RESEARCH ARTICLE

Development of Size Optimized Bromelain Loaded Nanocarriers by Box-Behnken Design

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ABSTRACT

Bromelain (BRN) is an extensive product of investigation, regarded as effective naturally produced anticancer agents. Heterogeneity of tumors amongst patients and within disease, generates a necessity of personalization of nanomedicine. The size of nanoparticle is found to be a significant target in enhancing precision therapeutics, by fondly accumulate it within the tumor microenvironment. The objective of the study is to achieve an optimum size of 50 to 100 nm BRN loaded nano carriers, to intensify the EPR effect and thereby overcome heterogeneity. Optimization of the nanoparticles commenced with the interrogation of effect by various formulation variables. The development of nanoparticles carried out by the nanoprecipitation method, where three independent variables, such as the amount of PLGA, Tween 80 and BRN are chosen after an overall screening and employed in Design Expert Software. The selected Box-Behnken design provides a total of 17 confirmatory runs at varied levels of independent variables and detected its influence on responses. The runs resulted in an optimized formula with the desired particle size of 78.64 ± 2.14 nm and a maximum entrapment efficiency of 89.14% at 24th hour. Then the selected formula characterized for polydispersity index, zeta potential, sanning electron microscopy, determination of drug content, study of *in-vitro* drug release etc.

Keywords: Breast cancer, Bromelain, Experimental design, Nanoparticles, Nanoprecipitation, Size.

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INTRODUCTION

Bromelain (BRN) is an effective anticancer agent,[1] "mainly grown in tropical and subtropical countries such as China, Indonesia, Thailand, India, and Philippines"etc. belongs to the family Bromeliaceae.[2] Past research authenticates that BRN recovered breast cancer in MDA-MB 231 and MCF-7 cell lines,[3] which can be used as a secure substitute as current cancer therapies divulged with countless adverse events and effective ones are extremely toxic.[4]

Breast cancer, persuades iniquitous death among women, regarded as heterogeneous disease, such that tumors vary amongst patients and diseases.[5,6] Heterogenous being, a number of molecularly distinct tumors emerges from breast epithelial cells.[7] Mammary cancer cells were detected with enhanced apoptotic activity on treatment with BRN.[8] *In-vivo* apoptotic studies on activity of BRN on both deliberately developed and aggressive tumors,[9] as well as the use of BRN in single or combined treatment reduce viability in cancer cells[10] were studied previously.

Current anticancer therapies are associated with several drawbacks such as lack of tissue selectivity, dose-limiting toxicity, and non-specific delivery of chemotherapeutic agents that potentially steer to the damage of tissues treated and at the same time, it potentially harms healthy cells. Insufficient delivery of drugs leads to tumor relapse. With this regard, it has been found that nanomedicine-based therapy are the best approach to overcome these limitations of conventional methods.

Targeted drug carriers could achieve a better targeting via a prolonged circulation, by providing enough time for their interaction with the target.[11] Nanomedicines preferentially accumulate in the tumor tissue because of EPR effect and can be specially attributed to its size which is an important physical property. Targeted delivery anticipated exclusively on the physical properties of both nano carriers and tumors. Expeditious growth of tumors may result in neo vasculature with extensive fenestrations and repressed lymphatic drainage. Here the nano carriers that do not traverse across normal healthy cells may get accumulated inside the target site. This accession of nanoparticles is termed as enhanced permeation and retention (EPR) effect. This is highly heterogeneous as in tumors, and varies with different stages of the tumor and

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among individuals.[12] The enhanced permeation and retention directs to an improved intra-tumor drug concentration and increased therapeutic efficacy with minimum side effects that occurs usually with traditional chemotherapy.[13]

In size, nanoparticles with a diameter less than 10 nm found to be rapidly eliminated by kidney, whereas activation of complement system transpires with those of size larger than 200 nm. The organ distribution of nanoparticles befall conditional on its size, with great culmination in liver and spleen. This sort of distribution is altered by tumor vasculature, characterized by broad intercellular gaps that permit larger nanoparticles to exit from the vessel. Too small nanoparticles can cause cytotoxicity.[14] Most approved nano medicines in current clinical use have size ranges within 100 to 200 nm, and now it is exhibited enhanced performance *in-vivo*, namely considerable tissue penetration and enhanced tumor inhibition, particularly those with size around 50 nm. The extremum of systemic nanomedicine to traverse tumor vasculature is 200 nm.[15]

The value is measured in terms of hydrodynamic diameter, diameter of a perfect sphere that would exhibit same hydrodynamic friction as molecule of interest, it gives information on inorganic core bounded with the coating material and solvent influenced by Brownian motion. Passive targeting achieved through physicochemical optimization of nanoparticles is regarded as one of the best strategies adopted to overcome the tumor barriers. Size is a major factor that affects the bio-fate of nanoparticles, as it influences renal filtration, efficacy, tumor targeting, intratumoral distribution etc. Particles of less than 5.5 nm may filter through kidney, and that less than 50 nm may get eliminated by liver as they get traversed through hepatic circulation and swallowed up by Kupffer cells. At the same time, size of greater than 200 nm retains in spleen by mechanical filtration, then cleared by phagocytic cells present in spleen. Size of 50 to 200 nm found to have longer circulation half-life and they could enter into the tumor tissue via its larger fenestrations, which is of 400 nm to 1-µm.[16]

Nanoparticles less than 100 nm passively target tumor via EPR effect, where particles of appropriate size can extravasate through tumor fenestrations and locally accumulated. Large surface area characterized by the small size results in substantial intervention with tumor cause enhanced cellular uptake and exhibits cytotoxicity.[17] Particle size of nanoparticles are nowadays an area of discussion as it possibly increases drug stability, improve curative response and reduce degradation metabolism.[18]

Nanoparticles bridle the constraints for free therapeutics and at the same time it could navigate systemic and cellular biological barriers that are heterogeneous among populations and diseases. Precision therapeutics is a measure that could repress patient heterogeneity by enhancing efficacy through personalized interventions.[14]

MATERIALS AND METHODS

Materials

BRN was received as a gift sample from Szymotech BioSolutions, Cochin, India. Poly lactic co-glycolic acid (PLGA) was acquired from nomisma healthcare, Vadodara, Gujarat, India. Tween 80, acetone, potassium dihydrogen orthophosphate, sodium hydroxide pellets and potassium bromide (KBr)-IR grade were purchased from Ron lab Chemicals, Cochin, India.

Assessment of Drug-excipient Compatibility Using FTIR Spectroscopy

The interaction between drug and additives used in the nanocarrier fabrication were determined with FTIR spectra where KBr- pressed pellet technique was used as sampling technique. In 1 mg of both finely grounded BRN and 1 mg of BRN-PLGA mixture each added to 0.2 g KBr IR grade. Semi-transparent pellets are then prepared out with hydraulic press. The pellets are then scanned over a spectrum of 4000 to 400 cm^{-1} at a resolution of 2 cm^{-1}.

Development of BRN Loaded-nanocarriers Using Nanoprecipitation Method

Dissolved PLGA in 5 mL acetone and Tween 80 was added into it, constitutes the organic phase. Aqueous phase is formed by dissolving a definite amount of BRN in distilled water. Organic phase is added to the aqueous phase drop wise under 2000 rpm in a magnetic stirrer at 37°C. Organic solvent is then allowed to evaporate under continuous stirring for 4 hours on magnetic stirrer. The suspension is then dilute with 25 mL distilled water and stirred in magnetic stirrer for further 10 minutes. Nanoparticles are then recovered by centrifugation in high-speed refrigerator ultracentrifuge for 1 hour at 15000 rpm, after that wash them three to four times and then resuspended in distilled water. It was then lyophilized and stored in a refrigerator.[19,20]

Formulation Optimization by Box-Behnken Design

Experimental design software used in the optimization was version 11 of Design expert software where the independent variables X1, X2 and X3 each at two coded levels, selected as the critical material attributes to provide a desirable design space and to enable feasible processing of nanoparticles. The effect of independent variables were studied on Y1 and Y2, the responses where Y1 denotes the particle size and Y2 denotes the entrapment efficiency. A product design space developed with all the constraints were built up, as shown in Table 1. 17 experimental runs were generated from the software. The experiment order were randomized to avoid bias and all other parameters kept constant to minimize fluctuations. Software generates 3D response surface plots, to analyse graphically the results and to investigate the extent to which factors interacted for each response.

Particle Size Determination and Estimation of Polydispersity Index

Dynamic light scattering technique is used to determine the average particle size (z-average) and the polydispersity index at room temperature, using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) and the results were obtained in triplicate.[21]

Determination of Entrapment Efficiency

Entrapment efficiency is evaluated by separating the BRN loaded nanoparticles from free BRN containing suspension by centrifugation. The suspension obtained through nanoprecipitation was centrifuged at 15000 rpm for 40 minutes in high speed refrigerated ultracentrifuge. Free BRN gets dissolved in supernatant and this was collected at the end of centrifugation, and then quantitatively measured using UV spectrophotometer at 207.6 nm.[21] The entrapment efficiency is then calculated as,

$$\text{Entrapment efficiency} = \frac{\text{Total amount of drug added(mg)} - \text{free unentrapped drug(mg)}}{\text{Total amount of drug added(mg)}} \times 100$$

Zeta Potential Determination

Zeta potential is a parameter that is characteristic of the colloidal stability of formulation and was estimated by an instrument Zetasizer Nano ZS (Malvern Instrument) and results were then read and obtained in triplicate.

Scanning Electron Microscopy (SEM)

SEM is generally helps in determining particle surface morphology of nanoparticles, and that of the optimized nanoparticles was found using JEOL, JSM-6390 LV, Tokyo, Japan. The evaluation was conducted at Sophisticated Test and Instrumentation Centre, CUSAT, Kochi, where SEM measurements were performed at 15kV accelerating voltage and photographs were taken at different magnifications and recorded.

Percentage yield of optimized formula

To determine the percentage yield of obtained nanoparticles, lyophilized product was collected and weighed carefully. Percentage yield of nanoparticles were calculated as,

$$\%\ \text{yield} = \frac{\textit{weight of lyophilized nanoparticles}}{\textit{weight of drug and polymer}} \times 100$$

In-vitro drug release study

A total of 10 mg of weighed formulation transferred to 100 mL glass beaker containing phosphate buffer pH 7.4 of 50 mL volume, and then kept in rotary shaker. Aliquots of 2 mL buffer were drawn out at predefined time intervals using 2 mL pipette and medium is replaced with same volume of buffer. The study is carried out for 24 hours. Amount of the released drug was established by observing the absorbance at 204.8 nm in UV spectrophotometer.[20]

Drug Release Kinetics Determination

To study the BRN release kinetics from nanoparticles, the *in-vitro* drug release data was tested for zero order equation, first order equation, Higuchi model and Korsmeyer-Peppas model by plotting graphs for the corresponding models and interpreting the release profile with these models.

Statistical Analysis

All results were recorded as mean ± standard deviation.

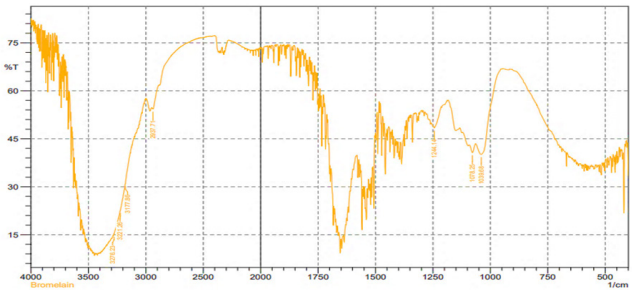

Figure 1: FTIR Spectrum of BRN

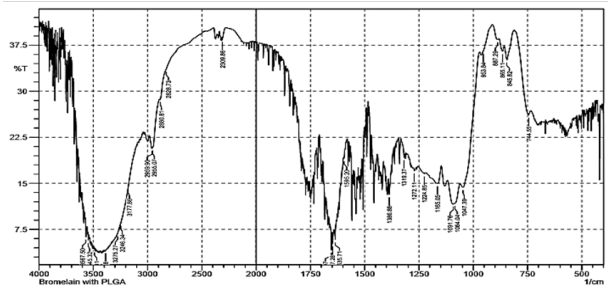

Figure 2: FTIR Spectrum of BRN-PLGA mixture

RESULTS AND DISCUSSION

FTIR is an outright instrument used as a compatibility screening tool,[22] where interactions are assessed based on band shift and widening in spectra.[23] Incompatibility studies are indispensable, as they designate the safety, efficacy, quality and elegance of the dosage form. The first step in interpreting an FTIR Spectra, is to identify the number of absorption bands in the entire spectrum. If the total number of absorption bands are less than five, then it is a simple organic compound, and if greater than five, it is a complex molecule.[24] The spectra are then obtained and the presence of more than five absorption bands indicates that BRN is a complex molecule.

FTIR spectrum of pure BRN is indicated in Figure 1. The medium absorption bands at 1039.68, 1078.25, 1244.14 cm^{-1} indicates C-N stretching, which clearly defines the existence of amine groups. While the broad absorption bands at 2937.71 cm^{-1} indicates N-H stretching and exhibits the presence of amine salt. Similarly strong absorption bands at 3221.26, 3276.23 cm^{-1} specifies the O-H stretching that demonstrates the presence of alcohol groups.

The FTIR spectrum of BRN-PLGA mixture is obtained and were indicated in Figure 2. The spectra do not exhibit any reduction in peak intensity, as well as no appearance of new peaks or fading of existing peaks. The interpretation descends into the conclusion that there is no significant interaction between BRN and PLGA, and is compatible to generate stable, effective and safe dosage form, in addition no molecular modifications are required.

BRN as in previous studies is a potential anticancer agent against diverse tumors. However, the limited effectiveness of BRN when given as such and the heterogeneity of tumors leads to the development of size optimized BRN nanoparticles through enhanced permeation and retention. BRN loaded nanoparticles were prepared by nanoprecipitation technology

Table 1: Product design space

Factors	*Levels*				
	-1	*+1*			
Concentration of polymer *(mg) (X1)*	80	120	Responses	Constraints	Optimization goals
Concentration of Tween 80 *(mg) (X2)*	10	30	Particle size (Y1)	Minimum	50-100nm
Concentration of BRN*(mg) (X3)*	10	30	Entrapment efficiency (Y2)	Maximum	Maximum

utilizing the drop-wise addition of organic phase into the aqueous phase were then subjected to magnetic stirring, followed by ultracentrifugation. The interfacial deposition of polymer arising from the evaporation of water-miscible solvent, acetone results in the formation of nanocarriers.[25] The recovered nanoparticles appeared as white sediment in the walls of vials, subjected to lyophilisation and then stored.

An overall screening was necessary to generate nanoparticles in appropriate amount and it helps to know the factor variability in producing nanoparticles, therefore as an initial step a few trial formulations are obtained and the factors were identified.

Optimization of nanoparticles is done by utilizing response surface methodology using Design Expert Software version.[11] Box-Behnken design is the selected response surface methodology design that requires fewer runs in an experiment dominated by three factors and useful when paramount treatment combinations are to be excluded, as in Table 1.

A sum of 17 confirmatory runs were done, to optimize the three independent variables and responses were recorded in Design-Expert software. Three dimensional response surface plots and the contour plots intrigued to elucidate the effect of independent factors on the dependent responses. A system always prefer a response corresponds to the desirability value of 1. Box-Behnken design is selected and a total of 17 runs has been obtained via software in order to find the interactions and to optimize the formulation.

Table 3: ANOVA model for the response, "encapsulation efficency"

Source	*Sum of Squares*	*df*	*Mean Square*	*F-value*	*p-value*	
Model	2085.07	3	695.02	4.63	0.0206	Significant
A-PLGA	298.17	1	298.17	1.99	0.1823	
B-Tween 80	47.87	1	47.87	0.3187	0.5820	
C-BRN	1739.03	1	1739.03	11.58	0.0047	
Residual	1952.56	13	150.20			
Lack of Fit	1596.47	9	177.39	1.99	0.2642	Non-significant
Pure Error	356.09	4	89.02			
Cor Total	4037.63	16				

Nanoparticles of lesser sizes may traverse the leaky vasculature and assimilate in that site due to enhanced permeability and retention effect (EPR). A 50 to 100 nm ranged particle size is desirable to achieve this effect. With these interpretations, the size of nanoparticle is a significant factor in achieving the desired accumulation to cure cancer, devised as the significant target of this study. Dynamic light scattering by Zetasizer was adapted to determine the particle size. Each measurement of the 17 confirmatory runs were done in triplicate, and the observed particle size, represented as 'z-average' ranged from 158.77 ± 31.03 nm to 1355.33 ± 174.00 nm.

The ANOVA analysis obtained from the software, represented in Table 2 indicates that the Model with F-value of 5.97 significant. In this case B, C, AB, AC are significant model terms. In the present case, a model reduction is not required, and hence the particle size is predominantly affected by B, C, AB and AC.

As in Figure 3, an increased concentration of surfactant would reduce the particle size, while a decreased surfactant concentration results in particles with greater particle size, even when keeping the polymer concentration constant. This indicates that the polymer do not have much effect on the particle size. Surfactants helps in stabilization of the dispersion in addition to reduce the particle size, by coating

Table 2: ANOVA model for the response "size"

Source	*Sum of Squares*	*df*	*Mean Square*	*F-value*	*p-value*	
Model	1.242E + 06	6	2.070E + 05	5.97	0.0070	significant
A-PLGA	50117.78	1	50117.78	1.44	0.2572	
B-Tween 80	2.698E + 05	1	2.698E + 05	7.77	0.0192	
C-BRN	2.946E + 05	1	2.946E + 05	8.49	0.0155	
AB	2.400E+05	1	2.400E + 05	6.92	0.0252	
AC	3.786E + 05	1	3.786E + 05	10.91	0.0080	
BC	9109.11	1	9109.11	0.2625	0.6195	
Residual	3.470E + 05	10	34704.15			
Lack of Fit	3.464E + 05	6	57727.46	341.22	< 0.0001	significant
Pure Error	676.72	4	169.18			
Cor Total	1.589E + 06	16				

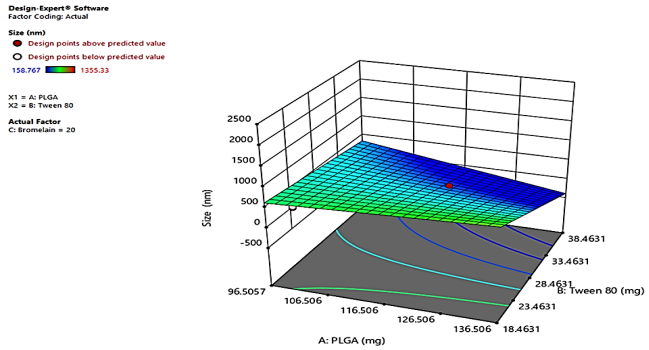

Figure 3: 3D Surface plots showing influence of particle size

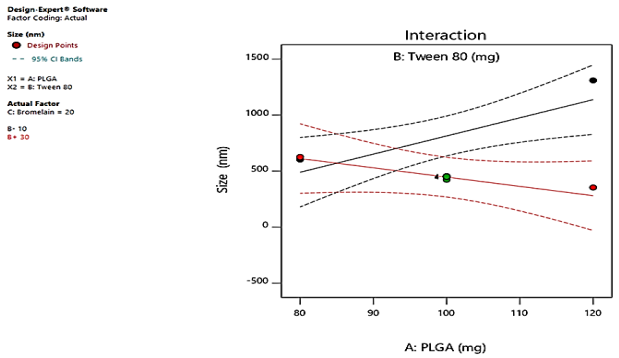

Figure 4: Interaction plot showing the effect of Tween 80 on particle size

a thin film over the surface preventing it from further growth and aggregation.

Increasing the quantity of Tween 80 from low to medium level had a positive impact on particle size, results in a drastic increase in the particle size. And later on further change in levels from 0 to + 1, results in a reduction of particle size. The interaction terms B, C, AB, and AC exhibits the pattern of impact of two variables on particle size concomitantly, as shown in Figure 4. It is important to optimize the particle size, as it is a pivotal factor in enhancing the EPR effect, in addition can avoid rapid clearance from the circulation. The target was to minimize particle size (50–100 nm) and maximize entrapment efficiency. The selected optimized formulation produces z-average, which denotes the particle size of 78.64 ± 2.14 nm, which is desirable.

The selected optimized formula is then subjected for the determination of encapsulation efficiency. ANOVA analysis of influence of factors on entrapment efficiency represents the data as shown in Table 3. The model is found to be significant with model F-value 4.63. C imparts a significant model term and regarded as an important fatcor. Values greater than 0.1000 indicate the model terms are not significant. A 1.99 Lack of Fit – F value implies that it is not significant relative to the pure error. Non-significant lack of fit is good and the model is fit.

The results indicated by Figure 5 and 6 that amount of surfactant is not a significant factor, such that it does not bring a drastic change in the encapsulation efficiency. Optimization is a best way to increase the loading efficiency without greater impact in alterations of physicochemical properties of drug substance.

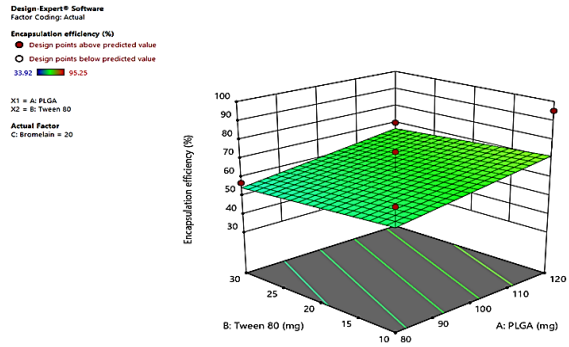

Figure 5: Response surface plots of selected independent variables on encapsulation efficiency

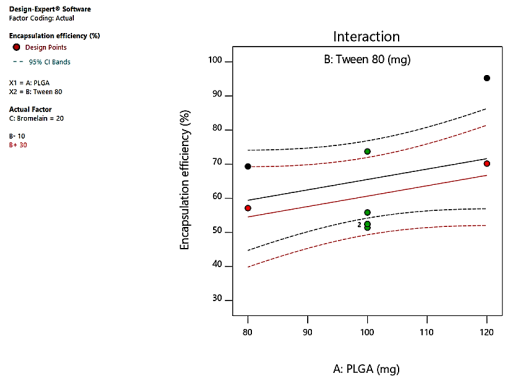

Figure 6: Interaction plots of selected independent variables on the encapsulation efficiency

At the intermediate level of BRN, with the increase in polymer concentration a gradual increase in entrapment efficiency is observed. It was observed that the optimized formula achieves 88.71% of encapsulation.

Value of 0.563 is considered to be acceptable as a value higher than 0.7 indicates very broad distribution and found to be suitable for DLS analysis, such that it is inferred that the formed particles are poly dispersed, as shown in Figure 7.

Zeta potential, denotes electro-kinetic potential and is related to colloidal stability. It is a benchmark for the extent of repulsion between consecutive and like charged particles. Colloids with high zeta potential, negative or positive are said to be of high stability, while a low value confers aggregation or flocculation.

Nanoparticles were estimated for zeta potential with the help of Zetasizer and were recorded. Zeta potential of a particle is an indication of overall surface charge in a medium within which it is dispersed. An average of -18.9 mV exhibits an increased stability of the formulation.

Samples are scanned and photographs were taken at magnifications 7000 and 10000Â by using JEOL, JSM-6390LV, Tokyo, Japan, as in figure 8. On observation, it is found that nanoparticles are surmised as cluster of spheres possessing a smooth surface, without any detectable cracks over it. The minimal solvent diffusion rate contributes the smooth and spherical particles.

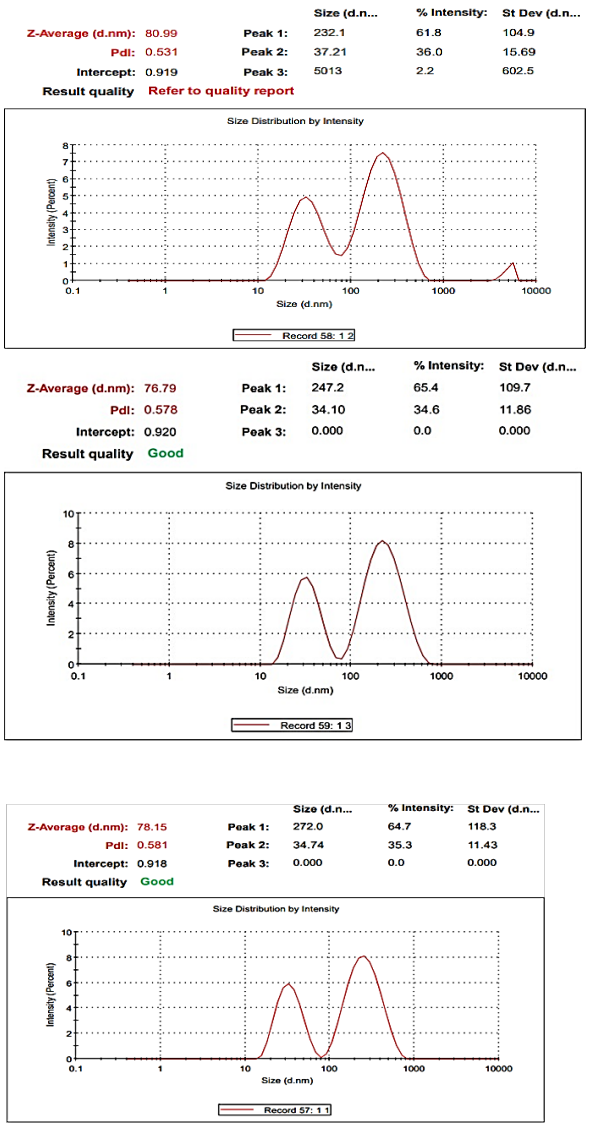

Figure 7: Observation of particle size and PdI of optimized formula

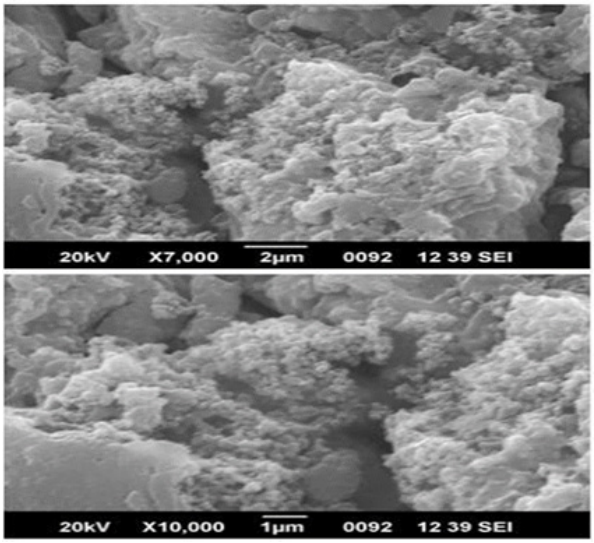

Figure 8: SEM Photographs of optimized nanoparticles

The lyophilized product were collected and weighed. On calculation, the particles exhibit a percentage yield of 85.57 ± 10.47%.

In-vitro drug release data shown initially a burst release of 17.59% within 1 hour, where it exhibits 89.14% at the 24th hour that confirms the sustained release of BRN from size optimized PLGA nanoparticles. The release pattern was then assessed by determining the drug release kinetics where the lyophilized formulation tested in zero order equation model, the first order equation model, Higuchi model and Korsmeyer-Peppas model. The nanoparticle was found to follow first order kinetics, where drug releases as a diffusion process obeying Fick's law. Active release of drug from nanoparticles is ruled by diffusion through polymer matrix and relaxation of the polymer chain that is supercase-II transport.

CONCLUSION

Effective drug delivery to target cells may achieve beneficial anticancer therapy, such that it could bypass the phagocytic uptake and several barriers along the tumor microenvironment. The particle size of 50 to 100 nm exhibited enhanced penetration ability as per previous studies. It is indispensable to optimize the formulation parameters as they affect the design aspects. This study manifests the benefit from optimizing the formulation parameters to produce desired nanoparticle with the minimum particle size of 78.64 nm and maximum entrapment efficiency of 88.71% with an *in-vitro* drug release of 89.14% which directly indicates a high drug release from the particles.

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