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Formulation and in vitro evaluation of topical nanosponge-based gel containing *cassia alata* leaves extract

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ABSTRACT

This abstract describes the formulation and in vitro testing of a topical nanosponge-based gel containing *Cassia alata* leaf extract, which is known for its powerful antibacterial capabilities. Active metabolites such alkaloids, flavonoids, glycosides, steroids, tannins, and phenols were discovered through qualitative phytochemical screening. Quantitative experiments measured total phenolic content (TPC) and total flavonoid content (TFC) using gallic acid and rutin as standards. Nanosponges made of ethyl cellulose and polyvinyl alcohol (PVA) showed promising drug transport properties. Scanning electron microscopy (SEM) validated the nanosponges' porous, spherical form, with an average particle size of 521.2 nm and a consistent zeta potential of 69.7 mV. The produced nanosponge gel had excellent viscosity, pH, and spreadability, which aided in drug release and permeability. Notably, the nanosponge formulation enhanced the *Cassia alata* extract's antibacterial efficacy against E. coli. The superior performance of the 1 mg/ml nanosponge gel highlights its potential for more effective bacterial infection treatment. The findings indicate that *Cassia alata* nanosponges may improve medication solubility and bioavailability, making them a good candidate for skin disease treatment, with the added benefits of lower dosage, administration frequency, and side effects.

Keywords: Nanosponge, *Cassia alata,* Antimicrobial Activity, *In vitro,* SEM

1. INTRODUCTION

Quality by Design: QbD was first described by famous quality professional Joseph M. Juran **(Joseph *et al.,* 1998).** Juran thought that quality could be planned, so that maximum quality crises and difficulties related to quality would reduce. His concept called "designed in" is used in QbD for optimization of process/product. According to ICH Q8 guideline, QbD is a systematic approach to pharmaceutical development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management

(**ICH Q8 2009**).QbD is a method of choice in formulation development to get robust and quality product followed by continuous improvement. QbD is widely used tool in formulation and development. It is particularly helpful in designing robust processes with well understood operational limits and their significance. Implementation of QbD is a complex and challenging work in pharmaceutical industry. Scientists have used QbD tools in development of various formulations such as tablet, emulsion, nanosuspension, liposomes, pellet preparations; gastroretentive tablets (**Charoo *et al.,* 2012**).

Herbal Plants: Plants with medicinal potentials and their secondary metabolites have been identified and implicated in dishes from the earliest annals of human habitancy; herbal medicine in ancient systems as well as advanced medicine has created one of the most important science bases for security in various lands of the mankind. For many of years, herbal plants have been used for distinct goals. Herbal plants are generally defined as one year gramineous herbs with not any strict contexture **(Singh *et al*., 2016).**

Nanosponge: Disease diagnosis, treatment, and prevention are starting to shift as a result of the broad variety of nanotechnology being developed. Medical technology has considerably benefited from the development of various nano-devices, which have greatly increased the effectiveness of many current medications and allowed for the creation of whole new therapeutic approaches. According to Pandey et al. (2019), nanosponge is a novel material that has a few nanometer-sized cavities that can hold different chemicals. These particles have the ability to transfer hydrophilic and lipophilic materials and make compounds that aren't very soluble in water more soluble **(Panda *et al*., 2015).**A naturally degradable scaffold-like structure the size of a virus is called a nanosponge. The long polymer strands are combined in solution with tiny molecules known as cross-linking, which are specific to particular regions of the polymer (**Kapileshwari *et al.,* 2020**).

2. MATERIAL AND METHODS

2.1 Chemicals

Ammonia, sodium hydroxide, nitroprusside, and glacial acetic acid were obtained from Merck, a reputable supplier of analytical reagents. Researchlab provided the petroleum ether, and Fizmerck provided the concentrated sulfuric acid. 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 sulfate solution.

2.2 Plant collection 300 grams of the medicinal herb *Cassia alata* were gathered. Following cleaning, plant components (leaves) were dried for three days at room temperature in the shade

and for a further three days at 45°C in an oven. Verification of the identity and purity of a chosen traditional plant - A plant taxonomist verified the identity and purity of the medicinal plant *Cassia alata*.

2.3 Extraction Plant material was extracted for the current investigation utilizing the Soxhlet apparatus and a continuous hot percolation process. *Cassia alata* powder is added to a thimble of a soxhlet device. Soxhlation was carried out at 60°C with a non-polar solvent such as petroleum ether. The plant material that had been exhausted (marc) was dried and then extracted again using methanol. Each solvent's soxhlation was continued until no discernible color change was seen in the siphon tube, and the extraction's completion was verified by the absence of any solvent residue upon evaporation. The obtained extracts were evaporated at 40°C in a Buchi-type rotating vacuum evaporator. Weighing the dried extract, we calculated the % yield for each extract using the following formula:

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

The prepared extracts were labeled and stored in an airtight container until further usage after being examined for organoleptic characteristics (percentage yield, color, and odor) **(Baidya *et al*., 2002).**

2.4 Phytochemical investigation

An investigation was carried out to determine the existence or disappearance of several phytoconstituents using thorough qualitative phytochemical analysis. Medical reactions to testing were based on colour intensity or precipitate formation. The following standard methods were followed **(Kokate *et al.,* 2000).**

2.5 Quantitative Phytochemical Estimation

2.5.1 Total Phenolic Content (TPC): The Folin-Ciocalteu Assay was used to ascertain the total phenolic content of the *Cassia alata* extract. Two milliliters of 7.5% sodium carbonate, 2.5 milliliters of Folin-Ciocalteu Reagent, and 0.2 milliliters of *Cassia alata* extract were combined. Distilled water was added to this combination to dilute it up to 7 mL. After the final solutions were let to remain at room temperature for two hours, the absorbance at 760 nm was determined using spectrophotometry. Gallic Acid Equivalent (GAE) mg/gm standard solutions were used to create calibration curves. Gallic aid was produced at concentrations of 20, 40, 60, 80, and 100 µg/mL. The reagent Folin-Ciocalteu is susceptible to reducing agents, such as polyphenols. When they react, they turn blue. This blue hue was determined using spectrophotometry. **(Tangco *et al.*, 2015).**

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2.5.2 Total Flavonoid Content (TFC): The aluminum chloride technique was used to determine the flavonoid concentration. A mixture of 0.5 ml of *Cassia alata* extract solution and 2 ml of distilled water was prepared. Following that, 0.15 ml of sodium nitrite (5%) was added and thoroughly stirred. Subsequently, add 0.15 ml of aluminum chloride (10%) and let it stand for an additional 6 minutes. Next, two milliliters of 4% sodium hydroxide were added. The blend was well blended and given a good shake. A UV spectrophotometer was used to assess the mixture's absorbance at 510 nm. Rutin Equivalent (RE) mg/gm standard solutions were used to create calibration curves. Rutin was produced at concentrations of 20, 40, 60, 80, and 100 µg/mL. Total flavonoid content was determined from the calibration curve and results were indicated as mg Rutin equivalent per gram dry extract weight**(Parthasarathy *et al.,* 2009).**

2.6 Development of extract loaded NS: "Hot melt method" used to prepare the nanosponges. Different ratios of β-CD polymer and DPC (diphenyl carbonate) cross-linking agent were synthesized. A surplus of DPC (between 50 and 200 mg) was melted at approximately 90 °C, and anhydrous β-CD (between 100 and 300 mg) was added to the hot melt. After that, a magnetic stirrer was used to continuously heat and agitate the reaction mixture for five hours. To guarantee that the crosslinking reaction between the substrate mixture (β-CD and DPC) was completed and NS was formed, the reaction was allowed to react for five hours. The mixture was then allowed to cool before being filtered **(Iriventi *et al.,* 2020).**

2.6.1 Loading of extract in Nanosponges

To prevent aggregation formation, precisely weighed volumes of produced NS (100 mg) were suspended in water (20 mL) and sonicated for a few minutes. The extract mixture in excess of 200 mg was distributed throughout this aqueous suspension. For a duration of one to four hours, the resulting suspension was continuously stirred to facilitate the complexation of CD NS and the integrated extract. Following the complexation reaction, the uncomplexed extract was extracted from the nano-suspension using a 10-minute centrifugation process. After the colloidal supernatant was collected, it was lyophilized to extract the NS, which were then kept at room temperature in a covered vacuum desiccator until further research was conducted **(Iriventi *et al.,* 2020).**

2.6.2 Composition of nanosponges formulation as per Design of expert (DOE) approach

Table 1: Composition of nanosponges formulation

S. No	Polymer (B-Cyclodextrin) mg	Cross linker (DPC) mg	Stirring time (hrs)	Extract (mg)	Temperature (°C)
1	50	175	1	200	90

2	175	50	1	200	90
3	300	175	1	200	90
4	175	300	3	200	90
5	175	300	1	200	90
6	300	300	2	200	90
7	50	300	2	200	90
8	50	50	2	200	90
9	300	175	3	200	90
10	50	175	3	200	90
11	300	50	2	200	90
12	175	50	3	200	90

2.7 Design of experiment

Design of experiment for the formulation of nanosponges was performed by Design Expert (Version 12.0.1.0) software. The quadratic response surfaces were represented by the second-order polynomial model.

2.7.1 Independent and Dependent variables

Table 2: Independent and Dependent variables

Independent variables	Dependent variables
(X1) Polymer (B-Cyclodextrin) (mg)	(Y1) Particle size (nm)
(X2) Cross-linker (DPC) (mg)	(Y2) Zeta potential (mV)
(X3) Stirring time (min.)	

2.7.2 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	50.00	300.00	-1 ↔ 50.00	+1 ↔300.00	175.00	106.60
B	Cross linker	mg	Numeric	50.00	300.00	-1 ↔ 50.00	+1 ↔300.00	175.00	106.60

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C	Stirring time	hrs	Numeric	1.0000	3.00	-1 ↔ 1.00	+1 ↔ 3.00	2.00	0.8528

2.8 Evaluation parameters of Nano sponge

2.8.1 Zeta potential and Particle size: In the present work, the Nanosponges was diluted 10 times with distilled water and analyzed by Zeta sizer Malvern instruments. All samples were sonicated for 5-10 minutes before zeta potential measurements **(Kumar *et al.*, 2018, Penjuri *et al.*, 2016).**

2.8.2 Scanning Electron Microscopic (SEM)

The morphological characteristics of the optimum extract loading were obtained using the electron beam from a scanning electron microscope. Using a vacuum-stopped sputter coater, nanosponges were coated with a thin layer (2–20 nm) of gold, palladium, or platinum. After the specimen had been pre-treated, it was exposed to an electron beam, which caused secondary electrons known as auger electrons to develop. The specimen's atoms interacted with the electron beam, and only the electrons dispersed at a 90° angle were chosen and processed further to obtain surface topography images using Rutherford and Kramer's Law **(Anwer *et al.,* 2019).**

2.9 Formulation of Nano sponges loaded Gel

Carbopol-934 was first mixed evenly at 600 rpm using a magnetic stirrer after being submerged in 50 mL of warm water (A) for two hours. To generate a stiff gel, 50 milliliters (B) of warm water, carboxymethyl cellulose, and methyl paraben were added and continuously mixed in a separate container. Stirring continuously was used to combine mixes A and B. Subsequently, a dropwise addition of tri-ethanol amine was made to neutralize the pH, and the dispersion was then combined with nano sponges of an optimum formulation to create gel. Propylene glycol, a permeability enhancer, was now added. Up till a lump-free, smooth gel was generated, the final dispersion was shaken **(Silpa *et al.*, 2021).**

2.9.1 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.	Nano sponges	1.0 gm

6.	Tri-ethanolamine	q.s
7.	Water	100 ml

2.10 Characterization of Nanosponges loaded Gel

- **Physical appearance:** The prepared Gel formulation was evaluated for appearance, Color, Odor, and homogeneity by visual observation **(Kumar and Eswaraiah 2020).**
- **PH:** pH of the formulation was determined by using Digital pH meter (EI) **(McGlynn, W. 2003).**
- **Viscosity:** The viscosity of the gel formulations was determined using Brookfield viscometer with spindle no.61 at 100 rpm at the temperature of 25^0C **(Monica and Gautami 2014)**
- **Spreadability:** An ideal topical gel should possess a sufficient spreading coefficient when applied or rubbed on the skin surface. Spreadability was determined by the following formula **(S= M*L/T) (Sandeep, D. S. 2020).**

2. 11 Anti-microbial Activity

2.11.1 Preparation of Nutrient Agar Media: 28 g of Nutrient Media was dissolved in 1 litre of distilled water. PH of media was checked before sterilization. Media was sterilized in autoclave at 121^{oC} at 15 lbs pressure for 15 minutes. Nutrient media was poured into plates and placed in the laminar air flow until the agar was get solidified.

2.11.2 Well Diffusion Assay: To summarize, the experiment successfully proved the antibacterial activity of multiple test solutions against E. coli. The creation of clean zones around the wells following incubation indicated that the chemicals tested—Nano sponges and Nano sponge-loaded gel (at concentrations of 0.5 mg/ml and 1 mg/ml)—had an inhibitory effect on bacterial growth. The zones of inhibition (ZOI) in millimeters allowed for a quantitative assessment of each test solution's antibacterial activity. This study reveals that nano sponges and nano sponge-loaded gels could be promising options for antibacterial therapies, with efficacy varied depending on dose and formulation. Further research could look into improving these chemicals and testing their efficiency against a wider spectrum of microbial strains **(Manandhar et al., 2019).**

2.12 Stability studies

The formulations were checked for evaluation parameter viscosity and pH studies at the interval of 30, 45, 60, 90 days (3 month) months. The formulation was tested for stability under accelerated storage condition for 3 months in accordance to International Conference on Harmonization (ICH) guidelines. (**Sharma *et al*., 2014**).

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3. RESULT AND DISCUSSION

3.1 Percentage Yield

Table 5: Percentage Yield of crude extracts of *Cassia alata* extract

S. No	Plant name	Solvent	Theoretical weight	Yield(gm)	% yield
1	*Cassia alata*	Pet ether	285	1.50	0.52%
2		Methanol	294	6.76	2.29%

3.2 Preliminary Phytochemical study

Table 6: Phytochemical testing of extract

S. No.	Experiment	Presence or absence of phytochemical test	
		Pet. Ether extract	Methanolic extract
1.	Alkaloids		
1.1	Dragendroff's test	Absent	**Present**
1.2	Mayer's reagent test	Absent	**Present**
1.3	Wagner's reagent test	Absent	**Present**
1.3	Hager's reagent test	Absent	**Present**
2.	Glycoside		
2.1	Borntrager test	Absent	**Present**
2.2	Legal's test	Absent	**Present**
2.3	Killer-Killiani test	Absent	**Present**
3.	Carbohydrates		
3.1	Molish's test	Present	**Absent**
3.2	Fehling's test	Present	**Absent**
3.3	Benedict's test	Present	**Absent**
3.4	Barfoed's test	Present	**Absent**
4.	Proteins and Amino Acids		
4.1	Biuret test	Absent	**Absent**
5.	Flavonoids		
5.1	Alkaline reagent test	Absent	**Present**
5.2	Lead Acetate test	Absent	**Present**

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6.	Tannin and Phenolic Compounds		
6.1	Ferric Chloride test	Absent	**Present**
7.	Saponin		
7.1	Foam test	Present	**Present**
8.	Test for Triterpenoids and Steroids		
8.1	Salkowski's test	Present	**Absent**
8.2	**Libbermann-Burchard's test**	**Present**	**Absent**

3.3 Quantitative Analysis

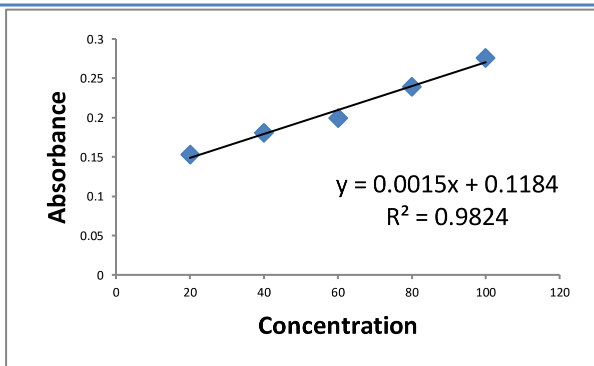
3.3.1 Total Phenolic content (TPC) and Total Flavonoids content (TFC) estimation

Table 7 Standard table for Gallic acid and Rutin

S. No.	Concentration (μg/ml)	Absorbance(Gallic acid)	Absorbance (Rutin)
1.	20	0.153	0.182
2.	40	0.181	0.203
3.	60	0.199	0.285
4.	80	0.239	0.322
5.	100	0.276	0.335

Table 8 Total Phenolic Content of extract *Cassia alata*

Extracts	Methanol
Total Phenolic content (mg/gm equivalent of Gallic acid)	56.33 mg/gm
Total Flavonoid content (mg/gm equivalent of rutin)	17.83 mg/gm



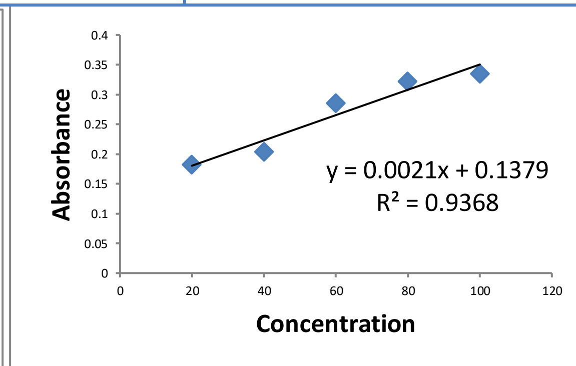


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Figure 1: Represent standard curve of Gallic acid

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

3.4.1 Independent variables and Dependent variables

Table 9: Independent variables and Dependent variables

S. No.	Independent variables		Dependent variables	
	Coding	**Variables**	**Coding**	**Variables**
1.	X1	A: Polymer (B-Cyclodextrin) (mg)	Y1	Particle size (nm)
2.	X2	B:Cross-linker (DPC) (mg)	Y2	Zeta potential (mV)
3.	X3	C:Stirring time (min.)		

3.4.3 Formulation trials as per Box–Behnken design

Table 10: Formulation trials

S. No	Polymer (B-Cyclodextrin) mg	Cross linker (DPC) mg	Stirring time (hrs)	Particle size (nm)	Zeta potential (mV)
1	50	175	1	863.2	72.3
2	175	50	1	256.8	74.6
3	300	175	1	321.5	69.1
4	175	300	3	197.6	94.6
5	175	300	1	489.1	88.2
6	300	300	2	538.9	96.8
7	50	300	2	976.8	97.1
8	50	50	2	812.5	90.7
9	300	175	3	218	78.5
10	50	175	3	551.2	89.3
11	300	50	2	847.2	68.3
12	175	50	3	772.6	65.8

3.4.4 Fit Summary

Table 11: Response 1: Particle size

Source	Sequential p-value	Adjusted R^2	Predicted R^2	
Linear	**0.0110**	**0.6333**	**0.3999**	**Suggested**

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2FI	0.7208	0.5407	-0.2025	
Quadratic	0.5474	0.4877	-1.2354	Aliased

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

3.5.1 Response 1: Particle size

Table 12: Response 1: Particle size (ANOVA for Linear model)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	5.275E+05	3	1.758E+05	7.33	0.0110	significant
A-Polymer	4.842E+05	1	4.842E+05	20.19	0.0020	
B-Cross linker	21352.11	1	21352.11	0.8905	0.3730	
C-Stirring time	21924.18	1	21924.18	0.9143	0.3670	
Residual	1.918E+05	8	23978.39			
Cor Total	7.193E+05	11				

3.5.2 Effect of formulation variables on Zeta potential

Table 13: Response 2: Zeta potential (Fit Summary)

Source	Sequential p-value	Adjusted R^2	Predicted R^2	
Linear	**0.0361**	**0.5004**	**0.1825**	**Suggested**
2FI	0.5120	0.4763	-0.3712	
Quadratic	0.1857	0.7159	-0.2399	Aliased

3.6 ANOVA for Linear model

3.6.1 Response 2: Zeta potential (ANOVA Linear model)

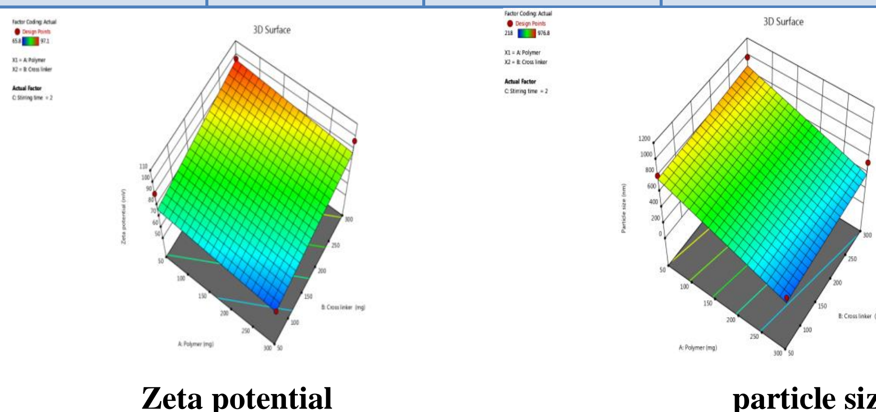
Table 14: Response 2: Zeta potential (ANOVA Linear model)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	987.27	3	329.09	4.67	0.0361	significant
A-Polymer	168.36	1	168.36	2.39	0.1607	
B-Cross linker	746.91	1	746.91	10.60	0.0116	
C-Stirring time	72.00	1	72.00	1.02	0.3416	
Residual	563.46	8	70.43			
Cor Total	1550.73	11				

3.6.2 Predicted and actual value of zeta potential and Particle size

Table 15: Predicted and actual value of zeta potential and Particle size

Formulation code	zeta potential		Particle size	
	Actual Value of zeta potential	Predicted Value of zeta potential	Actual Value of Particle size	Predicted Value of Particle size
NS 1	72.30	83.70	853.20	771.61
NS 2	74.60	69.45	256.80	473.94
NS 3	69.10	74.52	321.50	279.59
NS 4	94.60	94.77	597.60	681.96
NS 5	88.20	88.77	489.10	577.26
NS 6	96.80	87.18	538.90	383.60
NS 7	97.10	96.36	976.80	875.63
NS 8	90.70	77.03	812.50	772.30
NS 9	78.50	80.52	218.00	384.29
NS 10	89.30	89.70	751.20	876.31
NS 11	68.30	67.86	347.20	280.28
NS 12	65.80	75.45	772.60	578.64



Zeta potential **particle size**

Figure 2: Response surface plot showing combined effect of polymer and cross linker of nanosponges formulation

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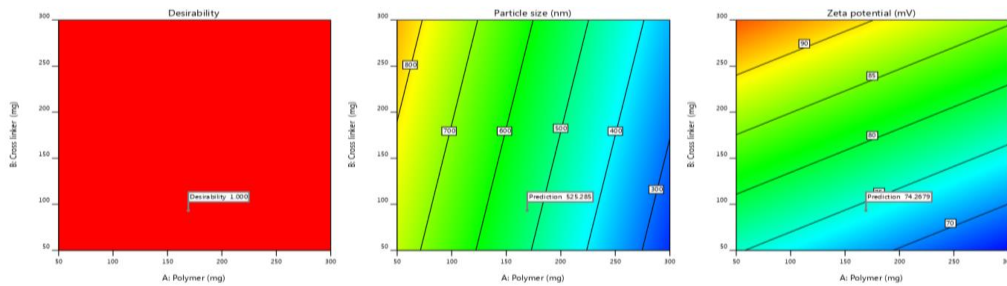

Figure 3: Response surface plot showing prediction data for optimization

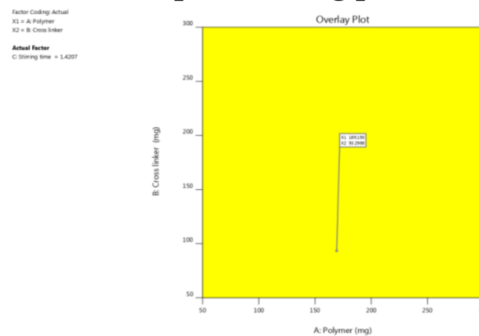

Figure 4: Overlay plot of optimization formulation

3.7 Optimized formula of nanosponges formulation

Table 16: Optimized formula of nanosponges formulation

S. No.	Polymer	Cross linker	Stirring time	Particle size	Zeta potential	Desirability	
1	**169.193**	**93.297**	**1.421**	**525.285**	**74.268**	**1.000**	**Selected**
2	50.000	175.000	1.000	771.612	83.696	1.000	
3	50.000	50.000	2.000	772.300	77.033	1.000	

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3.8 Characterization of optimized formulation

3.8.1 Particle Size

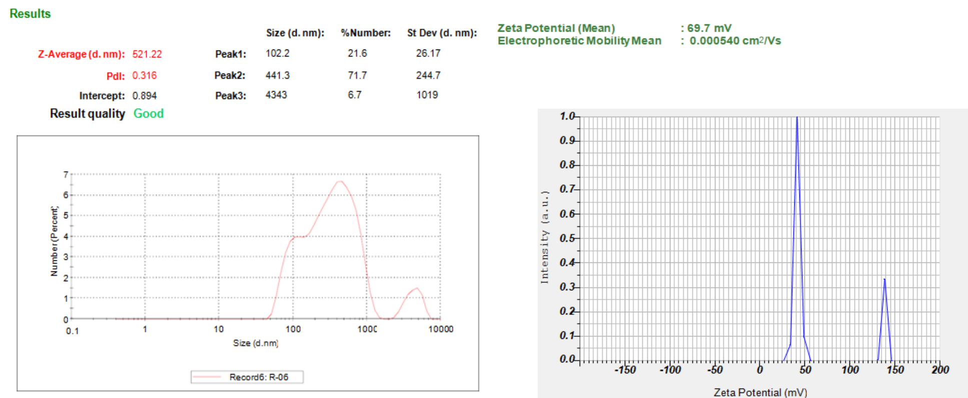

Figure 5: Particle size and Zeta potential

3.8.2 Particle size and Zeta potential

Table 17: Particle size

S. No	Formulation	Particle size (Predicted value)	Particle size (Actual value)
1.	**Particle size**	525.2 nm	521.2nm
2.	**Zeta potential**	74.2 mV	69.7 mV

3.8.4 Scanning electron microscope (SEM)

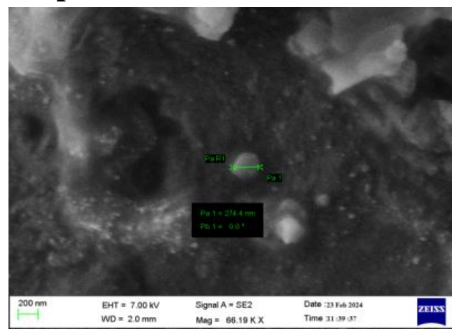

Figure 6: Scanning electron microscope (SEM)

3.9 Characterization of Nanosponges loaded gel and Viscosity PH and Spreadability of Gel

3.9.1 Physical appearance

Table 18: Physical appearance and Viscosity, pH, Spreadability

S. No	Parameter	Result
1.	Colour	Red to brown

S. No	Formulation	Viscosity	pH	Spreadability
1.	Gel	6142±0.39+	6.5	13.48

2.	Odour	Odourless
3.	Appearance	Semisolid
4.	Homogeneity	Homogeneous

3.10 Results of antimicrobial activity of nanosponges gel

3.10.1 Antimicrobial activity of Formulation against *E.coli*

S. No.	Sample Name	Zone of Inhibition (mm)
1.	Extract	7 mm
2.	Nanosponges gel (0.5mg/m	13 mm
3.	Nanosponges gel (1mg/ml)	18mm

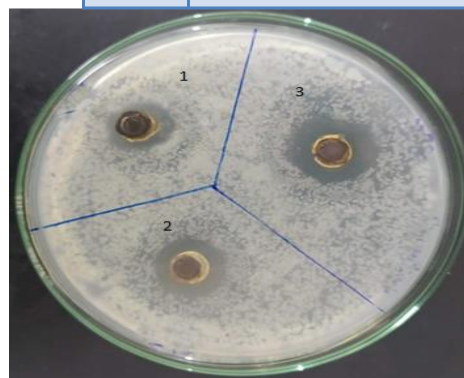

Figure 7: Antimicrobial activity

3.11 Stability study

Table 19: Stability Study of optimized formulation (Nanosponges gel)

S. No	Time (Days)	$25^0C\pm2\ ^0C$ and 60 ± 5% RH		$40^0C\pm2\ ^0C$ and 70 ±5% RH	
		Viscosity	pH	Viscosity	pH
1.	0	6142	6.5	6153	6.5
2.	30	6151	6.3	6144	6.5
3.	45	6158	6.3	6148	6.2
3.	60	6165	6.4	6130	6.2

4.	90	6170	6.3	6131	6.3

4. CONCLUSION

This work demonstrates the ability of Cassia alata-loaded nanosponges to increase the solubility and bioavailability of active chemicals, which is crucial for medicinal efficacy. The nanosponge formulation improves medication stability and bioavailability while simultaneously providing a controlled release mechanism that reduces drug toxicity, improves targeting, and minimizes adverse effects. These findings imply that *Cassia alata* nanosponges gel could be utilized to treat a variety of skin illnesses by delivering drugs directly to skin cells over time, reducing dosage requirements and administration frequency. The creation of such nanotechnology-based formulations marks a big step forward in addressing the obstacles associated with traditional medication delivery technologies.

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