Formulation and In-Vitro Evaluation of Clopidogrel Bisulphate Nanosuspension using Box Behnken Design.

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Abstract

In the current study an endeavour was made to formulate Clopidogrel Bisulphate (CLB) nanosuspension for enhancing aqueous solubility of CLB which displays poor bioavailability (<50%) by utilizing nanoprecipitation method, using Polyvinyl alcohol (PVA) as stabilizer optimized by polymer screening studies using different polymers with degree of precipitation as deciding factor. Box-Behnken design was used for optimization of CLB nanosuspension with temperature, drug: polymer and solvent:antisolvent as independent factors and %Drug release as a dependent factor, resulting in 13 formulation runs. Based on responses formulation was optimized (30^{o}C Temperature, 1:4 Drug:Polymer and 1:30 Solvent:Antisolvent). The 13 formulations were assessed for viscosity, drug content and drug entrapment which were in range of 2.01- 4.43cps, 61.2- 86.3% and 81.12- 90.36% respectively. All formulations were found to redisperse within seconds with no lag time. Optimized formulation showed entrapment efficiency of 89.5% with drug release of 88.4% at 90 min. which is close to the value predicted by design. Optimized formulation was likewise evaluated for - particle size, zeta potential and polydispersibility index (PDI) prior to and after subjecting to probe and bath sonication. Particle size was 2334 d.nm, 4.306 d.nm, 4.808 d.nm, zeta potential was 0.940mV, 2.14mV, 0.511mV and PDI was 0.410, 0.487, 0.369 respectively. Optimized formulation demonstrated a 2-fold increase compared to the pure drug. Other evaluation tests performed include viscosity and drug content which were identified to be 2.12cps and 89.78% respectively. The evaluation results indicate that the attempt for enhancement of aqueous solubility of CLB by nanonization technique was successful.

Keywords: Clopidogrel bisulphate, Nanosuspension, Nanoprecipitation method, Box-Behnken design, Polyvinyl alcohol.

INTRODUCTION

Oral administering is convenient and commonly used drug delivery route due to higher rate of patient compliance, cost-effectiveness and flexibility in dosage form.[(1)] However, the major problem associated with oral administration is poor bioavailability which depends upon aqueous solubility, permeability, dissolution rate etc. out of all these factors, low solubility and permeability are the most frequent factors.[(2)]

Poor aqueous solubility often requires higher doses to achieve the therapeutic plasma concentration following oral administration[(3).] This especially is difficult for drugs belonging to BCS class II (low solubility and high permeability), where the release of drug from dosage form is rate-limiting step.[(2)] So, enhancing solubility and dissolution rate of poorly soluble drugs and enhancing their permeability are the two major ways to enhance oral bioavailability.[(3)]

Various ways have been employed to tackle the inadequate aqueous solubility which include use of surfactants, lipid-based formulations, self-emulsifying drug delivery systems, cyclodextrins, cosolvents, amorphous solid dispersions and other techniques. [(2)(4)(5)(6)] (Figure 1.) gives the techniques for enhancing solubility.

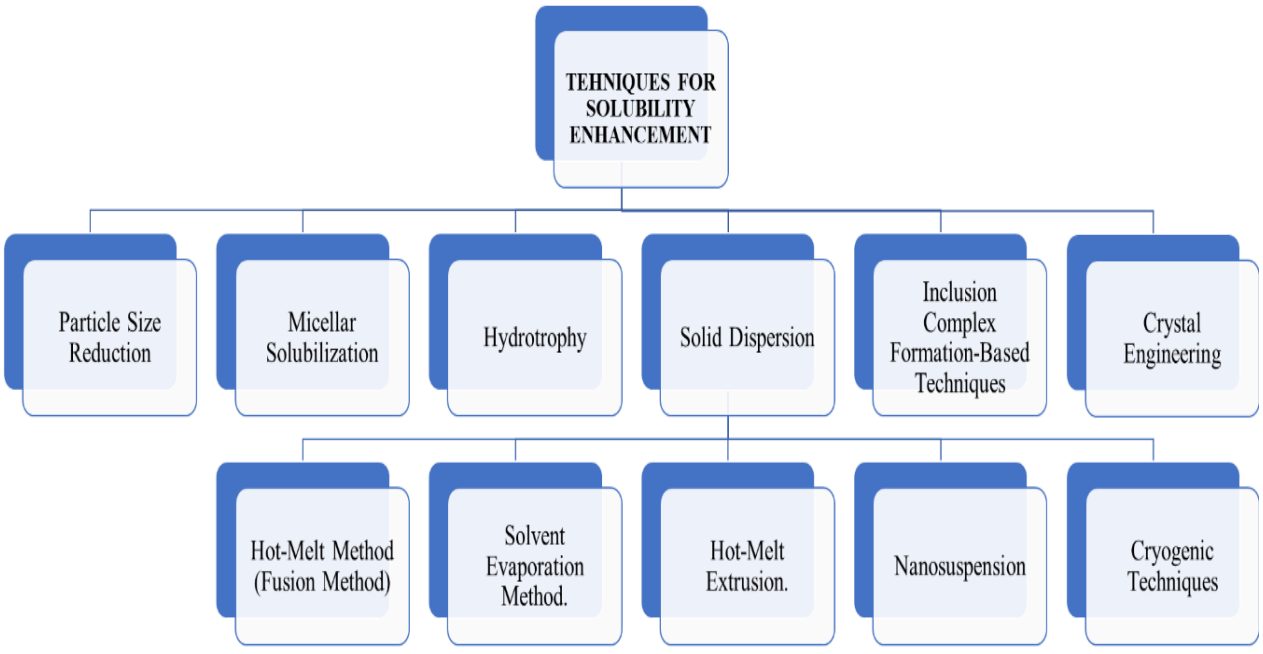


Figure 1. Techniques for solubility Enhancement.

Nanosuspensions are biphasic system comprising drug particles dispersed in aqueous medium of diameter <1 µm.[(7)]It is preferred for the drugs insoluble in water and for the compounds with high

log P value, high melting point and dose. Poor solubility of drugs in aqueous as well as in lipid media can be enhanced by utilising nanosuspensions. In nanosuspensions, overall bioavailability is enhanced by increasing surface area and saturation solubility via particle size reduction. Nanosuspension have demonstrated more prominent drug loading in contrast to other oral dosage forms, thus, lower dose is needed to acquire intended therapeutic effect and improved patient compliance. It is more suitable for compounds having high log P value, melting point and dose.[(4)(8)(9)]

Clopidogrel bisulphate is an anti-platelet agent belonging to thienopyridine class which is widely used in prevention of thrombotic event in cardiovascular disease.[(10)] It is indexed as class II agent (poorly water soluble and highly permeable). Due to poor water solubility, it is nearly insoluble in water at neutral pH thus, oral bioavailability of CLB is very low (<50%). [(11)] To confound this issue, one potential way is formulation of CLB using nanotechnology.

The present work aims to enhance aqueous solubility of CLB thereby its bioavailability by formulating it into nanosuspensions employing nanoprecipitation method and optimizing by utilising Box-Behnken design.

MATERIALS REQUIRED

Clopidogrel Bisulphate purchased from Suven Life Sciences, Ethanol from Emplura, Hydrochloric acid, PVA, PVP K-30, Polaxomer-407 from S.D. Fine Chemicals Ltd.

EXPERIMENTAL METHODOLOGY

Analytical Methodology for CLB

Standard calibration curve of CLB was generated in 0.1N HCl (pH 1.2). For determination of λ_{max} of CLB, 40 µg/ml of CLB was prepared and scanned using double beam spectrophotometer in spectrum mode from 400-200nm against 0.1N HCl as blank. The λ_{max} of CLB was 219 nm which was chosen for preparation of standard calibration curve.[(12)(13)] Calibration curve for CLB was plotted by preparing solutions with concentrations 5 - 40µg/ml and determining absorbance at 219 nm against 0.1N HCl.

Fourier Transform Infra-Red Spectroscopy Studies (FTIR)

The FTIR of CLB and CLB nanosuspensions were performed to characterize drug and check drug-excipient compatibilities. For drug, FTIR spectra were recorded by preparing KBr pellet, using a Shimadzu Corporation facility (Kyoto, Japan, model - 8400S). The pellet was mounted into sample holder and IR spectrum recorded from 4000 cm^{-1} to 400 cm^{-1}. The resultant spectrum was collated for spectral changes. For drug-excipient compatibility studies, Liquid FTIR was performed.[13][14]

Preliminary Trials

A. Selection of solvent system for CLB: Solubility of CLB was determined in purified water, ethanol, methanol and acetone. In 25ml of solvent 10mg of drug was added and sonicated for 30 minutes. If clear solution obtained, more 10mg was added and sonicated. For each solvent the procedure was repeated till no more drug could dissolve in 25ml of solvent.[15]

B. Polymers screening for formulating CLB Nanosuspensions: Different polymers viz., PVP K-30, PVA, Polaxamer-407 were screened for formulating nanosuspension under constant speed and run time. The polymer for the nanosuspension was chosen based on degree of precipitation.[16]

Preparation of CLB Nanosuspension

Preparation of CLB Nanosuspension by Nanoprecipitation Method is depicted in (Figure 2.)[16]

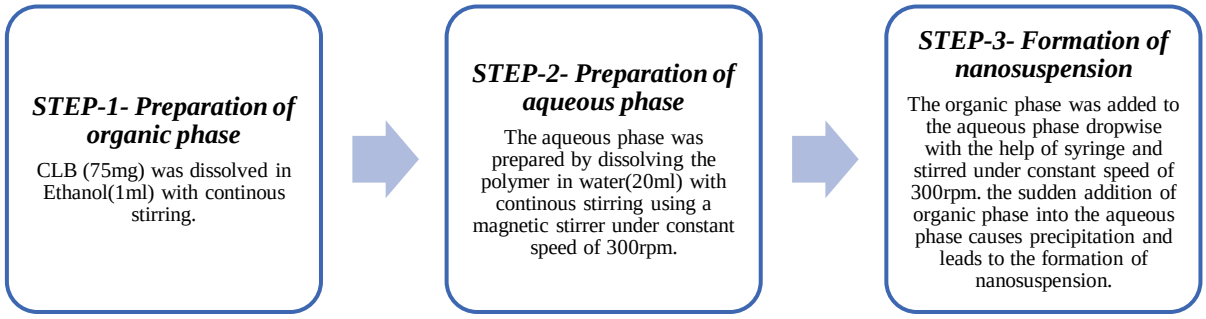


Figure 2. Flowchart depicting nanoprecipitation method.

Optimization of CLB Nanosuspensions by Box Behnken Design

A three-factor, three-level Box-Behnken design was selected for optimization to explore quadratic response surface using design expert 13 (version 11; Stat-Ease Inc, Minneapolis, MN).[5][17] Accordingly, the software has generated 13 formulation runs, for which *in-vitro* %drug release studies were executed. (Table 1.)

Table 1. Factors and factor levels of Box-Behnken design.

Independent factors	**Levels**		
	Low	**Medium**	**High**
	-1	0	1
A= Drug:Polymer	1:1	1:2.5	1:4
B=Temperature	30	50	70
C=Solvent: Antisolvent	1:10	1:20	1:30
Responses (Dependent factors)			
R1 – Dissolution (%)			

Evaluation of CLB Formulations

1. **Structure, morphology, particle size and polydispersibility index (PDI):** The structure and morphology was determined using scanning electron microscopy (SEM) while particle size and poly-dispersity index (PDI) of the nanosuspension was analysed using zetasizer (Zetasizer Ver. 7.12 Malvern instrument)[17]
2. **Zeta potential:** Zeta potential of optimized nanosuspension was estimated by zetasizer **(**Zetasizer Ver. 7.12 Malvern instruments) to measure stability of nanosuspensions by studying its colloidal property.[18]
3. **Determination of viscosity:** The viscosity of the formulated batches was determined using Brookfield Viscometer. The preparation was added to beaker and allowed to settle down for 30min. at 25±1ºC before measuring. Spindle no.64 was lowered carefully, rotated and viscosity noted. [19]
4. **Redispersibility**: The redispersibility of nanosuspensions was specified by tilting the vial bottle up and down with hand till the sediment was uniformly dispersed in the aqueous phase and number of times tilted was noted. [21][22]
5. **Drug content**: An amount of 0.2 ml nanosuspension, was diluted with 20 ml of 0.1 N HCl filtered and assayed utilising UV Spectrophotometer at 219 nm and formula (1) was used to determine actual drug content. [3]

$$\text{Drug content} = \frac{\text{Amount of drug}}{\text{Total drug}} \text{ X } 100 \qquad (1)$$

6. **Drug entrapment efficiency:** 1.5 ml of freshly prepared nanosuspension was centrifuged at 10,000rpm for 30min. utilising microcentrifuge. Then, 0.2ml of supernatant was diluted with

0.1N HCl and subjected to UV Spectrophotometer to measure amount of drug which was unincorporated. For calculating DEE, formula (2) is employe [3]

$$\text{Drug Entrapment Efficiency (DEE \%)} = \frac{\text{Actual amount of drug} - \text{Amount of free drug}}{\text{Actual amount of drug}} X100 \qquad (2)$$

7. **In-vitro dissolution studies:** A 4-5 cm long portion of dialysis tubing was made into a dialysis sac by folding and tying up one end of the tubing with thread. It was filled up with 5ml CLB nanosuspension and transferred into dissolution baskets. USP apparatus type II was used. For 90 minutes, at regular time intervals, 5ml samples were drawn from receptor compartment and replaced with fresh buffer. 0.45 µm Whatman filter paper was utilised to filter withdrawn samples and analysed using UV visible spectrophotometer at 219nm. [23] [24]
8. **Drug release kinetics study:** To find the release model that best fits the profile of drug release from nanosuspensions, the *in-vitro* release data was fitted into mathematical models. The calculation for regression coefficient (r^2) were carried out using MS-office excel. Based on the obtained r^2 value, mathematical model was interpreted.[25]

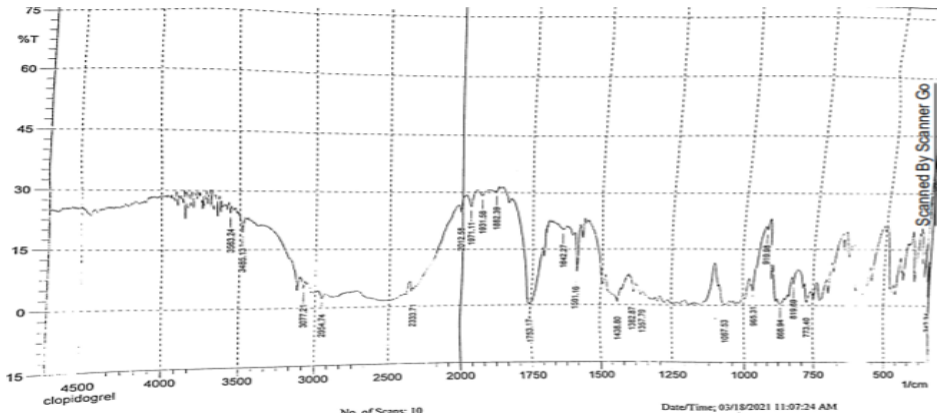
RESULTS AND DISCUSSIONS

Analytical Methodology of CLB

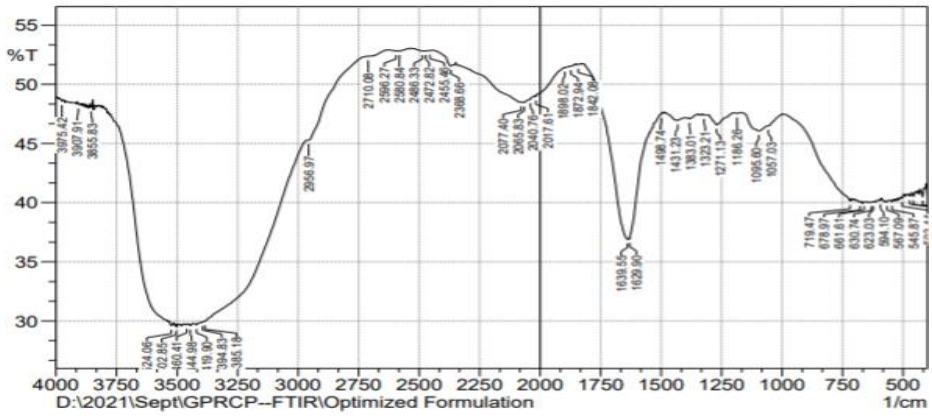
The λ_{max} of CLB was found to be 219 nm. Standard calibration curve was plotted and Beer Lambert's law was applicable in the concentration range of 5-40µg/ml with R^2 of 0.9993 in 0.1N HCl.

Fourier Transform Infra-Red (FTIR) Spectroscopy Studies

From the FTIR spectral interpretation, no interaction was found between pure drug (CLB) and excipients indicating drug to be compatible with excipients. **(**Figure 3.)



(a)



(b)

Figure 3. FTIR of a) Clopidogrel Bisulphate, b) Optimized formulation of CLB nanosuspension.

Preliminary trials

A. Selection of solvent system for CLB: Depending on solubility of CLB in diverse solvents, Ethanol was chosen for formulating nanosuspensions as 100mg of CLB dissolved easily in 25ml of ethanol. Whereas, in solvents like methanol and acetone only 60mg and 50mg of CLB was soluble respectively, in 25ml of solvent. In 25ml purified water, 10mg of CLB was partially soluble. Hence, ethanol was preferred for formulation of CLB nanosuspensions.

B. Polymers Screening for formulation of CLB Nanosuspensions: As depicted in (Table 2.), Precipitation was high for PVA and relatively low with PVP K30 and poloxamer-407. Hence, PVA was selected for formulation and evaluation of CLB nanosuspensions.

Table 2. Formulation of nanosuspension with different polymers.

Trials	Ethanol (ml)	Water (ml)	PVA (mg)	Poloxamer 407 (mg)	PVPK30 (mg)	Time (hrs)	Speed (rpm)	Temp (^{o}C)	Observations
T1	1	20	0.3	-	-	1	300	30	+
T2	1	20	0.6	-	-	1	300	30	+
T3	1	20	0.3	-	-	1	300	50	++
T4	1	20	0.6	-	-	1	300	50	++
T5	1	20	-	0.3	-	1	300	30	-
T6	1	20	-	0.6	-	1	300	30	-

T7	1	20	-	0.3	-	1	300	50	+
T8	1	20	-	0.6	-	1	300	50	-
T9	1	20	-	-	0.3	1	300	30	-
T10	1	20	-	-	0.6	1	300	30	+
T12	1	20	-	-	0.3	1	300	50	-
T13	1	20	-	-	0.6	1	300	50	-
Note: No precipitation seen (-); low precipitation (+); high precipitation (++)									

Optimization by Box-Behnken Design

Based upon the preliminary trials, temperature, Drug: Polymer and Solvent: Antisolvent were picked as independent factors in low, medium and high concentration and percentage drug release as the dependent factor as inputs to the Box-Behnken design expert software. The formulation runs obtained are displayed in (Table 3.)

Table 3. Formulation of CLB Nanosuspensions using Box-Behnken design.

Formulations	Drug : Polymer	Temperature	Solvent : Antisolvent
F1	1:2.5	70	1:30
F2	1:4	50	1:10
F3	1:2.5	30	1:10
F4	1:1	50	1:30
F5	1:1	70	1:20
F6	1:4	70	1:20
F7	1:2.5	30	1:30
F8	1:4	50	1:30
F9	1:4	30	1:20
F10	1:1	50	1:10
F11	1:1	30	1:20
F12	1:2.5	70	1:10
F13	1:2.5	50	1:20

In-vitro studies were carried for the formulations (F1-F13) in comparison with pure drug in 0.1N HCl for 90min. From the studies, it was noted that release of drug was maximum with F9 and F13,

i.e., 82.3% and 88.5% respectively, when compared to pure drug (48.29%). It showed 2-fold increase. Whereas the other formulations, didn't show a substantial increment in drug release. **(**Figure 4.)

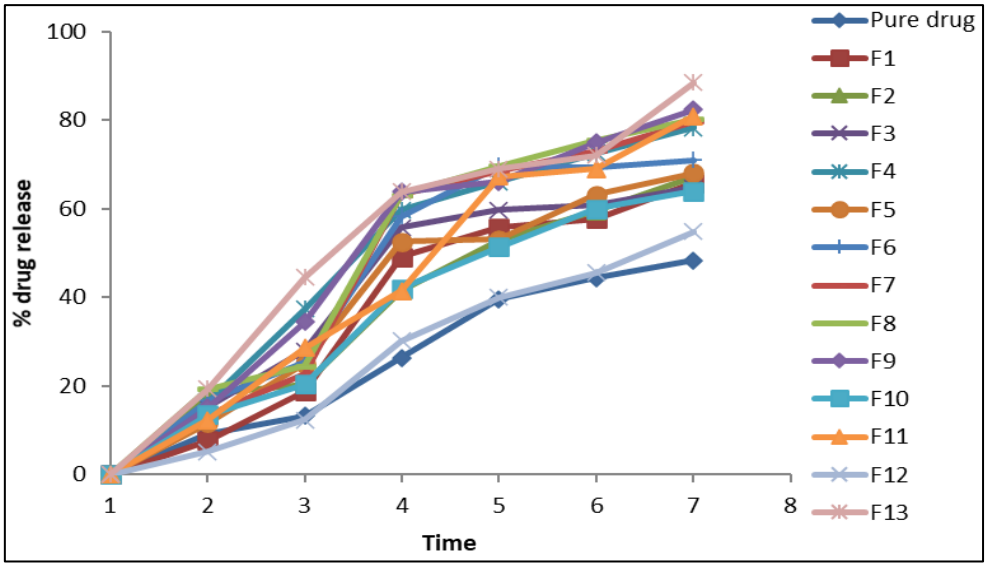


Figure 4. In-vitro dissolution studies of formulation runs.

1. **Fit summary:** Fit summary suggested model was linear for which adjusted R2 value is 0.4537 and predicted R2 value is 0.3281 for response 1 i.e.; drug release. There exists no lack of fit, p-value suggests that the model is significant and cubic model is aliased. (Table 4.)

Table 4. Fit summary for responses

Source	Sequential p-value	Adjusted R^2	Predicted R^2	
Linear	**0.0380**	**0.4537**	**0.3281**	**Suggested**
2FI	0.9951	0.1894	-0.2716	
Quadratic	**< 0.0001**	**0.9986**		**Suggested**
Cubic				Aliased

2. **Fit statistics:** The Predicted R^2 of 0.3281 is in reasonable agreement with the Adjusted R^2 of 0.4537; i.e., the difference is less than 0.2. Adeq. Precision measures the signal to noise ratio. A ratio >4 is preferable. A ratio of 6.426 suggests adequate signal. (Table 5.)

Table 5. Fit statistics for response

Std. Dev.	7.10	**R^2**	0.5903
Mean	72.71	**Adjusted R^2**	0.4537

C.V. %	9.77	**Predicted R^2**	0.3281
		Adeq. Precision	6.4263

3. **ANOVA summary:** The Model F-value of 4.32 implies the model is significant. Only a 3.80% chance that an F-value this large could be present due to noise. P-values less than 0.0500 indicate that the model terms are significant. In this case A and C are significant model terms. (Table 6.)

Table 6. ANOVA

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	654.39	3	218.13	4.32	0.0380	significant
A-Temperature	288.00	1	288.00	5.71	0.0406	
B-Drug: Polymer	11.28	1	11.28	0.2235	0.6476	
C-Solvent:Antisolvent	355.11	1	355.11	7.04	0.0264	
Residual	454.26	9	50.47			
Cor Total	1108.65	12				

4. **Predicted vs. Actual:** A graph was plotted for predicted vs. actual values for Response 1 (%Drug release) in MS-Excel to get the R^2 which was found to be 0.9954, indicating linearity between Predicted and actual values. **(**Figure 5.)

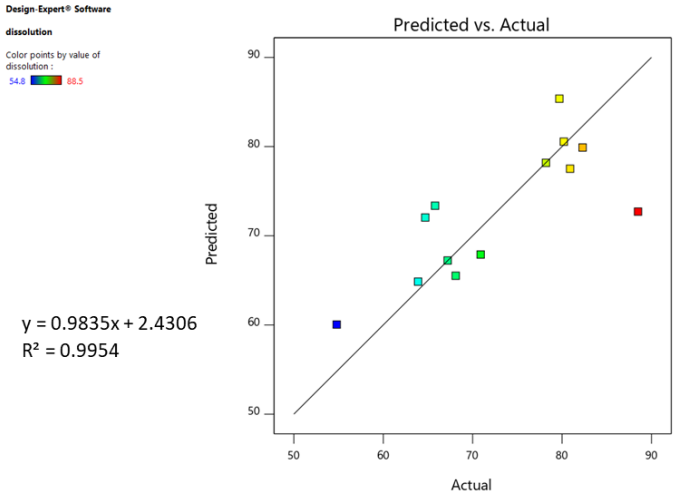


Figure 5. Predicted vs. Actual for the responses

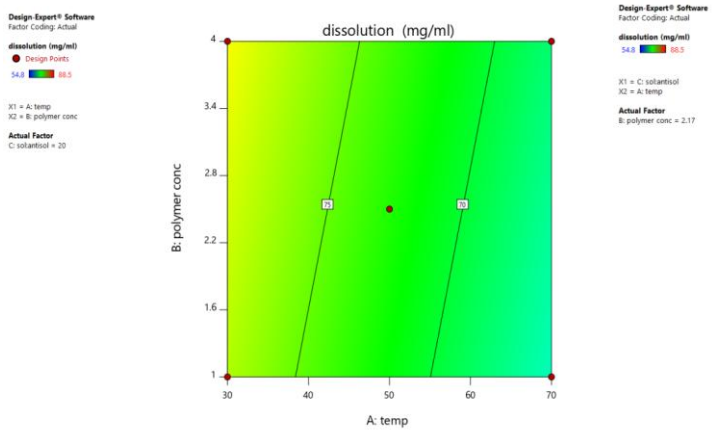
5. Model graphs:

a) Two-Dimensional counter plots: In **(**Figure 6a) Effect of temperature, ratio of drug: polymer on %Drug release can be visualised here %Drug release decreases as the temperature increases and as drug: polymer ratio increases. **(**Figure 6b) we can observe that %Drug release decreases as the temperature increases and vice versa. As ratio of solvent: antisolvent increases the dissolution increases.

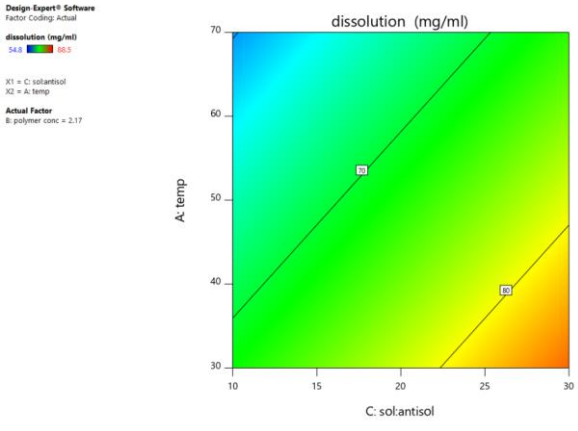
b) Three-Dimensional plots: From **(**Figure 7a), it is observed that blue colour is at minimum drug release (more temperature and less drug: polymer ratio). As drug release increases, gradually colour shifts from blue to red colour. Similar changes is observed in **(**Figure 7b), as ratio of solvent: antisolvent increases, the dissolution increases which is indicated by red colour and dissolution decreases, as the temperature increases which is indicated near blue colour.

c) Numerical optimization: Based on responses obtained, numerical optimization was done. 65 solutions were obtained with different ratios of drug: polymer, temperature, and solvent: antisolvent with little difference in concentration range. **(**Figure 8.) Out of 65 solutions, one solution was considered. Desirability of 0.942 indicates better response for predicted factors. **(**Figure 9.)

d) Graphical representation: In **(**Figure 10.), bright yellow is the acceptable criteria and unacceptable criteria is in grey colour. The numerical optimized solutions (flags) are displayed in the graph. Here, the predicted factor concentrations are same as numerical solution.

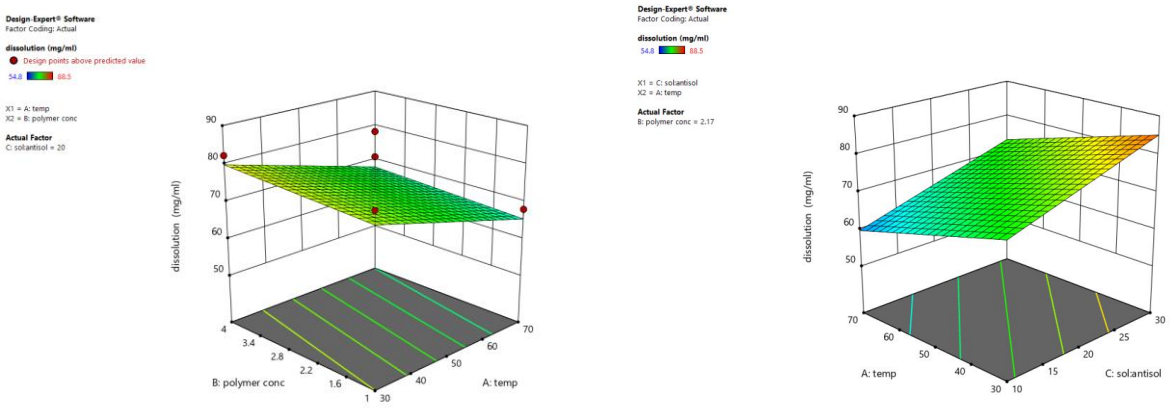


(a)



(b)

Figure 6. a) 2D Plot of Drug release plotted between drug: polymer and temperature b) 2D Plot of Drug release plotted between solvent: antisolvent and temperature.



(a) **(b)**

Figure 7. (a) 3D Plot of Drug release plotted between drug: polymer and temperature (b) 3D Plot of Drug release plotted between solvent: antisolvent and temperature.

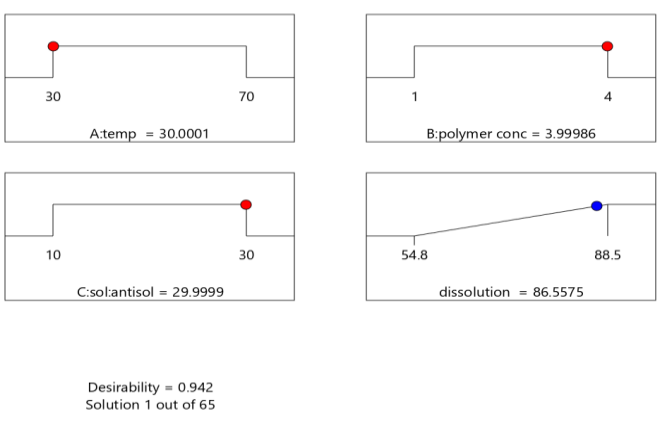


Figure 8. Numerical optimization

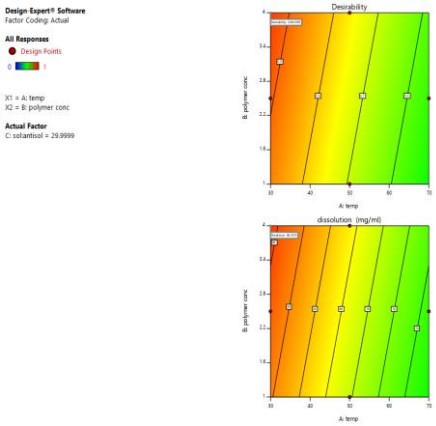


Figure 9. Desirability graphs

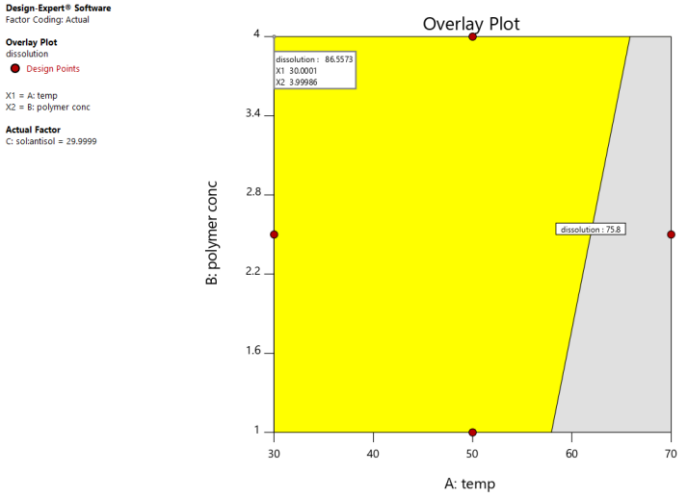


Figure 10. Overlay plot

Evaluation of CLB Nanosuspension

a) Viscosity: All the formulations (F1-F13) were evaluated utilizing Brookfield and the viscosity of nanosuspensions ranged from 2.01-4.43cps.

b) Redispersibility: All the nanosuspensions (F1-F13) redispersed quickly within few seconds.

c) Drug content: Drug content of CLB nanosuspension was estimated and found to be in 90.2-99.7% range.

d) Drug entrapment efficiency (DEE): Formulation F13 displayed highest DEE of 88.76% which was prepared at 50^0C temperature, solvent: antisolvent ratio of 1:20 and drug: polymer range of 1:2.

Table 7. Evaluation of viscosity, entrapment efficiency and drug content for the formulation runs.

Formulation	Redispersibility	Viscosity (cps)	Entrapment efficiency	Drug content
F1	Fast	2.70	72.76%	91.2±0.03
F2	Fast	3.47	81.12%	94.6±0.29
F3	Fast	3.24	78.97%	93.5±0.16
F4	Very fast	2.10	84.22%	99.7±0.32
F5	Fast	2.67	75.87%	92.1±0.41
F6	medium	4.01	78.11%	95.8±0.52
F7	Fast	2.20	80.03%	97.3±0.33
F8	Very fast	2.56	82.55%	92.7±0.28
F9	Fast	2.55	70.23%	90.2±0.42
F10	Very fast	3.72	80.23%	94.5±0.71
F11	Fast	2.01	71.35%	91.8±0.08
F12	medium	4.43	76.89%	96.3±0.22
F13	Very fast	2.86	80.76%	96.3±0.22

6. **Selection of the Optimized Formulation:** By using Numerical optimization and overlay plot the concentration of optimized formulation was obtained and shown in the (Table 8.)
   Factor A: Drug: Polymer: 1:4
   Factor B: Temperature: 30^0 C
   Factor C: Solvent: Antisolvent: 1:30

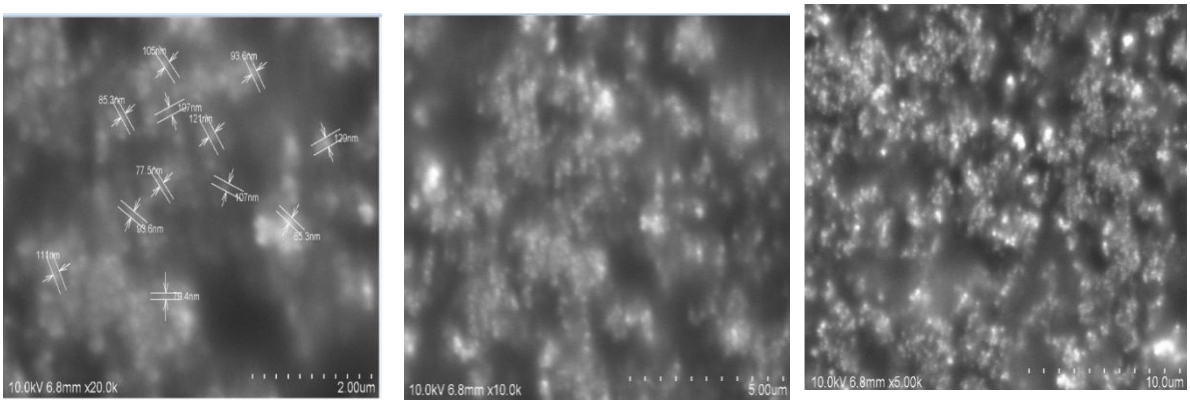
Table 8. Optimized formulation.

Ingredient	Quantity
Ethanol	1ml
Water	30ml
Clopidogrel Bisulphate	0.075gm
PVA	1.2gm
Temperature	30^0 C
Speed	500
Time	1.5hrs

Characterization of Optimized Nanosuspension Formulation

Optimized formulation was formulated and evaluated for particle size, zeta potential, entrapment efficiency and %Drug release.

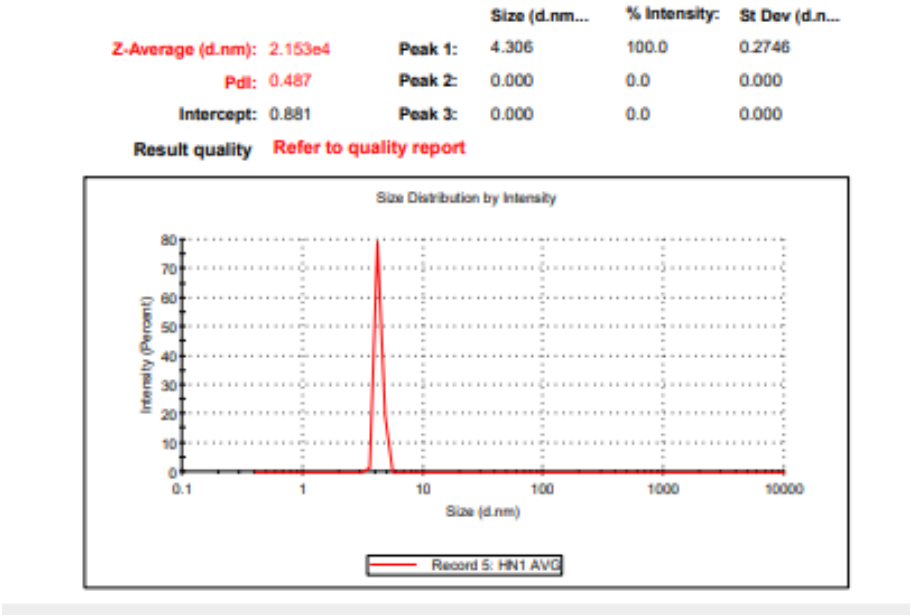
1. **Redispersibility:** The final optimized nanosuspensions were stored in room temperature. 24hrs later a thin layer of sediment was visualized and redispersed within few seconds of shaking the vial.
2. **Determination of viscosity:** Viscosity of 2.12cps was determined by Brookfield viscometer using spindle No. 64.
3. **Vesicle morphology using SEM:** The surface morphology was determined using SEM which gives three dimensional images of globules. Magnification was performed at 2µm, 5µm and 10µm**.** SEM images are shown in **(**Figure 11.)



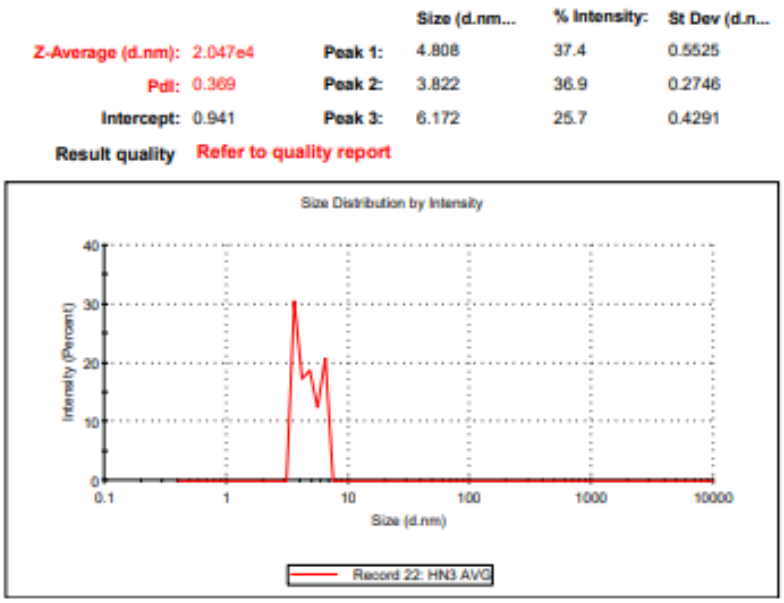
(a) **(b)** **(c)**

Figure 11. SEM images of optimized CLB nanosuspension.

4. **Particle size, polydispersity index (PDI) and zeta potential:** Optimized formulation was put for probe sonication, bath sonication and the particle sizes were compared with optimized formulation.



(a)



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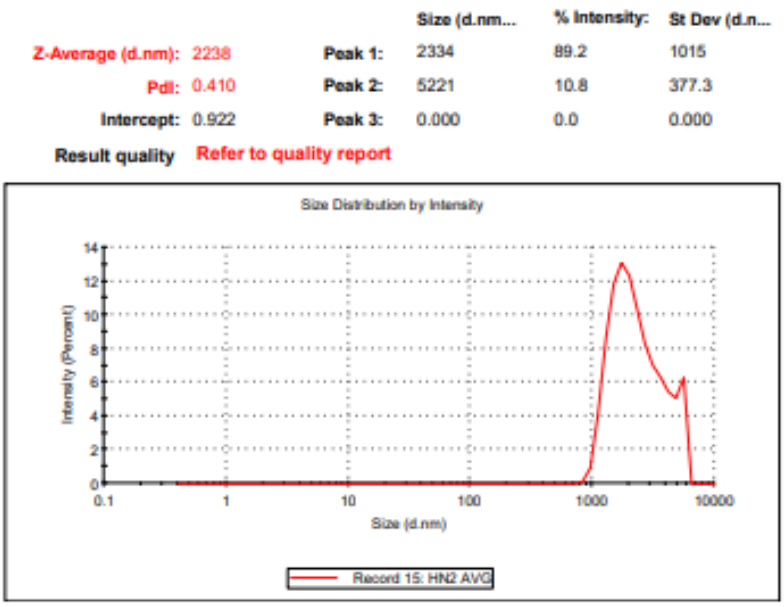

(c)

Figure **12. Size distribution report of (a) final optimized formulation subjected to probe sonication, (b) final optimized formulation subjected to bath sonication and (c) final optimized formulation**

The nanosuspension obtained had a diameter of 4.306d.nm, 4,808d.nm and 2334d.nm, PDI 0.487, 0.369 and 0.410 and zeta potential 2.14mV, 0.511mV and 0.940mV respectively for nanosuspension subjected to probe sonication, bath sonication and optimized nanosuspension, indicating the screened formulations were polydisperse in nature. Particle size analysis revealed that all the molecules in nanosuspensions were in nanometer size. **(**Figure 12.)

5. **Entrapment efficiency (DEE):** Optimized formulation showed DEE of 78.7%

6. **Drug content:** Final optimized formulation showed drug content of 99.78%.
7. ***In-vitro* dissolution studies of optimized formulations:** The drug release profile is displayed in **(**Figure 15.) Formulation showed 88.4% drug release at 90min.

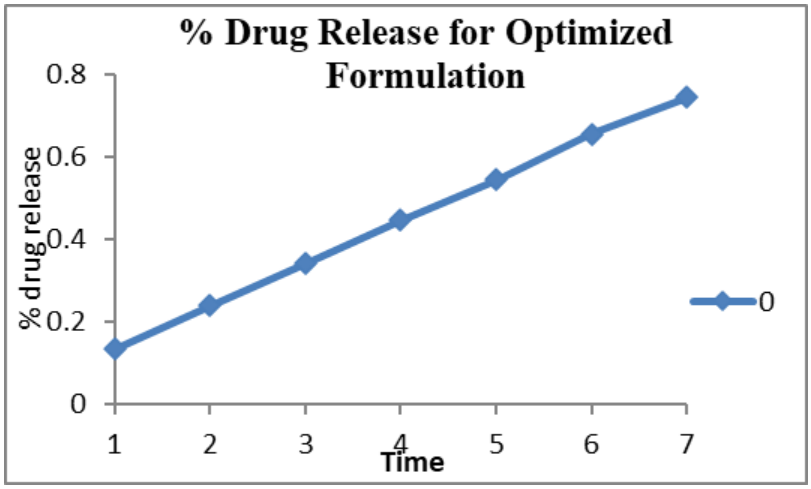

Figure 13. %Drug release of optimized formulation

8. **Model Dependent Kinetics:** The data of in-vitro release profile of optimized CLB nanosuspension showed best fit to Higuchi model (r^2=0.9523) and Korsmeyer's Peppas model (r^2=0.9748) as illustrated in **(**Figure 16.)

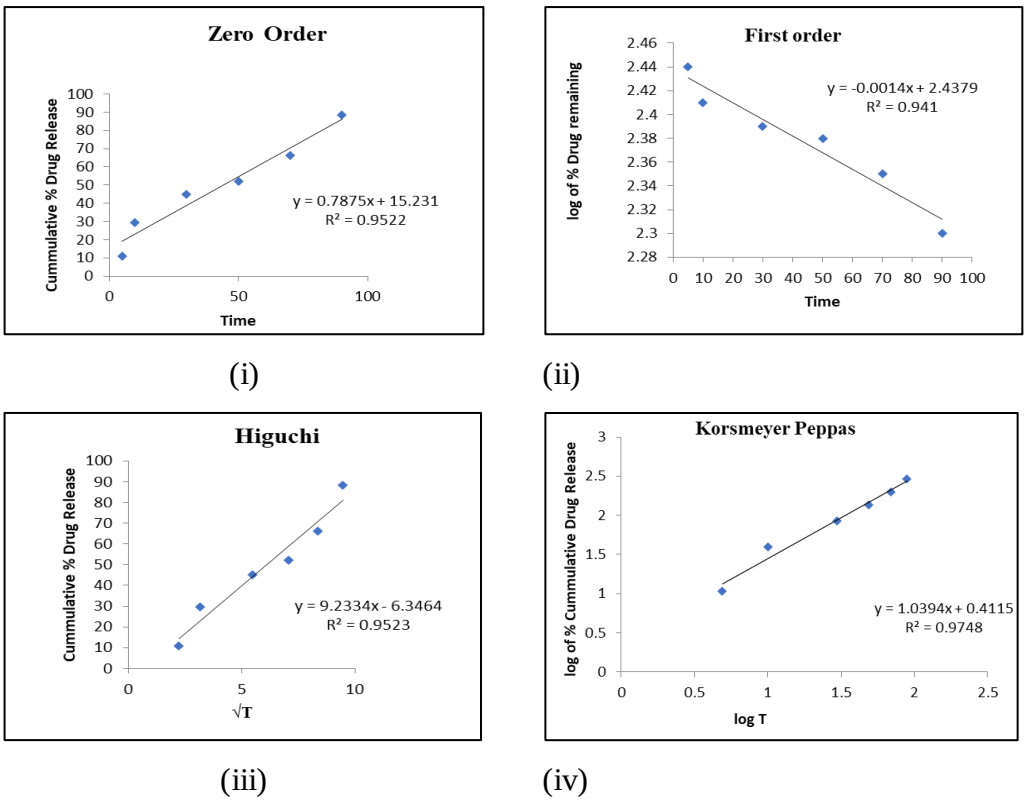

Figure 14. Model dependent kinetics of optimized formulation

CONCLUSION

Clopidogrel bisulphate is BCS class II drug i.e., poorly soluble with high permeability. Due to poor aqueous solubility, its reported bioavailability following oral administration is <50%. So, in the current work, nanosuspensions of CLB were formulated by nanoprecipitation method, optimized utilizing Box-Behnken design and evaluated to prove the attempt of enhancement of aqueous solubility of drug by nanonization technique.

Preformulation studies followed by selection of the suitable solvent, polymer screening were executed based on degree of precipitation, at constant time and speed (rpm).

Optimization of CLB nanosuspension was carried utilizing Box-behnken design with temperature, drug: polymer and solvent: antisolvent as independent factors and % drug release as dependent factor. The design resulted in 13 formulation runs for which effect of independent factors on % drug release was studied by conducting *in-vitro* dissolution studies which indicated F9 and F13 showed better drug release (82.3 and 88.5 respectively) compared to all 13 formulations. Based on responses obtained from numerical optimization and overlay plot, optimized formulation identified to be the one with temperature 30^0 C, drug: polymer 1:4 and solvent: antisolvent 1:30, which was then formulated and evaluated. The entrapment efficiency was found to be 78.7% and drug release 88.4% at 90min. exhibiting 2-fold increase compared to pure drug (49.8%). Other evaluation tests produced significant results.

FUTURE SCOPE

Stability studies and *in-vivo* studies (pharmacokinetic studies) need to be performed to completely establish the work.

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CONFLICT OF INTEREST

"All Authors declare that there is no conflict of interest"

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