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Original Article

# Sustainable Fabrication of Phycocyanin Encapsulated Nanoparticles: A Response Surface Optimization Impact

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## ABSTRACT

**Background:** Phycocyanin (Pc), a phycobiliprotein pigment, has therapeutic potential for treating inflammation, oxidative stress, neuroprotection, liver protection and cancer. Despite its therapeutic potential, its effectiveness is constrained by low bioavailability and low stability. **Objectives:** This study aimed to design and develop Phytocyanin-Loaded Nanoparticles (PcNPs) to enhance PC's stability and efficacy. **Materials and Methods:** PCNPs were prepared using a green ionotropic gelation method and optimized via a $3^2$ full factorial designs. The prepared trial batches of the nanoparticles were characterized particle size, polydispersity index, drug entrapment efficiency and drug content. The optimized PcNPs were evaluated by compatibility studies by using Transmission Electron Microscopy (TEM), Zeta potential, Infrared Spectrometry (IR), Differential Scanning Calorimetry (DSC), X-ray crystallography studies (X-RD), *in vitro* drug release study, *in vitro* antioxidant activity and stability studies. **Results:** PcNPs exhibited an amorphous character, as evidenced by the Differential Scanning Calorimetry (DSC) analysis. FTIR showed no interaction between Pc and polymer employed. The dissolution studies revealed enhance Pc release from PcNPs compared to the plain Pc following zero-order kinetics and Fickian diffusion. An *in vitro* antioxidant activity study revealed that PcNPs were superior to plain Pc and comparable to the standard drug ascorbic acid. Stability studies revealed that they remained sufficiently stable for period of 30 days at 4ºC and -20ºC, with no physical or chemical alterations in the formulation. **Conclusion:** The developed PcNPs showed an improved dissolution profile and promising antioxidant activity, making them a potential tool with enhanced stability and efficacy.

**Keywords:** Antioxidant activity, Ionotropic gelation method, Nanoparticles, Phycocyanin, Stability study.

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## INTRODUCTION

Many different nutrients, including vitamins, minerals, protein and γ-polyunsaturated fatty acids are found in Spirulina. Antibacterial, antifungal, antiparasitic and antiviral, properties are also present in Spirulina species.[1] By encouraging the development of lactobacillus, bifidobacterium and other favorable bacteria in the intestines, spirulina preparation can enhance the environment within. Spirulina has been the focus of various studies recently, revealing that it contains phenolic compounds, which typically have strong antioxidant properties. Phycobiliprotein pigment Phycocyanin (Pc), was isolated from Spirulina and to have strong antioxidant properties.[2,3] Pc is often taken as a nutritional supplement because it includes all of the essential amino acids. Human nourishment and health could greatly benefit from phycocyanin use. It is also utilized in immunoassays as a biochemical tracer due to its fluorescent qualities, which provide precise understanding of the different phases of the animal immunoassay procedure. It is a naturally occurring pigment that dissolves in water and is widely used as a food colouring, cosmetic preservative and medical indicative.[4-6] Additionally, due to its strong antioxidant, liver-protective, hepatoprotective, neuroprotective, anti-inflammation, anticancer and free radical-scavenging qualities can be used to treat diseases that are caused by oxidative stress. Nevertheless, phycocyanin's sensitivity to processing and storage conditions causes fluctuations, discoloration and precipitation, which restricts its use in a variety of fields. When processing phycocyanin as a natural pigment, it is crucial to regulate pH, light and temperature in order to enhance phycocyanin stability. Currently, stabilizers can be added to phycocyanin to increase its stability.[6,7]

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Phycocyanin Loaded Nanoparticles (PcNPs) were synthesized using a green ionotropic gelation method, which involved sodium alginate and calcium chloride with honey as the surfactant and stabilizer. Its solubility and compatibility limitations were studied carefully and water was finalized as the solvent for the formulation preparation. The principles of green chemistry can be effectively applied to production of Phycocyanin loaded nanoparticles (PcNPs) by using safer and greener manner that avoid organic solvents.[8]

Nanoparticles can be made to enhance the medications' therapeutic and pharmacological properties as well. Compared to other carrier systems, nanoparticles have greater advantages. One of the main benefits of using nanoparticles as a delivery system is their submicron size, which allows for extravasations and blockage of terminal blood vessels. Furthermore, a high therapeutic agent density can frequently be dissolved, dispersed, or encapsulated in these nanoparticles; the latter three states rely on the preparation method to produce distinct qualities and release characteristics of the entrapped material.[9] Polymeric nanoparticles, on the other hand, have the beneficial controlled release characteristics and provide some unique benefits of boosting the stability of medications and proteins. Additional characteristics of nanoparticles include their high physical stability, low number of excipients in their formulations, ease of preparation and potential for sustained drug release that may be useful in the management of long-term illnesses. By adjusting the particle's morphology and polymer composition, one can effectively tailor various controlled release characteristics, enabling sustained, moderate dosing over extended periods.[8,9]

The outcomes demonstrated that the generated nanoparticles might increase phycocyanin's stability. This research offers insights into potential applications within the food sector and beyond. Thus, the current research aimed to assess the potential of the Phycocyanin Loaded Nanoparticles (PcNPs) synthesized using a green ionotropic gelation method with less experiments and time, using factorial design for antioxidant activity.

## MATERIALS AND METHODS

### Materials

Drug Phycocyanin extract was extracted from *Spirulina platensis* by ultrasonic method, sodium alginate and calcium chloride were sourced from Analab Fine Chemicals, Mumbai, India. Honey was purchased from Dabur India Limited. For HPLC-grade water was utilized for the analysis, which was prepared in-house laboratory. Phycocyanin biomarker (purity 20%) was gifted from Sun Pure Extracts. All other chemicals and reagents used were of analytical grade.

### Characterization of drug

#### *Ultra violet-visible spectrophotometric method of analysis*

For the analytical study, an UV spectrophotometer (Jasco International Co. Ltd., Japan) with software was used. Absorption of 100 µg/mL solution of Phycocyanin was recorded on UV spectrophotometer. The standard solutions of Phycocyanin in water and phosphate buffer 7.4 solvent were scanned between 200-800 nm in UV spectrophotometer. The maximum absorbance was observed at 615 nm in distilled water and phosphate buffer 7.4. Thus, the working a max was chosen as 615 nm. This wavelength was used for further study. For the standard curve, different concentrations of standard Phycocyanin (100-600 µg/mL) were prepared using water and phosphate buffer 7.4. The peak of the standard Phycocyanin was compared with the peak of the Phycocyanin extract drug sample. The UV standard curve plotted in water and phosphate buffer 7.4 was used to determine entrapment efficiency, drug content and drug release.

### Preparation of Phycocyanin-Loaded Nanoparticles (PcNPs)

PcNPs were produced using the green ionotropic gelation method. Sodium alginate was suspended in deionized water (1% w/v) with honey added as a surfactant and stabilizer. Phycocyanin was then mixed with into this solution while stirring continuously. Aqueous calcium chloride (1% v/v) solution was placed drop by drop and the mixture was stirred, homogenized and sonicated (Labman scientific instruments). Various formulations of PcNPs were developed and optimized using two-factor, three levels $3^2$ Factorial design using Design Expert® software (Version 13, State-Ease Inc., Minneapolis, MN, USA). Sodium alginate (X1), calcium chloride (X2) was selected as independent variables, with their low (-1), medium (0) and high levels (+1) used to create 9 different formulations. The Entrapment Efficiency (EE) (Y1) and Particle Size (PS) (Y2) were the dependent variables as experimental design matrix shown in the Table 1. The coded levels of all 9 batches of Phycocyanin loaded nanoparticles (PcNPs) are outlined in Table 2.[10-12]

**Table 1: Experimental design matrix.**

| Independent (Input) Variables | Dependent (Responses) Variables |
|---|---|
| $X_1$ (Sodium alginate mg/mL). | $Y_1$-Entrapment efficiency (%). |
| $X_2$ (Calcium chloride mg/mL). | $Y_2$-Particle size (nm). |

## Evaluation of Phycocyanin extract loaded nanoparticles

### *Particle Size (PS) and Polydispersity Index (PDI)*

The mean PS and PDI of all the 9 PcNPs formulations were evaluated using a Nanophox (Sympatec GmbH, Germany) at a fixed dispersion angle of 90° at 25°C. The PDI, which indicates the thickness of particle size distribution, was measured at this angle which was obtained at an angle of 90°. The measurement of PS and PDI was conducted in triplicate for all formulations and were recorded.[13,14]

## Entrapment Efficiency (EE)

The entrapment efficiency of all the 9 PcNPs formulations was determined using an indirect centrifugation method (Remi C-24 plus). A 10 mL aliquot of the nanoparticle dispersion was centrifuged at 7,000 rpm for 40 min. After centrifugation, the supernatant was carefully collected and diluted with an appropriate solvent. The concentration of the free drug in the supernatant was then measured using UV-visible spectroscopy at a wavelength of 615 nm.[13,14]

Drug entrapment efficiency and are calculated by using the following equations:

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

## Drug content

Drug content of nanoparticle complex was assessed by suspending correctly weighed 1 mL of complex in 1 mL of 0.1% Tritron-X-100 and diluted to 10 mL with distilled water. After suitable dilution, the absorbance was measured using a UV Spectrophotometer (Jasco International Co. Ltd, Japan) at 615 nm to estimate drug content.

$$\text{Drug content} = \frac{(\text{Actual amount of drug})}{(\text{Theoretical amount of drug})} \times 100$$

## $3^2$ full factorial designs

$3^2$ full factorial designs were used in the development of PcNPs. This design involved 2 different factors sodium alginate (polymer) and calcium chloride (cross linker), which were each examined at 3 varying levels, labeled as -1, 0 and +1, respectively. Experimental trials were conducted for each of all possible 9 combinations of these factors. The dependent variables measured included entrapment efficiency, particle size and drug content, of the formulated nanoparticles. After applying the $3^2$ factorial designs, 9 batches were able to be successfully prepared with different proportions of excipients used. An optimized formulation was chosen by evaluating the highest entrapment efficiency, smallest particle size and high drug content. The experimental design was managed using Design Expert 13 (StatEase Minneapolis, MN, USA) for formulating the experiments. A total of 9 formulations were created as described in Table . The relationship between dependent and independent variables were analyzed and a significant model was achieved.[15,16]

## Characterization of an optimized batch of Phycocyanin-Loaded Nanoparticles (PcNPs)

The characterization of an optimized batch B7 of PcNPs was further studied for parameters like Transmission Electron Microscopy (TEM), zeta potential, Fourier-Transform Infrared Spectroscopy (FTIR) analysis, Differential Scanning Calorimetry (DSC), X-ray crystallography studies (X-RD), *In vitro* drug release studies and kinetic modeling of release profiles.

**Table 2: $3^2$ full factorial experimental design and the results of Particle size, Entrapment efficiency and Drug content of $B_1$-$B_9$ batches.**

| Batch | Coded values | | Actual values | | Responses | | | |
|---|---|---|---|---|---|---|---|---|
| | Sodium alginate conc. $X_1$ (mg) | Calcium chloride conc. $X_2$ (mg) | Sodium alginate conc. $X_1$ (mg) | Calcium chloride conc. $X_2$ (mg) | Entrapment Efficiency (%) | Particle Size (nm) | PDI | Drug content (mg) |
| $B_1$ | -1 | -1 | 250 | 250 | 62.32s±0.84 | 127.54±1.98 | 0.08 | 41.32 |
| $B_2$ | -1 | 0 | 250 | 500 | 59.86±0.56 | 211.95±2.35 | 0.08 | 38.71 |
| $B_3$ | -1 | +1 | 250 | 750 | 57.87±0.37 | 244.71±1.74 | 0.09 | 36.96 |
| $B_4$ | 0 | -1 | 500 | 250 | 65.09±0.97 | 102.49±1.26 | 0.09 | 44.17 |
| $B_5$ | 0 | 0 | 500 | 500 | 62.41±0.87 | 118.45±1.77 | 0.09 | 41.42 |
| $B_6$ | 0 | +1 | 500 | 750 | 61.50±0.86 | 170.22±1.05 | 0.09 | 40.35 |
| $B_7$ | +1 | -1 | 750 | 250 | 71.49±1.28 | 95.41±0.78 | 0.09 | 53.25 |
| $B_8$ | +1 | 0 | 750 | 500 | 65.68±0.66 | 95.53±2.15 | 0.08 | 45.25 |
| $B_9$ | +1 | +1 | 750 | 750 | 64.04±1.11 | 105.16±1.57 | 0.09 | 43.28 |

**Table 3: ANOVA study results Particle size (PS) ($Y_1$).**

| Source | Sum of squares | Df | Mean Square | F-value | *p*-value | Comments |
|---|---|---|---|---|---|---|
| Model | 116.86 | 3 | 38.95 | 32.99 | 0.0010 | Significant |
| $X_1$-SA | 74.62 | 1 | 74.62 | 63.20 | 0.0005 | |
| $X_2$-$Cacl_2$ | 39.99 | 1 | 39.99 | 33.87 | 0.0021 | |
| $X_1X_2$ | 2.25 | 1 | 2.25 | 1.91 | 0.2260 | |
| Residual | 5.90 | 5 | 1.18 | | | |
| Cor Total | 122.77 | 8 | | | | |

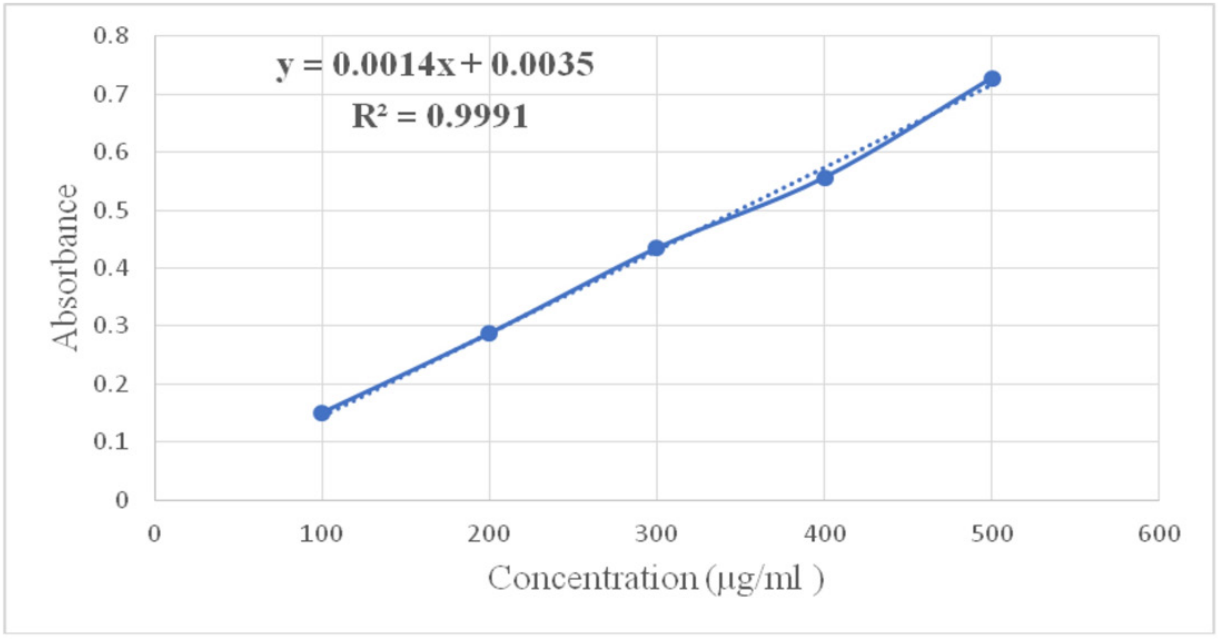


**Figure 1:** Calibration curve of Phycocyanin in distilled water.

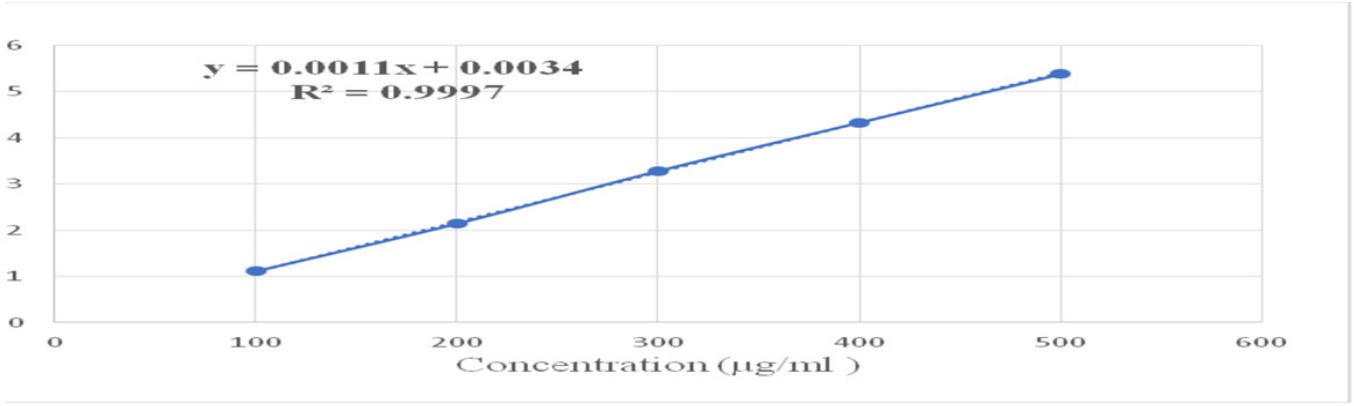


**Figure 2:** Calibration curve of Phycocyanin in phosphate buffer (pH 7.4).

## Transmission Electron Microscopy (TEM)

A Transmission Electron Microscopy (TEM) with a Hitachi H 7500 (Houston, Texas) was utilized to examine the morphology of the optimized batch B7 of PcNPs. the nanoparticle dispersion was applied onto Formvar-coated copper grids from Ted Pella, Inc, (Redding, CA) and permitted to equilibrate. Surplus fluid was extracted using filter paper and the samples were allowed to air-dry at room temperature for approximately 30 min. Images were captured using Digital Micrograph imaging software (Gatan, Inc., v1.82.366) at an acceleration voltage of 80 kV and a magnification of 60,000X.[17]

## Zeta Potential (ZP)

Zeta potential is the most vital parameter that provides insight into the surface charge of the nanoparticle formulations, which in turn indicates their physical stability. The higher the electrostatic repulsion between the particles more is the stability. The optimized batch $B_7$ of PcNPs was assessed using Beckman coulter Delsa TM Nano. Zeta potential was determined after the sample was diluted with water to a volume of 10 mL and transferred in 5 mL to a cuvette.

## Fourier-Transform Infrared spectroscopy (FTIR) Analysis

The interactions among the molecules in the formulation compounds were analysed to generate the FTIR spectra of phycocyanin, sodium alginate, calcium chloride, physical mixture and optimized batch B7 of PcNPs on an FTIR spectrophotometer (Bruker Alpha FTIR). The samples were dried to eliminate any residual moisture and individual spectra were recorded for both physical mixture and optimized batch B7 of PcNPs in the 4000 to 1000 cm -1 wavelength range.[17,18]

## Differential Scanning Calorimetry (DSC)

DSC is a thermo analytical method that calculates, the heat difference needed to raise the temperature of a sample in comparison to a reference, as a function of temperature. A curve showing heat flux versus temperature is the end product of DSC experimentation. In a nitrogen atmosphere, phycocyanin extract and optimized batch B7 of PcNPs were heated in an aluminum crimp cell. The heating process occurred at a rate of 10°C/min, spanning a temperature range from 0°C to 400°C (using a Metlar Toledo DSC 1). The peak transition onset temperatures were recorded using an analyzer.

## X-ray Crystallography studies (X-RD)

The phycocyanin, sodium alginate and optimized batch B7 of PcNPs was exposed to X-ray crystallographic studies (PW 1729, Philips).

## *In vitro* drug release studies and kinetic modelling of release profiles

To study the % cumulative drug release in dissolution medium, *in vitro* dissolution study was performed for the samples i.e., phycocyanin and optimized batch B7 of PcNPs at pH 1.2 and pH 7.4. Dissolution studies were conducted using a USP-II (paddle) dissolution apparatus (Lab India). Initially, 900 mL of 0.1N HCl, simulating gastric fluid at pH 1.2, was used for 2 hr. Following this, the dissolution medium was replaced with phosphate buffer, simulating intestinal fluid at pH 7.4, for the subsequent 12 hr.[19]

The experiment involved continuous stirring at 100 rpm for 12 hr, with the temperature maintained at 37°C. Samples extracted at specific intervals from 0 to 12 hr. At each specified interval, 10 mL aliquots were withdrawn from the release medium and replenished with an equal volume of fresh dissolution medium the samples underwent filtration using Whatman filter paper (45 µm) and were analyzed using a UV spectrophotometer (Jasco International Co. Ltd., Japan) at 615 nm. Each test was conducted three times and the average values were plotted against time. The outcomes were presented as the percentage of the cumulative drug released over time, with data reported as mean±standard deviation.[20]

**Table 4: ANOVA study results Entrapment Efficiency (EE) ($Y_2$).**

| Source | Sum of squares | $D_f$ | Mean Square | F-value | *p*-value | Comments |
|---|---|---|---|---|---|---|
| Model | 23033.14 | 3 | 7677.71 | 31.65 | 0.0011 | Significant |
| $X_1$-SA | 13833.60 | 1 | 13833.60 | 57.03 | 0.0006 | |
| $X_2$-$Cacl_2$ | 6314.77 | 1 | 6314.77 | 26.03 | 0.0038 | |
| $X_1X_2$ | 2884.76 | 1 | 2884.76 | 11.89 | 0.0183 | |
| Residual | 1212.84 | 5 | 242.57 | | | |
| Cor Total | 24245.97 | 8 | | | | |

**Table 5: Zeta Potential of nanoparticles optimize batch B7.**

| Sl. No. | Nanoparticles Batches | Zeta Potential mV |
|---|---|---|
| 1 | $B_7$ | -17.34 |

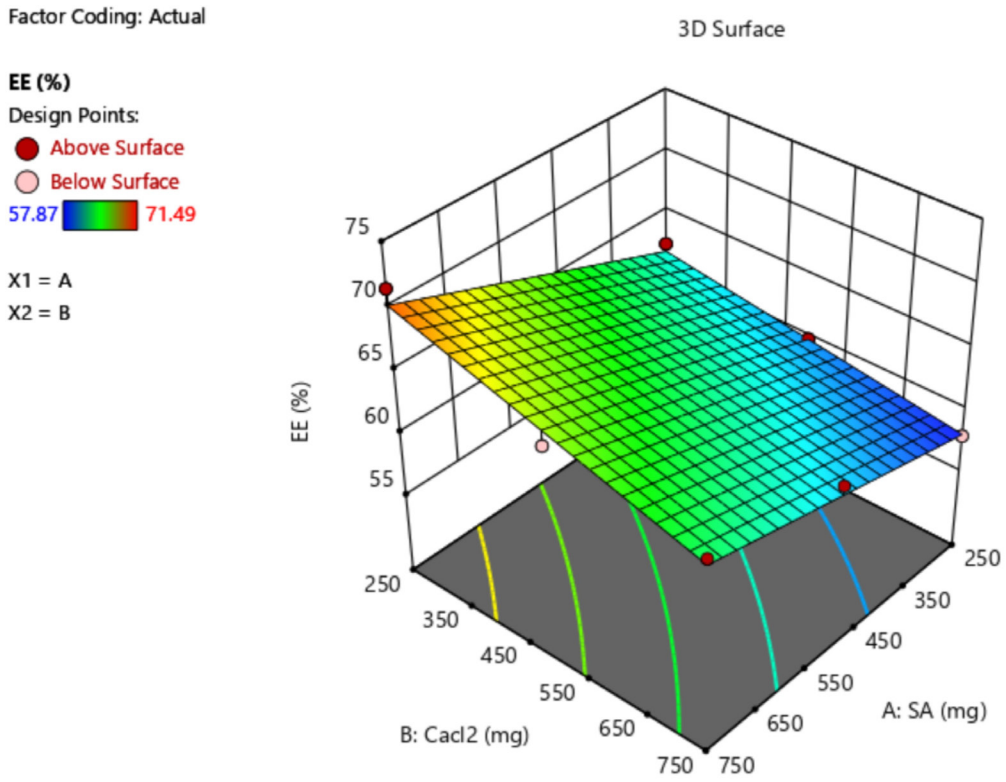


**Figure 3a:** 3D Response surface plot (% EE).

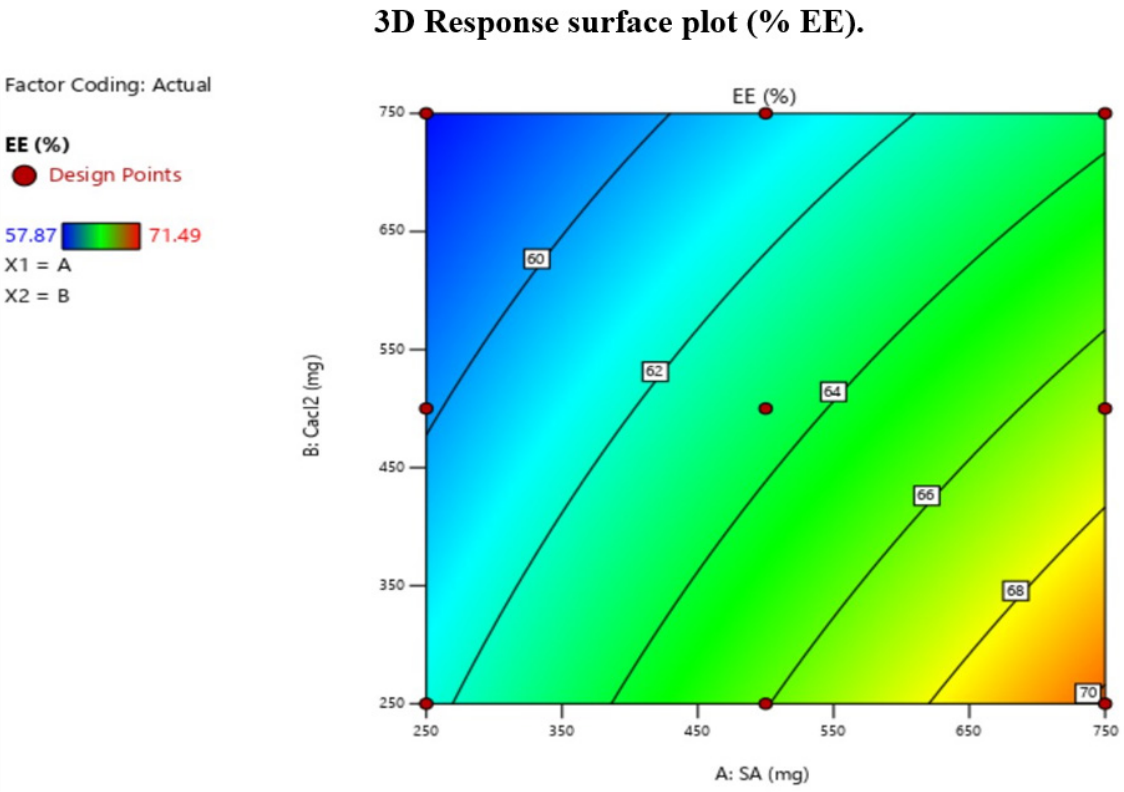


**Figure 3b:** 2-D contour plot for $Y_1$ (% EE).

**Table 6: FT-IR spectrum data of Phycocyanin.**

| Functional Group | Observed Frequency ($cm^{-1}$) | Reference Frequency ($cm^{-1}$) |
|---|---|---|
| OH stretch | 3492 | 3650-3400 |
| N-H group | 3270 | 3373-3422 |
| Carboxyl group | 2931,2907 | 2500-3300 |
| Alkyl C-H stretch | 2931,2907 | 2950-2850 |
| C=N | 1648 | 1650-1550 |
| Alkenyl C=C | 1985 | 2000-1660 |
| $CH_2$ bending | 1448 | Around 1465 |
| C-N | 1353,1239 | 1340-1250 |
| C-O stretch | 1089,1022 | 1100-1040 |
| OOPS bending | 993,844 | 1000-700 |

**Table 7: Antioxidant activity of Ascorbic acid, Phycocyanin and Optimize batch B7.**

| Formulation | Concentration | % Inhibition | $IC_{50}$ (mcg/mL) |
|---|---|---|---|
| Ascorbic acid | 12.5 ppm | 15.59±0.16 | 49.79 |
| | 50 ppm | 29.50±0.01 | |
| | 75 ppm | 42.20±0.05 | |
| | 100 ppm | 56.37±0.10 | |
| | 125 ppm | 71.10±0.11 | |
| Phycocyanin | 12.5 ppm | 8.71±0.11 | 38.60 |
| | 50 ppm | 16.75±0.09 | |
| | 75 ppm | 24.05±0.16 | |
| | 100 ppm | 31.29±0.05 | |
| | 125 ppm | 37.99±0.25 | |
| Batch B7 PcNPs | 12.5 ppm | 12.91±0.80$^{ns}$ | 47.79 |
| | 50 ppm | 21.52±0.68$^{*}$ | |
| | 75 ppm | 27.02±0.52$^{*}$ | |
| | 100 ppm | 35.24±0.51$^{*}$ | |
| | 125 ppm | 42.68±0.05$^{**}$ | |

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. When compared with Phycocyanin and Batch B7 PcNPs at different concentration point (in ppm). Values are represented as Mean±SEM. Data was analyzed using 2-way ANOVA followed by Tukey's multiple comparisons test.

Kinetic modeling of the release data for the optimized batch B7 of PcNPs was carried out by fitting the data to various kinetic equations, including zero-order, first-order, Higuchi and Korsmeyer-Peppas models. The most appropriate model was determined based on the correlation coefficient.[19,20]

## Antioxidant Activity

The evaluation of antioxidant activity was conducted using DPPH (2, 2-diphenyl-1-picrylhydrazyl) reagent and UV spectroscopy. DPPH (2 mg) was dissolved in 50 mL of methanol and stored in darkness at ambient temperature for 30 min. Methanol was used to prepare a 0.1 mM DPPH solution. Various concentrations (25-150 ppm) of the reference standard, Pc and optimized batch B7 of PcNPs were prepared from a 1 mg/mL stock solution. Ascorbic acid served as the reference standard, with its stock solution also prepared at 1 mg/mL. The DPPH solution was combined in equal volumes with the sample and standard solutions separately and then allowed to react in dark conditions. UV spectroscopy was employed to measure the absorbance at 517 nm. A similar procedure was followed for the blank solution. The experiment was repeated three times and the mean values were utilized for analysis.[20]

Data are presented as Mean±Standard Deviation (M±SD) of three parallel samples ($n$=3). Statistical significance was determined using a one-way ANOVA with a significance level of $p<0.05$, analysed using SPSS 25.0 software.

$$\%\ \text{DPPH Scavenging Effect} = \frac{\text{Absorbance of standard} - \text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100$$

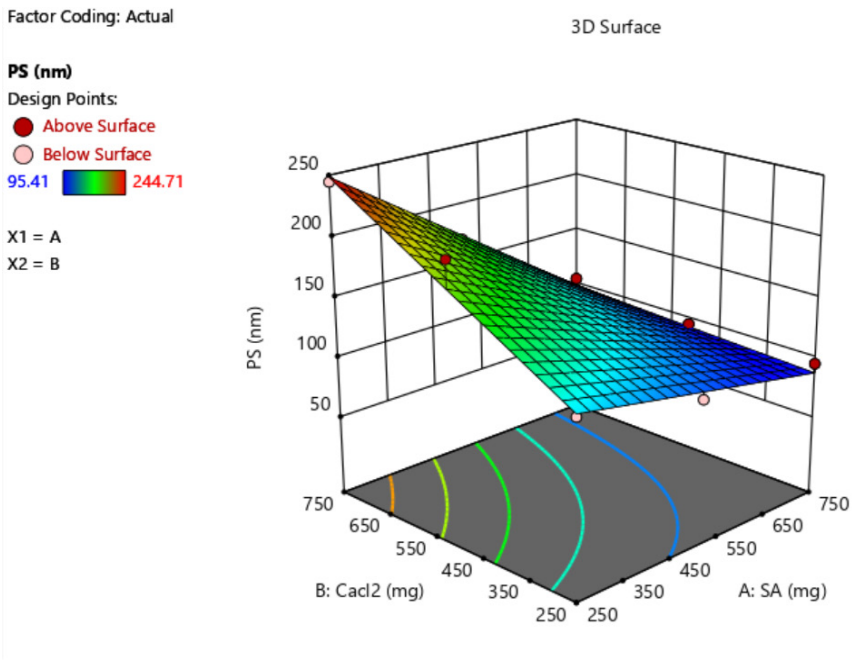


**Figure 4a:** 3D Response surface plot (PS).

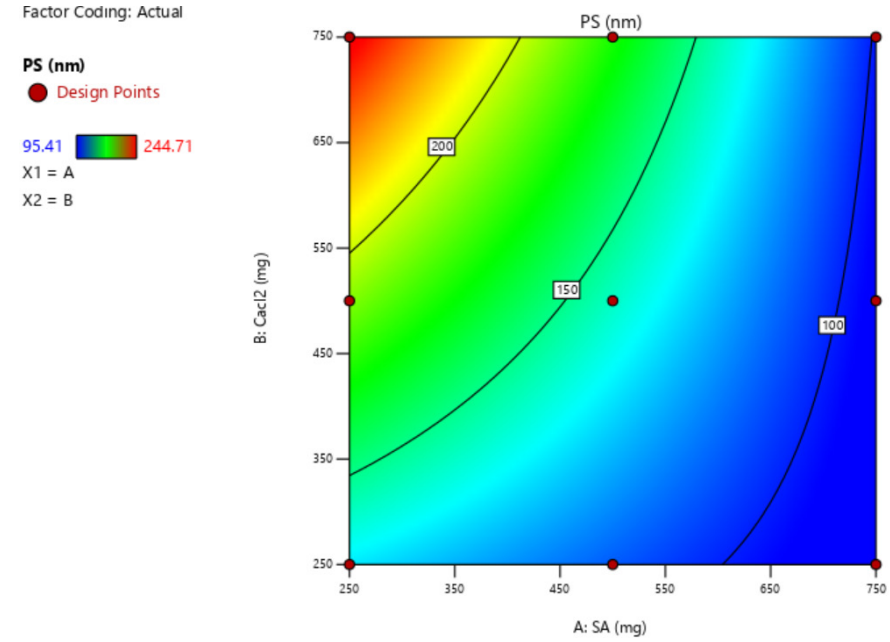


**Figure 4b:** 2-D contour plot for $Y_2$ (PS).

**Table 8: Stability result of particle size analysis and % entrapment efficiency of Phycocyanin loaded nanoparticles.**

| Days | 4°C (Stability condition) | | -20°C (Stability condition) | |
|---|---|---|---|---|
| | Size (nm) | EE (%) | Size (nm) | EE (%) |
| 0 | 95.41 | 71.49 | 95.41 | 71.49 |
| 7 | 95.41 | 71.49 | 95.41 | 71.49 |
| 14 | 95.41 | 71.49 | 95.41 | 71.49 |
| 21 | 95.41 | 71.49 | 95.41 | 71.49 |
| 30 | 95.43 | 71.47 | 95.43 | 71.47 |

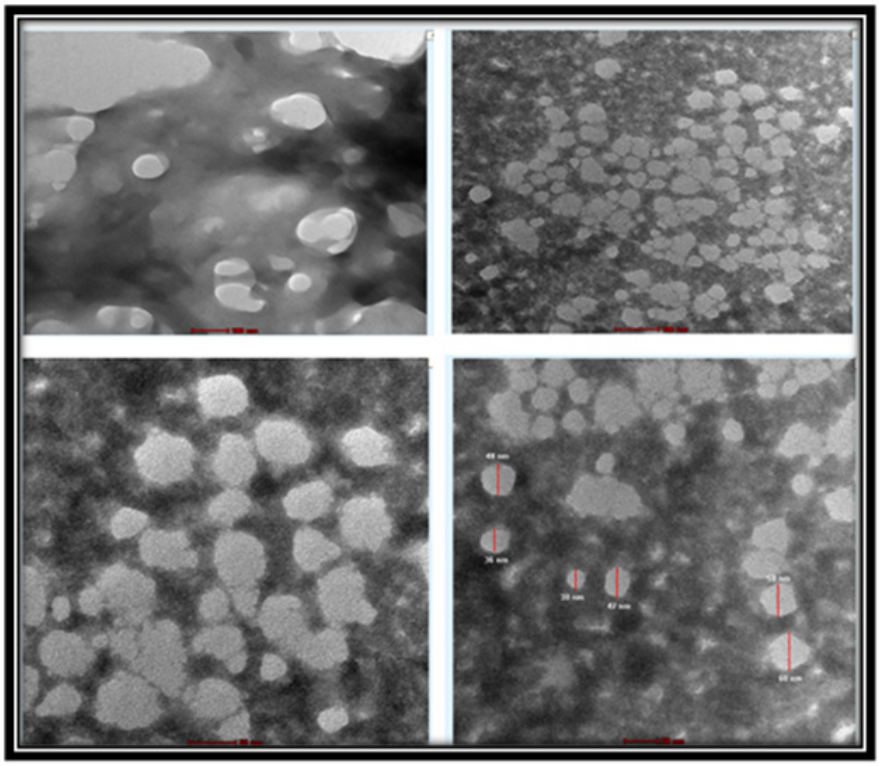

**Figure 5:** TEM of nanoparticles optimize batch B7.

## Stability Study

The optimized batch $B_7$ PcNPs were exposed to stability testing as per ICH guidelines. The optimized batch B7 (PcNPs) formulation was sealed and stored in a Deep freezer (Blue Star, Model-CHFK300DGS) at 4°C±1°C, 60±5% RH and -20°C±2°C, 60%±5% RH and room temperature +25±1°C for a time period of a month. The formulation was removed from time to time and estimated on 7th, 14th, 28th and 30th days. The stability studies of developed formulation were assessed using parameters such as physical appearance and entrapment efficiency.[21,22]

# RESULTS

The present study aimed to formulate nanoparticles using Pc through a green ionotropic gelation method and evaluate their antioxidant activity.

## Ultra violet-visible spectrophotometric method of analysis

### *Calibration curve of Phycocyanin in distilled water*

The calibration curve of Phycocyanin in distilled water and phosphate buffer pH 7.4 is shown in Figures 1 and 2.

## Evaluation of Phycocyanin extract loaded nanoparticles

### *Particle size and polydispersity index*

The mean particle size for $B_1$-$B_9$ batches ranged between 95.41-244.71 nm as seen in the Table 3. The formulated Phycocyanin Loaded Nanoparticles (PcNPs) had a mean PDI value≤0.09. Thus, demonstrating a homogeneous distribution of nanoparticles as depicted in Table 3.

Additionally, all formulations have a rigid size distribution, with Polydispersity Index (PI) ranging from 0.08 and 0.09.

## Entrapment Efficiency

Determining % entrapment efficiency is an important parameter with respect to nanoparticle's drug loading capacity. Entrapment efficiency ranged from 57.87-71.49%. As displayed in Table . As a result, the batch B7 had the highest recorded entrapment efficiency of 71.49±1.28%.

## Drug Content

The drug content of Phycocyanin loaded Nanoparticles (PcNPs) from batches $B_1$-$B_9$, ranged from 36.96-53.25 mg in the respective batches as depicted in Table . In batch $B_7$ and $B_8$ drug content and entrapment efficiency was found to be the highest.

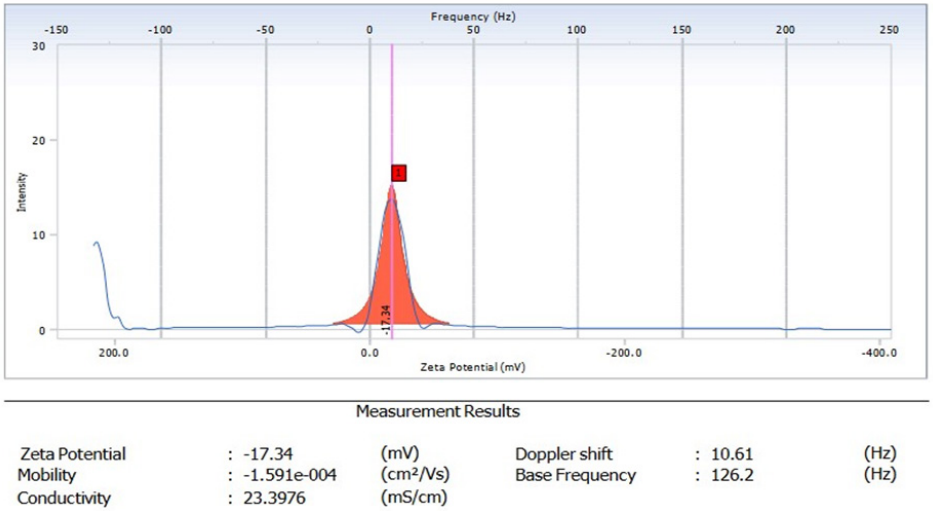


**Figure 6:** Zeta Potential of Phycocyanin loaded nanoparticles of optimize batch B7.

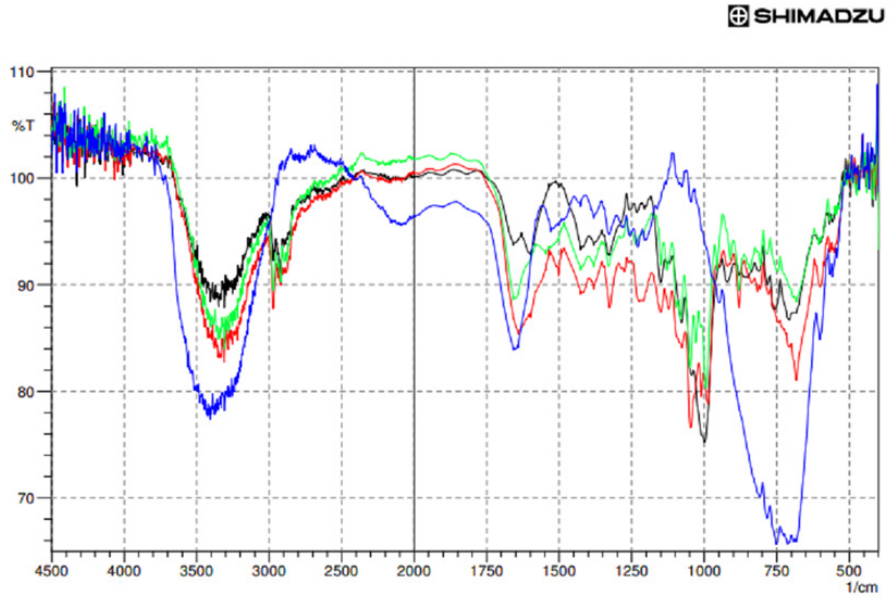


**Figure 7:** Infra-red spectra of (B) Phycocyanin+Sodium alginate+Calcium chloride, (C) Phycocyanin, (D) Phycocyanin loaded nanoparticles of optimize batch B7.

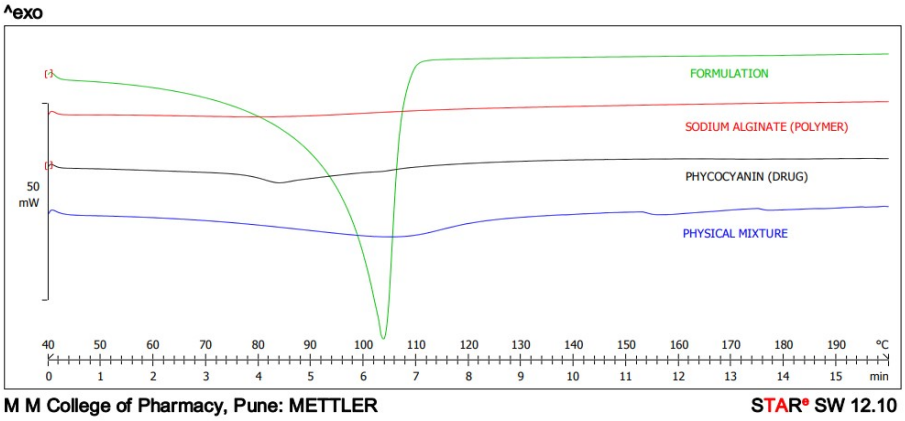


**Figure 8:** DSC overlay curves of (1) Phycocyanin loaded nanoparticles optimize batch B7, (2) Sodium alginate, (3) Phycocyanin and (4) Physical mixture.

## $3^2$ full factorial designs

The outcomes of the Design of Experiment (DOE) are mentioned in Tables 3 and 4 and Figures 3 and 4. After evaluation of all the 9 batches, it was concluded that batch B7 achieved the best outcomes and was deemed the optimized batch with respect to its particle size within the acceptable range according to the literature survey and the EE was also fairly acceptable for antioxidant formulations.

The present study used the Design Expert 13 software for the formulation design. Specifically, a $3^2$ factorial design is employed, which involves 2 independent variables: sodium alginate (polymer) and calcium chloride (cross linker), each with three different levels (-1, 0, +1) that are considered. Nine distinct formulations are developed with different ratios of the polymer and cross linker. The evaluation parameters of the formulations are then recorded in the software DOE and the results are used to determine the model's significance. The responses for particle size and entrapment efficiency are measured for all nine formulations and are presented in Tables 3 and 4, respectively. Additionally, response surfaces are depicted for entrapment efficiency (Figure 3a) and particle size (Figure 4a).

The entrapment efficiency for all 9 batches was observed in the range of 57.87% to 71.49%. Table 4 shows entrapment efficiency of all 9 batches. Maximum entrapment efficiency i.e. 71.49±1.28% was observed for B7 batch. The 3D response surface and contour plot showing the effect of varying proportions of independent variables ($X_1$ and $X_2$) on the response $Y_1$ (% EE) is shown in Figures 3a and 3b.

The particle size for all 9 batches was observed in the range of 95.41 to 211.92 nm. Table  shows particle size of all 9 batches. Maximum particle size i.e. 95.41±0.78 nm was observed for B7 batch. The 3D response surface and contour plots demonstrated the effect of varying proportions of independent variables ($X_1$ and $X_2$) impact the response $Y_2$ (PS) is illustrated in Figures 4a and 4b. The results of Analysis of Variance (ANOVA) are presented in Tables 3 and 4.

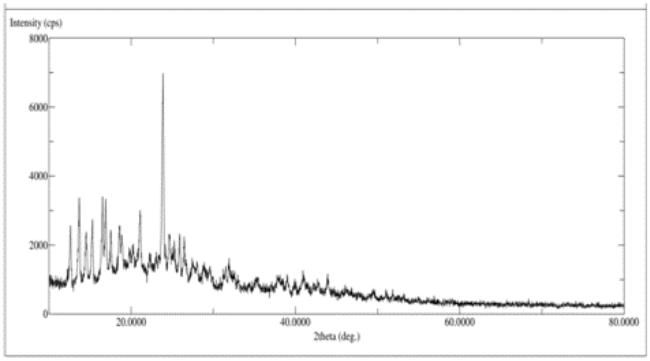


**Figure 9:**XRD graph of Phycocyanin.


**Figure 10:** XRD graph of Phycocyanin loaded nanoparticles of optimize batch B7.

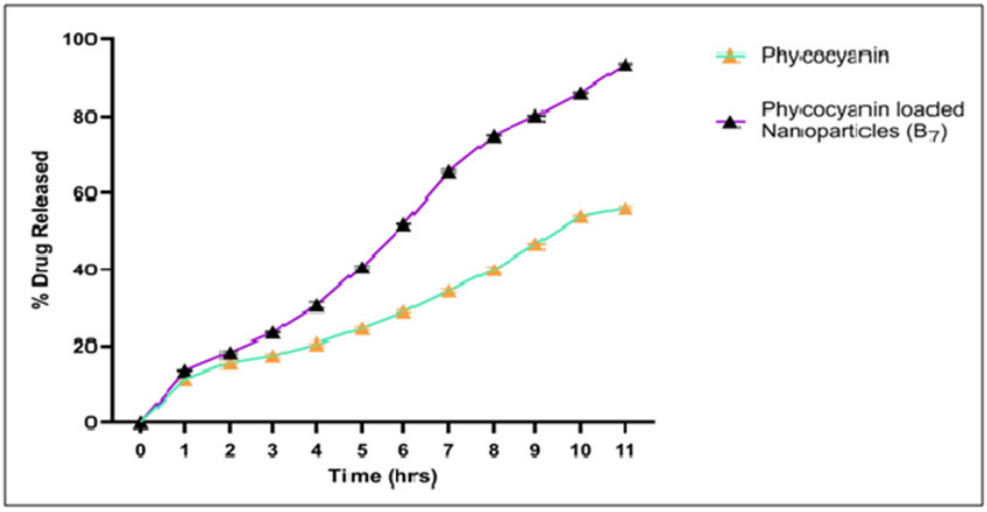


**Figure 11:** *In vitro* dissolution study and drug release of Phycocyanin loaded nanoparticles of optimize batch B7.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. When compared with Phycocyanin and Phycocyanin loaded Nanoparticles (B7) at different concentration point (in ppm). Values are represented as Mean+SEM. Data was analyzed using 2-way ANOVA followed by Tukey's multiple comparisons test.

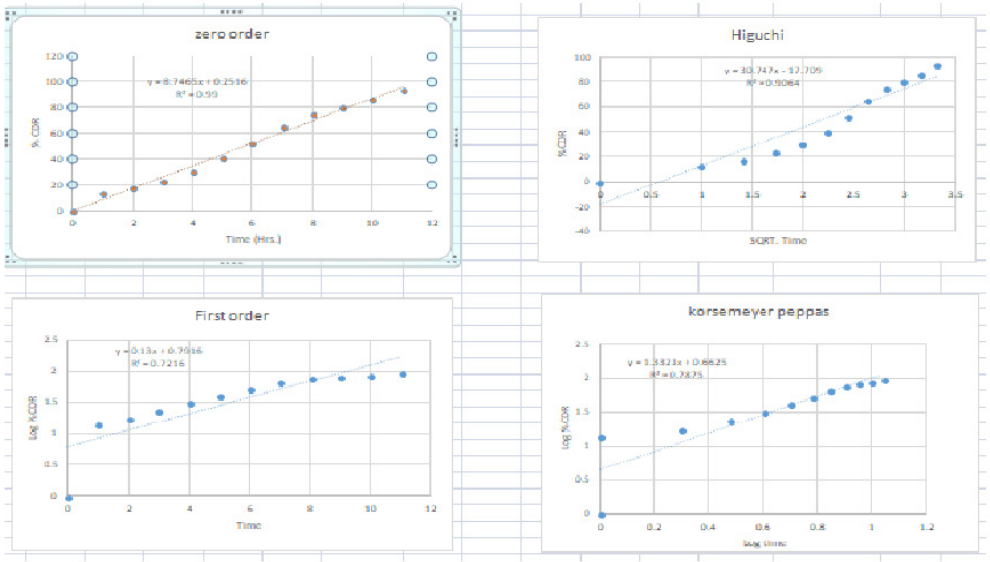


**Figure 12:** Kinetic order of Phycocyanin loaded nanoparticles of optimize batch B7.

## Characterization of an Optimized batch of Nanoparticles

Amongst all the batches, particle size and entrapment efficiency were selected as the key parameters for final formulation. Batch B7 PcNPs exhibited the smallest particle size, highest entrapment efficiency and greatest drug content and was selected as the optimized batch and preferred for further study.

## Morphology Study

The transmission electron microscope images of optimized batch B7 PcNPs as presented in Figure 5. The formulation exposed spherical particles with smooth surfaces and the particle size was examined to be 50 nm and 100 nm. The TEM images displayed smaller particles compared to those observed via dynamic light scattering. This discrepancy can be attributed to the dehydration of nanoparticles during the TEM sample preparation. Dynamic light scattering measures the hydrodynamic radius of the particles, while TEM provides the actual size of the particles.

## Zeta Potential (ZP)

The values for zeta potential of optimized batch B7 PcNPs was detailed in Table 5 and displayed in Figure 6.

## Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

FTIR spectrum of the optimized batch B7 PcNPs showed the retention of peak of physical mixture as well as drug. From this

it can be conclude that Phycocyanin was encapsulated by the polymer used to a good extent. Figure 7 shows infrared spectrum of Pc, sodium alginate, physical mixture and PcNPs.

## Differential Scanning Calorimetry (DSC)

The physical state of phycocyanin within the nanoparticles was analyzed using Differential Scanning Calorimetry (DSC). It significantly affects both *in vitro* and *in vivo* release characteristics. Phycocyanin displayed an endothermic peak at 84.89°C, corresponding to its melting point. Sodium alginate displayed a broad endothermic peak at 83.87°C, near its melting point. The physical mixture displayed an endothermic peak at 104.75°C, while the optimized batch B7 PcNPs had an endothermic peak at 106.91°C. The thermal behavior of the batch $B_7$ PcNPs revealed a slight shift in the Phycocyanin peak to 101.37°C presented in Figure 8.

## X-ray crystallography studies (X-RD)

The X-ray diffraction data are displayed in Figures 9 and 10.

## *In vitro* drug release studies and kinetic modelling of release profiles

The nanoparticles are expected to protect core material from the external environment and conditions present inside body at different time intervals or timeline. *In vitro* release studies of Pc and optimized batch B7 PcNPs were performed using gastric simulated fluid (0.1 N HCl) at pH 1.2 for the initial 2 hr, followed by phosphate buffer at pH 7.4 for the subsequent 12 hr. The results provided valuable insights into the drug release profile. Initially, less than 20% of the drug was released in the first two hr. From the 3rd to the 11th hr, the release rate remained relatively constant, ranging from 23% to 93%. However, during the final hr (11th to 12th hr), there was a drastic major release of the drug. 91% to 93% of the drug is released during this time frame rapidly within the 60 min window. Finally, the amount released was > 90% drug release from the batch B7 PcNPs as compared with Pc drug solution as shown in Figure 11.

The *in vitro* drug release profiles of the optimized batch B7 PcNPs were employed to various kinetic models (zero-order, first-order, Higuchi and Korsmeyer-Peppas), as illustrated in Figure 12.

## Antioxidant activity

PC's unique structure is responsible for the antioxidant activity. In the current study, the antioxidant activity of PcNPs was assessed using DPPH free radical scavenging assay. The antioxidant capacity results as of different samples at various concentrations are depicted in Table 7. The batch B7 PcNPs has 47.74 µg/mL and Phycocyanin has 38.60 µg/mL antioxidant activities while ascorbic acid has 49.79 µg/mL $IC_{50}$ value as depicted in Figure 13. As the sample concentrations increased, there was gradual enhancement in each sample's ability to scavenge the DPPH radicals displayed in Figure 14.

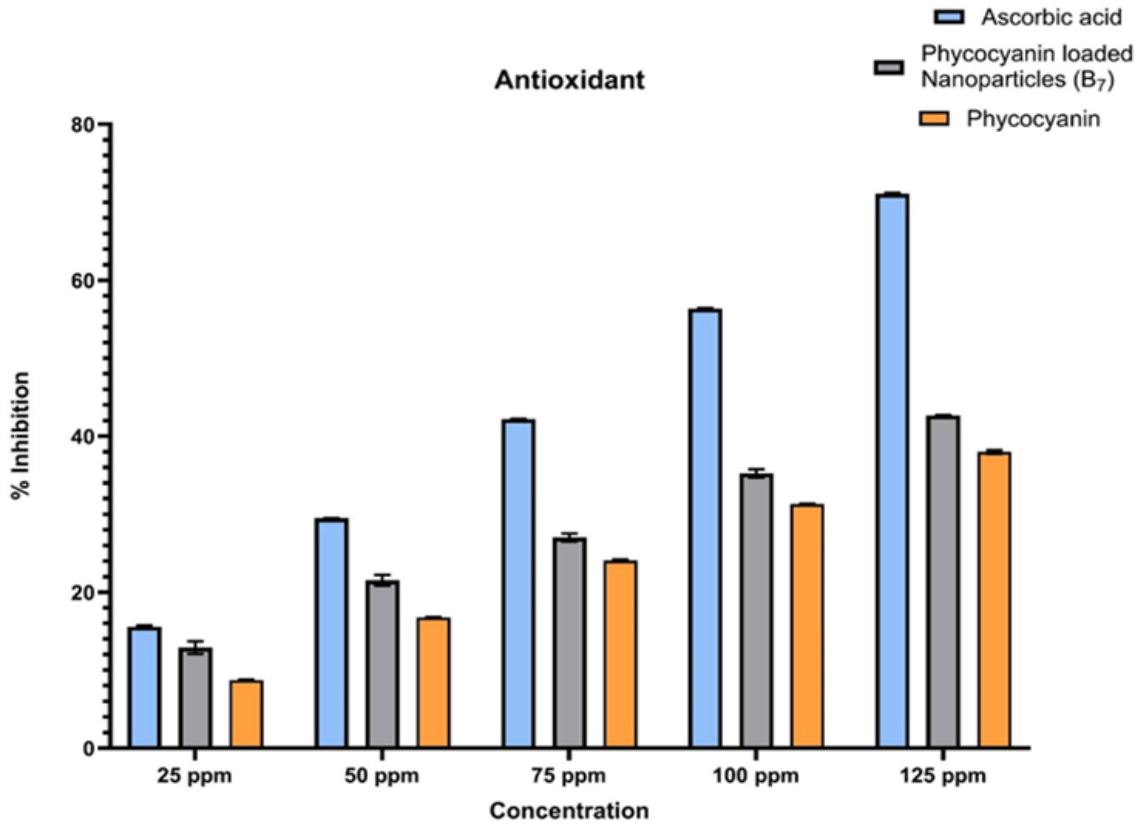


**Figure 13:** Graphical representation of Antioxidant activity.

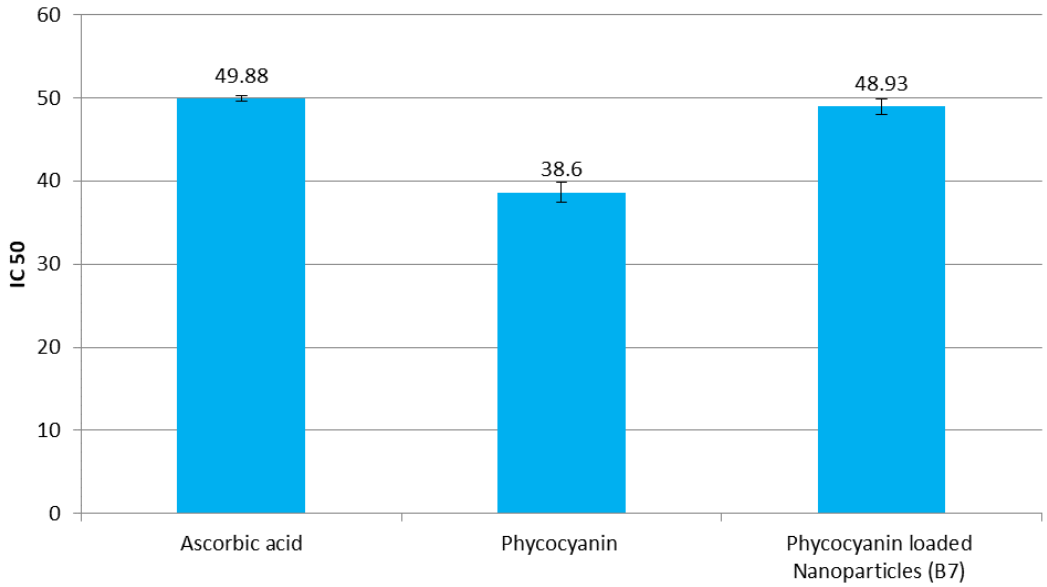


**Figure 14:** Graphical representation % Inhibition.

## Stability Study

The optimized batch B7 PcNPs were conducted for stability studies under various conditions: at 4°C±1°C with 60±5% Relative Humidity (RH), at -20°C±2°C with 60±5% RH and at room temperature for 30 days. The estimation was performed on 7th, 14th, 28th and 30th days for the parameters physical appearance and entrapment efficiency were performed. The results, detailed in Table 8, showed no significant change in physical appearance and entrapment efficiency for 4°C±1°C, 60±5% RH and -20°C±2°C, 60%±5% RH conditions.

## DISCUSSION

In the present work utilizes a green synthesis ionotropic gelation method employing sodium alginate and calcium chloride to formulate Pc nanoparticles were prepared by using green ionotropic method. Nine formulations were designed with varying levels of sodium alginate and calcium chloride and all other factors were kept unvaried during the study. The formulations were evaluated for particle size, polydispersity index, entrapment efficiency and drug content. Additionally, zeta potential, TEM, DSC, X-RD, *in vitro* drug release and *in vitro* antioxidant activity parameters of Pc were investigated for optimized formulations.

The calibration graph showed a linear relationship between absorbance and concentration in the region of 100-500 µg/mL in both water and phosphate buffer pH 7.4. The regression equation was determined to be y=0.0014x+0.0035 for water and y=0.0011x=0.0034 for phosphate buffer pH 7.4. Thus, indicating that the drug follows Beers-Lambert law.

From the result obtained from particle and polydispersity index shows that amount of sodium alginate (X1) and calcium chloride (X2) seems to impact the particle size. Results also showed that increasing sodium alginate results in a smaller particle size and with higher amounts of calcium chloride the particle size increases. The findings revealed a direct correlation between cross linker concentration and particle size, with the smallest size observed in nanoparticle formulations with the lowest cross linker concentration. In the evaluation parameter of entrapment efficiency, the formulations $B_2$ and $B_3$ illustrated least entrapment efficiency due to lower proportion of the sodium alginate present in formulation. Likewise, formulations $B_7$ and $B_8$ demonstrated maximum entrapment efficiency as the sodium alginate present were exactly in the opposite concentrations of their upper and lower limits as per the factorial design. The amount of sodium alginate (X1) and calcium chloride (X2) seems to impact the entrapment efficiency. Results revealed that increasing sodium alginate results in higher entrapment efficiency while the entrapment efficiency tends to decrease with more calcium chloride.

The drug loading was lowest in the formulations (B2, B3, B5 and B6) with high cross-linker and low polymer concentration. Conversely, the highest drug loading occurred in formulations (B1, B4, B7, B8) with low cross-linker and high polymer content. A higher polymer amount results in a more robust internal matrix structure, enhancing the ability to entrap Phycocyanin (Pc).

The amount of sodium alginate (X1) and calcium chloride (X2) seems to impact the drug content. Result revealed higher sodium alginate (X1) appears to be associated with higher drug content. The highest drug content is observed with 750 mg sodium

alginate (B7, B8, B9), with a peak of 53.25 mg in B7. Increasing the amount of calcium chloride (X2) does not consistently correlate with drug content. For instance, drug content decreases as calcium chloride increases from B1 to B3, but fluctuates in higher sodium alginate formulations (B7 to B9). This pattern suggests that sodium alginate might play a more significant role in drug content than calcium chloride and the optimal balance between these two factors is crucial for maximizing drug content.

The DOE results revealed that entrapment efficiency was affected by both the concentration and proportions of sodium alginate and calcium chloride. Therefore, % EE increases as the concentration of sodium alginate increases and concentration of calcium chloride decreases and the interaction of two factors reflects significantly for the decreases in % EE suggesting the antagonistic effect. The response surface plot for measured responses showed that the % entrapment efficiency increased as the concentration of sodium alginate ($X_1$) as well as decreased as the concentration of calcium chloride ($X_2$) was increased indicating the antagonistic effect. The effect of sodium alginate on %EE is more as compared to calcium chloride. Interaction of $X_1X_2$ affected negatively on %EE i.e., as the concentration of sodium alginate and calcium chloride increased %EE decreased significantly.

Particle size was also affected by both the concentration and proportions of sodium alginate and calcium chloride. Therefore, particle size decreases as the concentration of sodium alginate increases and the concentration of calcium chloride decrease. And the interaction of two factors reflects significant antagonistic effect for the decrease in particle size. The response surface plot indicated that as the concentration of sodium alginate ($X_1$) increased and concentration of calcium chloride ($X_2$) decreased, the particle size gets decreased. The interaction between $X_1$ and $X_2$ had a negative effect, suggesting an antagonistic influence on particle size i.e., as the concentration of sodium alginate increased and calcium chloride decreased particle size decreased significantly. Thus, data reveal that the quadratic model's p-value is 0.011 (<0.0500), indicating statistical significance. Therefore, determining the optimal ratio of the polymer to the cross-linker is required. The formulation B7 represents this optimum ratio. The Design of DoE surface response analysis for Y1 and Y2 revealed a significant model, with a p-value below 0.01%. The counter plot of the trails demonstrates that formulation B7 meets all the criteria more effectively than the other eight formulations and is thus chosen for additional investigation.

The formulation B7 exposed spherical particles with smooth surfaces determined by TEM. A negative zeta potential value as displayed in Figure 6 suggests that the optimized formulation is a stable. The spectral analysis revealed that Pc is characterized by type I and type II amine bonds, which are absorbed at 1541 $cm^{-1}$ and 1651 $cm^{-1}$, respectively. Additionally, the stretching and bending vibrations at 1381 $cm^{-1}$ correspond to the C-O-H bonds in phenolic groups. The intense bands observed at 1049 $cm^{-1}$ suggest the presence of inorganic sulfate, likely originating from the extraction process as showed in Table 6. In the sodium alginate spectrum, the 3452 $cm^{-1}$ peak observed is associated with hydroxyl groups (O-H) stretching vibration of while the peak at 1029 $cm^{-1}$ corresponds to carbonyl (C-O) bond stretching vibration. Additionally, the absorption bands at 1598 $cm^{-1}$ and 1419 $cm^{-1}$ are indicative of the asymmetric and symmetric of ester ($COO^-$) stretching groups, respectively. In the spectral analysis of PcNPs, all the distinguishing peaks of Pc were observed signifying that the drug is integrated with the polymer without any chemical interaction. The slight shift in Pc peak indicates no substantial interaction between the drug and the polymer analyzed by DSC. The XRD studies revealed that Phycocyanin (Pc) exhibits a highly crystalline nature, with a prominent peak at a specific 2θ value. In contrast, the Phycocyanin loaded nanoparticle formulation displayed a distorted peak, indicating an amorphous state with minimal structural change. The absence of distinctive peaks of Pc in optimized batch B7 PcNPs suggests molecular dispersion of the drug within the polymer matrix, resulting in complete drug amorphization. The *in vitro* drug release profile study can be attributed to the quantity of sodium alginate used; increasing its ratio relative to calcium chloride enhances the polymeric matrix's ability to facilitate drug dissolution and release. The best fit was observed with the zero-order model for the optimized nanoparticle batch ($R^2$=0.99). The Higuchi diffusion model describes the release of water-soluble drug from complexes, including liquid. Kinetic models such as zero order and Higuchi are suitable for controlled or sustained release. Observations concluded that the release pattern following the Fickian diffusion ($n$<0.5), signifying that diffusion is the predominant drug release mechanism. The controlled release formulation is expected to be effective for the intended therapeutic use, with a 93% drug release serving as a promising benchmark for the newly developed formulation. The dissolution studies have significantly contributed to understanding the drug release characteristics of the formulation.

The outcome of antioxidant activity demonstrated that all samples exhibited notable antioxidant capacity. However, the antioxidant activity of optimized batch B7 PcNPs was notably higher than that of the Pc and was comparable to the standard reference. Consequently, it can be implied that the antioxidant activity of B7 PcNPs with the addition of sodium alginate may be higher than that of PC. The end results confirm the Phycocyanin nanoparticles, has good the antioxidant activity. Thus, antioxidant investigational results show that Phycocyanin loaded nanoparticles PcNPs (B7 batch) is a potent natural antioxidant with potential applications across various fields.

In the stability study, a notable reduction in entrapment efficiency was observed at room temperature, suggesting some drug degradation under these conditions. This indicates that room temperature is not ideal for storing Phycocyanin

formulations. Therefore, storage at 4°C±1°C or -20°C±2°C with 60±5% RH is recommended for maintaining the stability of Phycocyanin-loaded nanoparticles over extended periods.

## CONCLUSION

The preformulation studies for phycocyanin were conducted prior to the nanoparticle formulation. The Phycocyanin loaded nanoparticles were successfully prepared using a green ionotropic gelation method. The $3^2$ full factorial designs were applied by using DoE software, resulting in 9 different formulations with varying in the polymer and cross linker ratios. The formulation B7 showed the superior performance across the tested parameters. The formulation batch B7 demonstrated the best results among the evaluated parameters, showing the smallest particle size and highest entrapment efficiency. This formulation was deemed the optimized batch and used for further assessment studies. The optimized batch Phycocyanin loaded nanoparticles exhibited near spherical morphology, amorphous character as evidenced by lack of distinct peaks in the Differential Scanning Calorimetry (DSC) analysis. Additionally, Fourier Transform Infrared Spectroscopy (FTIR) studies did not indicate any interaction between Pc and polymer employed. *In vitro* drug release studies showed that encapsulating the drug in the polymer matrix significantly improved release characteristics, following zero-order kinetics. The Phycocyanin loaded nanoparticles demonstrated greater antioxidant activity as compared with Phycocyanin. These nanoparticles exhibited good stability at 4°C and -20°C for a time period of 30 days.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interests or personal relationships that could have influenced the research presented in this paper.

## ABBREVIATIONS

**Pc:** Phycocyanin; **PcNPs:** Phycocyanin Loaded Nanoparticles; **TEM:** Transmission Electron Microscopy; **ZP:** Zeta potential; **IR:** Infrared Spectrometry; **DSC:** Differential Scanning Calorimetry; **XRD:** X-ray crystallography; **UV:** Ultra Violet Spectroscopy; **EE%:** Entrapment efficiency; **PS:** Particle Size; **PDI:** Polydispersity Index; **nm:** Nanometers; **DOE:** Design of Expert; **FT-IR:** Fourier Transform Infrared Spectroscopy; **DSC:** Diffraction scanning calorimetry; **XRD:** X-ray diffraction; **rpm:** Revolution Per Minute; **mL:** Millilitre; **µm:** Micrometer; **DPPH:** 2, 2-Diphenyl-1-Picrylhydrazyl; **ppm:** Parts Per million; **mg:** Milligram; **µg:** Microgram; **SD:** Standard deviation; **ANOVA:** Analysis of variance; **$X_1$:** Sodium Alginate; **$X_2$:** Calcium chloride; **RH:** Relative Humidity; **Conc:** Concentration; **min:** min.

## AUTHORS CONTRIBUTIONS

Mrs. Supriya Jagtap contributed to conducting experiments, proposing the study design, preparing and analyzing results, interpreting data and engaging in discussions. Mr. Sachin Vanve was involved in performing experiments, preparing and analyzing results, interpreting data. Dr. Kishor Gujar and Dr. Rajesh Khathuriya provided project administration and supervision. All authors reviewed and approved the final manuscript and are in agreement with its contents.

## SUMMARY

Phycocyanin (Pc), a phycobiliprotein pigment with therapeutic potential for treating inflammation, oxidative stress, neuroprotection, liver protection and cancer was used and investigated for nanoparticle formulation and antioxidant activity. Pc Loaded Nanoparticles (PcNPs) were prepared using a green ionotropic gelation method. The nine formulations prepared were optimized via a $3^2$ full factorial designs. The developed PcNPs showed an improved dissolution profile and promising antioxidant activity, making them a potential tool with enhanced stability and efficacy.

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