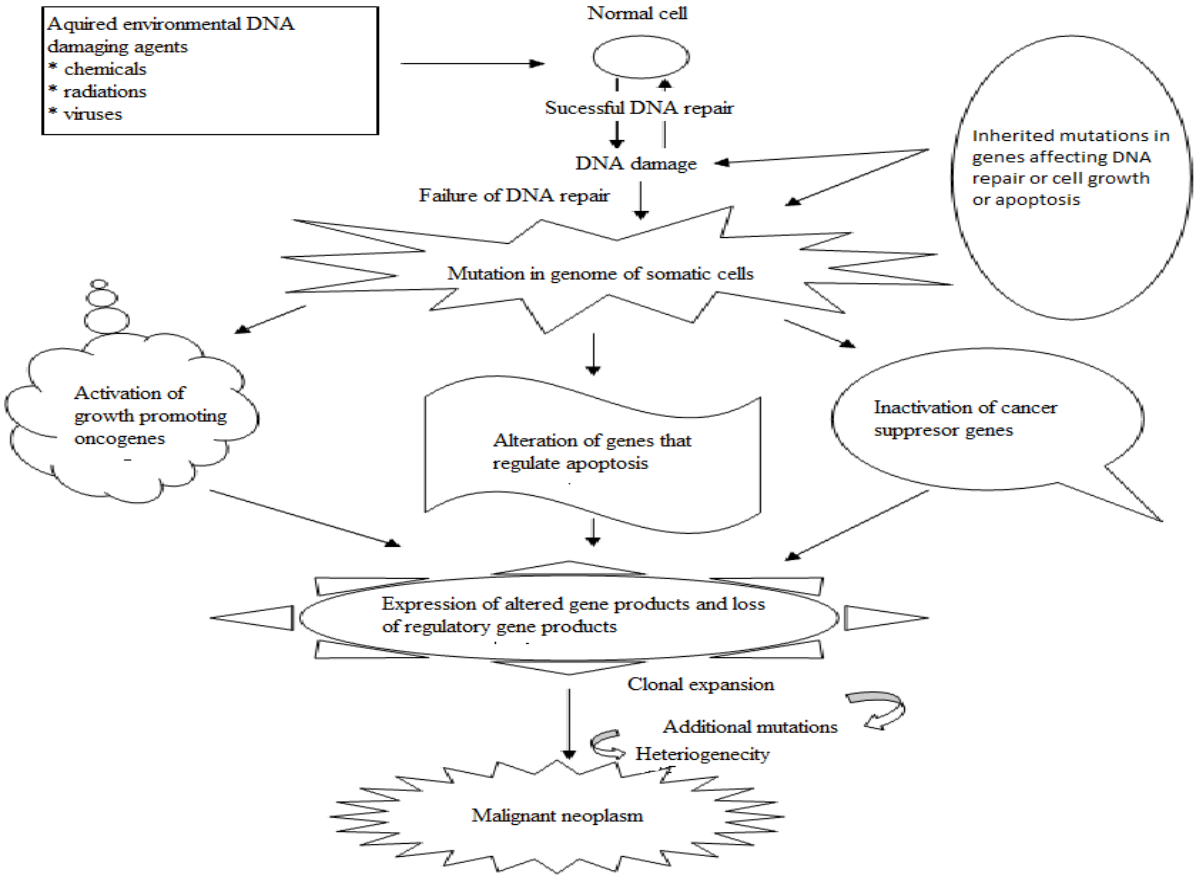
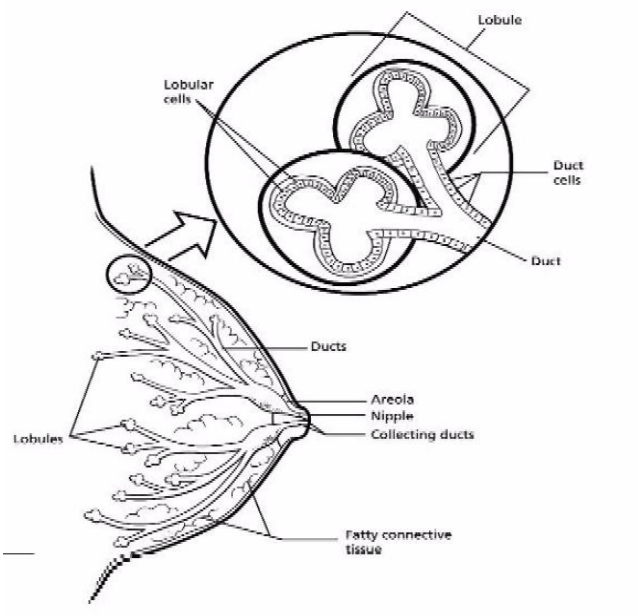


Fig 1: Molecular Pathogenesis of Cancer

Cancer being one of the dread diseases is classified into more than 200 types of which bladder, lung, breast, melanoma, colon and rectal, non-Hodgkin lymphoma endometrial, pancreatic, renal cell, prostate, leukemia and thyroid cancer are commonly diagnosed [3]. However, based on its histological pattern it is broadly grouped as Hematologic (ex: lymphoma, leukemia, etc.) and Solid tumour (ex: breast, neck, overian, and GIT cancer etc.) [4,5].

For the past few decades an extensive research in the field of cancer therapy evoked the specific pathways involved in tumour growth and progression. This mechanistic, had come with the target-based approach of chemotherapeutic with minimized toxicity to the normal cells and maximized therapeutic index. However, until the date, the overall effectiveness of targeted therapy in case of solid tumours has not achieved the robustness as that of hematologic cancer [6]. So, the present work is focussed on solid tumour and breast cancer is considered for the study has it is most commonly diagnosed; one in four cancer cases [7].

Breast Cancer (BC)



To understand the BC, one should have a basic knowledge on the normal female breast (Fig 2). It is made up of lobules (mammary glands), ducts (tiny tubes which carry milk from lobules to nipples), and stroma (fatty and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels). Most BC(s) begin in the cells that line the ducts (ductal cancers) and other in the cells that line the lobules (lobular cancers), while a small number start in other tissues.

Fig 2: Structure of Normal Breast.

BC is a malignant tumour that starts from the cells of the breast with or without invasion to the surrounding tissues or to the distant organs of the body. Women are more prone to develop this but men can also come across [8].

Classification of BC

1. Epithelial tumour
1.1 Invasive ductal carcinoma
1.2 Invasive lobular carcinoma
1.3 Tubular carcinoma
1.4 Invasive cribriform carcinoma
1.5 Medullary carcinoma
1.6 Mucinous carcinoma and other tumours with abundant mucin
1.6.1 Mucinous carcinoma
1.6.2 Cystadenocarcinoma and columnar cell mucinous carcinoma
1.6.3 Signet ring cell carcinoma
1.7 Neuroendocrine tumours
1.7.1 Solid neuroendocrine carcinoma
1.7.2 Atypical carcinoid tumour
1.7.3 Small cell/ Oat cell carcinoma
1.7.4 Large cell neuroendocrine carcinoma
1.8 Invasive papillary carcinoma
1.9 Invasive micropapillary carcinoma
1.10 Apocrine carcinoma
1.11 Metaplastic carcinoma
1.11.1 Pure epithelial metaplastic carcinoma
1.11.1.1 Squamous cell carcinoma
1.11.1.2 Adenocarcinoma with spindle cell metastasis.
1.11.1.3 Adenosquamous carcinoma
1.11.1.4 Mucoepidermoid carcinoma
1.11.2 Mixed epithelial meseochymal metaplastic carcinoma.
1.12 Lipid-rich carcinoma
1.13 Secretory carcinoma
1.14 Oncocytic carcinoma
1.15 Adenoid cystic carcinoma
1.16 Acinic cell carcinoma
1.17 Glycogen-rich clear cell carcinoma
1.18 Sebaceous carcinoma
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1.20 Lobular carcinoma *in situ*
1.21 Intraductal proliferative lesions
1.21.1 Usual ductal hyperplasia
1.21.2 Flat epithelial atypia
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1.24.1.3 Blunt duct adenosis
1.24.1.4 Microglandular adenosis
1.24.1.5 Adenomyoepithelial adenosis
1.24.2 Radial scar/ Complex sclerosing lesion
1.25 Adenomas
1.25.1 Tubular adenoma
1.25.2 Lactating adenoma
1.25.3 Apocrine adenoma
1.25.4 Pleomorphic adenoma
1.25.5 Ductal adenoma

2. Myoepithelial lesions
2.1 Myoepitheliosis
2.2 Adenomyoeithelial adenosis
2.3 Adenomyoepithelioma
2.4 Malignant myoepithelioma

3. Mesenchymal lesions
3.1 Haemangioma
3.2 Angiomatosis
3.3 Hemangioperi Cytoma
3.4 Pseudoangiomatosis stromal hyperplasia
3.5 Myo fibroblastoma
3.6 Fibromatosis (aggressive)
3.7 Inflammatory myofibroblastic tumour
3.8 Angiolipoma
3.9 Granular cell tumour
3.10 Neurofibroma
3.11 Schwannoma
3.12 Angiosarcoma
3.13 Liposarcoma
3.14 Rhabdomyo sarcoma
3.15 Osteosarcoma
3.16 Leiomyoma
3.17 Leiomyosarcoma

4. Fibroepithelial tumours
4.1 Fibroedenoma
4.2 Phyllodes tumour
4.2.1 Benign
4.2.2 Borderline
4.2.3 Malignant

5. Tumours of the nipple
5.1 Nipple adenoma
5.2 Syringomatous adenoma
5.3 Paget disease of the nipple

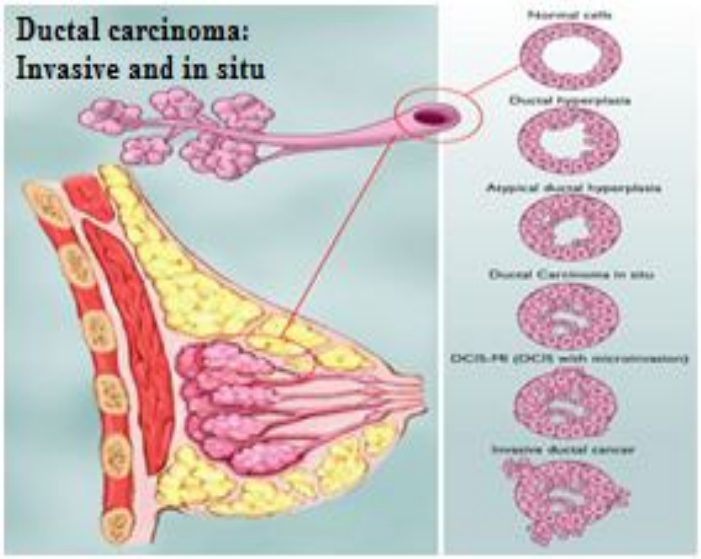
6. Malignant lymphoma
6.1 Diffuse large B-Cell lymphoma
6.2 Burkitt lymphoma
6.3 Extranodal marginal-zone B-cell lymphoma of MALT type
6.4 Follicular lymphoma

7. Metastatic tumours

8. Tumours of the male breast
8.1 Gynecomastia
8.2 Carcinoma
8.2.1 Invasive
8.2.2 *In situ*

Adopted from WHO, Tumours of the Breast and Female Genital organs, IARC press, 2003.

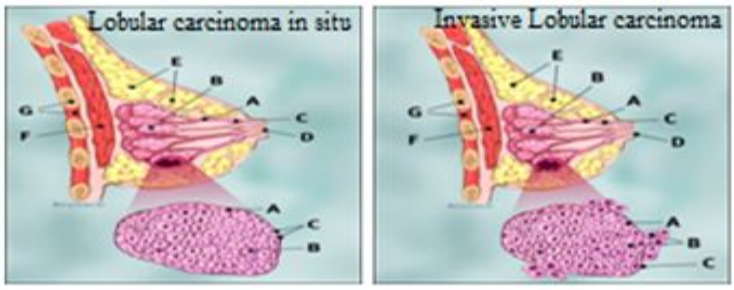
Though the WHO has classified the tumours of the breast as above, the most commonly diagnosed are as below



1. Ductal Carcinoma: starts in the cells that lines the breast's ducts located beneath the nipple and areola. About 85-90% of the cases are ductal carcinoma. Based on the invasive character of it, it's is grouped as ductal carcinoma *in situ* (non-invasive) and ductal carcinoma invasive.

Fig 3: Diagrammatic view of invasive and *in situ* ductal carcinoma.

2. Lobular carcinoma: starts in the cells that line the lobules or mammary glands and covers about 8% of the cases. Similar to ductal carcinoma it is grouped as lobular carcinoma *in situ* and lobular carcinoma invasive.



Breast profile: A ducts, B lobules, C dilated section of duct to hold milk, D nipple, E fat, F pactoralis major muscle, and G chest wall/rib cage. Enlarged section: A normal lobular cells, B lobular cancer cells, and C basement membrane. [Adopted from www.breastcancer.org/pictures/

Fig 4: Diagrammatic view of invasive and *in situ* lobular carcinoma

Apart from these we also come across inflammatory breast cancer (IBC) and pagets disease. IBC is the least common and most aggressive taking the form of sheets or nests instead of lumps in the soft tissues of the breast just under the skin. It can even appear on the skin [9]. Pagets disease is the cancer of the nipple/areola often looks like a skin rash, or rough skin. Being the least common it resembles eczema and can be itchy. The disease is named after Sir James Paget who first noticed the changes in the nipple and the underlying area [10].

Staging of BC – the currently accepted staging system for BC is TNM staging, which means "tumour, node, and metastasis. The T stage is numbered 1-4 and describes the size of tumour, where T1 represents tumour size of diameter <2 cm , T2 with >2 and <5cm, T3 measuring >5cm and T4 symbolize any size with extension to chest wall or skin, also covers IBC. In case of poor assessment it is denoted as Tx and T0 if it lacks the evidence of primary tumour. The N stage is numbered from 1-3 and describes the metastasis of BC from lymph nodes, where in Nx the lymph nodes cannot be assessed and in N0 metastatsis is not found. N1 symbolize the metastasis in 1-3 movable axillary lymph node(s) on the same side of the affected breast. N2 denotes the metastasis to fixed regional axillary lymph nodes (4-9) or metastasis to the internal mammary lymph nodes of the same side of the affected breast. N3 stands for metastasis (more than 10 lymph nodes) to supra or infraclavicular lymph nodes or to the internal mammary lymph nodes. The Stage M represents the metastases to other than breast and lymph nodes so called distant sites like lungs, brain, and bone etc., Where M0 is signified as no distinct metastasis and M1 as distinct metastasis with Mx remaining not to be assessed [11].

Oncogenes and tumour suppressor genes in BC

Oncogenes are the genes whose activation can contribute to development of cancer. Among the numerous oncogenes being characterized in the breast cancer the *HER-2*, and *c-myc* are the main ones.

HER-2 a human epithelial receptor 2 located on 17q chromosome binds to specific ligands and cascades the pathways like MAP (mitogen-activated protein) and PI3K/Akt. The pathways eventuate the proliferation, angiogenesis, cell-cell interaction, metastasis and resistance to apoptosis [12]. This gene is overexpressed in about 30% of invasive breast cancer. Trastuzumab, bevacizumab and pertuzumab monoclonal antibodies are widely used for impairment dimerization of this receptor by creating the steric hindrance [13,14]. *c-myc* oncogene localized to chromosome v8q24 encodes a nuclear phosphoprotein that acts as transcriptional regulator involved in cell proliferation, differention and apoptosis. Being overexpressed in 15-25% of BC cases it plays a role in hormone responsiveness and chemotherapy resistance [15,16].

Tumour suppressor genes are those whose loss of function promotes malignancy with affect of invasion and metastatic potential such as cell adhesion and regulation of protease activity. *p*53, *p*27, *BRCA* 1 and *BRCA* 2 are the most important suppressor genes studied in case of BC. *p*53 gene coding 393 kDa protein located on chromosome 17q regulates the cell division on normal condition, the mutation of it leads to cell proliferation. Around 20-30% of BC involves *p*53 mutation, 80% of which is missense or splice site mutation [17]. The gene p27 first isolated in 1993 belongs to the family cyclin-dependent protein kinase inhibitors. It inhibits the cell division by arresting the G_1 phase of the cell cycle. In BC diminished expression of it is associated with shorter overall survival and shorter time to progression [18].

BRCA 1 gene located on chromosome 17q21 account mutation in approximately 5% of BC in women less than 40 years of age but records up to 90% in familial cases. The lifetime risk of BRCA 1 mutation is estimated to 49-73% by age 50 and by age 70 it is 71-87%. In normal condition, this gene has shown to localize the genotoxic damage and regulate the transcription as well as repair the double strand DNA which is found to be lost in BC. The investigation for further culprits led to discovery of BRCA 2, a gene located on 13q12-13. Being structurally dissimilar it shares the many characteristic features with BRCA 1 [19].

Epidemiology and Etiology of BC

Although many epidemiological risk factors have been identified, the cause of any individual BC is often unknown. About 5% of the cases are attributed to hereditary syndromes with rest having no very well known etiology [20].

The various risk facors for BC are

1. Age: The incidence of BC increase with age doubling about every 10years till menopause. When compared to lung cancer it is higher in younger ages and most common in 50+ years.

2. Geography: the incidence varies worldwide, being lower in less developed countries compared to developed ones. USA stands the first among the countries with ratio of 1 in 8 (12.5%) women developing invasive BC in lifetime.

3. Age at menarche and menopause: women with early menarche (normal between 10-18years) and late menopause (normal between 45-55 years) have an increased risk of developing BC. Women having menopause after 55 years are twice likely to develop BC than those who have before 45 years.

4. Pregnancy: nulliparity and late age at first birth increase the chances of budding of BC. The women having first child after 30 years are twice prone to develop BC compared to those who have before 20years. The risk is more severe in those having after 35years.

5. Family history: The women with following criteria are at menace of developing BC
   - One first degree relative with bilateral breast or ovarian cancer before 50 years.
   - One first degree relative with BC within 40 years or one first degree male relative with BC at any age.
   - Two first/second degree relatives diagnosed for BC below 60 years.
   - Two first/second degree relatives diagnosed for ovarian cancer at any age.

   First degree relatives are mother, sister, daughter, brother, son or father.

   Second degree relatives are grandmother, grandfather, granddaughter, grandson, aunt or niece.

6. Benign breast disease: women with severe atypical epithelial hyperplasia, is at four to five times higher risk of developing BC than those who have not come across any proliferative changes in the breast. Women with this changes and a family history are at 9 fold higher risk.

7. Exposure to ionizing radiations specially during rapid breast formation increase the risk of developing BC in life time.

8. Life style: low level consumption of vitamin D, obesity, alcohol consumption, and smoking etc., amplify the threat of BC.

9. Oral contraceptives: women who use oral contraceptives before 20 years are at higher jeopardy than those who begin to use at older age.

10. Hormone replacement therapy: puts on the risk of developing BC by delaying menopause. It also increases the breast density and reduces the sensitivity and specificity of breast screening.

11. Lack or shorter duration of breast feeding enhances the risk of development of BC at menopause.

12. Physical characteristics:
    - Risk is greater in women who gain weight after age of 18 years.
    - Women being heavier has a child is at lower risk as estrogen level is low in presence of high fat.
    - Women with heavy bones, dense bones are likely to have high estrogen levels and are at risk for BC development.

13. Exposure to estrogen-like chemicals called xenoestrogens, pesticides like organochlorides (DDT) and its metabolities such as dieldrin, pyrethroids (eg. permethrin), diethyl stilbestrol etc augment the menace of BC.

14. Insulin-like growth factor 1 is an important hormone during development of womb and childhood. It has a property of increasing cell proliferation and presence of it in high concentration can lead to cancer. It may be one of the factors responsible for the association between the height and BC [21,22].

Diagnosis of BC

Breast self examination: the diagnosis of BC at the primary stage can reduce the risk of death in women. Women should undergo self-examination every month, followed with health professionals at least once in a year, as they are often not very proficient. The self-examination involves check for changes or redness in the nipple area, difference in size of breast, and appearance of skin at different positions. It also involves circular motion of fingers on breast with slight pressure followed by hard to feel the presence of any early unnoticed lump. The massage should cover entire area of breast from collar bone to underneath of breast and middle of chest to armpits.

Mammogram: is a very effective low-radiation (x-ray) screening method. This is not a full proof to examine BC as 25% of the cancer cases which are screened physically are missed.

Magnetic Resonance Imaging (MRI) and Ultrasound: these techniques can detect even very small tumours (less than half an inch) but very expensive and time consuming. Ultrasound plays a vital role in case of women with dense breast tissue.

Scintimammography: employs an injection of radioactive chemical, which is selectively taken up by tumour and gets revealed on mammograms. The method is accurate and eventually reduces the number of unnecessary invasive biopsies.

Biopsy: it is the microscopic examination of a tissue sample of the suspicious area. The different types of biopsies are like core, spring loaded, vacuum assisted and sential node biopsy. The core biopsy involves small incision and insertion of spring loaded hallow needle. The wire localization biopsy involves insertion of small wire hook to the suspicious tissue through a needle. The hook is used to localize and remove the lesion. In vacuum assisted biopsy tissue is removed on application of vacuum upon insertion of probe. For determination of spread of cancer beyond nodes sential node biopsy is applied which involves study of flow of radiolabel or dye substrate in lymphatic system on injection of it at tumour site [21].

Positron emission mammography: the technique record of gamma rays emitted by radioactive glucose tracer, as the glucose is absorbed and stored more readily in rapidly growing cells like cancer cells.

Thermography: a technique records the temperature of body by measuring the IR rays it emits. Malignant tissue generally has a higher temperature than normal because of its richer blood supply and higher metabolic rate.

Breast-specific gamma imaging: it employs a radiotracer technetium sestamibi which accumulates in mitochondria. Rapidly proliferating cells are rich in mitochondria and the tracer will concentrate there, emitting gamma rays that produce dark spots on a digital image [23].

Treatment of BC

The treatment is grouped mainly into 2 sections; a) local therapy (surgery and radiation) b) systemic therapy (hormone/adjuvant/neoadjuvant/biological/chemotherapy) [24].

Surgery – is an initial treatment in breast cancer in combination with other therapies. The treatment of BC involves 3 different types of surgeries [25,26].

1. Mastectomy – the simple mastectomy involves removal of entire breast, modified mastectomy includes removal of entire breast and lymph nodes under the arm whereas radical mastectomy covers the modified mastectomy including removal of underlying chest wall muscle.

2. Lumpectomy – this surgery removes only the breast lump and some normal tissue surrounding it. The post surgery is usually accompanied with radiation therapy.

3. Breast conserving surgery (Quadrantectomy) – the treatment involves removal of cancer and large area of the breast tissue, occasionally include some of the lining over the chest muscle.

Radiation therapy – the treatment applies high-energy rays (such as X-rays) to kill cancer cells or to shrink the tumour volume before surgery. Most often external beam radiation is applied which is much like regular x-ray for a longer time. In brachytherapy radioactive pellets are placed next to cancer in breast tissue [27,28].

Apart from several side-effects of the above therapies they (surgery and radiation) cannot be applied in case of invasive or metastasis tumours as the remedy remains localized. The most common side-effects of surgery are like pain, wound infection, mondor's disease, injuries to other structures, lymphedema, chronic breast edema and cellulitis, swelling of arm, bleeding, and reaction to anesthesia [27,29]. The radiation therapy can result in acute and late complications, skin erythema and fatigue, pneumonitis, pericarditis, rib fracture, brachial plexopathy and edema. The patient with left sided breast tumour may come across myocardial infarction [30].

Hormonal therapy: it is based on the fact that BC cells express receptors for sex hormones mainly estradiol and its growth is partly dependent on hormonal stimulation. The therapy is based on withdrawal of the growth stimulators either by reducing hormone production or by blocking receptor-ligand binding. The hormonal therapy is commonly prescribed in post-menopausal women with advanced BC. The therapy covers administration of anti-estrogens (tamoxifen/fulvestrant) or aromatase inhibitors (anastrozole/letrozole/exemestane) or progestogens like megestrol acetate or medroxyprogesterone acetate [31]. But the therapy is applicable only in hormonal BC cases. The major adverse effects of the therapy are arthralgia, diarrhea, gynaecologic symptoms, vaginal bleeding, muscle cramps, thromboembolic events and even osteoporosis and fractures. In some cases even cancer of endometrium, lung and melanoma developed [32-34].

Biological therapy: A member of epidermal growth factor receptor family called *HER2/neu* is overexpressed in 25-30% of human BC and is implicated as prognostic and predictive marker for BC [35]. Clinically chemotherapeutic regimens include several monoclonal antibodies like trastuzumab, cetuximab and bevacizumab that are directed against this domain. Apart from these monoclonal's several tyrosine kinase inhibitors like erlotinib, lapatinib, sunitinib, and soragenib also targets this protein [36]. Unfortunely the widespread use of these extraordinary agents are limited because of several reasons like a) only small number of patients can be treated; b) no beneficial effects to majority of patients who are *HER2* negative; and c) produce cardiotoxicity [37].

Chemotherapy – it employs the drug or combination of drugs called cytotoxic agents. They are given orally or by parental route [38]. The benefit of chemotherapy is dependent on multiple factors including the size of the cancer, the number of lymph nodes involved, presence of estrogen or progesterone receptors, and amount of *HER2*/*neu* proteins made by the cancer cells [39]. The therapy is considered when the surgery/radiation is not applicable, on the microscopic evidence of spread of cancer, or in combination of other therapeutic system for the better treatment [40]. In most of the cases, the treatment is more sensitive to combination of drugs than just one. A number of combinations can be used including anthracyclines, alkylators, antimetabolities, antimicrotubules, and antitumour antibiotics etc. The choice of therapy depends on the financial considerations and the preferences regarding the route and schedule of administration, and toxicity [41,42].

The chemotherapy before surgery is termed as neoadjuvant therapy and after surgery it is expressed as adjuvant therapy [43]. As like other therapies chemotherapy do posses side effects. The most common side effects are fatigue, nausea and vomiting, anxiety, sleep disturbances, change in bowel function, palpitation, nose bleeding, neurological and haematological toxicities and an altered sense of taste. These side effects are overwhelming [44,45].

Apart from this the selective toxicity to cancer cells cannot be achieved as the cells share the familiar features with the normal cells from which they are derived. The lacuna of selective toxicity increased toxicity of drug against normal tissues, bone marrow, GIT and hair follicles etc. Thereby, most of the commonly used chemotherapeutics like paclitaxel, docetaxel, 5-FU, cyclophosphamide and doxorubicin etc mean they are given at suboptimal doses, resulting in the eventual failure of the therapy; often accompanied by the development of resistance of tissue to drug further showing the metastasis [46,47]. Furthermore to avoid the side effects and to increase the selective toxicity; a novel or targeted drug delivery systems are implemented to increase the amount of drug reaching the cancer tissue. With early stated clinical benefits of chemotherapy an attempt as been made in the present work to develop a targeted drug delivery system.

Targeted therapy: the therapy has emerged with multiple definitions; the FDA has considered the therapy as a drug having specific reference to a previously approved diagnostic test that must be performed and measured has eligible candidate. The oncologists and scientists defined it as a drug with specific mechanism that acts on a well-defined target or biologic pathway. The research investigators believe as anticancer antibodies especially conjugated with cytotoxic drugs or radioisotopes or cellular poisons which seek out and eradicate the malignant cells bearing the target antigen.

The ideal anticancer target should - be crucial to the malignant phenotype; not express significantly in vital organs and tissues; be a biologically relevant molecule; be reproducibly measurable in readily obtained clinical samples; show clinical response in significant proportion of patients [48].

The targeting is mainly grouped as passive and active. In passive targeting the drug delivery system stays in the blood for long longer time and slowly accumulate in the cancer tissue which expresses leaky vasuculature. In cancer, the passive targeting take place in a non-specific way through interstitial space of tumour. The target system carrying the entrapped drugs, extravasate into the tumour interstitial space and release the active moiety increasing the local concentration of it significantly compared to conventional systemic therapy. This is also known as "enhanced permeation and retention". The larger tumours show poor vascularisation, especially inside the necrotic areas, which prevent the localization of the targeted drug delivery system. One approach suggested to overcome these limitations is the inclusion of a targeting ligand or antibody in polymer-drug conjugates. Initally, direct conjugation of an antibody to drug failed to show the superiority as a targeted delivery tool because of less number of drug molecule that can be loaded [49]. The recent developmenta have introduced a wide variety of drug delivery systems like multifunctional metal nanoparticles [50], microspheres [51], liposomes [52], erythrocytes [53], quantum dots [54], superparamagnetic iron oxide crystals [55], carbon nanofibres [56], carbon nanotubes [57], dendrimers [58], polymeric micelles [59], nanoparticles [60], lipopolyplexes [61], and nanocrystals [62] etc. to enhance the loading efficacy of the drug molecule. All these systems can be grouped under one section as polymer-based nanotechnology. Among the systems the polymeric nanoparticles is broadly investigated for the last several decades as drug carrier [63].

The polymeric-nanoparticle-based drug delivery provides many advantages, such as enhancing drug-therapeutic efficiency and pharmacological characteristics; improved solubility of poorly water-soluble drugs; modified pharmacokinetics; increased $t_{1/2}$ of drug by reducing immunogenicity, increased local concentration at the site of action; reduced side effects; improved bioavailability; diminished metabolism and enable controlled and/or sustained release [50].

For the last few decades several diverse studies had and are been carried out to target these polymeric nanoparticles to the cancer cells and achieved to increase their concentration significantly. The concept beyond the polymeric nanoparticles makes use of polymers that poses the affinity to the receptors that are expressed on the surface of cancer cells.

The rapidly expanding necrotic mass of tumour needs increased supply of nutrients. Increased rate of transcription, proliferation of tumour cells and altered morphology of blood vessels interpenetrating the tumour mass leads to expression of various receptors on the surface of these cells [64,65]. The receptors have two essential components i.e. extra-cellular ligand binding domain and an intracellular signal-transmitting domain. These receptors have specific binding sites so that, only the ligand of given orientation can be recognized and thus a specific ligand-carrier binds to specific receptor where the system is either internalized or liberate the therapeutic moiety at the extra-cellular site and the biological response is elicited [66]. The ligands should have affinity to selectively bind to biologically relevant target, and is the basic necessity when the matter concerns more specifically for the delivery of an anticancer drug. Various literatures have reviewed about the morphology of cancerous cells in all its existing forms like leukemic cells, sarcomas, and solid tumours and elucidated the receptors present on these heterogenic mass. These elucidations are very useful to provide the access of drugs, oligonucleotides, peptides etc. into the tumour vasculature for its complete eradication with no undue side effects. In order to replenish the needs of tumour cells, various receptors are over-expressed on their surface. These receptors help tumour mass by providing them with the bio-actives required for neo-vascularization. The nutrients and other molecules having affinity for their specific receptors move to the receptors, and once the molecules bind to them; the receptor–nutrient complex is internalized via receptor mediated endocytosis. Alternatively the delivery systems can be manipulated in such a manner that they mimic the nutrients, and hence gain access to these receptors. Various receptors present on the breast tumour cells are summarized as epidermal growth factor receptors [67], folate receptors [68], integrin receptors [69], FAS receptors [70], estrogen receptors [71], transferrin receptors [72], hyaluronan/CD44 receptors [73], progestrone receptors [74], and prolactin receptors [75] etc. Though the above receptors are been well targeted using the biocompatible or biodegradable ligands/polymers especially hydrophilic no or very less attempts are made to develop the dual ligand therapy. The molecular design of dual-ligand nanoconstruts can provide better applications administered *in vivo* as the cancerous cells posses multiple functional characteristics [76].

Thus the present dissertation is focused on dual ligand nanoparticulate therapy for the better treatment of BC using natural, biocompatible, biodegradable and hydrophilic polymers (Albumin, Gelatin and Hyaluronan). As the taxanes are widely applied in the treatment of BC they are selected as drug of choice (Paclitaxel and Docetaxel).

Literature review

LITERATURE REVIEW

The body of the section was undertaken to establish the current knowledge including substantial findings as well as theoretical and methodological contribution to the topic of dissertation. Some of the important findings to the topic are summarized below;

Castelli C et al, 2004 summarizes the immune mechanism in neoplasia. According to them majority of solid tumours express tumour-associated antigens for supporting their immunogenicity. The interaction between tumour cells and the immune system is a dynamic with both of them modulating and influencing each other. The tumour cells modulate their own phenotypes with ability to evade immune recognition. The immune system is also influenced directly by microenvironment generated by presence of growing tumour cells. The tumour cells also posses the ability to interfere with the host environment, turning off most of the mechanisms devoted to the control of tissue homeostasis, thus enabling tumour cell survival and progression *in vivo*. The negative effects detected in the immune system of tumour-bearing patients often go beyond the local immunosuppressive activity of a neoplastic lesion and suggest that a systemic modulator mechanism exists [77].

Hortobagyi GN 1994 observed combined modality therapy has the treatment of choice for patients with primary BC, including those with locally advanced BC. Primary or induction chemotherapy, followed by surgery or radiation therapy or both, and adjuvant systemic therapy is a generally accepted strategy. Most tumours responded with a more than 50% decrease in tumour size, and approximately 70% of patients experienced down-staging through primary chemotherapy. Breast conservation was possible for many patients with locally advanced BC, and almost all patients are rendered disease free initially. Primary chemotherapy was the initial choice of treatment for patients with locally advanced tumours, but it is unclear what the optimal sequence of subsequent therapies should be, or whether one or two local treatment modalities are necessary. The most dramatic example of the efficacy of these regimens was demonstrated in patients with inflammatory BC. Previously a universally fatal condition, the natural history of this disease has been changed dramatically by the introduction of these combined modality therapies. Five-year survival rates of 35-60% commonly were reported, and were likely that about one third of patients with this highly aggressive form of disease will survive beyond 10 years without recurrence. Combined modality therapy that includes primary chemotherapy provides appropriate local control, the

possibility of breast conservation therapy, and, probably, an increased survival rate, at least for some subsets of patients with locally advanced BC. The use of similar treatment strategies for early BC is currently under evaluation. Hormone therapy and combination chemotherapy represent the main treatment approaches to metastatic BC. Radiotherapy is also a useful palliative tool, especially for control of painful bone metastasis and central nervous system involvement. Patient and tumour characteristics help in the optimal selection of systemic therapy. Palliative therapy for symptomatic control and prolongation of life also preserved a good quality of life. Dose-intensive chemotherapy had been evaluated as a treatment to increase complete response rate and cause remission consolidation. Clinical trials on several new and effective cytotoxic agents, as well as new hormonal compounds have showed encouraging preliminary results [78].

Smith BL et al 2001 discussed the complication of breast surgery. As per authors surgical procedures performed for diagnosis and treatment of breast disease have become progressively less invasive. As an increasing proportion of breast surgery is performed on an outpatient basis, identification and management of postoperative problems have become a collaborative effort involving the surgeon, the patient, home care services and medical office staff. Although this shift to outpatient care has created a number of challenges, complication rates are no higher when breast procedures are performed on an outpatient or short-stay basis. Perioperative, short-term and long-term complications, including wound complications, injuries to adjacent structures, lymphedema, and pain syndromes were discussed [29].

Partridge AH et al 2004 compiled the sustained and long-term complications of adjuvant chemotherapy including information about risk factors, etiology, and incidence rates for specific complications. Interventions to ameliorate long-term complications were also addressed with benefits of adjuvant chemotherapy for early stage breast cancer (BC) on consideration of the potential risks and anticipated side effects. One should also consider the long-term risk factors of adjuvant therapy for a patient along with its acute side effects. Sustained or long-term effects of adjuvant chemotherapy usually have either a late onset or a sustained impact – often lasting for many years. In case of rare long-term complications, many years may elapse before any symptoms develop. The paper reported premature menopause/infertility, osteoporosis, weight gain, and quality of life as common complication; cardiac dysfunction and leukemia/myelodysplastic syndrome as rare complication; and neuropathy and cognitive dysfunction as additional [79].

Senkus-Konefka, E et al 2006, overview the contemporary knowledge on the toxicity of BC radiotherapy and discussed the possible preventive measures. Though the postoperative radiotherapy for BC is beneficial, the treatment is associated with several complications, which may affect patient quality of life and possibly survival. The possible long-term irradiation complications are cardiac and lung damage, lymphoedema, brachial plexopathy, impaired shoulder mobility and second malignancies. The risk of these complications is particularly high with old and suboptimal irradiation techniques and has decreased with the introduction of modern technologies in field of radiotherapy [80].

Gao J et al 2009 brought forth the examples with different types of nanomaterials acting inside cells and discussed the challenges and perspectives for their relevant biomedical applications. The successful and explosive developments of nanomaterials inevitably lead to their intersections with biology and medicine resulting in increased efforts on nanobiotechnology (or bionanotechnology) research. The piece of writing describes the applications of various types of nanomaterials like quantum dots, metallic nanoparticles (e.g. gold, silver and copper), nanorods, magnetic nanoparticles, nanowires, nanotubes, and nanofibres inside cells ranging from cell imaging and tracking to treatment especially in cancer. They summarized to concern the physiological stability of nanomaterials to extravasate blood vessels and penetrate interstitial spaces because of macrophage uptake [81].

Solanki A et al, 2008 focussed on nanotechnology for regenerative medicines and for stem cell imaging. The review focused challenges in regenerative medicine, applications of nanoparticles in stem cell biology and potential of nanotechnology approaches towards medicine. The literature revises on magnetic nanoparticles and quantum dots as primary nanoparticles in the field of regenerative medicines and cell imaging. The article also addressed the unique feature of nanomaterials mainly as a) must interact with proteins and cells without interfering biological activities; b) surface modification should not alter their physical properties; and c) should be nontoxic. They discussed that surface engineering and drug delivery via nanoparticles enable the modulation of stem cell pathways and improvement in therapeutic applications [82].

Nie S et al, 2007 describes cancer nanotechnology as an interdisciplinary area of research in science, engineering, and medicine with broad applications for molecular imaging, molecular diagnosis, and targeted therapy. The basic rationale of nanometer-sized particles such as

semiconductor quantum dots and iron oxide nanocrystals was optical, magnetic, or structural properties that were not available from molecules or bulk solids. When linked with tumour targeting ligands such as monoclonal antibodies, peptides, or small molecules, these nanoparticles can be used to target tumour antigens (biomarkers) as well as tumour vasculatures with high affinity and specificity. In the mesoscopic size range of 5–100 nm diameter, nanoparticles also have large surface area and functional groups for conjugating to multiple diagnostic (e.g., optical, radioisotopic, or magnetic) and therapeutic (e.g., anticancer) agents. Recent advances have led to bio-affinity nanoparticle probes for molecular and cellular imaging, targeted nanoparticle drugs for cancer therapy, and integrated nanodevices for early cancer detection and screening. These developments raise exciting opportunities for personalized oncology in which genetic and protein biomarkers are used to diagnose and treat cancer based on the molecular profile of individual patients [83].

Emerich DF et al reviewed the nanotechnology and medicine as a system/device manufactured at the molecular level, is a multidisciplinary scientific field undergoing explosive development. The genesis of nanotechnology can be traced to the promise of revolutionary advances across medicine, communications, genomics and robotics. On the surface, miniaturisation provides cost effective and more rapidly functioning mechanical, chemical and biological components. Less obvious though is the fact that nanometre sized objects posses remarkable self-ordering and assembly behaviours under the control of forces quite different from macro objects. These unique behaviours are what make nanotechnology possible, and by increasing our understanding of these processes, new approaches to enhance the quality of human life will surely be developed. A complete list of the potential applications of nanotechnology is too vast and diverse to discuss in detail, but without doubt one of the greatest values of nanotechnology will be in the development of new and effective medical treatments (nanomedicine). The review focused on the potential of nanotechnology in medicine, including the development of nanoparticles for diagnostic and screening purposes, artificial receptors, DNA sequencing using nanopores, manufacture of unique drug delivery systems, gene therapy applications and the enablement of tissue engineering [84].

Farrell et al, 2010 went through the generation of nanotechnology-based drug forumulations to deliver the material directly to the tumour site and eradicate selectively, sparing healthy cells. The nano-construct design of drug enhanced efficacy at lower doses compared to its free form with reduced side-effects. The study also focused on understanding the toxicity,

biodistribution and effective regulatory pathways with summarizing the promising developments in field of cancer diagnosis and treatment by nanotechnology-based drug delivery system [85].

Maharana BR et al, 2010 studied the various application of nanobiotechnology and summarized as an emerging field in treatment of diseases through drug delivery and tissue engineering. The nanobiotechnology based drug delivery system has overcome the problems such as poor solubility, drug instability in biological milieu, poor bioavailability and potential side effects. In the field of diagnosis nanotechnology exhibited tremendous potential for detecting biomarkers, pre-cancerous cells, fragment of virus or other indicators. They envisioned that the nanopatteining can directly induce stem cell differentiation and generate desired cell types or regulate cell behaviour within 3D scaffolds. The review also focused on nanobiotechnology for blood substitutes to overcome sterilization of RBC for HIV and hepatitis viruses and illustrates some of the substitutes under clinical trials. They also stated that the accidental contact during production of nanoparticles through the lungs, skin or GIT may cause altered gene expression, cytotoxicity, idiosyncrasy, and apoptosis of the cell if an individual is exposed for longer time [86].

Van der schaft DWJ et al 2001 focused on tumour vasulature targeting. The book chapter wraps functions of vascular endothelial cells in the body, molecular control of tumour growth-related angiogenesis, role of VEGF, FGF-2, integrins, ECM, and subendothelial support cells in proliferation of cancer. The chapter covered the angiogenesis assays and models with tumour vasculature targeting and pre-clinical experience. The section discloses the various tumours targeting epitopes such as CD44, Endosialin, MMP-2/MMP-9, VEGF, α_v integrins, TNFα and endostatin receptors etc. As per the targeting strategies the carrier molecule should posses a) selective binding with high affinity; b) undergo internalization; and c) intracellular routing favouring translocation of the targeting agent to the cytoplasm. The chapter summarizes the targeting induces tumour blood flow blockage and for complete eradication of cancer cells a combination of tumour vasculature directed strategies may come up with optimal treatment for clinical applications [87].

Sinha R et al 2006, refers nanotechnology to the interactions of cellular and molecular components and engineered materials-typically, clusters of atoms, molecules, and molecular fragments into incredibly small particles between 1 and 100 nm. Nanometer-sized particles have novel optical, electronic, and structural properties that are not available either in

individual molecules or bulk solids. The concept of nanoscale devices has led to the development of biodegradable self-assembled nanoparticles, which are being engineered for the targeted delivery of anticancer drugs and imaging contrast agents. Nanoconstructs should serve as customizable, targeted drug delivery vehicles capable of ferrying large doses of chemotherapeutic agents or therapeutic genes into malignant cells while sparing healthy cells. Such "smart" multifunctional nanodevices hold out the possibility of radically changing the practice of oncology, allowing easy detection and then followed by effective targeted therapeutics at the earliest stages of the disease. The article, briefly discussed the use of bioconjugated nanoparticles for the delivery and targeting (passive and active) of anticancer drugs along with physiologic and biologic properties of nanoparticles [88].

Bawarski WE et al 2008, discussed on the major nanopharmaceutical formulations as well as the impact of nanotechnology in future. The nanopharmaceuticals like liposomes, polymeric micelles, dendrimers, solid nanoparticles, colloidal gold nanoparticles, iron oxide crystals, and quantum dots have advanced for the past several years. It offers the ability to detect diseases or metastatic colonies at much earlier stages often in diagnosis of breast, lung, colon, prostate, and ovarian cancer. The review also focused polymers/block polymer used for formulation of nanopharmaceuticals and the unknown health effects that can only be imagined at present. Some of the possible toxic effects are cancer and dermatological disorders associated with an excess in iron, alteration of pigmentation, inflammation, porphyria and other consequences. The carbon nanotubes can lead to increased pulmonary toxicity due to oxidative stress. They can also cause granuloma [89].

De Jong WH et al 2008, provides an overview on some of the systems for drug delivery. The use of nanotechnology in medicine more specifically as drug delivery system is set to spread rapidly and many substances are under investigation for drug delivery especially in cancer therapy. Interestingly pharmaceutical sciences are using nanoparticles to reduce toxicity and side effects of the drugs and up to recently it was not realized that carrier systems themselves may impose risk to the patient. The kinds of hazards introduced by using nanoparticles for drug delivery are beyond that posed by conventional hazards imposed by chemicals. The toxicology of particulate matter differs from toxicology of substances as the composing chemical(s) may or may not be soluble in biological matrices, thus influencing greatly the potential exposure of various internal organs. However, absorbed species may also influence the potential toxicity of the particles. For nanoparticles the situation is different as their size

opens the potential for crossing the various biological barriers. From a positive viewpoint, especially the potential to cross the blood brain barrier may open new ways for drug delivery into the brain. In addition, the nanosize also allows for access into the cell and various cellular compartments including the nucleus. A multitude of substances are currently under investigation for the preparation of nanoparticles for drug delivery, varying from biological substances like albumin, gelatin and phospholipids for liposomes, and more substances of a chemical nature like various polymers and solid metal containing nanoparticles. It is obvious that the potential interaction with tissues/cells and the potential toxicity greatly depends on the actual composition of the nanoparticle formulation. Besides their potential benefits; the attention is drawn to the question how to proceed with the safety evaluation of the nanoparticle formulations for drug delivery. For such testing the lessons learned from particle toxicity as applied in inhalation toxicology may be of use. Although for pharmaceutical use the current requirements seem to be adequate to detect most of the adverse effects of nanoparticle formulations, it cannot be expected that all aspects of nanoparticle toxicology can be detected. So, probably more specific testing are needed [90].

Peer D et al 2007 examines the various nanocarriers like polymer-protein nanoconjugates, polymer-drug nanoconjugates, radio-immunoconjugates, liposomes, polymersomes, polymeric nanoparticles, nanoshells, nanocages, dendrimers, immunotoxins, immunopolymers and fusion proteins available for treatment of cancer. They also summarized the advantages of nanocarriers over free drugs. The nanocarriers protect the drug from premature degradation and interaction with biological environment. They enhance the absorption of drugs into selected tissue especially solid tumour and control the pharmacokinetic and tissue distribution profile with improved intracellular penetration. The nanocarriers bypass the activity of integral membrane proteins known as multidrug resistance transporters. For rapid and effective clinical translation, the nanocarrier should be made of biocompatible, well characterized and functionalized material. The carrier system should exhibit high differential uptake efficiency in the target cells over normal cells (or tissue). The material of interest should be soluble or colloidal under aqueous conditions at low rate of aggregation with extended shelf life and circulating half-life [91].

Zhang L et al 2008 studied the therapeutic applications and developments of nanoparticles in medicine and describe nanotechnology as the understanding and control of matter generally in the 1–100 nm dimension range. Nanomaterials have unique physicochemical properties, such

as ultra small size, large surface area and high reactivity compared to their bulk materials. The nanoparticles can overcome some of the limitations found in traditional therapeutic and diagnostic agents. The article focussed nanoparticle-based therapeutics approved for clinical use (Table 1), in clinical trials, and in preclinical development [92].

Table 1: Nanoparticle-based therapeutics for clinical use.

Composition	Trade name	Company	Indication	Route
Liposomal platforms				
Amphotericin B	Abelcet	Enzon	Fungal infections	i.v.
Amphotericin B	AmBisome	Gilead Sciences	Fungal and protozoal infections	i.v.
Cytarabine	DepoCyt	SkyePharma	Malignant lymphomatous meningitis	i.t.
Daunorubicin	DaunoXome	Gilead Sciences	HIV-related Kaposi's sarcoma	i.v.
Doxorubicin	Myocet	Zeneus	Combination therapy with cyclophosphamide in metastatic BC	i.v.
IRIV vaccine	Epaxal	Berna Biotech	Hepatitis A	i.m.
IRIV vaccine	Inflexal V	Berna Biotech	Influenza	i.m.
Morphine	DepoDur	SkyePharma, Endo	Postsurgical analgesia	Epidural
Verteporfin	Visudyne	QLT, Novartis	Age-related macular degeneration, pathologic myopia, ocular histoplasmosis	i.v.
Liposome-PEG doxorubicin	Doxil/Caelyx	Ortho Biotech,	Schering-Plough HIV-related Kaposi's sarcoma, metastatic BC, metastatic ovarian cancer	i.m.
Micellular estradiol	Estrasorb	Novavax	Menopausal therapy	Topical
Polymeric platforms				
L-Glutamic acid, L-alanine, L-lysine, and L-tyrosine copolymer	Copaxone	TEVA Pharmaceuticals.	Multiple sclerosis	s.c
Methoxy-PEG-poly(D,L-lactide) taxol	Genexol-PM	Samyang	Metastatic BC	i.v.
PEG–adenosine deaminase	Adagen	Enzon	Severe combined immunodeficiency disease associated with ADA deficiency	i.m.
PEG-anti-VEGF aptamer	Macugen	OSI Pharmaceuticals	Age-related macular degeneration	i.r.
PEG–a-interferon 2a	Pegasys Nektar,	Hoffmann-La Roche	Hepatitis B, hepatitis C	s.c.
PEG–GCSF	Neulasta	Amgen	Neutropenia associated with cancer Chemotherapy	s.c.
PEG–HGF	Somavert	Nektar, Pfizer	Acromegaly	s.c.
PEG–L-asparaginase	Oncaspar	Enzon	Acute lymphoblastic leukemia	i.v., i.m.
Poly(allylamine hydrochloride)	Renagel	Genzyme	End-stage renal disease	Oral
Other platforms				
Albumin-bound paclitaxel	Abraxane	Abraxis BioScience, AstraZeneca	Metastatic BC	i.v.
Nanocrystalline aprepitant	Emend	Elan, Merck	Antiemetic	Oral
Nanocrystalline fenofibrate	Tricor	Elan, Abbott	Anti-hyperlipidemic	Oral
Nanocrystalline sirolimus	Rapamune	Elan, Wyeth Pharmaceuticals	Immunosuppressant	Oral

Agarwal A et al 2008 reviews the scope of ligand based dendritic system as a prospective for delivery of anti-cancer drugs, via active targeting with interception of minimal side effects. Medications that can selectively target tumours at the same time avoid access of the drug to nontarget areas employing homing devices termed as ligands that can bind to specific epitopes expressed on the surface of the necrotic mass of cells. Molecular signatures for transferrin, epidermal growth factor, sialic lewis, and folic acid are expressed on the surface of the cancer cells. Dendrimers are nanosized, non-immunogenic, and hyper-branched vehicles that can be efficiently tailored for spatial distribution of bioactives, thereby reducing untoward

cytotoxicity on normal cells. These nanoparticulate drug delivery vehicles provide a unique platform that has precisely placed functional groups so that multiple copies of ligands can be attached to it for targeting to the tumour surface or neo-vascularizing vessels proliferating around these cells [64].

Parveen S et al 2008 overview the use of clinically applicable nanoparticles mainly for cancer therapy. The piece of writing focused on the different types of nanoscale polymer carriers used for the delivery of chemotherapeutic agents and the mechanisms that facilitate their targeted delivery to tumour cells. Cancer is an ever-increasing menace that needs to be curbed soon. Though chemotherapy is successful to some extent, the main drawbacks of chemotherapy is the limited accessibility of drugs to the tumour tissues requiring high doses, their intolerable toxicity, development of multiple drug resistance and their non-specific targeting. Nanoparticles an evolution of nanotechnology, have the potential to successfully address these problems related to drug delivery and retention and are considered potential candidates to carry drugs to the desired site of therapeutic action. The Poly(lactide-co-glycolide) and polylactide, Chitosan, Poly l-caprolactone, and Human serum albumin are some of the primarily focused polymers. They concluded, nanoparticles can serve to improve the therapeutic index of drug and can be applied effectively in case of drug molecules showing poor bioavailability or instability [93].

Sajja HK et al 2009, explores the development of nanoparticles with their potential for enabling and improving the targeted delivery of therapeutic agents. They also walk-around the limits of molecular diagnostics providing point-of-care diagnosis and more personalized medicine. Nanotechnology is a multidisciplinary scientific field undergoing explosive development. Nanometer-sized particles offer novel structural, optical and electronic properties that are not attainable with individual molecules or bulk solids. Advances in nanomedicine can be made by engineering biodegradable nanoparticles such as magnetic iron oxide nanoparticles, polymers, dendrimers and liposomes that are capable of targeted delivery of both imaging agents and anticancer drugs. This leads toward the concept and possibility of personalized medicine for the potential of early detection of cancer lesions, determination of molecular signatures of the tumour by non-invasive imaging and, most importantly, molecular targeted cancer therapy. Evidences suggests that the nanoparticles containing targeting molecules on their surface increase sensitivity and specificity in tumour detection by binding to the receptors expressed on the tumour cells. In comparison with other small molecule

contrast agents, the advantage of using nanoparticles is their large surface area and the possibility of surface modifications for further conjugation or encapsulation of large amounts of therapeutic agents. Targeted nanoparticles ferry large doses of therapeutic agents into malignant cells while sparing the normal healthy cells. Finally the author's wrap up as multifunctional nanodevices can hold the promise of significant improvement of current clinical management of cancer patients [94].

Reis CP et al 2006 studied polymeric nanoparticles as particulate carriers in pharmaceutical and medical field. They found nanoparticles as promising drug delivery systems as a result of their controlled- and sustained-release properties, subcellular size, and biocompatibility with tissue and cells. They also reviewed polymerization of monomers, emulsion polymerization, emulsion/solvent evaporation, solvent displacement and interfacial deposition, salting out, and emulsion/solvent diffusion as some of the important methods in preparation of nanoparticles. The advantages and disadvantages of method are presented according to a particular application [95].

Mohanraj VJ et al 2006 assessed the various aspects of nanoparticle formulation, characterization, effect of their characteristics and their applications in delivery of drug molecules and therapeutic genes. Nanoparticles as a drug delivery system provide several advantages like a) easy manipulation of size and surface to achieve passive and/or active drug targeting; b) control and sustain release of drug with altered organ distribution, enhanced therapeutic efficacy and reduced side effects; c) incorporation of drug at relatively high concentration into system without any chemical reaction; thus preserving activity of drug; d) site specific targeting; and e) application of system for various routes of administration including oral, nasal, parenteral, and intra-ocular etc. Variety of materials such as natural polymers, proteins, polysaccharides and synthetic polymers are applied in preparation of nanoparticles. The selection of matrix material is dependent on many factors including size of nanoparticles required; inherent property (solubility and stability) of drug; charge and permeability of polymeric molecule; degree of biodegradability, biocompatibility and toxicity; desired release profile; and antigenicity of final product [96].

Vauthier et al 2009 defined and explained the structural features of nanocapsules and nanospheres along with the different methods and principle involved in preparation of them. Polymer precipitation by solvent removal, emulsification-solvent evaporation, emulsification-

solvent diffusion, emulsification-reverse salting out, gelation of emulsion droplets, *in situ* polymerization, interfacial polycondensation, and nanoprecipitation are some of the techniques for preparation of nanoparticles. Many of these methods include two main steps; a) preparation of emulsified system, and b) precipitation or gelation of polymer or polymerization of monomer. The review also summarized the purification, sterilization, lyophilisation and concentration of nanoparticles. Finally they describe the methods to obtain the labelled nanoparticles for *in vitro* and *in vivo* investigation [97].

Li C et al 2008 provide the update on polymer-drug conjugates that had advanced into clinical trials. The polymer-drug conjugate drug delivery aimed at increased therapeutic index by making more drug molecules available at the diseased sites while reducing systemic drug exposure. The systems used synthetic water-soluble polymers as the drug carriers. The preclinical pharmacology and recent data in clinical trials with poly(l-glutamic acid)-paclitaxel (PG-TXL) was discussed with a summary of a variety of polymeric conjugates with chemotherapeutic agents. The early clinical trials of these polymer-drug conjugates demonstrated several advantages over the corresponding parent drugs, including fewer side effects, enhanced therapeutic efficacy, ease of drug administration, and improved patient compliance. Collectively, the data warranty the further clinical development of polymer-drug conjugates as a new class of anticancer agents. The other polymer-drug conjugates reported were HPMA-doxorubicin for lung and BC; HPMA-doxorubicin-galactosamine for hepatocellular carcinoma; HPMA-camptothecin, HPMA-paclitaxel, PEG-Camptothecin, PEG-SN38, Cyclodextrin-based polymer-CPT and carboxymethyldextran-exatecan for solid tumours; HPMA-platinate for ovarian, melanoma and colorectal cancers; polymeric-micelles for pancreatic cancers; PG-TXL for lung, overian, colorectal, breast and esophageal cancers; and PG-Camptothecin for colorectal, lung and overian cancers [98].

Kumari A et al 2010 made a study on biodegradable polymeric nanoparticles based drug delivery systems. The study reported the vast application of system due to its grand bioavailability, better encapsulation and control release and less toxic properties. Various nanoparticulate systems, general synthesis and encapsulation process, control release and improvement of therapeutic value of nanoencapsulated drugs were covered in the review. The revise highlighted the impact of nanoencapsulation of various disease related drugs on biodegradable nanoparticles such as PLGA, PLA, chitosan, gelatin, polycaprolactone and poly-alkyl-cyanoacrylates and also focused the release pattern of drug from the systems. The

nanoparticulate drug delivery systems seem to be a viable and promising strategy for the biopharmaceutical industry. They have advantages over conventional drug delivery systems. They can increase the bioavailability, solubility and permeability of many potent drugs which are otherwise difficult to deliver orally. The nanoparticulate drug delivery systems also reduce the drug dosage frequency and will increase the patient compliance. Nanoparticles can minimize some of these drugs unique problems by safeguarding stability and preserving their structure. In addition, nanoparticles provide ingenious treatment by enabling targeted delivery and controlled release [99].

Watkins G et al 2005 examined the transcript levels of SPARC and its presence in BC tissue to demonstrate a link existing between the levels of SPARC and the clinical outcome. SPARC (secreted protein acidic and rich in cystein), also known as osteonectin and BM40, is a 32 kDa secreted glycoprotein that interacts with extracellular matrix (ECM) proteins to promote adhesion of cells from the matrix, thereby inducing a biological state conducive to cell migration. SPARC is also thought to play an important role in tissue remodelling, angiogenesis, embryonic development and tumourigenesis. Protein levels of SPARC were assessed using immunohistochemistry. Transcript levels of SPARC were analysed using RT-PCR. The levels were correlated with nodal status, grade, prognosis and long-term survival (10 years). Transcript levels of SPARC were found to be significantly higher in tumour tissue when compared to normal breast tissue. The fact was mirrored when levels of SPARC in ductal, lobular and other types of tumour were compared. A high level of SPARC was also found in Grade 3 and TNM2 and TNM4 tumours. Node-positive tumours also exhibited higher levels of SPARC than node-negative tumours. It was noted that SPARC was present at high level in NPI 2 and NPI 3 tumours. Over a 6 year follow-up, high levels of SPARC was seen to be significantly associated with the overall survival of the patients (P=0.0198). However, there was no significant correlation with disease-free survival. It was concluded that SPARC played a crucial role in tumour development in BC and as such has a significant bearing on patient prognosis and long-term survival [100].

Dhanesuan N et al 2002 transfected the MDA-MB-231 BAG cell lines with SPARC to study its effect on BC using Tet-On inducible system. SPARC had multiple effects on cell behaviour. *In vitro*, its major functions are anti-adhesive and anti-proliferative associated with tissue remodelling and cancer. *In vivo* SPARC was over expressed in many cancers, including BC, and the effects of SPARC seem to be cell type-specific. By western analysis, the

background levels of SPARC were analyzed in the MDA-MB-231 BAG and clone X parental cells. The prominent induction of SPARC protein expression after doxycycline treatment in SPARC transfected clones X5, X21, X24 and X75 did not affect cell morphology or adhesiveness to collagens type I and IV, but slowed the rate of proliferation in adherent cultures. Cell cycle analysis showed that SPARC slowed the progression to S phase and doxycycline induction of SPARC slowed the rate of monolayer wound closure in the cultured wound healing assay. Thymidine inhibition of proliferation abrogated this effect, confirming that it was due to anti-proliferation. Consistent with this, it was unable to detect any differences in migration and Matrigel outgrowth analysis of doxycycline-stimulated cells. The SPARC was concluded as inhibitory to human BC cell proliferation with no stimulus to migration, in contrast to its stimulatory effects reported for melanoma and glioma cells [101].

Hsiao YH et al 2010, characterized the immunohistochemical distribution of SPARC in benign and malignant BC of different histologic types and its association with the outcome of invasive ductal carcinoma (IDC). A total of 286 patients of benign and malignant breast lesions between 1994 and 2005 were retrieved from National Taiwan University Hospital. Immunohistochemistry staining with SPARC was performed in tissue microarray or whole section. The association of expression of SPARC and cumulative overall survival of IDC patients were analyzed using Kaplan-Meier survival and Cox regression analysis. SPARC was not expressed in benign breast phylloides and all benign breast tumours, while expressed in 17.2% of IDC, 85% of metaplastic carcinoma of the breast (MCB), and all malignant breast phylloides. SPARC was strongly expressed in mesenchymal components of MCB and expression levels in epithelial components were variable. The correlation of positive expression of SPARC and poor long-term survival in IDC is significant ($p = 0.004$). Individuals with positive SPARC expression had 2.34 times higher hazard of death compared with those with negative SPARC expression after adjusting for factors including positive lymph node, TNM tumour stage, estrogen receptor, and progesterone receptor. SPARC can be a prognostic indicator for IDC [102].

Desai N et al 2006 studied the antitumour activity, intratumoural paclitaxel accumulation, and endothelial transport for ABI-007, an albumin-bound, 130-nm particle form of paclitaxel and Cremophor-based paclitaxel to exploit albumin receptor-mediated endothelial transport. Antitumour activity and mortality were assessed in nude mice bearing human tumour xenografts [lung (H522), breast (MX-1), ovarian (SK-OV-3), prostate (PC-3), and colon

(HT29)] treated with ABI-007 or Cremophor-based paclitaxel. Intratumoural paclitaxel concentrations (MX-1-tumoured mice) were compared for radiolabeled ABI-007 and Cremophor-based paclitaxel. *In vitro* endothelial transcytosis and Cremophor inhibition of paclitaxel binding to cells and albumin was compared for ABI-007 and Cremophor-based paclitaxel. Both ABI-007 and Cremophor-based paclitaxel caused tumour regression and prolonged survival; the order of sensitivity was lung > breast congruent with ovary > prostate > colon. The LD(50) and maximum tolerated dose for ABI-007 and Cremophor-based paclitaxel were 47 and 30 mg/kg/d and 30 and 13.4 mg/kg/d, respectively. At equitoxic dose, the ABI-007-treated groups showed more complete regressions, longer time to recurrence, longer doubling time, and prolonged survival. At equal dose, tumour paclitaxel area under the curve was 33% higher for ABI-007 versus Cremophor-based paclitaxel, indicating more effective intratumoural accumulation of ABI-007. Endothelial binding and transcytosis of paclitaxel were markedly higher for ABI-007 versus Cremophor-based paclitaxel, and this difference was abrogated by a known inhibitor of endothelial gp60 receptor/caveolar transport. In addition, Cremophor was found to inhibit binding of paclitaxel to endothelial cells and albumin. Enhanced endothelial cell binding and transcytosis for ABI-007 and inhibition by Cremophor in Cremophor-based paclitaxel may account in part for the greater efficacy and intratumour delivery of ABI-007 [103].

Zhao D et al 2010 prepared paclitaxel-loaded bovine serum albumin nanoparticles by desolvation technique and characterized for amount of folate conjugation, surface morphology, drug entrapment efficiency, drug loading efficiency, and release kinetics *in vitro*. The targeting effect was investigated *in vitro* by cancer cell uptake of fluorescein isothiocyanate-labelled nanoparticles. The spherical nanoparticles obtained were negatively charged with a zeta potential of about -30 mV, and characterized around 210 nm with a narrow size distribution. Drug entrapment efficiency and drug loading efficiency were approximately 95.3% and 27.2%, respectively. The amount of folate conjugation was 9.22 μg/mg of bovine serum albumin. The folate-decorated nanoparticles targeted a human prostate cancer cell line effectively [104].

Esmaeili F et al 2009 conjugated docetaxel (DTX) with human serum albumin via a succinic spacer to overcome the systemic toxicity of marketed formulation containing high concentration of tween 80, which is associated with several hypersensitivity reactions. A high-performance liquid chromatography method was developed for the determination of

DTX-albumin conjugate. T47D and SKOV3 cells were used for the evaluation of the *in vitro* cytotoxicity of the conjugate by MTT assay. Tissue distribution of conjugates was elucidated on balb/c mice upon intravenous administration. The albumin-conjugated formulation of DTX with the particle size of 90-110 nm showed enhanced solubility and *in vivo* characteristics and significantly higher cytotoxicity against tumour cells, for example, IC50 of 6.30 ± 0.73 nM for T47D cell line compared to free DTX with IC50 of 39.4 ± 1.75 nM. Conjugation also maintained DTX plasma level at 16.19% up to 2 h after injection compared with 2.51% for Taxotere, hence increasing the chance of nanoparticles uptake by tumour cells [105].

Naor B et al 1997 studied the structure, function and association of CD44 with the process of malignant. CD44 is a ubiquitous multistructural and multifunctional cells surface adhesion molecule involved in cell-cell and cell-matrix interactions. Twenty exons were involved in the genomic organization of this molecule. The first and the last 5 exons were constant, whereas the 10 exons located between are subjected to alternative splicing. Differential utilization of the 10 variable region exons, as well as variations in N-glycosylation, O-glycosylation, and glycosaminoglycanation (by heparan sulfate or chondroitin sulfate), generate multiple isoforms of different molecular sizes (85-230 kDa). As it is expressed mainly on cells of lymphohematopoietic origin, CD44s is also known as hematopoietic CD44. CD44s is a single-chain molecule composed of a distal extracellular domain (containing, the ligand-binding sites), a membrane-proximal region, a transmembrane-spanning domain, and a cytoplasmic tail. The molecular sequence displays high interspecies homology. The immunological activation of T lymphocytes and other leukocytes transiently up regulates the CD44 isoforms expressing variant exons. A CD44 isoform containing the last 3 exon products of the variable region is preferentially expressed on epithelial cells. The longest CD44 isoform expressing in tandem eight exons of the variable region was detected in keratinocytes. Hyaluronic acid (HA), an important component of the extracellular matrix (ECM), is the principal, but by no means the only, ligand of CD44. Other CD44 ligands include the ECM components collagen, fibronectin, laminin, and chondroitin sulfate. Mucosal addressin, serglycin, osteopontin, and the class II invariant chain (Ii) are additional, ECM-unrelated, ligands of the molecule. In many, but not in all cases, CD44 does not bind HA unless it is stimulated by phorbol esters, activated by agonistic anti-CD44 antibody, or deglycosylated (e.g., by tunicamycin). CD44 also participates in the uptake and intracellular degradation of HA, as well as in transmission of signals mediating hematopoiesis and apoptosis. Many of the neoplasms including gastrointestinal cancer, bladder cancer, uterine cervical cancer, BC and

non-Hodgkin's lymphoma express CD44. Hence CD44, particularly its variants, may be used as diagnostic or prognostic markers of at least some human malignant diseases. Furthermore, it had been shown in animal models that injection of reagents interfering with CD44-ligand interaction inhibits local tumour growth and metastatic spread. These findings suggest that CD44 may confer a growth advantage on some neoplastic cells and therefore, could be used as a target for cancer therapy. It is hoped that identification of CD44 variants expressed on cancer but not on normal cells will lead to the development of anti-CD44 reagents restricted to the neoplastic growth [106].

Ahrens et al 2001 observed the proteolytic cleavage of the extracellular domain of CD44 from the surface of cells in different cell types. The study revealed that serine proteases and metalloproteases are involved in the cleavage of CD44 from the surface of melanoma cells. To analyse a possible function of soluble CD44 a human malignant melanoma cell line was stably transfected with cDNAs encoding either wild type soluble CD44s or mutated forms with defective HA binding properties (CD44sR41A and CD44sR150A/R154A). Soluble CD44s almost completely inhibited hyaluronic acid (HA) binding by melanoma cells, whereas soluble CD44 mutated in the HA binding domain had no effect. When cultivated on HA, melanoma cell proliferation was induced by 30% for both the parental and the control transfected cells. The increase in proliferation was blocked completely in solCD44s-secreting transfectants, whereas solCD44sR41A and solCD44sR150A/R154A-secreting cells again showed HA-induced cell proliferation. The cell lines were subcutaneously injected into MF1 nu/nu mice to compare their growth as tumours *in vivo*. Compared to tumours derived from parental and control transfected cells, they observed a dramatic reduction of primary tumour growth with solCD44s expressing MM cells. Transfectants expressing solCD44s mutated in the HA binding domain in contrast developed fast-growing primary tumours. The results provided strong evidence that direct solCD44 interactions with HA interferes competitively with processes induced by HA binding to surface of CD44. Autocrine or drug-induced secretion of solCD44 by human melanoma cells may thus exert potent antitumoural effects *in vivo* [107].

Brown TJ 2008 postulates the action of Hyaluronan (HA) as a novel excipient, capable of improving the therapeutic index of the active anti-cancer agent. Despite advances in chemotherapeutic regimens, the treatment of metastatic cancer remains a challenge. A key problem with chemotherapy drugs is nonspecific drug distribution, resulting in low tumour

concentrations and systemic toxicity. The holy grail of clinical cancer research has been to establish more specific ways of directing therapeutics to tumours, whether through more targeted anti-cancer agents or via the method of delivery. Many tumour cells show up-regulated expression of receptors for the polysaccharide HA. This observation has led to the preclinical development of HA-cytotoxin bioconjugates that utilize HA as the tumour recognition moiety. The primary challenges have been organ-directed toxicity and limited efficacy. An alternative, simpler strategy has been to use the large volumetric domain of HA to entrain small chemotherapeutic drugs within the HA matrix. The resultant HA/drug formulation accumulates in the microvascular of the tumour, forming a microembolism that increases drug retention at the tumour site and allows for active tumour uptake through HA receptors. Clinical trials of HA formulations of 5-flurouracil, doxorubicin, methotrexate, and irinotecan have demonstrated that such formulations are safe and efficacious [108].

Gotte M et al 2006 summarized glycosaminoglycan's has major constituents of the cancer cell surface and the tumour stroma. The heparan sulfate degrading enzyme heparanase, hyaluronan (HA), and its receptor CD44 are up-regulated in BC, generating a microenvironment that promotes tumour progression and metastasis. Recent experimental and clinical evidence shows that heparanase, hyaluronan, and CD44 regulate cancer cell proliferation, migration, and invasion, as well as tumour-associated angiogenesis and are correlated with patient survival. The findings suggests the coupling of conventional chemotherapeutic drugs with HA allows for selective targeting of CD44-expressing cancers, thus lowering the dosage of anticancer medication administered and reducing unwanted side effects. Estrogen-antagonistic BC therapy using tamoxifen, fulvestrant, or aromatase inhibitors may lead to a down-regulation of heparanase expression. As heparanase, hyaluronan, and CD44 promote tumour cell proliferation, migration, and angiogenesis through different molecular mechanisms, simultaneous targeting of these three molecules may be expected to produce synergistic effects and prevent cancer cells from using alternative salvage pathways during treatment. Future studies will provide further insight into the pathophysiologic roles of these molecules and may offer important tools for BC management and prognostication [109].

Auzenne E et al 2007 initially evaluated the CD44 ligand hyaluronic acid (HA) as a backbone for paclitaxel (TXL) prodrugs for tumour targeting. HA-TXL was prepared by solvent evaporation technique. The *in vitro* cytotoxicity of HA-TXL against cell lines SKOV-

3ip and NMP-1 significantly blocked cell growth by preincubation with a molar excess of free HA. Female nude mice bearing implants of NMP-1 cells were treated with a single i.p sub-maximum tolerated dose of HA-TXL or Taxol. The effect of these regimens on intra peritoneal tumour burden was assessed by magnetic resonance (MR) imaging and even host survival study was adopted. NMP-1 xenografts were highly resistant to Taxol regimens, as host survival was only nominally improved compared to controls (tumour growth inhibition Treated over Control (T/C) value of approximately 120), whereas single-dose HA-TXL treatment significantly improved survival (T/C approximately 140; P = 0.004). In both NMP-1 and SKOV-3ip models, MR images of abdomens of HA-TXL-treated mice revealed markedly reduced tumour burdens compared to control mice [110].

Rivkin et al 2010 designed the hyaluronan (HA) coated paclitaxel (PTX) clusters to overcome the clinical limitations like poor solubility of the PTX and lack of selective strategy to tumour site. The clusters delivered PTX selectively into tumour cells in a CD44-dependent manner. Injected systemically to mice bearing solid tumours, the HA coated PTX clusters showed high safety profile and tumour accumulation. Tumour progression was exponential upon treatment with free PTX or the marketed formulations like Taxol and Abraxane respectively. Under the same conditions, the designed formulations induced tumour arrest and were as potent as a 4-fold higher Taxol dose. The findings suggest HA coated PTX clusters merit further investigation as vehicles for taxanes, and may be applicable as carriers [111].

Hyung W et al 2008 synthesized hyaluronic acid coated drug carriers (HCDCs) by chemical conjugation method for delivery of doxorubicin to CD44 expressed human BC cells. The X-ray photoelectron spectroscopy demonstrated the superior HA fixation amount and colloidal stability when compared with the nanoparticles by nanoprecipitation. The cytotoxicity of the HCDCs formulation accessed by the MTT assay against the higher CD44 expressed cell line (MDA-MB-231) and lower CD44 expressed cell line (ZR-75-1) of human BC demonstrated the excellent tumouricidal effect with predominant affinity to MDA-MB-231 cell line compared to ZR-75-1. The *in vitro* drug release from the HCDCs was sustained with 80% release on 14^{th} day [112].

Yadav Ak et al 2007 devised a novel hyaluronic acid-poly(ethylene glycol)-poly(lactide-co-glycolide) (HA-PEG-PLGA) copolymer and characterized it by infrared and nuclear magnetic resonance spectroscopy. The nanoparticles of doxorubicin (DOX)-loaded HA-PEG-PLGA

were prepared and compared with monomethoxy(polyethylene glycol) (MPEG)-PLGA nanoparticles. Nanoparticles were prepared using drug-to-polymer ratios of 1:1 to 1:3. Drug-to-polymer ratio of 1:1 was considered the optimum formulation on the basis of low particle size and high entrapment efficiency. The optimized nanoparticles were characterized for morphology, particle size measurements, differential scanning calorimetry, x-ray diffractometer measurement, drug content, hemolytic toxicity, subacute toxicity, and *in vitro* DOX release. The *in vitro* DOX release study was performed at pH 7.4 using a dialysis membrane. HA-PEG-PLGA nanoparticles were able to sustain the release for up to 15 days. The tissue distribution studies were performed with DOX-loaded HA-PEG-PLGA and MPEG-PLGA nanoparticles after intravenous (IV) injection in Ehrlich ascites tumour-bearing mice. The tissue distribution studies showed a higher concentration of DOX in the tumour as compared with MPEG-PLGA nanoparticles. The *in vivo* tumour inhibition study was also performed after iv injection of DOX-loaded HA-PEG-PLGA nanoparticles up to 15 days. DOX-loaded HA-PEG-PLGA nanoparticles were able to deliver a higher amount of DOX as compared with MPEG-PLGA nanoparticles. The DOX-loaded HA-PEG-PLGA nanoparticles reduced tumour volume significantly as compared with MPEG-PLGA nanoparticles [113].

Yadav AK et al 2008 prepared core-corona nanoparticles of doxorubicin (DOX) using hyaluronic acid-polyethyleneglycol-polycaprolactone (HA-PEG-PCL) copolymer for tumour targeting. Targeting efficiency of HA-PEG-PCL nanoparticles was compared with non-HA-containing nanoparticles (methoxy poly ethylene glycol (MPEG)-PCL). The copolymers were chemically synthesized and characterized by IR and NMR spectroscopies. The nanoparticles were characterized for shape and morphology by transmission electron microscopy, particle size, percentage of drug entrapment, and *in vitro* drug release profile. Differential scanning calorimetry and X-ray diffraction studies were also performed to appraise the crystalline or amorphous nature of DOX inside the polymer matrix. Formulations were prepared using different DOX:polymer ratios (1:1-1:3 w/w) and the optimum formulation with the drug:polymer ratio of 1:1 showed the mean particle size of 95 ± 5 nm and entrapment efficiency of 95.56% in the case of HA-PEG-PCL nanoparticles, while the values were 115 nm and 95.50%, respectively, in the case of MPEG-PCL nanoparticles. The HA-PEG-PCL nanoparticles could release DOX for up to 17 days, whereas the MPEG-PCL nanoparticles could release it for up to 14 days. The hemolytic toxicity and hematological studies confirmed that both DOX-loaded HA-PEG-PCL and MPEG-PCL nanoparticles were safe and suitable for sustained and targeted drug delivery. The tissue distribution study and tumour growth

inhibition were performed after intravenous injection of nanoparticles in Ehrlich ascites tumour (EAT)-bearing mice. The nanoparticles of HA-PEG-PCL copolymer accomplishes efficient delivery of DOX in EAT tumour when compared with the MPEG-PCL nanoparticles by the process of receptor-mediated endocytosis, as well as enhanced permeability and retention effect [114].

Hanemaaijer R et al 2000 assayed the activity of MMP-2 and MMP-9 (also known as gelatinase A and B) in malignant and benign BC. The activities of MMP's significantly increased in malignant compared to benign and were thought to be causally involved in cancer invasion and metastasis. In normal as well as in malignant tissue, both these MMPs occur in multiple forms such as inactive, precursors, active enzymes and enzyme-inhibitor complexes. The assessment of potential prognostic values of MMP-2 and -9 in different cancers may play a vital role in design of MMP inhibitors for preventing cancer metastasis [115].

Farias et al 2000 verified the matrix metalloproteinases (MMP) activity in healthy volunteers and cancer patients by gelatin zymography. The euglobulin plasma fraction of activity of 82 healthy controls, 66 patients with benign diseases and 149 patients with breast, lung, colon, or brain cancer was collected for the study. The assay showed four gelatinolytic bands of 62, 92, 120 and 200kDa. The value for 92 kDa-MMP activity was significantly higher in breast and lung cancer patients compared to the healthy volunteers. Interestingly the colon cancer or glioma patients did not show any difference at significance level in MMP activity compared to healthy volunteers. The multivariate clinicopathological parameters such as age, stage, tumour size, number of positive lymph nodes, histologic grade and type, nuclear grade or mitotic index did not predict the plasma MMP-9 activity. They reported 92 kDa-MMP activity in plasma euglobulin fraction can be potentially used as tumour marker in breast and lung cancer [116].

Kim KJ et al 1999 proposed a targeting drug carrier for all-trans-retinoic acid by using angiogenesis one of the specific physiological properties of cancer cells. The PEGylated gelatin nanoparticles of 176nm were prepared by coupling of gelatin with deoxycholic acid followed by surface coating with polyethylene glycol to reduce RES uptake. The feasibility of the nanoparticles as a targeting drug carrier was proved by degradation of nanoparticles by collagenase IV. The release pattern of drug was evaluated with and without the enzyme in the

release media. Only twelve percent of drug was released at the end of an hour in absence of enzyme. In presence of enzyme a burst effect was seen with release of 40% of the total drug content within 10min followed by continuous release up to 75% for 5 hr. The results showed that the formulation could be used as targeting drug delivery system using angiogenesis as a specific physiological property of cancer cells [117].

Aim and Objectives

AIM AND OBJECTIVES

The section provides the main concept expected for the design of the work along with assumptions taken into consideration. The chapter also cover the complete plan involved in execution of work.

Extensive research for the past several decades in the field of cancer targeted drug therapy has proved the ligands hyaluronan, albumin and gelatin has drug carriers for enhancing the concentration of drug at tumour site. The hyaluronan binds the CD44 receptors, albumin gets attached to the SPARC protein and gelatin gets linked with MMPs which are highly present on cancer cell surface, thereby enhancing the local concentration of conjugated chemotherapeutic agent. These ligand binding sites vary from patient to patient and cell to cell which may make the therapy expensive as it become necessary to know the type of receptor present on the cells for adopting the treatment. However in most of the cases either of these binding sites are seen which made us to orient towards the dual ligand targeting therapy which may minimize the variation in targeting of drug from patient to patient. Even theoretically it sounds beneficial for better treatment of breast cancer as simultaneous blockage of CD44 receptors cum SPARC or MMPs is expected with dual ligand targeting therapy. This simultaneous blockage may further enhance the local concentration of chemotherapeutic agent given by binding to one of this ligand.

Thus the aim of the present study was to develop a stable dual ligand nanoparticulate drug delivery system of paclitaxel and docetaxel using the natural, biodegradable and biocompatible hydrophilic polymers like hyaluronan, albumin and gelatin for the better treatment of breast cancer. The combination of ligands was hyaluronan-albumin and hyaluronan-gelatin.

The further objectives of the work were as below

1) To characterise the developed dual ligand nanoparticulate drug delivery system in terms of its *in vitro* and *in vivo* parameters and compare with that of marketed formulations.
2) To enhance the solubility of the above drugs.

3) To increase the biological half-life of the drug by reducing immunogenicity thereby prolonging the action of the drug at the site of interest.

4) To reduce the side effects of the currently available conventional formulations by the elimination of pharmacological active excipients like Cremophor-EL and Tween 80.

5) To enable controlled and/or sustained release.

6) To improve the therapeutic efficacy of the drug by enhancing its local concentration at the tumor site.

7) To reduce the natural cytotoxicity by minimizing the distribution to non-targeted tissues.

To attain the aim and objective, the work was planned in below pattern

1. **Literature Survey**: All the available sources like media, news papers, magazines, text books, journals and CD- roms were browsed to gain the thorough knowledge on the BC. It also covered the complete concept of pharmaceutical, pharmacological, and analytical techniques required in development and evaluation of the formulations.

2. **Preformulation:** The section covered the HPLC analytical and bioanalytical method development for the drugs and study of its compatibility with selected polymers by FT-IR peak matching technique and thermal analysis (DSC). The solubility profile of drug in various solvents (aqueous and organic) was also estimated as described by USP and IP.

3. **Formulation of Nanoparticles**: a simple precipitation cum solvent evaporation technique was applied to develop various batches of dual ligand nanoparticulate drug delivery system.

4. **Characterization of Nanoparticles**: The formulations prepared were characterized for below parameters
   a) Process yield – by gravimetric technique.
   b) pH – by hydrogen ion activity measurement.
   c) Particle size – by lazer scattering technique.
   d) Zetapotential – by application of electric field across the particulate dispersion.
   e) Loading and entrapment efficacy – by HPLC (reverse phase mode).

f) Solubility profile – by general USP/IP method
g) Study of crystallanity – by thermal analysis (DSC).
h) Surface morphology – by topographical image of electron-electron interaction (SEM).
i) *In vitro* release pattern – by dialysis bag technique.
j) Accelerated stability studies – at exaggerated storage conditions.
k) *In vitro* cytotoxicity studies – by SRB assay.
l) *In vivo* studies – pharmacokinetics and biodistribution on female SD rats.
m) Statistical tool – GraphPad prism (ANOVA and Student-t test were applied).

5. **Results:** compilation of experimental facts and figures
6. **Discussion:** study of the experimental facts and figures.
7. **Summary:** brief description of the entire work.
8. **Conclusion**: based on the compilation and interpretation of experimental data.

Drug and Polymer profile

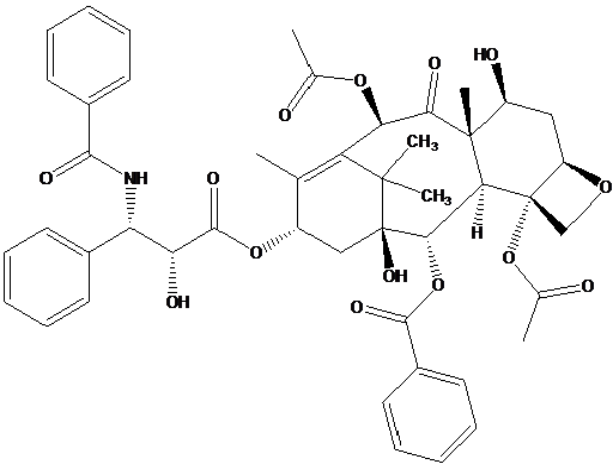
DRUG AND POLYMER PROFILE

The chapter outlines the characteristic features of taxanes (Paclitaxel and Docetaxel) and the biocompatible polymers (Hyaluronan, Albumin and Gelatin).

Paclitaxel (PTX)

PTX commonly known as taxol is a complex tetracyclic diterpene naturally found in barks and needles of pacific yew tree *Taxus bravifolia*. The cytotoxic nature of PTX was first demonstrated in the year 1964, followed by its isolation in 1966 and structural elucidation in 1971 [118].

Since, large numbers of *Taxus bravifolia* trees are required to treat an individual cancer patient, the basic molecule baccatin extracted from leaves of related species *Taxus baccata* is semisynthetically linked to an ester fragment to yield PTX [119].



PTX is off-white crystalline powder insoluble in water with molecular formula $C_{47}H_{51}NO_{14}$ and chemically it is 5β,20-Epoxy 1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one 4,10-diacetate 2-benzoate 13-ester with (2R,3S)-N-benzoyl-3-phenylisoserine (Fig 5) [120].

Fig 5: Molecular structure of Paclitaxel

PTX exhibit has needle-shaped crystals with melting point ranging from 213-216°C with average molecular weight of 853.9. Practically it is insoluble in water and soluble in organic solvents like alcohol, acetone, DMSO and chloroform etc [121,122].

Indication: Used in the treatment of ovarian cancer, breast cancer (BC), non-small cell and small cell lung cancer, head and neck cancer, bladder cancer, endometrial cancer, esophageal cancer, cervical cancer, and AIDS-related Kaposi's sarcoma [123].

Dosage: The optimal dosage regimen of PTX ranging from 100-250 mg/m^2 is administered by an i.v. infusion for 4-6 cycles at interval of 21 days. The greater toxicity was observed on exceeding the dose level of 175 mg/m^2 [124-126].

Pharmacology: PTX is an antimicrotubule antineoplastic agent. It inhibits cell replication by disrupting the dynamic equilibrium within the microtubule system and blocking the cells in the late G_2 phase and M phase of the cell cycle. Evidences also suggest that PTX may also induce cell death by triggering apoptosis. In addition, PTX enhances the effect of ionizing radiations as it blocks cells in G_2 phase, the phase in which the cells are most radiosensitive. PTX is indicated as first-line and subsequent therapy for the treatment of advanced carcinoma of the ovary, and other various cancers including BC [127,128].

Mechanism of Action: PTX interferes with the normal function of microtubule growth. This destroys the cell's ability to use its cytoskeleton in a flexible manner. Specifically, PTX binds to the β subunit of tubulin. Tubulin is the "building block" of mictotubules, and the binding of PTX locks these building blocks in place. The resulting microtubule/PTX complex does not have the ability to disassemble. This adversely affects cell function because the shortening and lengthening of microtubules (termed dynamic instability) is necessary for their function as a transportation highway for the cell. Chromosomes, for example, rely upon this property of microtubules during mitosis. Further research has indicated that PTX induces programmed cell death (apoptosis) in cancer cells by binding to an apoptosis stopping protein called *Bcl-2* (B-cell leukemia 2) and thus arresting its function [121].

Caution:

- Hematological effects and infectious complications – the major adverse effect is bone marrow suppression manifested by neutropenia, leukopenia, thrombocytopenia and anaemia. The infectious complications are associated with fever including sepsis, pneumonia and peritonitis [129,130].
- Sensitivity reaction: Patients treated with PTX are generally associated with hypersensitivity reactions manifested by diffuse and intense erythroderma, pruritus, chest tightness, severe back pain, dyspnea, bradycardia, tachycardia, extreme anxiety, and the development of hypotension or hypertension. Even deaths were documented due to PTX-associated hypersensitivity reaction. The prophylaxis of the

hypersensitivity reaction consisted of dexamethasone (either 20 mg orally the night before treatment and the morning of treatment or 20 mg intravenously before 30 minutes of PTX delivery) plus diphenhydramine (50 mg) and famotidine (20 mg) i.v., 30 minutes before chemotherapy [131].

- Effect on Nervous system: PTX causes peripheral neuropathy associated with symptoms like numbness, paresthesias, and burning pain in glove-and-stocking distribution. It also rarely results in seizures, syncope, ataxia and neuroencephalopathy [132].
- PTX deviate the normal sinus rhythm of heart by disturbing the generation or conduction of electric impulses that spread to atria and ventricles [133].
- PTX induces gastrointestinal complications like nausea, vomiting, diarrhoea, mucositis, anorexia, pancreatitis, and neutropenic enterocolitis etc [134-136].
- Dermatological complications – alopecia and onycholysis are the most common reported complications of dermatology along with other side-effects like skin reaction, fluid-retention syndrome and stomatitis etc [137].
- Respiratory complications – PTX causes dyspnea, cough, hypoxemia, pneumonitis, pulmanory fibrosis and pulmonary infiltrates. Severe pneumonitis and pulmonary fibrosis resulting in death has also been reported [138].
- PTX is known to cause ocular complications like cystic maculopath, loss of visual acuity, flashing lights and photosensitivity, and keratopathy [139].
- The other complications involved with PTX are hepatic necrosis, hepatic encephalopathy, hepatic veno-occlusive disease, acute renal failure [140], fatal hepatic coma [141], arthralgia, myalgia [142], systemic lupus erythematosus, malaise [143], and asthenia/fatigue [137].

Precautions and Contraindications:

- The patient should be prior treated with corticosteroids (dexamethasone, methylpredisolone, and predisone), H_1 receptor antagonist (diphenhydramine, and chlorpheniramine), N-methyl-D-aspartate receptor antagonist orphenadrine and/or H2 receptor antagonists (cimetidine and ranitidine) as a prophylaxis of PTX induced hypersensitivity reaction [144,145].
- PTX should not be administered for patients with baseline neutrophil count less than 1500/mm^3 [146].

- PTX is avoided in pregnancy or in case of women with childbearing potentiality as it can cross placenta and may induce medullar toxicity and myelosuppression in neonate. The distribution of PTX to breast milk is unclear but better to avoid its use during breastfeeding [147-149].

Pharmacokinetics:

PTX exhibit non-linear dose dependent pharmacokinetics which is further compounded in conventional formulations due to presence of cremophor EL. The pharmacokinetic model of PTX indicated saturable distribution and elimination process. The clinical implications of disproportionate increase or decrease in area under the curve with time and the peak plasma concentration are observed with escalations or reductions in dose of PTX. Depending on the dose and duration of infusion with strong protein binding the PTX gets widely distributed to body fluids and tissues [150].

The PTX undergo phase I metabolism in liver to yield a major metabolite 6-α-hydroxypaclitaxel by hydroxylation at 6-α position of baccatin-III ring on action of cyto-P 450 isoenzyme CYP2C8. The CYP3A4/CYP3A5 yields a minor metabolite called 3'-p-hydroxyphenylpaclitaxel by hydroxylation at para position of 3' of the phenyl side chain. The biotransformation rate depends on the physiologic state of the liver. The above two metabolites further undergo metabolism to yield 6-α-hydroxy-3'-hydroxypaclitaxel which is also seen in blood with the above 2 hydroxy metabolites to small extent [151,152].

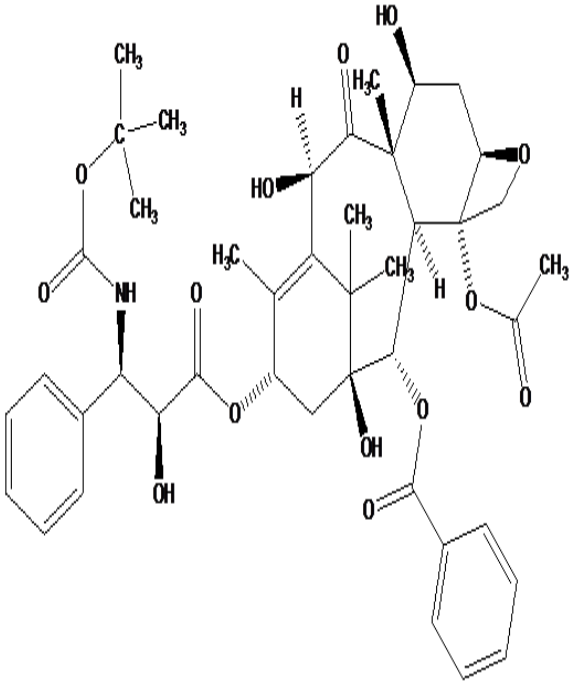
The basic mode of elimination of PTX and its metabolites is hepatic metabolism followed by biliary excretion. The unchanged drug excretion in urine is generally less than 14% of the administered dose with average distribution half-life of 0.34 hr and an elimination half-life of 5.8 - 7.58 hours. The assay of PTX by radioactivity quantification had paradoxical results to the results obtained by HPLC with reverence to elimination with a marginal increase in half-life by almost 13hrs [153].

Toxicity: Rat (ipr) LD_{50}=32530 µg/kg. Symptoms of overdose include bone marrow suppression, peripheral neurotoxicity, and mucositis. Overdoses in pediatric patients may be associated with acute ethanol toxicity.

Synonyms: 7-Epipaclitaxel, 7-Epitaxol, 7-epi-Paclitaxel and 7-epi-Taxol [121].

Docetaxel (DTX)

DTX commonly known as taxotere is a tetracyclic diterpene obtained semisynthetically from the needles of european yew tree *Taxus baccata* [154]. The DTX, an analogue of PTX was first synthesized by Francoise Gueritte-Voegelein and Daniel Guenard in 1984. Later in 1987, Rhone-Poulenc selected DTX for clinical development [155,156].



DTX occur as a white to almost white crystalline powder. It is practically insoluble in water with molecular formula $C_{43}H_{53}NO_{14}$ and chemically it is (2R,3S)-N-carboxy-3-phenylisoserine,N-*tert*-butyl ester, 13-ester with 5β-20-epoxy-1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one 4-acetate 2-benzoate, trihydrate (Fig 6). The DTX exhibit needle-shaped crystals with melting point ranging from 230-233°C with average molecular weight of 807.87. Practically it is insoluble in water and soluble in organic solvents like alcohol, acetone, DMSO and chloroform etc.

Fig 6: Molecular structure of Docetaxel

Indication: DTX is widely used in the treatment of patients with locally advanced or metastatic BC (in case of failure to anthracycline regimen). It is also used in treatment of other primary tumours like anal, gastric, head and neck, colon, and non-small lung cell cancer [157,158].

Dosage: The optimal dosage regimen of DTX ranging from 60-100 mg/m^2 is administered by an i.v. infusion for 4-6 cycles at interval of 21 days [159]. In case of toxicity, dosage modification is considered and the therapy is withheld until neutrophil counts are at least 1.5 x 10^9/L and platelet count exceeds 100 x 10^9/L [160].

Pharmacology: DTX is a taxoid antineoplastic agent. It promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cellular functions. In addition, DTX induces abnormal arrays or "bundles" of microtubules throughout the cell cycle and multiple asters of microtubules during mitosis.

Mechanism of Action: DTX interferes with the normal function of microtubule growth. It also shows promising clinical results in combination of other molecules like trastuzumab, cetuximab, farnesyl transferase inhibitors, capecitabine, and augmerosen whereas with drugs like colchicine it acts by arresting their function. This destroys the cell's ability to use its cytoskeleton in a flexible manner. Specifically, DTX binds to the β-subunit of tubulin. Tubulin is the "building block" of mictotubules, and the binding of DTX locks these building blocks in place. The resulting microtubule/DTX complex does not have the ability to disassemble. This adversely affects cell function because the shortening and lengthening of microtubules (termed dynamic instability) is necessary for their function as a transportation highway for the cell. Chromosomes, for example, rely upon this property of microtubules during mitosis. Further research has indicated that DTX induces programmed cell death (apoptosis) in cancer cells by binding to an apoptosis stopping protein called Bcl-2 (B-cell leukemia 2) and thus arresting its function [161,162].

The DTX being an analogue of PTX, show similar cautions, precautions and contraindications [163].

Pharmacokinetics:

DTX exhibit linear pharmacokinetic profile with high inter and intra-patient variability [164,165]. The area under the plasma concentration-time curve was directly proportion to the dose of DTX. It has a high volume of distribution and clearance with extensive binding (> 95% of dose) to plasma proteins like lipoproteins, albumin and alpha-1 acid glycoprotein. Among the plasma proteins the alpha-1 acid glycoprotein shows high affinity to DTX which is influenced by the presence of formulation vehicle polysorbate 80 [166,167].

The basic mode of elimination of DTX and its metabolites is hepatic metabolism followed by biliary excretion via faeces. The cytochrome P450 3A4 isozymes play a major role in hydroxylation of tert-butoxy function in the C_{13} side chain followed by a cyclization reaction. The four principal metabolites of the pathway are generally represented as M1, M2, M3 and M4 [168]. The DTX undergo oxidation to yield a metabolite M2 (primary alcohol) which undergo further oxidation via an intermediate aldehyde to produce two cyclic hydroxyoxazolidinones which are diastereomers (M1 and M3). An additional oxidation of intermediate aldehyde results in unstable carboxylic acid derivative from which the oxazolidinedione (M4) forms by cyclization. Among the four the M4 is the main metabolite and accounts in large fraction [169]. The weekly dose of 30-36 mg/m^2 given as i.v. infusion for 1 to 2 h, a three phase disposition profile was observed with plasma time-course data model. The alpha (distribution), beta and gamma (elimination) half-life of 4.3 ± 1.5 min, 43.8 ± 21.0 min, and 15.7 ± 7.4 h respectively was recorded for the model. When 48 h time-point was included, the pharmacokinetic parameters were changed with the alpha, beta and gamma half-life of 4.7 ± 1.9 min, 52.8 ± 28.8 min, and 25.4±20.1 h respectively. (170].

Toxicity: Oral LD_{50} in rat is >2000 mg/kg. Anticipated complications of overdosage include: bone marrow suppression, peripheral neurotoxicity, and mucositis. In two reports of overdose, one patient received 150 mg/m^2 and the other received 200 mg/m^2 as 1-hour infusions. Both patients experienced severe neutropenia, mild asthenia, cutaneous reactions, and mild paresthesia, and recovered without incident of death.

Synonyms: Docetaxel anhydrous, Docetaxel Trihydrate, or TXL [171].

Bovine Serum Albumin (BSA)

BSA also known as "Fraction V" is the most common type of albumin used in biochemical applications and development of pharmaceutical formulations. It generally regarded as albumin. The serum albumins secreted by liver belong to a multigene family of proteins that include α-fetoprotein and human group-specific component (Gc) or vitamin D-binding protein [172].

Physical and Chemical properties:

The BSA has almost same molecular weight (66 KDa), isoelectric point (at pH 4.8), globular dimensions and other physical characteristics as human serum albumin [173]. It is highly water soluble with considerable structural stability. It makes up to 60% of plasma proteins and attributes colloidal osmotic pressure of plasma [174]. Being acidic in nature it exhibits the half life of 19 days and shows no deleterious effects even at 60°C for about 10 h [175]. Albumin consists of 585 amino acids with a total of 17 disulphide bridges, one thiol (Cys 34), and a tryptophan (Trp 214). The disulphides are positioned in a repeating series of nine loop-link-loop structures centred on eight sequential Cys-Cys pairs. The protein is derived from several sources like bovine, rat, human, sheep, salmon, frog, mouse, pig and sea lamprey. All of these share high sequence homology with characteristic repeating series of disulphide bridges [172].

Clinical Usage: Albumin is used in several pathological conditions like liver cirrhosis and ascites, bacterial peritonitis [176], nephrotic syndrome [177], menetriers [178], hyperbilirubinemia in severe jaundice and renal failure conditions [179,180], and a very rare case of analbuminemia (no plasma albumin) etc [181].

Apart from the above clinical application it plays an important role in transport of hormones, enzymes, fatty acids, metal ions and medicinal products. It is also used as a therapeutic agent in maintainence and restoration of circutlating blood volume in situations such as trauma, surgery, blood loss, burn management and plasma exchange. It is also used as isotonic solution at concentration of 4-5% in case of hypovolemia [174].

As a drug carrier it accumulates in malignant and inflamed tissue due to an absent or defective lymphatic drainage associated with leaky capillary. As an alternate to drug targeting, conjugating therapeutic peptides the albumin improves the pharmacokinetic profile of many drugs by altering their half life and tissue distribution [175]. The albumin crosses the endothelial barriers by binding to the receptor gp60 which further initiates transcytosis by activation caveolin-1. Activation of caveolin-1 results in internalization of the albumin-receptor complex followed by subsequent transport of albumin-bound products into the tumour cell interstitium. The tumour targeting capacity of albumin may also be due to its affinity to SPARC (secreted protein acidic and rich in cysteine) which is enormously expressed on tumour cell surface [182].

Biodegradation:

Albumin being the biodegradable monomeric protein undergoes fragmentation in renal proximal tubule of kidney. The low molecular fragments (amino acids) of albumin are usually excreted through urine with very less around 2% of intact albumin. The body can also take up these fragments for further utilization by the process of tubular reabsorption [183].

Gelatin

Gelatin is a natural, biocompatible, nontoxic, edible, and inexpensive macromolecule (protein) with wide application in pharmaceutical, medical, and cosmetic products. It is also known as Byco, Cryogel, Instagel or Solugel.

Physical and Chemical properties: Gelatin occurs as light amber to faintly yellow colored, vitreous, brittle solid. It is odourless, tasteless with molecular weight ranging from 15 to 250 KDa. It is soluble in glycerine, acid, alkali and hot water. With moisture content of 9-11% w/w it often swells and softens in water and record a viscosity of 4.3 to 4.7 mPa for a aqueous solution of 6.67% w/v at 60°C [184]. Gelatin is a protein obtained from the partial acid or alkali hydrolysis of skin and bones of animals (mainly bovine and pork). The gelatin is characterized as type A and B based on its isoelectric point and the process of hydrolysis. The type A gelatin is derived by acid hydrolysis with pH of 3.8 – 6 and the isoelectric point of 6-8 whereas, the type B is obtained by alkali hydrolysis with pH of 5-7.4 and isoelectric point of 4.7 – 5.3. Generally, the gelatin used in pharmaceutical industries is a blend of these two types [185]. The solubility of gelatin in water is due to formation of hydrogen bonds between the polar groups like OH, CO, and NH_2 with water. The hydrophilicity can also be influenced due to NH_3 and COO^- groups. The model monomeric structure of Gelatin is $–(Gly\text{-}X\text{-}Pro)_n-$ or $–(Gly\text{-}X\text{-}Hypro)_n-$ where x is an amino acid. Gly, Pro, Hypro stands for glycin, proline and hydroxyproline respectively [186].

Applications and Uses in Pharmaceutical Technology:

Gelatin being a prime ingredient of capsules (hard and soft) is widely applied as a biodegradable matrix material in preparation of implantable delivery system along with clinical applications [187-190].

It is widely applied in zymography to estimate the extracellular matrix enzymes and to determine the proteolytic activity in cells and tissues [191-193].

It is extensively used in several formulation like tablets [194,195], inhalants [196-198], suppositories and pessaries [199,200], injections [201], and topical preparations [202] etc.

Clinically the gelatin (4%) is used has plasma substitute (volume expander) in case of surgical blood loss [203] though its anaphylactoid reactions with release of histamine are reported

[204]. It is also used in design of medical device (ocular insert-Gelfoam) intended for application to bleeding surfaces as haemostatic. The device being compatible with many drugs like pilocarpine, phenylephdrine, and tropicamide etc absorbs water and becomes soft and pliable [205]. The absorbable gelatin powder is injected in management of transient vocal cord paralysis [201].

Gelatin has also been functionalized in the field of dental science and orthopaedics with its application in preparation of plaques and cements [206-208].

Gelatin is also broadly applied in the field of novel drug delivery systems like hydrogels [209], films [210], microspheres [211], and nanoparticles [212,213] etc., for the delivery of medicinal products.

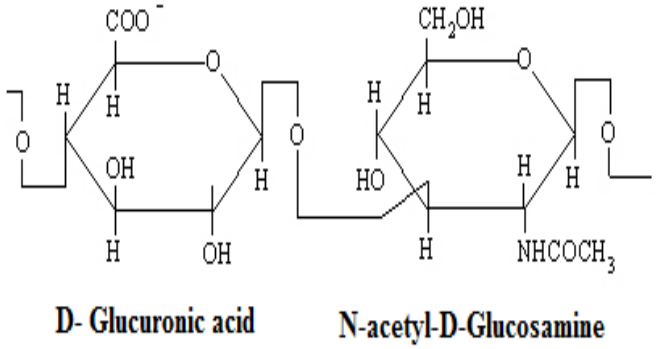
Distribution and Clearance:

The distribution and clearance of the gelatin is mainly dependent upon its molecular weight. As the molecular weight of gelatin increases, the excretion of it decreases with accumulation in liver. Irrespective of the molecular weight it gets cleared from the body via urine as unchanged within hours [214].

Hyaluronan (HA)

HA also termed as Hyaluronic acid is a naturally occurring polysaccharide commercially available as sodium salt. It appears as white to off-white powder or granules. It promotes motility, adhesion and proliferation of cells thereby playing roles in morphogenesis, wound healing and metastasis [215].

Physical and Chemical Properties.



HA is a water soluble non-sulfated glycosaminoglycan, widely distributed throughout the extracellular matrix of all connective tissues in human and other animals [216].The molecular weight of HA depends on the number of repeating disaccharide units (Fig 7).

Fig 7: Molecular structure of disaccharide units of Hyaluronan

The monosaccharides D-glucuronic acid and N-acetyl-D-glucosamine are linked together by altering $\beta_{1,3}$ and $\beta_{1,4}$ glycosidic bonds with empirical formula $(C_{14}H_{20}NO_{11}.Na)_n$. Commercially the HA is available as sodium salt with molecular weight ranging from 500 to 6000 KDa. It is estimated that the HA with molecular weight around 1000KDa contain approximately 2,500 disaccharide units [217,218]. The HA behaves as a polyelectrolyte in solution forming a viscous gel and is negatively charged at physiological pH due to presence of carboxyl group of the glucuronic acid [219].

Applications and Uses in Pharmaceutical Technology

HA synthesized in a unique manner by a family of HA synthases exert biological effect by binding to cellular receptors in treatment of cerebrovascular diseases like cerebral ischemia (stroke) [220].

The aerosolized HA with proinflammatory and anti-inflammatory activities has shown beneficial results in experimental lung injuries like pulmanory emphysema, airway hyperreactivity, and acute lung injury [221].

HA and its derivatives stimulates the healing process of tympanomastoid surgery by re-epithelialization on application with no adverse events [222].

Experimental results suggests that the zinc salt of HA reduces the time of healing of neuropathic and neuroischemic diabetes foot ulcers significantly with no local and systemic side effects [223].

Therapeutically HA is applied as surgical aid in ophthalmology to increase the success rate of trabeculotomy procedure in patients with congenital glaucoma by prevention of postoperative haemorrhage, adhesion of the incision lips or fibroblastic proliferation [224]. HA is also reported for the repair of corneal lacerations [225].

Clinically it is widely used in treatment of osteoarthritis in the knee and is an effective treatment for arthritic pain [226].

The HA is also used in cosmetology and reported to moisturize and restore elasticity of skin thereby achieving antiwrinkle effect. The HA based formulations are also capable of protecting skin against ultraviolet irradiation due its free radical scavenging property. HA is also a material of choice for use in cosmetic soft tissue and dermal correction [218,227].

HA is widely used as vehicle for delivery of several drugs via various routes with numerous applications. It is used to enhance the bioavailability and the ocular residence time of pilocarpine, tropicamide, gentamycin, timolol, and tobramycin etc. It acts as nasal bioadhesive vehicle to enhance the bioavailability of gentamycin, vasopressin and xylometazoline. It alters the absorption and dissolution rate of insulin when given pulmonary and in form of implant. The dissolution rate of plasmid DNA and monoclonal antibodies is altered. It is also applied in delivery of anticancer drugs to the site of interest [227].

In treatment of cancer the HA is widely applied as a vehicle to target the chemotherapeutic agents to the site of action by targeting the HA receptors CD44/Receptor for HA mediated mobility (RHAMM) [228]. These receptors are found at much higher concentration at the tumour invasion front than in central areas of tumour [229]. The HA also regress the tumour by several different mechanisms like a) cleavage of HA to its oligomers to inhibit tumour growth; b) mask the membrane binding sites of tumour cells and thereby disrupt cell-extracellular matrix adhesion leading to apoptosis;. c) disruption of interaction between cell

membrane CD44 and the extracellular matrix, thus altering the signals necessary for proliferation or creating an environment that favours apoptosis and d) by affecting host resistance with generation of immune responses, antibodies production, macrophages infiltration and NK activity such as TNF-α secretion and expression of CD69 through protein tyrosine-kinase pathway [230-234].

Biodegradation

In mammals, the HA gets degraded by the action of enzymes hyase (hyaluronidase), β-d-glucuronidase and β-N-acetyl-hexosaminidase. These enzymes are found in various forms, intracellularly and in serum. The hyase cleaves the HA into oligosaccharides which gets further fragmented by β-d-glucuronidase and β-N-acetyl-hexosaminidase. The hydrolysis of HA lowers its viscosity and enhances the tissue permeability. With the wide tissue and intracellular fluid distribution the HA undergo renal, hepatic, pulmonary, and GIT excretion [235].

Materials and Instruments

MATERIALS AND INSTRUMENTS

The section presents the list of chemicals and reagents along with the instruments used throughout the dissertation work along with their standards and the vendor.

Chemicals and reagents:

Paclitaxel	: M/s. Cipla Pvt Ltd, Mumbai, India
Docetaxel	: M/s. Cipla Pvt Ltd, Mumbai, India.
Hyaluronan	: Sigma Chemicals & Co (Fluka), USA.
Gelatin	: SD Fine-Chem Ltd, Mumbai, India.
Bovine serum albumin (Fraction V)	: Sigma Chemicals & Co, USA.
Acetone (LR grade)	: Ranbaxy Fine Chemicals, New Delhi
Acetonitrile (HPLC)	: Rankem Fine Chem Ltd, New Delhi, India.
Dialysis membrane	: Himedia Laboratories Pvt Ltd, Mumbai, India.
Dichloro methane	: Rankem Fine Chemicals Ltd, New Delhi, India.
Diethyl ether	: Qualigens Fine Chemicals, Mumbai, India.
Glacial acetic acid	: Qualigens Fine Chemicals, Mumbai, India.
Hydrochloric acid	: Qualigens Fine Chemicals, Mumbai, India.
Methanol (HPLC)	: Himedia Laboratories Pvt Ltd, Mumbai, India.
Orthophosphoric acid (LR grade)	: E Merck Ltd, Mumbai, India.
Perchloric acid	: Qualigens Fine Chemicals, Mumbai, India.
Potassium bromide (IR grade)	: E. Merck (India) Ltd, Mumbai, India.
Potassium dihydrogen phosphate	: SD Fine-Chem Ltd, Mumbai, India.
Sodium chloride	: SD Fine-Chem Ltd, Mumbai, India.
Sodium hydroxide	: Qualigens Fine Chemicals, Mumbai, India.
Triethyl amine (LR grade)	: SD Fine-Chem Ltd, Mumbai, India.
Tween 80	: Ranbaxy Fine Chemicals Ltd, New Delhi, India.
Zinc sulphate	: SD Fine-Chem Ltd, Mumbai, India
Bovine calf serum	: PAA Laboratories, GmbH, Austria.
Trichloro acetic acid	: Acros Organics, New Jerscy, USA.
Sulfrhodamine-B	: Sigma Chemicals & Co, St. Louis, USA.
Acetic acid	: Qualigens Fine Chemicals, Mumbai, India.
Tris base	: Sigma Chemicals & Co, St. Louis, USA.
MEM medium	: Himedia Laboratories Pvt Ltd, Mumbai, India.

L-glutamine	: Sigma Chemicals & Co, St. Louis, USA.
Fetal bovine Serum	: PAA Laboratories, GmbH, Austria.
Streptomycin	: Himedia Laboratories Pvt Ltd, Mumbai, India.
Amphoterecin B	: Himedia Laboratories Pvt Ltd, Mumbai, India.
MNU	: Sigma Chemicals and Co, St. Louis, USA.
Sodium EDTA (AR)	: Himedia Laboratories Pvt Ltd, Mumbai, India.

Instruments:

Deep Freezer (HDF – 1703)	: Labline Instruments, Cochin, India
DSC (Q-200)	: TA Instruments Division, Waters (India) Pvt Ltd. Bangalore
ELISA reader	: Bio-rad, model 550.
Freeze dryer (Lyophilizer)	: Christ alpha 1-2, Wertheim, Germany.
FTIR Spectrophotometer	: Perkin Elmer, USA.
Hot air oven (NSW 142)	: NSW India
Magnetic stirrer	: Raagaa Industries, Chennai, India.
Micropipette	: Scoorex, Switzerland.
Orbitary shaker (KS 4000i)	: IKA India Pvt Ltd, Bangalore.
Particle size analyzer (Malvern Nano-ZS)	: Malvern Instruments, UK.
pH meter (pH 510)	: Eutech Instruments, Singapore.
Research Centrifuge (R23)	: Remi Instruments, Mumbai, India.
Scanning Electron Microscope	: JEOL-JSM-5610LV, Japan.
Single Pan Digital Balance (BT 224S)	: Sartorious Mechatronics India Pvt Ltd, Bangalore.
Ultra Fast Liquid Chromatography	: Schimadzu Corporation, Kyoto, Japan.
UV Spectrophotometer	: Shimadzu Spectrophotometer, Japan.
Zeta Sizer (Malvern Nano-ZS)	: Malvern Instruments, UK.

Methodology

METHODOLOGY

The section is instantiated and materialized by a set of methods, techniques and tools. It covers procedure, pre- and post-formulation characterization of the nanoparticles.

I) Preformulation studies: involves the development of analytical and bio-analytical techniques and the curve of linearity, study of solubility profile of the paclitaxel (PTX) and docetaxel (DTX), and the study of incompatibility profile between the drug and polymer of choice.

1. Development of an Analytical and Bio-analytical method - by HPLC

The HPLC analytical and bio-analytical method was developed to characterize and estimate the drug in dosage form, *in vitro* and *in vivo* media. The principle involved in the method was either adsorption or partition. The methods were developed using shimadzu LC20AD as the system and the LC solutions as the software. C_{18} column was used as a stationary phase with ACN and 0.1% TEA pH 3.5 as mobile phase at ratio of 70:30 v/v for analytical and 54:46 v/v for bio-analytical technique at flow rate of 1 and 1.4 ml/min respectively. The pH of TEA was adjusted with orthophosphoric acid.

Curve of Linearity:

For development of the analytical curve, the drug (PTX and DTX) was dissolved separately in ACN to get a concentration of 1 mg/ml solution. The further dilution of the same were made with the composition of the mobile phase and the range of concentration considered for PTX was 200, 20, 2, 0.2, 0.02, and 0.002 µg/ml and for DTX the range was 1000, 100, 10, 1, 0.1, 0.01 µg/ml.

The bio-analytical curve of PTX was developed by spiking 0.5 ml of aliquot concentration of it to a mixture of 0.5 ml of internal standard solution of DTX, 0.5 ml of blank plasma and the 0.5 ml of methanol (protein precipitating agent). The spiking was done such a way that the samples produced a concentration of 500, 50, 5, 0.5, 0.05, and 0.005 µg/ml respectively keeping the concentration of internal standard constant (500 µg/ml). The same procedure was applied to develop the bio-analytical curve of DTX considering PTX as internal standard.

2. Solubility Studies:

The solubility profile of PTX and DTX was determined as described by Ooya with slight modification [236]. The sample (10 mg) was added to the screw capped vials each containing fixed volume (2 ml) of various solvents and stirred at 150 rpm in an orbitary shaker at 37±2 °C. The solvent of choice was water, phosphate buffer pH7.4, and buffer with different ratio of tween 80 (0.1-1 %w/v). Samples were taken at 48 h, centrifuged and filtered through 0.2 µm filters (Millipore, USA) and analyzed for respective drug content by HPLC. The study was carried thrice for each solvent system.

3. Incompatibility Studies by FT-IR:

The drug polymer interaction study was carried out by Fourier-transfer infrared spectrum analysis. The Fourier-transfer infrared spectra's of dry samples maintained at isothermal stress conditions were recorded on Shimadzu FTIR 8400S. The KBr pellet method was employed as FTIR sampling technique. The samples - pure drug and the physical blend of drug and polymer stored at isothermal stress condition were mixed with KBr (IR grade) at ratio of 1:5 by weight. A thin layer pellets were prepared by subjecting samples to the hydraulic press at 10000-12000 Kg/cm^2 pressure. The analysis was carried in the frequency range between 4000-400 cm^{-1} with 4 cm^{-1} resolution. The results were the mean of 6 scans. The result of the study was further confirmed by DSC.

4. Incompatibility Studies by DSC:

The physical status of PTX and DTX along with the combination of polymers albumin, gelatin and hyaluronan stored at isothermal stress conditions was investigated by DSC prior calibrated with indium as a standard with melting point at 156.63 °C. The samples (pure drug, pure polymers and the physical blend) were accurately weighed (2-3 mg) and heated in closed aluminium crimped cells at rate of 10 °C/min between 20 to 225 °C under purge of dry nitrogen at flow rate of 50 ml/min.

II) Formulation of Nanoparticles:

The formulations were developed by simple precipitation cum solvent evaporation technique and the process of optimization involved trial and error method. Several batches of formulations were prepared by altering the polymer to drug ratio and the procedure is as below;

1. Preparation of Hyaluronan-Albumin Nanoparticles:

The organic phase of the drug in DCM was added dropwise to the aqueous polymeric mixture containing of 0.25% w/v hyaluronan in 0.1M NaCl and 0.25% w/v albumin in water. The rate of addition was maintained at 2 ml/min at a distance of 1-2 cm from the surface of the aqueous solution. The process of addition was carried under continuous stirring using magnetic stirrer to develop the milky colloidal solution at 600 rpm for 24h. Various batches were prepared by varying the drug to polymer ratio. The drug to polymer ratio was maintained at 1:1 in case of batch A1 and A2 whereas with batch A3 and A4 the ratio was adjusted to 1:2. In case of batch A5 and A6 the 1:2 ratio was maintained changing the concentration of polymeric mixture to 0.5% w/v hyaluronan and 0.5% w/v of albumin. The batch A1, A3, and A5 contain PTX and the A2, A4 and A6 restrain DTX. All the ratio of drug to polymer corresponds to weight by weight.

2. Preparation of Hyaluronan-Gelatin Nanoparticles:

The organic phase of the drug in DCM was added drop wise to the aqueous polymeric mixture containing of 0.25% w/v hyaluronan in 0.1M NaCl and 0.25% w/v gelatin in water. The rate of addition was maintained at 2 ml/min at a distance of 1-2 cm from the surface of the aqueous solution. The process of addition was carried under continuous stirring using magnetic stirrer to develop the milky colloidal solution at 600 rpm for 24h. Various batches were prepared by varying the drug to polymer ratio. The drug to polymer ratio was maintained at 1:1 in case of batch G1 and G2 whereas with batch G3 and G4 the ratio was adjusted to 1:2. In case of G5 and G6 the 1:2 ratio was maintained with changing the concentration of polymeric mixture to 0.5% w/v hyaluronan and 0.5% w/v of Gelatin. The batch G1, G3, and G5 contain PTX and the G2, G4 and G6 hold back DTX. All the ratio of drug to polymer corresponds to weight by weight.

III) Characterization of Nanoparticles:

1. Process yield:

The process yield of the batches was calculated by gravimetric method [237]. The suspensions were centrifuged at 7500 rpm for 30 min at room temperature and the sediments were lyophilized. The mean % process yield of all the batches with standard deviation (n=3) was determined from the below formula.

$$\% \text{ Process yield} = \frac{\text{Weight of Nanoparticles}}{\text{Total Solid Weight}} \times 100$$

2. pH of the formulations:

The pH of the colloidal suspensions was determined by direct immersion of the silver-silver chloride electrode of a calibrated pH meter at room temperature.

3. Determination of Particle size:

The size analysis of nanoparticles was performed by laser scattering technique using Malvern Nano-ZS. The nanoparticles were dispersed in an aqueous solution and taken in a sample dispersion unit and stirred during the process to minimize the inter particle interaction. The particle size analysis spectrum is a mean observation of three scans performed at 25 °C. In addition to the mean particle size, the system reports a polydispersity index.

4. Zeta potential:

The zeta potential of the formulations was measured using Malvern Nano-ZS. The analysis was performed twenty times after 6-8 folds dilution with water to result in optimum signal intensity at 25 °C. The average value was recorded and used for further interpretation.

5. Determination of loading and entrapment efficiency:

The content of PTX and DTX in the nanoparticles was assayed by HPLC analytical technique. Briefly around 5 mg of particles of each batch was dissolved in 2 ml of methanol (HPLC grade) under vigorous vortexing. The solution was centrifuged and the supernatant was further diluted with mobile phase to ascertain the AUC of them fall in the linearity range. The analysis was performed in triplicate for each batch. The loading and entrapment efficiency was calculated from the below given formula and expressed as percentage [238].

$$\text{Loading efficiency (\%)} = \frac{\text{Actual drug content}}{\text{Amount of drug loaded nanoparticles}} \times 100$$

$$\text{Entrapment efficacy (\%)} = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} \times 100$$

6. Study of crystallanity:

The crystalline phase of the drug in particles was studied by subjecting 2-3 mg of the nanoparticles to the thermal analysis by DSC. The samples were heated in closed aluminium crimped cells at rate of 10 °C/min from 20 to 225 °C under purge of dry nitrogen at flow rate of 50 ml/min. Prior to analysis, the DSC was calibrated with standard indium with melting point at 156.65 °C.

7. Solubility profile of Nanoparticles:

The solubility profile of nanoparticles containing 10 mg of PTX and DTX was determined as described earlier. The PBS pH 7.4 with 1% w/v tween 80 was used has solubilising media for PTX nanoparticles whereas, for DTX nanoparticles it was PBS pH 7.4 with 0.5% w/v tween 80.

8. Surface morphology:

The nanoparticles were imaged by a scanning electron microscope (SEM) at an accelerating voltage of 20 kV. To avoid the misconception of overlay of particles with agglomeration the particles were dispersed in water and few drops of dispersion were fixed on to stub by a double-sided sticky carbon tape. The dispersion was air-dried and coated with gold layer for 60 sec before scanning for an image.

9. ***In Vitro*** **release studies:**

The *in vitro* drug release was performed by dialysis bag method. Nanoparticles containing approximately 25 mg of the drug were dispersed in 5 ml of respective release medium. The release studies for PTX batches were carried out in phosphate buffer saline (pH 7.4) with 1% w/v tween 80 and for DTX the volume of tween 80 was reduced to 0.5 %w/v. The dispersion of nanoparticles was placed in cellulose dialysis bag (pore size 2.4 nm) and sealed at both the ends. The bag was later immersed in 50 ml of dissolution medium has receptor compartment, which was maintained at 37 ± 2 °C with constant stirring at 100 rpm throughout the process. At given time intervals (Table 8 and 9) from 30 min to 24 h, 5 ml of sample was withdrawn from the receptor compartment for analysis and replaced with fresh medium. The tween 80 was used to enhance the solubility of drug thereby maintaining the sink condition and to prevent the binding of drug to the compartment surface. The receptor compartment was covered to prevent the loss of medium during the studies [239].

10. ***In vitro*** **cytotoxicity studies – SRB assay:**

The monolayer cell culture of MDA-MB-231 was trypsinized and cell count was adjusted to 1.0×10^5 cells/ml using medium containing 10% new-born calf serum. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off and washed once with medium followed by addition of 100 µl of different extract concentrations to the cells in microtitre plates. The plates were then incubated at 37 °C for 3 days in 5% CO_2 atmosphere, microscopic examination was carried out and observation were recorded every 24 h. After 72 h, 25 µl of 50% trichloro acetic acid was added to the wells gently in such a way that it forms a thin layer over the extract to form an overall concentration of 10 %. The plates were incubated at 4 °C for 1 h and then plates were flicked and washed five times with water to remove traces of medium, extract and serum, and air-dried. They were stained with SRB for 30 min., the unbound dye was then removed by rapidly washing for four times with 1% acetic acid. The plates were then air-dried. Tris base (10 mM, 100 µl) was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 min. The absorbance (OD) was measured using microplate reader (BIO-RAD) at a wavelength of 540 nm. The percentage growth inhibition was calculated using following formula and concentration of

drug or test extract needed to inhibit cell growth by 50 % (IC_{50}) values were generated from the dose-response curve for each cell line [240]. The study was performed thrice for all the batches analyzed.

$$\% \text{ Growth Inhibition } (IC_{50}) = 100 - \left\{ \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \right) \right\} \times 100$$

11. Accelerated Stability studies:

The studies were carried for batches A3, G3, A4 and G4 as per WHO guidelines for zone IV (hot and humid). The studies were carried at 25 ± 2 °C at 60 ± 5% RH. The entrapment efficiency and the pH of the product was evaluated as a function of the storage time on sampling interval of first, second and third month. On sampling interval of 6th month even the particle size and *in vitro* release studies was determined as an additional functions [241,242]. The procedure for all the functions studied remains the same as that stated earlier.

12. *In vivo* studies:

12. A. Pharmacokinetics studies:

The studies were carried on female SD rats with prior permission of committee for the purpose of control and supervision of experiments on animals (CPCSEA). SD rats (220±20g) of 8 weeks old were supplied by the laboratory animal centre of JSS College of Pharmacy, Ooty. The animals were used following the guidelines of institutional animal ethics committee (IAEC). The animals were acclimatized at the temperature of 25 ± 2 °C and relative humidity of 60 ± 5% under natural light/dark condition for at least 24 h before dosing (i.v. injection); the animals had free access to food and water.

Thirty six animals were divided into six individual groups, each containing six animals to investigate the pharmacokinetic parameters of the formulations after single i.v. injection via tail vein. The dose per kg of animal was calculated based on the ratio of body surface area with comparison to human from the given formula [243,244]. The various pharmacokinetic parameters were determined on application of plasma drug concentration to database WinNonlin version 5. To determine the blood pharmacokinetic parameters for the batches PMF, A3, G3, DMF, A4 and G4 the blood sample (0.4 ml) was withdrawn into vials pre-treated with sodium EDTA. The blood was withdrawn from the eye of the rat anaesthetized

by diethyl ether at regular interval of time from 0.5 to 24 h (Table 12 and 13). The plasma was separated by centrifugation and stored at -20 °C till analysis.

$$\text{Animal dose (mg/kg)} = \frac{\text{Human effective dose (mg/m}^2\text{)}}{37\,[\text{Animal Wt (kg)/Human Wt (kg)}]^{0.33}}$$

12. B. Biodistribution studies:

Thirty six female Sprague-Dawley rats (body weight: 220±20gms) were employed for the study. Tumour was induced by i.p. administration of N-nitroso-N-methyl urea (NMU) dissolved in 0.85% w/v NaCl and acidified at pH 5 (acetic acid) at dose of 50 mg/kg. Animals were examined for palpation of tumour mass regularly thrice a week and tumour volume was measured with vernier calliper. The tumour volume was calculated from the equation [113].

Tumour Volume = (Length x Width^2) / 2.

At tumour volume of 100 mm^3 the animals were grouped and treated at similar manner as mentioned in pharmacokinetics. At the interval of 1 and 2 h, three animals of each group were sacrificed by deep ether anaesthesia and different organs like heart, kidney, lungs, brain, liver and tumor was isolated, washed with physiological solution and blotted on filter paper. The samples were frozen at -20°C till analysis. The 100 ± 5 mg of organ was homogenised with 5ml of ACN, centrifuged and subjected to HPLC analysis [156,245].

13. Statistical analysis:

The statistical significance was studied using GraphPad Prism 5 has statistical tool. ANOVA (one way and two way) and student-t test was applied as the test of tool. The differences between the groups analyzed were considered to be significant at a level of $p<0.05$ (95% confidence interval). Based on the threshold significance level, it is considered as extremely significant for $p<0.001$, very significant for p value 0.001 to 0.01. The p value above 0.05 is termed as non significant [246,247].

Results

RESULTS

The section provides the practical conditions and the outcome of the experimental procedures applied or studied.

Development of an Analytical and Bio-analytical method - by HPLC.

As the molecular weight of the paclitaxel (PTX) and docetaxel (DTX) is less than 2000, reverse phase mode is used for the analysis with C18 as the column [248]. The HPLC instrumental conditions like stationary phase, mobile phase, column condition, and mobile phase flow rate etc applied for development of analytical and bio-analytical method are shown in Table 2. The bio-analytical method for PTX and DTX was developed using vice-versa as internal standard (Fig 8). The Fig 9 and 10 provides the chromatogram view of PTX and DTX at analytical method conditions. The Fig 11 and 12 represents the blank samples at bio-analytical and analytical conditions. The Fig 13 records the PDA spectra indicating the lamda max (λ_{max}) at which system records the maximum peak intensity for drug.

Table 2: HPLC instrumental conditions and parameters for Analytical and Bio-analytical method for PTX and DTX.

Parameters	Analytical method	Bio-analytical method
Mode	Reverse phase	Reverse phase
Column	C18 (Purospher star)	C18 (Purospher star)
Column-Physical condition	Pore size 5µm, dimension 250x4.6mm, temperature 20°C	Pore size 5µm, dimension 250x4.6mm, temperature 20°C
Mobile phase	ACN:0.1% TEA pH 3.5 (70:30 v/v)	ACN:0.1% TEA pH 3.5 (54:46 v/v)
Flow rate	1ml/min	1.4ml/min
Internal standard	Nil	DTX for PTX analysis & vice-versa.
λ_{max}	227nm	227nm
Injection volume	20µl	20µl
Programme	Linear gradient elution	Linear gradient elution
Recovery studies	Nil	Protein precipitation technique
Retention	5.4min for DTX and 5.8min for PTX	8.3min for DTX and 9.9min for PTX
Detector	PDA with manual injector	PDA with manual injector

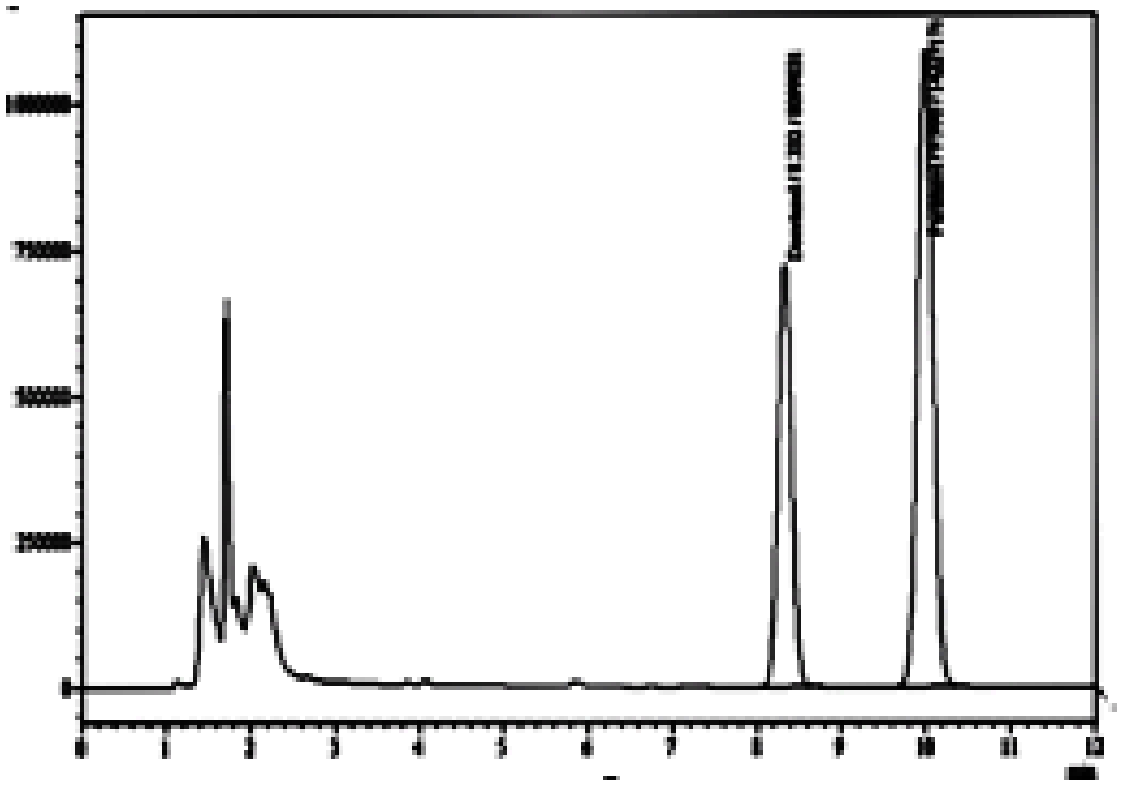


Fig 8: Bio-analytical HPLC spectrum of PTX and DTX in rat plasma.

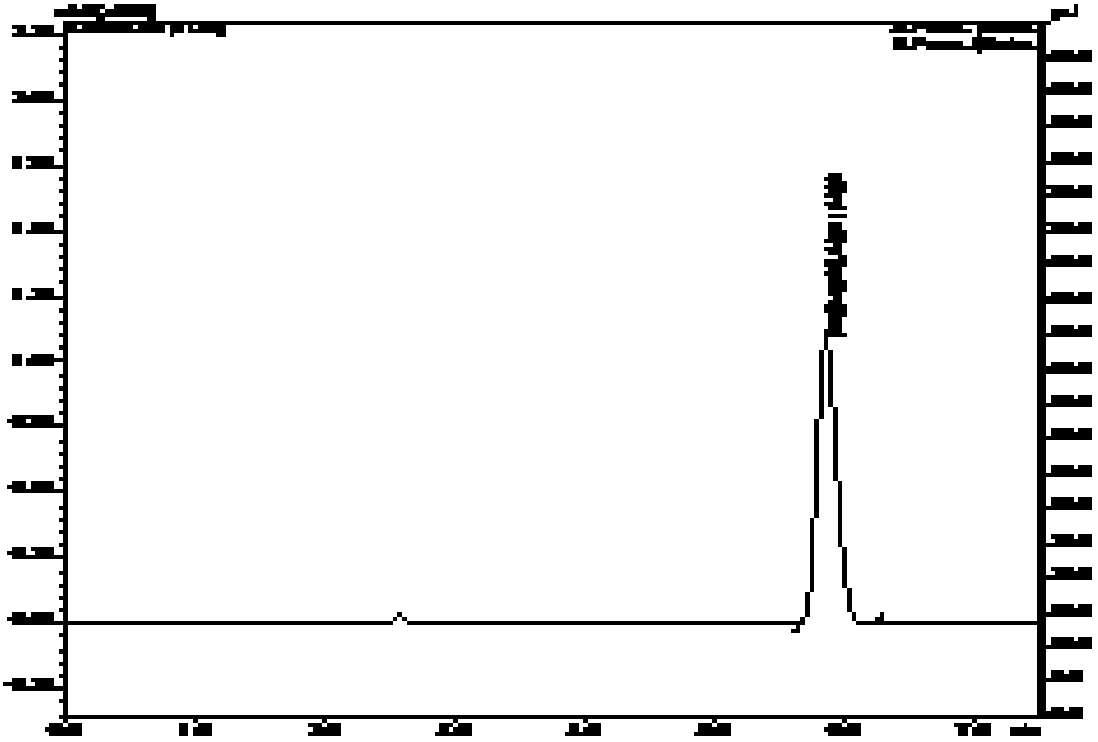


Fig 9: HPLC spectrum of PTX at specified analytical conditions.

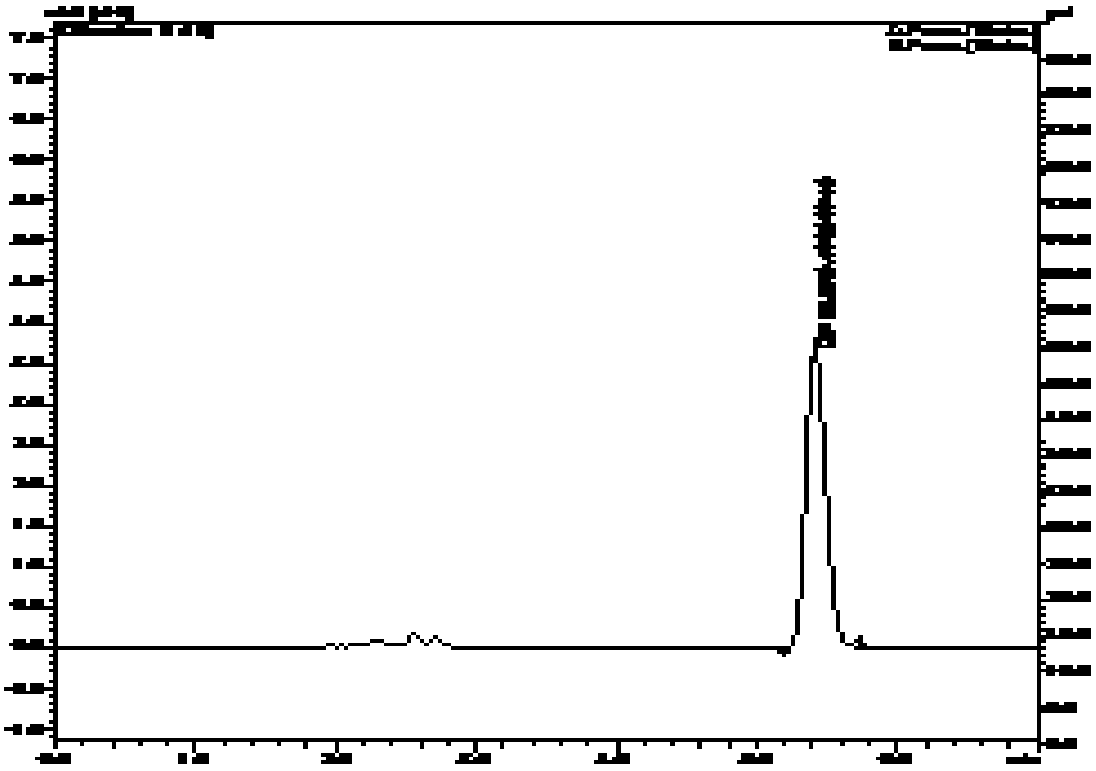


Fig 10: HPLC spectrum of DTX at specified analytical conditions.

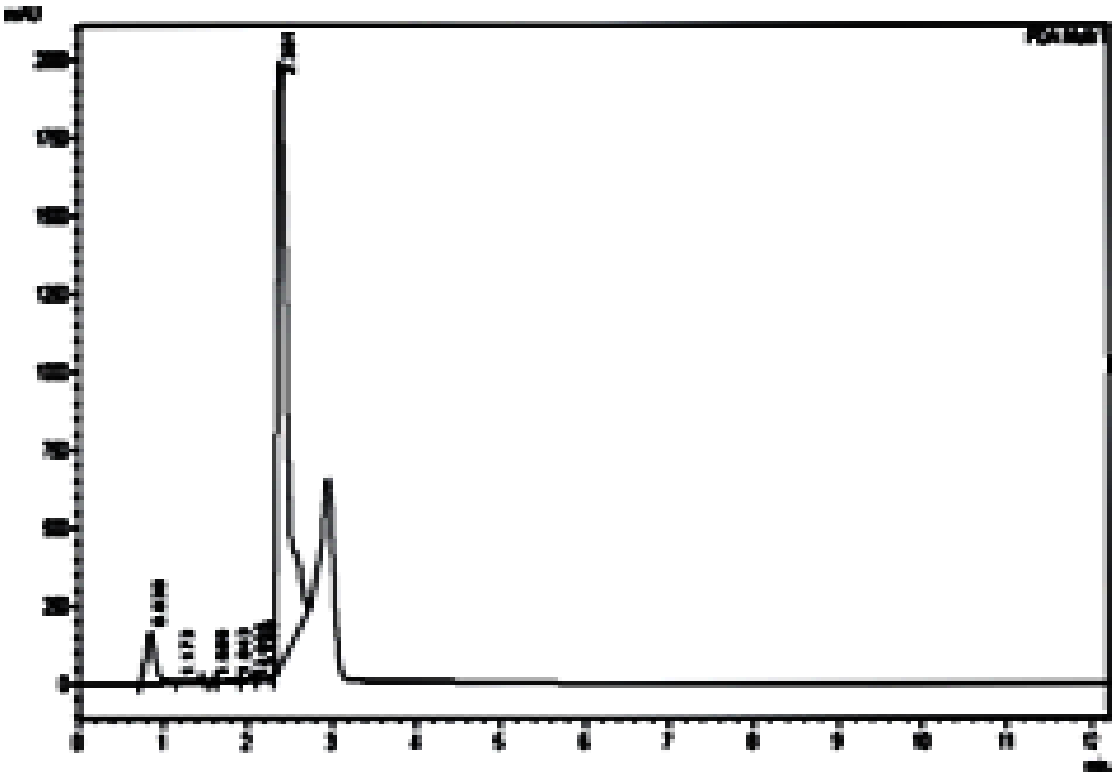


Fig 11: HPLC spectrum of blank plasma at specified bio-analytical conditions.

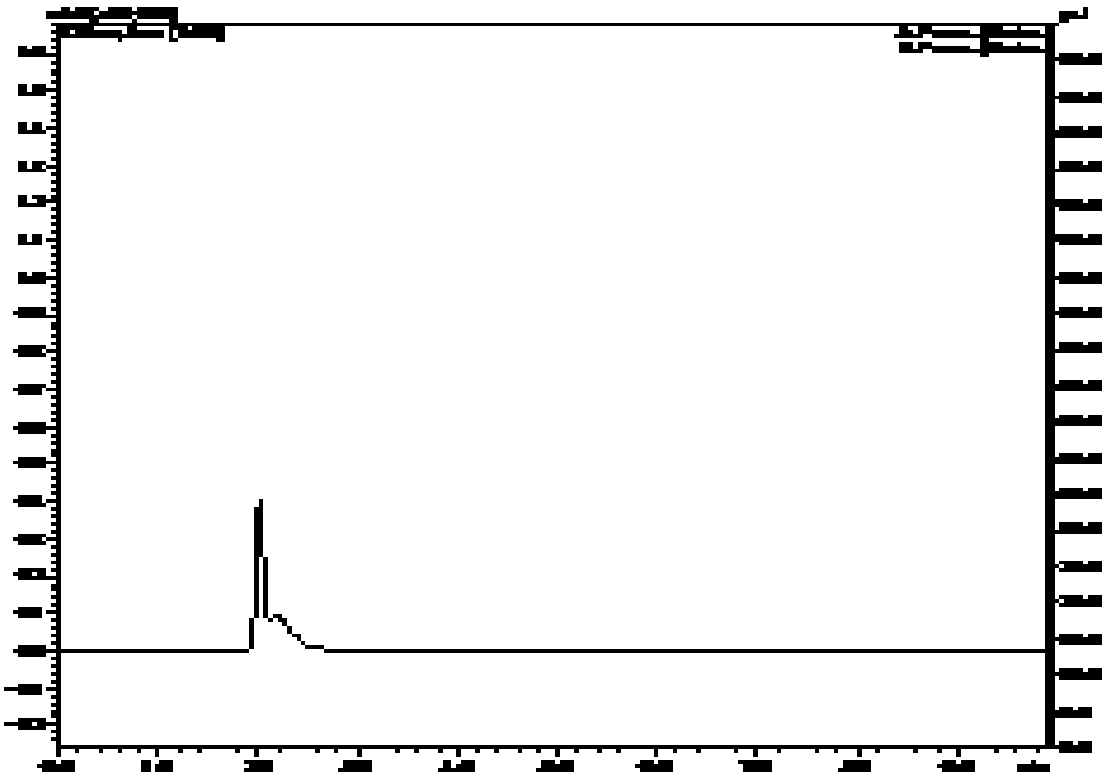


Fig 12: HPLC spectrum of blank at specified analytical conditions.

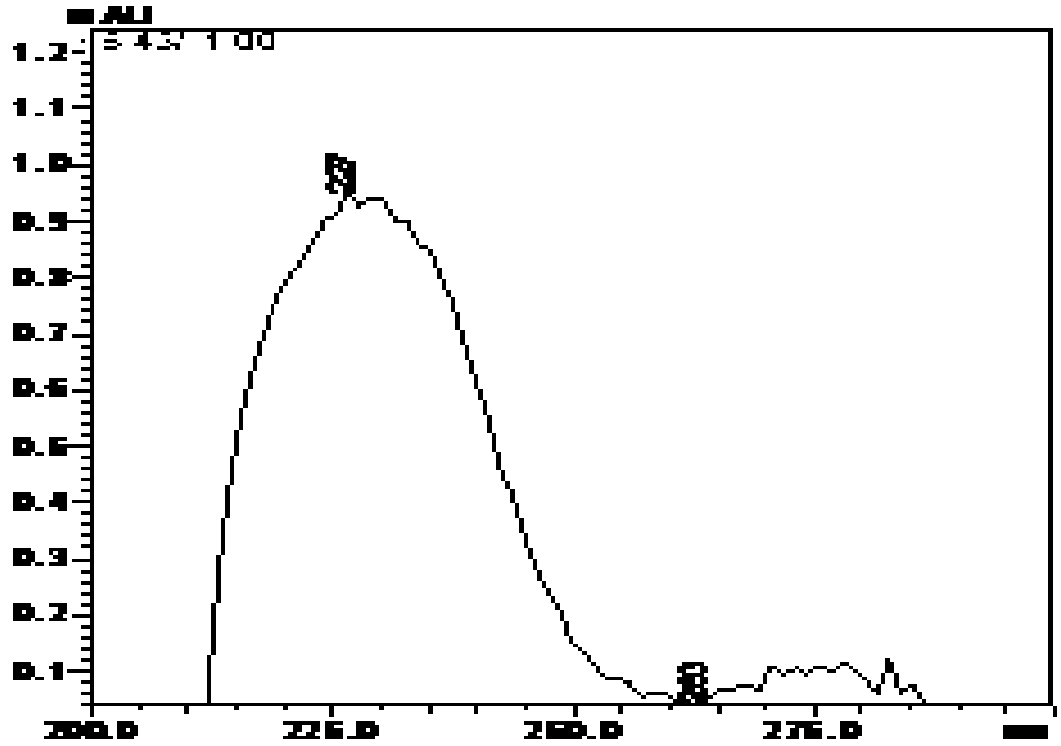


Fig 13: PDA spectrum of drug (PTX or DTX) – registered λ_{max} (227nm).

Curve of Linearity:

The analytical curve of linearity was plotted by taking the AUC of the aliquot dilutions of PTX and DTX and for that of bio-analytical the response factor is taken into consideration. The aliquot dilutions for the PTX ranged from 200 to 0.002µg/ml and for DTX it ranged from 1000 to 0.01µg/ml. The Fig 14 to 17 provides the curve of linearity (analytical and bio-analytical). The response factor was calculated by taking the ratio of AUC of sample to the AUC of internal standard. The each point of the curves represents the mean value of three observations.

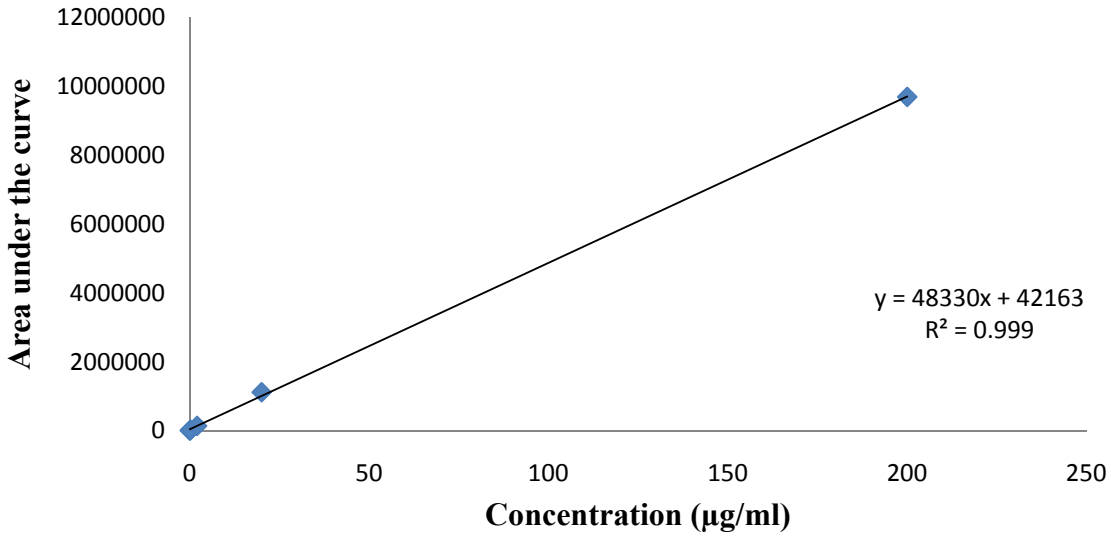


Fig 14: Paclitaxel analytical curve

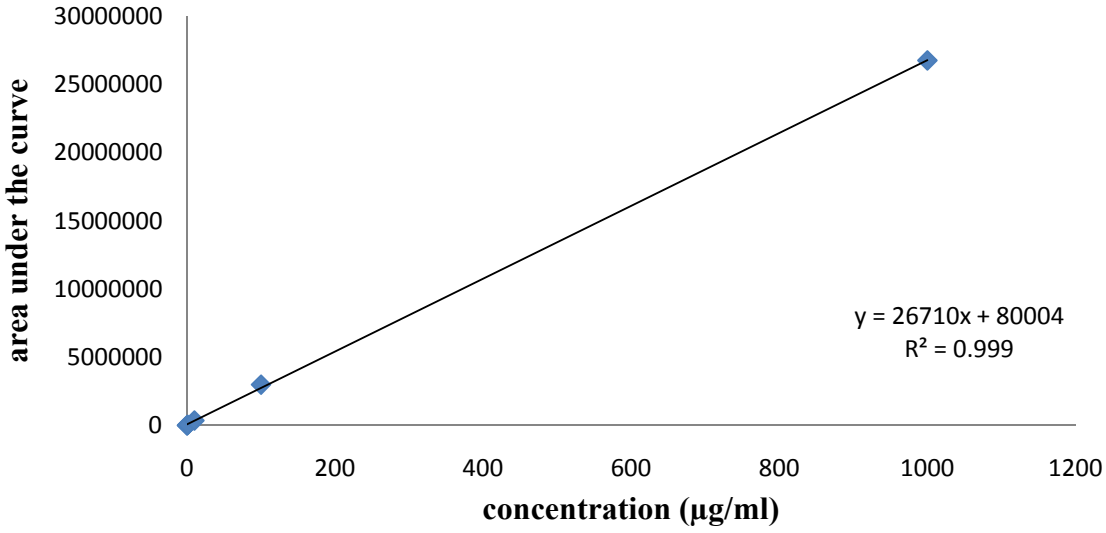


Fig 15: Docetaxel analytical curve

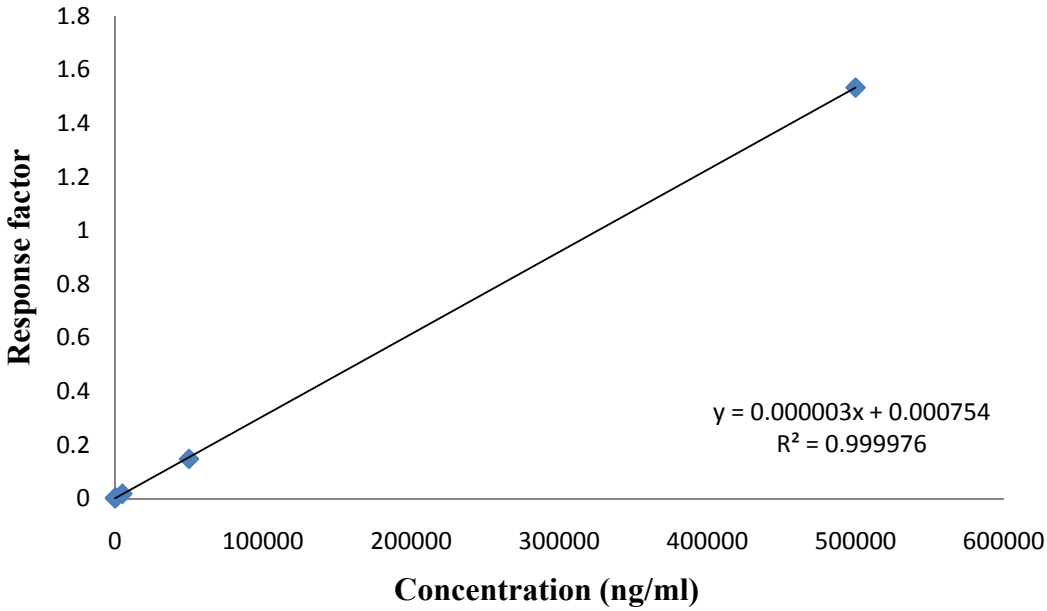


Fig 16: Bio-analytical curve of Paclitaxel

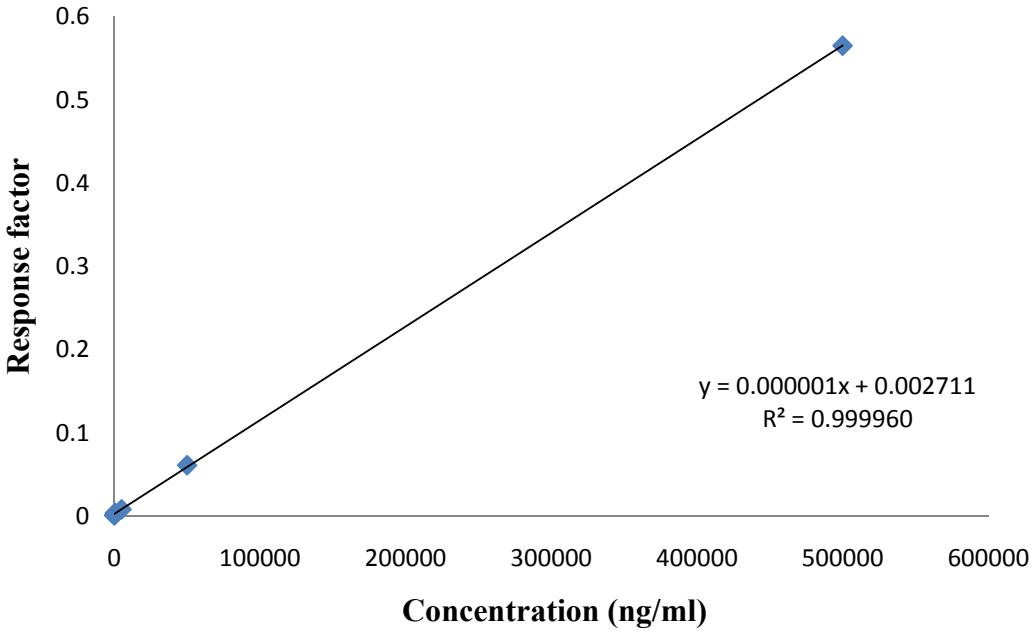


Fig 17: Bio-analytical curve of Docetaxel

Solubility:

The solubility of drug is neither a standard nor test for purity; it just provides the primary information which plays a key role in-and-out of the process of formulation. The statement of solubility is indicated by a descriptive phrase and intended to apply at 25±5 °C [249]. Since the release studies of the formulation are to be carried at body temperature, the solubility of the drug was estimated at 37±2 °C. The Table 3 provides the solubility profile of the PTX and DTX which is diagrammatically represented in Fig 18a and b.

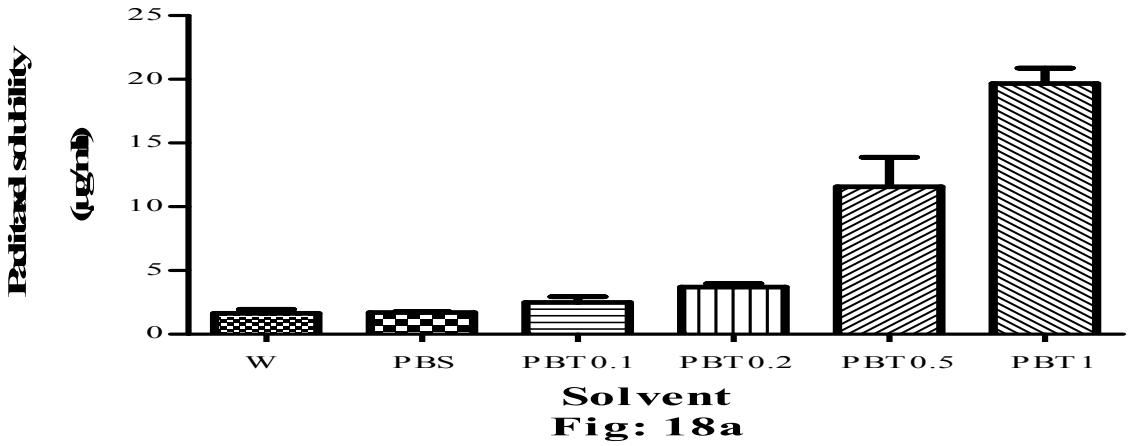


Fig: 18a

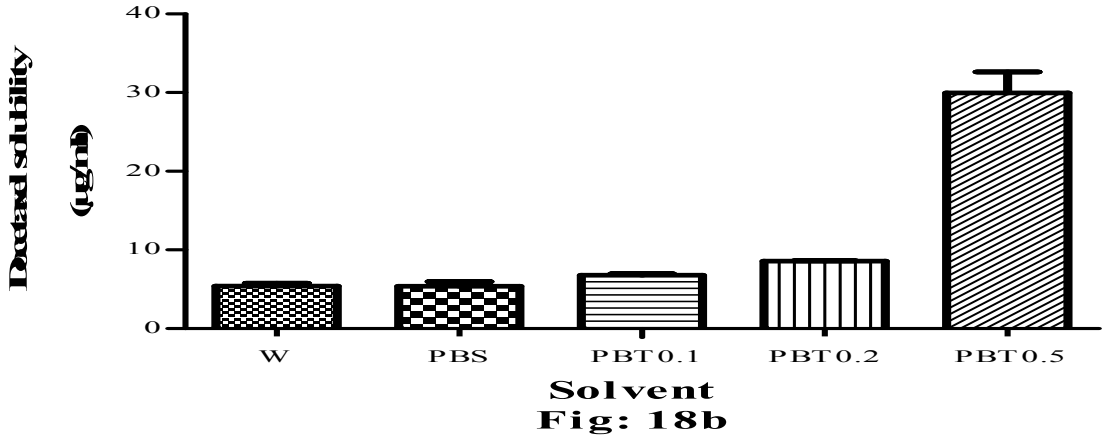


Fig: 18b

Fig 18a and b: Diagrammatic representation of solubility profile of PTX and DTX with bar indicating the SD of three observations. The W represent water, PBS symbolize phosphate buffer saline pH7.4, PBT0.1 to PBT1 stands for phosphate buffer saline with 0.1, 0.2, 0.5 and 1%w/v tween 80 respectively.

Table 3: Solubility profile of PTX and DTX in water and phosphate buffer saline (PBS) pH 7.4 with or without tween 80.

Solute	Solvent system	Concentration µg/ml	Solubility profile
PTX	Water	1.66±0.08	Practically insoluble
	PBS pH 7.4	1.73±0.09	Practically insoluble
	PBS pH7.4 +0.1% w/v tween 80	2.51±0.42	Practically insoluble
	PBS pH7.4 + 0.2% w/v tween 80	3.72±0.6	Practically insoluble
	PBS pH7.4 + 0.5% w/v tween 80	11.62±1.45	Very slightly soluble
	PBS pH7.4 + 1% w/v tween 80	19.77±0.33	Very slightly soluble [250]
DTX	Water	5.44±0.35	Practically insoluble
	PBS pH 7.4	5.39±0.61	Practically insoluble
	PBS pH7.4 +0.1% w/v tween 80	6.80±0.21	Practically insoluble
	PBS pH7.4 + 0.2% w/v tween 80	8.60±0.04	Practically insoluble
	PBS pH7.4 + 0.5% w/v tween 80	30.05±2.67	Very slightly soluble

PTX – Paclitaxel

DTX – Docetaxel

PBS – Phosphate buffer saline

Incompatibility studies by FTIR

The spectra's were recorded at the room temperature to verify the alterations in frequency and intensity of bands of pure drug in presence of polymer of choice (Hyaluronan-Albumin and Hyaluronan-gelatin). The Fig 19 to 24 provides the spectral view of the samples.

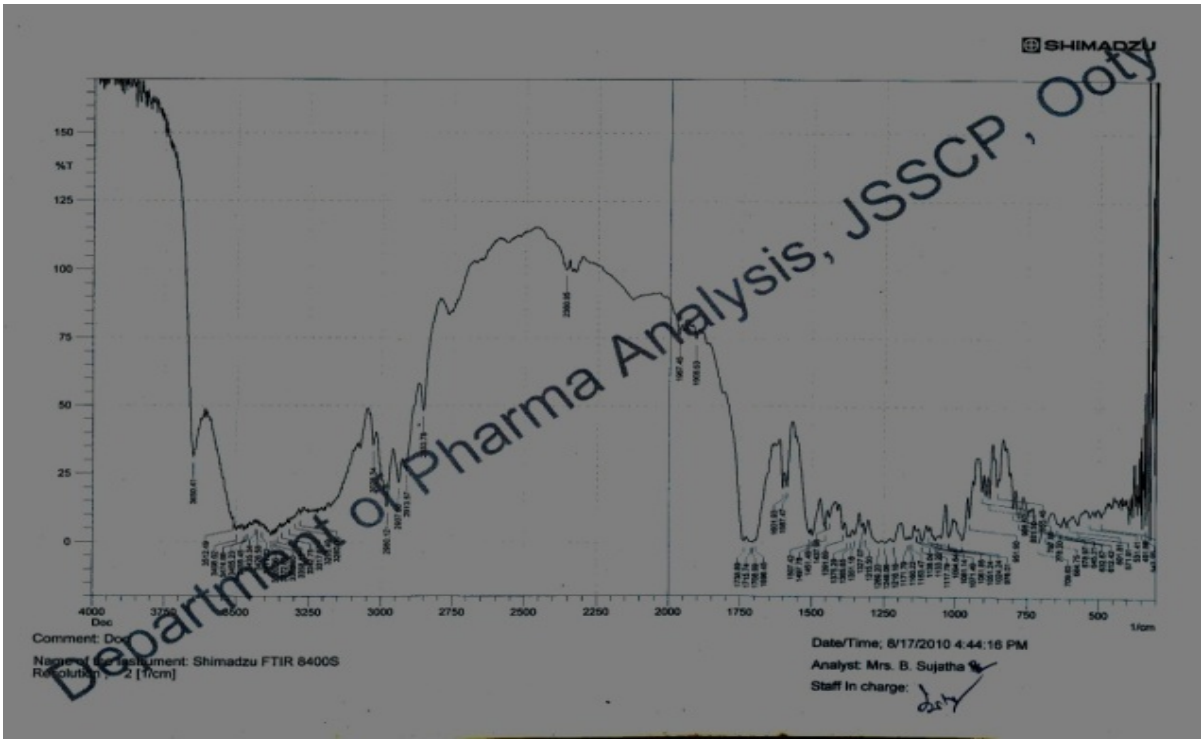


Fig 19: IR spectrum of pure DTX.

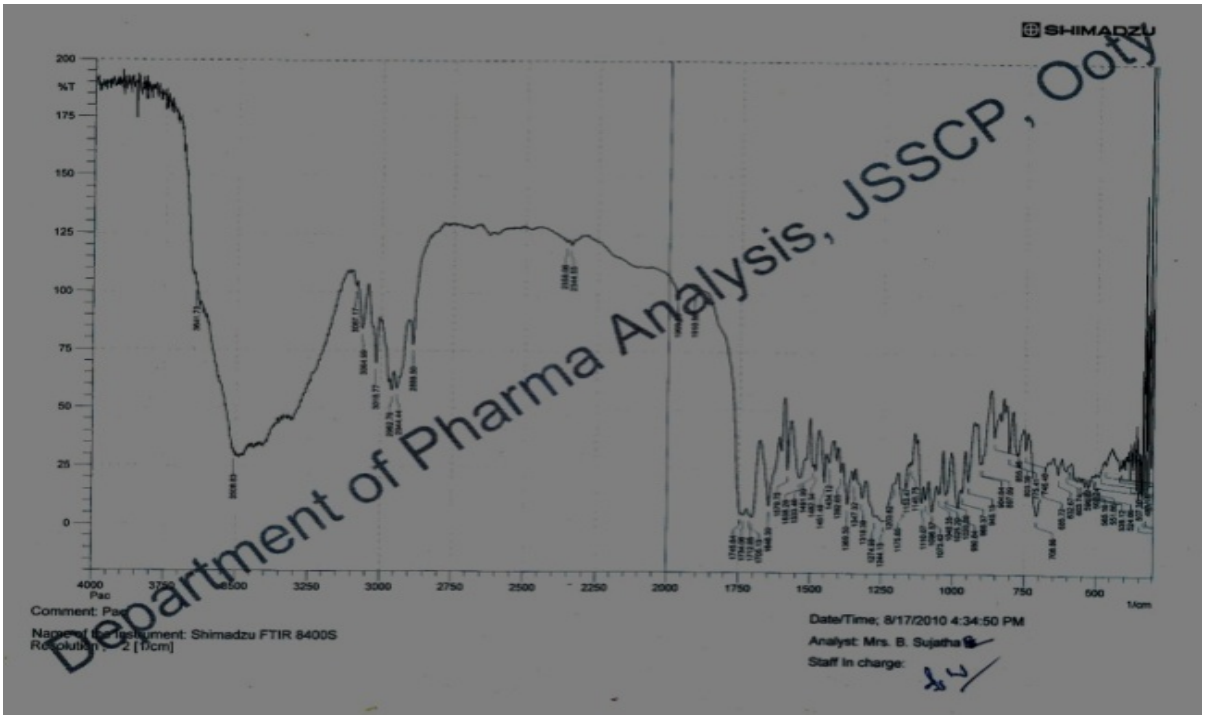


Fig 20: IR spectrum of pure PTX.

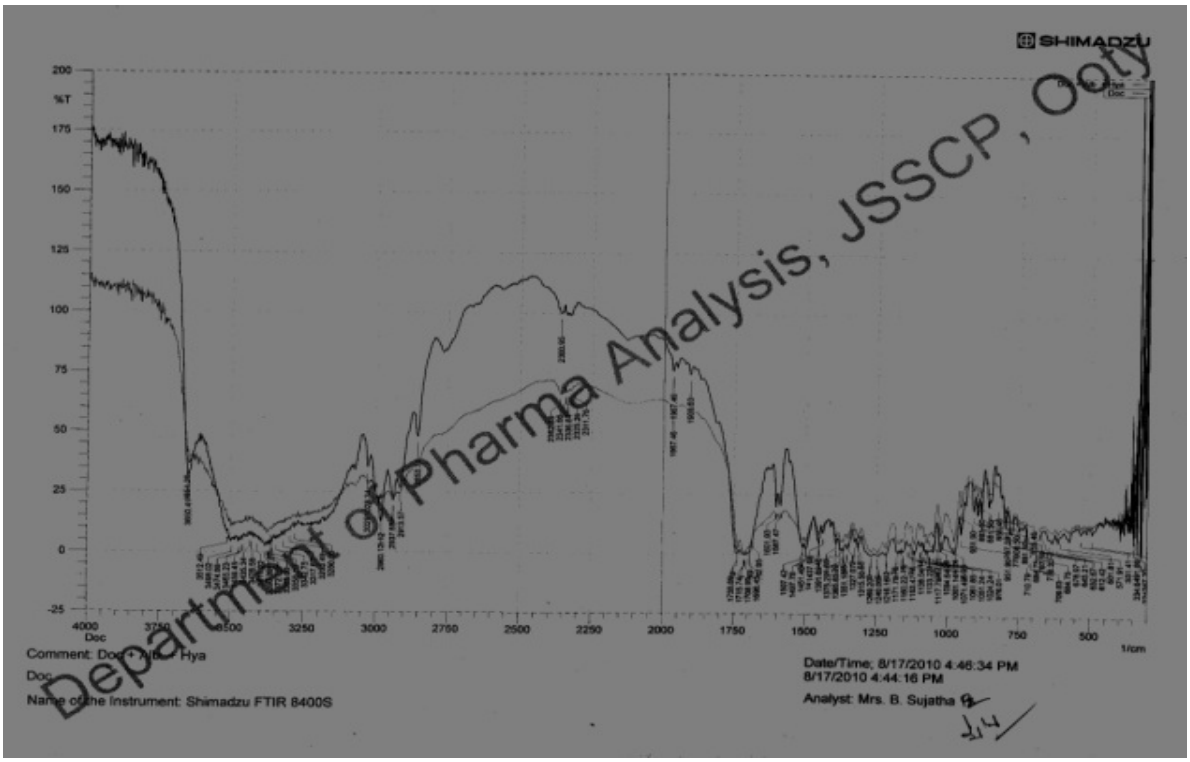


Fig 21: IR spectrum - overlay of pure DTX with its physical blend of HA and albumin.

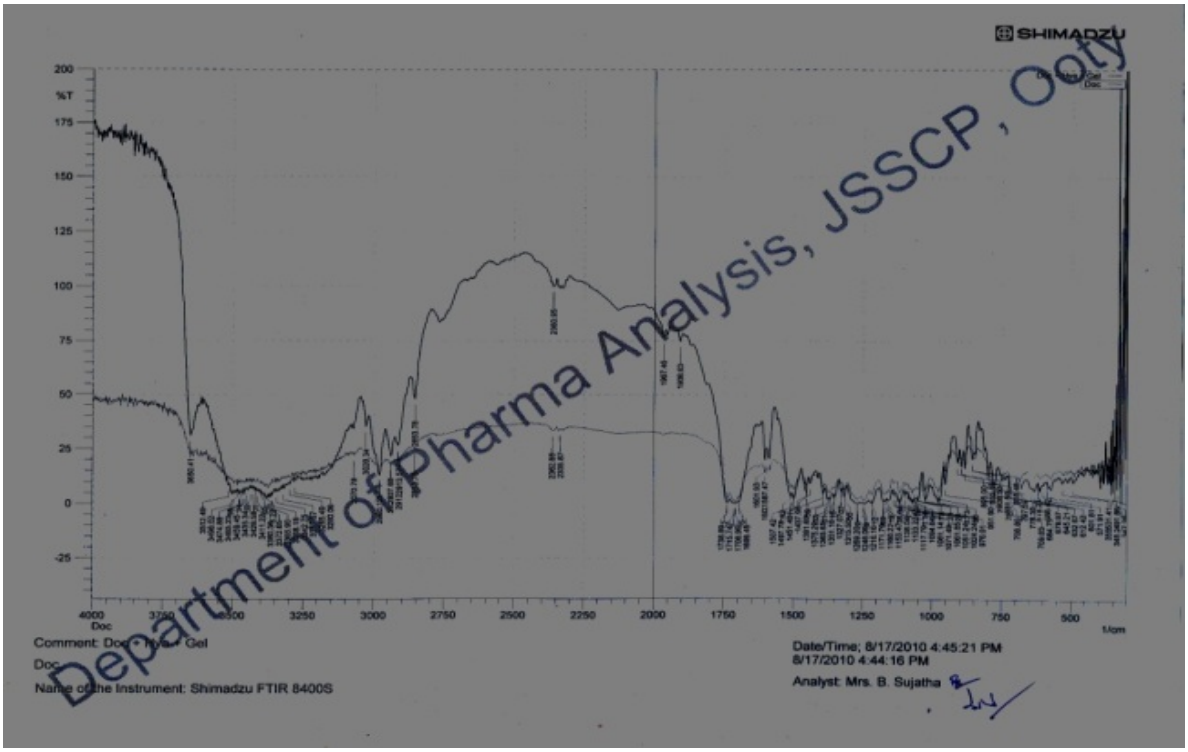


Fig 22: IR spectrum - overlay of pure DTX with its physical blend of HA and gelatin.

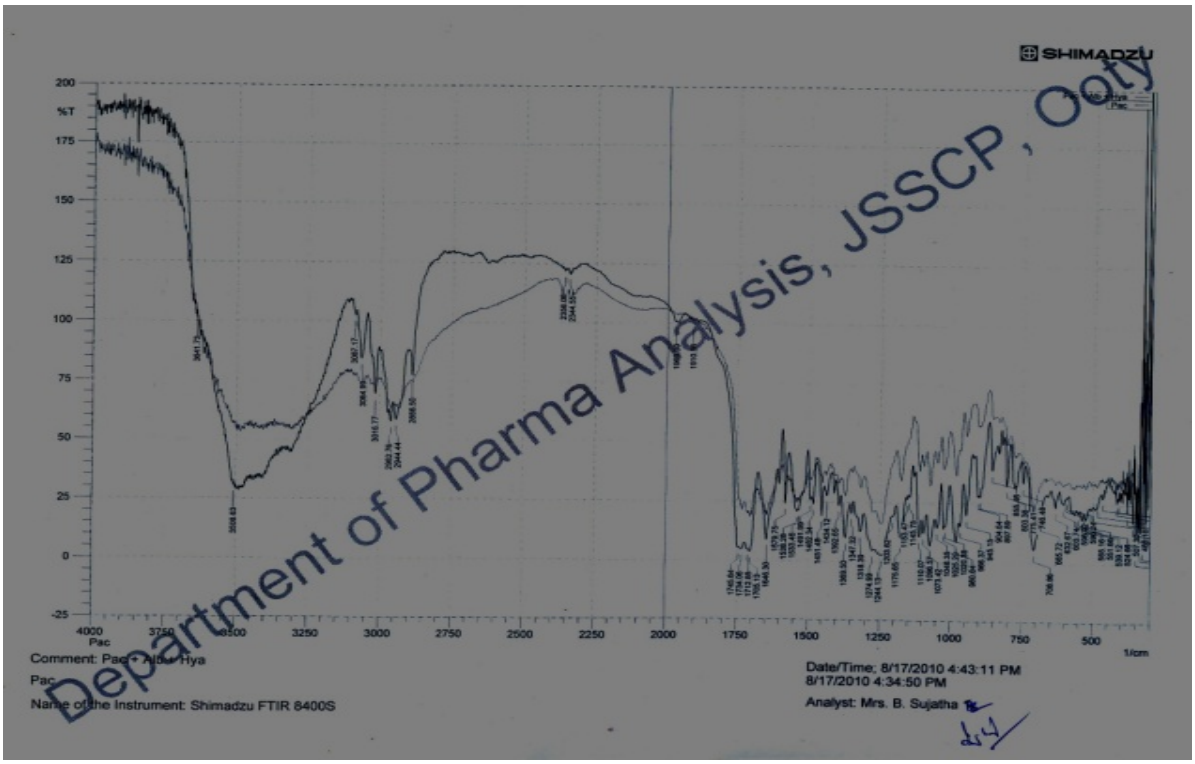


Fig 23: IR spectrum - overlay of pure PTX with its physical blend of HA and albumin

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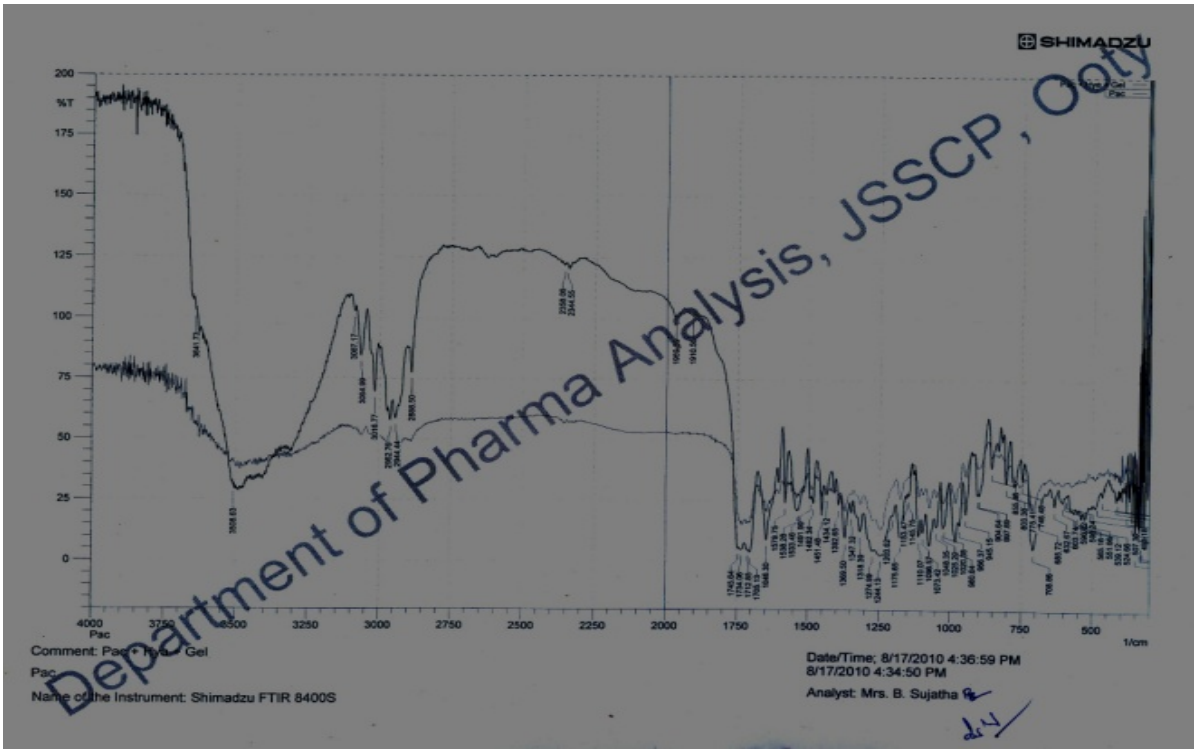

Fig 24: IR spectrum - overlay of pure PTX with its physical blend of HA and gelatin.

The DTX being the analogue of PTX showed almost same FTIR characteristics peaks. The main characteristic peaks recorded for PTX are as follows; the –N-H/O-H stretching was assigned at frequency 3350-3300 cm^{-1}. The CH_3/C-H stretching bands were observed in range 3087-2888 cm^{-1}. The characteristic peak of C=O ester stretching was monitored at frequency array of 1745-1705 cm^{-1}. The band at 1646, 1451 and 1369 cm^{-1} represents C=C stretching/N-H bend/C=O amide stretching, -CH_2 scissoring and C-H rock/CH_3 deformation respectively. The band at 1274 cm^{-1} represents C-N stretch and the absorption peaks in range of 1020-1096 signalize the C-O, -COO stretch. The characteristic peaks of C-H in-plane deformations, =C-H bend/N-H wag/C-C stretching, -C-H out-of-plane/C-C=O deformation were screened at frequency range of 945-803, 708, and 685 cm^{-1} correspondingly [120,251-253].

Incompatibility studies by DSC

DSC is a well-established method of thermal analysis within the pharmaceutical sciences. It is often used as a qualitative technique to characterize physical and chemical events via change in either enthalpy or heat capacity of a sample [254]. The measurement of the enthalpy of fusion is the prime basis of this method. The Fig 25 to 28 represents the DSC thermograms of the drug, polymer and their physical mixture stored at isothermal stress conditions.

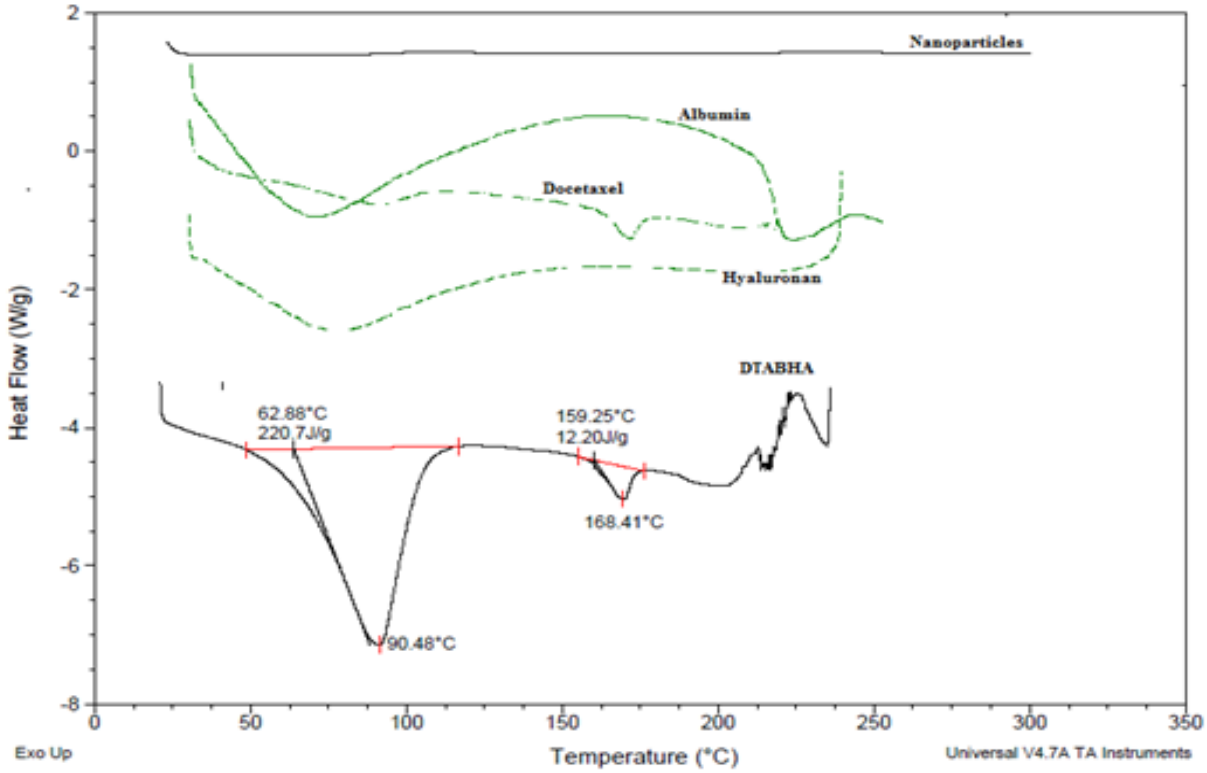

Fig 25: DSC thermal spectra of Hyaluronan-Albumin DTX nanoparticles, pure DTX, albumin, hyaluronan, and DTABHA (physical blend of DTX, albumin and hyaluronan).

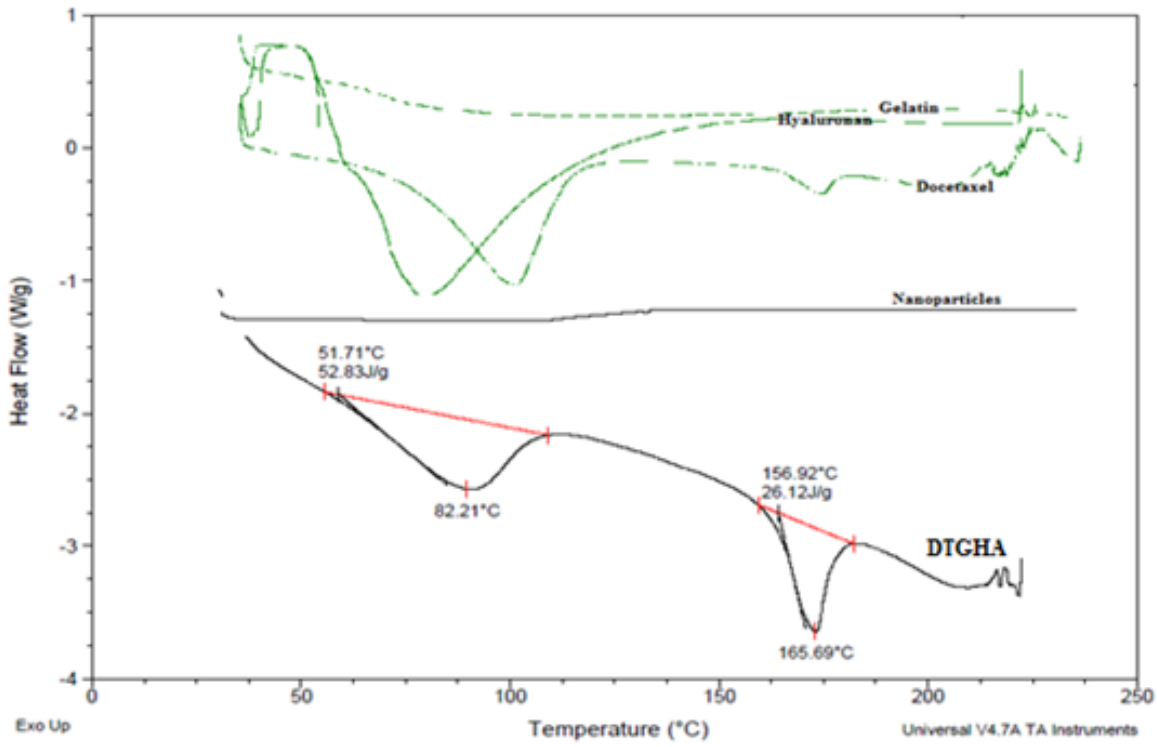

Fig 26: DSC thermal spectra of Hyaluronan-Gelatin DTX nanoparticles, pure DTX, gelatin, hyaluronan, and DTGHA (physical blend of DTX, gelatin and hyaluronan).

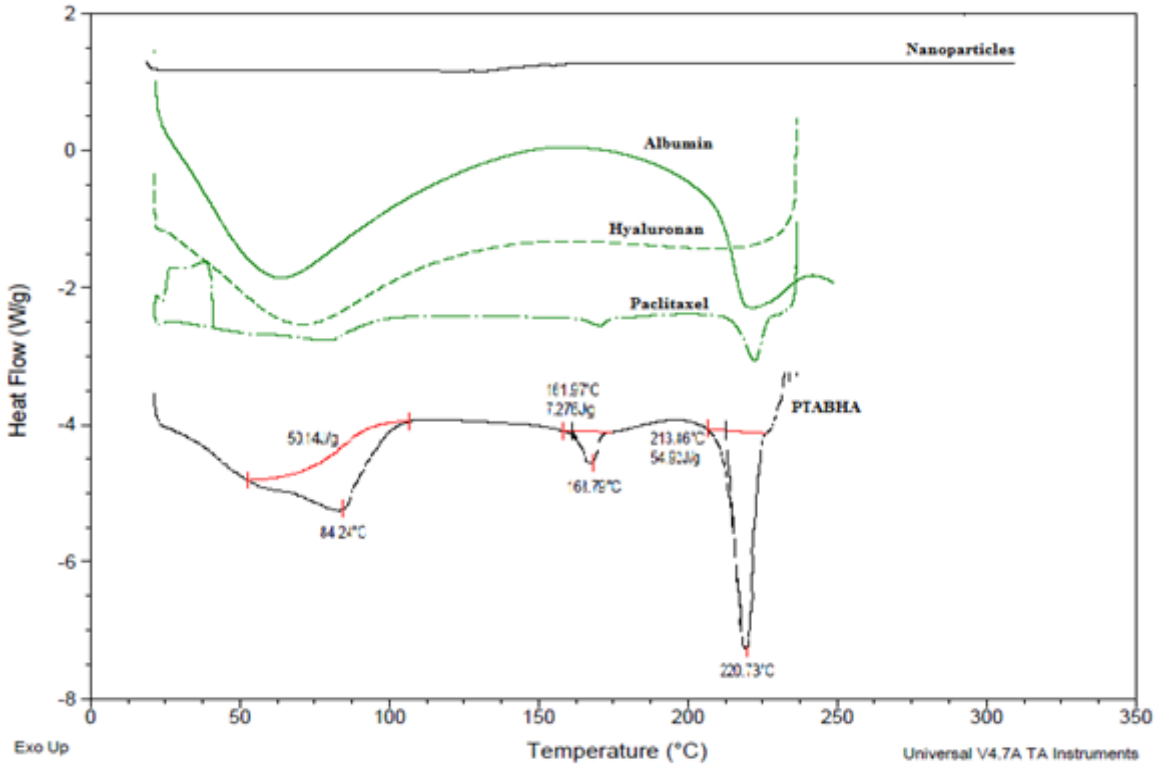

Fig 27: DSC thermal spectra of Hyaluronan-Albumin PTX nanoparticles, pure PTX, albumin, hyaluronan, and PTABHA (physical blend of PTX, albumin and hyaluronan).

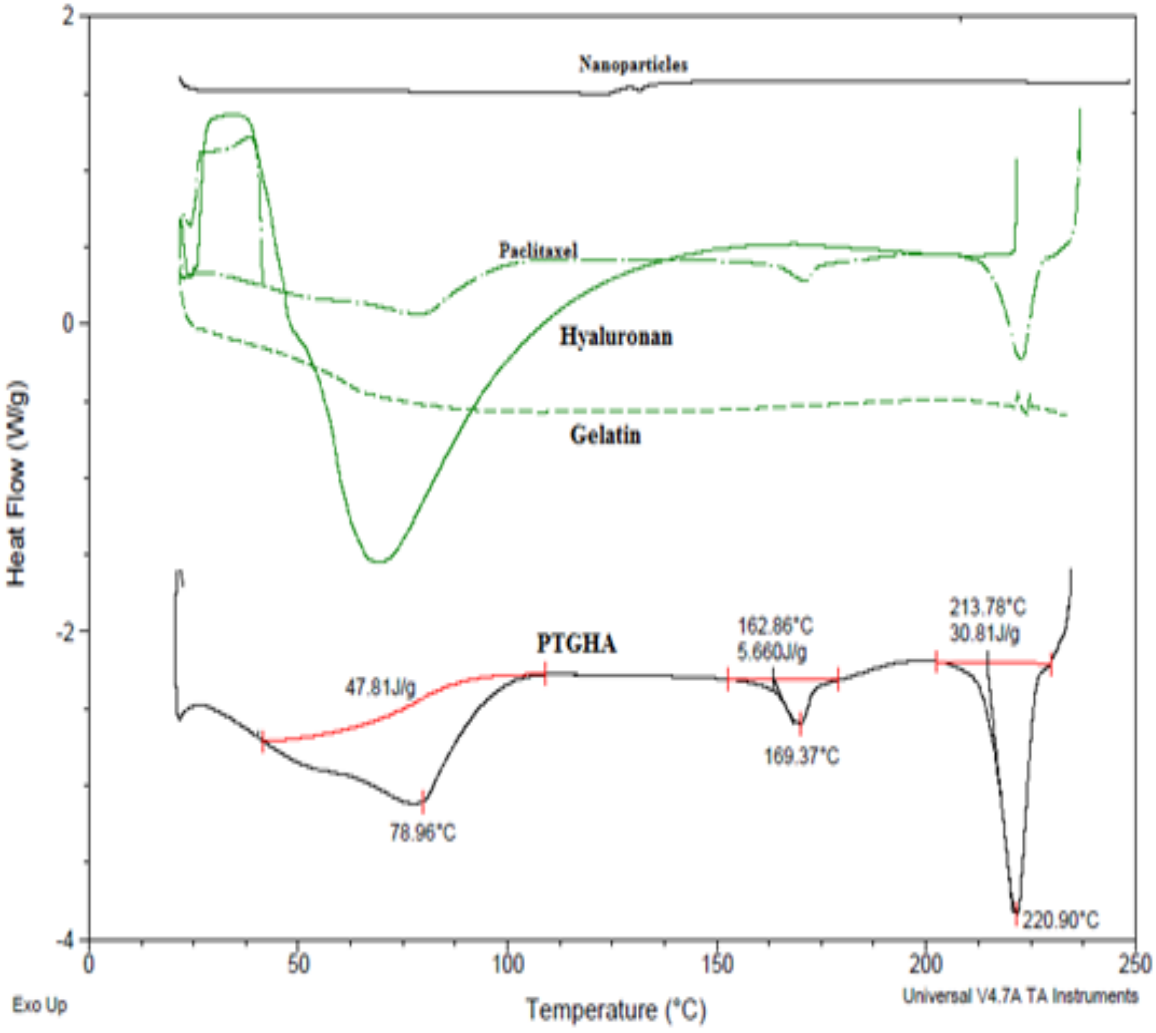

Fig 28: DSC thermal spectra of Hyaluronan-Gelatin PTX nanoparticles, pure PTX, gelatin, hyaluronan, and PTGHA (physical blend of PTX, gelatin and hyaluronan).

Characterization of Nanoparticles:

The exact mechanism beyond the formulation of nanoparticles remains unclear and is hypothesized, may be due to change in pH as the above polymers forms complex at pH 6.8 or interaction of some appropriate counter ions [255,256]. The distance and flow rate of the drug solution added to aqueous polymeric mixture during the process was maintained to minimize the broad deviation in size distribution. To ensure complete removal of organic solvent the formulations were subjected to vacuum evaporation for 1 hr before centrifugation. The formulations were further characterized for process yield, pH, particle size, zeta potential, loading and entrapment efficacy, entrapment pattern, solubility profile, surface morphology, *in vitro* release behavior, *in vitro* cytotoxicity, stability studies, and *in vivo* studies (pharmacokinetics and biodistribution).

Process yield

The process yield for all the batches was calculated using the formula mentioned in methodology. The Table 4 provides the mean % process yield of all the batches with standard deviation (n=3).

Table 4: Percentage process yield of formulated batches. 'A' represents for hyaluronan-albumin nanoparticulate system and 'G' stands for hyaluronan-gelatin nanoparticulate system.

Batches of PTX	Process yield (%)	Batches of DTX	Process yield (%)
A1	78.78 ± 2.8	**A2**	74.51 ± 4.54
A3	77.13 ± 3.76	**A4**	76.68 ± 8.27
A5	74.95 ± 8.80	**A6**	75.32 ± 0.78
G1	82.13 ± 3.41	**G2**	75.55 ± 2.23
G3	70.16 ± 1.61	**G4**	79.85 ± 2.28
G5	76.58 ± 3.36	**G6**	70.82 ± 0.58

pH of the formulations:

The pH for all the batches were recorded as described in methodology. The pH of all the formulations including A5, A6, G5 and G6 was around 6.8 to 7.0. The Table 5 provides the pH of rest of the batches.

Particle size:

The determination of particle size is significant in pharmaceuticals as it is inversely related to the cellular uptake; smaller the sizes better the uptake with higher retention capacity [257]. The Fig 29-36 shows the size distribution of the nanoparticles by intensity and the Table 5 provides the average size of the particles along with polydispersity index.

Table 5: Particle size and polydispersity index, Surface charge, and pH of hyaluronan-albumin PTX (A1 and A3), hyaluronan-gelatin PTX (G1 and G3), hyaluronan-albumin DTX (A2 and A4), and hyaluronan-gelatin DTX (G2 and G4) nanoparticles respectively.

Batches	Mean particle size (nm)	Polydispersity Index	Zetapotential (mV)	pH
A1	25.23	0.353	-17.8	6.8
A2	29.02	0.437	-18.4	6.7
A3	36.11	1	-18.3	6.8
A4	69.28	0.270	-18.6	6.9
G1	23.27	1	-13.7	6.8
G2	40.11	0.524	-12.9	7.0
G3	46.64	0.183	-13.6	7.0
G4	53.81	0.213	-13.7	6.9

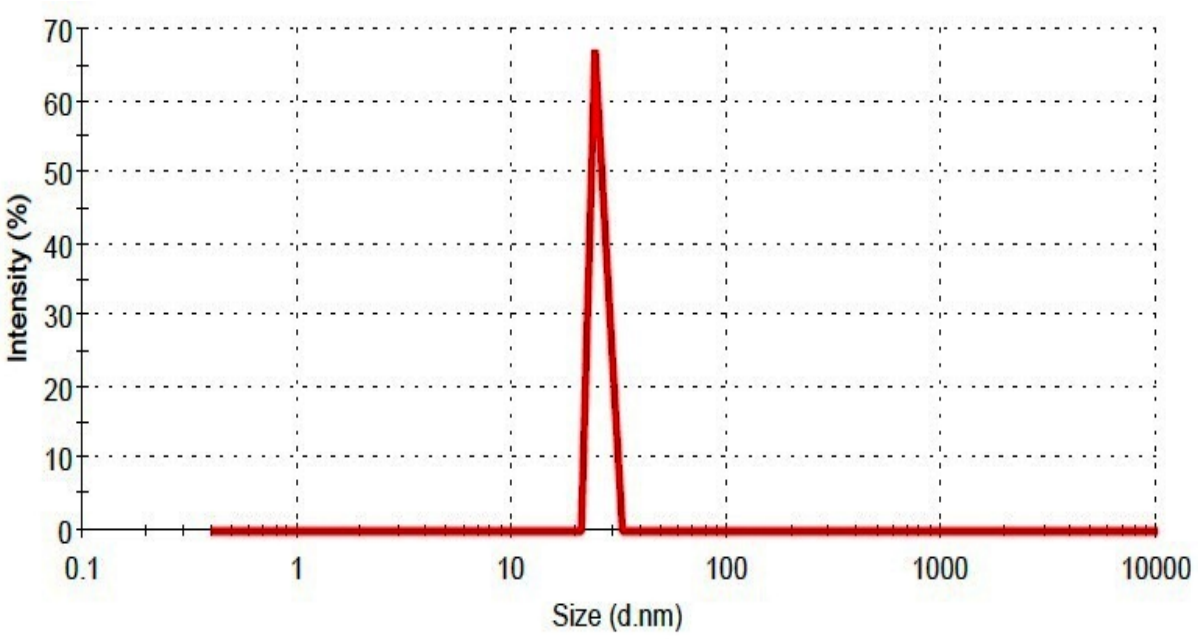

Fig 29: Particle size distribution of hyaluronan-albumin PTX nanoparticles (A1 batch).

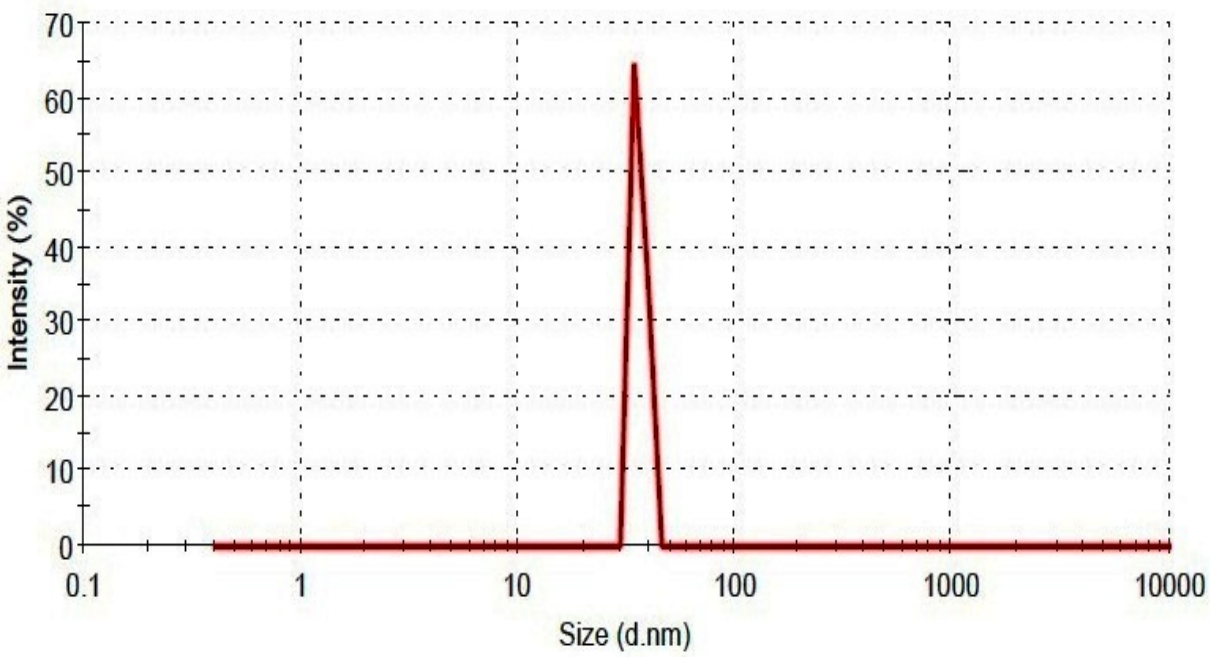

Fig 30: Particle size distribution of hyaluronan-albumin PTX nanoparticles (A3 batch).

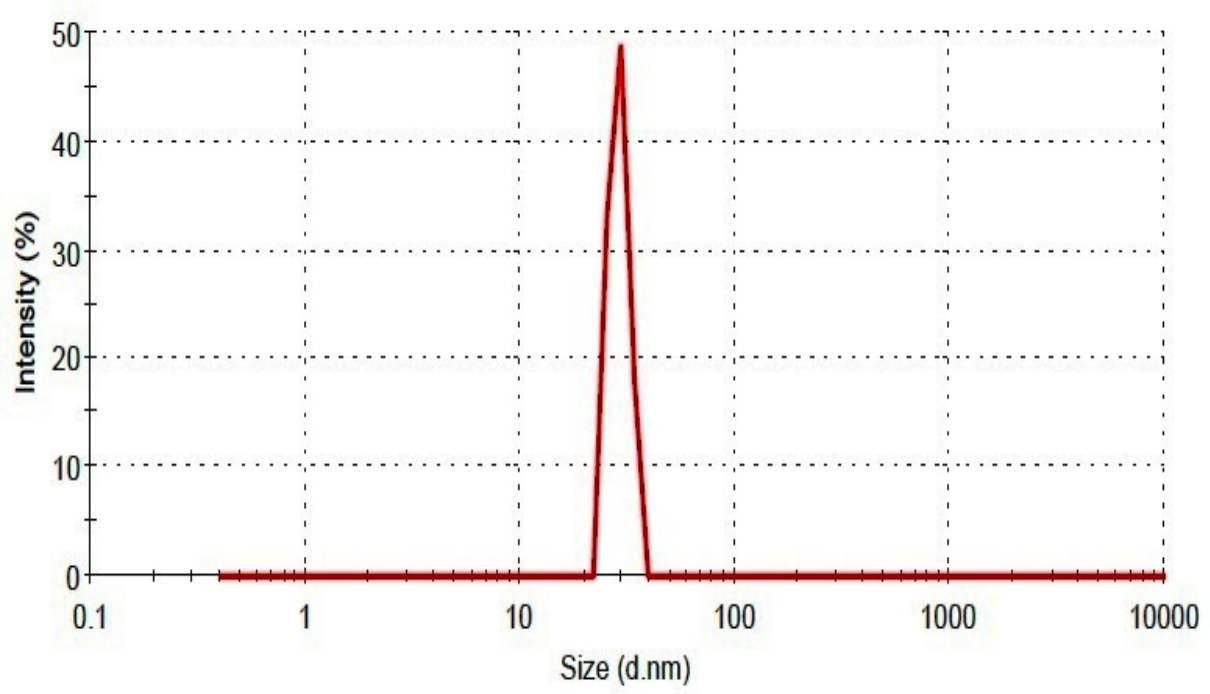

Fig 31: Particle size distribution of hyaluronan-albumin DTX nanoparticles (A2 batch).

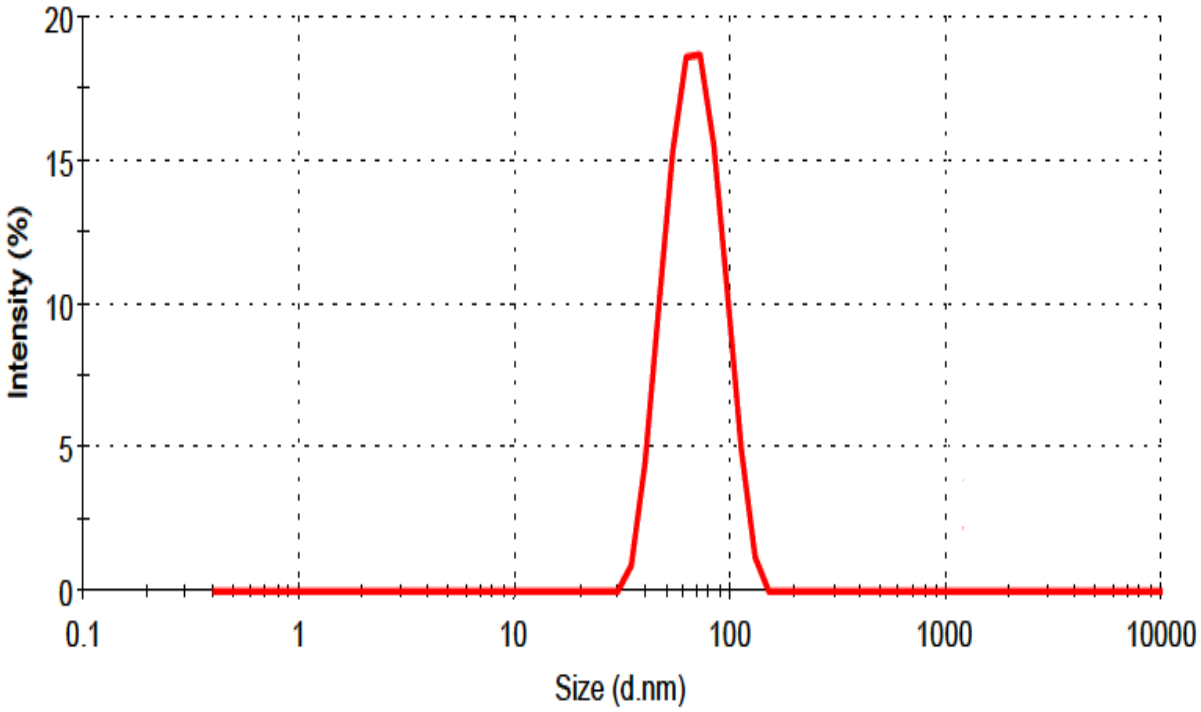

Fig 32: Particle size distribution of hyaluronan-albumin DTX nanoparticles (A4 batch).

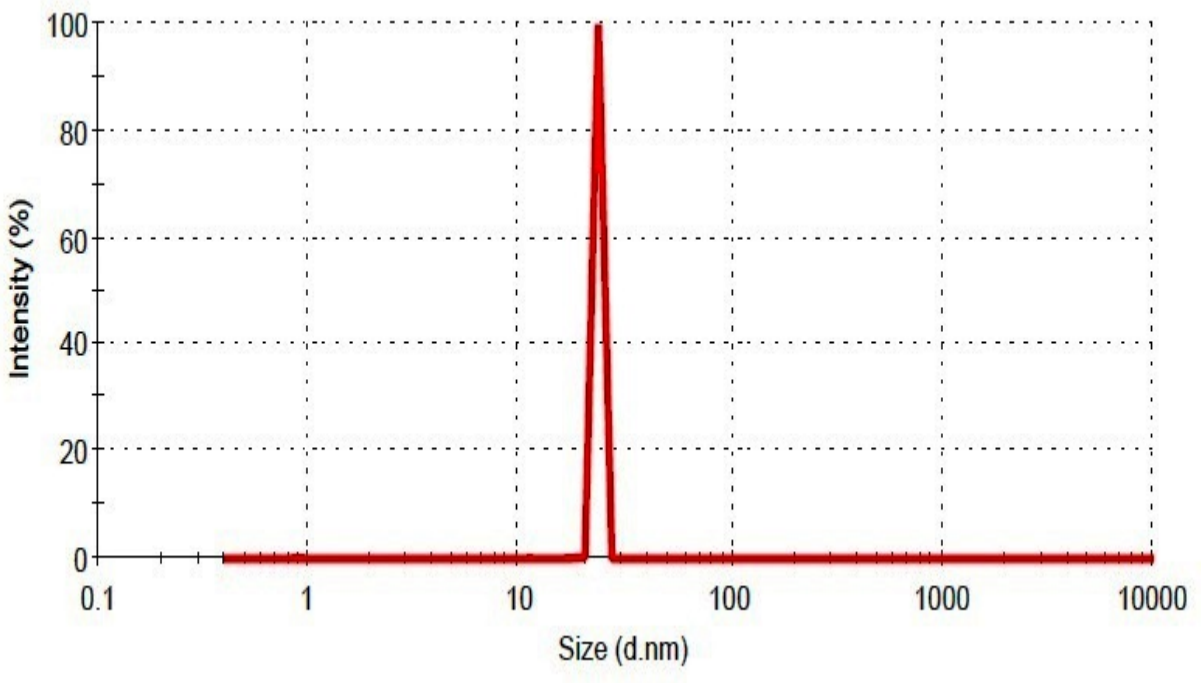

Fig 33: Particle size distribution of hyaluronan-gelatin PTX nanoparticles (G1 batch).

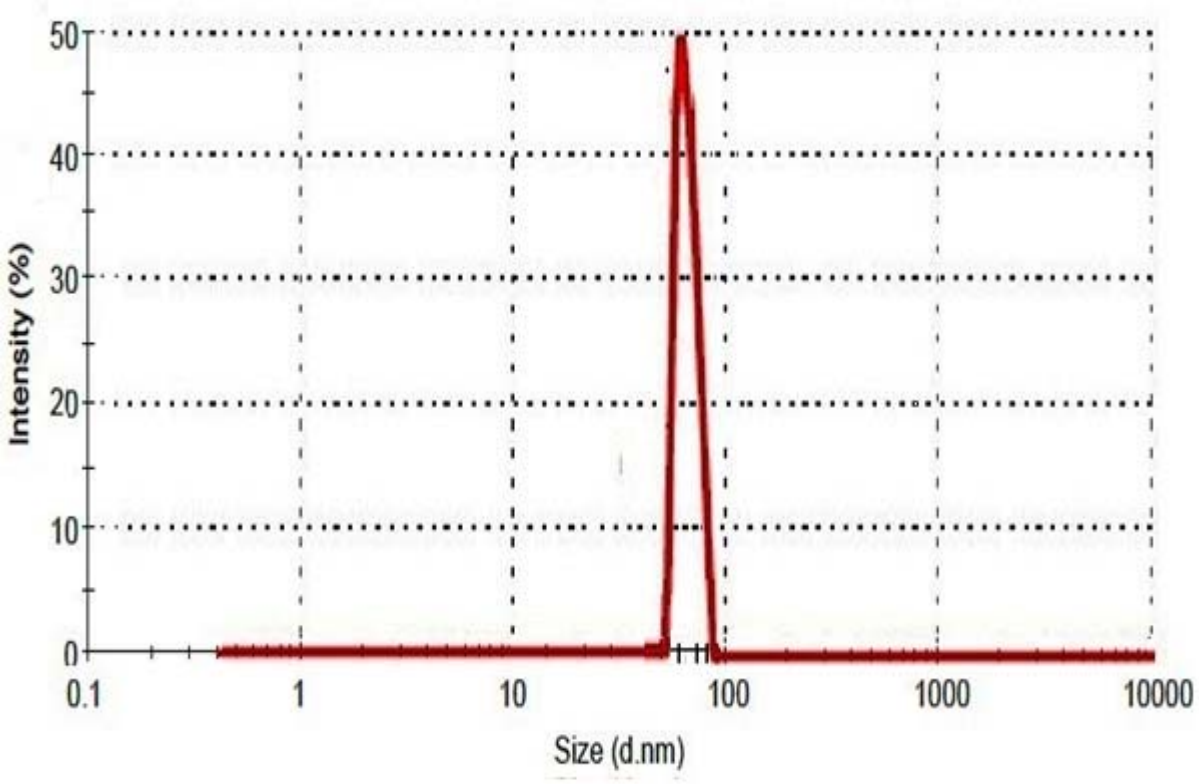

Fig 34: Particle size distribution of hyaluronan-gelatin PTX nanoparticles (G3 batch).

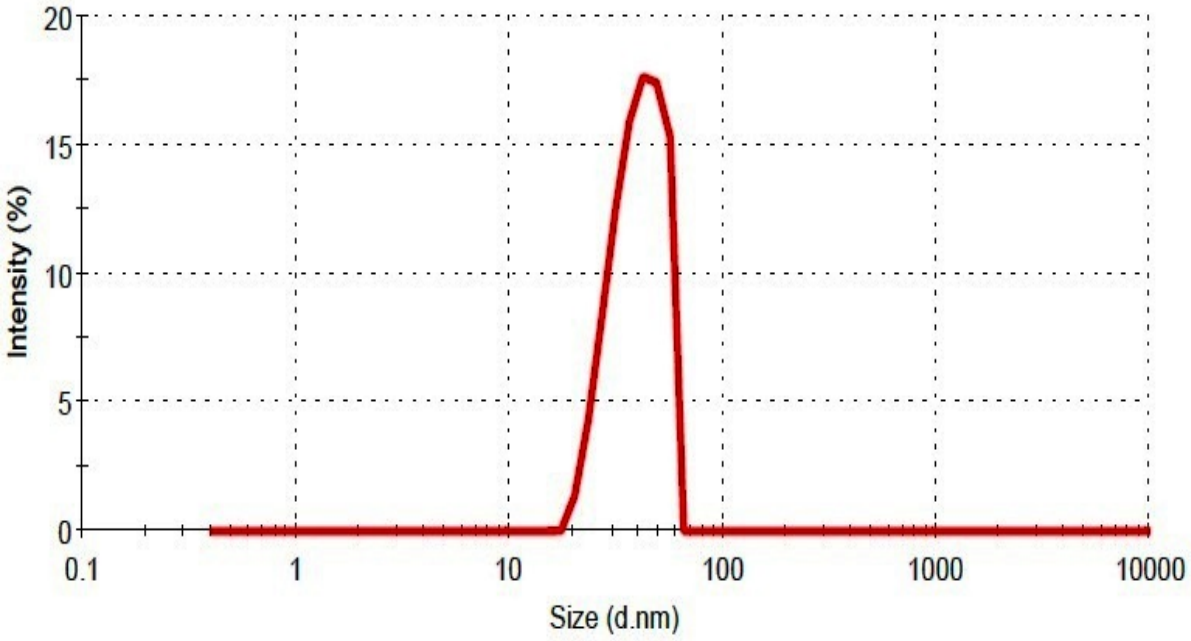

Fig 35: Particle size distribution of hyaluronan-gelatin DTX nanoparticles (G2 batch).

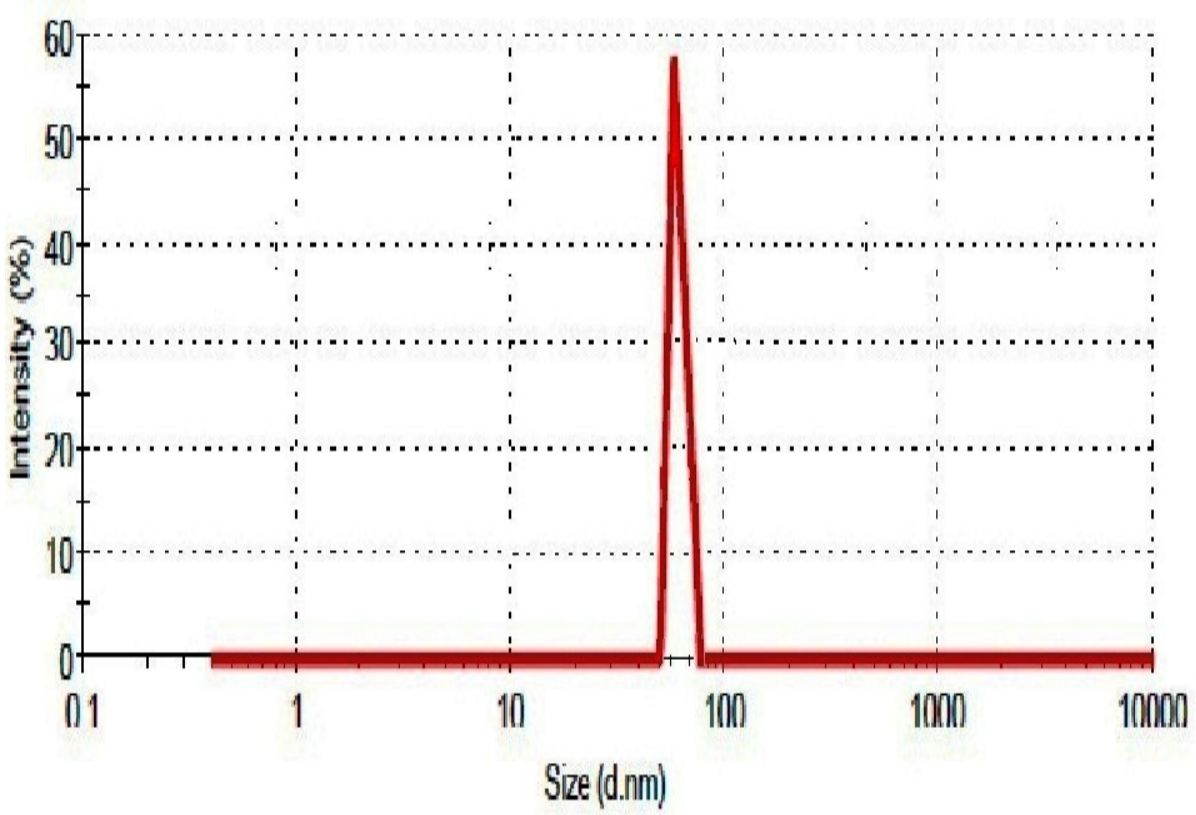

Fig 36: Particle size distribution of hyaluronan-gelatin DTX nanoparticles (G4 batch).

Zetapotential

The charge on the particles is an important parameter in hypothesis of cellular uptake. The electric charge on both HA-albumin and HA-gelatin nanoparticles containing PTX or DTX were found to be negative. An average zeta potential for the particles prepared are shown in Table 5. There was no much variation in the charge among the batches of HA-gelatin, even same was seen in case of nanoparticles containing HA-albumin, which may be because of their constant concentration.

Loading and entrapment efficacy:

The loading and the entrapment efficiency was calculated as mentioned in methodology and the mean value of all the batches with standard deviation (n=3) is given in Table 6.

Table 6: Loading and entrapment efficacy of batches A1 to A4 and G1 to G4.

Batches of PTX	Loading efficacy (%)	Entrapment efficacy (%)	Batches of DTX	Loading efficacy (%)	Entrapment efficacy (%)
A1	20.54±1.28	64.91±4.06	**A2**	18.81±1.08	59.47±3.41
A3	16.55±0.98	88.07±5.25	**A4**	15.10±1.22	80.36±6.52
G1	20.56±1.41	64.04±4.41	**G2**	18.41±1.20	57.35±3.74
G3	16.49±1.15	86.24±6.06	**G4**	15.91±0.40	83.20±2.11

There was no significance difference in neither loading nor entrapment efficacy between the batch A1 & G1 and A3 & G3. But the increase in the polymer concentration decreases loading and increases the entrapment efficacy significantly with p value <0.05 for both HA-albumin and HA-gelatin nanoparticles of PTX. The statistical significance was determined by application of one-way ANOVA. Similar results were observed for the batches of DTX. The Fig 37a and b gives the comparative percentage loading efficacy of various batches whereas Fig 38a and b provides the comparative percentage entrapment efficacy.

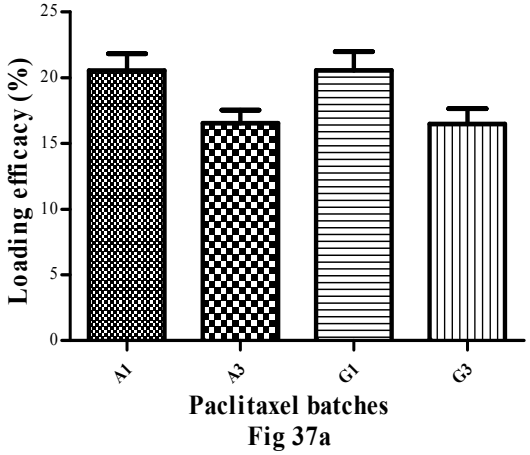

Fig 37a

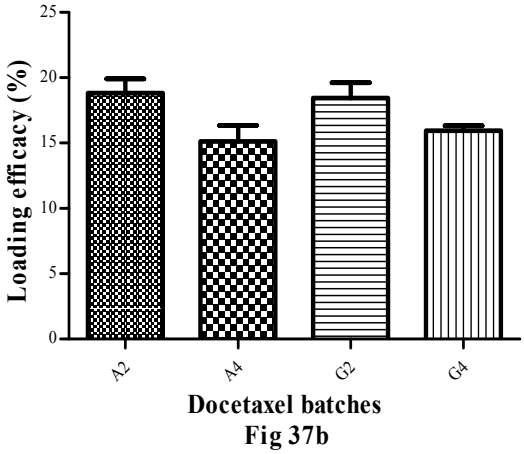

Fig 37b

Fig 37a and b: Diagrammatic representation of loading efficacy of PTX and DTX in HA-albumin and HA-gelatin nanoparticulate batches. The bar represents the SD of three observations.

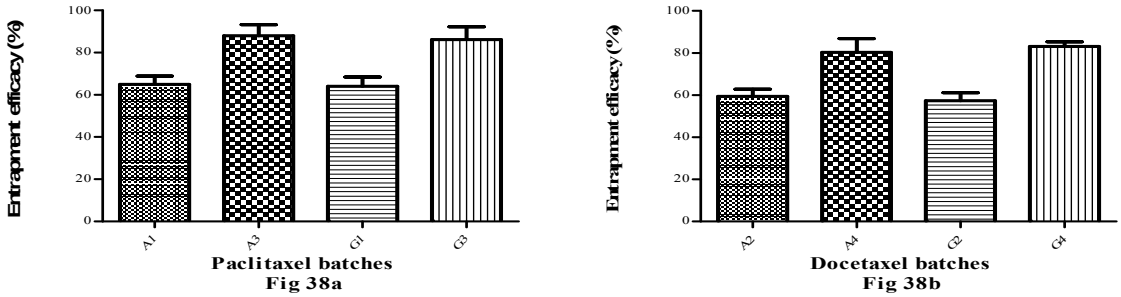

Fig 38a and b: Diagrammatic representation of entrapment efficacy of PTX and DTX HA-albumin and HA-gelatin batches. The bar indicates the SD of three observations.

Study of Crystallanity:

It was essential to determine the nature of crystalline phase in nanoparticles has the orderly and disorderly phase affects the solubility phenomena of the drug. The Fig 25-28 furnishes the DSC thermogram of the HA-albumin DTX, HA-gelatin DTX, HA-albumin PTX, and HA-gelatin PTX nanoparticles respectively. The endothermic peak of drug was absent in all the thermogram.

Solubility profile of Nanoparticles:

The nanoparticles significantly enhanced the solubility of both the drugs. The nanoparticulate systems HA-albumin and HA-gelatin of drug neither PTX nor DTX reported no significant difference in solubility of drug on application of one-way ANOVA. The nanoparticlulate systems enhanced the solubility of drug with p value less than 0.001 on comparison to the pure drug. The Fig 39 provides the diagrammatic representation of solubility of nanoparticles to pure drug. The analysis was performed thrice and the mean value with standard deviation is given in Table 7. The A1, A2, G1 and G2 batches were not considered for the study has they show lower entrapment value for drug on comparison to A3, A4, G3, and G4 batches.

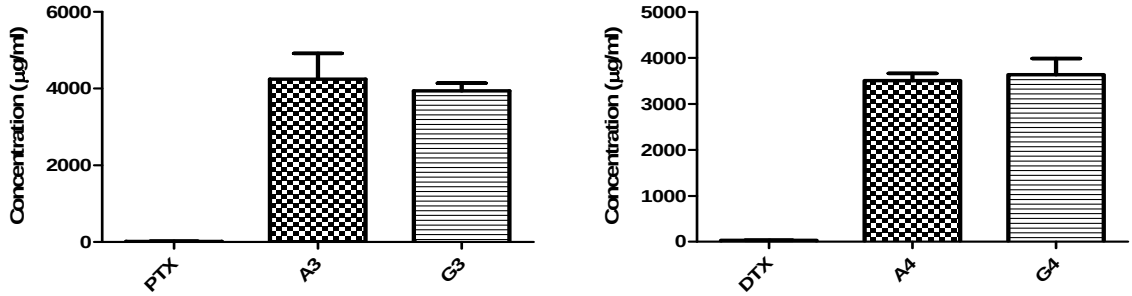

Fig 39: Diagrammatic comparison of solubility profile nanoparticles with their respective pure drug.

Table 7: Solubility profile of nanoparticles of PTX and DTX. All the values represents the mean and SD of three observations.

Solvent system	Solute	Concentration (µg/ml)	Solubility profile
	PTX	19.77 ± 0.33	Very slightly soluble
PBS pH7.4 with	A3	4243.75 ± 673.46	Slightly soluble
1% w/v tween 80	G3	3940.73 ± 201.28	Slightly soluble
	DTX	30.05 ± 2.67	Very slightly soluble
PBS pH7.4 with	A4	3505.15 ± 155.24	Slightly soluble
0.5% w/v tween 80	G4	3630.80 ± 356.60	Slightly soluble

Surface Morphology:

Regardless to the mode of administration the shape of the particles affect the velocity, diffusion and adhesion to walls in blood vessels. The Fig 40-47 illustrate that the particles were almost spherical and smooth textured.

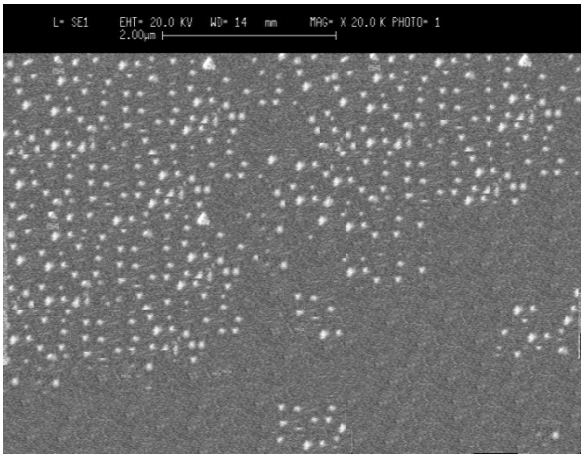

Fig 40: SEM image of A1 batch nanoparticles

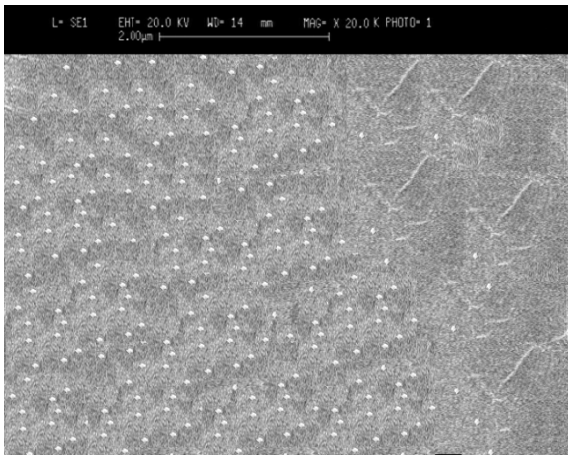

Fig 41: SEM image of A2 batch nanoparticles

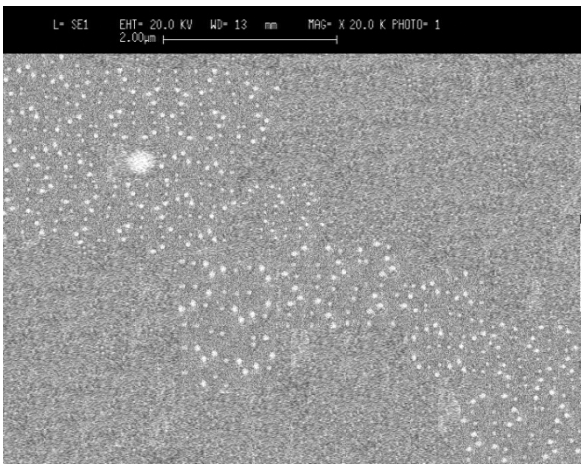

Fig 42: SEM image of A3 batch nanoparticles

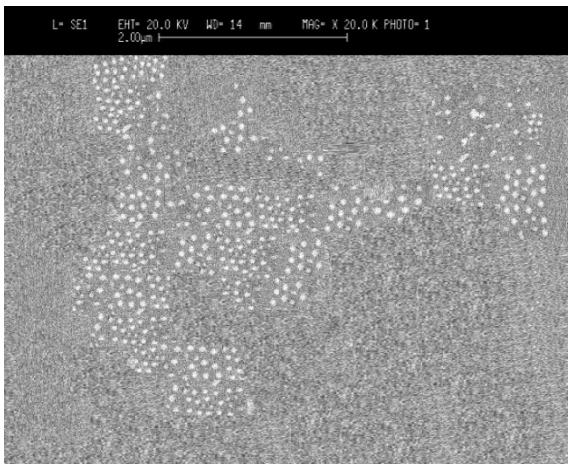

Fig 43: SEM image of A4 batch nanoparticles

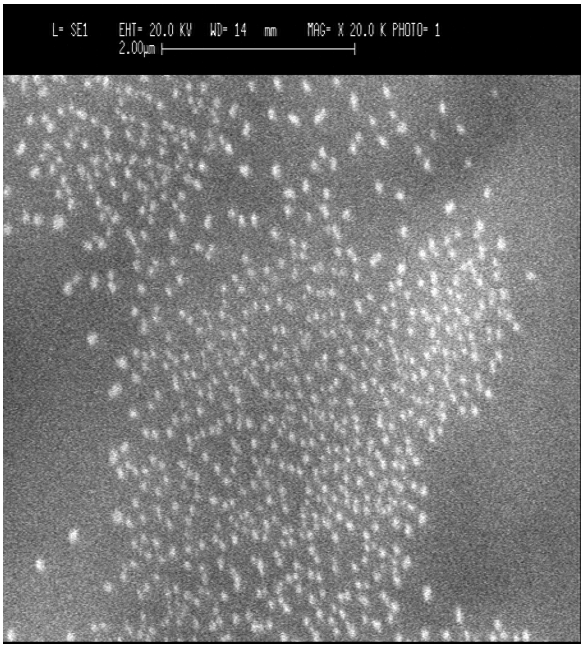

Fig 44: SEM image of G1 batch nanoparticles

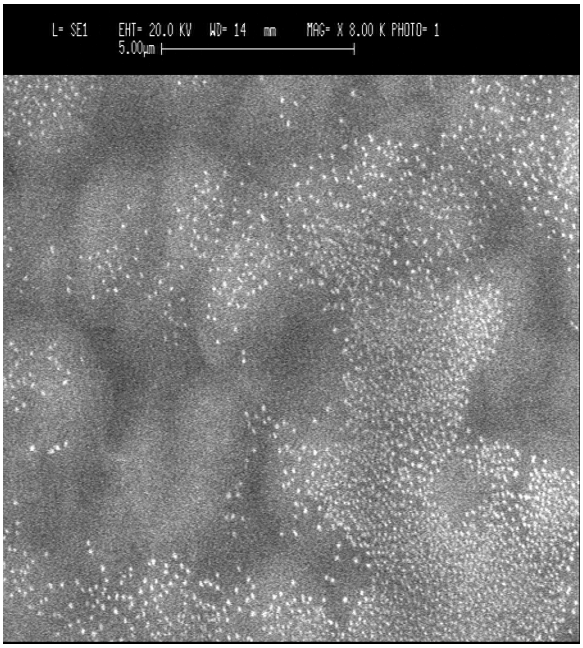

Fig 45: SEM image of G2 batch nanoparticles

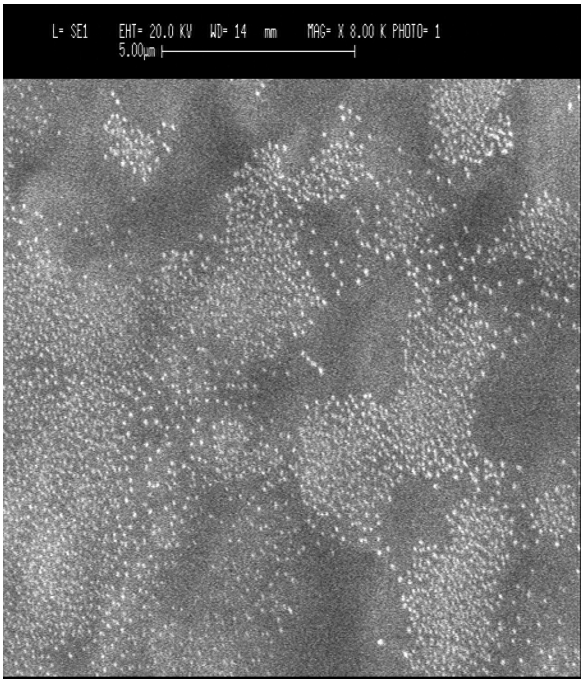

Fig 46: SEM image of G3 batch nanoparticles

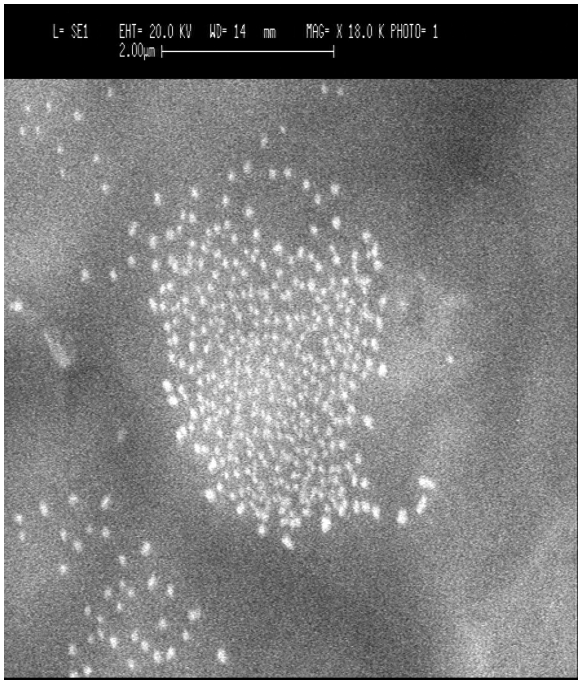

Fig 47: SEM image of G4 batch nanoparticles

***In vitro* release:**

The general phenomena of increase in particle size reduce the release rate was observed in the work, but the changes were not significant. The release of the drug was predicted due to formation of pores by the hydrophilic polymers with increasing the contact to the aqueous media [258,259]. As the concentration of the hydrophilic polymers is maintained same, the rate of pore formation is theorized to be similar which might have influenced the non-significant release rate. However slight variation observed with change in ratio of drug to polymer might be because of the path of diffusion (Table 8 and 9). The formulation with ratio of 1:2 showed slight slower release when compared to 1:1 because of the much deeper path taken by the drug moiety to diffuse. The data of the *in vitro* release studies were plotted in various kinetics models (Fig 48-57) like a) zero order (cumulative amount of drug release vs. time); b) first order (log cumulative percentage drug remaining vs. time) c) Higuchi (cumulative percentage of drug release vs. square root of time) d) Krosmeyer Peppas (log cumulative percentage drug release vs. log time) and e) Hixson-Crowell root model (cube root percentage of the drug remaining vs. time) to study the release behaviour. The Table 10 provides the slope (n) and the regression coefficient (r^2) of the batches A1 to A4 and G1 to G4. The formulations containing gelatin had relatively slower release of the drug on comparison to albumin containing batches and is expected because of the compounded gel consistency by gelatin along with hyaluronan.

Table 8: *In vitro* release profile of PTX from different batches. All the values are mean±SD (n=3).

Time (h)	Batches			
	A1 (%)	A3 (%)	G1 (%)	G3 (%)
0.5	3.53 ± 0.45	2.89 ± 0.78	2.43 ± 0.83	2.15 ± 0.54
1.0	4.36 ± 0.92	3.58 ± 0.53	3.52 ± 1.83	3.90 ± 0.86
2.0	8.06 ± 1.59	7.62 ± 1.37	10.77 ± 1.37	8.66 ± 0.96
4.0	24.92 ± 3.28	28.61 ± 6.28	18.94 ± 2.73	17.50 ± 3.61
6.0	60.75 ± 3.38	35.18 ± 0.49	33.44 ± 7.19	22.30 ± 2.66
8.0	37.05 ± 3.16	56.09 ± 1.87	45.53 ± 0.98	35.50 ± 5.33
12.0	70.54 ± 6.13	65.61 ± 3.49	56.26 ± 7.12	47.64 ± 5.85
16.0	80.68 ± 3.34	76.64 ± 2.44	61.40 ± 7.77	58.91 ± 3.65
24.0	87.75 ± 1.65	84.67 ± 1.19	72.46 ± 6.84	63.80 ± 5.28

Table 9: *In vitro* release profile of DTX from different batches. All the values are mean±SD (n=3).

Time (h)	Batches			
	A2 (%)	A4 (%)	G2 (%)	G4 (%)
0.5	3.28 ± 2.07	4.34 ± 2.70	3.24 ± 0.04	3.56 ± 0.61
1.0	5.89 ± 1.52	7.64 ± 1.44	4.69 ± 0.85	4.79 ± 0.42
2.0	14.45 ± 2.73	10.07 ± 1.88	7.87 ± 1.48	6.43 ± 0.58
4.0	34.27 ± 3.74	20.71 ± 5.66	27.69 ± 8.31	19.53 ± 3.75
6.0	43.13 ± 1.72	35.16 ± 7.13	35.62 ± 2.06	31.44 ± 2.22
8.0	59.02 ± 7.27	47.88 ± 3.28	45.00 ± 2.95	40.14 ± 1.67
12.0	75.97 ± 4.32	63.47 ± 8.68	54.79 ± 2.81	51.26 ± 4.22
16.0	84.12 ± 2.19	71.46 ± 3.96	66.17 ± 3.29	55.69 ± 2.40
24.0	92.85 ± 3.60	84.28 ± 6.15	81.31 ± 3.29	71.68 ± 3.49

Table 10: Slope (n) and the regression coefficients (r^2) of PTX and DTX batches with respect to various mathematical models.

Batches	Mathematical models					
	Zero order	First order	Higuchi	Krosmeyer-Peppas		Hixson-Crowell
	r^2	r^2	r^2	r^2	n	r^2
A1	0.880	0.963	0.938	0.924	1.008	0.943
A3	0.890	0.975	0.948	0.946	1.152	0.954
G1	0.886	0.929	0.941	0.942	1.085	0.953
G3	0.917	0.941	0.942	0.981	0.981	0.936
A2	0.883	0.958	0.959	0.925	1.130	0.961
A4	0.916	0.975	0.948	0.946	1.152	0.955
G2	0.925	0.929	0.964	0.942	1.085	0.974
G4	0.929	0.941	0.961	0.981	0.985	0.967

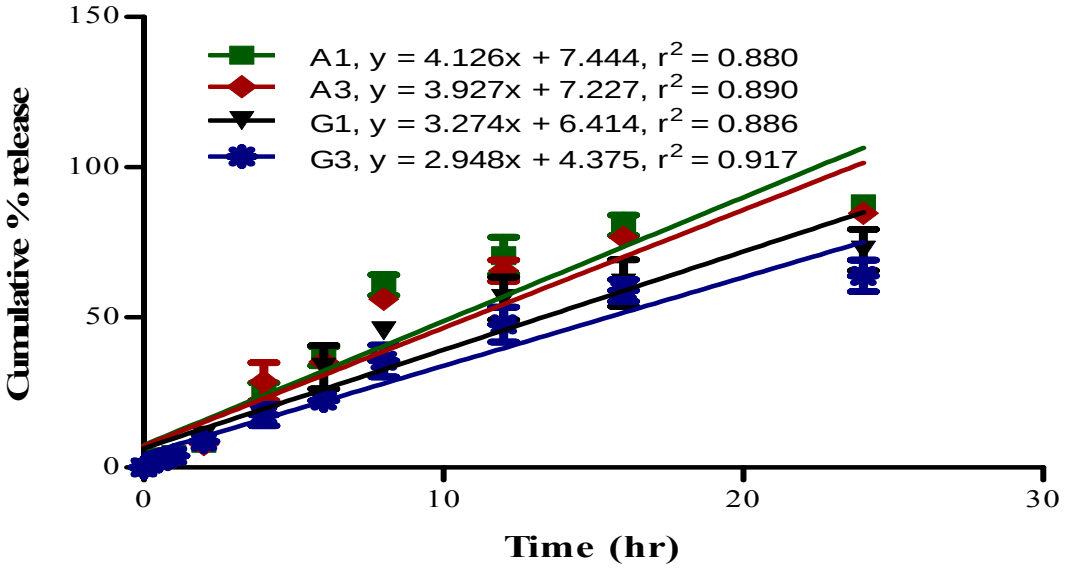

Fig 48: Zero order release kinetics of paclitaxel. A1 and A3 represent HA-albumin paclitaxel nanoparticles. G1 and G3 represent HA-gelatin paclitaxel nanoparticles. Each point symbolizes the mean of 3 observations with bar indicating the standard deviation.

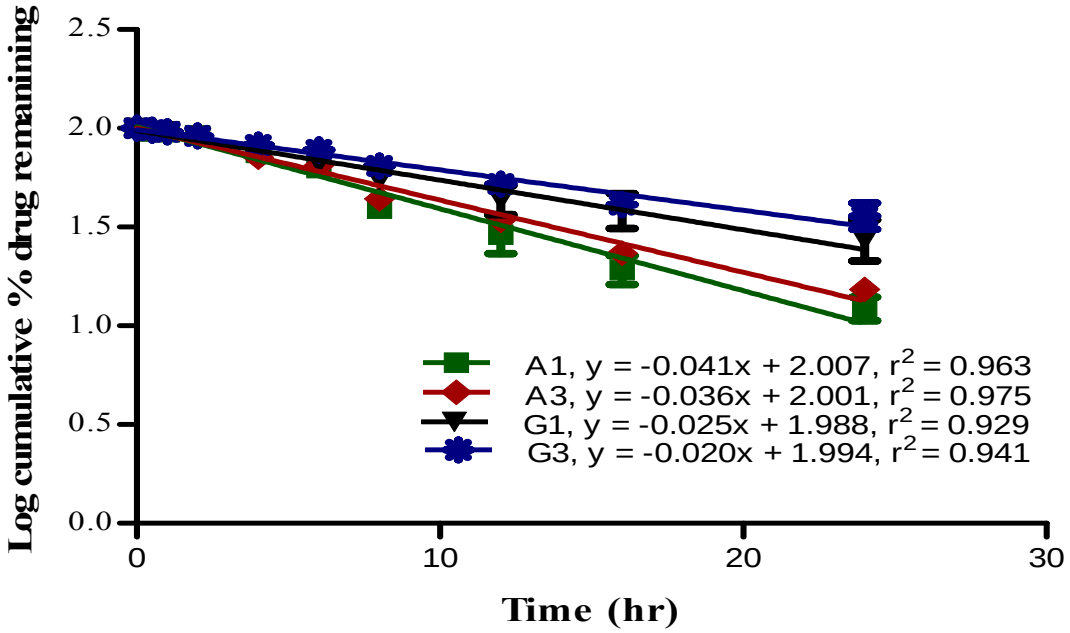

Fig 49: First order release kinetics of paclitaxel. A1 and A3 represent HA-albumin paclitaxel nanoparticles. G1 and G3 represent HA-gelatin paclitaxel nanoparticles. Each point symbolizes the mean of 3 observations with bar indicating the standard deviation.

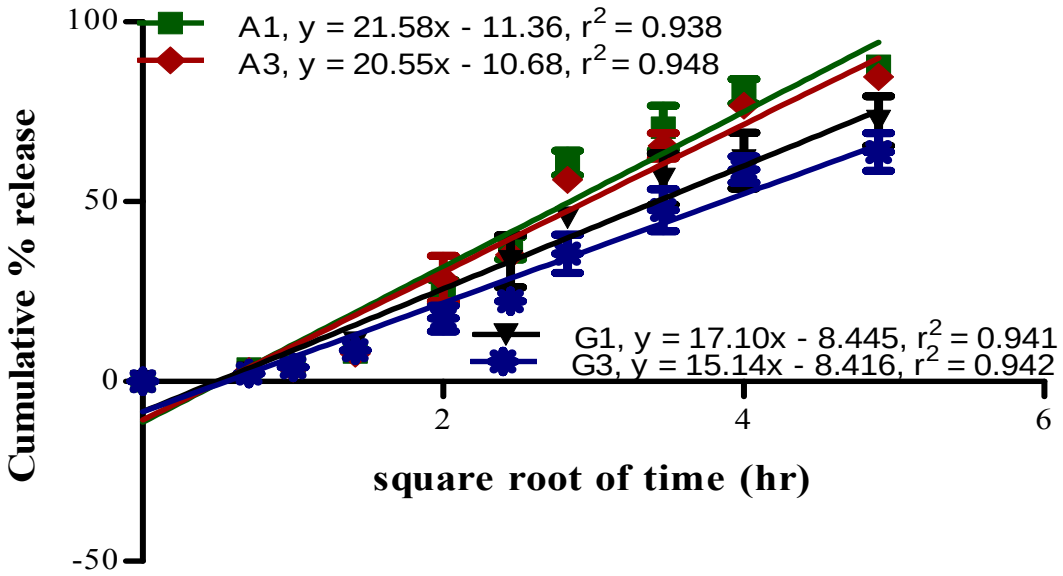

Fig 50: Higuchi model release kinetics of paclitaxel. A1 and A3 represent HA-albumin paclitaxel nanoparticles. G1 and G3 represent HA-gelatin paclitaxel nanoparticles. Each point symbolizes the mean of 3 observations with bar indicating the standard deviation.

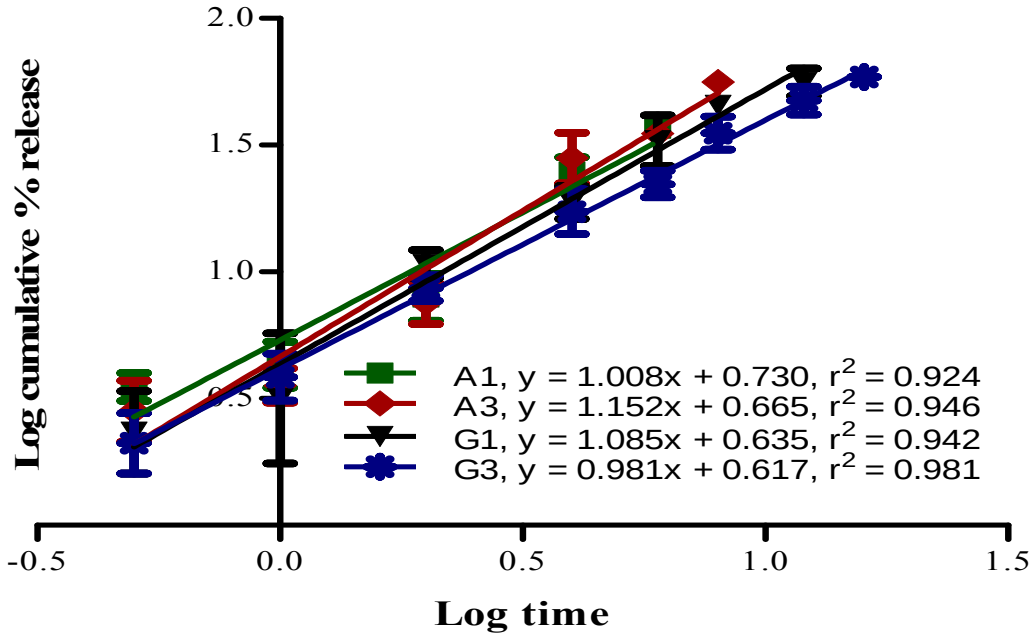

Fig 51: Kroysmeyer-Peppas model release kinetics of paclitaxel. A1 and A3 represent HA-albumin paclitaxel nanoparticles. G1 and G3 represent HA-gelatin paclitaxel nanoparticles. Each point symbolizes the mean of 3 observations with bar indicating the standard deviation.

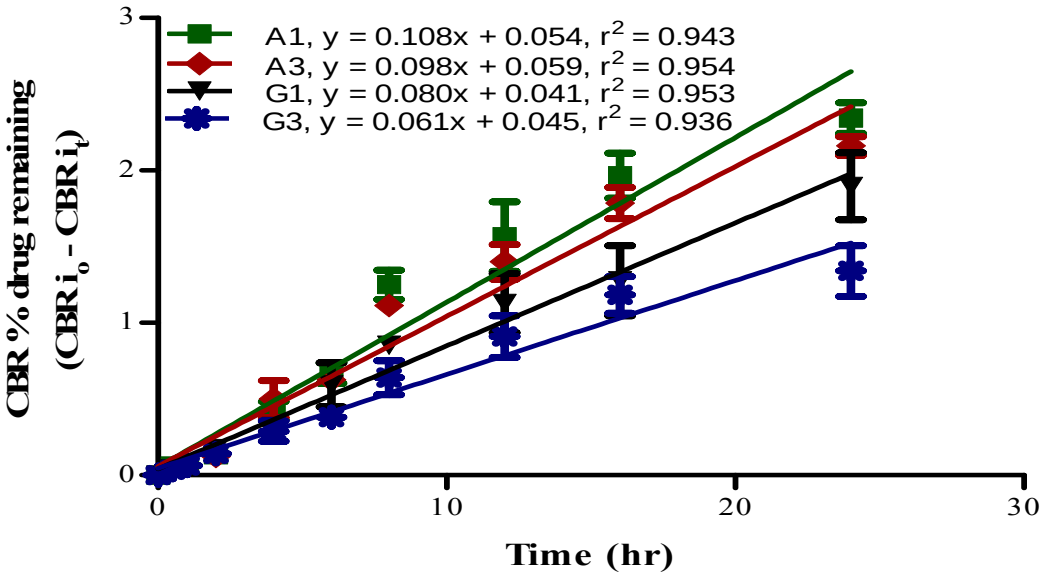

Fig 52: Hixson-Crowell model release kinetics of paclitaxel. A1 and A3 represent HA-albumin paclitaxel nanoparticles. G1 and G3 represent HA-gelatin paclitaxel nanoparticles. Each point symbolizes the mean of 3 observations with bar indicating the standard deviation.

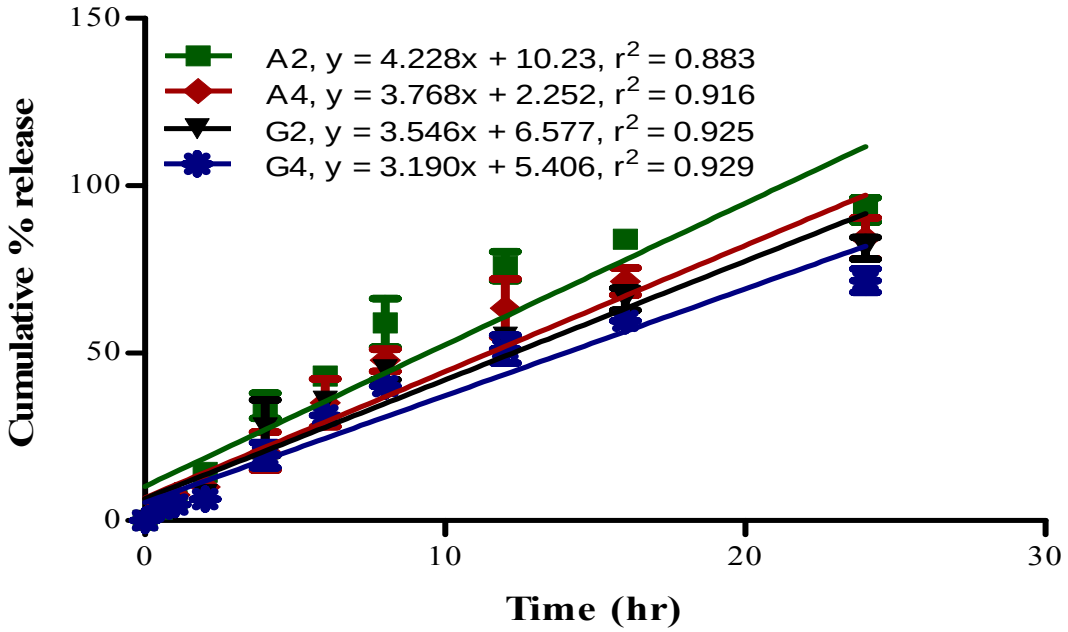

Fig 53: Zero order release kinetics of docetaxel. A2 and A4 represent HA-albumin docetaxel nanoparticles. G2 and G4 represent HA-gelatin docetaxel nanoparticles. Each point symbolizes the mean of 3 observations with bar indicating the standard deviation.

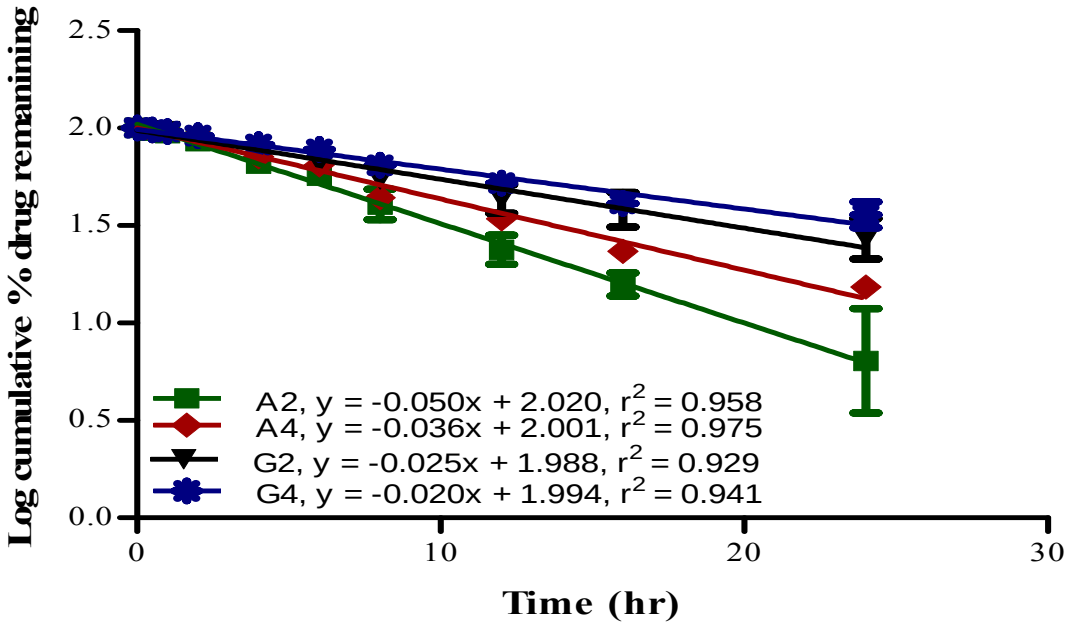

Fig 54: First order release kinetics of docetaxel. A2 and A4 represent HA-albumin docetaxel nanoparticles. G2 and G4 represent HA-gelatin docetaxel nanoparticles. Each point symbolizes the mean of 3 observations with bar indicating the standard deviation.

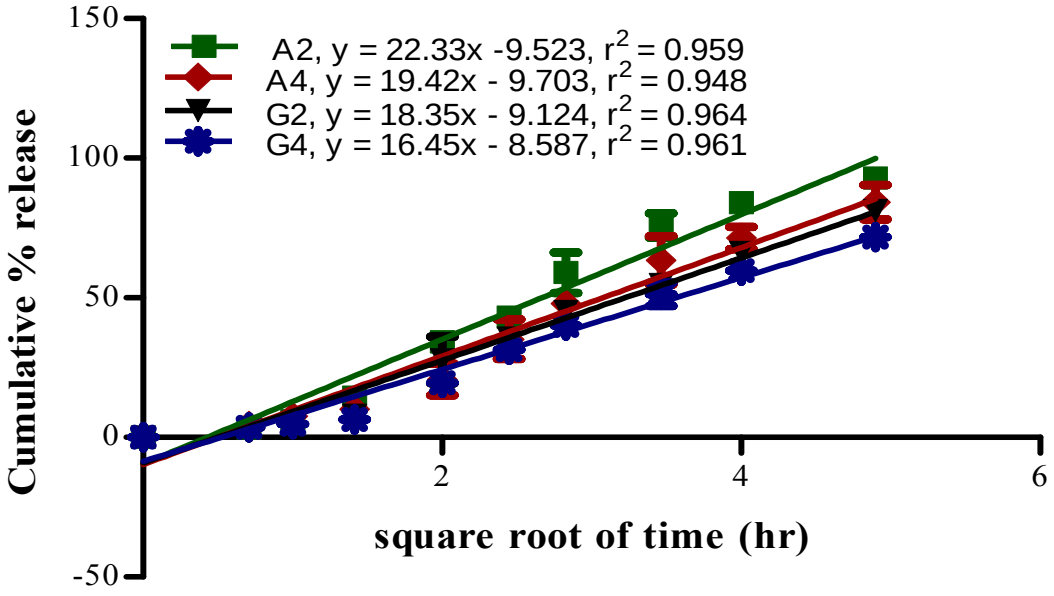

Fig 55: Higuchi model release kinetics of docetaxel. A2 and A4 represent HA-albumin docetaxel nanoparticles. G2 and G4 represent HA-gelatin docetaxel nanoparticles. Each point symbolizes the mean of 3 observations with bar indicating the standard deviation.

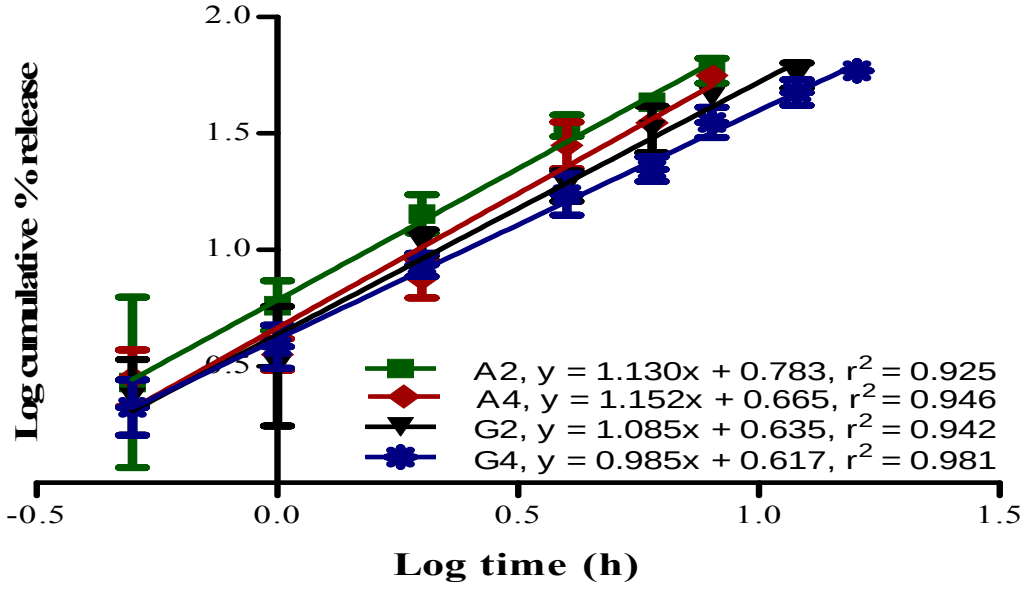

Fig 56: Kroysmeyer-Peppas model release kinetics of docetaxel. A2 and A4 represent HA-albumin docetaxel nanoparticles. G2 and G4 represent HA-gelatin docetaxel nanoparticles. Each point symbolizes the mean of 3 observations with bar indicating the standard deviation.

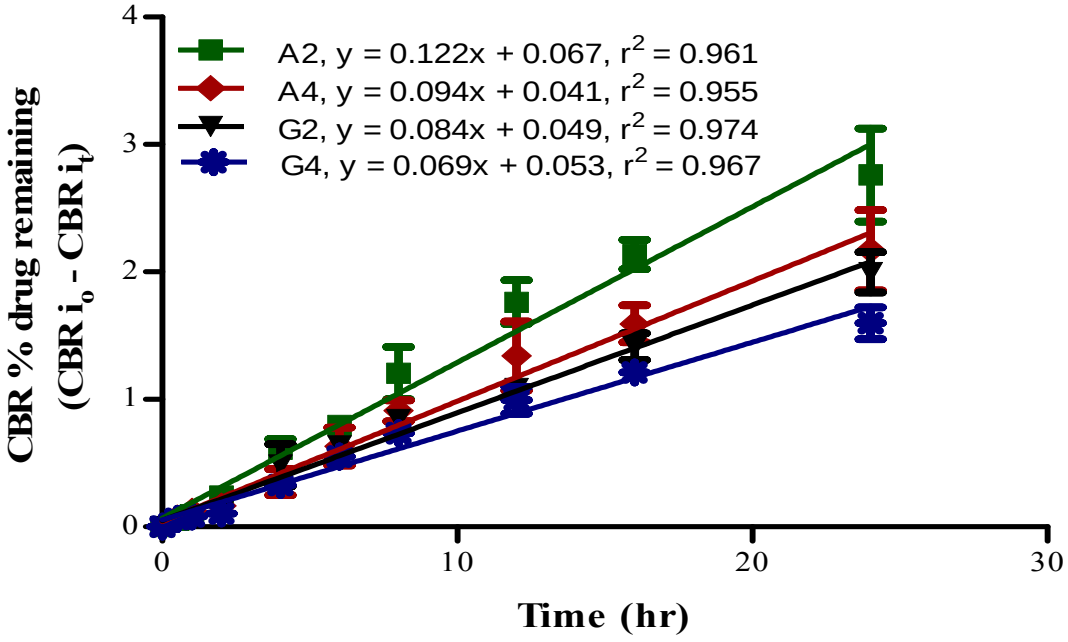

Fig 57: Hixson-Crowell model release kinetics of docetaxel. A2 and A4 represent HA-albumin docetaxel nanoparticles. G2 and G4 represent HA-gelatin docetaxel nanoparticles. Each point symbolizes the mean of 3 observations with bar indicating the standard deviation.

***In Vitro* Cytotoxicity studies:**

The basic principal involved in the SRB assay is interaction of a bright pink aminoxanthine dyne with the basic amino acids (protein) of viable cells to provide a sensitive index of cellular protein content. The color sensitive index of cellular protein content was linear over a cell density range of at least two orders of magnitude. The color development was rapid, stable and visible. The developed color was measured over a broad range of visible wavelength in spectrophotometer (540nm). The Fig 58 provides the appearance of normal cancerous MDA-MB-231 and that of 100% cytotoxic cells, whereas Fig 59 correlates the cytotoxicity of the formulations in comparison to respective pure drug.

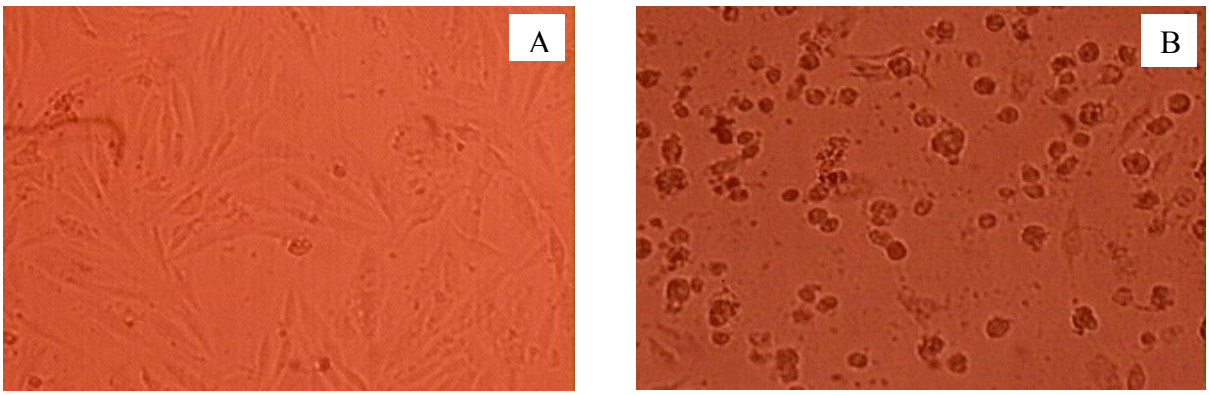

Fig 58: *In Vitro* cytotoxicity A) Normal cell culture of MDA-MB-231; B) 100% cytotoxicity of the MDA-MB-231 cell lines.

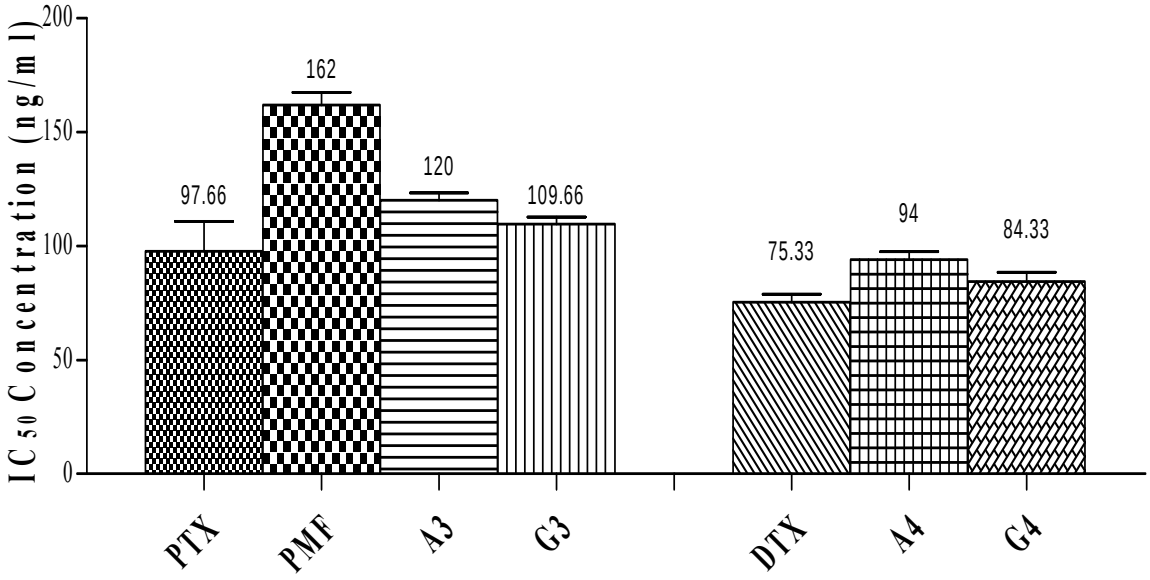

Fig 59: *In vitro* cytotoxicity – inhibitory concentration of representative formulations to kill the MDA-MB-231 breast cancer cells by 50% of its initial viable count.

Accelerated stability studies:

The WHO defines the stability as the drug/formulation to retain the chemical, physical, microbial and biopharmaceutical properties within the specified limits throughout the shelf life. The accelerated stability testing is defined as the studies designed to increase the rate of chemical degradation and physical changes of a drug or product by using exaggerated storage conditions as part of the formal stability testing programme. The data of studies is given in Table 11. The studies were carried at 25 ± 2 °C as we feel products to be stored at refrigerated conditions during their shelf-life as the formulations contain biodegradable natural polymers especially hyaluronan [260].

Table 11: Accelerated stability conditions and the functional parameters evaluated. All the values represent the mean of three observations with SD.

Parameters	Batches	Sampling interval (Month)				
		0	1	2	3	6
Entrapment efficacy (%)	A3	88.07±5.25	87.26±2.70	84.50±4.21	86.75±2.30	85.62±0.71
	G3	86.24±6.06	89.36±0.52	88.83±3.05	87.75±1.85	86.32±1.22
	A4	80.36±6.52	75.53±2.31	76.34±2.19	82.23±2.51	80.00±1.84
	G4	83.20±2.11	83.37±0.43	80.90±1.03	78.85±3.11	81.35±1.10
pH	A3	6.8	6.9	7.0	6.9	6.8
	G3	6.9	6.9	6.8	7.0	6.8
	A4	7.0	6.9	6.8	6.8	6.9
	G4	6.9	7.0	7.0	6.8	6.8
Average particle size (nm)	A3	36.11	---	---	---	35.52
	G3	46.64	---	---	---	47.01
	A4	69.28	---	---	---	72.34
	G4	53.81	---	---	---	51.89
Cumulative % release (24^{th} h)	A3	84.67±1.19	---	---	---	86.34±2.65
	G3	63.80±5.28	---	---	---	67.79±1.05
	A4	84.28±6.15	---	---	---	80.57±3.48
	G4	71.68±3.49	---	---	---	75.47±6.62

Pharmacokinetic studies:

As described in methodology the animals were grouped for the investigation of pharmacokinetics of the selected batches. The group one received paclitaxel marketed formulation (PMF) at dose of 44.75mg/kg (approximately 45mg/kg). A similar equivalent dose was maintained for A3 and G3 formulations to have a better comparison of pharmacokinetic data. The A3 and G3 were administered to group two and three respectively. The group four to six received the DTX formulations; DMF (docetaxel marketed formulation), A4 and G4 respectively at dose of 17.2mg/kg (approximately 17mg/kg). The A3, G3, A4 and G4 formulations were sterilized by membrane filtration (Whatman Membra-fil pore size 0.22μ). The membranes were than dispersed in acetonitrile which was further analyzed by HPLC (analytical method). The HPLC chromatogram did not furnish drug peak symbolizing the complete filtration without membrane blockage by the particles.

The plasma concentration of the PTX (PMF, A3 and G3) and DTX (DMF, A4, and G4) are given in Table 12 and 13 respectively. The table 14 and 15 provides the blood pharmacokinetic parameters for PTX and DTX formulations which are calculated on application of database WinNonlin version 5.

Table 12: Blood plasma concentration of PMF, A3 and G3 formulations. All the values indicates mean ± SD (n=6).

Time (h)	Concentration (μg/mL)		
	PMF	A3	G3
0.5	49.396 ± 5.592	48.322 ± 5.982	46.306 ± 3.156
1.0	44.970 ± 5.810	39.970 ± 4.536	37.748 ± 4.160
2.0	40.692 ± 6.522	35.766 ± 4.406	32.702 ± 1.838
4.0	36.322 ± 8.916	31.822 ± 4.936	27.174 ± 2.112
6.0	29.267 ± 5.886	27.026 ± 3.340	23.470 ± 2.622
8.0	24.508 ± 3.035	21.359 ± 3.858	19.341 ± 2.547
12.0	16.952 ± 2.803	19.844 ± 2.446	18.067 ± 1.901
24.0	15.376 ± 2.654	16.681 ± 2.341	16.142 ± 1.563

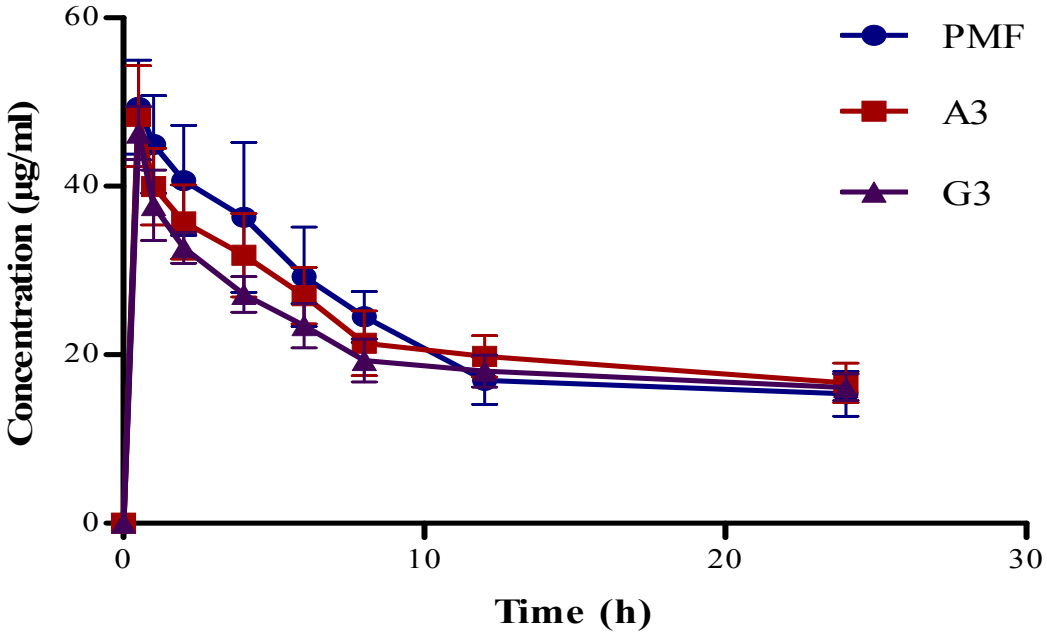

Fig 60: Plasma decay curve of PTX associated to formulation PMF, A3, and G3 respectively following i.v. injection. Results represent the mean of six observations with SD (bars).

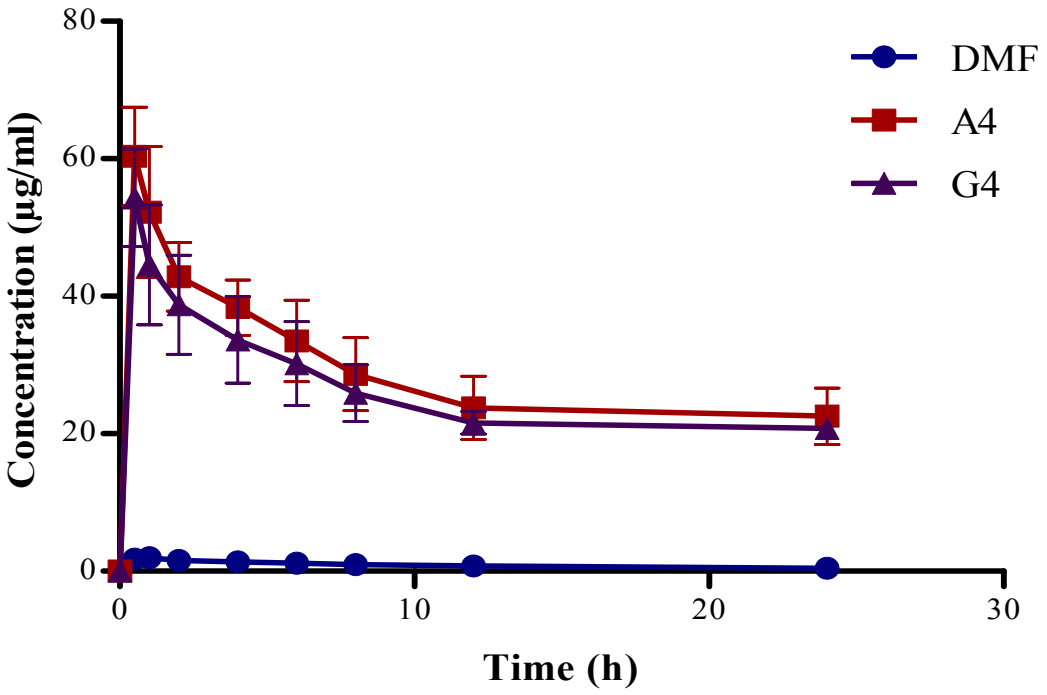

Fig 61: Plasma decay curve of DTX associated to formulation - DMF, A4, and G4 respectively following i.v. injection. Results represent the mean of six observations with SD (bars).

Table 13: Blood plasma concentration of DMF, A4 and G4 formulations. All the values indicates mean ± SD (n=6).

Time (h)	Concentration (µg/mL)		
	DMF	A4	G4
0.5	1.783 ± 0.629	60.344 ± 7.157	54.322 ± 7.121
1.0	1.935 ± 0.268	52.239 ± 9.547	44.544 ± 8.744
2.0	1.563 ± 0.109	42.844 ± 4.987	38.778 ± 7.195
4.0	1.345 ± 0.118	38.344 ± 4.013	33.650 ± 6.290
6.0	1.163 ± 0.177	33.498 ± 5.944	30.200 ± 6.118
8.0	0.985 ± 0.182	28.666 ± 5.269	25.894 ± 4.124
12.0	0.784 ± 0.188	23.788 ± 4.597	21.572 ± 1.633
24.0	0.462 ± 0.203	22.535 ± 4.095	20.773 ± 1.508

Table 14: Blood pharmacokinetic parameters of PTX formulations.

Variables	Formulations		
	PMF	**A3**	**G3**
C_0 (µg/mL)	54.379 ± 6.272	58.584 ± 9.082	57.244 ± 6.712
C_{max} (µg/mL)	49.396 ± 5.592	48.322 ± 5.982	46.306 ± 3.156
T_{max} (h)	0.5	0.5	0.5
$T_{1/2}$ (h)	16.712 ± 9.249	36.218 ± 11.267[a]	69.348 ± 18.617[c,*]
AUC_{0-24} (µg.h/mL)	565.631 ± 75.624	563.05 ± 72.306	515.53 ± 46.772
$AUC_{0-\infty}$ (µg.h/mL)	943.709 ± 225.687	1448.482 ± 414.526[a]	2116.298 ± 380.426[c,*]
K_e (h^{-1})	0.048 ± 0.016	0.021 ± 0.01[c]	0.010 ± 0.003[c]
V_d (mL/kg)	1107.049 ± 338.42	1616.174 ± 277.745[a]	2107.605 ± 298.777[c,*]
CL (mL/h.kg)	50.108 ± 12.539	33.477 ± 10.449[b]	21.878 ± 4.152[c]
MRT (h)	9.255 ± 0.587	9.853 ± 0.182[a]	10.003 ± 0.119[b]

All the values are mean of six observations ± standard deviation. The [a], [b], and [c] indicates statistical significance with $p < 0.05$, < 0.01 and < 0.001 respectively on comparison to the value of PMF. The [*] symbolizes the statistical difference between the batch A3 and G3 with p value < 0.05. C_0 – Initial plasma concentration; C_{max} – Peak plasma concentration; T_{max} – Time to attain C_{max}; $T_{1/2}$ – Half-life; AUC – Area under curve; K_e – Elimination rate constant; V_d – Volume of distribution; CL – Total body clearance and MRT – Mean residence time.

Table 15: Blood pharmacokinetic parameters of DTX formulations.

Variables	Formulations		
	DMF	A4	G4
C_0 (µg/mL)	1.976 ± 0.981	73.069 ± 13.97[c]	66.757 ± 7.661[c]
C_{max} (µg/mL)	2.067 ± 0.335	62.066 ± 6.735[c]	54.322 ± 7.121[c,*]
T_{max} (h)	0.92 ± 0.2	0.58 ± 0.2[b]	0.5[b]
$T_{1/2}$ (h)	14.807 ± 6.427	38.917 ± 21.255[a]	54.795 ± 59.084[c,*]
AUC_{0-24} (µg.h/mL)	22.2 ± 4.356	707.08 ± 93.704[c]	638.02 ± 69.371[c]
$AUC_{0-\infty}$ (µg.h/mL)	33.52 ± 13.451	1983.533 ± 738.944[b]	2215.382 ± 1602.26[b]
K_e ($h^{-1)}$	0.0535 ± 0.02	0.023 ± 0.012[a]	0.027 ± 0.017[a]
V_d (mL/kg)	10823.25 ± 942.658	460.497 ± 120.62[c]	491.558 ± 204.983[c]
CL (mL/h.kg)	578.161 ± 233.781	9.847 ± 4.262[c]	10.565 ± 4.964[c]
MRT (h)	8.738 ± 0.878	10.011 ± 0.521[b]	10.181 ± 0.411[b]

All the values are mean of six observations ± standard deviation. The [a, b,] and [c] indicates statistical significance with p <0.05, <0.01 and <0.001 respectively on comparison to the corresponding value of DMF. The [*] symbolize statistical difference between the batch G4 and A4 with p value <0.05. C_0 – Initial plasma concentration; C_{max} – Peak plasma concentration; T_{max} – Time to attain C_{max}; $T_{1/2}$ – Half-life; AUC – Area under curve; K_e – Elimination rate constant; V_d – Volume of distribution; CL – Total body clearance and MRT – Mean residence time.

The C_{max} for all the PTX formulations were observed immediately (at 0.5h) after their injection with multiphasic decline in their concentration (Fig 60). The DTX formulations recorded C_{max} within an hour of injection with similar phase of turndown in concentration (Fig 61). The A3 and G3 formulations produced the significant difference in PK parameters such as half-life, $AUC_{0-\infty}$, K_e, V_d, CL and MRT on comparison to the respective results of PMF (Table 14). The A4 and G4 formulations documented significant difference among all the analyzed PK parameters on comparison to respective results of DMF (Table 15). Although the volume of distribution of PTX exceeded total body water (0.67 L/kg) the readings were relatively lower suggesting strictly plasma volume distribution [261,262]. On administration of drug of dose D the drug molecules gets distributed throughout the body. These molecules stay in the body for various time period with some getting eliminated immediately followed by other. The term mean residence time (MRT) describes the average time period for all the

drug molecules to transit in body. The formulations A4 and G4 had relatively lower volume of distribution on comparison to A3 and G3 which may be mainly because of reduced lipophilicity of the drug. The significant enhancement in V_d of DTX in case of DMF is correlated to the pure diffusion of drug to the tissue and extravascular compartment.

Biodistribution:

The samples of PTX and DTX formulations were injected to SD rats induced with tumour (Fig 62) at the dose mentioned earlier for investigation of pharmacokinetic parameters. The Table 16 and 17 provides the concentration of each formulation per gram of organ of interest at the end of first and second hour of treatment. The antitumor efficacy of the formulation depends on the amount and exposure time of it at the tumour site [263]. As discussed earlier the polymer of choice HA, albumin and gelatin have the ability to clinch the antitumour activity by deliverance of drug to the site of need. To investigate the efficacy of A3, G3, A4 and G4 and compare (Fig 63) with their respective batch of PMF and DMF the tissue of tumour was isolated. Further to revise the possible mechanism of clearance along with its effect on major organs; the heart, kidney, lungs, spleen, brain and liver were collected for estimation of drug and compared with corresponding commercial batches (Fig 64-69).

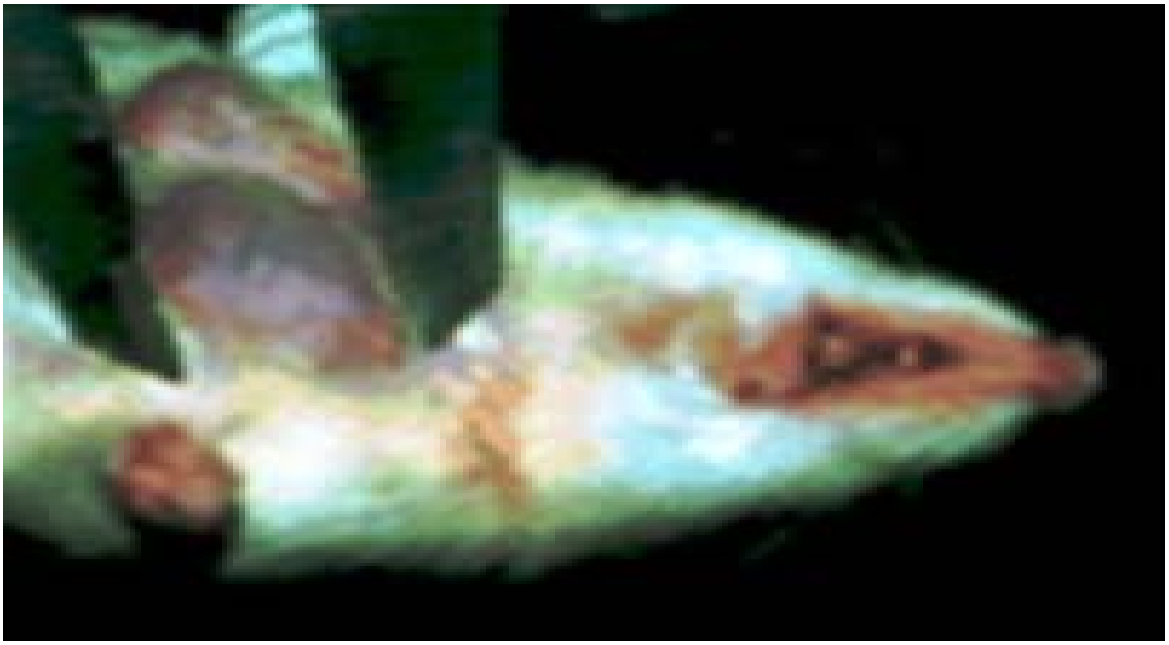

Fig 62: NMU induced breast cancerous SD rat.

Table 16: Biodistribution study of PTX formulations in NMU induced breast cancerous SD rats weighing 200±20 g. All the values are mean ± SD (n=3).

Organs	Time (min)	PMF (µg/g)	A3 (µg/g)	G3 (µg/g)
Tumor	60	514.00 ± 30.22	778.37 ± 88.88	830.55 ± 99.07
	120	440.50 ± 42.88	583.21 ± 78.65	747.88 ± 65.35
Heart	60	0.51 ± 0.38	0.53 ± 0.21	0.51 ± 0.21
	120	0.49 ± 0.30	0.35 ± 0.15	0.53 ± 0.09
Kidney	60	391.48 ± 78.45	584.89 ± 111.07	747.95 ± 74.55
	120	487.84 ± 60.67	725.18 ± 145.32	832.88 ± 96.33
Lungs	60	1267.60 ± 163.11	982.83 ± 104.59	905.34 ± 97.33
	120	872.89 ± 96.33	964.46 ± 88.12	811.10 ± 97.97
Spleen	60	544.83 ± 36.98	435.52 ± 66.66	445.27 ± 54.49
	120	678.97 ± 41.14	507.17 ± 19.89	491.93 ± 34.48
Brain	60	0.016 ± 0.005	0.015 ± 0.006	0.017 ± 0.006
	120	0.012 ± 0.004	0.014 ± 0.006	0.016 ± 0.005
Liver	60	175.79 ± 21.86	158.13 ± 17.98	154.42 ± 23.33
	120	195.61 ± 32.43	172.87 ± 31.08	167.11 ± 17.61

Table 17: Biodistribution study of DTX formulations in NMU induced breast cancerous SD rats weighing 200±20 g. All the values are mean ± SD (n=3).

Organs	Time (min)	DMF (µg/g)	A4 (µg/g)	G4 (µg/g)
Tumor	60	14.21 ± 1.05	157.15 ± 23.43	201.35 ± 44.88
	120	13.87 ± 0.96	154.66 ± 18.22	187.65 ± 34.22
Heart	60	14.75 ± 3.7	0.53 ± 0.31	0.44 ± 0.13
	120	12.67 ± 5.6	0.37 ± 0.11	0.39 ± 0.20
Kidney	60	145.94 ± 8.45	189.75 ± 38.88	165.18 ± 43.11
	120	149.83 ± 7.21	225.52 ± 32.05	185.85 ± 22.87
Lungs	60	198.94 ± 12.52	359.46 ± 53.91	362.72 ± 25.83
	120	194.76 ± 10.33	348.07 ± 47.33	325.61 ± 27.66
Spleen	60	22.48 ± 3.69	145.25 ± 33.75	123.11 ± 22.45
	120	18.87 ± 4.14	147.5 ± 10.05	133.45 ± 16.01
Brain	60	0.007 ± 0.002	0.021 ± 0.002	0.022 ± 0.004
	120	0.006 ± 0.002	0.023 ± 0.002	0.023 ± 0.004
Liver	60	175.69 ± 12.06	57.67 ± 9.89	45.75 ± 10.22
	120	174.98 ± 10.67	62.87 ± 18.81	55.45 ± 9.75

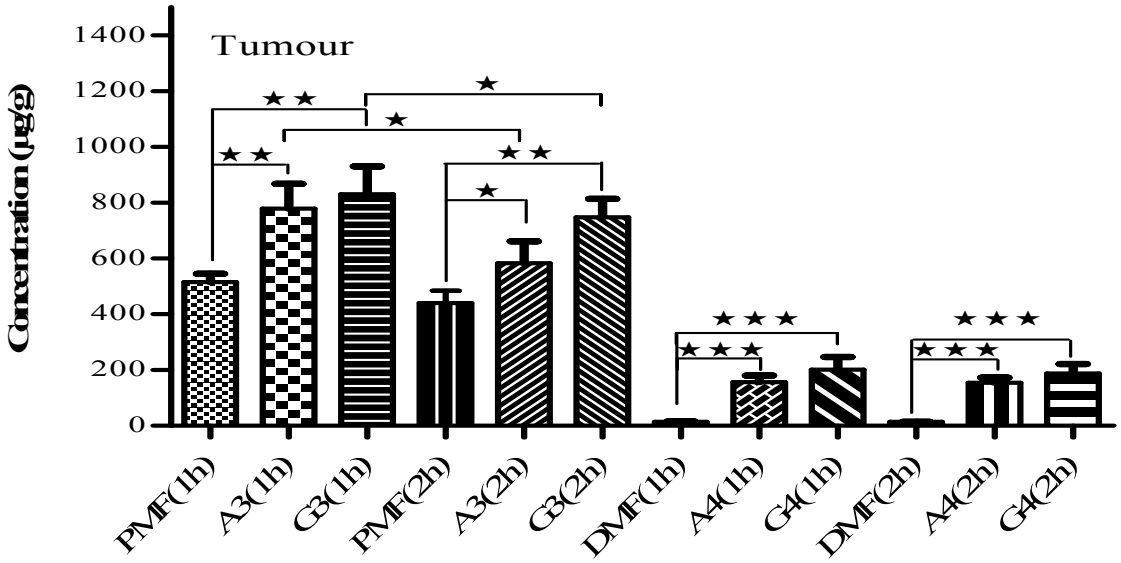

Fig 63: Concentration of Paclitaxel and Docetaxel at tumour site after i.v. injection of PMF, A3, G3, DMF, A4, and G4. PMF – Paclitaxel marketed formulation, DMF – Docetaxel marketed formulation. 1h and 2h represents time of sacrifice of animal. All the values are mean of 3 observations with bar representing the SD. ★ represent p <0.05, ★★ indicates p <0.01, and ★★★ symbolize p <0.001.

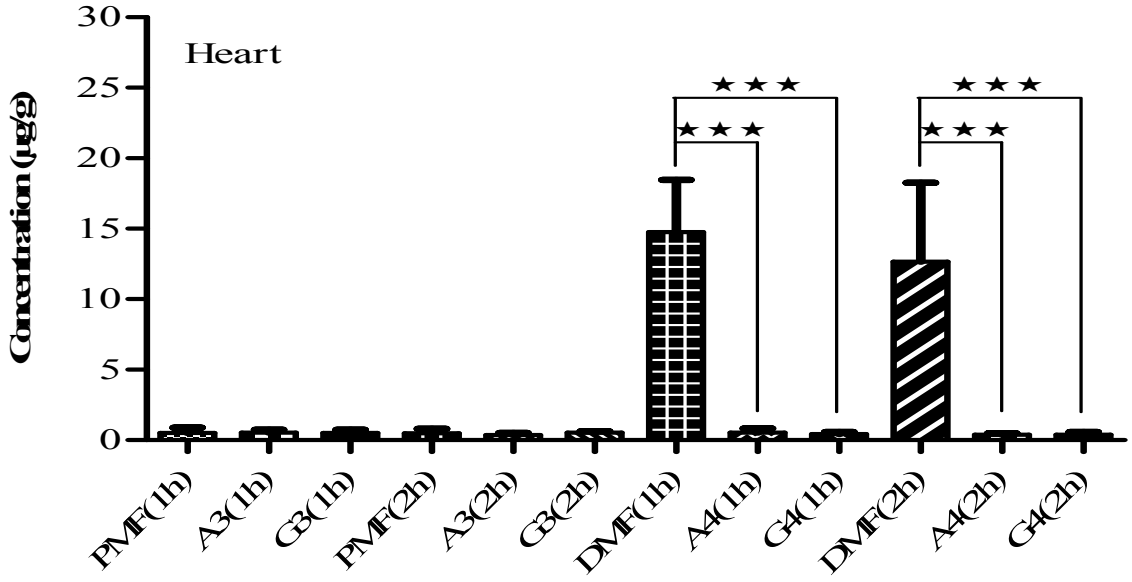

Fig 64: Concentration of Paclitaxel and Docetaxel in heart after i.v. injection of PMF, A3, G3, DMF, A4, and G4. PMF – Paclitaxel marketed formulation, DMF – Docetaxel marketed formulation. 1h and 2h represents time of sacrifice of animal. All the values are mean of 3 observations with bar representing the SD. ★ represent p <0.05, ★★ indicates p <0.01, and ★★★ symbolize p <0.001.

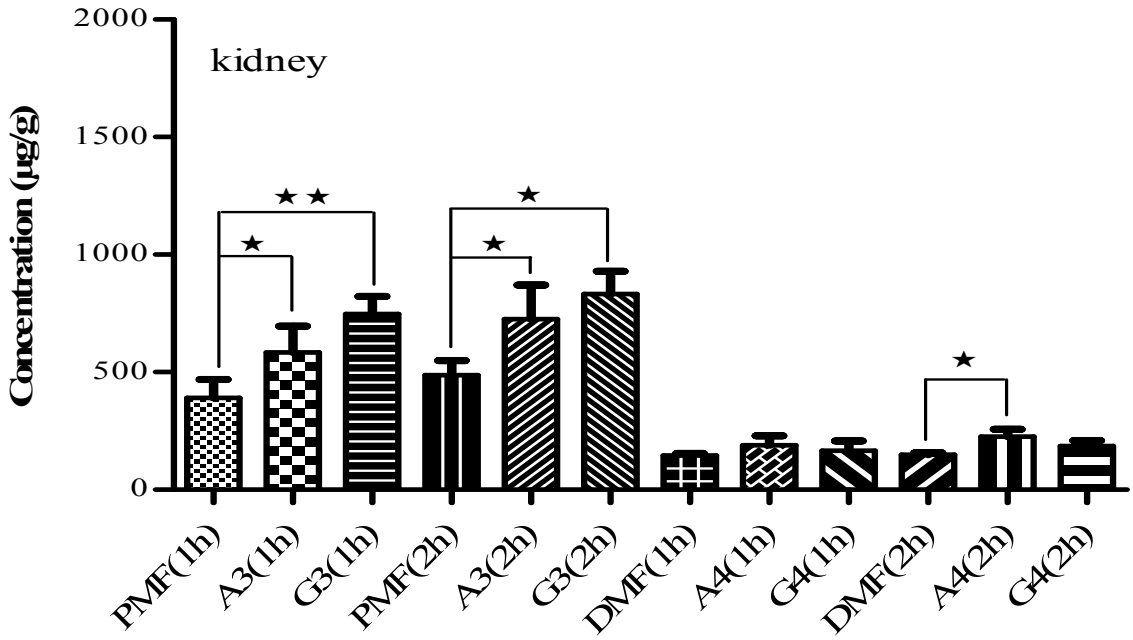

Fig 65: Concentration of Paclitaxel and Docetaxel in kidney after i.v. injection of PMF, A3, G3, DMF, A4, and G4. PMF – Paclitaxel marketed formulation, DMF – Docetaxel marketed formulation. 1h and 2h represents time of sacrifice of animal. All the values are mean of 3 observations with bar representing the SD. ★ represent p <0.05, ★★ indicates p <0.01, and ★★★ symbolize p <0.001.

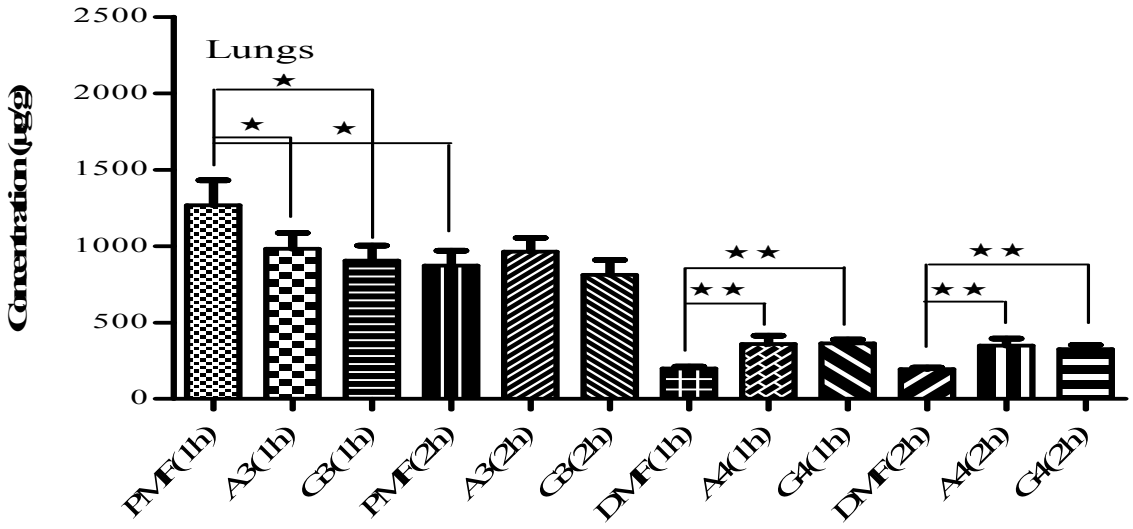

Fig 66: Concentration of Paclitaxel and Docetaxel in lungs after i.v. injection of PMF, A3, G3, DMF, A4, and G4. PMF – Paclitaxel marketed formulation, DMF – Docetaxel marketed formulation. 1h and 2h represents time of sacrifice of animal. All the values are mean of 3 observations with bar representing the SD. ★ represent p <0.05, ★★ indicates p <0.01, and ★★★ symbolize p <0.001.

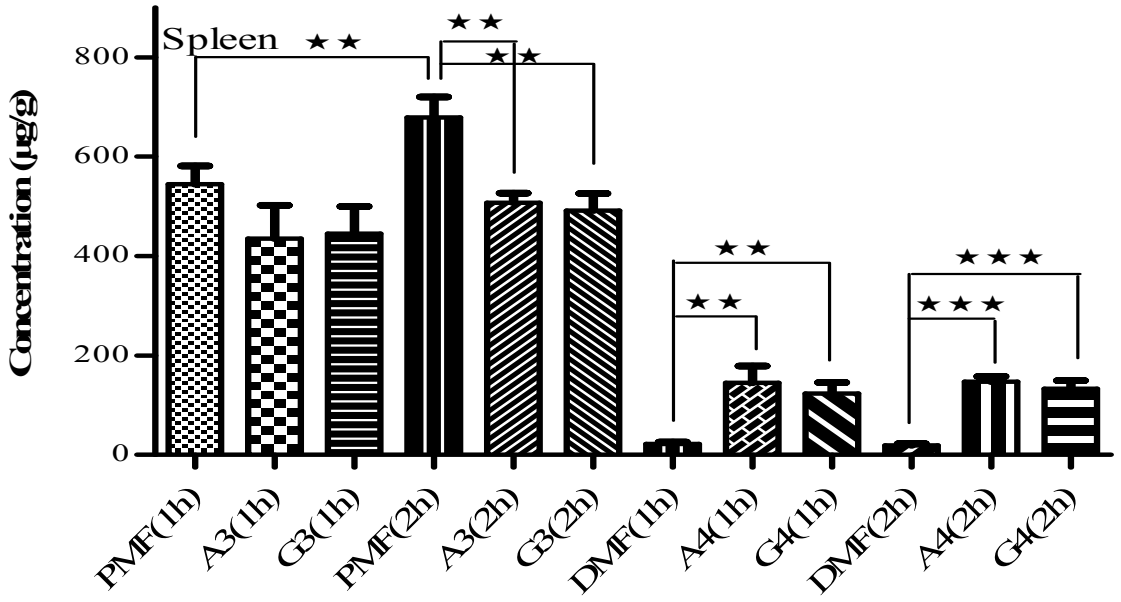

Fig 67: Concentration of Paclitaxel and Docetaxel in spleen after i.v. injection of PMF, A3, G3, DMF, A4, and G4. PMF – Paclitaxel marketed formulation, DMF – Docetaxel marketed formulation. 1h and 2h represents time of sacrifice of animal. All the values are mean of 3 observations with bar representing the SD. ★ represent p <0.05, ★★ indicates p <0.01, and ★★★ symbolize p <0.001.

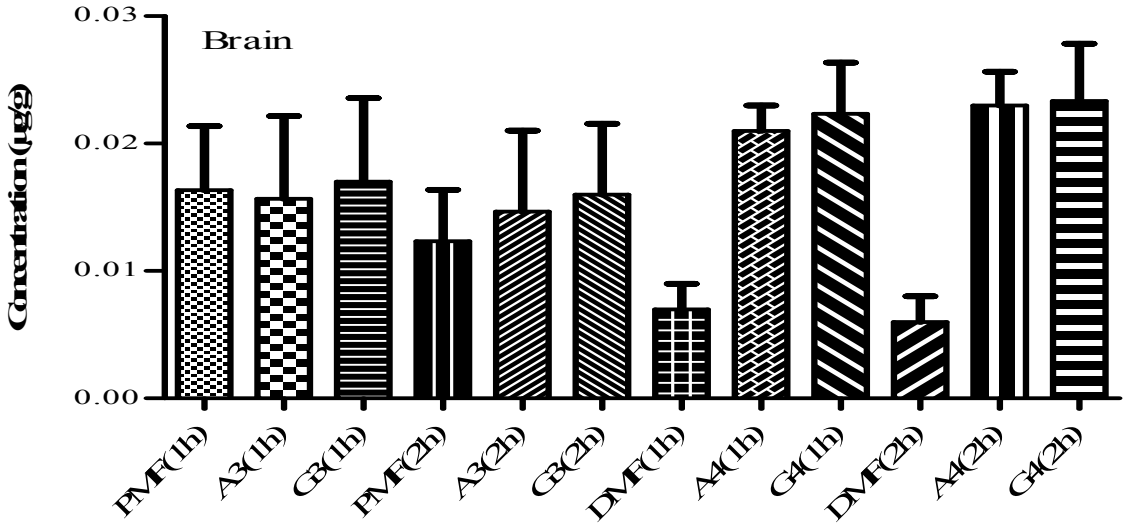

Fig 68: Concentration of Paclitaxel and Docetaxel in brain after i.v. injection of PMF, A3, G3, DMF, A4, and G4. PMF – Paclitaxel marketed formulation, DMF – Docetaxel marketed formulation. 1h and 2h represents time of sacrifice of animal. All the values are mean of 3 observations with bar representing the SD. ★ represent p <0.05, ★★ indicates p <0.01, and ★★★ symbolize p <0.001.

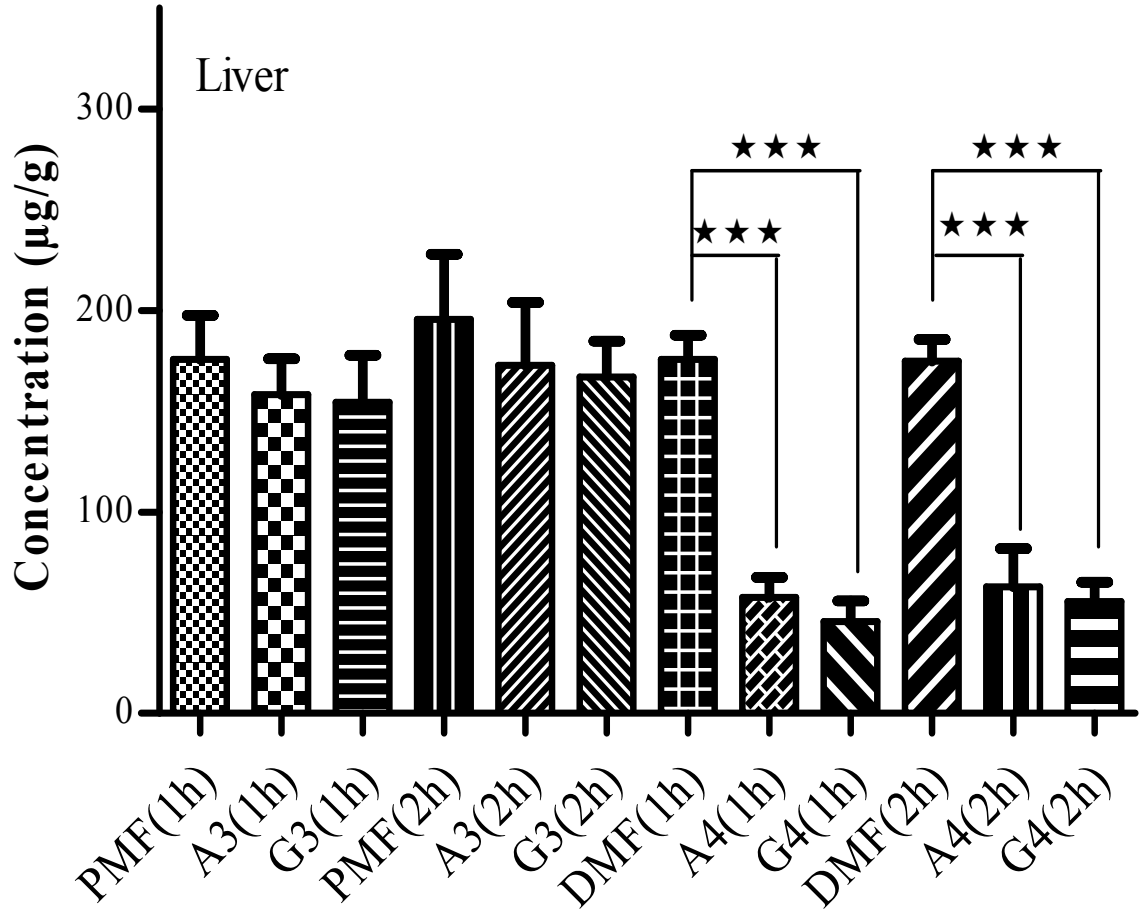

Fig 69: Concentration of Paclitaxel and Docetaxel in liver after i.v. injection of PMF, A3, G3, DMF, A4, and G4. PMF – Paclitaxel marketed formulation, DMF – Docetaxel marketed formulation. 1h and 2h represents time of sacrifice of animal. All the values are mean of 3 observations with bar representing the SD. ★ represent p <0.05, ★★ indicates p <0.01, and ★★★ symbolize p <0.001.

Discussion

DISCUSSION

The chapter provides an additional support with pragmatic or hypothetical reasons beyond the experimental findings.

Development of Analytical and Bio-analytical techniques:

As a part of process validation of bio-analytical method the solid phase extraction, liquid-liquid extraction and protein precipitation technique were applied to determine the limit of detection for paclitaxel (PTX) and docetaxel (DTX). The limit of detection for PTX and DTX by solid phase extraction technique was 0.002 and 0.003 µg/ml respectively. The protein precipitation technique also reported the same limits when methanol was used as a protein precipitation agent. The liquid-liquid extraction technique accounts much higher limit of detection for PTX (10 ng/ml) and DTX (16 ng/ml). Thus the further validation of method was carried by protein precipitation technique since the solid phase extraction is time consuming with multi purification steps. Initially, at flow rate of 1mL/min, the mobile phase of 54:46 % v/v ratio (ACN: 0.1% TEA pH 3.5) reported the runtime of 16min with retention time of 11.6 and 14.8 min respectively for DTX and PTX. To reduce the run time the flow rate of mobile phase was enhanced to 1.4 ml/min. No attempt was made for further enhancement of flow rate of the mobile phase as it may build pressure on the pump. The better resolution factor was observed at the ratio of 54% of ACN and 46% of 0.1% TEA (pH 3.5). Increase in percentage of ACN show a faster elution with poor resolution. The broad peaks were observed with enhanced run time with a decrease in the ratio of ACN. The taxanes were highly sensitive to the wavelength of 227nm as observed by other workers without any interference of mobile phase or the other excipients involved in process.

The so developed HPLC based bio-analytical method by protein precipitation agent was sensitive and simple. The design was further applied for investigation of pharmacokinetic and biodistribution profile of PTX and DTX. Among the protein precipitation reagents (ACN, $ZnSO_4$, Methanol, Tungstic acid and $HClO_4$) the methanol provided the better intensity with at most sensitivity to solid phase extraction technique. The method was also reproducible and repeatable with robust stability of component in the matrix of plasma. Though the system was UFLC, the application of column C18 made no changes in retention time of drug peaks as reported earlier [264-266], .thus UFLC is expected to behave as normal HPLC. Even the analytical method was reproducible. The analytical method was sensitive enough to measure

the concentration of PTX and DTX up to 2 ng/ml concentration. In bio-analytical method the limit of detection for DTX was 5 ng/ml whereas for PTX it was 2 ng/ml. The increase in limit of detection for DTX may be due to less recovery of drug from the plasma during the extraction process.

Curve of Linearity:

Both the analytical and bio-analytical curve of PTX and DTX were developed by taking the mean of 3 observations. In all the cases the linearity was observed with regression value of 0.999 and the respective slope values. Within the range of study the response factor/AUC increased linearly with increase in concentration and better accuracy was recorded with minimum fluctuations. The technique was suitable for further analytical and bio-analytical work with the application of equation $y = mx \pm c$ generated. y denotes AUC in case of analytical method and response factor in case of bio-analytical; m represents slope; and c stands for y intercept.

Solubility:

PTX belonging to BCS class IV is a non-ionic molecule and is practically insoluble in water at 37 °C and expected to remain same at 25 °C. The equilibrium solubility is as low as 1.66±0.08 µg/ml. The DTX a derivative of it had a better solubility which may be due to modification at C10 and C13 [119,267].On the other hand the DTX still posses the very low equilibrium concentration (5.44±0.05 µg/ml). The change in pH did not alter the profile of solubility of PTX and DTX significantly. The p value in case of PTX was 0.70 and that of DTX is 0.90 respectively. So the non ionic surfactant tween 80, also called as polysorbate 80 which is widely used as solubilising agent is made used to alter the solubility profile. The Fig 18a and b shows the linear increase in aqueous solubility of PTX and DTX with increase in concentration of tween 80. Tween 80 being the mixture of polyoxyethylene 20 sorbita monoleates is a yellow viscous liquid at room temperature. It increases the wettability and surface availability of the drug to the medium. The wetting property is greatly influenced by the decreased interfacial tension between the medium and the drug [268]. The solubility profile of PTX and DTX was not determined in organic solvents like methanol, acetone, acetonitrile, DMSO, dicholoromethane and ethanol etc as it is reported that they are freely soluble in the organic solvents [269].

Incompatibility studies –FTIR:

The spectral analysis by peak matching technique indicated that the physical mixture of drug and polymer exhibit the specific functional groups with almost same chemical characteristics observed for pure drugs (DTX and PTX). The study suggests that the polymer of choice albumin, gelatin and hyaluronan do not bring the molecular changes of the drug with alteration in its molecular behaviour. The study was further supported by differential scanning calorimetry.

Incompatibility studies – DSC:

The isothermal stress testing (IST) involves the storage of drug-excipient blend with or without moisture at high temperature for a specific period of time normally a month to accelerate drug ageing and interaction with excipients [271]. For IST, the 5mg of the drug PTX and DTX were weighed into ria vial along with equal quantity of mixture of polymers. The mixture was vertexed for 2 min to ensure uniform mixing. The ria vials were than screw capped and stored at 40°C for a month. The pure drug and the pure polymers were stored separately as control at the normal room condition. Samples were examined periodically for any unusual colour change. After the test period the samples were taken for thermal analysis by DSC. The DSC thermogram of the DTX and PTX shows the endothermic melting peaks at 165-169°C and 220-221°C respectively. In all the drug-polymer blends, the melting endothermic peaks of DTX and PTX were well preserved with little change. The minor changes in the melting endotherm are attributed to mixing process which lowers the purity of each component but not a representative of incompatability. Slight variations in the peak shape and melting point may also be triggered by varying sample geometry [271]. In some cases, the shape of the DSC thermogram of the pure excipient differed from that of the mixture. This may be due to variations in the quantities of excipients used for the analysis. The peak shape and enthalpy depend on quantity of material used [272]. Therefore, peak transition temperatures were taken into consideration for DSC thermograms. The polymers did not show any characteristic endotherms, instead a broad endotherm was observed below 100°C which is attributed to evaporation of the absorbed water. As the melting endotherms of the drug (PTX and DTX) are well preserved in all the cases it is concluded that the drug is stable with the choice of polymer.

Process yield and pH:

The mean process yield was greater than 70% for all the batches. Though it sounds to be good percentage the loss of rest attributes the lacuna of complete collection of particles during the entire process of centrifugation, freeze drying and weighing. As the pH of all the formulations was almost neutral, the batches were considered to be non-irritant to the biological system. As the cytoplasmic pH of the tumour cells are slightly acidic around 6.8±0.1 the formulations were expected to deposit selectively at the site [273,274].

Particle size:

The batch A1 to A4 and G1 to G4 reported particle size of less than 100nm, which clearly specify the process adopted was suitable for preparation of nanoparticles. The system reported the polydispersity index from 0.124 to 1. A polydispersity index of 1 indicates large variation in particle size. As the size variation is reduced the polydispersity index decreases certifying the homogenicity of particle size distribution [275]. It was observed the particle size increased significantly ($p<0.001$) with increase in drug polymer ratio. This difference may be because of increased void space in central core of particle thus aimed to have higher entrapment of the drug.

The batches A5, A6, G5 and G6 reported the particle size greater than the rest of the batches on microscopic observation which may be due to resistance offered by the polymer hyaluronan which as a characteristic feature of forming gel with increase in concentration. According to USFDA, the term nanoparticle is applied to any particulate system lying within the size of 100nm [276]. The batches (A5, A6, G5 and G6) also showed separation of drug with appearance of needle shaped crystals which were predicted to be because of evaporation of solvent DCM before it comes in contact with the polymeric mixture due to its increased viscosity. Thus the formulations A5, A6, G5 and G6 were omitted for particle size measurement by Malvern Nano-ZS and also from further characteristic studies.

The size of particles also influences the targeting by avoiding the process of opsonisation by RES or kupffer cells as they lack sensing upon reduction of size below 100nm [277]. The other mechanism involved in better concentration of particles at the tumour site is; devoid of macrophages or RES or kupffer cells at the site compared to normal tissue. It was also observed that the metastatic lymph nodes lack macrophages potentiating the accumulation of particles with absence of clearance [278,279]. It's not only the size but also the unique

molecular pathology of the tumour vessels that attribute to the accumulation of nanoparticles at the tumour vesicle [257].

Zetapotential:

Even though there is no specific standard value for the zetapotential it is believed that the charge greater than ±20 provides the better stability to the nanoparticles. In the present case, the albumin formulations showed more or less nearer charge whereas the gelatin formulations had relatively smaller values. The lack of effect of charge can be explained by the presence of other ions (H^+, Cl^-, Na^+, and OH^-) in the medium, which can shield the charge on the particle surface [280]. Even the dispersion medium being acidic (pH 5.5-6.2) can attribute to the charge especially in case of gelatin.

The cellular surface being negatively charged generally bind to the cationic particles by electrostatic interaction but in most of the cases the binding is unspecific. The negatively charged particles are taken up by the cells and found to be superior in cellular uptake compared to the cationic particles [281]. Even earlier research has reported the negatively charged particles possess better half-life than the positively charged. It was also noted that the phagocyte uptake is more for strong negative charged particles [282]. Thus the anionic particles of our findings possessing the mild charge were aimed to target or exploit the cancer cells with better half-life and reduced phagocytic uptake.

Loading and entrapment efficacy:

The loading efficacy is an important parameter since higher drug entrapment could help to decrease the dose of nanoparticles used for further *in vivo* experiments. The entrapment efficacy is important in developing drug carriers since too low entrapment will prevent the mass production of drug loaded devices, especially for expensive drugs such as PTX and DTX. The high entrapment efficacy is attributed to the physicochemical characteristics of the drug. It is predicted that the high entrapment efficacy of the drug in the formulations is mainly due to diffusion of organic solvent leaving behind the drug in the polymer matrix on precipitation. It is also noted that increase in drug to polymer mass decreases the loading efficacy and increases the entrapment efficacy. The increased entrapment may be mainly because of availability of large molecule of polymer to interact with the drug. The increased polymeric concentration delays the drug diffusion through the polymeric membrane thus the

drug does not get escaped easily during the process and the entrapment efficiency remains high.

Study of crystallanity:

The crystalline form of the drug results in sharp endothermic peaks whereas the amorphous nature of it does not produce any endothermic peaks [283,284]. The absence of endothermic peaks both in case of PTX and DTX nanoparticles suggests the absence of crystalline drug in the nanoparticles, at least at the particle surface level. Therefore, it is concluded that the drug in the nanoparticles is in disordered crystalline phase of a molecular dispersion.

Solubility profile of nanoparticles:

The significant enhancement in solubility of drug in form of nanoparticles is attributed to its size which provides the better surface area for wettability of the drug. Even the disordered crystalline phase of the molecular dispersion may feature its solubility significantly at a confidence interval of 99.9% [285,286]. The volume of dissolution media is decided based on these studies. The study was carried only for batches with high drug entrapment assuming that the batches with low drug entrapment will be soluble at the set experimental conditions.

Surface Morphology:

The spherical particles play an important task in biological system, due to which the movement of them would be easier compared to non-spherical which may tumble in transportation in body. The irregularity in shape makes the particle to get filtered by liver or spleen as they get compounded at the site [277]. The spherical particles highlighted with recognizing moieties provide the better adhesion to the vascular cells because of their larger surface area and can also influence the internalization of particles [287]. Disparate to these the macrophage phagocytosis, the actin dependent uptake of particles by immune cells is undesirable but the hydrophilic nature of the particles can influence to overcome the phenomena of opsonisation [288]. Unlikely the spherical particle can also attribute to clearance of the drug due to shear stress exhibited by the blood flow because of their free flow nature. Irrespective to this shear stress the concentration of particles was high at the tumour site which may be due to high affinity of the particles to sensors present on the surface of tumour cells or due to their binding to the cellular receptors expressed by them in abundance.

Another important finding in the field of nanotechnology is the surface nature of the particles which dominate the property of wettability by altering the surface energy. It has been reported in earlier research works that the particles with smooth texture offer the better contact angle with water compared to the rough textured particles. Thus this may be one of the reasons for improved solubility of the drug in current findings [289]. Unlike, to the better contact angle the smooth textured particles result in stronger adhesion with higher surface energy [290]. Hence, the particles of our finding being smooth may feature the earlier results.

***In vitro* release:**

The release of drug is dependent on the various factors such as solubility of drug, desorption, drug diffusion, and particle matrix degradation or erosion etc. The slope of the appropriate plots provides the respective release constants and the Table 10 provides the regression coefficient (r^2) for various mathematical models. The curvilinear plot produced by the zero order kinetic's model suggests that the release process is dependent on the amount of the drug available for dissolution and diffusion. This is further confirmed by first order release kinetics with better r^2 value. From the poor r^2 obtained from the plot of higuchi it was difficult to judge the predominant process involved in release of drug is either diffusion oriented or not. Thus the Krosmeyer-peppas was applied to confirm the mechanism involved in the release. Data for the first 60% of drug release was plotted in Korsmeyer-peppas model. The model explains the release pattern based on the slope value obtained for the linear plots of release data.

The slope value of 0.5 suggests that the movement of the drug mainly takes up diffusion process (Fickian) and reverts to profile of higuchi (case I). For value of $n > 0.5$ and < 0.89 it is considered as non-Fickian or anomalous, the mass transfer of drug follows theory of combination of diffusion and erosion of polymeric matrix. In case II (zero order) the release is independent of concentration and the n takes a value of 0.89. If the $n > 0.89$, the molecule take a path of pure relaxation controlled delivery and is assigned as super case II transport [291].

Since the results showed the super case II transport with non-Fickian release behaviour, it is predicted that the release of PTX or DTX from the particles was controlled by a swelling of the polymer matrix especially hyaluronan followed by solvent penetration to diffusion the drug. Similar result was document for the release of insulin and it was seen that increase in ratio of hyaluronan provided the extended release of insulin [292].

Rani et al reviewed that in the swelling controlled release system the drug dispersed within a glossy polymer shows no diffusion initially upon contact with solvent system. As the solvent penetrates, the transition state of the polymer gets lowered due to relaxation of its chains due to which the drug diffuse out, which is further controlled by viscosity and the portion of glossy to rubbery nature of polymer [293].

As it is noted that not more than 10% of the drug is released at end of 2 hrs it is concluded that, most of the drug is entrapped within the particles. The behaviour is also found beneficial as route of administration takes i.v., during which the drug is not released into the saline and thus expected to get targeted at high concentration.

Since the release mechanism is controlled by the polymer relaxation, a model of Hixon-Crowel cube root law was applied to study the changes in surface area and diameter of the particles. The poor r^2 (< 0.97) calculated between the cube root percentage of the drug remaining in the system and time confirms not much change with the surface pattern of the particles confirming no noticeable changes occur in the size. Thus the size of particles is expected to be maintained for longer time in the biological system.

***In vitro* Cytotoxicity:**

From the results, it was evident that the PMF exhibited near 40% lower cytotoxicity compared to the pure drug and the value was significantly different ($p<0.01$). The large difference is mainly due to poor uptake of PMF by the ER negative MDA-MB-231 cells as they do not posses SPARC or osteonectin receptors [294]. But still the product exhibited the cytotoxicity which is expected due to its size which attributes to the penetration of it to interstitium of cell through leaky vasculature [295]. Whereas, designed dual ligand formulation A3 showed only 13.79% of difference compared to pure drug and its IC_{50} dose differed significantly compared to PMF ($p<0.01$).

The cytotoxicity results were correlated to mechanism of cellular uptake of formulations. The predicted mechanism of cell uptake by the formulations were pure diffusion in case of pure drug, penetration of leaky vasculature for PMF, receptor mediated endocytosis for A3 and G3 which contain HA as one of the component. The receptor mediated endocytosis is favoured by the presence of CD44 receptors on the MDA-MB-231 cellular surface. Though there was no significant difference in IC_{50} dose between A3 and G3, the A3 had statistical difference

($p<0.05$) with pure drug which was not seen with G3 (7.41% higher). Even in case of DTX formulations similar results were observed with p value of <0.05 in case of A4 and >0.05 in case of G4 compared to pure drug. The difference in percentage of cytotoxicity for A4 and G4 compared to pure DTX was +19.99 and +9.70 respectively.

For both PTX and DTX formulations; the gelatin incorporated batches were more potent than the albumin bearing batches. The difference is mainly because of cell surface expression of MMP-2 and MMP-9 metalloproteinases which can degrade the gelatin. The degradation of gelatin might have made the drug to diffuse intracellular at faster rate upon uptake [296,297]. The results also illustrates that the formulations of DTX possess high cytotoxicity compared to PTX with supporting the earlier research stating DTX is more potent than PTX. The potency exhibits significant difference ($p<0.05$) [298]. The precipitation of DMF during the experimental process, the reason beyond which remained unclear made us to omit the batch from the study.

Accelerated stability studies:

As discussed earlier with no significant change in the release pattern, the batches with high entrapment efficiency were considered for the stability studies. The stability of the pharmaceuticals remains one of the important criteria as it should reach the consumer in an active form. As the deterioration of the product due to its changes in physical, chemical and microbiological properties reduces the therapeutic value with increased toxicity, 90% of the label claim is the minimum acceptable potency level in general [299]. In case of nanoparticles, aside to its potency there is chance of increase in size due to agglomeration of particles. Thus size analysis as a part of stability study becomes the must. Since the batches are preferred to be stored at refrigerated conditions to avoid the degradation of body component hyaluronan, the accelerated stability studies were carried at room temperature. The application of One-Way ANOVA and student-t test for the results of the functions analyzed at point of time of analysis graded no significant difference ($p>0.05$) on comparison to initial data (Zero day). The non-significant difference in the results for the parameters analyzed, suggests that the batches of PTX and DTX are stable at the end of the study period even at accelerated condition.

Pharmacokinetic Studies:

The formulations are considered to be 100% bioavailable as they are given as i.v. injection. All the batches showed initial rapid decline in plasma concentration followed by slow elimination. A study reports initial decrease in the plasma concentration of PMF was significantly lower than the solvent-based PTX suggesting the faster distribution of PMF than of solvent-based PTX [300]. The batches of dual ligand therapy (A3 and G3) are expected to show the same effect with non significant C_{max} on application of One-Way ANOVA followed by Newman-Keuls multiple comparison test. The slight decrease in plasma concentrations in case of A3 and G3 with comparison to PMF suggested better distribution of them. The rate of distribution follow the order G3>A3>PMF. As it practically impossible to access the concentration of drug in body at zero time, the C_0 is calculated on extrapolation of plasma drug decay curve. Though the C_0 is greater than C_{max} it not assigned as maximum plasma concentration as it is a hypothetical value obtained by the software.

The dual ligand therapy batches (A4 and G4) had high significant difference in C_{max} compared to its respective marketed formulation (DMF). The difference correspondingly correlates the volume of distribution and rate of clearance. The better the C_{max}, smaller the volume of distribution with lower the rate of clearance. As expected the difference in rate of clearance affected the half life of drug significantly with inverse proportionality. The volume of distribution slightly lesser than the total body water offers the particle to remain in blood stream for longer time [301]. The high volume of distribution of DMF represents its distribution into tissues leading to toxicity.

The nanoparticle reduces the rate of metabolism and was predicted as one of the reason for statistical difference in half-life of the formulated batches of DTX on comparison to DMF [302]. The statistical difference in half-life between DMF and A4 was pragmatic on application of tool student-t test (Unpaired – two tailed) with p value <0.05. The similar results were documented between the batch A4 and G4. The scrutiny between the batches DMF and G4 reported more predominant significant difference with $p<0.001$. The reason behind the non-significant report by statistical tool One-Way ANOVA for the half-life of the batches DMF, A4 and G4 might be the huge range of experimental findings, the explanation beyond which remains mysterious. The significant change in MRT and the $AUC_{0-\infty}$ suggests that the formulations are stable in plasma for longer time with orientation to antitumour activity.

The half-life of the PTX is mainly dependent on the rate of metabolism [303]. The significant difference in half life of A3 and G3 on comparison to PMF indicates that their stability in plasma for longer time. This may favour the pharmacological activity with ability to orient towards site of action [304]. The statistical difference in the elimination rate constant suggests the release of drug is controlled, thereby avoiding its affect on normal tissue. The slow release of drug from the polymeric matrix due to resistance offered by the HA in case of A3 might have delayed its elimination. The *in vitro* release studies suggest the gel like consistency of gelatin further delay the release with supplement to HA resistance. The delayed half-life is accomplished with enhanced release of drug at the tumour site. This reduces the exposure of formulations to normal tissues which are potentially associated with a more favourable toxicity profile for conventional formulations [262].

The total body clearance (CL) of the drug refers to the overall elimination rate comprised of sum of all elimination processes occurring in all organs in all its forms (metabolites or free drug) [305]. The CL of PTX decreased with increase in V_d. The odd findings may recommend the nonlinear pharmacokinetic profile of PTX [306]. However, the results were supportive to the recorded half-life, that is lower the clearance better the half-life. Though there was no significant difference in the AUC_{0-24}, the relatively lower value reflects improved tissue distribution of the G3 and A3 [307]. The prolonged MRT indicated that the A3 and G3 possessed the long-circulating property with slow elimination than PMF, which may favour to target the tumour sites. The significant increase in the $AUC_{0-\infty}$ for the batch A3 and G3 may be due to the sustained release of PTX and the decrease in time-dependent excretion from the body. The prolonged circulation in the blood compartment strongly suggests that A3 and G3 are capable of avoiding reticuloendothelial system uptake. The similar findings were observed in case of A4 and G4 on comparison to DMF [308]. The G3 had better circulation than the A3 with p value of <0.01. The prolonged circulation may enhance the permeability and retention effect in solid tumours contributing to the improved tumour activity [309]. The prolonged circulation of formulations may also impart the antitumour efficacy in cancer by passive targeting. Many studies have shown the polymeric carriers with average size below 100nm have high potential for prolonged circulation in the blood followed by accumulation at tumour site [310,311].

Biodistribution:

Initially 10 animals per group (six groups) of analysis were taken for induction of cancer. The animals of each group had irregularity in development of cancer with only 36 animals (overall) developing cancer. The reason beyond the irregularity in development of cancer attributes the immune system of animal. The animals in which cancer was not developed were discarded from the investigation. These 36 animals were grouped into 6 groups and were treated with formulations similarly as mentioned in pharmacokinetic studies.

The distribution of PMF, A3 and G3 to liver had no significant change. The relative low concentration of G3 followed by A3 and PMF suggests the reduced rate of metabolism (dependent on release rate of drug) which might have enhanced the half-life of the formulations. Similar findings were observed for DTX formulations with minimum concentration of G4 followed by A4 and DMF. The difference in concentration of A4 and DMF, and G4 and DMF was significant enough. The statistical difference may also be due to low surface charge of particles with high hydrophilicity which evade the recognization of liver macrophages and thus reduced the hepatic deposition [96,312].

Correspondingly in spleen there was no significant difference for PMF, A3 and G3 at 1st hr which was observed in case of DTX but at 2nd hr the changes were observed which may be due to macrophage uptake. A high relative concentration of formulations in spleen was considered to be consequence of prolonged systemic circulation of formulations. Similar observation was made by Sheng et al 2009 for PLA-PEG nanoparticles [313]. It was also observed that the PMF concentration varied significantly with time and may be due to enhanced uptake of it followed by additional deposition. The larger particle size of the formulation PMF (approximately 136nm) compared to A3 and G3 (< 100nm) might have led to improved uptake [314]. The concentration of A4 and G4 batches in spleen was remarkable compared to DMF, but still the batches had better circulation indicating that liver plays major role in metabolism of drug.

The study reports the peaked high concentration for A4 and G4 in lungs at 1st and 2nd hr on comparison to DMF whereas the in case of PTX formulations concentration of A3 and G3 peaked only at 1st hour because of its significant drop in concentration at 2nd hr compared to PMF. The relative deposition in lungs might be due to high solute permeability, large surface area, and limited proteolytic activity [315]. The bioadhesive property of hyaluronan might have rendered the resistance to the significant drop in concentration of A3, A4, G3 and G4 at 2 hr as that of PMF. However it does imply the release of drug as it is expected to be cleared with time.

In kidney the distribution of A3 and G3 was significantly different on comparison to PMF which could be due to driven force of it for elimination of polymer (hyaluronan, albumin and gelatin). The prolonged half-life of the formulations suggests that the driven force is not strong enough to evade these polymers. As they are bound intact with each other and drug expected to remain unharmed and undergo recirculation in blood stream.

The DTX formulations (A4 and G4) significantly peak lower concentration than DMF suggesting that the particles are flushed at greater rate in heart and thereby expected to have reduced drug-associated cardiac toxicity [316]. The concentration of all the nanoformulations was almost same. The brain penetration can be considered almost nil however a slight increase in penetration of A4 and G4 compared to DMF is expected because of the nanostructure. The difference in penetration was not noteworthy.

Apart from the above findings the significant change in concentration of drug was observed at tissue (tumour) of interest. In case of PTX, the dual ligand drug delivery system (A3 and G3) had enhanced the concentration of drug momentously. Though the concentration of drug (in case of A3 and G3) falls geometrically at 2^{nd} hour it was still at higher concentration compared to its single ligand drug delivery system (PMF). The difference in concentration of A3 and G3 with time may be due to recirculation of particles upon blockage of all binding sites like osteonectin, MMP, and CD44 receptors. In case of DTX batches the change in concentration with time was not noteworthy between A4 and G4 even though the statistical differences were reported at 1^{st} and 2^{nd} hour on comparison to DMF. This property of difference in PTX and DTX concentration in A3, G3, A4 and G4 at tumour site with respect to time can be correlated to their dose of administration. The dose of DTX was relatively half of PTX and thus the concentration of dual ligands were expected to be half which might have brought the no significant change in concentration of drug with time.

The bio-distribution assessment suggests that the dual ligand drug delivery formulations A3, G3, A4 and G4 possessed higher tumour uptake. This may count for the better therapeutic efficacy than their respective commercial batches. The release of drug is expected to occur directly in the cancer cells because of the structural changes of receptors present on cancerous cell surface [111]. Despite the fact that the MMPs are stimulated by presence of SPARC [317] the results show that the G3 and G4 formulations are trapped at higher concentration than A3 and A4. The mechanism beyond this remained unclear with no noteworthy difference. The effect of hyaluronan is considered to be equally effective in all the formulations of study as the concentration is maintained at same proportion.

Summary

SUMMARY

The section outlines the concept, techniques, and outcome of the entire dissertation work to gist the reader in short period of time.

Cancer literally meaning tumour in medical usage has to be curbed soon. Among the various types of cancers the breast tumour is commonly diagnosed in women but can also occur in men. The treatment of breast cancer involves several therapies like surgery, radiation, hormonal, biological, targeted, and chemotherapy. Generally the chemotherapy applied has adjuvant to all the other therapies make use of drug or combination of drugs called cytotoxic agents. These drugs act by killing the cells but they cannot differentiate the cancerous cells from the normal ones, thereby leading to adverse effects with minimized antitumour efficacy.

For the past few decades the attempts are been made to enhance the antitumour efficacy of these cytotoxic agents by application of novel drug delivery systems like liposomes, quantum dots, carbon nanotubes and nanofibres, microspheres, nanocrystals, and nanoparticles etc. Among these systems the polymeric nanoparticles are widely investigated for their several advantages like a) enhanced drug therapeutic efficacy; b) increased local concentration at site of action; c) improved solubility of poor water soluble drugs; d) modified pharmacokinetics; e) prolonged half-life of drug; f) diminished metabolism; and g) controlled or sustained release etc.

The concept beyond the application of polymeric nanoparticles is usage of polymers that posses affinity to the receptors expressed by cancerous cells. Though the research had revealed several receptors and attempts are made to target these receptors no or very less effort has been made to develop dual ligand nanoparticulate system. The dual ligand nanoparticulate system sounds beneficial in cancer treatment as the cancerous cells possess multi-functional characteristics. Thus in the present work an attempt was made to develop dual ligand nanoparticulate system of paclitaxel (PTX) and docetaxel (DTX) using a natural occurring biocompatible, biodegradable, and hydrophilic polymers like albumin, gelatin, and hyaluronan (HA). In this system simultaneous targeting of CD44 receptors and SPARC protein by HA and albumin is expected whereas in case of HA-gelatin combination the CD44 receptors and MMPs are the targets.

With the extensive literature survey the preformulation studies was carried out initially. As a part of development and manufacture of formulations, the analytical and bio-analytical method was developed using UFLC, which worked as normal HPLC as the conditions of HPLC was maintained. Both the analytical and bio-analytical methods were suitable for estimation of drug without any interference of polymer or plasma components at wavelength of 227nm. The C_{18} column was used as stationary phase with mixture of ACN and 0.1% TEA (pH 3.5) as mobile phase. The ratio of mobile phase ACN: 0.1% TEA was maintained at 70:30 and 54:46 v/v for analytical and bio-analytical respectively. The curve of linearity was developed for both analytical and bio-analytical method by plotting AUC versus concentration and response factor versus concentration respectively.

The solubility study of the drug (PTX and DTX) was carried at 37±2°C in water, phosphate buffer saline pH 7.4 and buffer with tween 80. The concentration of tween 80 was varied from 0.1% to 1% w/v. Both the drugs were practically insoluble in water and buffer but the solubility was enhanced upon addition of tween 80. The PTX solubility in buffer was enhanced nearly by 11.5 times in presence of 1% w/v tween 80. The DTX solubility was increased by 5.5 times in presence of 0.5% w/v tween 80.

To study the mode of compatibility between drug and polymer of choice, the FTIR and DSC was applied after storing the physical blend of drug and polymer at isothermal stress conditions. The study revealed the polymers were compatible with drug. With acceptable preformulation parameters the dual ligand nanoparticulate system (HA-Albumin and HA-Gelatin) of PTX and DTX were formulated by simple precipitation cum solvent evaporation technique. Various batches from A1 to A6 and G1 to G6 were prepared to understand the process parameters. The A1, A3 and A5 represent HA-Albumin PTX formulations. The A2, A4, and A6 represent HA-Albumin DTX formulations. Similarly the G1, G3, and G5 represent the HA-Gelatin PTX formulations and the HA-Gelatin DTX formulations are symbolized as G3, G4, and G6.

The so obtained formulations were subjected to *in vitro* characteristic studies like the process yield, pH, particle size, zeta potential, entrapment efficacy, crystallanity, solubility, surface morphology, release pattern, cytotoxicity, and accelerated stability studies. As a part of *in vivo* characterization the pharmacokinetic and bio-distribution studies were conducted and compared with respective commercial samples.

The process yield for all the batches was more than 70% with pH ranging from 6.8-7. The particle size of batch A5, A6, G5 and G6 was not recorded as they showed separation of drug on microscopic observation. The rest of the batches recorded the particle size below 100nm (as per USFDA regulations for nanoparticles) ranging from 23.27 to 69.28nm on an average.

The zeta potential for HA-Albumin batches ranged from -17.8 to -18.6 whereas for HA-Gelatin batches it was -12.9 to -13.7. The entrapment efficacy for HA-Albumin nanoparticles increased by 22 to 23% on change in ratio of drug: polymer to 1:2 from 1:1. With the similar changes the HA-Gelatin formulations increased the entrapment efficacy by 21 to 25.85%.

The study of crystallanity by DSC revealed that drug was present in disordered phase in nanoparticles. The nanoparticle formulations significantly enhanced the solubility of drug and the particles were found to be almost spherical in shape. The release pattern of drug from formulations was carried by dialysis bag method at 37±2°C using 50 mL of phosphate buffer pH 7.4 containing 1% w/v tween 80 as the media for PTX formulations. In case of DTX the concentration of tween 80 was reduced to 0.5% w/v. The media was stirred at rate of 50 rpm. At the end of 24 hr the release of drug from A1, A2, A3, A4, G1, G2, G3, and G4 batch was 87.75, 92.85, 84.67, 84.28, 72.46, 81.31, 63.80 and 71.68 % respectively on an average of three observations.

To understand the release behavior, mathematical models like Zero order, First order, Higuchi, Peppas and Hixson-crowell study designs were applied. The release pattern of drug from formulations followed super case II transport in non-fickian manner. As there was no significant change in percentage release of drug at the end of 24 hr with change in drug polymer ratio the A3, G3, A4 and G4 batches were selected for further studies as they showed high drug entrapment efficacy.

The *in vitro* cytotoxicity on MDA-MB-231 cell lines for A3, G3, A4, and G4 batches was carried by SRB-assay. The IC_{50} value for pure PTX, PMF, A3 and G3 was 97.66, 162, 120, 109.66 ng/ml on an average whereas for DTX, A4, and G4 it was 75.33, 94, 84.33 ng/ml respectively. The IC_{50} value for DMF was not determined as it was precipitating during the process of analysis. As the formulations are recommended to refrigeration during storage the accelerated stability studies were carried at 25±2°C at 60±5% RH. The entrapment efficacy, pH, average particle size, cumulative % release were determined as function of analysis to

study the stability of formulations. There was no significant change in results of above parameters even at the end of 6 months on comparison to their day zero values. Thus formulations are termed to be stable.

The female SD rats of 8 weeks old weighing 200±20g were dividing into six groups each carrying six animals for investigation of pharmacokinetic profile of formulations on single i.v injection of PMF, A3, G3, DMF, A4, and G4 respectively. The dose of PTX formulations was 45mg/kg and that of DTX formulations was 17mg/kg. The C_0, C_{max}, T_{max}, $T_{1/2}$, AUC_{0-24}, AUC_{0-inf}, K_e, V_d, CL and MRT were evaluated for all the formulations from their drug plasma decay curves on application of statistical tool winNonlin version 5.

The C_{max} was insignificant in case of PTX formulations but the A3 and G3 had significant difference in their half-life, AUC_{0-inf}, CL, V_d, and MRT on comparison to PMF values. The half-life of PMF, A3, and G3 was 16.71±9.2, 36.21±11.26, 69.34±18.61 hr respectively. The same parameters were calculated for DTX formulations and all the parameters were statistically different for A4 and G4 on comparison to DMF. The half-life of DMF, A4 and G4 was 14.80±6.42, 38.91±21.25, 54.79±59.08 respectively.

Similar grouping was made for bio-distribution studies in NMU induced breast cancerous SD rats with each group carrying 6 animals. The animals were treated at similar dosage as mentioned in Pharmacokinetic studies. At the end of 1st and 2nd of administration of formulation the three animals of each group were sacrificed and various organs like tumour, kidney, liver, heart, lungs, brain and spleen was collected and estimated for concentration of drug. The dual ligand drug delivery system had enhanced the concentration of drug at tumour site significantly thereby account the better therapeutic efficacy of PTX and DTX.

Conclusion

CONCLUSION

The section states our opinion on this dissertation work titled **Nanoparticles Mediated Targeted Drug Delivery System of Some Antineoplastic Agents for the Treatment of Breast Cancer with regard to its aim and objective**.

We conclude the work as below;

- The various batches of dual ligand nanoparticulate drug delivery system (Hyaluronan-Albumin and Hyaluronan-Gelatin) of paclitaxel (A1, A3, G1, and G3) and docetaxel (A2, A4, G2, and G4) were successfully developed by simple precipitation cum solvent evaporation technique for better treatment of breast cancer.

- All the batches were characterized for process yield, particle size, surface charge and morphology, loading and entrapment efficacy, solubility and crystallanity phase, and *in vitro* release pattern. Based on *in vitro* release profile and entrapment efficacy the A3, A4, G3, and G4 were subjected for cytotoxicity and stability studies. The pharmacokinetics and biodistributions studies were conducted for A3, A4, G3 and G4 batches for their *in vivo* characterization followed by comparison with their respective commercial batches.

- With process yield of more than 70% the formulations were almost neutral (pH 6.8-7) and thus remain non-irritant to biological system with selective deposition at the site of action.

- All the batches from A1 to A4 and G1 to G4 suits the term “nanoparticle” defined by USFDA.

- The mild negative charge of particles might have exploited them to cancerous cells with better half-life and reduced phagocytic uptake.

- The loading efficacy decreased with enhancement in entrapment efficacy on increase in drug to polymer mass ensuring poor escape of drug from particulate system.

- The disordered crystalline phase (amorphous) of drug certified by absence of DSC endothermic peaks ensures nil or minimum concentration of drug on the surface of particles.

- The nanoparticulate system enhanced the solubility of drug by providing better surface area for wettability of molecule with disordered crystalline phase.

- The spherical shape of the particles might have rendered the reduction in filtration of particles by liver. The smooth texture of the particles might have provided additional benefit to solubility profile by providing better contact angle with the solvent of choice.

- The rate of metabolism of drug is expected to be minimized as the concentration of nanoparticulate system is significantly lower in liver. The low surface charge and the hydrophilicity of the nanoparticle might have contributed to reduced hepatic deposition and may also reduce the immunogenicity.

- As the formulations are target specific the natural cytotoxicity lead by drug can be minimized.

- The half-life of the formulations was significantly enhanced and was found focused to the site of action and thus expected to have prolonged action at the site with delayed release of drug as polymers retard the discharge of drug molecule.

- As the formulations were found to be less deposited in heart and showed poor permeability to brain, any drug related CVS and CNS side effects could be minimized.

- As the formulations are free of cremophor-EL and tween 80, the excipient associated side effects of the currently available conventional formulations can be prevailed.

Overall, the dual ligand nanoparticulate drug delivery system HA-albumin and HA-gelatin nanoparticles of PTX or DTX significantly gets targeted to the tumour site with prolonged half-life and better solubility. The simultaneous blockage of CD44 receptors and SPARC protein by nanoparticles containing HA and albumin might have influenced the significant change in their concentration at tumour site. In case of formulations containing combination of HA and gelatin, the MMPs are the target molecules along with CD44. The gelatin focuses these MMPs. Thus the formulations are expected to have better therapeutic efficacy in treatment of breast cancer. However, further research at the pre-clinical or clinical level is in need to understand the status of any synergistic action involved as the hyaluronan can readily retard the growth of cancer. The gelatin given as external source may also attribute to the synergism as degradation of extravascular component is prevented. Further investigations on tumour reduction efficacy, survival rate, and targeting efficacy towards various cell lines (*in vivo*) etc may orient these dual drug delivery systems to commercial application.

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ANNEXURE I – LIST OF ABBREVIATIONS

%	Percentage
°C	Degree centigrade
µl	Microlitre
ACN	Acetonitrile
ANOVA	Analysis of Variance
AUC	Area under curve
BC	Breast cancer
BCS	Biopharmaceutics Classification System
cm	Centimetre
CNS	Central Nervous System
CVS	Cardiovascular System
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
DSC	Differential Scanning Calorimeter
DTX	Docetaxel
DMF	Docetaxel marketed formulation
EDTA	Ethylenediamine tetraacetic acid
ER	Estrogen
FTIR	Fourier transform infra red
g	grams
h	hour
HA	Hyaluronan
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HPLC	High performance liquid chromatography
i.v	Intravenous
IC	Inhibitory Concentration
IST	Isothermal stress testing
KBr	Potassium bromide
KDa	Kilo Dalton
Kg	Kilogram

m^2	Meter square
mcg or µg	Microgram
µl	microlitre
mM	milli molar
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
MMP	Matrix metalloproteinase
PTX	Paclitaxel
PMF	Paclitaxel marketed formulation
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometer
NMU	N-Nitrosomethyl Urea
p value	Probability factor
r^2	Regression coefficient
RH	Relative humidity
rmp	Rotation per minute
SD	Standard deviation
SEM	Scanning Electron Microscopy
SPARC	Secreted protein acidic and rich in cysteine
SRB	Sulforhodamine B
TEA	Triethyl amine
UFLC	Ultra fast Liquid Chromatography
UV	Ultra violet
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight
$ZnSO_4$	Zinc Sulphate

ANNEXURE II

J.S.S. College of Pharmacy, Ootacamund, Tamil Nadu, India.
Committee for the Purpose of control and Supervision of Experiments on Animals (CPCSEA)
Institutional Animal Ethics committee (IAEC).

CERTIFICATE

Title of the Project: Nanoparticles Mediated Targeted Drug Delivery System of Some Neoplastic Agents for the Treatment of Breast Cancer.

Proposal Number: JSSCP/IAEC/PH.D/PH-CEUTICS/02/2009-2010.

Date received after modification (if any):

Date received after second modification: 22.09.09

Approval date: 30.09.09

Animals: ~~Wistar rats~~/ Albino mice
SD.RATS Rabbits / Guinea pigs

No. of animals sanctioned: 48

Male/Female

Expiry date (Termination of the Project): 3MONTHS.

Name of IAEC/CPCSEA chairperson: **Prof. K. Elango**

Signature of Chairperson
Date:

(Prof. K. Elango)
Chairperson
Institutional Animal Ethics Committee
JSS College of Pharmacy
Rocklands, Ooty-643 001

ANNEXURE III – PATENTS, PUBLICATIONS AND PRESENTATIONS

Patent: Filed

1. **Compositions and methods of delivery pharmacological agents. Application No: 3667/CHE/2010., dated: 06/12/2010., Govt of India.**

Publications:

1. **N Manjunatha,** G Prakash Naidu, Vasanti Sutrave, Kalpesh Patel, MK Samanta. Preparation and Evaluation of Liposomes of an Antiviral Drug. Ind J Novel Drug Del. 2009;1(1):25-31.
2. SB Chandramohan, **N Manjunatha**, Kalpesh Patel, MK Samanta, Shamala Bhaskaran. Design and Development of Oral Sustained *In Situ* Gelling System of Famotidine. Ind J Novel Drug Del. 2009;1(1):42-46.

Communicated Papers:

1. Consecutive assessments of Taxanes by Prominence UFLC.
2. Establishment of PK parameters for Herbal Extract of *Atropa Belladona.*
3. Caffeine PK Arbitration in Tablet Dosage Form of Extract of Green Tea Leaves and Analogy with Synthetic Form
4. Establishment of Pharmacokinetic Parameters forthe Herbal Drug Containing Forskolin

Presentations:

1. Design and Development of Triphala Fast Dispersable Tablets and its Characterization. Rizwanbasha K, Shanmukha Srinivas M, Kanwale S Ramrao, **Manjunatha N**, Senthil V, Samanta MK. International Conference on Herbal Drugs; Present and Future Prospects. 4-5 Dec 2009,

2. Effect of Various super disintegrants and a comparative study of formulation technology for design and development of Triphala dispersable tablets. Shanmukha Srinivas, Rizwanbasha K, **Manjunatha N,** Santosh K Ramrao, Ganesh GNK, Samanta MK. International Conference on Herbal Drugs; Present and Future Prospects. 4-5 Dec 2009.

3. Effect of superdisintegrant on the release rate of drug from MCC pellets and its PK study. **Manjunatha Narayanappa**, Nilkant More Shivajirao, Samanta MK. Polymer congress, APA-2009. Dec 17-20, 2009. New Delhi, India. Organised by Asian Polymer Association, SCS Division of Polymers and Colloids, IIT Delhi, Indonesian Polymer Association, European Polymer Federation.

4. Formulation and evaluation (in vitro and in vivo) of oral control release theophylline gastroretention mucoadhesive tablets. Rizwanbasha K, Senthil V, Suresh Kumar R, Nagaswami Venkatesh, Ganesh GNK, **Manjunatha N.** Abstract code: PP069. International Conference on Drug Delivery 2010. 29th – 30th Jan, 2010. Co sponsored by Department of Science and Technology, Department of Biotechnology, Council of Scientific and Industrial Research, Indian Council of Medical Research. Organised by PSG College of Pharmacy, Coimbatore, Tamil Nadu, India.

5. Solubility Enhancement and Pharmacokinetic Evaluation of Forskolin in tablet formulation from herbal extract. Tiwari Sindhu, **Manjunatha N,** Rathi Varun, Pathak Deepa, Prasad Vishnu, Samanta Malay K. Abstract No: PP 130. International Conference on Drug Delivery 2010. 29th – 30th Jan, 2010. Co sponsored by Department of Science and Technology, Department of Biotechnology, Council of Scientific and Industrial Research, Indian Council of Medical Research. Organised by PSG College of Pharmacy, Coimbatore, Tamil Nadu, India.

6. Design and Evaluation of Novel Drug Delivery System of a Proton Pump Inhibitor. Narendra Babu Bhavana, CVVC Nagaraju, **Manjunatha N,** Nagabasayya R Chikkamath. Abstract no: PP 270. International Conference on Drug Delivery 2010. 29th – 30th Jan, 2010. Co sponsored by Department of Science and Technology, Department of Biotechnology, Council of Scientific and Industrial Research, Indian Council of Medical Research. Organised by PSG College of Pharmacy, Coimbatore, Tamil Nadu, India.

7. Transdermal Therapeutic System for Novel Non-Steroidal Anti-inflammatory Drug – "Design and Standardisation. Pankaj Masih, Srinivas Murthy Sammata, **Manjunatha N,** Vasanti S, Samanta MK. Abstract code: PP 354. International Conference on Drug Delivery 2010. 29th – 30th Jan, 2010. Co sponsored by Department of Science and

Technology, Department of Biotechnology, Council of Scientific and Industrial Research, Indian Council of Medical Research. Organised by PSG College of Pharmacy, Coimbatore, Tamil Nadu, India.

8. Biodistribution and Antitumor activity of PLGA anchored Magnetic Nanoparticles of Paclitaxel. **Manjunatha N,** Kalpesh Patel, Sarika N, Samanta MK. Abstract code: PP 047. International Conference on Drug Delivery 2010. 29th – 30th Jan, 2010. Co sponsored by Department of Science and Technology, Department of Biotechnology, Council of Scientific and Industrial Research, Indian Council of Medical Research. Organised by PSG College of Pharmacy, Coimbatore, Tamil Nadu, India.

9. Studies on Formulation and In Vitro Evaluation of Mucoadhesive Famotidine Microcapsules. Shashanka S, Nandhakishore P, Jawahar N, **Manjunatha N.** Abstract code: PP 185. International Conference on Drug Delivery 2010. 29th – 30th Jan, 2010. Co sponsored by Department of Science and Technology, Department of Biotechnology, Council of Scientific and Industrial Research, Indian Council of Medical Research. Organised by PSG College of Pharmacy, Coimbatore, Tamil Nadu, India.

10. Development and Evaluation of Hyaluronan-Albumin Nano Aggregates of Paclitaxel. **Manjunatha Narayanappa**, Kalpesh Patel, Rizwan Basha K, Anil Dubala, Malay K Samanta. 2010. 3rd Summer School on Nanotechnology in Advanced Drug Delivery. 23rd – 27th Aug, 2010. NIPER.