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OPTIMIZATION, CHARACTERIZATION AND PHARMACOLOGICAL ACTIVITY OF BERBERINE LOADED NANOPARTICLES - AN APPROACH TO IMPROVE THE EFFICACY OF ANTICONVULSANT AND ANTIOXIDANT ACTIVITY

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ABSTRACT: Berberine, an isoquinoline alkaloid that has better anticonvulsant and antioxidant activities, results in less efficacy due to poor solubility, poor permeability, poor stability and poor drug delivery to the brain. Hence, this study aim at improving Anticonvulsant and Antioxidant activities of Berberine by formulating nanoparticles with three-factor two-level boxbehnken with design expert software 6.1. In this study, the particle size and drug release percentage were considered as dependent variables, whereas the concentration of HPMC, PEG and Sonication time was considered as independent variables. Similarly, sodium caprate was separately optimized by Franz diffusion study to enhance the permeability, and thus nanoparticles were formulated with solvent evaporation method. Prepared nanoparticles were characterized and evaluated. FTIR, DSC and the Computer-based drug interaction revealed that drug encapsulated with HPMC and PEG was compatible with each other. Developed nanoparticles were found to be below 500nm. Zeta potential of nanoparticles was -35.9mV which indicated that the nanoparticles were stable; MES study, Rotarod study, and biochemical study results revealed that the anticonvulsant activity and antioxidant activity were improved in the optimized nanoparticles rather than the pure drug. Thus the overall result showed that the optimized nanoparticles act as a better candidate with improved efficacy.

INTRODUCTION:

Research Background: Berberine is an isoquinoline alkaloid obtained from Chinese herbs Hydrastis Canadensis and captis chinesis [1, 2]. It is a promising candidate to exert a boosting antiepileptic effect.

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This alkaloid that could be found in many citrus fruits such as grapefruit, berries, peaches, *etc.*, has various pharmacological activities [3]. Various researches had described its neuroprotectivity and antioxidant activities [4, 5, 6, 7, 8, 9].

Berberine exerts the activities (antioxidant and neuroprotectivity) *via*: free radical scavenging and lipid peroxidation inhibition, nitric oxide modulation, potassium channel restriction, and NMDAR (also known as: "NMDA receptor" or "N-methyl-D-aspartate receptor") channel current inhibition in the brain [11]. Though Berberine owns antioxidant [10] and anticonvulsant activities, it does

not exert its full potency in the brain due to its poor permeability, poor solubility, and poor stability [12].

Apart from the above-mentioned factors, the blood-brain barrier (BBB) is another restricting factor that also prevents the entry of 'Neuro drugs' to the brain [13]. Since delivering the Berberine drug is also considered a challenging process, this research has been designed in such a manner that aims at improving the antioxidant and anti-convulsant activity. To achieve the proposed aim, 'Nanotechnology' was adopted with incorporation of Solubility and Permeability enhancement approaches. Since, Nanotechnology is a unique drug delivery system to the brain [14, 15] 'Solvent Evaporation' with 'Probe Sonication' method was employed to produce the nanoparticles. Nanoparticles were then optimized for the experiment by utilizing the 3-factor 2-level of "Box-Behnken," a unique statistical model. Similarly, to evaluate the outcome of the independent process components (*i.e.*, Sonication time (C), PEG concentration (B), and HPMC concentration (A)) against the responses (such as: In-vitro release and particle size in nm) BBD-based optimization process was engaged. Lastly, the optimized formulations were chosen *via* plotting through methods like contour plots and 3D-response surface plots.

PEG and Sodium caprate were used as solubility and permeability enhancers. Sodium caprate increased the bioavailability by opening the tight junctions of BBB through contraction of the calmodulin-dependent actin and cadherin endocytosis [16 - 18]. Hydroxypropyl methylcellulose that has biocompatible and biodegradable property helps to design the controlled-release dosage-forms and the drug is often released by diffusion [19, 20]. PEG, along with HPMC, enhances the bioavailability of Berberine by prolonging the *in-vivo* circulation period [21]. MES study and Rotarod study helps to know the efficacy of anticonvulsant activity, whereas the antioxidant activity is confirmed through the estimation of oxidative parameters.

MATERIALS AND METHODS:

Research Methodology:

Design: Current experimentation study adapts experimental research as its design since the researcher makes use of laboratory experiments towards exploring the effects and causes of the drugs utilized.

Research Approach: Quantitative approach has been utilized by the researcher; since the study is based on exploration techniques, quantitative study would be the suitable approach.

Sampling:

- Hydroxypropyl Methylcellulose was obtained from Medopharm, Chennai (India), at free of cost.
- Berberine Chloride and Sodium Caprate were procured via Sigma Chemical-Co.in USA.
- PEG 6000 was procured as a sample gift from Matrix (India).
- Ethanol and Methanol were procured through Merck in India.

Other reagents (Analytical grade) were purchased from SD Fine chem (Bangalore).

Experimental Section: In this section, various analyses/ studies that had been conducted for the study will be elaborately explained. The analyses such as: pre-formulation study, permeability study, formulation of Berberine Nanoparticles would be explained under the section for better clarification and understanding.

Pre-formulation Study: Generally, basic studies like solubility study, permeability study, formulation of particles/ nanoparticles, evaluation studies, *etc*. would be focused under the experimental section. Since the study analyses the Berberine nanoparticles, permeability study (egg membrane preparation), formulation of Berberine nanoparticles, and other relevant analyses would be studied.

Permeability Study: [22] Egg membrane Preparation: Egg membrane preparation process was carried out by immersing and soaking the egg with 0.1NHCl over a night (whole night); later, the membrane of the egg was completely peeled off for the permeability test. Permeability study was carried out to optimize the concentration of permeability enhancer with Franz diffusion cells at

37°C. Once the egg membrane has been equilibrated with phosphate buffer (6.8) it was mounted between donor and receptor cells. On the other hand Donor cell Sodium caprate and Berberine were taken in different ratios (1:0.2, 1:0.4, 1:0.8, and 1:1) one after the other with 2ml of Phosphate buffer; whereas PBS filled receptor compartments were then stirred by the magnetic bar (coated bar). The processed samples were then taken from the receptor compartments at predetermined time intervals and replaced with the same volume of phosphate buffer. The maximum permeated drug was estimated at 345nm with Shimadzu UV-1800 Visible Spectrophotometer.

Experimental Design: According to the DOE (*i.e.*, design of experiments) principles, the methodology utilizes various kinds of experimental models, response mapping, and generating mathematical relationships of polynomials. The study also adapts BBD, which is considered as one of the most efficient DOE methods [23].

The model of non-linear quadratic is generated as:

$$Yo = bo + b1X1 + b2X2 + b3X3 + b12X1X2 + b13X1X3 + b23X2X3 + b11X2\,1 + b22X2\,2 + b33X23$$

(Where the variables: 'b0'= an intercept; 'Yo'= dependent; 'b1' to 'b33'= regression coefficients, that are calculated from the 'examined experimental values' of 'Y'; X1, X2, X3 =independent variables' coded levels. Similarly, the terms X1, X2 and Xi (i = 1, 2 or 3) represents quadratic terms and the interactions, respectively)

Quadratic model of response surface was carried out towards the formulation variables' optimization in which the design consisted of two stages (high and low); similarly to optimize the selected variables of independent formulation (*i.e.* (a) polymer concentration as X1; (b) concentration of PEG as X2 and (c) Sonication time as X3) a total of 17 runs were carried out (refer **Table 1**). The outcomes of the factors were observed with the values of PS and Y1 (particle size) where the Y2 (*in-vitro* releases) are the variables of formulation responses. To analyze the diagrammatic depiction of the response values, 'Contour plots' and 'three-dimensional (3D) graph' of response surface were also generated, which acts as a supportive representation in elucidating the association in-between: the variables (dependent and independent) and the responses.

TABLE 1: RESPONSE SURFACE QUADRATIC MODEL – BOX BEHNKEN DESIGN

Runs	Concentration of HPMC as Factor 1 (A)	Concentration of PEG as Factor 2 (B)	Sonication timeas Factor 3 (C)	Particle size (in nm) as Response1	*In-vitro* release (in %) as Response2
1	0.5	1.5	60	752	75.1
2	0.88	1	60	998.8	76.1
3	0.5	1	90	961.7	72.3
4	0.88	1	60	998.8	76.1
5	1.25	1	30	804.7	89.1
6	0.88	1	60	998.8	76.1
7	0.5	0.5	60	998.8	70.3
8	1.25	0.5	60	567	82.7
9	0.88	1.5	30	1676	78.2
10	1.25	1.5	60	471	98.5
11	0.88	0.5	30	1045	74.8
12	1.25	1	90	459.6	96.2
13	0.88	1.5	90	961	81
14	0.5	1	30	1676	70.6
15	0.88	1	60	998.8	76.1
16	0.88	0.5	90	892.4	74.3
17	0.88	1	60	998.8	76.1

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded
A	Concentration of HPMC	mg	Numeric	0.5	1.25	-1	1
B	Concentration of PEG	mg	Numeric	0.5	1.50	-1	1
C	Sonication time	sec	Numeric	30	90	-1	1

Statistical Analysis: The experimental designs' statistical relationship of the independent variables and dependant variables were adopted by utilizing the Software's design and expert version 6.1. (State-Ease Inc). The fit-quality (the experimental data) from the polynomial equation model was articulated by the coefficients of adjusted $R^2$and determination R^2.

Contrarily, ANOVA (analysis of variance) was adopted for analysis where each coefficient significance term along with lack of fit via the suggested model is evaluated through F-value and p-value with a confidence level of 95%.

Statistical Optimization: After statistically analyzing the data through experimental design, a statistical-based arithmetical optimization was adopted by applying D (desirability function) that eventually transforms the response values into 1 and 0; where 0 = response's non-acceptable value and 1 = values of ideal target [24]. Finally, optimization was exemplified through graphs/ plots (contour and 3D response surface) portraying the target areas and desirability fractions.

Preparation of Berberine Nanoparticles: By making use of the selected optimized independent variables, Berberine nanoparticles were prepared by solvent evaporation method [25]. Initially, accurately weighed quantities of Berberine were dissolved in ethanol, which later was added to aqueous-HPMC, PEG6000, and sodium caprate solution and kept in magnetic stirrer until its mixed thoroughly into a uniform mixture. The resultant mixture was placed in a rotary evaporator at a speed of 80rpm at 60 ºC. Then it was subjected to probe sonication and centrifugation at 10,000rpm for 15 min. The resultant sediment was then dried and refined (*i.e.* it contains Berberine nanoparticles).

Evaluation Studies: In this section, various Spectroscopic studies and other analyses, like UV Spectroscopy, FTIR spectrum studies, SEM analysis, etc would be focused on attaining accurate solutions.

Spectroscopic Studies:

UV Spectroscopy (Determination of λ_{max}): Berberine stock solution was prepared with methanol. Dilutions were made with methanol at the concentration level of 10mcg/ml. At the range of 200-400nm the UV spectrum was recorded by using a Shimadzu spectrophotometer.

Standard Calibration Curve of Berberine: Accurately weighed Berberine (50mg) was liquefied in 50ml methanol. The dilutions were made between the range 10-50µg/ml and the maximum absorbance level was observed at 345nm. Absorbance values of the Y-axis were plotted alongside the concentration of the X-axis.

FTIR Spectrum: FTIR studies were conducted on the Berberine (pure drug) and its nanoparticles in order to help to confirm the significant interaction of the drugs Berberine and HPMC. The FTIR spectrums of pure Berberine and nanoparticles were then recorded by Shimadzu Fourier Transform Infrared Spectrophotometer (Shimadzu UV-1800). On the contrary, the KBr disks were formulated by compressing the powders at 5tons of pressure for about 5 minutes in a hydraulic press. Major readings of the spectrum were attained in between 400-4000/cm.

SEM Analysis: The diluted Berberine nanoparticles were examined under a Scanning electron microscope (Zeiss, model EVO 18) to study the complete morphological classification. The nanoparticles were then attached at the top of the SEM aluminium stubs with the carbon-tape and then it was coated with gold by utilizing a sputter oater (Electron Microscopy Sciences; 550X model). The Berberine nanoparticles were collected in the expansion chamber, and the SEM images of different regions were obtained and examined. The process was carried out at a high vacuum range of 300mTorr, and the whole SEM analysis was carried out at 10kv (i.e., an accelerating voltage).

Evaluation of Particle Size and Zeta Potential: Malvern Zetasizer determined the zeta potential of Berberine nanoparticles by utilizing the Mastersizer-2000 method (Malvern in the UK) of laser-light scattering. Later on, the sonication process through diluted nanoparticles of 1ml was accurately conducted for roughly 30sec and positioned cautiously in the Mastersizer cell. Thus particle size along with zeta potential was obtained.

Analysis of DSC Thermograms: DSC analysis process through Q-2000 DSC instrument (TA

Instruments in USA) was carried out. Dry Nitrogen purged DSC cell (at 50ml/min) was then weighed and sampled (3 -5 mg) so that it could be placed in a standard aluminium pan at 25-300 °C as its temperature with 10°C/min as its heating rate. Thermograms of Berberine pure drug and nanoparticles were then recorded based upon the sample's melting point [26].

Computer-Based Drug Interaction Study: The interaction studies between the drug and polymer was carried out at BIOVIA discovery studio of 2017 platform. The Berberine (cid: 2353) and polymer HPMC (cid: 57503849) were gathered from the Pub-chem database. Initially, grids are produced around the polymer at the determined coordinates of Z (-0.0842), Y (3.542), and X (1.152); Berberine drug is then docked around the polymer; finally, the steady complex will be obtained and saved for the purpose of interaction analysis. The CHARM-based algorithm is an effective technique for analyzing the interaction between the drug complex and the polymer, and hence it is utilized here.

Determination of Drug Loading & Encapsulation Efficiency (% EE): Obtained Berberine nanoparticles were then centrifuged at the range of 10000rpm for about 15minutes towards separating the un-entrapped drugs. Free drugs presented in the supernatant were then examined and determined by utilizing the UV-spectrophotometer. Methanol was utilized for the necessary dilution process, and the solution was then filtered where the absorbance rate was measured at 345nm.

The drug content was calculated from the standard curve:

% Drug entrapment = calculated drug concentration (C_{total} – C_{free}) × 100 / Theoretical drug concentration (C_{total})

% Drug loading = Total Drug Weight × 100 / Total Nanoparticles weight

Study on *in-vitro* Dissolution: Test-apparatus of paddle-type (Labinda, Disso) dissolution was adopted in the *in-vitro* dissolution analysis. In the selected dissolution chamber, accurately 900ml of dissolution media (0.1 NHCl of 1.2pH and Phosphate buffer pH 6.8) was taken and sustained at 37°C±0.5°C. The obtained samples were inhibited at predestined times, up to 24 h. Contrarily, equal sum of buffer was substituted and the obtained samples were filtered, and the resulted dilution was processed in 10mcg/ml concentration; finally, the drug release was estimated at 345nm by Spectrophotometric method [27].

Drug Release Kinetics: To establish the drug release, various kinetic methods such-as: first order, zero order, etc. were adapted from the study of Higuchi, and also the Korsmeyer-Peppas equations were obtained too. The percent release was plotted against time to get *in-vitro* release plots similarly, the log percent remaining was plotted against time to get first order plot; additionally, the percent release was plotted beside the times' square-root towards attaining Higuchi plot. The % log cumulative of the drug release was marked along the log time (in hrs) towards attaining the Korsmeyer-Peppas plot.

Pharmacological Investigation:

Studies on Acute Toxicity: Acute toxicity analysis was executed towards ascertaining a safer dosage of Berberine nanoparticles. For experimental and investigation purposes, healthy Wistar albino rats were chosen with the Institutional Animal Ethical Committee's approval (IAEC/CHITRA KARTHIKEYINI.S/AU/1524559770/Ph.D/KMCP/40/2018) that was obtained exclusively for the use of experimentation on live animals and the study's design. Berberine nanoparticles were carefully suspended under administered doses, such as 5, 50, 300, and 2000mg/kg. The control group received an equal volume of vehicles, and the observations were recorded systematically and continuously as per the OECD guideline 423. The animals were then monitored for 14 continuous days, and the toxicological effect was estimated on the source of mortality.

Evaluation of Anticonvulsant Activity by MES Study: [28] Groups of 4 rats (*i.e.*, 6-Wistar albino rats – male) of 150 to 180g were utilized for the experimentations.

Group-A received the vehicle, Group-B received Phenytoin 25 mg/kg as a standard; Group C1 and C2 received pure Berberine 25mg/kg and Berberine nanoparticles 5 mg/kg, respectively. The test was officially initiated after the oral treatment (*i.e.*, an

hour from oral treatment) with nanoparticles or the vehicle. The intensity of the stimulus 45mA, 50Hz for 0.2s was applied by pinna electrodes. The test animals/subjects were closely observed for 2 min; subsequently, the tonic hindlimb extensor's (THLE) onset and period were calculated. Finally, the convulsion inhibition % was calculated.

Rota Rod for Muscle Coordination: [29] Four groups of 6 rats respectively were taken for the study, and they were vigorously trained for a week for about 15minutes/day on Rota-rod. The test animals were finally evaluated towards analyzing the motor coordination with time intervals of 30mins and 60mins; right after the primary initiation of the drugs (standard and test). When each subject falls off the Rotarod experiment for the very first time, it would be noted along with the consecutive occasions of falls, during the period of 5mins test; it would be considered toxic when the subjects fall off the Rotarod approximately thrice or more.

Biochemical Estimation:

Tissue Preparation: Animals were extremely anesthetized with chloral hydrate - 10% for 350mg/kg, intraperitoneally and executed by instant decapitating. Subject's brains were swiftly dissected for removal of cortex region towards determining the activities of MDA, SOD, GSH, and CAT. Samples of the brain were later weighed and stored at -70°C, prior to the thorough analyses. Samples of each brain were homogenized with phosphate buffer pH 7.4.

Estimation of Glutathione Reductase (GRD): [30] Glutathione reductase was assayed based on decreases in absorbance caused by oxidation of NADPH. 1unit of enzyme activity is articulated as nmoles (*i.e.* Nanomoles) of oxidized NADPH per min/mg of protein.

$$Enzyme\ activity\ (M/min/ml) = \frac{A_{340/min}\ X\ Vt}{\varepsilon\ X\ d\ X\ Vs}$$

Lipid Peroxidase (LPO) Estimation: [31] TBARS method was used to determine the level of lipid peroxide. The lipid peroxide levels were articulated as nmoles of wet tissue through the MDA released/g.

$$The\ concentration\ of\ MDA = \frac{Absorbance\ at\ 532\ nm}{L\ x\ C} \times D$$

Superoxide Dismutase (SOD) Estimation: [32] The SOD activity analysis is particularly based upon the SOD ability towards inhibiting the alkaline pH via epinephrine auto-oxidation. 1unit of SOD is considered as the necessary amount of required enzyme towards producing 50% of epinephrine auto-oxidation inhibition.

Calculation:

$$\%\ Inhibition = \frac{\Delta A_{480nm}/min\ Uninhibited - \Delta A_{480nm}/min\ inhibited}{\Delta A_{480nm}/min\ Uninhibited - \Delta A_{480nm}/min\ Blank} \times 100$$

Estimation of Catalase (CAT): [33] The level of catalase was estimated on the basis of the disappearance of hydrogen peroxide (H_2O_2), which was measured spectro-photometrically at 240nm. Catalase activity was later articulated through units/mg of protein.

$$K = \frac{Vt}{Vs} \times \frac{2.3}{\Delta t} \times Log\ \frac{A1}{A2} \times 60$$

RESULTS AND DISCUSSION:

Pre-formulation Study: Under this section, a group of various and relevant studies was carried out to validate the experiments and the findings gathered through the study.

Permeability Study: Permeability study was separately carried out with Franz diffusion cell to optimize the permeability enhancer (sodium caprate) concentration. The resulted data proved that 1% sodium caprate increases the permeability of Berberine. Thus the optimized concentration of sodium caprate that was added during the preparation of nanoparticles was optimized by boxbehnken method.

Optimization of Process Variables of Berberine Nanoparticles: On the basis of RSM box Behnken 17 formulations were prepared, and responses were evaluated for the three-factor two response set-up. **Table 1** shows the result for all the batches of Berberine nanoparticles that were evaluated for the release of *in-vitro* and the size of particles. Results that were obtained with wide variation clearly confirmed that chosen dependant variables were closely relying upon the chosen independent variables. The mathematical relationship for responses particle size (Y1) and *in-vitro* release (Y2) is presented through the polynomial equation where A, B, and C represent HPMC concentration,

PEG concentration, and Sonication time. The synergistic effect is indicated through positive value, whereas the antagonistic effect is indicated through negative value. **Fig. 1** depicts the relationship of parameters depicted in the three-dimensional graph forms.

The ANOVA was built entirely, and similarly, the p-value, R-value, and R^2-value were obtained too. A small value of "*Prob>F*" (p-value less than 0.05) explains a considerable model upon the response. Since the p-value is below 0.05, the quadratic model is suggested. Predicted R^2 and adjusted R2 lies within 0.2 with each other, which indicates that the selected model was significant. The adjusted R^2 is the amount of variation around the mean.

An evaluation was carried out towards analyzing the correlation of the data (*i.e.*, predicted versus experimental of particle-size responses and *in-vitro* release) **Fig. 1**. As per the acquired information, the well-distributed data points were extremely near towards a straight line of R^2 (value of 0.8890, 0.9744) that in turn recommended an exceptional association between values of predicted and experimental responses; similarly,, the primary suppositions of the above-mentioned analysis was thus appropriate. The outcomes thus indicate that the preferred quadratic model was adequate in presuming the variable's response of experimental data.

Model Sufficiency: A diagnostic plot was obtained between the predicted and experimental value that in turn aided to predict the model suitability. **Fig. 1** shows the actual versus predicted plots for *in-vitro* release and particle size, and the points that are closer to the diagonal line clearly show the model fits the experimental data.

Effect of Process Parameters on Responses: 3D surface plots were represented, and the optimum concentration was established by examining the independent variables' response's effects.

Effect of Process Parameters on Particle Size: Uniform spherical size plays an important role in nanoparticles drug release. Accordingly, the factors such as minimum size and spherical shape were considered as a primary aim of this optimization study.

Based on the multiple linear regression analysis, Y1 equation goes as:

Particle size = +998.80-260.77* A+44.60* B-240.88*C-234.85*A2-66.75* B2+211.55* C2+37.70*A*B+92.30*A*C-140.60*B*C

Table 2 shows the summary of ANOVA for comparison of the three independent parameters on particle size. The particle size ranges from 459.60nm to 1676nm and "Model F-Value" as this large might result from noise with 1.24% possibility. "Prob>F" values that are lesser than 0.0500 are normally considered as significant model terms. Here, the significant model terms are: A, C, A^2 and C^2 Regression coefficient was 0.8890. The predicted regression coefficient shows logical agreement towards the variable of adjusted R^2. Basically, a ratio with a greater value of 4 is desired, and as the obtained ratio was 9.496, it specifies a sufficient signal that the adapted model could be utilized towards navigating a design space.

TABLE 2: ANOVA - QUADRATIC MODEL (RESPONSE SURFACE) – PARTICLE SIZE

Source	Sum of Squares	DF	Mean Squares	F-Value	Prob>F	
Model	1.56E+06	9	1.74E+05	6.23	0.0124	significant
A	5.44E+05	1	5.44E+05	19.53	0.0031	
B	15913.28	1	15913.28	0.57	0.4744	
C	4.64E+05	1	4.64E+05	16.66	0.0047	
A2	2.32E+05	1	2.32E+05	8.34	0.0234	
B2	18760.26	1	18760.26	0.67	0.4389	
C2	1.88E+05	1	1.88E+05	6.77	0.0354	
AB	5685.16	1	5685.16	0.2	0.6651	
AC	34077.16	1	34077.16	1.22	0.3052	
BC	79073.44	1	79073.44	2.84	0.1359	
Residual	1.95E+05	7	27853.17			
Lack of Fit	1.95E+05	3	64990.72			
Pure error	0	4	0			
Cor Total	1.76E+06	16				

However, the Nanoparticles prepared with a low concentration of polymer were rough and irregular in shape due to poor encapsulation. **Fig. 1** shows the complete encapsulation of spherical morphology found with medium concentration; also, larger diameter nanoparticles were found with a higher concentration of polymer.

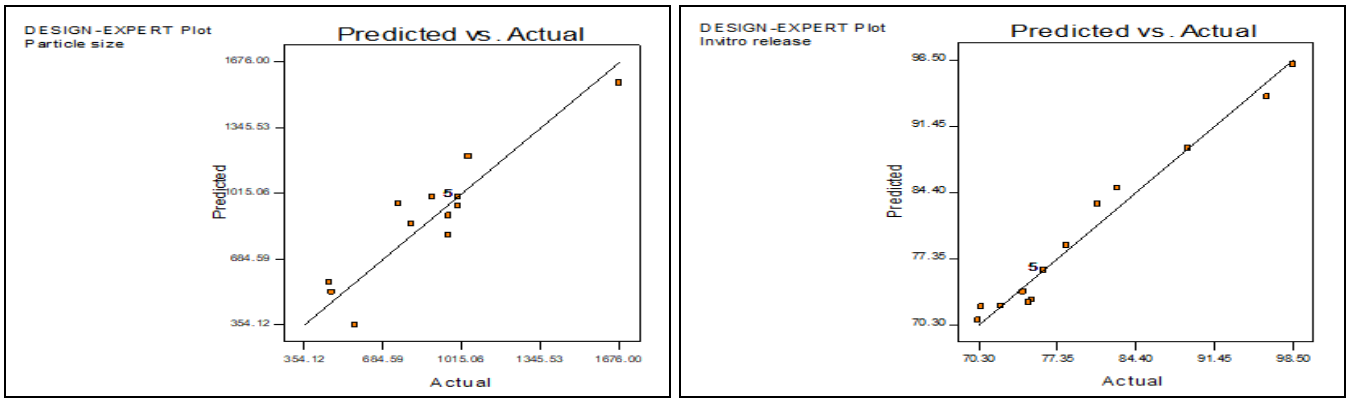


FIG. 1(A): PREDICTED VS ACTUAL GRAPH FOR PARTICLE SIZE AND *IN-VITRO* RELEASE

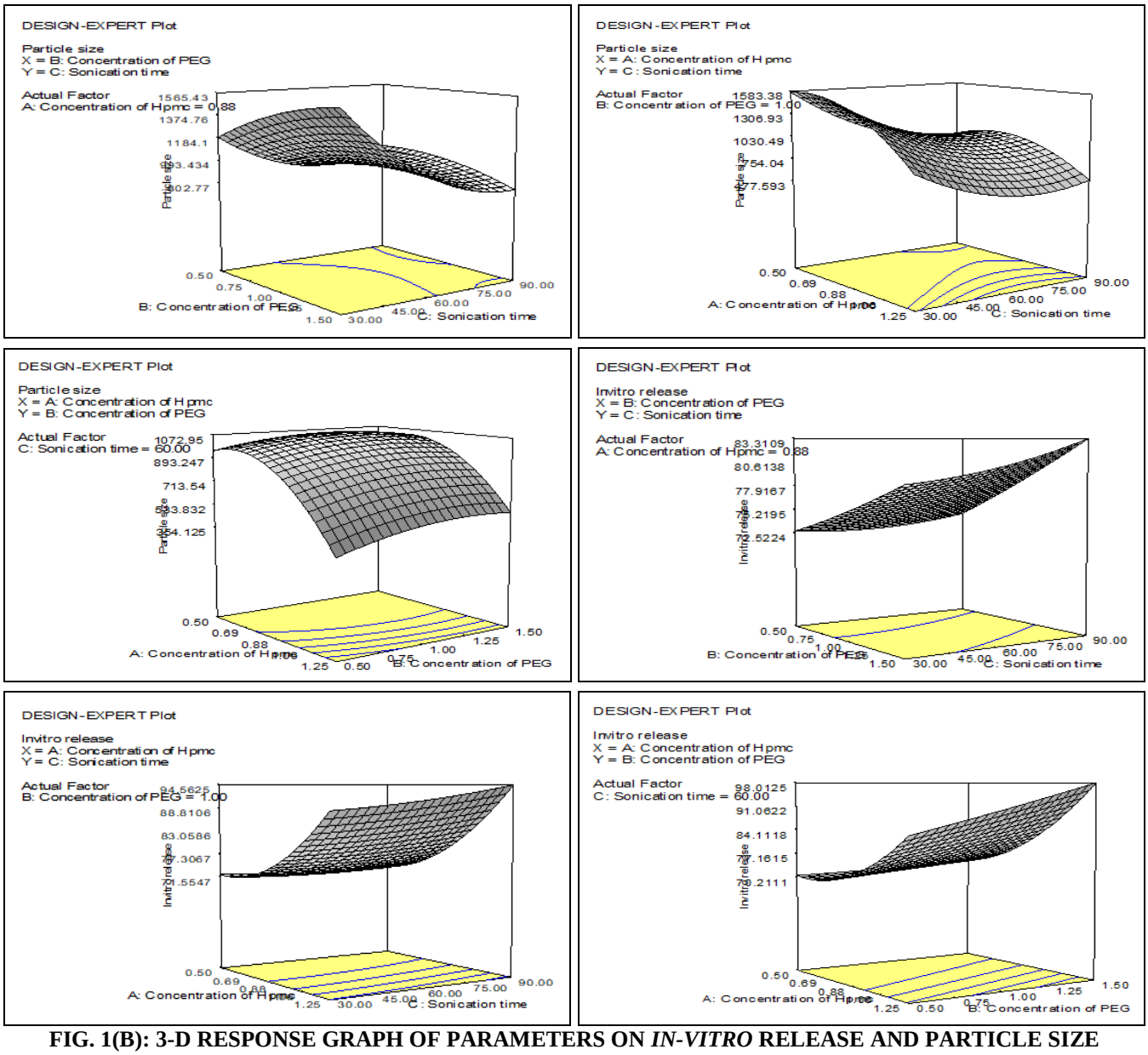


FIG. 1(B): 3-D RESPONSE GRAPH OF PARAMETERS ON *IN-VITRO* RELEASE AND PARTICLE SIZE

***In-vitro* Release Effects:** Drug release is quite important in a dependent variable that has been chosen for optimization in this analysis. As observed, the obtained response increased when the solubility increased; thus, it has been identified through a response that increasing the concentration of variable PEG (B) has a positive outcome upon the release of drugs. Whereas the variables A & C are inversely proportional to the response and have a negative effect on the release, thus favouring the controlled and prolonged releases. The numerical association as a polynomial equation for Y2 (drug release) is:

In-vitro release (Y2) = +76.10+9.78*A+3.84*B+1.39*C+ 5.26*A^2+0.29*B^2+0.69*C^2+2.75*A*B+1.35 * A * C+0.83 *B*C

As per the ANOVA study, the obtained values were elucidated. The % release ranged from 70.30 to 98.5%. F-value (33.64) thus indicates that adopted model is considerable; similarly, a "Model F-Value" as this large might result from noise with 0.01% possibility. "Prob>F" values that are lesser than 0.0500 are normally considered as significant model terms. On-other-hand coefficient R^2's value (0.9774) represents the appropriate adjustment of the recommended model towards the investigational data as a high inevitability; thus A, B, $A^{2,}$ and AB are accepted as important models. As mentioned earlier, a ratio greater than 4 is commonly desired, and since the obtained ratio was 18.971, it signifies an ample signal that implemented model could be utilized towards navigating the design space. Mathematical optimization was conducted following the statistical analysis towards all the responses aiming at selecting the most optimum formulation for preparation of Berberine nanoparticles, with the desired response by using the desirability approach. A constraint to increase the *In-vitro* release and minimize the size of the nanoparticles was selected; the independent variables were optimized by using design expert 6.1, based on the desirability.

Table 3 shows the summary of ANOVA for comparison of all three parameters on the *in-vitro* release. The selected optimum-process variables for preparation of optimized nanoparticles were: A of 1.06mg, B of 1.18mg, C of 50.27sec, and optimized nanoparticles were evaluated for: nm of particle sizes and % of *in-vitro* releases. Predicted outcomes of the variables, particle size with 995.28nm and *in-vitro* releases with 76.23%, were noted before experimentation. Later, the experimentation results showed that the *in-vitro* release was 98.5% and particle size was 471nm, respectively.

TABLE 3: ANOVA - QUADRATIC MODEL: RESPONSE SURFACE - *IN-VITRO* RELEASE

Source	Sum of Squares	DF	Mean Squares	F-Value	Prob>F	
Model	1060.03	9	117.78	33.64	< 0.0001	significant
A	764.41	1	764.41	218.33	< 0.0001	
B	117.81	1	117.81	33.65	0.0007	
C	15.4	1	15.4	4.4	0.0742	
A2	116.61	1	116.61	33.31	0.0007	
B2	0.35	1	0.35	0.099	0.7617	
C2	1.99	1	1.99	0.57	0.4755	
AB	30.25	1	30.25	8.64	0.0217	
AC	7.29	1	7.29	2.08	0.1922	
BC	2.72	1	2.72	0.78	0.4071	
Residual	24.51	7	3.5			
Lack of Fit	24.51	3	8.17			
Pure error	0	4	0			
Cor Total	1084.54	16				

FTIR Study: FTIR spectrums study was conducted upon the pure Berberine and its nanoparticles. The absorption bands were found at a wavenumber of 3540.02cm^{-1}, 1442.75cm^{-1}, 1274.95cm^{-1}, 422.41cm^{-1} in the spectrum where Berberine was missing in Berberine nanoparticles. At the peak, 1731.6cm^{-1}, 2912.95$cm^{-1,}$ and 2848.35cm^{-1} in FTIR spectrum, the Berberine nanoparticles represent the carbonyl c=o, stretching of the PEG methyl and propyl group of HPMC. Thus it has been demonstrated that the drug was encapsulated by HPMC, and there was no significant interaction between the drug and carriers (refer **Fig. 2a**).

DSC Study: Thermal analysis DSC study was also carried out on pure drugs and their nanoparticles. Characteristic Endothermic peak of Berberine was noted at 199°C, which was on the contrary not present in Nanoparticles. Thus the disappearance of peak clearly proved that Berberine drug was encapsulated by HPMC (refer **Fig. 2b**).

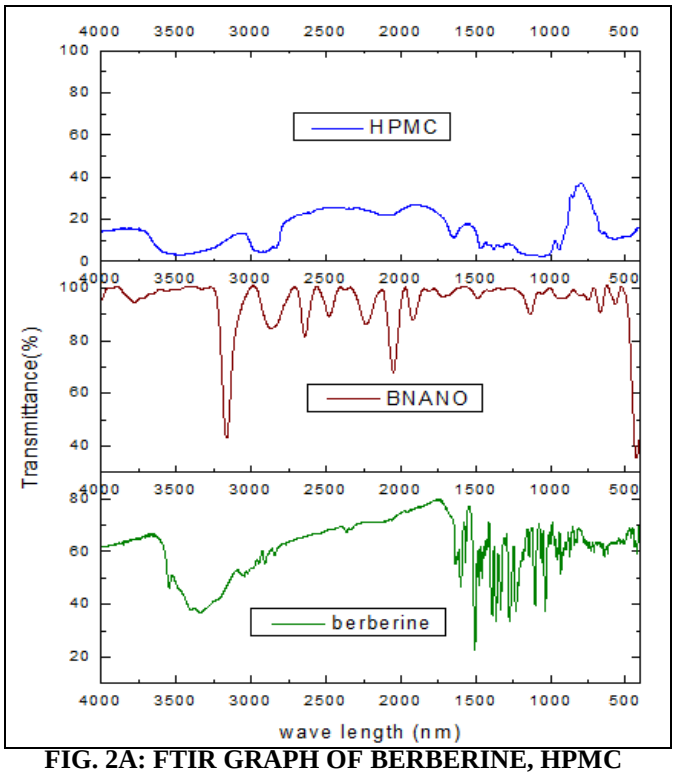


FIG. 2A: FTIR GRAPH OF BERBERINE, HPMC AND BERBERINE NANOPARTICLES

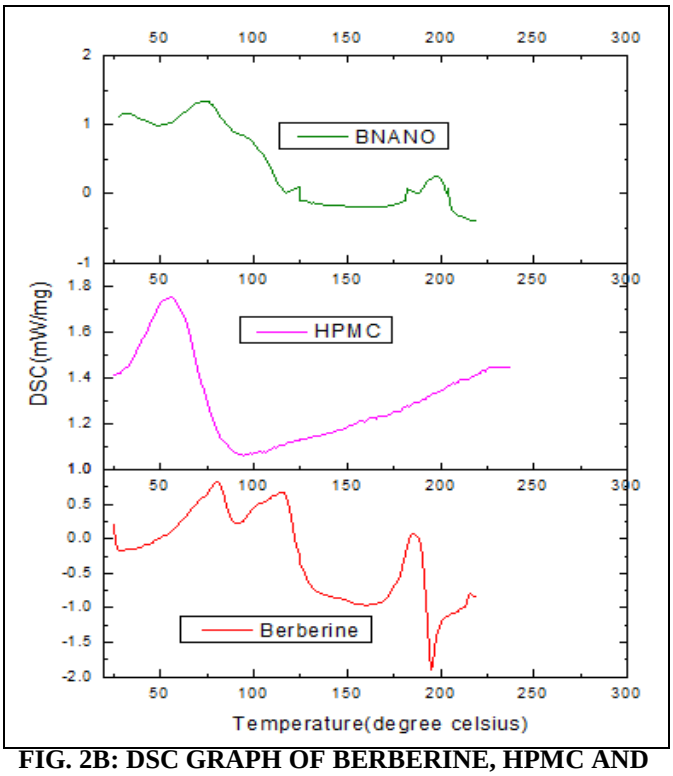


FIG. 2B: DSC GRAPH OF BERBERINE, HPMC AND BERBERINE NANOPARTICLES

Zeta Potential and Particle Size: Average particle size of optimized Berberine nanoparticles was 471nm, and the Particle dispersion index (PDI) was 0.592; particle size distribution by zeta-sizer also proved that all the particles of optimized formulation were found to be smooth, uniform, spherical in shape and exists within the range of 100-500nm. The Zeta potential of Berberine nano-particles was -35.9mv.

Narrow particle size distribution and negative side of zeta potential indicate that system obtained by solvent evaporation method keeps the nanoparticles thermodynamically stable. All the nanoparticles existing below 500nm were circulated in the RES system for a longer time, and the yield prolonged the drug delivery. Particle size and zeta potential were depicted in **Fig. 3**.

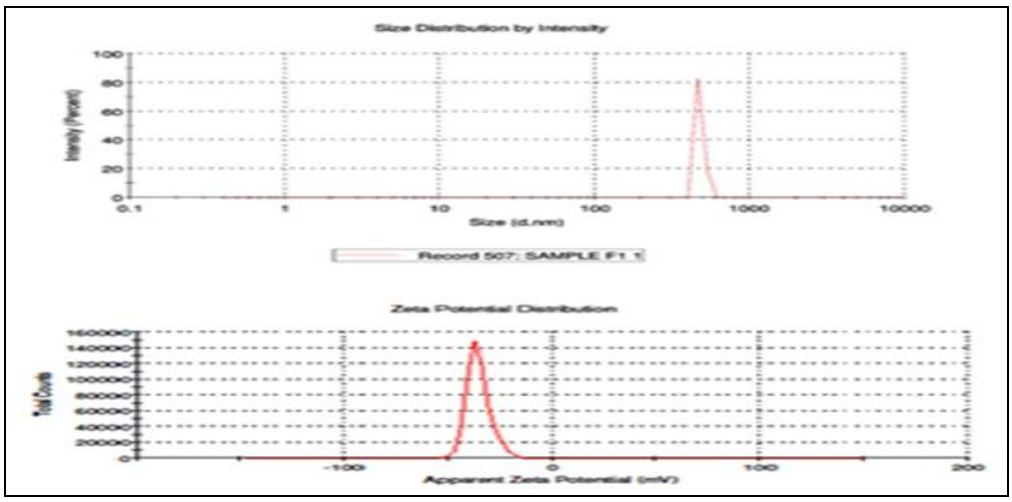


FIG. 3: DISTRIBUTION OF THE PARTICLE SIZE AND OPTIMIZED ZETA POTENTIAL - BERBERINE NANOPARTICLES

SEM Study: Surface Morphology was found by SEM and TEM photographs which are depicted in **Fig. 4**. The study proved that particles were in nano-level and they were in uniform-spherical shape.

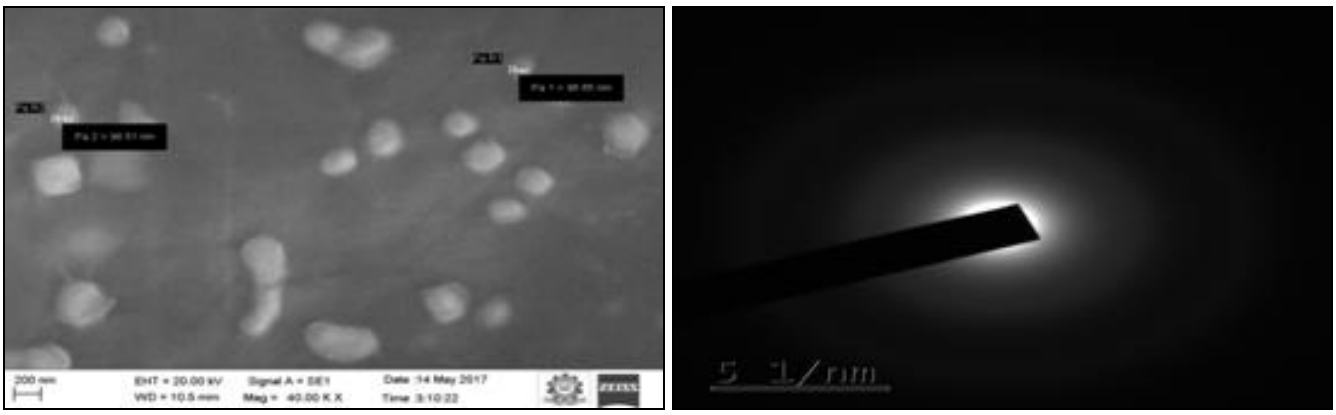


FIG. 4: SEM AND TEM IMAGES OF OPTIMIZED BERBERINE NANOPARTICLES

Computer-Based Drug Interaction: The computer-based interaction studies between the polymer and drugs showed the molecular level insight view and stability nature of the complex. Results of the interaction studies showed that both the drugs (Berberine and phenytoin) resulted in good interaction with the polymer. Analyzing the HPMC polymer and Berberine also revealed that the three interactions are formed in the complex, where: Two hydrogens in the dioxal ring of the Berberine forming hydrogen bond with the oxygen atoms of HPMC and One hydrophobic π interaction was generated between benzene ring of Berberine and hydrogen atom of polymer (**Fig. 5a** & **5b**). Similarly, the phenytoin formed three hydrogen-based bond interactions with the range of 2.06 - 2.60Å distance and One-Hydrophobic π interaction with the polymer **Fig. 5**. The results clearly proved that both the drugs formed a stable complex with HPMC polymer.

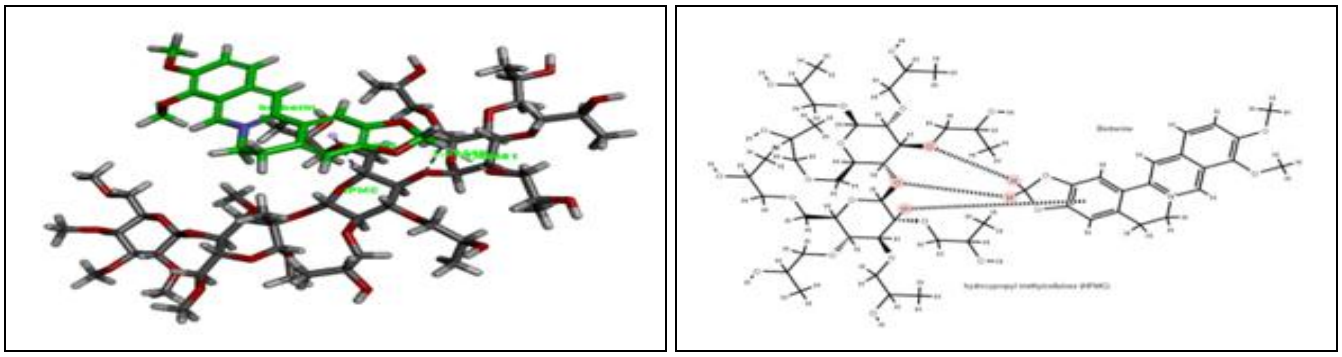


FIG. 5: 3D AND 2D INTERACTION PATTERN OF THE HPMC POLYMER AND BERBERINE

Molecular Dynamic (MD) Simulation/ interaction of Berberine Nanoparticles: Molecular dynamic study of Berberine nanoparticles was evaluated by using BIOVIA Discovery Studio and 2017 and Materials Studio 7.0 software. In this analysis, the MD simulations and docking studies were utilized towards investigating the Berberine-HPMC-PEG nanoparticles formation and the interactions between PEG and also the details of Berberine drugs in the molecular level.

The key purpose of this investigation was to authorize the new MD simulations procedure, which was tested on the drug-polymer such as Berberine-HPMC-PEG. Prior MD simulations, the Forcefield (COMPASS) was analyzed for Berberine, HPMC and PEG individually. It showed the Forcite energy as: 4092113472.7683Kcal/mol (Berberine), 131430524.73022Kcal/mol (HPMC) and 7953.42717Kcal/mol (PEG). Berberine-HPMC-PEG molecules are built in an amorphous cell protocol in order to simulate the interaction within the Berberine **Fig. 6a**.

The Berberine molecule forms van-der Waals energy of 565.235 with HPMC and PEG

molecules. The aromatic rings in the Berberine molecules form the highest van-der Waals of energy with hydrophobic groups of the HPMC and PEG molecules. Similarly, the two aromatic rings of the Phenytoin molecule forms van der Waals of the energy of 716.991 with HPMC and PEG molecules **Fig. 6b**. During the molecular interaction analysis, the H-bond of both the HPMC and PEG molecules were fixed as a stable condition to improve the Berberine and Phenytoin interactions; additionally, the involvement of the van der Waals interactions between the drug and polymers is accountable towards the formations of nanoparticles.

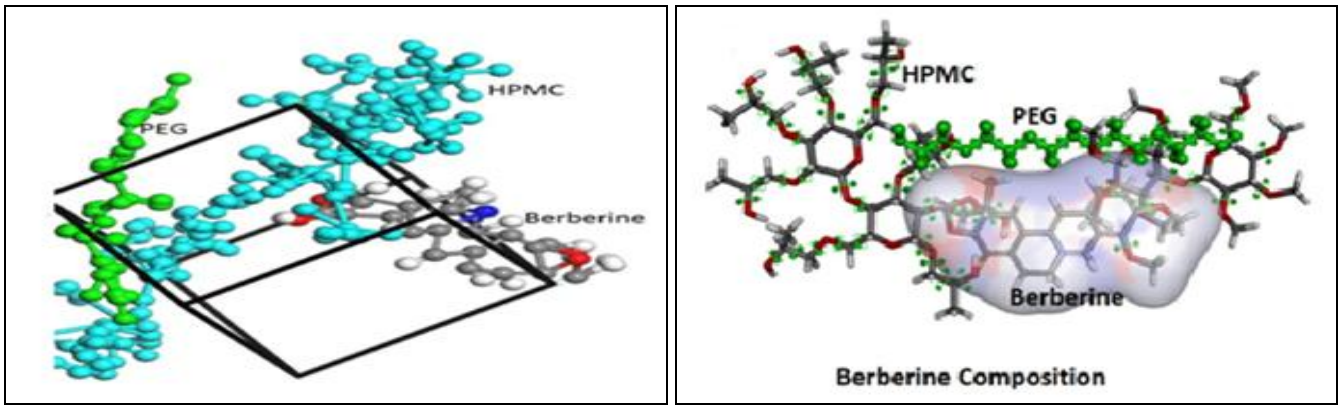


FIG. 6A: FORMATION OF MOLECULAR INTERACTIONS BETWEEN (A) BERBERINE NANOPARTICLES SIMULATED BY AMORPHOUS CELL MODULES CONSTRUCTION AND PACKING METHOD USING MATERIAL STUDIO SOFTWARE; FIG. 6B: VAN DER WAALS INTERACTIONS BETWEEN (A) BERBERINE COMPOSITIONS

Entrapment Efficiency and Drug Loading: In this analysis, drugs entrapped in HPMC, depending upon the solubility and polarity properties were examined. The results/ data showed that if the particle sizes increase, the entrapment efficiency will decrease. Drug loading and entrapment efficiency is an important parameter for nanoparticles. Drug loading and entrapment efficiency of optimized Berberine nanoparticles were 25.74% and 90.1%, respectively. Thus, it shows good electrostatic interaction between the drugs and the polymer.

***In-vitro* Release Examination:** This process was performed upon pure Berberine and Berberine nanoparticles in pH 1.2 and 6.8 medium, respectively. The results were gathered (refer **Fig. 7**) and it suggested that the optimized formulation selected by boxbehnken method exhibited a controlled remarkable dissolution rate in both acidic and alkaline media. The dissolution profile of nanoparticles showed that the release rate was significantly improved, and it releases the drug slowly up to 24 h, and the percentage of release was 98.5%. Thus, it can be proved that nano-drug delivery is an effective strategy towards enhancing the dissolution rate of poorly soluble drugs where the dissolution rate increases in acidic media than alkaline media.

Drug Release Kinetics: Drug release examination with various kinetic models (*i.e.* first order, zero order, *etc*) was adapted from the Higuchi study; also, the Korsmeyer-Peppas equations were obtained too.

The percent release was plotted against time towards obtaining the zero-order plots. Similarly, the log percent remaining was plotted against time towards obtaining the first-order plot. Additionally, the percent release was plotted along the time's square root towards attaining the Higuchi plot. Lastly, drug release's log of cumulative % was plotted beside log time (in hrs), towards attaining the Korsmeyer-Peppas plot.

Optimized Berberine Formulations have the highest regression coefficients (0.976) towards Higuchi's model, indicating Fickian diffusion. The diffusion mechanism was established by fitting the data into the Korsmeyer-Peppas equation and thus the regression coefficients of the Korsmeyer-Peppas plot was attained at 0.898. The analysis showed a good linearity and the diffusion was identified as the drug release's predominant mechanism. Release exponent (n) values of Berberine nanoparticles were 0.354, and the results indicated that n-value is around 0.5. Henceforth, it is proved that the drug release follows Fickian diffusion (refer **Fig. 7**).

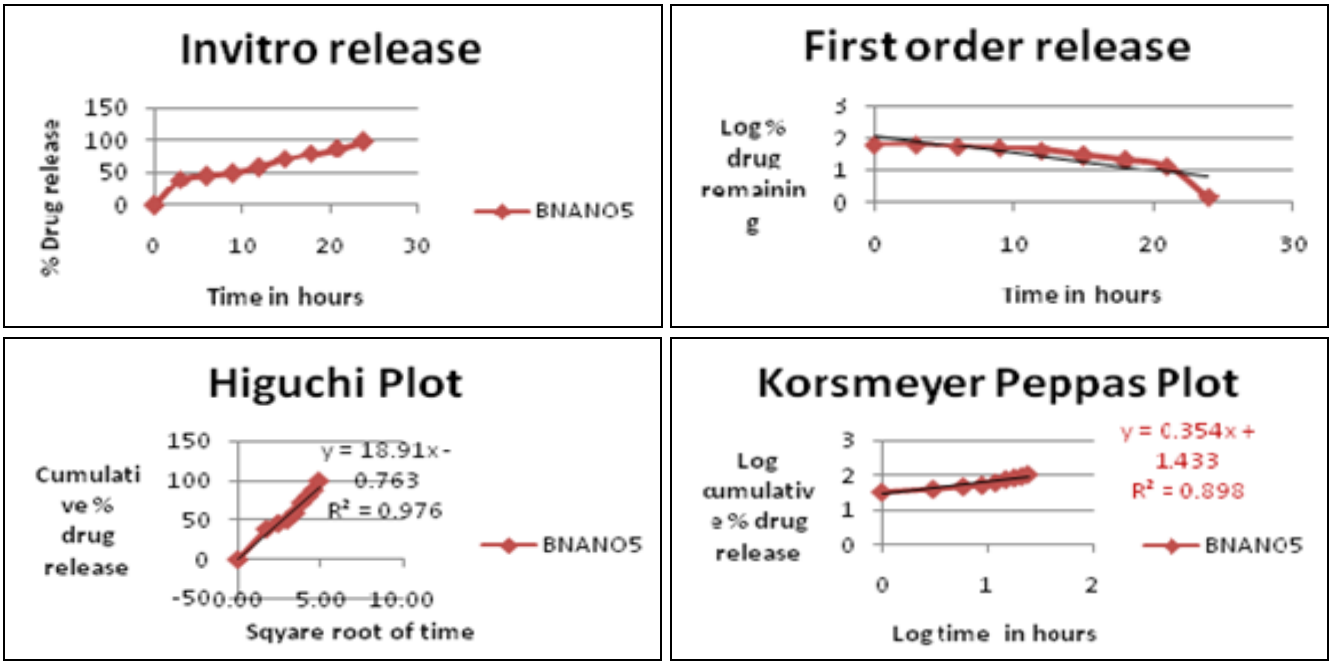


FIG. 7: *IN-VITRO* RELEASE, FIRST-ORDER, KORSMEYER PEPPAS PLOT AND HIGUCHI PLOT OF BERBERINE NANOPARTICLES

Pharmacological Evaluation: This evaluation analysis section has similar studies and techniques as above towards proving that the experimented data are valid and reliable.

Acute Toxicity: After drug administration, animals were observed for the next 14 days. Interpretations were recorded systematically and individually; similarly, records were maintained for each subject, individually.

Changes in mucous membranes and eyes, skin/ fur, central-nervous and autonomic systems, behavioral patterns and somatomotor activity, circulatory, respiratory, *etc*. were cautiously observed and noted/recorded. In acute toxicity study, Phenytoin and Berberine nanoparticles were found to be toxic at 1000mg/kg in albino rats, and it was found to be safe up to 5mg/kg.

MES Study: The hindlimb tonic extension (HLTE) occurrence was a positive parameter for MES(40). HLTE abolition was taken as a protection against MES seizures. HLTE occurrences and duration of HLTE (in seconds) were recorded and maintained. Statistical analysis was performed using "Graphpad Prism 5" (San Diego, California, USA). For comparison between groups, one-way ANOVA was utilized with *post-hoc* Turkey's test where $P<0.05$ was considered as significant.

The tonic hindlimb extension is suppressed by anti-epileptic drugs. THLE onset of actions and duration of actions were recorded for standard and test drugs such as phenytoin nanoparticles, Berberine nanoparticles, and Capsules (phenytoin and Berberine combination). When compared with control, individual nanoparticles significantly reduced THLE duration ($P<0.001$) than pure drug. Similarly, combination nanoparticles reduced the THLE duration than individual nanoparticles and inhibited the remarkable convulsion level. Thus the study showed more efficacies which in turn acts as an improved efficacy for capsules loaded nanoparticles. The THLE onset and duration along with inhibition percentages of convulsions, are tabulated for references (refer to **Table 4**).

TABLE 4: EFFECT OF PHENYTOIN, BERBERINE AND BERBERINE NANOPARTICLES ON MES INDUCED CONVULSION IN RATS

Treatment	Onset of THLE (s)	Duration of THLE (s)	Percentage of inhibitions of Convulsions
Control	1.90 ± 0.05	101.0± 4.58	-
Phenytoin 25mg/kg	2.73 ± 0.29	57.67 ± 3.84***	43.33%
Berberine 25mg/kg	3.26 ± 0.27 *	68.33± 2.02 ***	32.67%
Berberine Nano 5mg/kg	5.40 ± 0.40 ***	57.33± 2.72 ***	43.67%

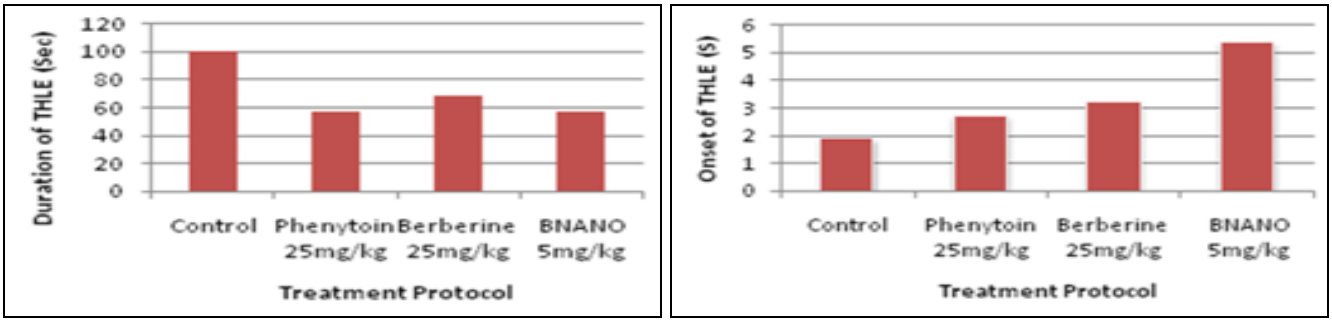


FIG. 8: ONSET AND DURATION OF THE FOR CONTROL, PHENYTOIN, BERBERINE AND BERBERINE NANOPARTICLES

Rota Rod for Muscle Coordination: Rotarod study was conducted towards the estimation of muscle relaxant activity. The standard Diazepam treated animals were fallen down in shorter time. The result revealed that mean time of fall was increased for berberine nanoparticles at 30min and 60 min which represents that the nanoparticles have higher efficacy than pure berberine (refer **Table 5**).

TABLE 5: MUSCLE RELAXANT ACTIVITY - ROTA ROD STUDY

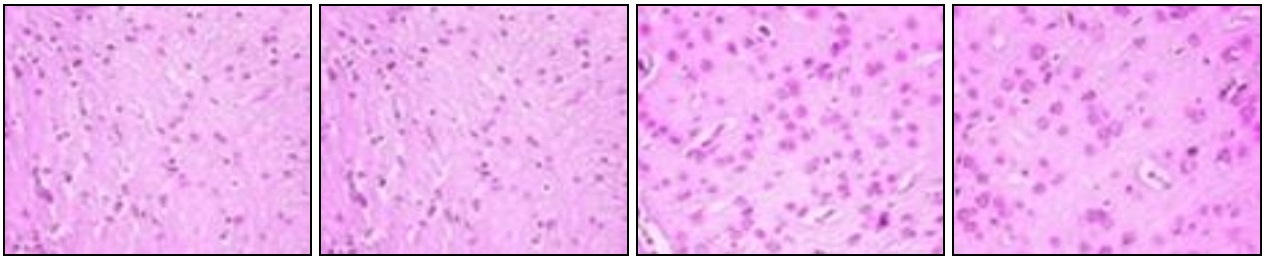
Group & Dose	Rotarod fall apparatus test - (Mean Time in sec)	
	30	60
Normal saline (0.5% Nacl.,i.p.)	215.16 ± 5.16	200.28 ± 6.16
Diazepam	29.16 ± 9.45***	13.45 ± 4.86 ***
Berberine 25 mg/kg .,ip	192.16± 4.28	188. 27± 2.65
Berberine Nano	203.16 ± 3.89	211.18± 5.92

Biochemical Study: Oxidative parameters such as SOD, TBARS, CAT, and GSH were analyzed; the obtained results in the table (refer **Table 6**) shows that the values were greater for Berberine nanoparticles than pure drug. Thus it indicates that antioxidant efficacy was improved for optimized.

TABLE 6: BIOCHEMICAL STUDY

Treatment	GSH (nmoles/g of tissue)	SOD (units/min/mg of protein)	TBARS (nmoles/mg of protein)	CAT (mg of protein/min)
Normal saline (0.5% Nacl., i.p.)	22.42± 6.42	4.12± 2.08	4.85±0.12	0.98± 1.26
Phenytoin 25 mg/kg., ip	31.26±2.49 **	7.92± 1.94 **	1.59±0.96 ***	2.93± 0.93 ***
Berberine 25 mg/kg ., ip	32.18± 8.16 **	7.43± 2.96 **	1.98± 0.23 ***	2.81± 1.34 ***
Berberine nanoparticles 5mg/kg	39.31±8.34***	7.58± 6.02 **	2.12± 0.12***	2.98± 1.45 ***

Histopathological Study: In the Control cortex regions, the albino rats showed a decrease in neuronal cells and increased chromatolysis and pyknotic nuclei. Phenytoin 25mg/kg., ip and Berberine 25mg/kg.,ip significantly decreased: the neuronal cell death, brain cell oedema, chromatolysis, and pyknotic nuclei in all regions when compared to the control rats cortex regions. Individual nanoparticles treated rats showed a significant decrease in cell edema and neuronal cell degeneration in all regions when compared to the control rats. Thus the combination of phenytoin and Berberine nanoparticles treated rats showed a significant decrease in neuronal cell death with mild edematous nuclei in all regions when compared against the control rats.



CONTROL **PHENYTOIN** **BERBERINE** **BERBERINE NANO**

FIG. 9: HISTOPATHOLOGICAL STUDY – ALBINO RATS' BRAIN CORTEX REGION

CONCLUSION: In this research, Optimized controlled release Berberine nanoparticles were prepared with improved efficacy by the Boxbehnken method. In order to accomplish the goal/ proposed aim, permeability enhancement and solubility enhancement techniques were adopted. Physicochemical characterization study revealed that prepared nanoparticles were spherical in shape and existed in the nano-level. Similarly, MES and Rotarod study showed that developed nanoparticles are more efficacious, and its 24 h drug release design reduces the dosage frequency. Thus the overall result proves that the developed nanoparticles have certainly improved the efficacy of anticonvulsant and antioxidant activity than the pure drug.

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CONFLICTS OF INTEREST: The authors declared that there are no conflicts of interest in this study.

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