RESEARCH ARTICLE

Design and development of plumbagin loaded poly (ε -caprolactone) nanoparticles for improved cytotoxicity

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ABSTRACT

Poly (ε -caprolactone) nanoparticles were developed as a nanocarrier for the delivery of natural naphthoquinones plumbagin for improvement in its solubility, drug release profile and *in vitro* cytotoxicity. Plumbagin loaded polymeric nanoparticle system was fabricated by nanoprecipitation method and the composition was optimized using factorial design approach. Nanoparticles showed particle size and encapsulation efficiency of 186 ± 1 to 300 ± 3 nm and 65.00+ 1.50 to 74.00+ 1.80% respectively. Optimization was carried out and optimized formulation showed sustained drug release over a period of 24 h. Concentration at total inhibition of cell growth (TGI) occurs was decreased by 56.95 % for PNP as compared to Plumbagin in MCF-7 cells indicates improved cytotoxicity of Plumbagin. The formulation development study proven that the developed PNP system exhibited improved solubility, sustained drug release, enhanced in vitro cytotoxicity in MCF-7 cell lines in comparison with Plumbagin. Thus the designed formulation approach can be further developed as novel carrier for plumbagin to enhance its biopharmaceutical properties.

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INTRODUCTION

Plumbago zeylanica linn (family *plumbaginaceae*) which is also well known as chitraka is widely reported in traditional system of medicine in India for wide range of therapeutic potential [1]. It has traditional applications as digestive aid, abortificient, laxative, anti arthritic, stimulant are well established. Total around 280 species are reported for family *Plumbaginaceae* out of which *Plumbago indica* L and *P. zeylanica* L are well distributed and widely used for traditional applications in India. The key chemical constituents like terpenoids, flavonoids, steroids and naphthoquinones are specifically reported for its therapeutic value [2]. Specifically cytotoxic potential of *P. zeylanica* was reported by multiple studies on different cancer cell lines [1, 2]. Plumbagin (PBG) is one of the important chemical constituent from plant *P. zeylanica* which is well reported for modulation in cell proliferation and carcinogenesis. The cytotoxic

activity is regulated by NF-κB activation. This leads to gene suppression [3]. Some more mechanisms like effects on inhibition on cell cycle, cell proliferation, induction of apoptosis are also reported [4]. For the evidence of *in vivo* aspect, ethanol extract *P. zeylanica* is reported for cytotoxic potential in Ehrlich Ascites Carcinoma model with the higher content of terpenoids and flavonoids it is also demonstrated for reduction in lipid peroxidation level [5]. The natural naphthoquinone plumbagin (PBG; 5-hydroxy2-methyl-1, 4-naphthoquinone) isolated form roots of *P. zeylanica* is key moiety which is well reported for variety of biological effects including anti-inflammatory, anti-bacterial and anti-atherosclerotic [6-8].

The bioactive constituents derived/isolated from natural sources are well proven for its relatively good biocompatibility and safety [6]. However, these moieties generally possess some issues related

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to the low solubility; bioavailability and stability which constrain their therapeutic use. Therefore, several approaches can be applied to overcome these issues. Nanotechnology as a one of the significant approaches have been well established for enhance solubility, better efficacy, dose reduction, safety and as well for patient compliance [9-13].

Cancer is commonly known as uncontrolled growth of cells which tend to develop lumps and if it is not treated on specific stage leads to cause death [14, 15]. PBG is reported for inhibition of growth for various types of cancers by activation of NF-kB and proto-oncogene Bcl-2 [16].

Despite of the good cytotoxic potential, PBG posses less bioavailability (oral- 39%) because of high (log P = 3.04) lipophilicity and less water solubility (79.3 ± 1.7 µg/ml); these factors considered as major challenges in formulation design [17]. Besides that considering dose requirement, high dose is required to reach therapeutic efficacy that leads to some toxicity and side effects [8].

Various approaches are reported in order to increase drug solubility including cyclodextrin complex formation solid dispersions or some chemical modifications. But there are various limitations associated with this approaches [9]. Polymeric nanoparticle system is one of the approaches established to overcome the limitations. The polymeric nanoparticles composed of biocompatible and degradable material have been investigated to achieve increased drug solubility, drug targeting by enhanced permeability and retention effect, prolonging blood circulating time and increase drug residence time at specific site [10]. Poly (ε-caprolactone) (PCL) is one of the biodegradable polymer most commonly used for pharmaceutical

applications [19-21] PCL has various advantages such as biocompatibility, biodegradation and applicability in formulation development [22] PCL has been reported widely for nanoparticulate drug delivery because of its various advantageous properties such as high hydrophobicity, high permeability, and biodegradability. Its biodegradation end product 6hydroxycaproic acid is neutral, which does not disturb the pH balance [23].

Quality by design approach helps researchers to understand the effects of critical variables in quality product development. Nanoparticle formulations can be developed using DoE approach to identify critical process parameters, reduce number of experiments, and study the interactions between formulation variables [18]. The present study has been designed to develop plumbagin loaded PCL nanoparticles using Design of Experiment approach to investigate its formulation characteristics and *in vitro* anticancer efficacy.

MATERIALS AND METHODS

Materials

Pluronic® F-127, Poly-ε-caprolactone, mw 65,000 Da) and dialysis bag were procured from Sigma Aldrich, Bangalore, India. Acetone GR grade, sodium hydroxide and potassium di-hydrogen phosphate from Merck India. All other chemical reagents used were of Analytical grade.

Preparation of Plumbagin loaded polymeric nanoparticles (PNP)

Polymer (PCL) and surfactant (Pluronic® F-127) were dissolved in 2 ml of acetone in which further plumbagin was dissolved as per composition showed in Table 1. Organic phase was added into

Table 1: The effect of formulation variable composition on PS and EE

Batches	Coded levels (X1, X)	PCL (mg; X1)	PF-127 (mg; X2)	DC %	PS (nm; Y1)	EE (%; Y2)
F1	-1, -1	50.00	25.00	90± 0.45	186± 1	65± 1.50
F2	-1, 0	50.00	50.00	92.4± 0.50	198± 2	66.12± 1.30
F3	-1, 1	50.00	75.00	93.57± 0.55	210± 2	69.04± 1.90
F4	0, -1	75.00	25.00	97.1± 0.80	160± 1	74± 1.80
F5	0, 0	75.00	50.00	96.1± 0.45	173± 1	73.9± 1.20
F6	0, 1	75.00	75.00	94.5± 0.90	204± 2	73.85± 1.50
F7	1, -1	100.00	25.00	95.2± 0.75	260± 2	73.87± 1.20
F8	1, 0	100.00	50.00	95.48± 0.90	289± 2	73.88± 1.80
F9	1, 1	100.00	75.00	94.2± 0.95	300± 3	71.5± 1.90

10 ml distilled water at injection rate 10 ml/min under continuous magnetic stirring at 1800 rpm for 2 h at 40°C. To remove copolymer aggregates the suspension was filtered through 0.45 μ membrane filter. Concentrations of PCL and Pluronic® F127 (PF 127) were also optimized obtain stable suspension [10].

Particle size, Drug content and Encapsulation Efficiency (EE)

The particle size (PS) of developed formulations was determined by laser diffraction using Malvern 2000 SM at a 90° scattering angle. The average particle size was determined,d (0.9) μm. The concentration of PBG in the nanoparticles was determined by measuring its absorbance at 419.5 nm on a double beam UV–VIS spectrophotometer. The nanoparticle solution was suitability diluted with ethanol prior to determination. The percent drug content (DC) was calculated according to the equation:

DC (%) = (Amount of PBG in nanoparticle/ Amount of PBG and polymer) × 100

Encapsulation efficiency (EE) was determined by centrifugation method (12,000 rpm; 2 h) at 40⁰C. The disruption of sediment nanoparticles were carried by dissolving it in acetone to release PBG; which further diluted with phosphate buffer pH 7.4 and analyzed by UV–VIS spectrophotometer. The percent EE was calculated using Equation:

EE (%) = Amount of PBG entrapped in the nanoparticle/ Initial amount of the PBG added × 100

DoE approach

For determination of optimized nanoparticle formulation Design of experiment approach was used. Particle size and Encapsulation efficiency values were measured after collecting the nanoparticles. For the statistical design, Design Expert® Version 12.0 was used and PS and EE were selected as dependent variables while the amount of polymer (PCL) and amount of surfactant (PF 127) were selected as independent variables. 3^2 (3 levels, 2 factors) factorial design was used, thus a total number of 9 set of system were performed.

Optimization of PBG-loaded nanoparticles by 3^2 factorial design

Preliminary experiments helped in understa-

nding the variables that affected the characteristics and utility of the drug loaded polymeric nanoparticles. The amount of PCL (X1) and PF 127 (X2) concentration were identified as crucial factors in determining the properties of the drug loaded polymeric nanoparticles. Thus, a 3^2 factorial design was implied for optimization of composition to study effect of X1 and X2 on PS and EE with response surface plot.

TEM Study

TEM study was carried out with Zeiss EM 109 with 50000X magnification to study of surface morphology of nanoparticle. Carbon film covered copper grid method was used for study. Sample was examined and photographed.

In vitro drug release study

The *in vitro* release of PBG was carried out by dialysis bag diffusion technique [10]. 2 mg of PBG equivalent formulation and 2 mg PBG solution as control was sealed in dialysis bag and immersed into 50 ml of release medium (PBS 7.4) at 37 ± 0.5°C with constant magnetic stirring at 100 rpm. Samples were withdrawn for analysis at specific time interval with maintaining sink condition. Absorbance was recorded by UV analysis [24].

Hemolysis study

The hemolysis study was carried out on Plumbagin and PNP formulationby following the method reported by love *et al.*2012. Study was carried out with concentrations range 0.25, 0.5, 0.75, 1 and 1.5 mg/mL. The analysis was carried out at absorbance 453 nm using UV analysis [25].

In vitro cytotoxicity studies

In vitro cytotoxicity of plumbagin and optimized formulation (PNP) was performed on breast cancer MCF-7 cell line with sulforhodamine B (SRB) assay. The study performed as per the procedure described by Bothiraja et al, 2013 at ACTREC Mumbai, India. PNP was diluted concentration ranges of 10, 20, 40 and 80μg/ml which were analyzed for cytotoxicity study. Absorbance was measured at a wavelength of 540 nm [25, 26].

RESULTS AND DISCUSSION

In present study, PBG-loaded PCL and PF 127 nanoparticles (PNP) have been developed by considering its safety. Nanotechnology based drug delivery systems has proven for improving

the solubility, efficacy and safety of various drugs over the past few decades [27-29]. In this study, PBG loaded polymeric nanoparticles have been developed and investigated as a nano carrier in order to improve its solubility, to achieve sustained release effect. The effect of the PBG loaded polymeric nanoparticles composition on Particle size and encapsulation efficiency was studied by DoE approach.

PS, DC and EE

The mean PBG nanoparticles size was in the range of 160 ± 1- 300 ± 3nm which was observed to be affected by formulation variables. The DC and EE were observed in range 90 ± 0.45 - 97.1 ± 0.80 % and 65 ± 1.50 - 74 ± 1.80% respectively for PNP (Table 1).A good fit (r² for PS = 0.9798 and EE = 0.9545 for PNP) was observed for the PS and EE the independent variables. The particle size affects the site specific drug delivery in terms of minimizing uptake by non-targeted cells or increasing the particle uptake either by targeted cells.

Optimization of nanoparticles by 3² factorial designs

Particle size and encapsulation efficiency are the major important factors considered in formulation development of nanoparticles. Primary assessment of the concentrations of polymers and surfactants were done for obtaining non-aggregating, non-sedimenting polymeric nanoparticles. After preliminary studies, Factorial design was used to optimize final proportions of polymer and surfactant (Table 1). Two factor three level designs were used with nine batches and response obtained was shown in Table 1. Data further analyzed for multiple regression analysis with Design Expert® Version 12.0 and data are summarized in Table 2.

From the factorial design study, it has been observed that positive coefficients for X1 and X2 showed a favorable effect on the mean PS and EE with the PCL and PF127. The PCL and PF 127 had a curvilinear effect on the PS and EE as seen in surface plot (Fig. 1). Based on the results of the factorial design, the solution for optimum batch selection with highest desirability was obtained with F5 so it was selected as an optimized formulation and further evaluated for various parameters.

TEM study

Surface morphology study of the PNP was carried out by TEM analysis as shown in Fig. 2. Spherical shaped freely dispersed nanoparticles were observed. No any drug crystallized particles were observed.

Hemolysis study

The result indicates that hemolysis rate of PNP was observed at 2.2 % up to 1.5 mg/mL concentration. From the hematological data it can be concluded that PNP showed better effects with less hemolysis within acceptable range so that can be consider as safe for *in vivo* applications

In vitro release study

In vitro release study revealed that PBG freely diffused in solution with 97.00 % release in 6 hr. Whereas, Plumbagin from PNP showed a biphasic release pattern with initial burst release (38.00%) within the first 6 h followed by sustained release up to 24 hr. The basic drug release mechanism includes diffusion, degradation and erosion out of which multiple mechanisms may involved in drug release for developed system. The burst release observed in initial stage may be due to drug

Table 2: Multiple regression analysis data with optimized batch solution

Sr. No.	Final Equation in Terms of Coded Factors		
	PS	EE	
1	+179.00	+73.98	
2	+42.50	+3.18	*A
3	+18.00	+0.2533	*B
4	+4.00	-1.60	*AB
5	+61.50	-4.02	*A²
6	+0.0000	-0.0900	*B²

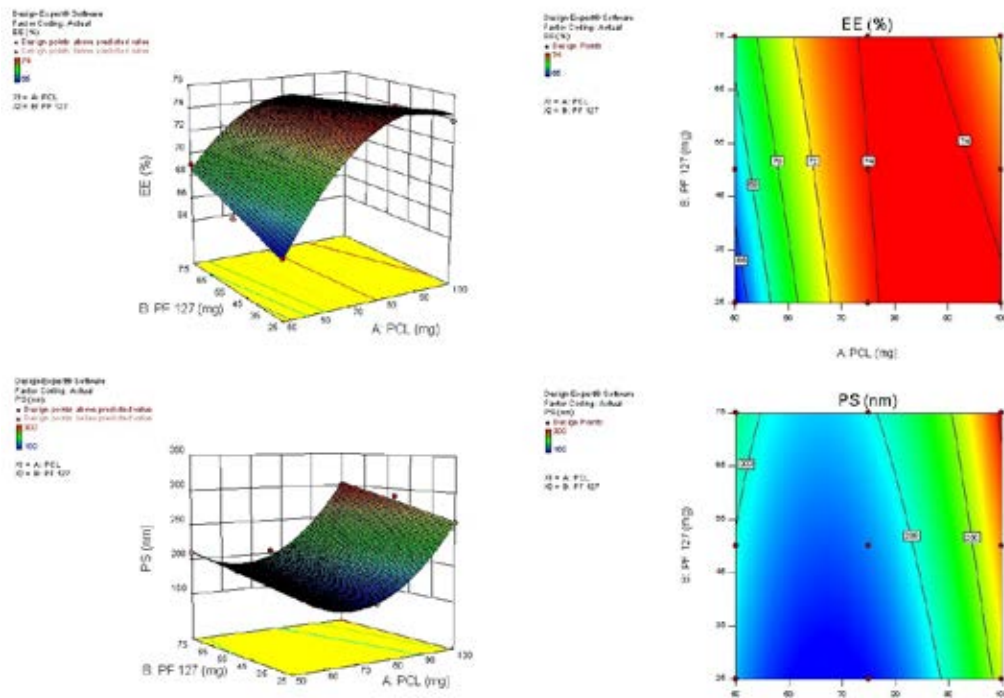| Optimized batch Solution					
PCL (mg)	PF 127 (mg)	DC (%)	EE (%)	PS (nm)	Desirability
82.852	52.056	96.470	74.559	200.000	1.000

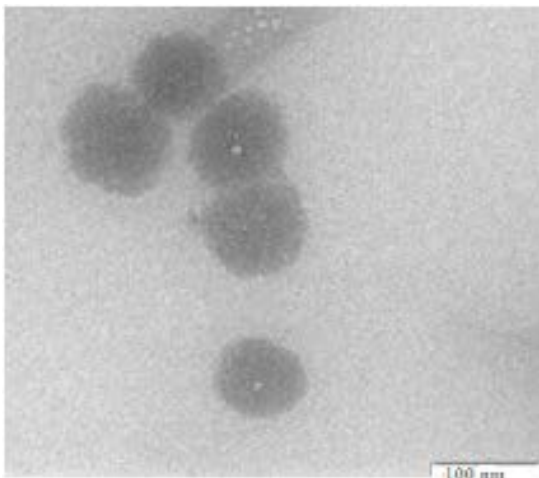Fig. 1: Response surface plot showing effects of formulation variables

Fig. 2: Transmission Electron Microscopy study of PNP

Table 3: *in-vitro* cytotoxicity study against MCF-7 Cell line

Group	MCF7 Cell line	
	TGI (µg/ml)	GI50 (µg/ml)
PNP	9.6 ± 0.020	<10
Plumbagin	22.3 ± 0.010	<10
Oncocristin inj. (marketed)	14.7 ± 0.010	<10
ADR (Doxorubicin)	<10	<10

adsorbed at surface or non encapsulated drug in solvent. Further entrapped drug showed sustained release pattern which is involved in maintenance of specific amount of drug at site.

In vitro cytotoxicity

In vitro cytotoxicity of PNP was carried out on MCF-7 by SRB assay. Results shown in Table 3; Fig. 4

indicated that PNP displayed better anticancer activity than PBG. TGI value of PNP was observed 9.6 ±0.020 µg/ml while for PBG it was 22.3 ±0.010 µg/ml. Results and microscopic images showed that in Fig. 4, .cells were appeared dense are represents normal control MCF-7, while in case PBG and PNP treated cells were appeared to be less dense and rounded.

The enhanced anticancer efficacy may be due to high cellular uptake of PNP through process of phagocytosis which resulted in the enhanced permeability of the cell membrane to PBG that allows sufficient drug concentration inside the cells. Therefore, PNP might serve as a good carrier to enhance cytotoxocity of PBG. The lower anticancer activity of free PBG may be due to P-glycoprotein pumps efflux. The GI50 value for
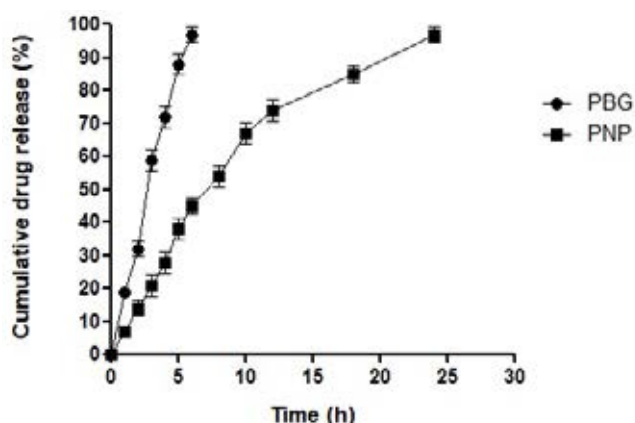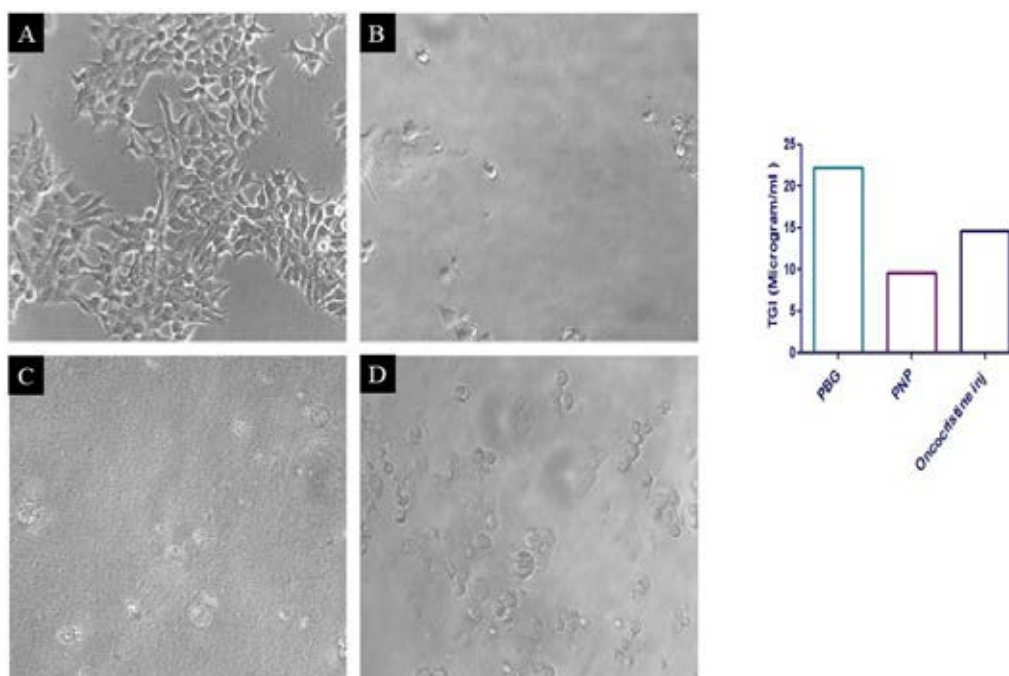
Fig. 3: In vitro drug release study

Fig. 4: In vitro cytotoxicity study A. Normal Control MCF-7 Cells (More density), B. Plumbagin (Rounded cells with less density), C. PNP (Rounded cells with less density), D. Oncocristine Inj. (Rounded cells with less density)

both PBG and PNP was observed to be less than 10 μg/ml as that of ADR (Doxorubicin) indicates good cytotoxic potential. PNP also compared with Oncocristin inj. (marketed formulation) which showed TGI value 14.7 + 0.010 μg/ml. The reason for enhanced cytotoxicity may be attributed to increased solubility, enhanced permeability and retention (EPR) effect because of reduction in particle size and developed formulation as a nanocarrier.

CONCLUSION

Nanoparticle formulations composed of PCL and PF-127 (PNP) as a nanocarrier for the delivery of an anticancer bioactive, PBG. Developed formulation displayed desirable characteristics in terms of PS, excellent EE, sustained release, biosafety and improved cytotoxicity as compare to plumbagin.Investigated drug delivery system can be further developed as novel dosage form to enhance biopharmaceutical aspect and therapeutic efficacy of PBG in therapy.

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CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

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