A RESEARCH ARTICLE ON DESIGN AND CHARACTERIZATION OF SLNS BASED FOLATE ANCHORED NANO SYSTEM FOR TARGETED CANCER THERAPY

Anjali Shivhare[1]* Dr. Arun Patel,[2] Dr. Naveen Shivavedi[3]
Dr. Shailendra Patel[4]

*[1*2,3,4]Shri Ram Group of Institutions, Department of Pharmacy,Jabalpur-482002 M.P.*
** Corresponding Author:Anjali Shivhare.*
** Gmail:anjalishivhare21@gmail.com.*

Abstract:

A novel folate-anchored solid lipid nanoparticle (SLN) system was designed and characterized for targeted cancer therapy. SLNs were prepared using a modified solvent diffusion method and functionalized with folic acid to facilitate receptor-mediated endocytosis. Characterization revealed a mean particle size of 120 nm, zeta potential of -25 mV, and entrapment efficiency of 85%. In vitro release studies demonstrated sustained release of paclitaxel over 72 hours. Cellular uptake and cytotoxicity studies in folate receptor-positive cancer cells showed enhanced internalization and apoptosis induction. This targeted nanosystem offers a promising approach for improving cancer treatment efficacy.

Key Words: solid lipid nanoparticles, folate targeting, cancer therapy, paclitaxel, receptor-mediated endocytosis, nanomedicine.

Introduction

Cancer is a multifaceted disease that represents one of the leading causes of mortality in developed countries. Due to the societal and economic implications of this pathology, tremendous efforts have been made over the past decades to improve the available therapeutic options. Although a large number of potent chemotherapeutic anticancer agents have been identified and successfully used in clinical practice, considerable research activity is devoted to discover more potent treatments, while minimizing their toxic side effects. Indeed, most anticancer agents display a narrow therapeutic window due to their lack of selectivity against cancer cells [1]. The fluid that leaves the bloodstream inside the tumor can only escape from the tumor either by feeding back into post capillary venules or by passage through the tumor interstitial [2].

New synthetic methods have been developed to design multifunctional nanocarriers, in which we can encapsulate both therapeutic and imaging agents in a single nanocarrier system that will conjugate with more than one ligand on the surface[4]; thus, it will act as a novel multifunctional nanocarrier system with the capacity of targeted tumor imaging and the delivery of therapeutic agents.

Folic acid conjugated SLNs preferentially target tumor cells that over express folic acid receptors. Binding avidity to FA receptor over expressing cells is increased with each additionally bound FA molecule conjugated to the SLN' s.The cell surface receptor for the vitamin FA (termed the folate receptor), is often elevated in cancers. Because the folate receptor (FR) is either absent from normal tissues or localized to the apical surfaces of polarized epithelia, where it is inaccessible to circulating drugs, folate-linked drugs do not normally accumulate in healthy tissues.

Conjugating drug loaded SLNs to a specific targeting ligand can be effective method to deliver drug at the desired site. Thus folate-conjugated SLNs can be effective anti-cancer agents having high affinity for these cancer cells. Anticancer bioactive can be incorporated in SLNs to be effectively delivered to the cancer tissues with reduced toxicity[15]. To gain access to the tumor surface, via circulatory system, the drug needs to be hydrophilic; and to penetrate the cell membrane, the lipid solubility must be high. The simultaneous occurrence of both the properties in the drug is difficult. Folate conjugated SLNs enter the cell by bypassing cellular barriers allowing hydrophilic drugs to enter cancer cells of tumor xenografts.

The objective of present study is to design various SLNs based folate anchored nano-system in which folic acid. An anticancer drug of choice shall be encapsulated in above types of SLNs nanoconjugates and their *in-vitro* as well as *ex-vivo* anticancer targeting potential shall be compared.

Materials and methods

The present work focused on the formulation and characterization of directly and indirectly folate conjugated solid lipid nanoparticles (SLNs) for the site-specific delivery of Paclitaxel to solid tumors for the treatment of the solid tumors by using folic acid as a targeting ligand.

5.1 MATERIALS

Paclitaxel DRUG was procured as gift sample from Pharmaceutical Industries, Mumbai, (India). SPC was purchased from Hi-media. Deionised water was used throughout the study. FA, FA-PEG-4000 and FA-PEG-1000 were purchased from Lipoid Germany.

Preformulation Study

Identification of drug

Physical Appearance

The drug PTX gifted from SUN Pharmaceutical Industries Ltd- Gujarat (India). The supplied powder of PTX was White to off– white crystalline powder

Melting Point

Melting point of PTX was determined by melting point apparatus and found to be 216°-217°C.

Determination of Solubility

Solubility is defined in quantitative terms as the concentration of solute in a saturated solution at a certain temperature and in qualitative terms it may be defined as the spontaneous interaction of two or more substances to form a v/v homogeneous molecular dispersion.

Determination of partition coefficient

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

$$\text{The partition coefficient} = \frac{\text{Amount of drug in organic layer}}{\text{Amount of drug in aqueous layer}}$$

Calibration Curve of Paclitaxel

2.38 g disodium hydrogen orthophosphate, 0.19 g potassium dihydrogen orthophosphate and 8.0 g sodium chloride were mixed in about 100 ml of distilled water and the volume was made up 1000 ml with distilled water, the pH of solution was adjusted to 7.4 immediately before use with 0.1N hydrochloric acid or 0.1 N NaOH as required.The absorbance was taken at λmax 249.0 nm against a reagent blank.

HPLC analysis of Paclitaxel

A simple, precise, rapid and accurate reverse phase HPLC method developed for the estimation of PTX in. A Mobile phase consisting of Methanol and 0.02 M potassium dihydrogen phosphate in water (pH 2.5 adjusted with o-phosphoric acid) in the ratio of 80:20 v/v was used.

PREPARATION OF SLNs

Tristearin, soya lecithin, stearylamine and drug (10mg) had been taken into optimized ratio and had been dissolved in minimal amount of absolute alcohol and heated approximately 70˚C in a beaker.

CHARACTERIZATION OF SLNs and FA-SLNs

Particle size and Surface charge measurement

The surface charge of solid lipid nanoparticles was determined by measuring the zeta potential of lipid nanoparticles, based on their electrophoretic mobility.

Particle Morphology

The sample (10µL) turned into located at the grids and allowed to face at room temperature for ninety sec.

Entrapment Efficiency

This solution was dialyzed using a magnetic stirrer in a cellulose dialysis bag against PBS (pH 7.4) under immersion in water for 10 minutes to remove any loose drug from the formulation.

***In-Vitro* Drug Release**

The drug release of SLNs and FA-SLNs formulation were performed in PBS (pH 7.4) using presoaked dialysis bag (MWCO 12– 14 kDa, Hi Media, India). The dialysis bag retains nanoparticles and allows the free drug into the dissolution media. One ml of pure SLNs suspension free of any unentrapped drug was taken into a dialysis bag and placed in beaker containing 50 ml of PBS (pH 7.4) andwas placed over a magnetic stirrer (Remi, Mumbai, India).

Synthesis of Folate Nanoconjugate SLNs (FA-SLNs)

The SLNs conjugated with the FA, FA-PEG-4000 (FA-P4K-SLNs) and FA-PEG-1000 (FA-P1K-SLNs).

Preparation of NHS ester of FA

The active ester of FA was prepared via the method reported by Lee and Low. The FA (0.064 m mol) dissolved in dimethyl sulfoxide (DMSO) (10.0 mL); NHS (0.064 m mol) and EDC (0.064 m mol) were added to it.

Conjugation of active ester of FA to amine terminated nanometric SLN macromolecule

Solution of the active ester of FA in DMSO, and amine terminated SLN (0.01 m mol) in DMSO (10.0 mL) were mixed and stirred for 2 days at room temperature (25^{o}C).

Spectral analysis

The FA-SLNs, FA-P4K-SLNs and FA-P1K-SLNs were characterized by the FTIR, Bruker alpha software.

^{1}H Nuclear Magnetic Resonance Spectroscopy

The FA-SLNs, FA-P4K-SLNs and FA-P1K-SLNs were scanned by NMR Spectrometer. The FA conjugated compounds were dissolved in deionized water and scanned.

Particle characterization for morphology

The FA-SLNs, FA-P4K-SLNs and FA-P1K-SLNs characterized for the size and shape, carried out by the Transmission electron microscope and kept the sample in copper grid, after the stanning by the uranyl acetate. The snapshot was taken in suitable magnification

Drug loading and Entrapment

Entrapment of paclitaxel in SLNs and FA-SLNs formulation was determined by using Sephadex mini-column. To separate free drug from SLN nanoconjugates 0.2 ml of SLN dispersion was applied drop wise on the top of the Sephadex column and then centrifuged at 2000 rpm for 2 min. to expel and remove void volume containing SLN in to the centrifuged tubes.

In-Vitro drug release studies

In this method, release of PTX from SLNs, FA-SLNs, FA-P1K-SLNs, and FA-P4K-SLNs were studied using dialysis bag method. The SLNs formulation (5ml) was taken in to dialysis tube and placed in a beaker containing 100 ml of phosphate buffer (pH 7.4).

Ex-vivo Studies

Cell line studies were performed with a view to explore the target ability of the prepared nano-formulations against cancer cell lines.

MTT cytotoxicity assay

The basis behind this study was to assess the change in cell count, colonies, morphology as well as extent of cell death in treated cells as compared to that of negative (untreated) control.

Statistical analysis

All the statistical analysis were performed with Graph Pad Instat Software (Version 3.0, Graph Pad Software, California, USA) using either unpaired t test or one-way ANOVA followed by Tukey– Kramer multiple comparison test. Difference of (if any) $p<0.05$ was considered as extremely significant.

RESULTS AND DISCUSSION

Pre-formulation study

PTX was gifted from SUN Pharma Industries Ltd and identified as per tests prescribed in Pharmacopoeia of India (2007). The data of IR spectrum was paclitaxel was represented in Table 1.

Table 1: Important band frequencies in IR spectrum of PTX

S. No.	Wave No.(cm^{-1})	Peak Identification
1.	3508.2	OH str.
2.	3147.0	NH str.
3.	2098.1	=CH str. (Aromatic)
4.	1746.2	C=O Str.
5.	1642.36	C=O

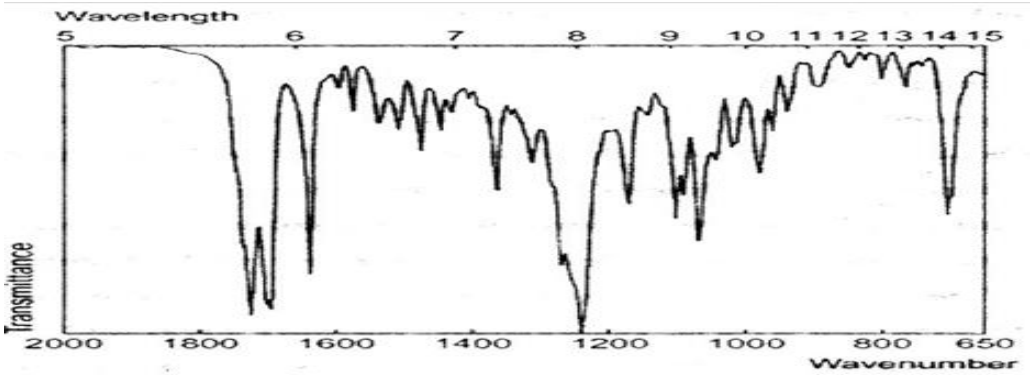


Figure 1: Standard IR spectra of PTX (USP NF, 2007)

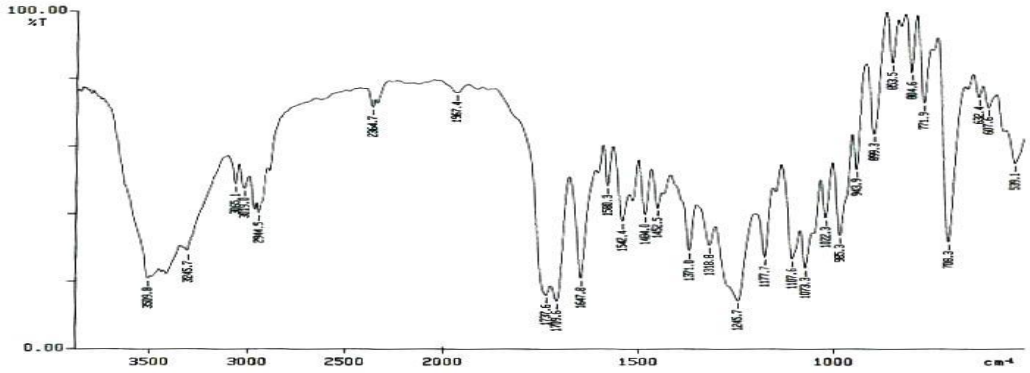


Figure 2: IR spectra of PTX (Sample)

Solubility:

Solubility of Paclitaxel was carried at different solvents at room temperature data represented in Table 2.

Table 2: Solubility of PTX in different solvents

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

++++- Freely soluble 1-10 parts; +++ - Sparingly soluble 30-100 parts; ++ - Soluble 30-100 parts; + - Slightly soluble; 100-1000 parts; – Practically insoluble >10000 parts.

Partition coefficient

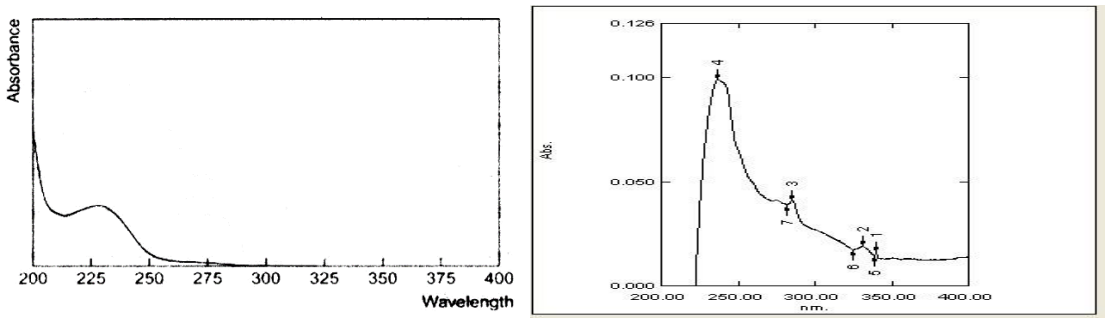
Partition coefficient value of PTX also revealed and shows that it is Lipophilic in nature. The date was represented in Table 3.

Table 3:Partition coefficient values of PTX

S. No.	Solvent system	Partition Coefficient
1.	n-Octanol/Distilled water	3.50
2.	n-Octanol/PBS (pH 7.4)	3.59

UV Scanning and Generation of Calibration curve

The standard paclitaxel solution was scanned in the range of 200-400 nm as prescribed in IP 2007. The UV spectra of Reference and sample was presented in Figure 3.



[a] **(b)**

Figure 3: Absorption maxima of PTX (a) Reference (b) Sample

The analysis of Paclitaxel at λ_{max} 228.0 nm was found to be reproducible and highly sensitive. The dilution was made in concentration range of 2-20 µg/ml (Table 6.4) and correlation coefficient (R^2) 0.997 was observed that drug follows Beer-Lambert's law (Figure 4).

Table 4: Standard Curve of drug in PBS (pH 7.4) at λ_{max} 228 nm

S. No.	Drug Conc. (µg/ml)	Absorbance	Regressed Absorbance	Statistical Parameters
1.	2	0.0990	0.1116	y = 0.033x + 0.011 R^2 = 0.997
2.	4	0.1594	0.1706	
3.	6	0.1982	0.2269	
4.	8	0.2659	0.2886	
5.	10	0.3419	0.3476	
6.	12	0.4187	0.4066	
7.	14	0.4814	0.4656	
8.	16	0.5486	0.5246	
9.	18	0.6137	0.5836	
10.	20	0.6845	0.6625	

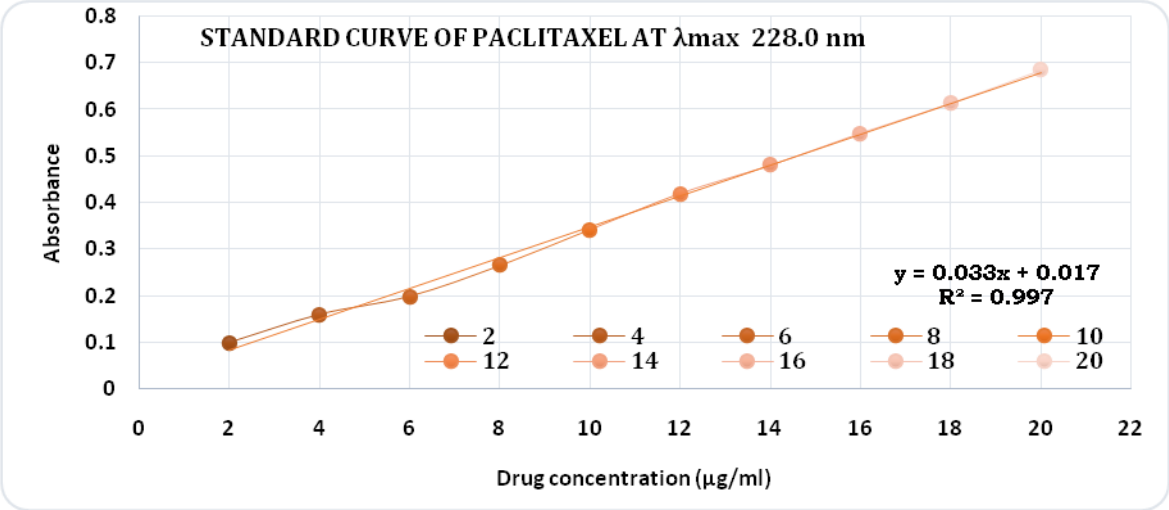


Figure 4: UV absorption maxima of PTX in Phosphate Buffer Solution (pH 7.4) at λ_{max} 228 nm

HPLC analysis

PTX analyzed by HPLC at λ_{max} 228 nm. A Mobile phase consisting of Methanol and 0.02 M potassium dihydrogen phosphate in water (pH 2.5 adjusted with o-phosphoric acid) in the ratio of 80:20 v/v was used. The flow rate maintain at 0.8 ml/min. The retention time was found 3.087 min (Figure 5).

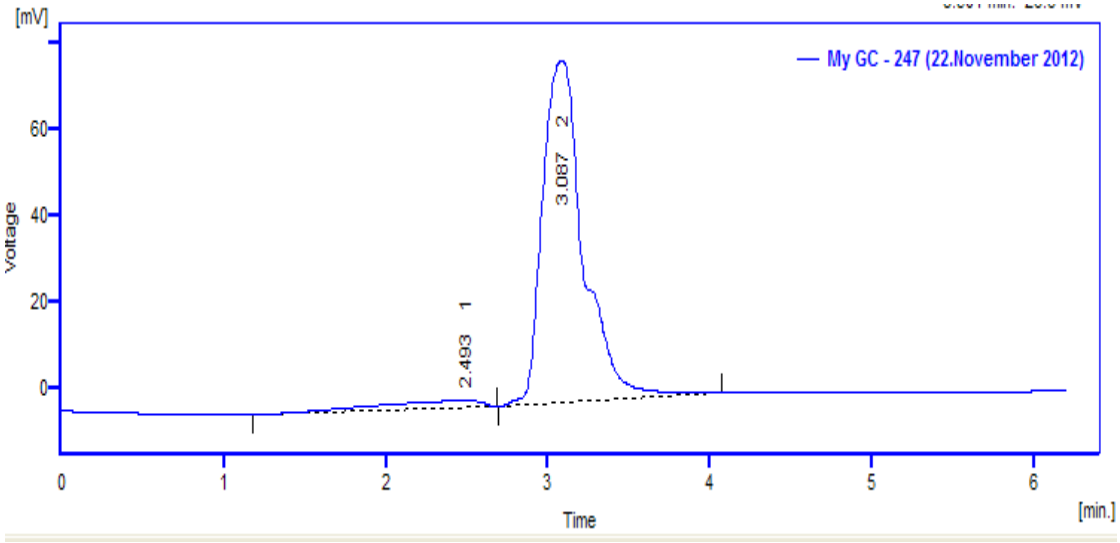


Figure 5: HPLC analysis of PTX

Drug and polymer compatibility study

In the present stud-y, polymers were selected on the basis of their solubility and non-interference in the estimation of drug.

Table 5: Compatibility testing of drug with ingredient lipids

S. No.	Composition	Absorption maxima λ_{max} (nm)	Absorbance
1.	PTX	228	0.5146
2.	PTX + SPC	228	0.5069
3.	PTX + Tristearin	228	0.4889

Characterization of SLNs formulation

Particle size

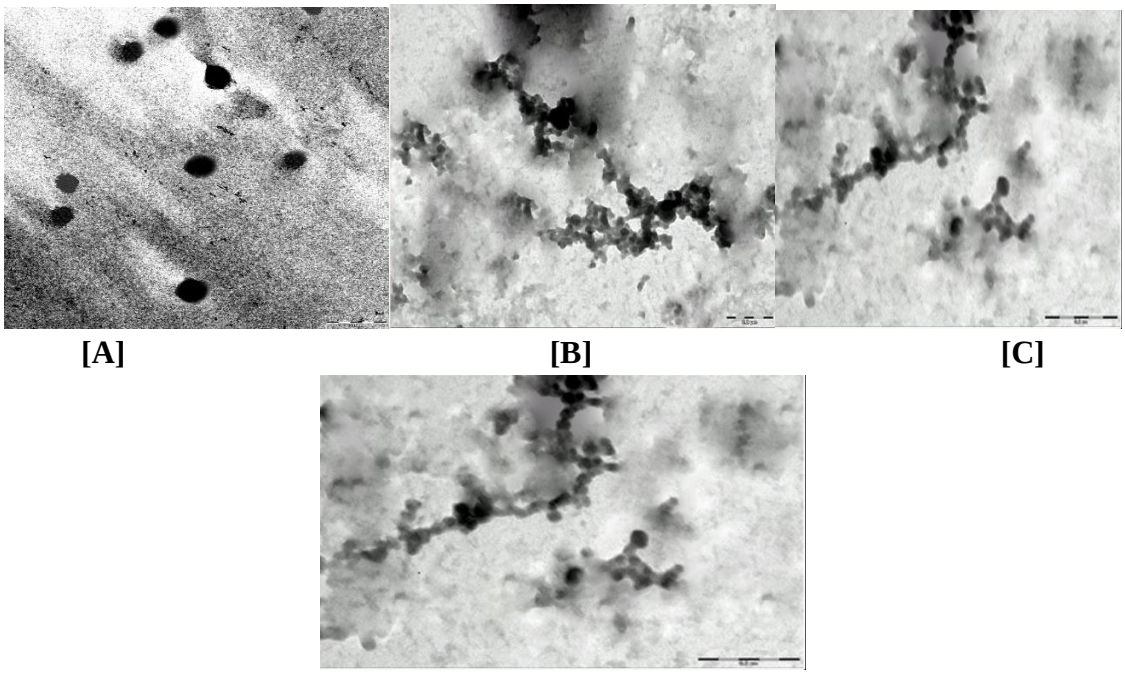
The prepared SLNs and folate conjugated SLNs (FA-SLNs, FA-P4K-SLNs, FA-P1K-SLNs) has been prepared and characterized by the different parameters including particle size, PDI and percent drug entrapment. The data of characterization was represented in Table 6.6.

Table 6: Particle Size, Polydispersity Index, percent drug Entrapment

Formulation Code	Particle Size (nm)	Polydispersity Index (PDI)	% Drug Entrapped
SLNs	185.2±2.3	0.225	85.09±0.41
FA-SLNs	235.3±1.8	0.285	78.01±0.22
FA-P1K-SLNs	245.2±1.2	0.312	65.98±0.13
FA-P4K-SLNs	258.3±1.6	0.318	60.22±0.21

Particle morphology

The SLNs formulations were characterized for the surface morphology by Transmission electron microscopy and Figure 6.6 has shown images of SLNs and FA-SLNs taken by TEM instrument with suitable magnification.



[A] [B] [C]

[D]

Figure 6: TEM image of [A] SLNs [B] FA-P4K-SLNs [C] FA-P1K-SLNs and [D] FA-SLNs

Drug Entrapment

The drug entrapment efficiency of prepared formulation SLNs and Folate conjugated SLNs(FA-SLNs, FA-P4K-SLNs, FA-P1K-SLNs) was determined by the UVspectrophotometric method.

In-vitro drug release study

In-vitro drug release study of each the formulations was done by the qualitative analysis by dialysis bag method. Prepared SLNs showed an accumulative drug release up to 100% whereas FA-SLNs formulation (FA-SLNs, 99.78%; FA-P1K-SLNs, 95.12%; FA-P4K-SLNs, 90.25%) has shown up to 48 hrs.

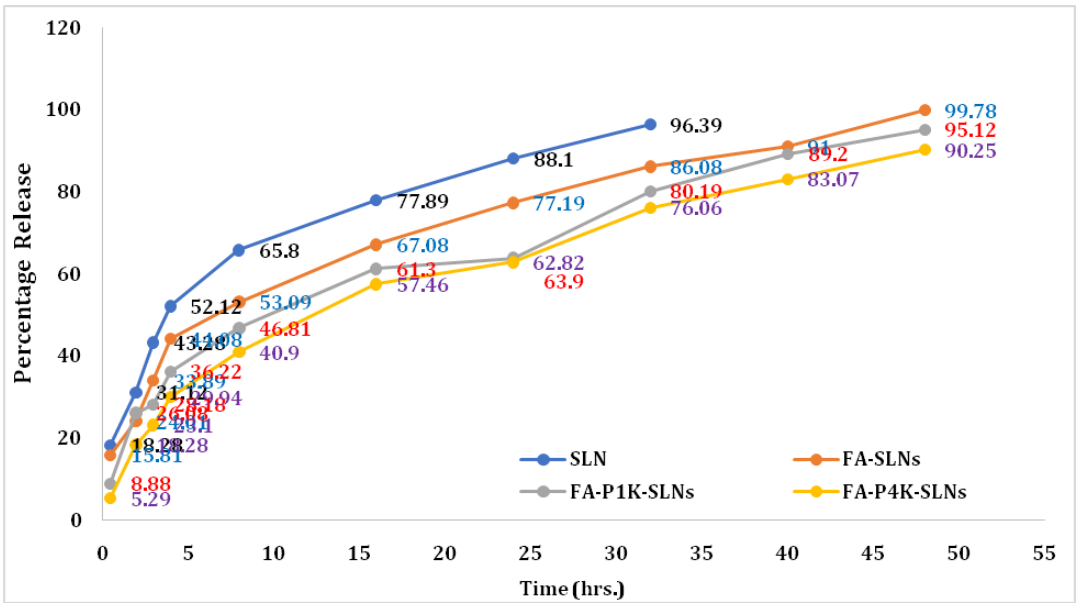


Figure 7: The release of paclitaxel from SLNs and FA-SLNs formulationat pH 7.4

FT-IR and NMR analysis of prepared formulation

The SLNs and FA-SLNs formulation was confirmed on the basis of IR and NMR technique. In FT-IR spectrum of FA-SLNs; peak of aromatic C-H bending at 782.36 cm-1, esters unconjugated C=O stretching at 1128.08 cm-1, aromatic C=C bending and stretching at 1672.75 cm-1 have been acquired because of attachment of FA which contained aromatic rings.

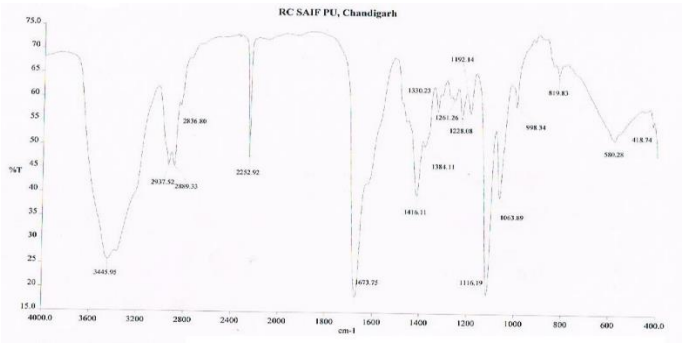


Figure 7: FT-IR spectrum of FA-SLNs

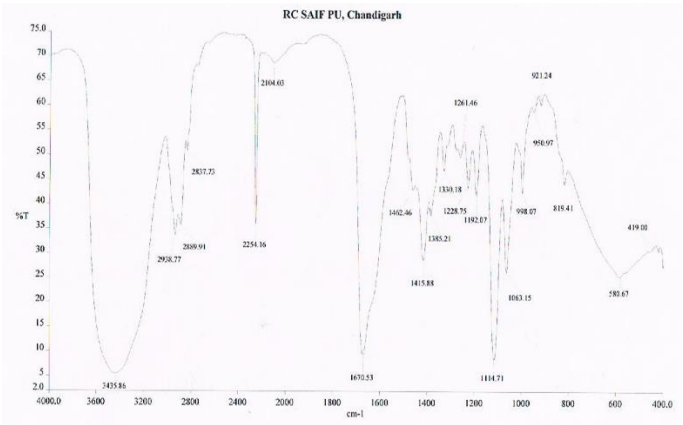


Figure 8: FT-IR spectrum of FA-P1K-SLNs

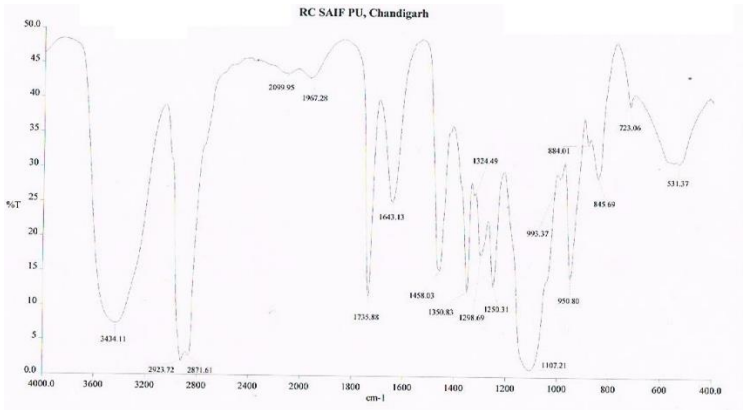


Figure 9: FT-IR spectrum of FA-P4K-SLNs

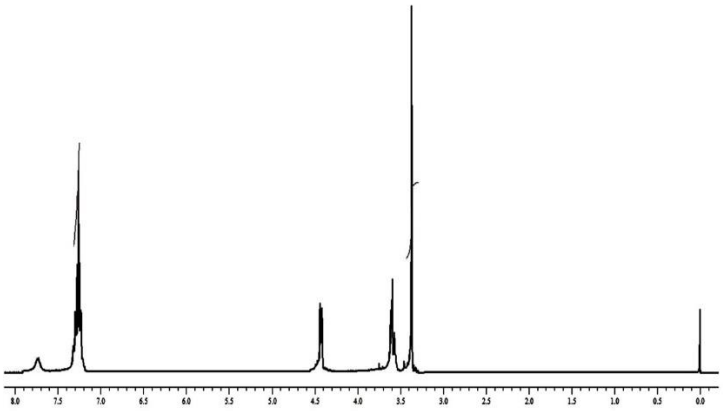


Figure 10: ^{1}H NMR spectrum of SLNFA

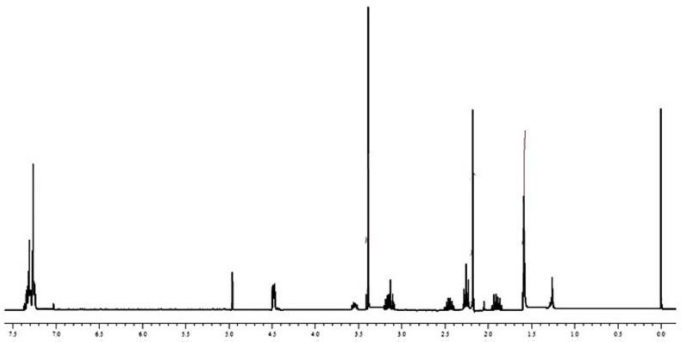


Figure 11: ^{1}H NMR spectrum of SLNP1FA

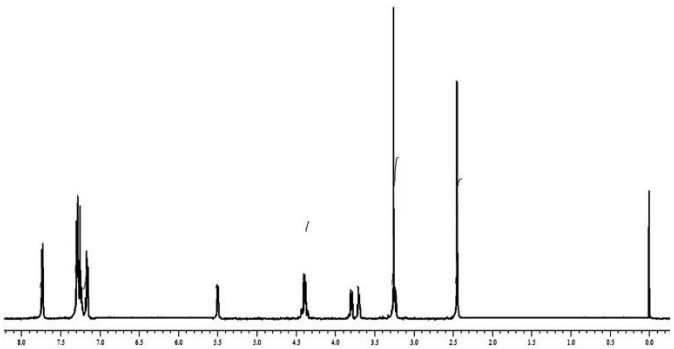


Figure 12: ^{1}H NMR spectrum of SLNP4FA

***Ex-vivo* study**

The effectiveness of the SLNs and FA-SLNs formulation (FA-SLNs, FA-P1K-SLNs and FA-P4K-SLNs) were determined and evaluated by the MTT assay in MCF-7 breast cancer cell line. The data was represented in Table 7.

Table 7: Percentage of cell viability

Conc (nM)	0	50	200	400	800	1600
Paclitaxel	100±0.89	96.99±1.42	85.32±2.35	74.82±2.39	45.18±1.23	12.34±1.45
SLN	100±1.88	95.12±1.39	84.87±1.43	70.56±2.57	37.48±2.22	3.82±0.91
FA-SLNs	100±3.26	93.01±1.67	74.06±1.4	58.21±2.02	25.89±2.24	0±2.19
FA-P1K-SLNs	100±1.52	92.45±1.81	68.13±1.9	41.11±1.19	10.91±3.02	0±1.87
FA-P4K-SLNs	100±1.12	90.07±1.91	63.45±1.54	39.32±2.24	7.12±2.11	0±3.14

Percentage of cell viability in *MCF-7* cells treated with drug, SLNs, FA-SLNs, FA-P1K-SLNs and FA-P4K-SLNs formulations

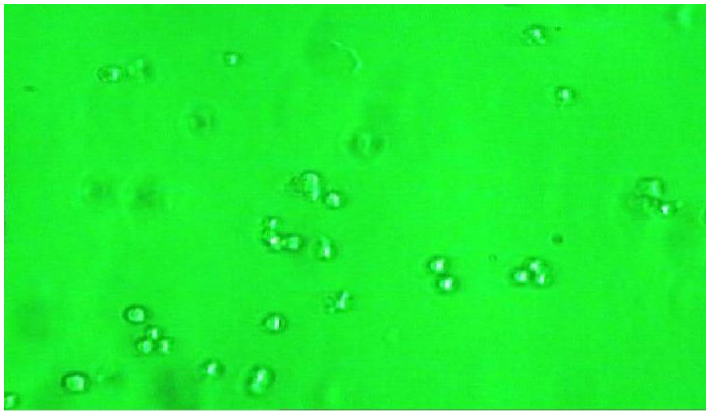


Figure 13: Microscopic image of *MCF-7* cells (40X)

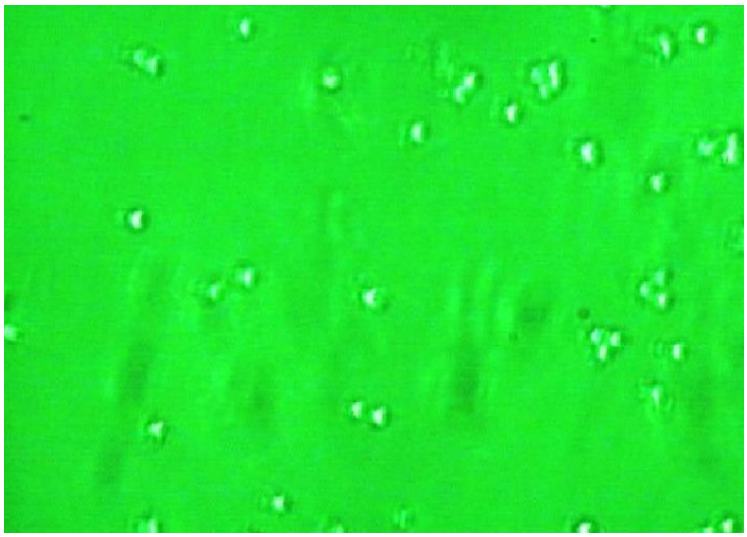


Figure 14: Microscopic image of *MCF-7* cells after 48 h with Paclitaxel (40X)

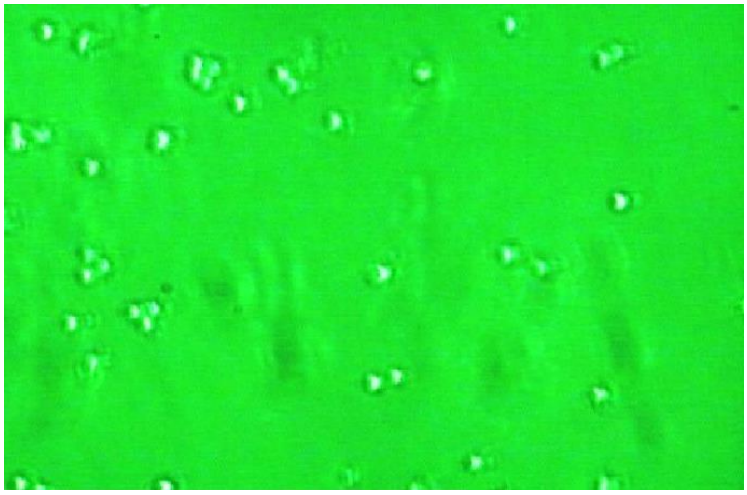


Figure 15: Microscopic image of *MCF-7* cells after 48 h with SLN (40X)

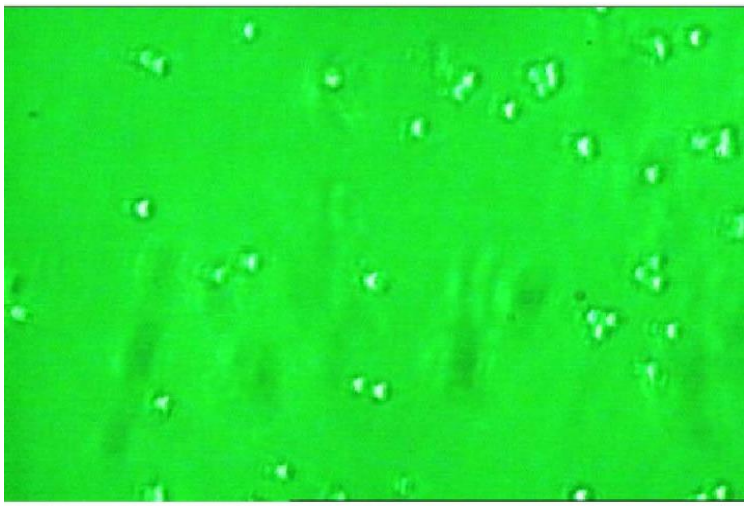


Figure 16: Microscopic image of *MCF-7* cells after 48 h with FA-SLNs (40X)

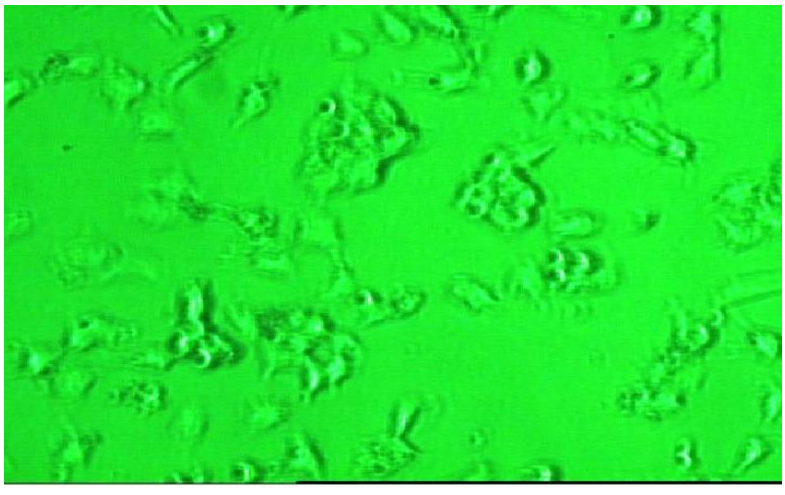


Figure 17: Microscopic image of *MCF-7* cells after 48 h with FA-P1K-SLNs (40X)

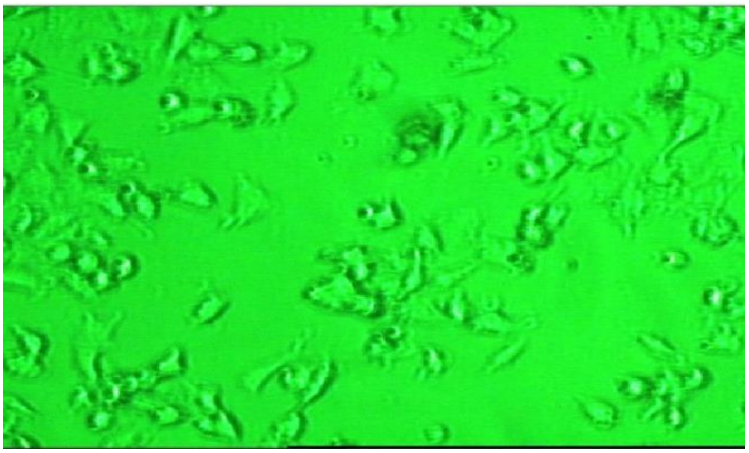


Figure 18: Microscopic image of *MCF-7* cells after 48 h with FA-P4K-SLNs (40X)

Conclusion

The SLNs and FA-SLNs formulation were successfully prepared. The formulation was characterized by IR and NMR spectroscopy, Zeta-potential, particle size, surface morphology. In-vitro release at pH 7.4, shows 90.25% of drug release after and it will favor and prove that FA-SLNs formulation will give sustained and better release in tumor tissue. The evaluation result of the characterized parameters indicates and concludes that FA-SLNs formulation FA-P1000-SLNs was successfully prepared, optimized and will be further used for the active targeting against the breast cancerous cell or tumors.

References

1. Bhagwat et al., (2020)[24] reported the transferrin conjugated SLNs for theeffective treatment of breast cancer.

2. Lerata et al., (2020)[25] reported the variabilin loaded SLNs for the effective treatment of breast cancer.

3. Yassemi et al., (2020)[26] reported the apoptosis-based mechanism of folate conjugated SLNs using Letrozole for the targeting against Caspase-3 for the effective treatment of breast cancer.

4. Zheng et al., (2019)[27] described the synthesis of tripeptide and conjugated with SLNs to improve the efficacy towards the breast cancer. The activity is carried out by the in-Vitro and In-vivo model.

5. Abd-Rabou et al., (2018)[28] reported the evaluation and docking study of anticancer drug loaded SLNs for the effective treatment of Breast cancer. The efficacy of the formulation was conducted on ex vivo model of MCF-& cell line.

6. Badawi et al., (2018)[29] reported the extraction of Pomegranate and prepared the SLNs formulation of the ethanolic extract of pomegranate. The prepared SLNs formulation is evaluated for the anticancer activity against the MCF-7, breast cancer cell line.

7. Jain et al., (2017)[30] reported the preparation of lycopene based nanoparticle for enhanced MCF-7 targeting by suing the *Ex-vivo* method.

8. Kani et al., (2016)[31] reported the anticancer activity of chemical synthesized SLNs of MCF-7 cancer cell line.

9. Yuan, H.; Miao, J.; Du, Y.Z.; You, J.; Hu, F.Q.; Zeng, S. Cellular uptake of solid lipid nanoparticles and cytotoxicity of encapsulated paclitaxel in A549 cancer cells. *Int. J. Pharm.,* 2008, *348*(1-2), 137-145.

10. Mussi, S.V.; Silva, R.C.; Oliveira, M.C.; Lucci, C.M.; Azevedo, R.B.; Ferreira, L.A. New approach to improve encapsulation and antitumor activity of doxorubicin loaded in solid lipid nanoparticles. *Eur. J. Pharm. Sci.,* 2013, *48*(1-2), 282-290.

11. Yang, T.; Sheng, H.H.; Feng, N.P.; Wei, H.; Wang, Z.T.; Wang, C.H. Preparation of andrographolide-loaded solid lipid nanoparticles and their *in vitro* and *in vivo* evaluations: Characteristics, release, absorption, transports, pharmacokinetics, and antihyperlipidemic activity. *J. Pharm. Sci.,* 2013, *102*(12), 4414-4425.

12. Sahu, P.K.; Mishra, D.K.; Jain, N.; Rajoriya, V.; Jain, A.K. Mannosylated solid lipid nanoparticles for lung-targeted delivery of paclitaxel. *Drug Dev. Ind. Pharm.,* 2015, *41*(4), 640-649.

13. Mosallaei, N.; Jaafari, M.R.; Hanafi-Bojd, M.Y.; Golmohammadzadeh, S.; Malaekeh-Nikouei, B. Docetaxel-loaded solid lipid nanoparticles: preparation, characterization, *in vitro*, and *in vivo* evaluations. *J. Pharm. Sci.,* 2013, *102*(6), 1994-2004.

14. Shenoy, V.S.; Gude, R.P.; Murthy, R.S.R. Paclitaxel-loaded glyceryl palmitostearate nanoparticles: *in vitro* release and cytotoxic activity. *J. Drug Target.,* 2009, *17*(4), 304-310.

15. Müller C,Schibli R. Folic acid conjugates for nuclear imaging of folate receptor-positive cancer. J. Nucl. Med.,2011;52(1):1-4.

16. Carbone C,Campisi A,Musumeci T,Raciti G, Bonfanti R, Puglisi G. FA-loaded lipid drug delivery systems: Preparation, characterization and biological studies. Eur. J. Pharm. Sci., 2014;52:12-20.

17. Mansoori GA, Brandenburg KS,Shakeri-Zadeh A. A comparative study of two folate-conjugated gold nanoparticles for cancer nanotechnology applications. Cancer2010;2(4):1911-1928.

18. Liu Z, Zhong Z, Peng G, Wang S, Du X, Yan D, Zhang Z, He Q, Liu J. Folate receptor mediated intracellular gene delivery using the charge changing solid lipid nanoparticles. Drug Deliv.2009;16(6):341-347.

19. Bilthariya U, Jain N,Rajoriya V, Jain AK. Folate-conjugated albumin nanoparticles for rheumatoid arthritis-targeted delivery of etoricoxib. Drug Dev. Ind. Pharm.,2015;41(1): 95-104.

20. Dubey RD,Alam N,Saneja A,Khare V, Kumar A,Vaidh S, Mahajan G, Sharma PR, Singh SK,Mondhe DM, Gupta PN. Development and evaluation of folate functionalized albumin nanoparticles for targeted delivery of gemcitabine. Int. J. Pharm.2015;4*92*(1-2): 80-91.

21. Gawde KA,Kesharwani P, SauS, Sarkar FH,Padhye S, Kashaw SK,Iyer AK. Synthesis and characterization of folate decorated albumin bio-conjugate nanoparticles loaded with a synthetic curcumin difluorinated analogue. J. Colloid Interface Sci.,2017;496:290-299.

22. Li R,Eun JS, Lee MK. Pharmacokinetics and biodistribution of paclitaxel loaded in pegylated solid lipid nanoparticles after intravenous administration. Arch. Pharm. Res., 2011;*34*(2):331-337.