IJBPAS, October, 2022, 11(10): 4758-4778

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

OPTIMIZATION OF GOAT INTESTINAL PERMEABILITY OF BERBERINE CHLORIDE IN PRESENCE OF NATURAL BIOENHANCER PIPERINE USING 3^2 FULL FACTORIAL DESIGN

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Received 20th Jan. 2022; Revised 24th March 2022; Accepted 13th April 2022; Available online 1st Oct. 2022

https://doi.org/10.31032/IJBPAS/2022/11.10.6497

ABSTRACT

Present study was aimed to exploit 3^2 full factorial design in optimizing goat intestinal permeability of poorly permeable berberine chloride (BBC) on pre-treatment with bioenhancer piperine. For the optimization of concentration of piperine and pre-treatment time, Design-Expert software was used to predict the response % cumulative drug release (% CDR) of BBC across membrane. Effect of piperine was investigated at 3 disparate concentrations (2, 6 and 10 mg) and 3 disparate time of pre-treatment (30, 45 and 60 min). Furthermore, apparent permeability, flux and enhancement ratio were investigated. Additionally, optimized batch was screened for *in-vitro* anticancer activity on K562, A459 and Hela cancer cell lines. It was noticed that, with decrease in both concentration of piperine and pre-treatment time has positive influence on permeability parameters of BBC. Maximum value of 63.72±1.16 %CDR was obtained at 30 min pre-treatment time with 2 mg piperine over control 8.49±1.45 %CDR. Further, optimized batch showed extremely remarkable enhancement in i*n-vitro* anticancer activity over control. In brief, piperine mediated inhibition of intestinal multidrug efflux pump P-glycoprotein (P-gp) might be solely accountable for

permeability improvement of BBC. The limitation of poor intestinal permeability of BBC could be successfully overcome by pre-treatment with piperine.

Keywords*:* Poorly permeable drug, Bioenhancer, Franz diffusion study, *ex-vivo* permeation data, goat intestinal membrane permeability, response surface analysis, in-vitro anticancer activity

1. INTRODUCTION

Poor intestinal permeability of systemically acting drugs resulted into its low oral bioavailability. In addition, incomplete oral bioavailability leads to the variable plasma concentrations of drug resulting into wasting of much of an oral dose that makes the treatment costlier especially in case of expensive and rare drugs. Therefore, the maximization of intestinal permeability and accordingly its oral bioavailability is an interesting goal during drug design and development, and in clinical drug therapeutics. This encountered problem can be solved through several approaches like improvement in the physicochemical properties like surface area, partition coefficient, number of hydrogen bonding functional groups, molecular weight etc., of poorly permeable drug molecule. But physico-chemical characteristics of drug candidate are quite tough to modify as it requires balancing biological activity and intestinal permeability properties **[1]**.

A separate novel and effective strategy is to optimize the permeability characteristics and pharmacokinetic parameters such as bioavailability through use of the drug with herbal bioenhancers (Biopotentiators/Bioavailability enhancers). Administration of natural, safe, effective and economical bioenhancers along with drug has shown significant enhancement in the bioavailability profile of so many poorly bioavailable drugs. Use of bioenhancers leads to the lowering of dose as well as frequency of dosing of potents and nutraceuticals, so ultimately reduces the drug-resistance, toxicity and shortens the period of treatment and it makes the treatment cost effective. This integration of traditional Ayurvedic and allopathic treatment strategy imparts multidirectional benefits and satisfies all necessary criteria of safe and ideal combination without causing toxicity **[2, 3]**.

With various types of bioenhancers available, one of the effective and low cost bioenhancer piperine was selected. As piperine is one of the natural, safe and effective permeation enhancer having p-gp inhibitory activity which also works through inhibition of drug metabolizing enzymes like CYP 3A4. Piperine mediated bioavailability enhancing action is partly due to increased blood supply in enteric

vessels due to local vasodilatation. It also interacts with small intestinal membrane cells and enhancing their amino acid absorbing capacity. Due to its easy partitioning, it can modulate membrane dynamics, thus helping to efficiently permeate drugs across barriers **[4]**. Thereby, taking into account the favorable impacts of these bioenhancers, it was deemed worth exploring the permeability behaviour and the anticancer activity of poorly permeable natural anticancer berberine chloride (BBC) in the existence of bioenhancer piperine.

Berberine chloride (BBC) is a natural isoquinoline alkaloid having manifold promising therapeutic activities like anti-hypertensive, hepatoprotective, anti-inflammatory, hypolipidemic, anti-retroviral, anti-oxidant, hypoglycaemic, anti-malarial, anti-arrhythmic, anti-neoplastic and anti-secretory activity etc. **[5, 6]**. It has been practiced in Ayurveda and traditional Chinese medicine for millennia. Due to cost effectiveness, minimal toxic impact and innumerable therapeutic actions, recently it has gained remarkable curiosity and tremendous attention. In spite of its significant activities, its oral use has been severely curtailed as it shows extremely low and variable plasma concentrations in humans with an absolute oral bioavailability of less than 1 % **[7]**. In clinical emergencies, it takes a massive dose (up to 1.5 g per day) that could cause detrimental effects on gastrointestinal tract **[8]**.

The key factors for poor membrane permeability and subsequently poor bioavailability of drug are the prevalence of cytochrome P450 enzymes in the gut / liver which are accountable for presystemic drug metabolism, impaired absorption, predominant tissue distribution, drug excretion into the lumen, bile or urine because of the presence of certain efflux transporters such as P- glycoprotein (P-gp) ascribes to poor absorption & flactuating plasma level following oral administration. It's a substrate for the gastrointestinal efflux pump P-glycoprotein (P-gp), which serves as a barrier to BBC uptake **[7, 9]**. This problem could be solved with the use of various permeability enhancement techniques to improve drug permeation transiently.

It has been postulated that co-administration or pre-administration of BBC with permeability enhancers might boost its permeability, blood levels, pharmacological activities, and reduce gastric negative consequences **[10]**.

However, in the existence of piperine on goat intestinal membrane neither of the studies has been published to optimize the ex- vivo permeability characteristics of BBC employing Franz diffusion cell. In current investigation, a 3^2 full factorial

design approach was employed to optimize the influence of pre-treatment of piperine on permeability behavior of BBC and it was utilized to minimize number of trials to get maximum data on permeability behavior of BBC. Following that, the optimized batch was evaluated for anticancer efficacy (in vitro) across various cancer cell lines.

MATERIALS AND METHODS

2.1 Materials

Sample of BBC was gladly bestowed by Indo German Alkaloids, Mumbai, India. Piperine was bought from Loba Chemie Pvt. Ltd. India. The research experiment was carried out using deionized double distilled water. Fresh goat intestine was obtained from a nearby slaughterhouse and used within 1 hour after the goat being killed **[11]**. All other chemicals and solvents were of analytical grade.

2.2 Preparation for ex-vivo Permeation Study

2.2.1 Receptor fluid

Phosphate buffer pH 7.4 was utilized as a receptor fluid and was made according to Indian Pharmacopoeia Monograph.

2.2.2 Goat intestinal membrane model

For *ex-vivo* permeation studies, freshly dissected goat intestinal membrane has been used as goat jejunum is a reliable and good predictor of human per-oral absorption **[12]**. It was prepared by cut with 3.2 cm^2 area and 500-600 µm thickness, washed with phosphate buffer pH 7.4 and kept alive by supplying oxygen through aerator in phosphate buffer (pH 7.4). Intestinal membrane was pre-treated with three disparate concentrations of piperine for three disparate interval, i.e. for 30 min., 45 min. and 60 min.

2.2.3 Control Sample

For the current study, a BBC dose of 10 mg was employed as a control sample.

2.2.4 Test Sample

Study plan for pre-treatment process, variables and levels from the experimental design approach by response surface analysis is shown in **Table 1**. The similar dose for BBC (10 mg) was kept for whole study.

2.3 Design of experiment for optimization study

To explore the impact of independent variables on permeability profile of BBC 3^2 full factorial design approach was employed. In current outlook, two factors were investigated at three disparate levels and nine possible combos were practically investigated. The 2 independent variables investigated were concentration of piperine (X_1) and pre-treatment time of intestinal membrane with piperine (X_2). The dependent variable i.e. response was chosen as % CDR (Cumulative Drug Release). The study

levels and process variables were established from earlier trials.

2.3.1 Response surface analysis

For optimization of experiment, STATEASE software (design expert, version 9.0.4.1) was used to carry out a 3^2 full factorial design by response surface methodology. Two factor three level design was used for optimizing permeability parameters by exploring the quadratic response surfaces and constructing second order polynomial model. Polynomial equation involving individual factors was selected based on R^2 analysis, model analysis, lack of fit and predicted residual sum of squares (PRESS) for measured response % CDR. To fit the surface in following term a quadratic equation given by the design was used

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2 + b_4X_1^2 + b_5X_2^2 \quad (1)$$

Where Y is response, b_0 is intercept, b_1 to b_5 are regression coefficients, X_1 and X_2 are individual effects, X_1^2 and X_2^2 are quadratic effects, X_1X_2 is interaction effect. One way ANOVA was applied to determine the significance of the model ($p < 0.05$). All the responses (% CDR), ($n = 3$) were expressed as mean ± standard deviation (SD).

2.3.2 Optimization and validation of model

For the optimization of concentration of piperine and pre-treatment time, Design-Expert software was used to build a mathematical model to predict the response %cumulative drug release (%CDR) of BBC across goat intestinal membrane. The influence of independent variables on dependent variable can be described by a contour plot and the ANOVA presented by software can be used to evaluate statistically significant factors. Design-Expert software provided comparisons of several statistically significant terms and R^2 value can be employed to test the best fitting mathematical models. Optimized batch will be batch giving maximum value of responses (O-1) which will be selected for check point analysis and *ex-vivo* permeation study of selected optimized batch was performed. The resulting response was compared quantitatively to the predicted value, and the prediction error was computed.

2.4 Ex- vivo permeability study

Assessment of ex-vivo permeability behavior of BBC in existence (co-administration) and non-existence (control) of piperine on freshly dissected goat jejunum membrane was investigated employing a Franz diffusion cell in a phosphate buffer pH 7.4. The goat intestinal membrane was firmly anchored between the donor and the receiving chamber (capacity 12 ml) of the diffusion cell with an upward orientation of the

mucosal side of membrane model. For diffusion of BBC across the intestinal mucosa, area of about 3.14 cm^2 was kept accessible. As a receptor fluid, phosphate buffer pH 7.4 maintained at 37 ± 1°C was used throughout the experiment which was agitated with teflon-coated magnetic stirring bead at 100 r / min. Test and control samples were applied on the donor side of diffusion cell, and at each 30 min time interval 2 ml aliquots were withdrawn from receiver chamber up to 6 h maintaining sink conditions throughout the experiment. UV spectrophotometer was used to analyze the aliquots at 341 nm and the cumulative amount of drug permeated was computed (n=3).

2.5 Data analysis

From ex-vivo permeability studies data obtained in triplicate for each test and control samples were utilized to calculate permeability metrics like % CDR, apparent permeability (Papp), Flux (J) and enhancement ratio (ER) by using standard formulae **[12, 13]**

Permeability coefficient (apparent permeability)-

$$Papp\left(\frac{cm}{s}\right) = \left(\frac{VA}{[area \times time]}\right) \times \left(\frac{[Drug]acceptor}{[Drug]donar}\right)$$

Where, VA= volume in acceptor chamber, Area= intestinal membrane surface area, Time= total transport time

$$Flux(J)\left(\frac{\frac{mg}{cm2}}{hr}\right) = \frac{mass\,diffusing}{surface\,area \times time}$$

$$Enhancement\,Ratio\,(ER) = Papp\,of\,combination/Papp\,of\,control$$

2.6 In vitro anticancer activity

In vitro anticancer activity of BBC, piperine, and optimized PreP1 batch (cancer cell lines pre-treated with 2 mg piperine for 30 min, then treating with BBC), were investigated on different cancer cell lines A459 (Lung Cancer), K562 (Leukemia) and Hela (Cervical Cancer) by MTT assay. Cells were seeded in triplicate at a density of approximately 5×10^3 cells/well for overnight in a 96 -well flat-bottom micro plate, maintained at 37°C in 95% humidity and 5% CO_2. Samples of different concentration were treated and cells were incubated for another 24 hours. Cells were washed twice with the solution of phosphate buffer, and 20 µl of the MTT staining solution (5mg/ml in phosphate buffer solution) was added to each well and at 37°C plate was incubated. After 4 h, in order to dissolve the formazan crystals,100 µl of di- methyl sulfoxide (DMSO) was added to each well and using micro plate reader absorbance was recorded at 570 nm

[14, 15]. Graph pad prism version 5.1 was used to compute the IC50 of compound, and mean cell viability was calculated using formula

$$\%\ cell\ viability = (mean\ OD\ of\ test\ compound \div mean\ OD\ of\ negative\ control) \times 100$$

3 RESULTS AND DISCUSSION

The findings of ex-vivo permeability behaviour of BBC in existence (pre-treatment) and non-existence (control BBC) of piperine is shown in **Table 2**. In the present goat intestinal permeability assessment, during the pre-treatment phase, it was discovered that the permeability of BBC was significantly enhanced (63.72 ± 1.16 %CDR) at 2 mg piperine pre-treated for 30 min (PreP1 batch) (optimized batch) while the minimum value (26.17±1.27 % CDR) was observed at 10 mg piperine pre-treated for 60 min (PreP9 batch), as compared to control sample BBC (8.49±1.45 % CDR at 10 mg). The **Table 3** shows values of % CDR, Papp, J and ER calculated upto 6 h for all samples. The impact of desperate pre-treatment time of piperine on % CDR of BBC is shown in **Figure 1, 2 and 3**. Papp and enhancement ratio of BBC on pre-treatment with piperine is shown in **Figure 4**.

The membrane permeability of the control sample BBC was only 8.49 ± 1.45 % CDR. The previously disclosed affecting factors for poor permeation across membrane, poor bioavailability as well as stability related problem of BBC were, low apparent oil-water partition coefficient (IgPapp= - 1.08), log P value (-1.5), influence of structural components present in BBC i.e. presence of hydrophobic properties like two methoxy groups and a quaternary ammonium cation that shows high binding affinity to the gastro-intestinal multidrug efflux pump P-gp. Earlier it was identified that BBC is a P-gp efflux pump substrate having a high affinity towards it, attributable to prevalence of cationic group in structure **[16, 17]**. It was indicated in the Caco-2 cell line work that, when synthetic inhibitors of P-gp efflux pump (cyclosporine and verapamil) co-administered with BBC, reported a dramatic enhancement in BBC absorption **[16]**, exemplifying engagement of P-gp pump in efflux of BBC back into intestinal lumen, demonstrating the primary cause for its poor permeability **[7]**.

In consonance with literature reports, co-administration or pre-treatment of BBC together with natural bioenhancer quercetin has really shown marked improvement in permeability profile of BBC **[18, 19]** implying that bioenhancer facilitated P-gp inhibition could be able to boost permeability profile of BBC.

As per literature review piperine inhibits human Pgp and CYP3A4 proteins that leads to first-pass clearance of many medications and that makes it an important

enhancer for enhancing the bioavailability of a variety of drugs against different diseases **[20]**. Literature review reveals that, co-administration of piperine (10 mg / kg orally) along with several drug molecules, such as ciprofloxacin, gatifloxacin, ginsenoside Rh2, atenolol, fexofenadiene, phenytoin etc., has demonstrated notable enhancement in pharmacokinetic characteristics of drug **[21-26]**. Also pre-treatment study of mice and healthy human volunteers with piperine (present as a food constituent) for 30 min has shown significant enhancement in the pharmacokinetic profile of phenytoin **[27]**. It was also reported that, pre-treatment of piperine along with certain drug candidates like domperidone, **[20]** diclofenac **[28]** etc. showed marked improvement in the pharmacokinetic profile of drug. Piperine has been shown to impact plasma levels of P-gp substrates and CYP3A4 substrates in humans, particularly when these medications are taken orally. Drug metabolizing enzymes like CYP1A1, CYP1B1, CYP1B2, CYP2E1, and CYP3A4 etc get inhibited by piperine thus improves bioavailability and therapeutic efficacy of various medications via its action on drug metabolism. It was noticed that, enhancement in the pharmacokinetics of drug was due to the cumulative influence of piperine on drug absorption kinetics and drug metabolism inhibitory effect. They recommended that piperine mediated P-gp efflux pump inhibition was predominantly attributed to intensified oral drug permeability and consequently bioavailability **[4, 29]**.

Similarly, in the current permeability work on goat intestinal membrane, increase in the permeability of BBC during pre-treatment with piperine was found. The decrease in the concentration of piperine (2 mg) and pre-treatment time (30 min) was noticed to have a beneficial influence on the drug permeability profile. Papp, J and ER were also observed to be substantially increased at optimized experimental conditions relative to the control **(Figure 4)**. This could be ascribed to piperine-mediated suppression of the P-gp efflux pump contributing to an improvement in the BBC permeability profile.

Employing factorial design approach by response surface analysis, pre-treatment study was performed. Design-Expert software was employed to build mathematical model to predict the response. The regression equation (2), the three-dimensional response surface plot **(Figure 5)** and corresponding contour plot **(Figure 6)** indicates, decrease in concentration of piperine and pre-treatment time has positive impact on % CDR, while the increase in concentration and pre-

treatment time by piperine had a negative impact on % CDR.

Table 4 presents results of model analysis, R2 analysis, and predicted residual sum of square (PRESS) value for measured responses. The software generated quadratic model was found to be statistically valid (R^2 0.9964) and significant (F 167.28), exhibiting excellent predictive ability (R^2 0.9576). **Table 5** indicates outcome of one-way ANOVA ($p < 0.05$) used for statistical model evaluation. Significant difference was found between group means than the expected value from the observed "F-value" (higher than 1.0).

The model equation was derived from the experimental design, and the response surface data were fitted in Equation (1) and the fitting models for response (% CDR) was given in Equation (2) suggesting an empirical relationship among dependent and independent variables in coded unit. Regression equation of the fitted models.

$$Y_1 = + 30.09 - 8.03\ X_1 - 9.95\ X_2 + 3.08\ X_1^2 + 5.23\ X_2^2 + 6.45\ X_1\ X_2 \quad (2)$$

Polynomial equation generated was used to optimize permeability behaviour of BBC for the response Y (% CDR). From the desirability criteria, the optimal value of response was obtained. Optimized batch (0-1) was selected for check point analysis and optimization capacity of generated model was evaluated by investigation of ex vivo permeability study. **Table 6** shows outputs of tests for confirmation of optimization capability.

In- vitro anticancer activity

Anticancer activity of optimized batch PreP1, BBC, and piperine against disparate cancer cell lines K562, A459, and Hela was determined and IC_{50} values of compounds in µg/ml **(Table 7)** and their mean cell viability are shown in **Tables 8 and 9**. Mean cell viability of optimized batch BBC, piperine and PreP1 at desperate concentrations on desperate cancer cell lines has been presented in **Figure 7, 8 and 9** respectively. Extremely significant enhancement in anticancer activity of optimized batch was recorded in case of all three cell lines as compared with drug BBC. IC_{50} value and mean cell viability of optimized batch PreP1 was found to be significantly decreased as compared to BBC and piperine alone in case of all three cell lines.

MDR (multidrug resistance) is a severe complication in cancer that limits the use of chemotherapy for successful cancer treatment **[30, 31]**. Overexpression of P-gp is sometimes blamed for the highly invasive phenotype associated with malignancies (also known as MDR1 or ABCB1). By serving as an ATP-dependent efflux pump for a wide spectrum of

chemotherapeutic drugs, P-gp confers resistance to cancer cells **[32]**.

Piperine is a powerful P-gp and MRP-1 inhibitor **[33]**. In P-gp overexpressing KB (cervical) and SW480 (colon) cancer cells, co-administration of piperine with specific medicines (like paclitaxel, vincristine, or colchicine) was shown to reverse drug resistance. Piperine also has chemopreventive benefits by inhibiting the drug metabolising enzyme system's metabolic activation of some pro-carcinogens (DME) **[34]**. It was originally considered that stimulating glutathione-metabolizing enzymes (e.g., GPx, GR, and G6PDH) protects cells from reactive metabolites like ROS and ultimate reactive forms of carcinogens. Piperine causes a significant enhancement in glutathione-metabolizing enzymes (GPx, GR, and G6PDH), taking into account it's action in the prevention of cancer **[35]**. Thus, enhancement in the anticancer activity of optimized batch might be due to synergistic anticancer effects produced by both BBC and P-gp inhibitor piperine.

Table 1: Experimental plan, factors and levels from the 3^2 factorial design for pre-treatment study

Sample code	Coded value		Actual value	
	X1 Concentration	X2 time	X1 Piperine (mg)	X2 Pre-treatment time (min)
PreP1	-1	-1	2	30
PreP2	0	-1	6	30
PreP3	+1	-1	10	30
PreP4	-1	0	2	45
PreP5	0	0	6	45
PreP6	+1	0	10	45
PreP7	-1	+1	2	60
PreP8	0	+1	6	60
PreP9	+1	+1	10	60

Table 2: %CDR ± SD of BBC from control and pre-administration study with piperine

Time (h)	BBC	PreP1	PreP2	PreP3	PreP4	PreP5	PreP6	PreP7	PreP8	PreP9
0.5	0.28±0.03	4.99±1.11	1.84±0.30	2.53±0.78	3.92±0.69	3.43±1.18	2.61±0.73	2.77±1.04	3.05±0.57	3.47±0.81
1	0.83 ±0.20	9.88±1.09	3.48±0.61	4.83±0.80	7.81±0.91	4.75±1.32	3.50±0.83	5.06±1.10	4.70±0.97	4.16±1.09
1.5	1.39±0.38	13.33±0.98	7.92±0.71	7.15±0.82	10.93± 1.48	7.53±1.29	4.75±0.95	7.33±1.09	7.08±1.20	5.00±0.89
2	1.97±0.05	19.23±1.08	13.42±1.30	9.15±1.34	15.25± 1.21	9.51±1.38	8.73±0.87	9.45±1.90	9.11±0.74	7.95±0.49
2.5	2.34±0.15	24.90±1.34	11.32±1.23	13.50± 1.52	17.47± 0.95	11.33± 1.90	11.76± 1.38	13.97± 1.20	13.38± 1.73	10.38± 0.77
3	2.95±0.00	30.46±1.31	18.90±0.98	19.90± 1.00	22.78± 0.95	15.37± 1.24	14.79± 1.11	19.05± 0.63	15.87± 1.30	13.39± 0.48
3.5	3.50±0.14	34.65±0.63	23.79±0.39	25.62± 1.08	27.81± 1.02	18.41± 0.59	17.59± 0.94	21.54± 0.34	18.30± 0.96	14.98± 0.18
4	4.50±0.41	45.54±0.64	25.66±1.10	30.10± 0.30	32.61± 1.43	21.51± 1.18	19.81± 1.02	27.51± 0.66	20.46± 0.65	17.38± 0.38
4.5	5.53±0.30	49.70±0.42	30.88±0.29	34.57± 0.34	35.54± 1.51	24.84± 0.87	22.65± 0.88	30.91± 0.86	22.31± 0.63	20.86± 0.74
5	6.95±1.10	53.74±0.02	35.05±1.48	37.12± 0.39	38.57± 0.77	27.68± 0.89	25.70± 0.36	33.94± 0.90	24.15± 0.42	23.68± 0.66
5.5	7.70±1.28	58.35±0.96	42.94±0.28	38.77± 0.38	16.64 ± 1.59	29.17± 0.71	26.93± 0.43	35.55± 0.82	25.89± 0.58	25.22± 1.21
6	8.49±1.45	63.72±1.16	44.82±0.47	40.57± 0.23	18.03 ± 1.74	30.45± 0.53	28.05± 0.33	37.01± 0.88	27.06± 0.36	26.17± 1.27

Table 3: Experimental plan and observed response values with *ex- vivo* permeability profiles from 3^2 full factorial design

Samples	Coded value X1	Coded value X2	Actual value X1	Actual value X2	%CDR	Papp $\times 10^{-7}$cm/s	Flux J mg/cm^2/h	ER
BBC	------	------	------	------	8.49±1.45	6.37	0.0451	------
PreP1	-1	-1	2	30	63.72±1.16	74.30	0.3381	11.6641
PreP2	0	-1	6	30	44.82±0.47	54.84	0.2420	8.6091
PreP3	+1	-1	10	30	40.57±0.23	47.76	0.2155	7.4976
PreP4	-1	0	2	45	42.25±1.03	47.76	0.2245	7.4976
PreP5	0	0	6	45	30.45±0.53	35.38	0.1614	5.5542
PreP6	+1	0	10	45	28.05±0.33	33.61	0.1486	5.2763
PreP7	-1	+1	2	60	37.01±0.88	44.22	0.1964	6.9419
PreP8	0	+1	6	60	27.06±0.36	30.07	0.3066	4.7206
PreP9	+1	+1	10	60	26.17±1.27	31.84	0.1391	4.9984

Pre: pre-treatment; BBC: berberine chloride, P: Piperine; X1- concentration of piperine (mg); X2- time of pre-treatment,(min); %CDR- percentage cumulative drug release; Papp-apparent permeability coefficient, *j*- flux i.e. amount of drug permeated through a unit area in a unit of time; ER- enhancement ratio; PreP1: Pre-treatment with 2 mg piperine for 30 min; PreP2: Pre-treatment with 6 mg piperine for 30 min; PreP3: Pre-treatment with 10 mg piperine for 30 min.; PreP4: Pre-treatment with 2 mg piperine for 45 min; PreP5: Pre-treatment with 6 mg piperine for 45 min; PreP6: Pre-treatment with 10 mg piperine for 45 min; PreP7: Pre-treatment with 2 mg piperine for 60 min; PreP8: Pre-treatment with 6 mg piperine for 60 min; PreP9: Pre-treatment with 10 mg piperine for 60 min.

Table 4: Outcome of model analysis, r^2 analysis and press value for measured response

Coefficients	Values	Parameters	Values
b_o	+30.09	R^2	0.9964
b_1	-8.03	Adjusted R^2	0.9905
b_2	-9.95	Predicted R^2	0.9576
b_3	+3.08	Adeq Precision	37.7429
b_4	+5.23	Std. Dev.	1.18
b_5	+6.45	Press	49.25
F value	167.28	p value	< 0.05

Table 5: Summary of ANOVA for the response parameters

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model for Y (% CDR)	1157.36	5	231.47	167.28	0.0007	significant
X_1-conc	387.05	1	387.05	279.71	0.0005	
X_2-time	594.41	1	594.41	429.57	0.0002	
X_1X_2	37.88	1	37.88	27.38	0.0136	
X_1^2	54.81	1	54.81	39.61	0.0081	
X_2^2	83.20	1	83.20	60.13	0.0045	

X_1 and X_2 represent the main effects (factors); X_1^2 and X_2^2 are the quadratic effect; X_1X_2 is the interaction effect; %CDR- percentage cumulative drug release.

Table 6: Result of experiment for confirming optimization capability

Code	Factor		Response % CDR		
	Conc (mg)	Time (min)	Predicted	Obsereved	Error (%)
O-1	2	30	62.84	61.60±0.66	1.97

O-1: optimized batch one; %CDR- percentage cumulative drug release; observed response values: mean ± SD (n= 3): Error (%) = [difference between predicted value and observed value/predicted value]×100.

Table 7: IC_{50} value of compounds in µg/ml

Compound	A549	K562	Hela
BBC	33.19	73.90	4.618
Piperine	59.81	57.69	31.07
PreP1	18.62	44.21	2.556

BBC: berberine chloride, PreP1: optimized batch no.1 pre-treated, cell lines A459 (Lung Cancer), K562 (Leukaemia) and Hela (Cervical Cancer)

Table 8: Mean cell viability of BBC and optimized batch Prep1 at different concentrations on different cancer cell lines

	A549		K562		Hela	
Conc µl/ml	BBC	PreP1	BBC	PreP1	BBC	PreP1
250	23.27	18.26	41.48	20.10	8.77	19.12
125	38.78	23.03	44.91	36.22	15.51	24.61
62.5	44.30	44.03	49.56	46.42	21.15	29.15
31.25	49.50	46.42	56.66	56.21	22.60	32.45
15.625	58.98	52.15	62.68	61.93	32.33	35.27
7.8125	65.57	56.44	71.09	78.64	44.35	42.32
NC	100					

BBC: berberine chloride, P: piperine, PreP1-Optimized batch, cell lines A459 (Lung Cancer), K562 (Leukaemia) and Hela (Cervical Cancer); NC: Negative control

Table 9: Mean cell viability of piperine at different concentrations on different cancer cell lines

Conc. Piperine µl/ml	A549	K562	Hela
50	54.65	55.49	42.89
25	76.25	63.48	54.70
12.5	85.92	81.51	62.69
6.125	93.68	86.99	79.29
3.0625	97.02	94.36	92.73
1.5313	99.40	97.65	95.78
NC	100		

Cell lines A459 (Lung Cancer), K562 (Leukaemia) and Hela (Cervical Cancer); NC: Negative control

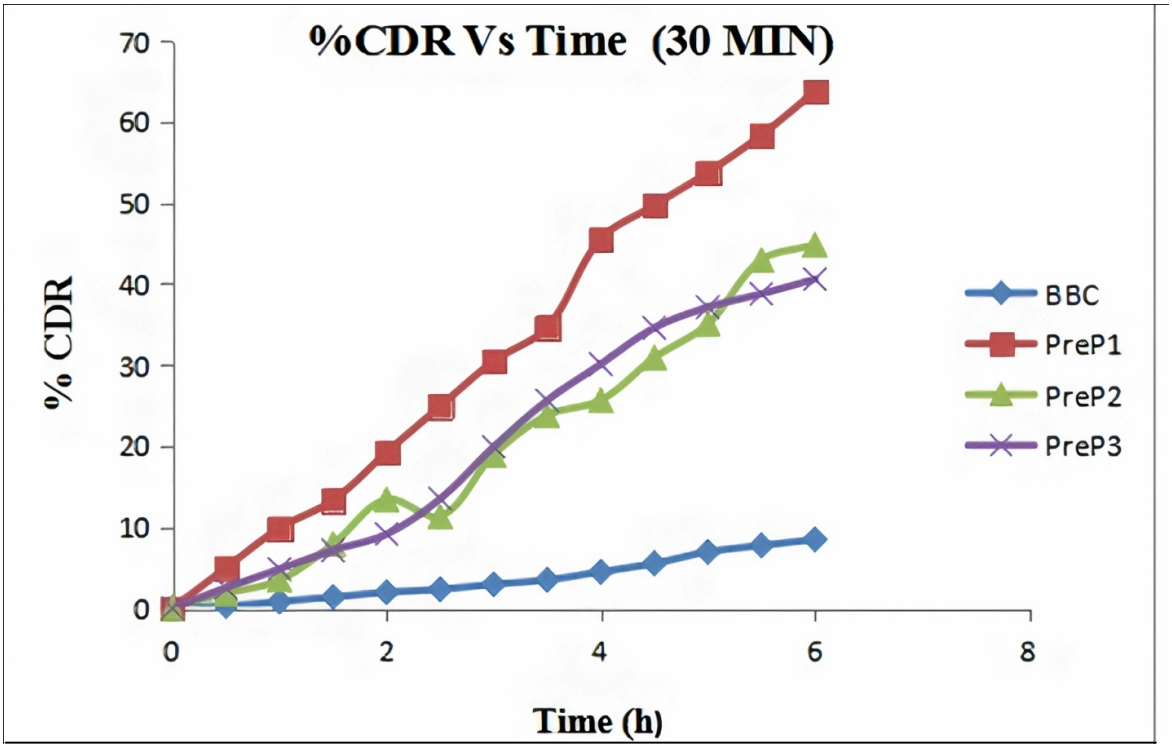

Figure 1: Effect of pre-treatment of Piperine for 30 min on % CDR

% CDR: Percentage Cumulative drug release; BBC: Berberine Chloride; PreP1: Pre-treatment with 2 mg piperine for 30 min; PreP2: Pre-treatment with 6 mg piperine for 30 min; PreP3: Pre-treatment with 10 mg piperine for 30 min.

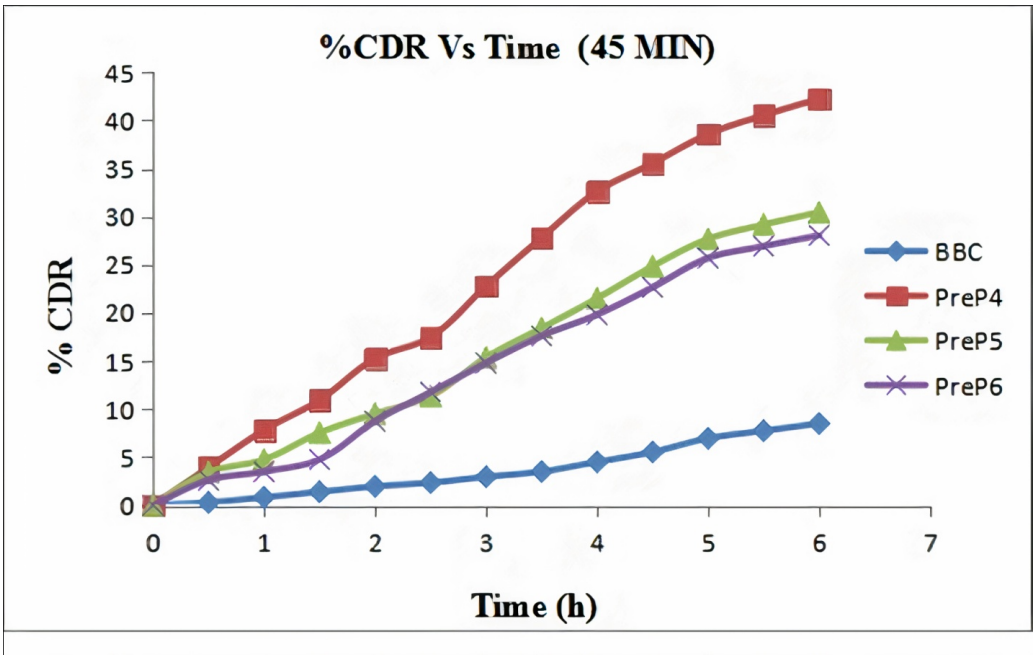

Figure 2: Effect of pre-treatment of piperine for 45 min on % CDR

% CDR: Percentage Cumulative drug release; BBC: Berberine Chloride; PreP4: Pre-treatment with 2 mg piperine for 45 min; PreP5: Pre-treatment with 6 mg piperine for 45 min; PreP6: Pre-treatment with 10 mg piperine for 45 min.

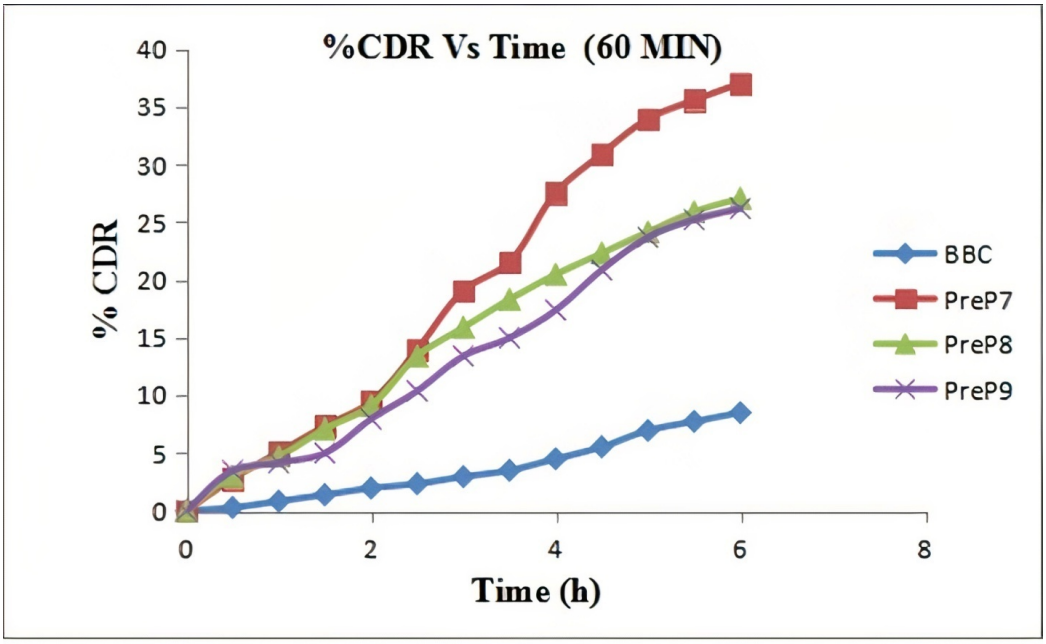

Figure 3: Effect of pre-treatment of piperine for 60 min on % CDR

% CDR: Percentage Cumulative drug release; BBC: Berberine Chloride; PreP7: Pre-treatment with 2 mg piperine for 60 min; PreP8: Pre-treatment with 6 mg piperine for 60 min; PreP9: Pre-treatment with 10 mg piperine for 60 min.

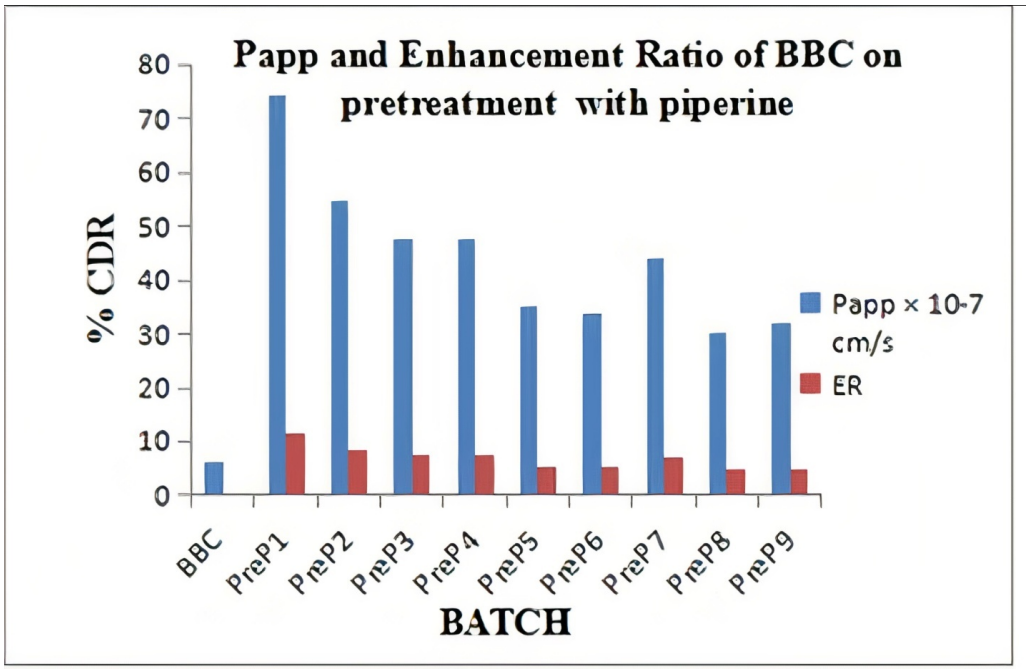

Figure 4: Papp and Enhancement Ratio of BBC on pre-treatment with piperine
% CDR: Percentage Cumulative drug release; BBC: Berberine Chloride; Papp-apparent permeability coefficient; ER- enhancement ratio. PreP1: Pre-treatment with 2 mg piperine for 30 min; PreP2: Pre-treatment with 6 mg piperine for 30 min; PreP3: Pre-treatment with 10 mg piperine for 30 min.; PreP4: Pre-treatment with 2 mg piperine for 45 min; PreP5: Pre-treatment with 6 mg piperine for 45 min; PreP6: Pre-treatment with 10 mg piperine for 45 min; PreP7: Pre-treatment with 2 mg piperine for 60 min; PreP8: Pre-treatment with 6 mg piperine for 60 min; PreP9: Pre-treatment with 10 mg piperine for 60 min.

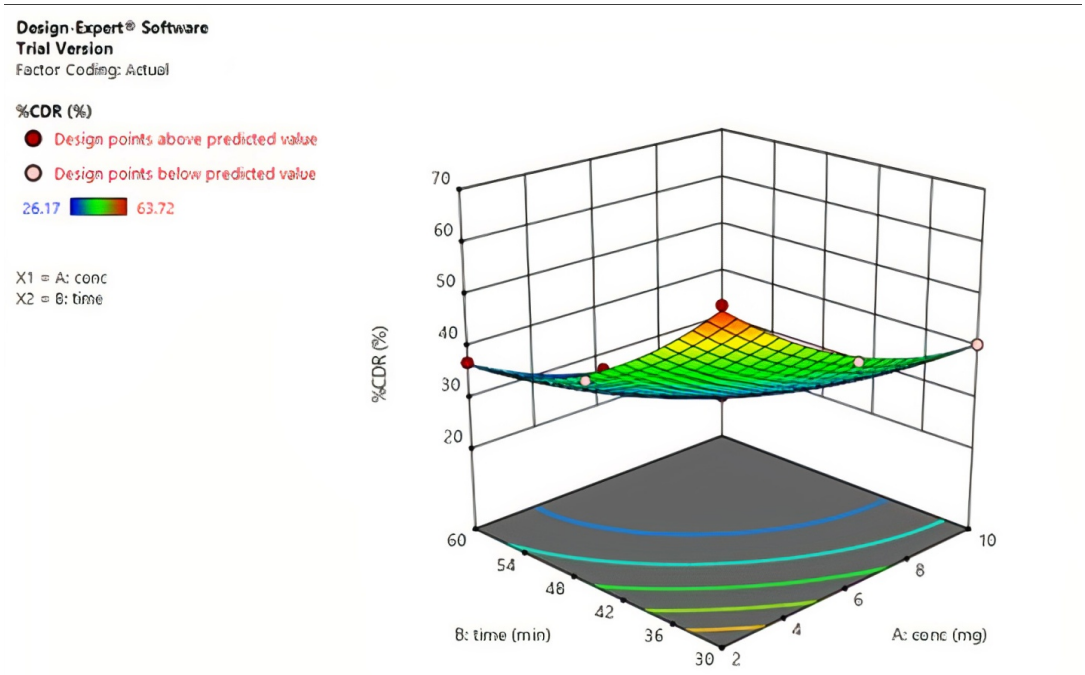

Figure 5: Effect of concentration of piperine and time on % CDR presented by response surface plot.
CDR: Cumulative drug release

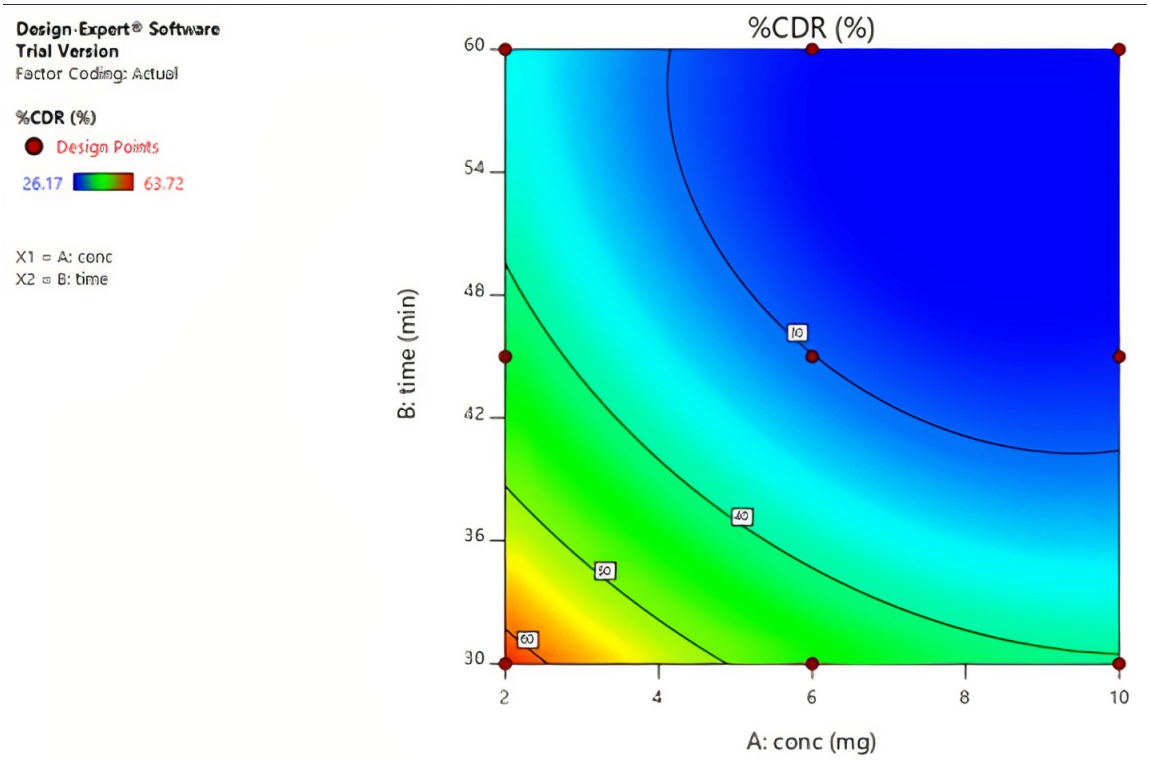

Figure 6: Effect of concentration of piperine and time on % CDR presented by contour plot.
CDR: Cumulative drug release

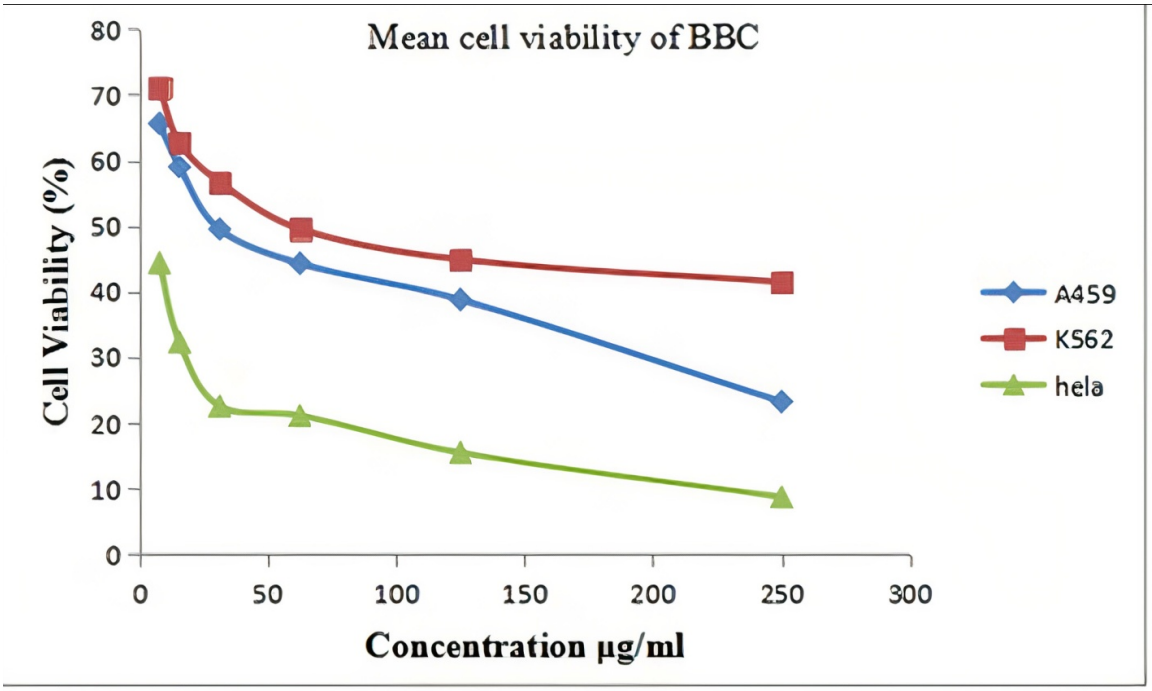

Figure 7: Mean cell viability of BBC at different concentrations on different cancer cell lines; BBC: berberine chloride; cell lines: A459 (Lung Cancer), K562 (Leukaemia) and Hela (Cervical Cancer)

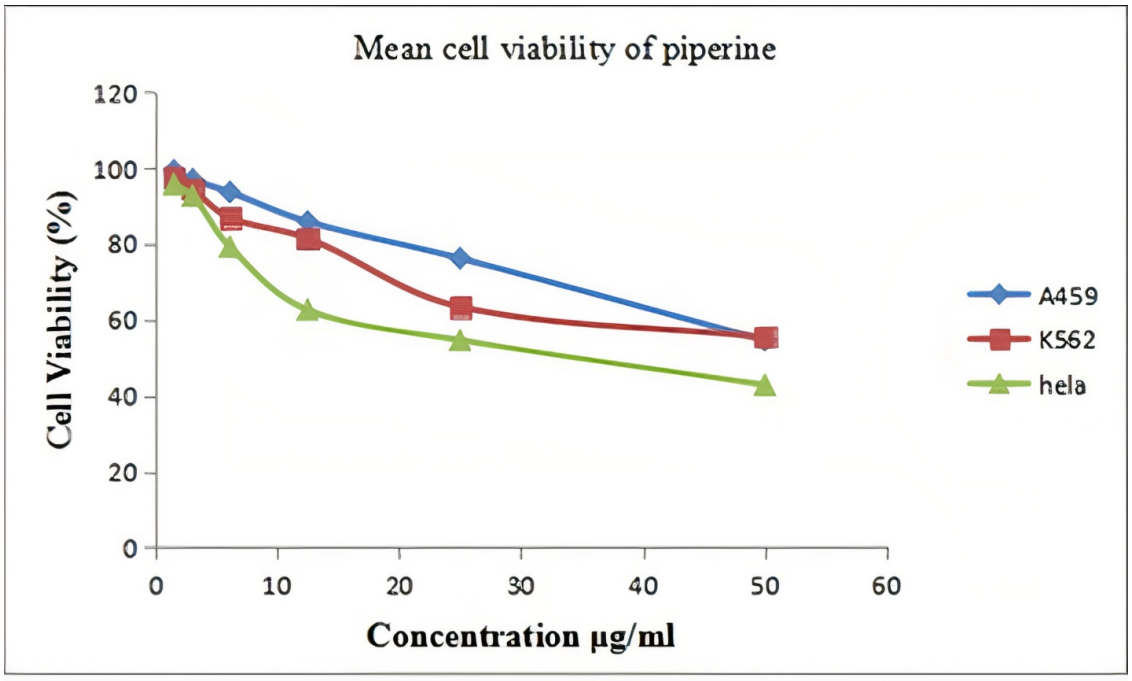

Figure 8: Mean cell viability of piperine at different concentrations on different cancer cell lines. Cell lines: A459 (Lung Cancer), K562 (Leukaemia) and Hela (Cervical Cancer)

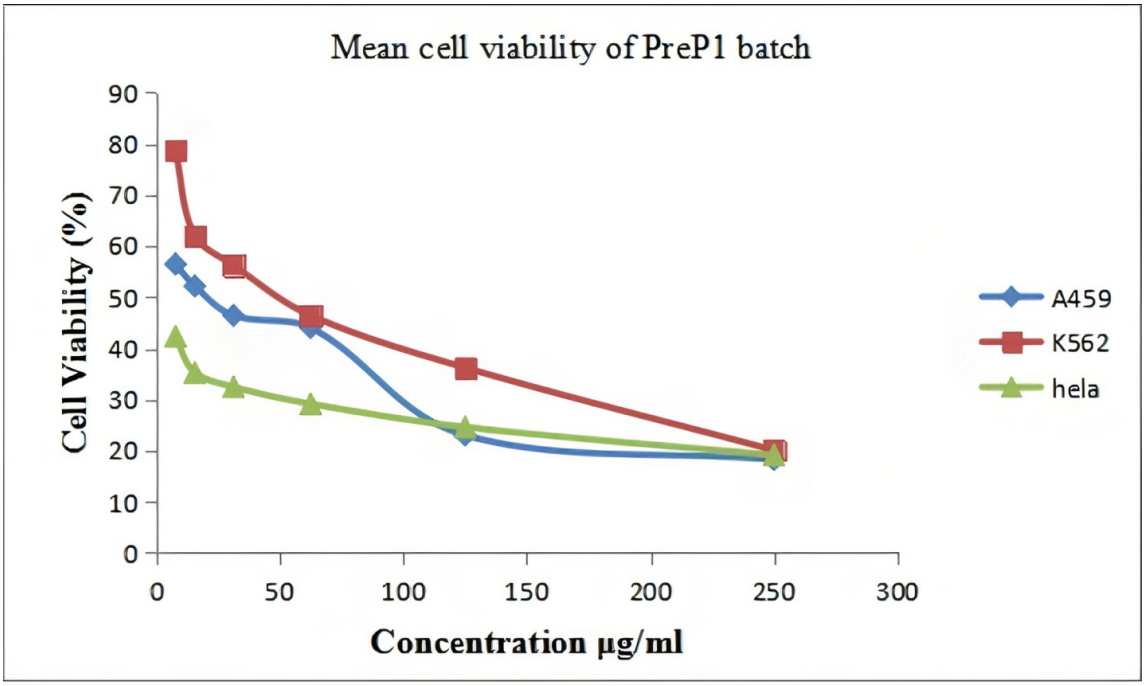

Figure 9: Mean cell viability of optimized batch PreP1 at different concentrations on different cancer cell lines; PreP1: Pre-treatment with 2 mg piperine for 30 min; cell lines: A459 (Lung Cancer), K562 (Leukaemia) and Hela (Cervical Cancer)

4. CONCLUSION

In the present ex- vivo permeability studies of BBC in existence and non-existence of bioenhancer piperine, resulted in rational optimization of PreP1 batch through application of 3^2 full factorial design approach. In additament, in- vitro anticancer cell line investigations indicated extremely remarkable anticancer activity of optimized batch over parent drug. It could be concluded that, the limitation of poor intestinal permeability of natural anticancer BBC could be successfully overcome by pre-treatment with bioenhancer piperine. The enhanced permeability of BBC during pre-treatment with piperine might be due to piperine mediated inhibition of the P-gp efflux pump which might be solely accountable for permeability improvement of BBC.

ACKNOWLEDGEMENTS

Authors are thankful to Principal, Government College of Pharmacy, Karad, Maharashtra, India for providing laboratory facilities and constant encouragement. Authors are grateful to Maratha Mandal's NGH Institute of Dental Sciences and Research Centre, Belgaum, Karnataka, India for performing in-vitro anticancer activity on cell lines. Authors are also grateful to Dr. Shivajirao Kadam College of Pharmacy, Kasabe Digraj, Sangli, Maharashtra, India for constant support and motivation.

CONFLICT OF INTEREST

The author(s) declare(s) that they have no declaration of interests to disclose.

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