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QUALITY BY DESIGN (QBD) ASSISTED FORMULATION AND DEVELOPMENT OF BETA- CYCLODEXTRIN BASED NANOSPONGES GEL CONTAINING BRYOPHYLLUM PINNATUM EXTRACT AND ITS ANTI-MICROBIAL ACTIVITY

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ABSTRACT
The study investigates the development of a beta-cyclodextrin-based nanosponges gel with *Bryophyllum pinnatum* extract to enhance its antibacterial properties. The extract contains bioactive components such as alkaloids, flavonoids, glycosides, steroids, tannins, and phenols. The nanosponges were created using beta-cyclodextrin as a polymer and diphenyl carbonate as a crosslinker, resulting in a spherical, porous shape. The nanosponges showed stable particle sizes, with an average particle size of 192.01 nm and a zeta potential of -1.2 mV. The gel formulation had high permeability and controlled release qualities. Antimicrobial tests showed substantial action against *Escherichia coli*, with concentration-dependent inhibition peaking at a minimum inhibitory concentration of 1.5 mg/mL. The study demonstrates the potential of nanosponge-based drug delivery systems to enhance the therapeutic efficacy of plant extracts, making them valuable for future biomedical applications.

Keywords:
Nanosponges, Antimicrobial activity, *Bryophyllum pinnatum*, beta-cyclodextrin, polymer, Diphenyl carbonate

1. INTRODUCTION
Pharmaceutical Quality by Design: ICH Q8 defines quality as the suitability, of either a drug substance or drug product for its intended use. This term includes such attributes as the identity, strength and purity. Pharmaceutical QbD is a systematic, scientific, risk based, holistic and proactive approach to pharmaceutical development that begins with predefined objectives and emphases product and processes understanding and process control. It means designing and developing formulations and manufacturing processes to ensure predefined product quality objectives **(Candy et al., 2006).**
Herbal medicines: Herbal medicines contain an active ingredient, aerial or underground parts of plants as their petal or seeds materials or combinations thereof, whether in the crude state or as plant preparations. In traditional medicine, the leaves of *Bryophyllum pinnatum* have been reported to possess antimicrobial activity **(Mehta and Bhatt 1952)**
Nanosponges: Nanosponges are colloidal structures that contain solid tiny particles with cavities and mesh-like network to encapsulate wide varieties of substances like antineoplastic, protein and peptide, volatile oil, DNA, etc. Nanosponges act as 3D networks with a backbone of naturally long-length polyester. Nanosponge attaches on the surface of desired target site during their circulation in body and releases the drug in a controlled and predicted manner **(Dubey et al., 2017).** Cyclodextrin nanosponges developed as a oxygen delivery system by suspending their subtypes i.e. α-, β- and γ-cyclodextrins in water, saturated with oxygen and characterized by *in vivo* studies **(Trotta et al., 2016).**

2. MATERIAL AND METHODS
2.1 Chemical
Petroleum ether, Copper sulphate, was obtained from Ranken, and Methanol was obtained from Molychem, a reputable supplier of analytical reagents. Himedia provided the Beta-cyclodextrin (β-CD), and Spectrochem provided the concentrated Diphenyl carbonate (DPC). Molychem provided the

ethanol, while Clorofiltind supplied the concentrated hydrochloric acid and 95% alcohol, along with chloroform. Himedia supplied the magnesium, and Rankem provided the 1% copper sulphate solution.

2.2 Plant collection

The medicinal plant *Bryophyllum pinnatum* (300 gm) was harvested. After cleaning, plant parts (leaves) were dried in the shade at room temperature for three days, followed by oven drying at 45°C until completely dry. Authentication of chosen traditional plants - A plant taxonomist authenticated the medicinal plant *Bryophyllum pinnatum* to ensure its identity and purity.

2.3 Extraction

Using Soxhlet apparatus and the continuous hot percolation process, plant material was extracted for the current experiment. Powdered *Bryophyllum pinnatum* was added to a thimble used for Soxhlet equipment. Petroleum ether was used as the non-polar solvent for soxhlation, which was carried out at 60°C. Marc, the exhausted plant material, was dried before being extracted again using methanol. Each solvent was subjected to soxhlation until there was no longer any discernible color change in the siphon tube. The extraction process was considered complete when there was no solvent left behind after it evaporated. In a rotary vacuum evaporator of the Buchi type, the extracted materials were evaporated at 40°C. Following the weight of the dried extract, the following formula was used to determine the percentage yield of each extract:

$$\% \text{ Yield} = \frac{\text{Weight of extract}}{\text{Weight of Plant Material used}} \times 100$$

After the produced extracts were checked for organoleptic properties (color, odor, and % yield), they were labeled and placed in an airtight container for later use **(Baidya *et al*., 2002).**

2.4 Phytochemical investigation

An experiment was conducted employing comprehensive qualitative phytochemical analysis to ascertain the presence or absence of multiple phytoconstituents. Based on precipitate development or color intensity, medical responses to tests were determined. The normal procedures listed below were applied **(Kokate *et al.*, 2000).**

2.5 Quantitative Phytochemical Estimation

2.5.1 Total Phenolic Content

The Folin-Ciocalteu Assay was used to assess the total phenolic content of *Bryophyllum pinnatum* extract. The process comprised combining 0.2 mL of *Bryophyllum pinnatum* extract from a stock solution with 2.5 mL of Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate. Distilled water was used to dilute the mixture to a total amount of 7 ml. The solutions were left at room temperature for two hours before being measured at 760 nm with a spectrophotometer. Calibration curves were created using standard Gallic Acid Equivalent (GAE) solutions with concentrations ranging from 20 to 100 µg/ml. The Folin-Ciocalteu reagent reacts with reducing compounds, including polyphenols, producing a blue tint that may be quantitatively quantified to determine the phenolic concentration **(Tangco et al., 2015).**

2.5.2 Total Flavonoid Content

The flavonoid concentration of *Bryophyllum pinnatum* extract was determined using the aluminum chloride method. To conduct this experiment, 0.5 ml of the plant extract was combined with 2 ml of distilled water. Then, 0.15 ml of 5% sodium nitrite was added to the mixture and stirred. After 6 minutes of waiting, 0.15 mL of 10% aluminum chloride was added, followed by another 6 minutes of waiting. Then, 2 ml of 4% sodium hydroxide was added, and the mixture was agitated firmly. The absorbance of the final solution was measured at 510 nm with a UV spectrophotometer. Calibration curves were constructed with Rutin as the standard at doses ranging from 20 to 100 µg/ml. The calibration curve was used to calculate total flavonoid concentration, which was then expressed as mg Rutin equivalent per gram dry extract weight **(Parthasarathy et al., 2009).**

2.6 Formulation of β-cyclodextrin Nanosponges

Beta-cyclodextrin Nanosponges were made by 'hot melt method'. Various ratios comprising Diphenyl carbonate (DPC cross-linker) and Beta- cyclodextrin (β-CD polymer) (Ratios of DPC and β-CD are

given in table no.4) were chosen for the nanosponges preparation. Anhydrous polymer (β-CD) and crosslinker (DPC) (finely homogenised) were gradually heated (90 to 100 ºC) with magnetic stirring for 1to 3 hours. The substrate solution (β-CD and DMC) was set aside for 1 to 3 hours to finish the cross-linking reactions, which produced nanosponges. The reaction mixture that was produced was then allowed to cool to room temperature. To remove unmodified β-CD, the sample underwent multiple washings with twice-distilled water. After being dried at 40 ºC, the resulting placebo nanosponges were kept in a desiccator until needed again **(Penjuri et al., 2016).**

Figure 1: Nanosponges

2.7 Extract loading in nanosponges

Freeze-drying was used to add the drug to the nanosponges that had been created. A magnetic stirrer was used to evenly disperse 1 g of placebo NS in 50 mL of double-distilled water. The aforementioned dispersion includes approximately 100 milligrams of medicine. The resultant suspensions were centrifuged for 10 minutes at 2000 rpm to extract the medication, which had become trapped as a residue beneath the extremely colloidal supernatant. The supernatant was then lyophilized at -81 °C with a force of 0.0010 mbar. The dried NS powder that was contaminated with medicines was saved for later use **(Sharma and Pathak 2011).**

2.8 Composition of nanosponges formulation

Table 1: Composition of nanosponges formulation

S. No	Formulations Code	Beta-cyclodextrin-Polymer (mg) X1	Diphenyl carbonate-Cross linker (DPC) (mg) X2	Stirring time (hrs) X3	Drug (1:1 Ratio) (mg)	Temperature (ºC)
1	F1	300	125	1	100	90 to 100
2	F2	100	125	3	100	90 to 100
3	F3	200	50	1	100	90 to 100
4	F4	300	50	2	100	90 to 100
5	F5	100	50	2	100	90 to 100
6	F6	200	200	1	100	90 to 100
7	F7	100	125	1	100	90 to 100
8	F8	100	200	2	100	90 to 100
9	F9	300	200	2	100	90 to 100
10	F10	300	125	3	100	90 to 100
11	F11	200	200	3	100	90 to 100
12	F12	200	50	3	100	90 to 100

2.9 Design of experiment

The experiment for the composition of nanosponges was designed using the Design Expert (Version 12.0.1.0) application. The quadratic response surfaces were modeled using a second-order polynomial approach.

2.9.1 Independent and Dependent variables

Table 2: Independent and Dependent variables

Independent variables	Dependent variables
(X1) Polymer (mg)	(Y1) Particle size (nm)
(X2) Cross linker (mg)	(Y2) EE (%)
(X3) Stirring time(hrs)	

2.9.2 Values of variables

Table 3: Values of variables

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Polymer	mg	Numeric	100.00	300.00	-1 ↔ 100.00	+1 ↔ 300.00	200.00	85.28
B	Cross-linker	mg	Numeric	50.00	200.00	-1 ↔ 50.00	+1 ↔ 200.00	125.00	63.96
C	Stirring time	hrs	Numeric	1.0000	3.00	-1 ↔ 1.00	+1 ↔ 3.00	2.00	0.8528

2.10 Evaluation parameters of nanosponge

2.10.1 Zeta potential and Particle size

The Malvern Zeta sizer (Malvern Instruments) was used to measure the size of the nanosponges **(Swetha et al., 2011).**

2.10.2 Scanning Electron Microscopic (SEM)

The morphological properties of drug-loaded nanosponges were identified utilizing an electron beam from a scanning electron microscope. A thin metal coating was applied, and secondary electrons were produced. Rutherford and Kramer's Law were used to choose dispersed electrons **(Abbas et al., 2019).**

2.11 Creation of Gel Loaded Nanosponges

Carbopol-934 was submerged in warm water for two hours, and then mixed with carboxymethyl cellulose & methyl paraben in room temperature water. The pH was adjusted with tri-ethanol amine, and the dispersion was combined with nanosponges to create a gel with propylene glycol as a permeability booster **(Silpa et al., 2021).**

Figure 2: Nanosponge gel

2.11.1 Composition of gel formulation

Table 4: Composition of gel formulation

S. No	Excipients	Quantity (gm)
1.	Carbopol 934	1.00 gm
2.	Carboxymethyl cellulose	1.00 gm
3.	Propylene glycol	0.5 ml
4.	Methyl paraben	0.2 ml
5.	Nanosponges	1.0 gm
6.	Tri-ethanolamine	q.s
7.	Water	100 ml

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2.12 Characterization of nanosponges loaded Gel

2.12.1 Physical appearance

Physical appearance Visual observation was used to assess the produced Gel formulation's appearance, color, odor, and homogeneity **(McGlynn 2003).**

2.12.2 pH

Using a digital pH measuring device (EI), the formulation's pH was measured **(Monica and Gautami 2021).**

2.12.3 Viscosity

This Brookfield viscometer (spindle no. 61) was used at 250 degrees Celsius and 100 rpm to test the gel compositions' consistency **(Sandeep 2020).**

2.12.4 Spreadability

When applied or massaged into the skin's surface, the optimum topical gel would have a high spreading coefficient. This was tested by putting approximately 1g of composition on a Slide made of glass. The gel was sandwiched between the two glass slides and spread at a predetermined distance when a 50 mg mass was placed on top of another glass slide that had the same length. It was noted how long it took the gel to move a specific distance from its starting point. The following formula was utilized to ascertain spreadability **(Sharma et al., 2014).**

$$S= M*L/T$$

3. RESULT AND DISCUSSION

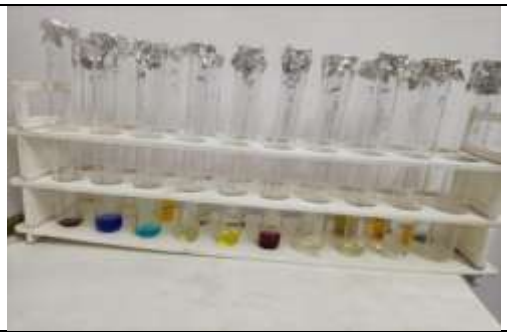
3.1 Percentage Yield

Table 5: Percentage Yield of crude extracts of *Bryophyllum pinnatum* extract

S. No	Plant name	Solvent	Theoretical weight	Yield(gm)	% yield
1	*Bryophyllum pinnatum*	Pet ether	298	1.47	0.49%
2		Methanol	286.21	6.60	2.30%

Figure 3: Extract pet. Ether

3.2 Preliminary Phytochemical study

Test with petroleum ether extract	Test with methanolic extract
	

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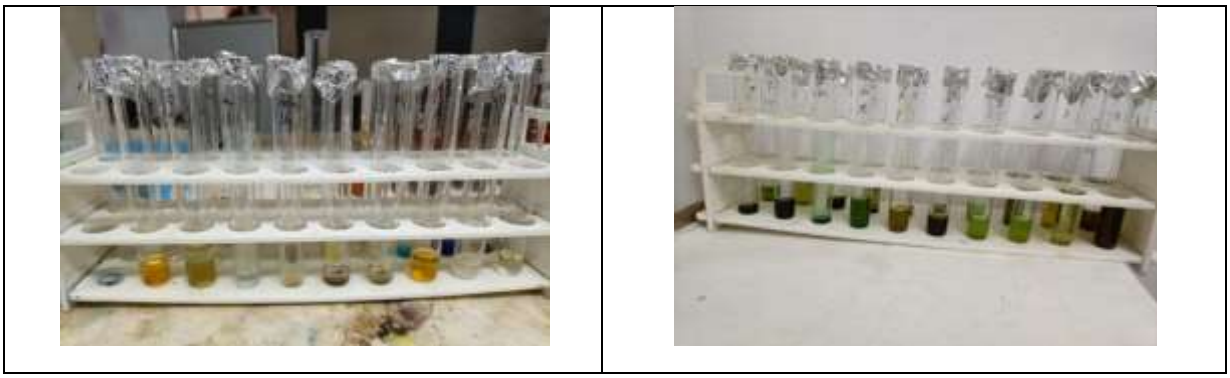

3.3 Quantitative Analysis

Table 6: Total Phenolic content (TPC) and Total Flavonoids content (TPC) estimation

S. No.	Concentration (µg/ml)	Absorbance (TPC)	Absorbance (TFC)
1.	20	0.153	0.182
2.	40	0.183	0.203
3.	60	0.196	0.284
4.	80	0.239	0.321
5.	100	0.280	0.335

Table 7: Total Phenolic content and Total Flavonoid Content of extract *Bryophyllu pinnatum*

Extracts	**Methanol**
Total Phenolic content (mg/gm equivalent of Gallic acid)	54.66 mg/gm
Total Flavonoid content (mg/gm equivalent of rutin)	17.66 mg/gm

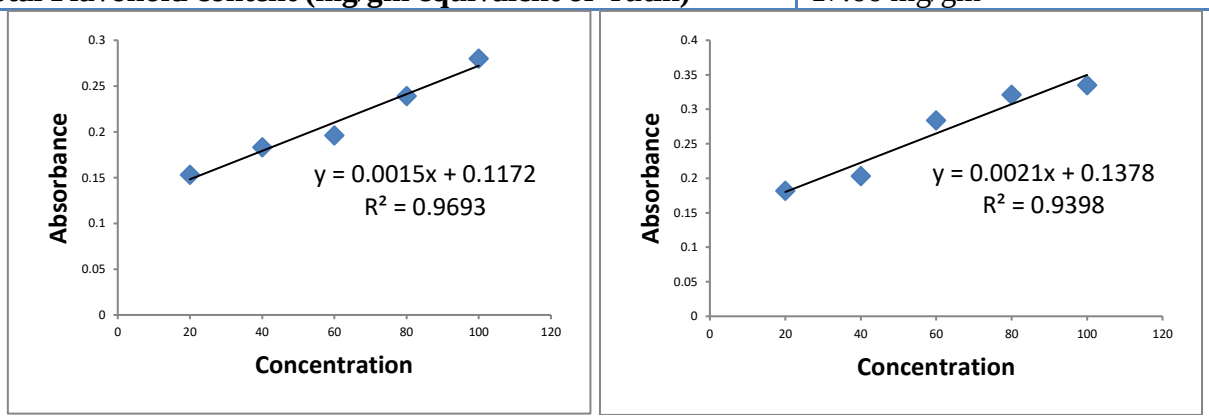

Figure 4: Represent standard curve of Gallic acid and Rutin

3.4 Optimization of formulation by design of expert (DOE) software

Table 8: Build information of DOE software

File Version	**12.0.1.0**		
Study Type	Response Surface	**Subtype**	Randomized
Design Type	Box-Behnken	**Runs**	12
Design Model	Quadratic	**Blocks**	No Blocks

Table 9: Independent variables

File Version	**12.0.1.0**		
Study Type	Response Surface	**Subtype**	Randomized
Design Type	Box-Behnken	**Runs**	12
Design Model	Quadratic	**Blocks**	No Blocks

Build Time (ms)	2.00		

Table 10: Dependent variables

S. No	Coding	Variables
1.	Y1	Particle size (nm)
2.	Y2	Zeta potential (mV)

Table 11: Formulation trials as per Box–Behnken design and Evaluation parameter

S. No	Formulation trials					Evaluation parameter	
	Beta-cyclodextrin-Polymer (mg) X1	Diphenyl carbonate-Cross linker (DPC) (mg) X2	Stirring time (hrs) X3	Extract (mg)	Temperature (°C)	Particle size (nm)	Zeta potential
1	200	200	3	200	90 to 100	124.64	-1
2	300	125	3	200	90 to 100	254.36	-1.2
3	300	125	1	200	90 to 100	860.96	-0.8
4	100	125	3	200	90 to 100	178.25	-0.7
5	300	50	2	200	90 to 100	492.83	-0.9
6	200	50	3	200	90 to 100	274.89	-1.3
7	200	50	1	200	90 to 100	775.21	-0.5
8	300	200	2	200	90 to 100	406.75	-1.5
9	100	50	2	200	90 to 100	492.18	-0.4
10	100	200	2	200	90 to 100	509.79	-0.3
11	200	200	1	200	90 to 100	924.04	-0.1
12	100	125	1	200	90 to 100	810.11	-0.2

3.4.1 Fit Summary

Table 12: Response 1: Particle size

Source	Sequential p-value	Adjusted R^2	Predicted R^2	
Linear	**< 0.0001**	**0.9358**	**0.8950**	**Suggested**
2FI	0.1374	0.9629	0.9029	
Quadratic	0.3898	0.9670	0.8561	Aliased

3.5 Effect of formulation variables on Particle size (ANOVA for linear model)

3.5.1 Response 1: Particle size

Table 13: Response 1: Particle size (ANOVA for Linear model)

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	8.060E+05	2.687E+05	54.46	< 0.0001	significant
A-Polymer	75.46	75.46	0.0153	0.9046	
B-Cross linker	610.58	610.58	0.1238	0.7341	
C-Stirring time	8.053E+05	8.053E+05	163.25	< 0.0001	
Residual	39464.22	4933.03			
Cor Total	8.454E+05				

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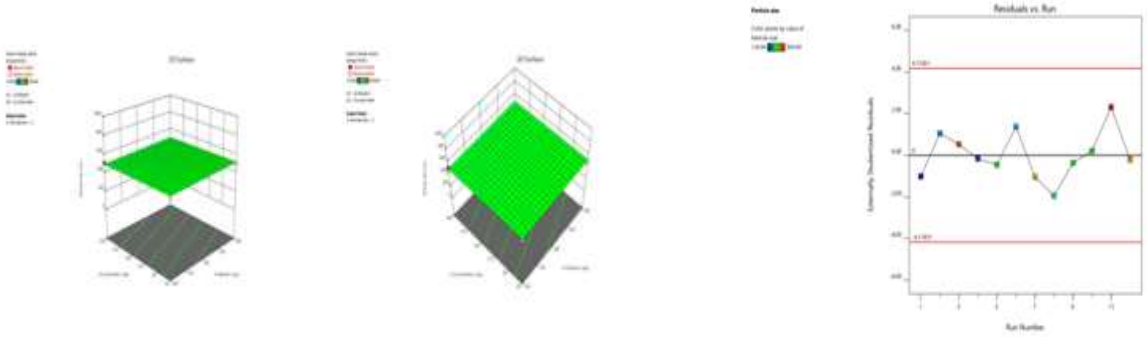

Figure 5: Response surface plot showing combined effect of polymer and cross linker on particle size of Nanosponges

3.5.2 Impact of formulation variables on Zeta potential

Table 14: Response 2: Zeta potential (Fit Summary)

Source	Sequential p-value	Adjusted R^2	Predicted R^2	
Linear	**0.0032**	**0.7339**	**0.5645**	**Suggested**
2FI	0.6002	0.6978	0.2089	
Quadratic	0.9681	0.5072	-1.1506	Aliased

3.6 ANOVA for Linear model

3.6.1 Response 2: Zeta potential (ANOVA Linear model)

Table 15: Response 2: Zeta potential (ANOVA Linear model)

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	1.83	0.6100	11.11	0.0032	significant
A-Polymer	0.9800	0.9800	17.85	0.0029	
B-Cross linker	0.0050	0.0050	0.0911	0.7705	
C-Stirring time	0.8450	0.8450	15.39	0.0044	
Residual	0.4392	0.0549			
Cor Total	2.27				

Table 16: Predicted value and actual value of all formulations of Particle size and Zeta potential

Formulations	Particle size		Zeta potential	
	Actual Value	Predicted Value	Actual Value	Predicted Value
F1	124.64	182.66	-1.0000	-1.04
F2	254.36	194.47	-1.20	-1.42
F3	860.96	829.01	-0.8000	-0.7667
F4	178.25	188.32	-0.7000	-0.7167
F5	492.83	520.48	-0.9000	-1.12
F6	274.89	200.13	-1.30	-1.09
F7	775.21	834.68	-0.5000	-0.4417
F8	406.75	503.00	-1.50	-1.07
F9	492.18	514.33	-0.4000	-0.4167
F10	509.79	496.86	-0.3000	-0.3667
F11	924.04	817.20	-0.1000	-0.3917
F12	810.11	822.87	-0.2000	-0.0667

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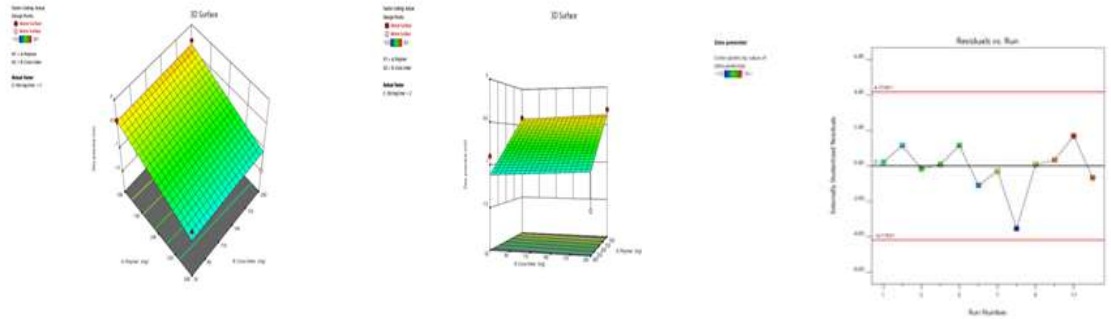


Figure 6: Response surface plot showing combined effect of polymer and cross linker on entrapment efficiency of nanosponges formulation.

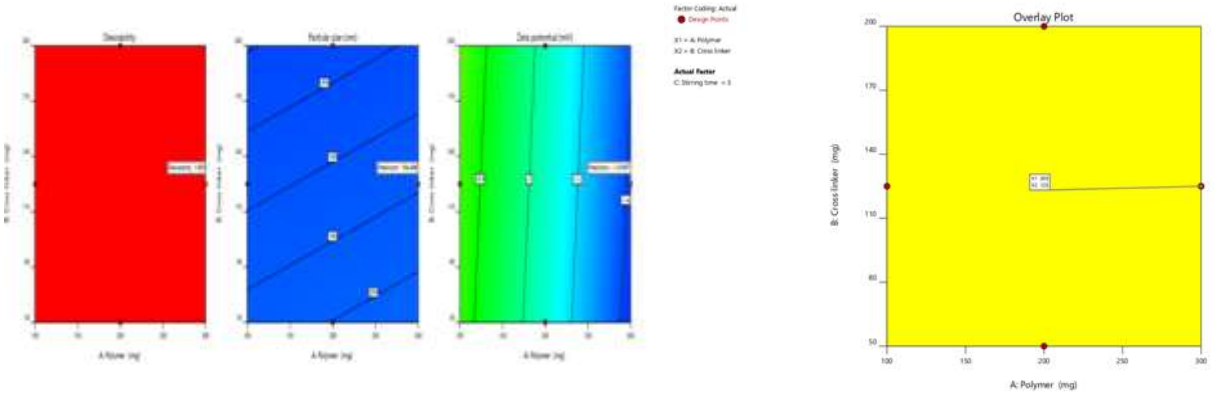


Figure 7: Response surface plot showing prediction data for optimization and Overlay plot of optimization formulation

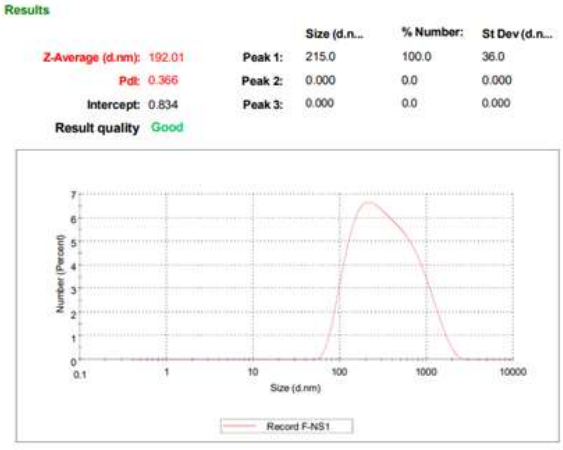
3.7 Optimized formula of Nanosponges formulation

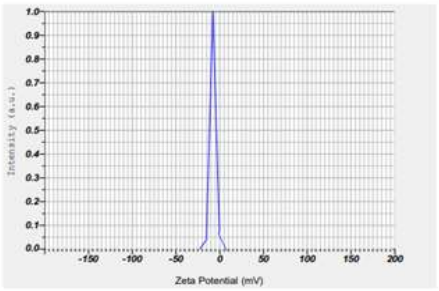
Table 17: Optimized formula of Nano sponges formulation

S.No	Polymer	Cross-linker	Stirring time	Particle size	Zeta potential	Extract (mg)	
1	**300.000**	**125.000**	**3.000**	**194.466**	**-1.417**	**200**	**Selected**
2	111.320	116.378	2.882	227.095	-0.721	200	
3	142.019	108.049	1.613	631.621	-0.419	200	

3.8 Characterization of optimized formulation

3.8.1 Particle Size and Zeta potential





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S. No	Formulation	Particle size		Zeta potential	
		(Predicted value)	(Actual value)	(Predicted value)	(Actual value)
1.	Nanosponges	194.4	192.01 nm	- 1.4 mV	-1.2mV

3.9 Scanning Electron Microscope (SEM)

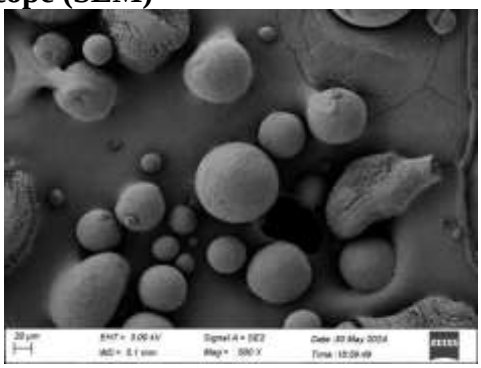

Figure 8: Scanning electron microscope (SEM)

3.10 Characterization of Nano sponges loaded gel

3.10.1 Physical appearance

S.No	Parameter	Result
1.	Colour	White to brown
2.	Odour	Odorless
3.	Appearance	Transparent
4.	Homogeneity	Homogeneous

Table 18: Viscosity, pH and Spredability

S. No	Formulation	Viscosity	pH	Spredability
1.	Gel	6107±0.42	6.1	11.02

Figure 9: Viscometer pH meter

3.11 Results of antimicrobial activity of nanosponges formulation

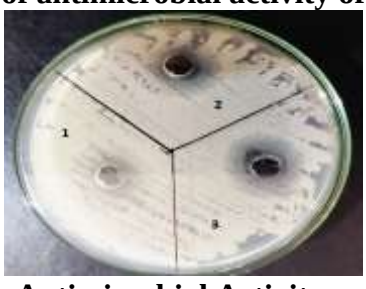

Figure 10: Antimicrobial Activity

S. No	Sample name	Zone of Inhibition (mm)
1	Extract (1mg/ml)	7 mm
2	Formulation (1.0mg/ml)	10 mm
3	Formulation (1.5mg/ml)	13 mm

4. SUMMARY AND CONCLUSION

The qualitative phytochemical screening of *Bryophyllum pinnatum* has confirmed the presence of bioactive metabolites such as alkaloids, flavonoids, glycosides, steroids, tannins, and phenols. Quantitative assays for total phenolic content (TPC) is 54.66 mg/gm and total flavonoid content (TFC) is 17.66 mg/gm were successfully performed using gallic acid and rutin as standards. Initial trials indicated that the chosen polymers are suitable for the formation of extract-loaded nanosponges, resulting in high-quality formulations. The use of diphenyl carbonate as a crosslinker and beta-cyclodextrin as a polymer was particularly effective, producing nanosponges with a porous, smooth surface morphology and spherical shape, as confirmed by scanning electron microscopy (SEM). Particle size analysis revealed an average size of 192.01 nm, and zeta potential measurements showed stability with a value of -1.2 mV. The nanosponges gel formulation demonstrated desirable properties, including a viscosity of 6107±0.42 cps, a pH of 6.1, and good spreadability of 11.02 cm, contributing to its strong permeability and release characteristics. The antimicrobial activity test showed that the gel containing *B. pinnatum* leaf extract exhibited concentration-dependent activity against *Escherichia coli*, with the highest activity observed at a minimum inhibitory concentration of 1.5 mg/ml. These findings suggest that the nanosponge-based delivery system significantly enhances the bioavailability of *Bryophyllum pinnatum* extract. Overall, the study demonstrates that nanosponge technology could be a valuable tool for improving the therapeutic efficacy of natural extracts, providing a promising approach for future drug delivery applications.

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