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Preparation, Characterization And Anti-cancer Screening Of Pectin Coated PTX-Chitosan Nano-Lipid-Construct For Management Of Liver Cancer.

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Abstract
Hepatocellular carcinoma is the most common type of primary liver cancer. Liver cancer happens when liver cells develop mutations in their DNA. Paclitaxel is a natural diterpenoid compound with antitumor activity. The main objective of study was to preparation, characterization and anticancer screening of pectin coated Paclitaxel-chitosan nano-lipid-construct for the management of liver cancer. In this study, we performed physicochemical characterization of the drug and pre-formulations study, and prepared and optimized pectin coated nano lipid formulation. Nano lipid particles were characterized by size and shape, scanning electron microscopy, particle size distribution, Zeta potential measurement, entrapment efficiency and ex-vivo study in HeLa cells. The pectin coated PTX-chitosan NLCs (P-PCNLCs) has successfully prepared by Ion gelation method and characterized for the particle size, zeta-potential, polydispersity index and surface morphology. The P-PCNLCs having the particle size 180.9±.12 nm that is suitable for the active targeting towards the cancer sites as well as for the engulfment by the carrier protein present in cell surface. The entrapment efficiency of the particle was 86.23±0.32%. The ex-vivo study data are in agreement with strategies that active targeting provides the promising result of cure and treatment of cancerous cell. The cytotoxicity of P-PCNLCs was significantly increased compared to PTX. Nano-lipid-construct exhibited a remarkably higher uptake in the HeLa cells compared to that of free drug (PTX).The nanoscale effect and targeting effect of P-PCNLCs could increase the drug accumulation in tumor cells, which leads to high drug concentrations in tumor cells and strongly inhibiting cell proliferation.

Key words: Liver cancer; Nano lipid particles; Paclitaxel; Ex-vivo study; Zeta potential measurement; Electron microscopy.

Introduction

Liver cancer is the growth and spread of unhealthy cells in the liver. Cancer that starts in the liver is called primary liver cancer. Cancer that spreads to the liver from another organ is called metastatic liver cancer. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults, and is the most common cause of death in people with cirrhosis. It occurs in the setting of chronic liver inflammation, and is most closely linked to chronic viral hepatitis infection (hepatitis B or C) or exposure to toxins such as alcohol or aflatoxin. Certain diseases, such as hemochromatosis and alpha 1-antitrypsin deficiency, markedly increase the risk of developing HCC. Metabolic syndrome and NASH are also increasingly recognized as risk factors for HCC. As with any cancer, the treatment and prognosis of HCC vary depending on the specifics of tumor histology, size, how far the cancer has spread, and overall health[1].

The vast majority of HCC occurs in Asia and sub-Saharan Africa, in countries where hepatitis B infection is endemic and many are infected from birth. The incidence of HCC in the United States and other developing countries is increasing due to an increase in hepatitis C virus infections. It is more common in males than females[2]. The liver is a reddish-brown, wedge-shaped organ with four lobes of unequal size and shape. A human liver normally weighs approximately 1.5 kg (3.3 lb), and has a width of about 15 cm (6 in). It is both the heaviest internal organ and the largest gland in the human body. Located in the right upper quadrant of the abdominal cavity, it rests just below the diaphragm, to the right of the stomach and overlies the gallbladder. The liver is connected to two large blood vessels: the hepatic artery and the portal vein and common hepatic duct. The hepatic artery carries oxygen-rich blood from the aorta via the celiac plexus, whereas the portal vein carries blood rich in digested nutrients from the entire gastrointestinal tract and also from the spleen and pancreas. These blood vessels subdivide into small capillaries known as liver sinusoids, which then lead to lobules. The liver is made up of many different cell types.

Hepatocytes represent 60 % of the liver‘s cells and about 80 % of the liver ‘s total cell mass. Most of the liver‘s synthetic and metabolic capabilities stem from the work of hepatocytes. The main function of hepatocytes is to participate in lipid, carbohydrate and protein metabolism. They also produce serum proteins such as albumin and coagulation factors. Furthermore, hepatocytes produce and secrete bile as well as detoxify and excrete cholesterol, steroid hormones and xenobiotic drugs. Numerous xenobiotics are metabolized by the mixed functions of mono-oxidases found in hepatocytes.

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Hepatocellular carcinoma, like any other cancer, develops when epigenetic alterations and mutations affecting the cellular machinery cause the cell to replicate at a higher rate and/or result in the cell avoiding apoptosis. In particular, chronic infections of hepatitis B and/or C can aid the development of hepatocellular carcinoma by repeatedly causing the body's own immune system to attack the liver cells, some of which are infected by the virus. Activated immune-system inflammatory cells release free radicals, such as reactive oxygen species and nitric oxide reactive species, which in turn can cause DNA damage and lead to carcinogenic gene mutation. Reactive oxygen species also cause epigenetic alterations at the sites of DNA repair).

While this constant cycle of damage followed by repair can lead to mistakes during repair, which in turn lead to carcinogenesis, this hypothesis is more applicable, at present, to hepatitis C. Chronic hepatitis C causes HCC through the stage of cirrhosis. In chronic hepatitis B, however, the integration of the viral genome into infected cells can directly induce a non-cirrhotic liver to develop HCC. Alternatively, repeated consumption of large amounts of ethanol can have a similar effect. The toxin aflatoxin from certain Aspergillus species of fungi is a carcinogen and aids carcinogenesis of hepatocellular cancer by building up in the liver.

Various new approaches have been developed for the treatment of liver cancer such as ligand-based targeting on receptors. For targeted drug delivery, various carrier systems can be used such as liposome, nano lipid construct, solid lipid nanoparticle and nanosphere etc. Carrier system can be used to target the receptor or disease site and reduced the dose of drugs and side effects of drug, which improved drug efficacy and bioavailability and also improved the pharmacokinetics and pharmacodynamics property.

Paclitaxel is an important new anti-neoplastic agent that has generated a considerable amount of scientific interest from many disciplines since it entered clinical trials in the early 1980s. It is a natural diterpenoid compound with antitumor activity. It is the number one anti-cancer drug in the battle of 'we human' against cancer as it is an anti-microtubule agent. It constitutes about 22% of all major cancer chemotherapeutic drugs in the world market. Paclitaxel success is largely due to its unique mechanism of action tubulin polymerization and its excellent ability to work in combination with other anticancer agents.

Materials and methods

Pre-formulation Studies of paclitaxel

Pre-formulation studies are needed to ensure the development of a stable as well as therapeutically effective and safe dosage form. It is a stage of development during which the physicochemical properties of drug substances and its interaction with various formulation components was checked for the assessment.

Identification of Drug

Physical Appearance

The drug paclitaxel gifted from Neon Laboratories, Mumbai. The physical characteristics was reported in Table 1.

Table 1 Physical Appearance

Physical Appearances (Drug- Paclitaxel)	
Color	White to Off-white
Odor	Odorless
State	Crystalline powder
Hygroscopicity	Non-hygroscopic

Melting Point

A capillary melting point apparatus was used for melting point determination of Paclitaxel after filling the drug powder in a capillary and then capillary tube (heat-sealed at one end).

Solubility study

The spontaneous interaction of two or more substances to form a homogeneous molecular dispersion is called as solubility in the solvent.

Table 2: Solubility profile of paclitaxel

S. No.	**Solvent**	**Solubility**
1.	Distilled Water	**----**
2.	PBS (pH 7.4)	**----**
3.	Ethanol	**++++**
4.	Chloroform	**++++**
5.	Diethyl ether	**++**
6.	Dimethyl sulfoxide(DMSO)	**++++**

++++: Very Soluble (1-10 parts of solvent); +++: Soluble (10-30 parts of solvent); ++: Sparingly soluble (30-100 parts of solvent); ------Insoluble (<10, 000 parts of solvent)

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Partition Coefficient studies

The partition behavior of drug was examined in n-Octanol: Water, and n-Octanol: PBS (pH 7.4) systems.

$$\textbf{Partition coefficient (K)} = \frac{\textbf{Concentartion of drug in organic phase}}{\textbf{Concentartion of drug in aqueous phase}}$$

Table 3: Partition coefficient of paclitaxel

Solvent system	Partition coefficient
n- Octanol: PBS (pH7.4)	3.78
n- Octanol : Water	3.70

Determination of λmax

UV-visible spectrophotometry has been used as a tool for identification of various drugs to obtain specific information related to chromophoric part of the molecule**.**

Absorbance maxima (λmax) of paclitaxel PBS 7.4 and PBS 6.8

Drug (10 mg) was accurately weighed and dissolved in 100 ml of PBS (pH 7.4) : methanol (7:3) & PBS (pH 6.8) : methanol (7:3) the volume was made up to 100 ml using 100ml volumetric flask.

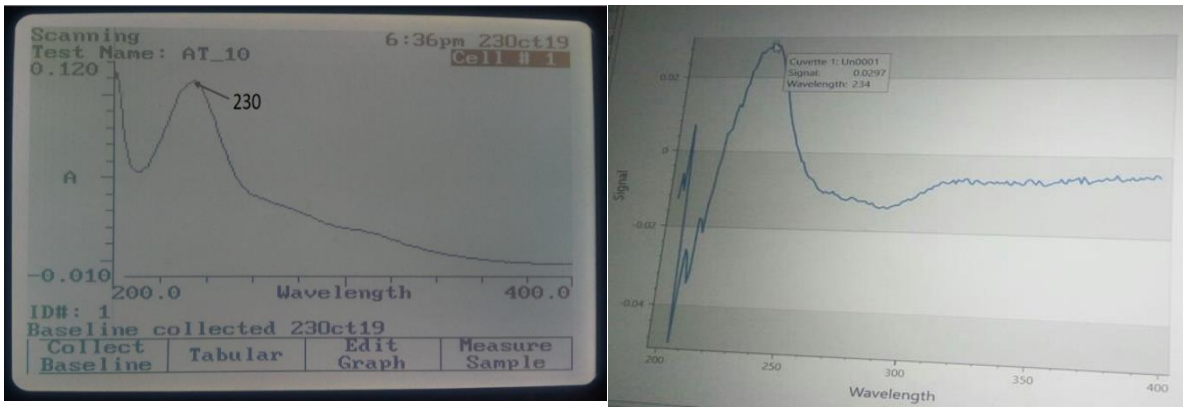

Figure 1: UV Scan of PTX in [a] Methanol: PBS 7.4 and [b] Methanol: PBS 6.8.

Preparation of Standard Curve of Paclitaxel

Standard curve of paclitaxel was determined by spectrophotometrically based on UV absorption in a mixture of methanol: PBS (pH 7.4) (30:70) at λmax 230 nm for the quantitative estimation of drug.

Preparation of Standard Curve of Paclitaxel in Methanol: PBS (30:70) pH 6.8 at λmax 234 nm

Paclitaxel was accurately weighed i.e. 10 mg in 10 ml of methanol and volume was made up to 100 ml with mixture of methanol: PBS (pH 6.8) (30:70) in 100 ml volumetric flask. This resulted in 100 µg/ml stock solution so from that aliquots of 0.2 ml, 0.4 ml, up to 2 ml was prepared and volume was made up to 10 ml volumetric flask with the mixture of methanol:PBS pH 6.8 (30:70).

Table 4: Standard curve data of paclitaxel in PBS (6.8) solution

Concentration(µg/ml)	Absorbance
2	0.07
4	0.112
6	0.152
8	0.195
10	0.241
12	0.289
14	0.332
16	0.374
18	0.425
20	0.471

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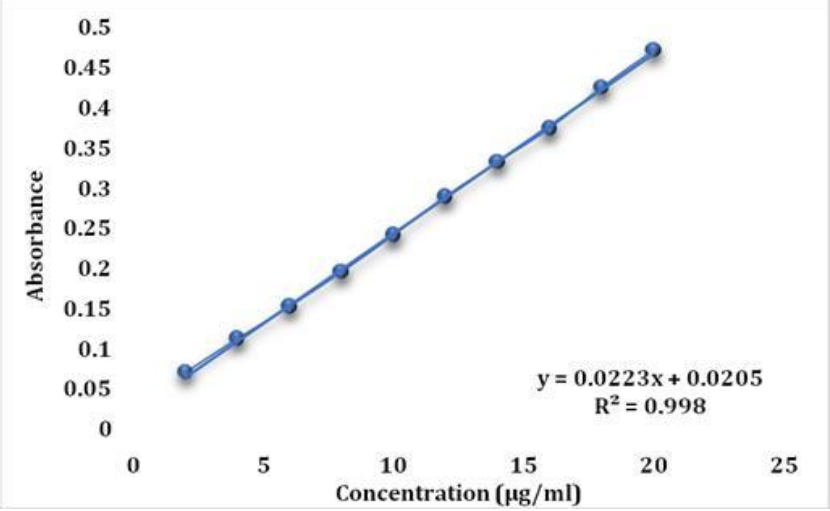

Figure 4.2 (a): Calibration curve of paclitaxel in PBS (6.8) solution at λmax=234nm

Preparation of Standard Curve of Paclitaxel in Methanol: PBS (30:70) pH 7.4 at λmax 230 nm
Drug (10 mg) was accurately weighed and dissolved in PBS (7.4) solution (100 ml) in a volumetric flask. This resulted in 100 µg/ml stock solutions.

Table 5: Standard curve data of paclitaxel in PBS (7.4) solution.

Concentration(µg/ml)	Absorbance
2	0.019
4	0.088
6	0.154
8	0.213
10	0.286
12	0.357
14	0.421
16	0.487
18	0.552
20	0.623

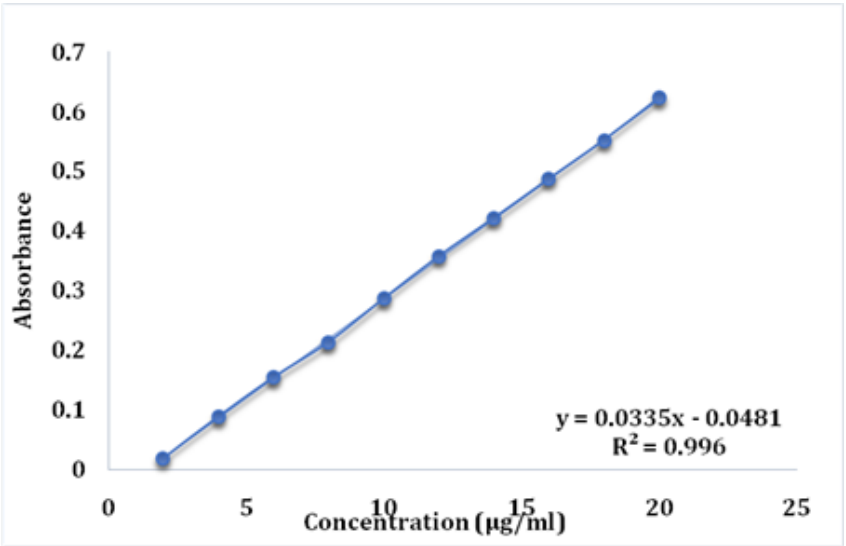

Figure : Calibration curve of paclitaxel in PBS (7.4) solution at λmax=230 nm.

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Formulation and Optimization

Materials

Paclitaxel was obtained as a gift sample from Neon Laboratories, Mumbai. Chitosan, pectin, glutaraldehyde solution, Span-80, MgCl2•6H2O CaCl2•2H2O, tween 80, liquid paraffin, acetone and methanol were purchased form Sigma Aldrich, Mumbai, India.

Methodology

Preparation of pectin coated PTX-chitosan NLCs (P-PCNLCs)

Conjugation of Paclitaxel loaded chitosan nano lipid construct with pectin was prepared using the method which was described by Hwang & Shin in 2018, which involves two steps.

Step 1: Preparation of Paclitaxel loaded chitosan Nano lipid construct

A solution of 1% chitosan was prepared by adding the specified quantity of chitosan to acetic acid solution (2.4% v/v) followed by stirring for one hour. 20 ml of sodium tripolyphosphate (TPP) solution was placed in a beaker and 10 ml chitosan solution was added by means of a glass syringe attached with an 18 G needle.

Step 2: Preparation of pectin coated PTX-chitosan NLCs (P-PCNLCs)

In this step0.5% (w/v) of pectin solution was dissolved in tween 80 (0.7ml) and was stirring, the dried Nano lipid construct were poured into the solution & added glutaraldehyde, followed by continuous stirring at 5000 rpm for 30 minutes and then entire mixture was centrifuged.

Optimization

Various variables which are affected the preparation and properties of nano lipid construct like process variables, formulation variables were optimized and studied.

Table : Optimization of Polymer concentration

Formulation code	Polymer Conc. (%w/v)	TPP Conc. (%w/v)	Stirring Speed (rpm)	Stirring Time (hour)	Particle Size (nm)	PDI	% EE
P1	0.25%	1%	500	3	168.2	0.541	82.3
P2	**0.50%**	**1%**	**500**	**3**	**179.6**	**0.382**	**87.4**
P3	0.75%	1%	500	3	192.5	0.423	78.8
P4	1%	1%	500	3	197.1	0.594	84.7

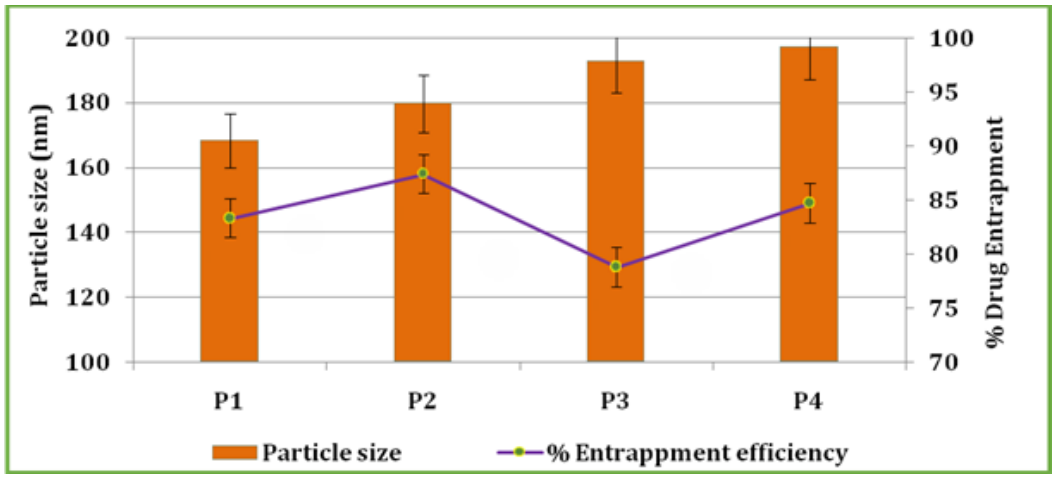

Figure 5.1: Effect of Polymer concentration on particle size and % entrapment efficiency.

Table : Optimization of TPP concentration

Formulation code	Polymer Conc (%w/v)	TPP Conc(%w/v)	Stirring Speed(rpm)	Stirring Time (hour)	Particle Size (nm)	PDI	% EE
P2S1	0.5%	1%	500	3	193.3	0.564	78.6
P2S2	**0.5%**	**1.5%**	**500**	**3**	**175.5**	**0.358**	**83.3**
P2S3	0.5%	2%	500	3	188.1	0.498	77.9
P2S4	0.5%	2.5%	500	3	197.2	0.439	79.5

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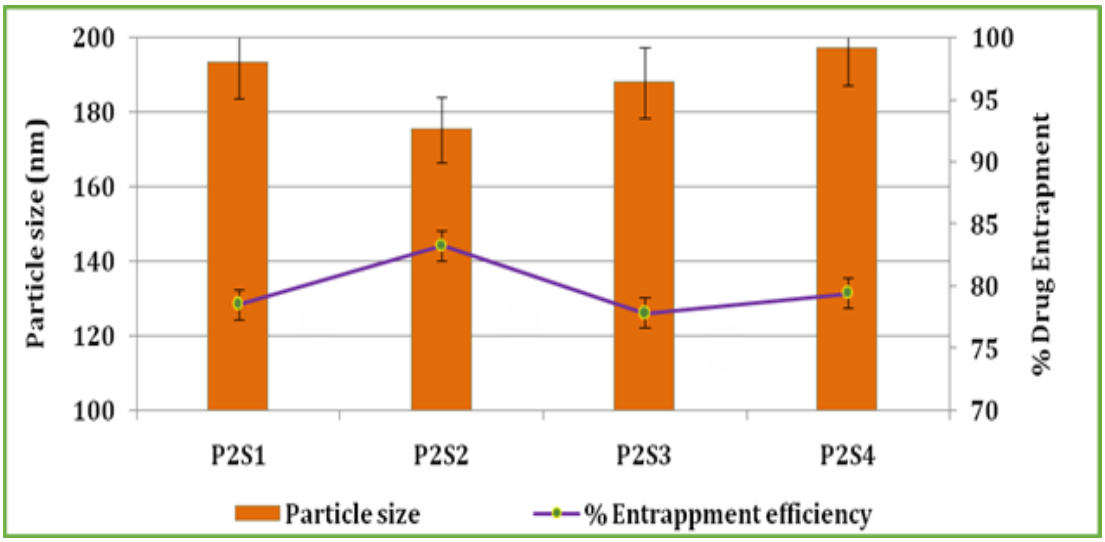

Figure : Effect of TPP concentration on particle size and % entrapment efficiency.

Preparation of pectin coated PTX-chitosan NLCs (P-PCNLCs)

In this step0.5% (w/v) of pectin solution was dissolved in tween 80 (0.7ml) and was stirring, the dried Nano lipid construct were poured into the solution & added glutaraldehyde, followed by continuous stirring at 5000 rpm for 30 minutes and then entire mixture was centrifuged.

RESULT AND DISCUSSION

Preparation of Pectin coated PTX-chitosan Nano lipid construct was carried out by using ion gelation method. The PTX were mixed into acetic acid solution of chitosan. The 20 ml of sodium tripolyphosphate (TPP) solution was placed in a beaker and 10 ml chitosan solution was added by means of a glass syringe attached with an 18 G needle. Stirring was done for 120 hrs. to obtain the nano lipid construct in the wet state. Filtration, using Whatman filter paper was done to separate the nano lipid construct. To the particles in the filter paper 10 ml of acetone was added. Finally, the nano lipid construct were transferred into 20ml of acetone in a beaker and mixed for a few hours. Drying was carried out by placing the particles in a petridish at room temperature for 18 h to obtain the chitosan nano lipid construct.

To optimized the various formulation variables i.e. polymer concentration, cross linking agent i.e. TPP concentration and process variables i.e. stirring time and stirring speed. Polymer concentrations was optimized by varying concentration from 0.25%-1% and then optimize the TPP concentration from 1% to 2.5 %, stirring time from 2-4 hour and finally optimized stirring speed from 500-2000 rpm. Firstly, optimized the Chitosan (polymer) concentration and select the suitable concentration of polymer as 0.50% w/v (P2) on the basis of particle size, PDI and % entrapment efficiency of PTX. Then optimize the concentration of TPP, the effect of TPP concentration was observed on the basis of particle size and % entrapment efficiency, at 1.5% concentration decrease the particle size and %EE further enhance the concentration of TPP then increase the particle size. Hence it obtained P2S2 optimized formulation, selected the optimize formulation further optimization of process variables and finally optimized the formulation P2S2R3T3 showed the average particle size, PDI, % entrapment efficiency were found to 180nm, 0.316, % entrapment efficiency of PTX in optimized formulation 86%.

Characterization of prepared Pectin coated PTX-chitosan Nano lipid construct

Particle size and surface charge determination

The particle size and zeta potential of Nano Lipid Construct were carried out using (Zeta Sizer Nano Series, Backman coulter DelsaTM Nano). The particle size distribution is determined by the average size (diameter) and polydispersity index of Nano Lipid Construct also reported in the same. Each sample was diluted ten times with filtered double distilled water to avoid multi- scattering phenomena and placed in disposable sizing cuvette. Analysis was performed in triplicate and the results were expressed as mean ± SD (Table 5.5, Fig 5.5 (A and B).

Table : Particle size, Zeta Potential and % entrapment efficiency of formulations

Formulation code	Particle size	PDI	Zeta potential(mV)	% Entrapment efficiency
P2S2R3T3	180.9	0.316	6.24	86.23

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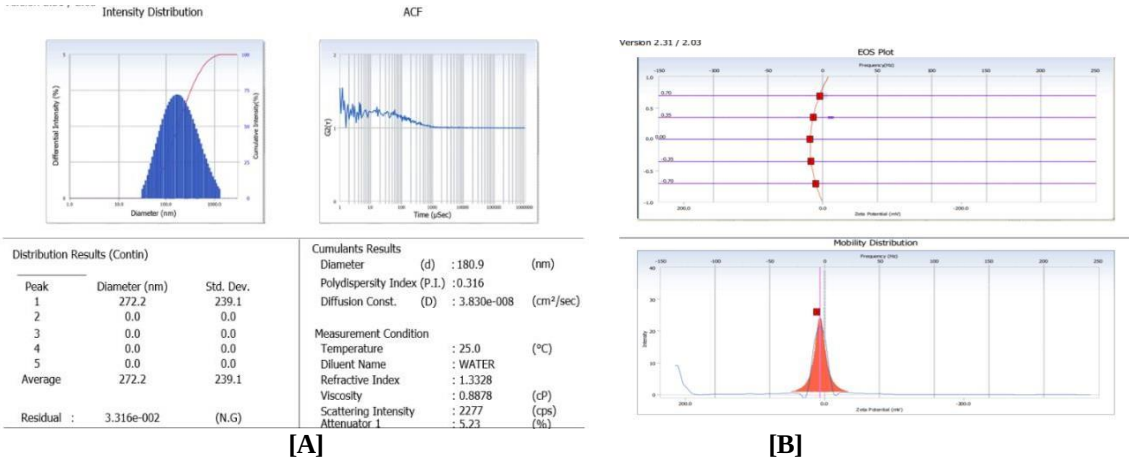

[A] **[B]**

Figure: The Particle size and Polydispersity Index of pectin coated PTX-chitosan NLCs (P-PCNLCs)

Scanning Electron Microscopy (SEM)

Particle morphology of nano lipid construct was analyzed by SEM and it was also used to measure the size ofnano lipid constructupto nanometric range.

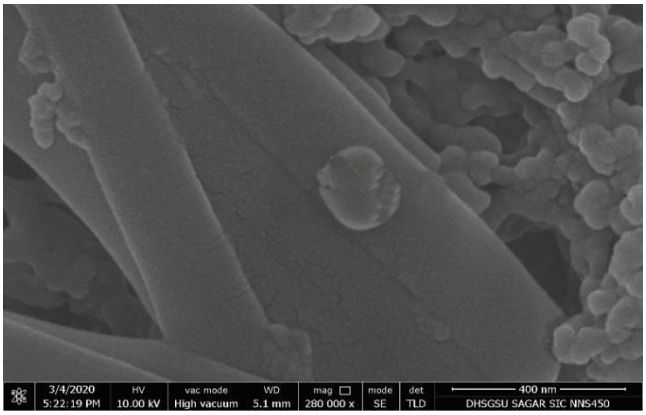

Figure : SEM photomicrographs of pectin coated PTX-chitosan NLCs (P-PCNLCs)

Ex-vivo study on HeLa cell

Cell culture

HeLa human cervical carcinoma cells obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Chengdu KeLong Chemical Co., Ltd.).

In-vitro cytotoxicity study

The cytotoxicity of pectin coated PTX-chitosan NLCs (P-PCNLCs) was measured by performing an MTT assay.

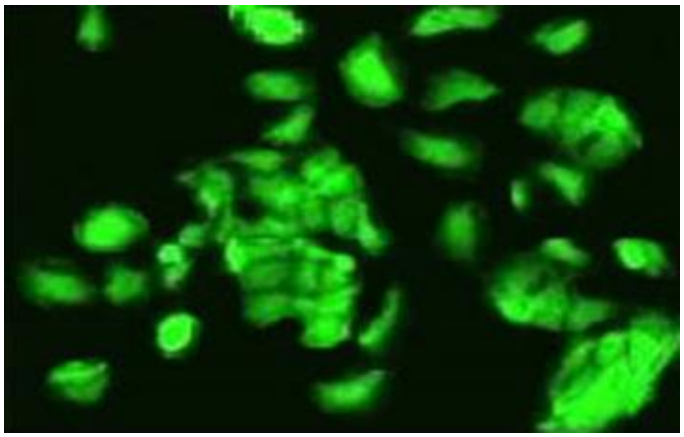

Figure: Cellular uptake of Pectin coated PTX-chitosan Nano lipid construct (P-PCNLCs)

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Table : Cytotoxicity of different formulations of Paclitaxel on HeLa cell line

Concentration (nm)	Percentage of cell viability	
	P-PCNLCs	PTX
5	65	70
25	52	68
75	46	65
125	42	58
250	35	52

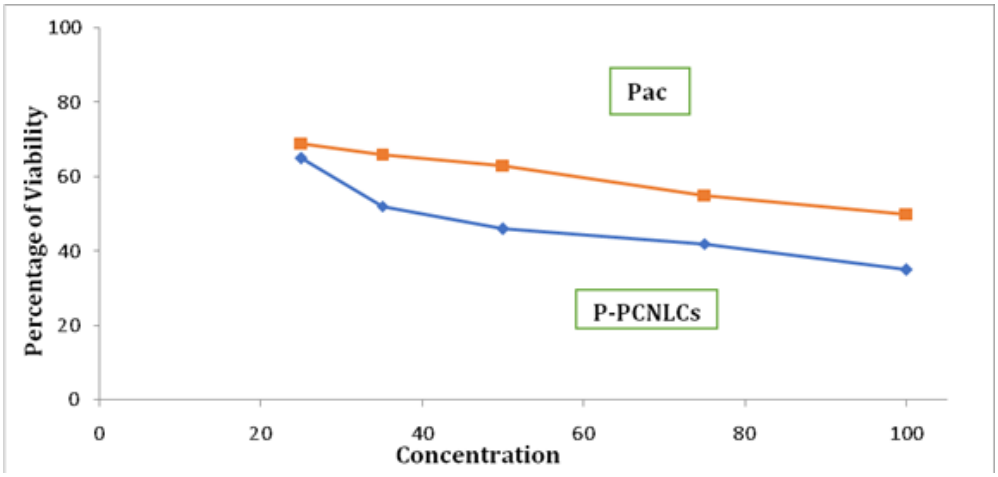

Figure : Percentage of cell viability on HeLa cell line by drug and formulation.

Conclusion

The pectin coated PTX-chitosan NLCs (P-PCNLCs) has successfully prepared by Ion gelation method and characterized for the particle size, zeta-potential, poly dispersity index and surface morphology. The P-PCNLCs having the particle size 180.9±.12 nm that is suitable for the active targeting towards the cancer sites as well as for the engulfment by the carrier protein present in cell surface. The entrapment efficiency of the particle was 86.23±0.32%. The ex-vivo study data are in agreement with strategies that active targeting provides the promising result of cure and treatment of cancerous cell. The cytotoxicity of P-PCNLCs was significantly increased compared to PTX. Nano-lipid-construct exhibited a remarkably higher uptake in the HeLa cells compared to that of free drug (PTX).The nanoscale effect and targeting effect of P-PCNLCs could increase the drug accumulation in tumor cells, which leads to high drug concentrations in tumor cells and strongly inhibiting cell proliferation.

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