Formulation Development of Novel Curcumin Analogue Loaded Non-aqueous Gel and Curcumin Analogue Loaded Nanoparticle (CA-NP) Gel for Topical Use and *In-vitro* Antioxidant Study

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

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ABSTRACT

In this paper we have reported development of suitable dosage form for novel curcumin analogue synthesized in our laboratory. The work involves preformulation studies of synthesized curcumin analogue followed by preparation and optimization of non-aqueous gel and curcumin loaded (CA-NP) nanoparticle gel. The formulated gels were observed visually for clarity, homogeneity, and phase separation. They were tested for their appearance and presence of any aggregates. Curcumin analogue loaded PLGA nanoparticles were prepared by using the nanoprecipitation - solvent evaporation method and further optimized. *In-vitro* antioxidant activity of formulation was then evaluated using DPPH radical scavenging activity. The gel exhibited good antioxidant activity with IC_{50} value of 5.39 μg/ml.

Keywords: Curcumin analogue; Non-aqeuous gel; PLGA nanoparticle; antioxidant activity.

1. INTRODUCTION

Curcumin is very versatile in showing biological activities ranging from treatment for skin wounds to angiogenic effect. It is also well accepted fact that curcuminoids have many limitations in terms of metabolism, low bioavailability, inadequate absorption, systemic elimination. Hence, we

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aimed in our last paper [1] to develop various analogues of curcumin synthetically, followed by optimization. Out of the synthesized analogues in previous work p-nitro contacting analogue was found to be equipotent with Adriamycin standard when evaluated for anticancer potential using SRB assay against HepG2 and MCF7 cell lines. Encouraged by results of anticancer activity we decided to develop a suitable formulation of analogue.

In the present work, curcumin analogue was synthesized by using corresponding aldehyde acetone, and boron trifluoride etherate [1]. The obtained crude product was purified by column chromatography using a suitable solvent system. The preformulation studies were then carried out helped to ensure the stability and solubility of curcumin and its analogue. Drug-excipient compatibility studies were performed. Due to no water solubility, non-aqueous gel formulation was selected for topical drug delivery systems. The gel was optimized by using various concentrations of Carbopol. Optimized concentration of Carbopol was selected. Nanoparticle of curcumin analogue was prepared by nanoprecipitation- solvent evaporation method, using biodegradable polymer and surfactant. The curcumin analogue nanoparticles were incorporated in optimized non -aqueous gel base and evaluated. The formulation was further optimized by performing various evaluation tests e.g.- spreadability, drug content, *in-vitro* drug release studies, and viscosity. The obtained nanoparticle formulation was evaluated on the following parameters: particle size, drug entrapment efficiency, different concentrations of surfactant. Optimized batch was selected for further evaluations. Antioxidant activity was then evaluated using DPPH radical scavenging assay.

2. MATERIALS AND METHODS

All the reagents and substrates were used as received, in their commercially available form and purchased from Bhavi Chem, Mumbai, Sigma-Aldrich, India and High-Media, Mumbai. Curcumin was obtained as gift sample from Arjuna extract, Mumbai. Polymers were obtained as gift sample from Carbion and Mohini Oragnics Ltd. All the solvents were used as received without further purification. All the reactions were monitored by thin-layer chromatography on silica gel plates (GF 254) and visualized with UV light. Melting points of the compounds were determined on DBK Prog. melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}CNMR spectra were recorded in CDCl$_3$, DMSO-d6, on a Bruker Avance II 400 MHz spectrometer with tetramethylsilane (TMS) as an internal reference at SAIF, Panjab University, Chandigarh, India. The chemical shifts are given in δ (ppm) referenced to the respective solvent peak and coupling constants are reported in Hz. Mass spectra were recorded on Agilent MSD at NMIMS University, Mumbai, India. The purity of complex is done by DSC at BVCOP, Navi Mumbai. Compounds were characterized by ^{1}H NMR, ^{13}CNMR and HRMS. UV-VIS spectra were recorded using Jasco V-750 UV Visible spectrophotometer. Particle size was determined by HORIBA nanoPartica-SZ-100V2 series analyzer. Remi R-8C Laboratory Centrifuge was also used during the study.

2. EXPERIMENTAL WORK

2.1 Synthesis of Curcumin Analogue

Fig. 1 gives the pictorial representation of scheme adopted for synthesis of curcumin analogue developed in our laboratory [1].
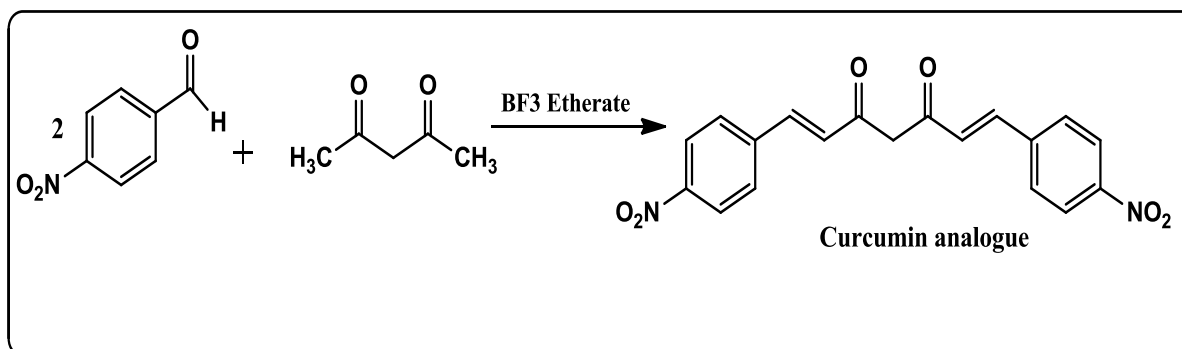

Fig. 1. Scheme for curcmin analogue synthesis

2.2 Preformulation Studies

Pre-formulation studies are the first step in the rational development of the dosage form of a drug substance. It can be defined as the investigation of the physical and chemical properties of drugs alone and when combined with excipients.

Preformulation investigations are designed to identify those physicochemical properties and excipients that may influence the formulation design, method of manufacture, and pharmacokinetic- biopharmaceutical properties of the resulting products.

Hence, preformulation studies on the synthesized sample of the drug were performed for identification and compatibility studies [2,3,4].

Organoleptic properties: The sample of curcumin analogue was studied for organoleptic characters.

Authentication of surcumin analogue: Curcumin analogue was identified and confirmed by its melting point, FTIR, ^{1}H NMR, [13] C NMR, Mass, and DSC spectra and were found to be within the limits as per specifications [1].

Solubility study of curcumin analogue: The solubility study aimed to decide or select a solvent for drug extraction during % entrapment efficiency determination. The solubility study of curcumin analogue was determined in water, ethanol, ethyl acetate, chloroform, acetone, DCM, DMSO, 0.1 N HCl, 0.1N NaOH, and methanol.

UV spectrum of curcumin analogue in methanol: Accurately weighed and transferred 10mg of curcumin analogue was dissolved in sufficient amount of methanol and the volume was made up to mark with methanol in 10 ml volumetric flask. 1ml of the resulting solution was diluted in order to achieve the final concentration of 10µg/ml using methanol. The prepared solution was scanned in the range of 200-800 nm keeping methanol as a blank by UV-VIS spectrophotometer in order to determine λ_{max}.

Construction of calibration curve in methanol for estimation of drug in developed formulations: Accurately weighed 10 mg of curcumin analogue was dissolved in 10ml methanol and the resulted solution was diluted sufficiently in methanol to get the final concentration of 10µg/ml. From this solution, different dilutions were prepared with methanol in the concentration range of 1 -12 µg/ml. The absorbance of these solutions was measured at 265 nm using methanol as a blank solution by UV-Visible spectrophotometer. Absorbance values were plotted against concentration to obtain the standard graph.

Construction of calibration curve in phosphate buffer for estimation of drug in developed formulations: 10 mg of curcumin analogue was weighed accurately and dissolved in 10ml phosphate buffer pH 7.4 and diluted further to get the solution of 100 µg/ml. Different dilutions were then prepared with phosphate buffer pH 7.4 in the concentration range of 5-40 µg/ml. The absorbance of these solutions was measured at 265 nm using phosphate buffer pH 7.4 as a blank solution by UV visible spectrophotometer. The absorbance value was plotted against the concentration to obtain the standard graph.

2.3 Stability Profile of the Drug in Various Buffers

The stability of curcumin analogue and curcumin was studied in various buffers. Buffers of various physiological pH were prepared according to procedures mentioned in USP. 100 ppm solution of curcumin analogue was prepared as a stock solution. From this 1 ml of the solution was diluted to 10 ml with buffers of different pH. The solutions were maintained at 37°C for 24 hrs. The absorbance was recorded at every 1 hr. up to 5 hrs. and thereafter at the end of 24 hrs. till 7 days and at 14th day.

For studying the effect of formulation matrix on UV absorbance, the UV scan of curcumin analogue, placebo nanoparticle, curcumin analogue loaded nanoparticle and placebo gel were taken and examined for excipient interference if any.

2.4 Anti-Oxidant Study by DPPH Radical Scavenging Method

The *In-vitro* Antioxidant study of the curcumin analogue gel was carried out by DPPH radical scavenging method Curcumin was used as standard.

The formula for calculating % reduction is as follows

% inhibition = $\frac{[AO-A1]/}{AO}$] X 100

Equation 1: % Inhibition by DPPH assay: Where, A_O is the absorbance of the control and A_1 is the absorbance of the samples.

Preparation of curcumin stock: 5 mg of Curcumin powder was dissolved in a 10 ml volumetric flask and the volume was made with quantity of methanol up to 10 ml. 0.1ml of resulting solution was then diluted to 10 ml using methanol.

Preparation of DPPH stock: 1 mg of DPPH powder was dissolved in sufficient methanol to make final volume 10 ml.

Preparation of test solution stock: The amount of the medicated gel was equivalent to 5 mg of curcumin analogue was weighed and mixed with methanol to obtain a 1000 ppm solution. Final dilutions were made to obtain 10 ppm methanolic solution.

Procedure: To a set of test tubes containing 3 ml methanol, 100 µl of DPPH reagent was added. The initial absorbance was measured at 517 nm. To this different volume (5-120µl) of drug solution was added. The percentage reduction of absorbance of each solution was taken 4 minutes after the addition of each test solution. Similarly, curcumin (0.05mg/ml) was added in the range of 5-120µl, and absorbance at 517nm was recorded. A blank solution containing 100µl of above DPPH in 3 ml methanol was maintained throughout the experiment and absorbance was recorded every 30 minutes up to 3 hrs.

2.5 Drug –Excipient Compatibility Study by FTIR

The crystal of FTIR-ATR was cleaned with isopropanol on cellulose tissues. The background was measured with an ATIR unit. The dry sample of curcumin analogue was placed on the crystal ensuring good contact and then measured. The entire operation was conducted under controlled humidity. The sample was then scanned over a range of 36000-600 cm^{-1}.

2.6 Preliminary Screening of Excipients

The preliminary study was carried out for the screening of the gelling agent and optimization of its concentration as well as the selection of solvent for dispersion of polymer.

Screening of gelling agent: A gelling agent would be natural, synthetic, or semi-synthetic polymer small molecules dispersed into an organic, inorganic, or aqueous solvent. The polymer in gels acts as the backbone of the gel matrix.

Carbopol 934 was screened in different concentrations based on the physical properties of the gel, consistency, and appearance. The concentration of selected polymers was decided to depend upon its gel-forming capacity with a suitable solvent.

Screening of solvent: Curcumin analogue is water-insoluble therefore the gel containing water as a solvent for dispersion of polymer cannot be used. The non-aqueous solvent was the ideal choice for the dispersion of polymeric agents. Glycerine, Propylene glycol, Polyethylene glycol 400 was selected based on literature data for the dispersion of polymers. Each polymer from the selected list was dispersed overnight in presence of these solvents and evaluated for good polymer dispersibility.

2.7 Formulation and Development

PART A

Preparation of Curcumin analogue loaded non-aqueous gel: Accurately weighed and transferred quantity of polymer was dispersed in an adequate amount of PEG-400; it was mixed gently and kept overnight for soaking. Drug was dissolved in a sufficient quantity of PEG-400 with the help of magnetic stirrer, preservatives: methylparaben (0.05%) and propylparaben (0.02%) were added to this solution. To the resulting clear solution soaked carbopol was added then and mixture was stirred using magnetic stirrer for sufficient time. The volume was made up to quantity sufficient to 10 gm amount using PEG -400. It was again mixed using a magnetic stirrer to get a uniform gel-like consistency. The prepared gel was allowed to stand until all the entrapped air was no more visible. The formulated gel was then filled into the wide-mouth bottle and labelled accordingly [5, 6]. Similarly, the gel containing a nano-particle was prepared.

Formulation optimization of curcumin analogue loaded non -aqueous gel: All

polymers were evaluated at different concentrations like 1%, 2%, and 3%. The suitable concentration of carbopol was chosen for formulation purposes. The time for mixing of carbopol dispersion and solutions of drug plus preservatives was optimized depending on the experimental trials [7].

Evaluation of curcumin analogue loaded topical gel formulation

Physical appearance: The formulated gels were observed visually for clarity, homogeneity, and phase separation. They were tested for their appearance and presence of any aggregates.

Consistency: The consistency of each formulated gel was inspected.

Viscosity: The viscosity of formulated gels was determined using Brookfield's viscometer with a spindle number 6 having a speed of 100 rpm at $25^{o}C$ for 5 minutes in 25 ml beaker and the corresponding dial reading on the viscometer was noted.

Viscosity can be calculated by using the following formula

Dial reading X Factor= Viscosity in Centipoise (mPas)

Equation 2: Viscosity formula

pH: The pH of the formulated gels was measured using a digital calomel glass electrode (pH meter). The electrode was calibrated using the standard solution of pH 4, 7 and 9.2. All the measurements were done at room temperature [8].

Spreadability: A sample of 2.5 g of each formulation was placed between two slides.100 gm. weight was placed on the upper slide for 1 minute to compress the formulation to the uniform thickness. Then 50 gm. weight was placed on the pan and the time was noted for the upper slide (movable) to separate from the fixed slides.

Spreadability can be calculated by using the following formula.

Spreadability (S) in g.cm/sec=$\frac{M \, x \, L}{T}$

Equation 3: Formula for spreadability

Where M= Mass (50 gm) is tied to the upper slide.
L= Length (30 cm) of lower glass slide
T= Time (second) needed to separate the slides.

Drug content/ Assay of gels: Topical gel equivalent to 5mg of drug was weighed and added to 10 ml volumetric flask containing methanol. The mixture was sonicated for 10 min and filtered under gravity through Whatman filter paper, from this 0.2 ml of solution was pipetted out and diluted to 10ml with methanol. The absorbance of this solution was taken at 265 nm using methanol as a blank. Drug content was calculated using the C.C of curcumin analogue in methanol [9, 10].

***In-vitro* drug release:** The *in-vitro* diffusion study was carried out using Franz diffusion cell. The receptor compartment was filled with 20ml of phosphate buffer pH 7.4 Curcumin analogue was kept in the donor compartment over a dialysis membrane. The aliquot of 1 ml was taken in a suitable interval of time and analysed using UV spectroscopy.

Details of permeation studies for topical gel: The *in-vitro* permeation studies were carried out using Franz diffusion cell. In which cellulose membrane was used for study. 1g of topical gel was spread on a cellulose membrane, previously soaked overnight in the release media. Receptor compartment was filled with 25 ml of release media and rotated on 50 rpm and 10 ml aliquots were withdrawn by replacing equal volume of fresh release medium. The sample were analysed by UV at its λ $_{max}$, and drug concentration was determined from the calibration curve.

PART B

Curcumin analogue Loaded Nanoparticles and Curcumin analogue loaded Nanoparticles as Topical Gel.

Preparation of Curcumin Analogue Loaded PLGA Nanoparticles: Curcumin analogue loaded PLGA nanoparticles were prepared using the nanoprecipitation -solvent evaporation method. Accurately weighed PLGA was dissolved in a sufficient amount of acetone to get a uniform PLGA solution. To this solution, 10 mg of curcumin analogue dissolved in the minimum amount of acetone was added and stirred for 5 minutes. This solution was added dropwise to the aqueous solution containing different concentrations of poloxamer 407 (0.1-2% w/v), over 10 min. on a magnetic stirrer plate operated

at 800 rpm. Within a few minutes, precipitation of nanoparticles was observed in the aqueous layer. This suspension was stirred at room temperature for 24 hrs. to evaporate the acetone solvent completely PLGA NPs were obtained by centrifugation at 15000 rpm. The developed nanoparticles (referred as CA-NP hereafter) were then evaluated for various parameters.

Optimization using 2^3 factorial designs: A 2^3 full factorial design was employed to study the effect of independent variables i.e., the ratio of drug: polymer, stirring rate, and concentration of surfactant on dependent variables i.e., entrapment efficiency and particle size. The probe sonication time was maintained at constant [Tables 1, 2].
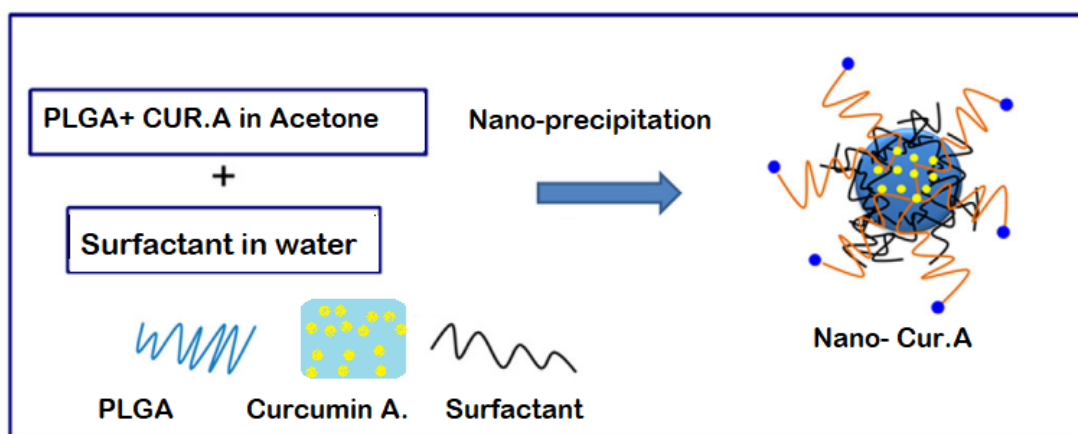

Fig. 2. Schematic representation of nano-curcumin analogue formulation using the nano-precipitation method

Table 1. Associated variables and their corresponding levels

Factor	Associated variables	Lower level	Upper level
Stirring rate	X_1	600 rpm	800 rpm
Concentration of surfactant	X_2	0.5	1
The ratio of drug: polymer	X_3	1:1	2:1

Table 2. Outline for 2^3 factorial design

Batch	Stirring rate (rpm)	Concentration of surfactant	The ratio of drug: polymer
F2	800	1	2:1
F3	800	0.5	2:1
F4	600	0.5	1:1
F5	800	1	1:1
F6	600	1	1:1
F7	800	0.5	1:1
F8	600	1	2:1

Evaluation of factorial design batches

Determination of drug entrapment efficiency: The entrapment efficiency of CA-NP was determined by the centrifugation method using Remi R-8C Laboratory Centrifuge. Nanoparticles were transferred into the polypropylene centrifuge tube. The tube was then subjected to centrifugation at 15,000 rpm for 30 min at 4°C and the supernatant and pellets were separated. The mixture of methanol and DCM was added to the nanoparticle pellet to allow the opening of the polymer and dissolution of the drug. These are then suitably diluted with methanol. The resulting clear solution was then analyzed for drug content by UV spectroscopy method at wavelength 265 nm. Percentage Entrapment efficiency was calculated by using the following formula [11].

Percentage entrapment efficiency (% EE) = $\frac{\text{Amt.entrapped drug}}{\text{Total drug added}}$ X 100

Equation 3: % Entrapment efficiency formula

Size and Size Distribution: Particle size was determined using HORIBA nano Partica-SZ-100V2 series analyser working on the principle of dynamic light scattering.

Selection of optimized batch: Designer Expert 13 software provided 10 new batches; batch with the highest desirability value was selected and performed experimentally.

Evaluation of nanoparticles

Particle size analysis and Polydispersity Index: Particle size and Polydispersity index were determined by using the particle size analyser.

Drug content: The amount of drug contained in CA-NP was determined by dissolving 20 mg of the sediment in 20 ml of methanol: DCM (1:1). It was diluted appropriately and analysed for drug content by UV spectroscopy method at wavelength 265 nm.

Surface morphology: The surface morphology of the particle was determined by Cryo-SEM. A sample is mounted on the plunger and the plunger is dipped in liquid nitrogen for freezing. Then it is transferred to the preparation chamber where temperature is maintained at -190°C. The sample is sublimated at (-90°C to -100°C) for a few minutes. Once the sample has been sufficiently sublimated it can then be coated with the fine layer of platinum. After coating, the sample is transferred to the SEM stage for Imaging.

***In-vitro* drug release:** The *in-vitro* diffusion study was carried out using Franz diffusion cell. The receptor compartment was filled with 20ml of phosphate buffer pH 7.4, CA-NP was kept in donor compartment over a dialysis membrane. The aliquot of 1 ml was taken in a suitable interval of time and analysed using UV spectroscopy.

Preparation and evaluations of CA-NP as Topical Gel: Similarly prepared and evaluated like curcumin analogue.

2.8 Stability Studies of the Optimized Formulations

Stability studies were carried out on optimized gels at 40 ± 5 % RH for 3 months. It is performed to provide evidence on how the quality of drug substances or drug products varies with time under the influence of environmental factors such as temperature, humidity, and light [12,13]. The following parameters were evaluated physical appearance, pH, spreadability, viscosity, drug content.

3. RESULTS AND DISCUSSION

The curcumin analogue was synthesized as per the reported procedure and characterized by melting point, FTIR, ^{1}H NMR, ^{13}CNMR and DSC technique and found to be comparable with literature reported values [1].

Part A

Preformulation study of curcumin analogue

Organoleptic properties of curcumin analogue: It is a pale yellow coloured amorphous powder having no characteristic odour.

Solubility study of curcumin analogue: The solubility study aimed to decide or select a solvent for drug extraction during % entrapment efficiency determination. The solubility study of curcumin analogue was determined in water, ethanol, ethyl acetate, chloroform, acetone, DCM, DMSO, and methanol. Results are summarized in Table 3.

Table 3. Solubility of curcumin in various solvents

Sr. No.	Solvents	Solubility
1.	Water	Insoluble
4.	Methanol	2.1mg/ml
5.	Ethanol	3.2mg/ml
6.	Acetone	100mg/ml
7.	Ethyl acetate	5mg/ml
8.	Chloroform	Insoluble
9.	DCM	1mg/ml
10.	DMSO	100mg/ml

Determination of λ_{max} of curcumin analogue in methanol: The spectrum of curcumin analogue in methanol is represented in given Fig. 3. After studying the UV- spectra of the drug, it was found that it shows maximum absorbance at 265 nm in methanol.
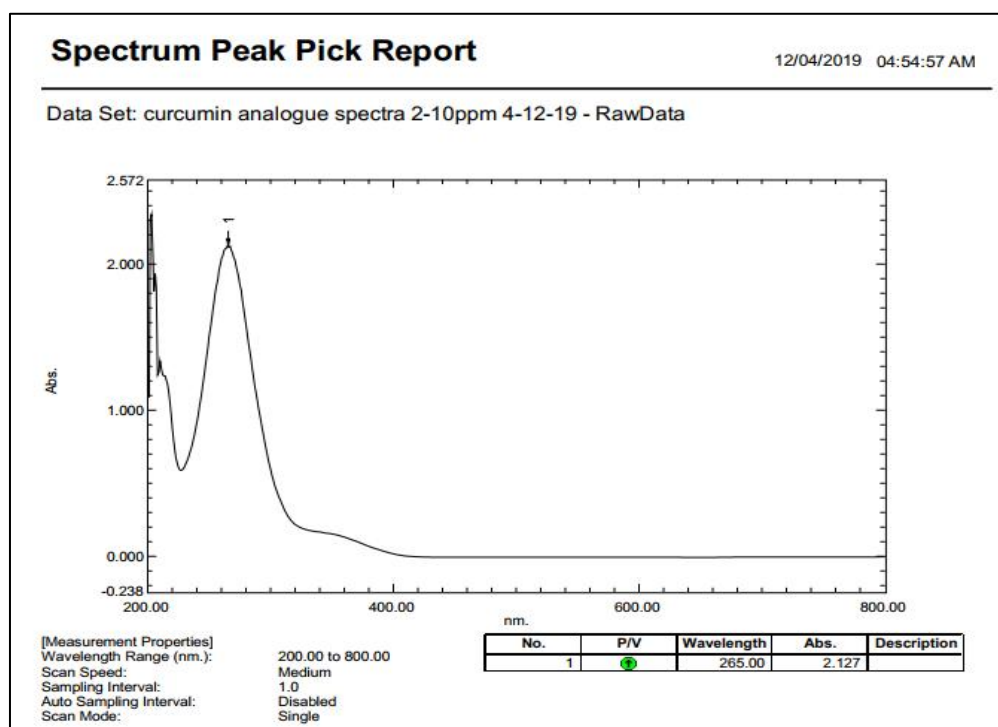
Fig. 3. UV-VIS spectra of curcumin analogue

Calibration curve of curcumin analogue in methanol: The concentration range of 0.2-14 µg / ml of the drug was selected for the development of the standard curve in methanol. The value of R^2 was found to be 0.9856 indicating that the relation of drug concentration and absorbance was linear. The standard curve is represented in Fig. 4.

Calibration curve of curcumin analogue in phosphate buffer pH 7.4: The concentration range of 0.5-10 µg / ml of the drug was selected for the development of the standard curve in phosphate buffer 7.4. The value of R^2 was found to be 0.9779. The standard curve is represented in Fig. 5.

Stability profile of the drug at various physiological pH: Stability study of curcumin analogue was carried out in different pH of buffer 1.2, 2.1, 4.5, 5.5, 6.8, 7.4. Stability was identified when 10 ppm of the drug was maintained at $37^{\circ}C \pm 2\,C$ and until 5 hrs. and absorbance was measured at each hour. At the end of the 24 hrs. again the absorbance was determined. It was observed that the drug degrades slowly in acidic medium and rapidly in alkaline medium, the absorbance of 10 ppm solutions of curcumin analogue in various buffers at $37^{\circ}C \pm 2C$ until 24th hrs. decreases as pH increases from acidic to alkaline medium (Table 4).

Anti-Oxidant study by DPPH radical scavenging method of curcumin analogue gel: The *in-vitro* antioxidant activity was carried out by DPPH radical scavenging activity method. Standard used as curcumin. The standard curcumin shows a scavenging activity of 83.89% at the concentration of 100 µg/ml whereas the synthesized curcumin analogue gel shows a scavenging activity of 95.79 % at the concentration of 100 µg/ml. Graph of overlay anti-oxidant activity of curcumin and curcumin analogue gel was represented in Fig. 6. The curcumin analogue gel was found to be more potent with IC_{50} value of 5.39 µg/ml whereas curcumin shows IC_{50} value of 9.39 µg/ml.

Drug excipient compatibility studies

FTIR spectroscopy: The compatibility studies were carried out using FTIR. The physical mixture of drug and different individual excipients used in formulations were used to study the compatibility. The peaks for the major functional groups of curcumin analogue like C=O, NO_2 were found unaffected in physical mixture with the excipient. This indicates that the drug and all excipients are compatible with each other. The FTIR spectrum of all the tested samples is shown in Figs. 7, 8.
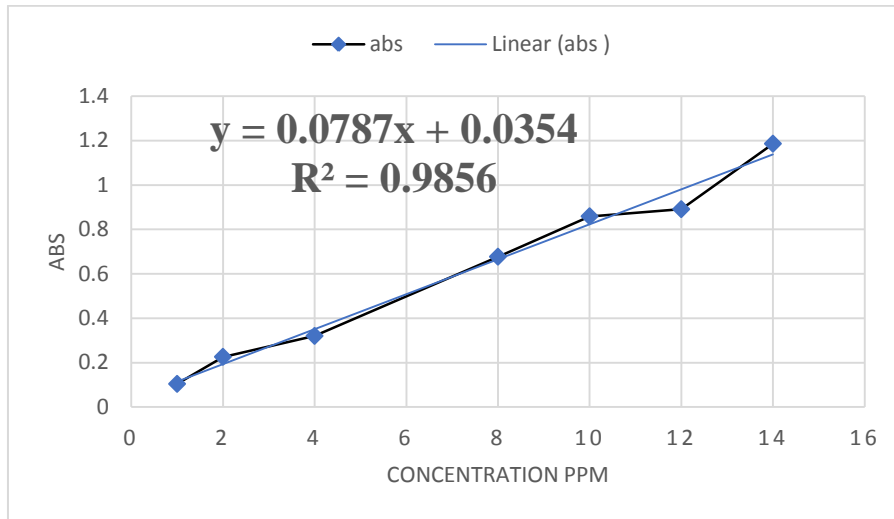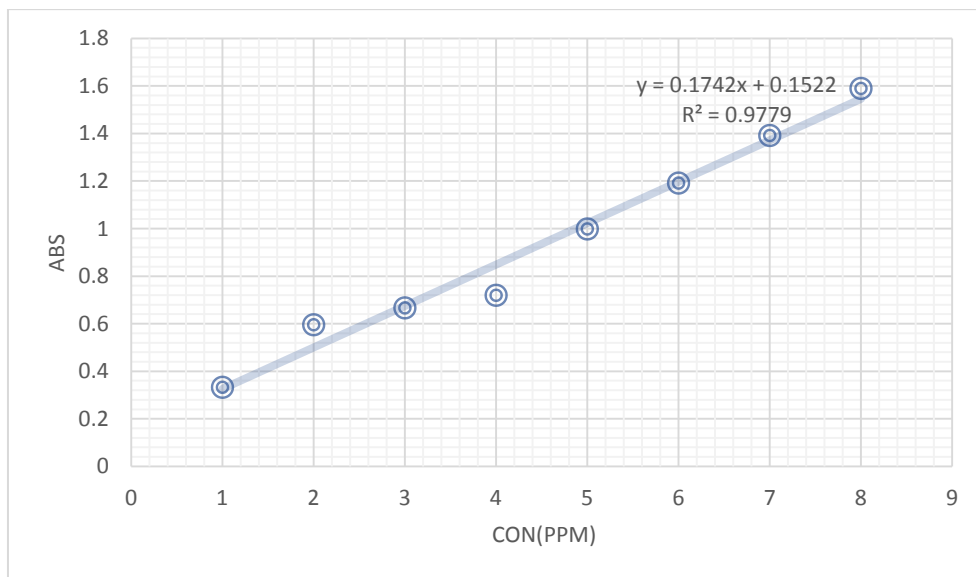
Fig. 4. Calibration curve in methanol

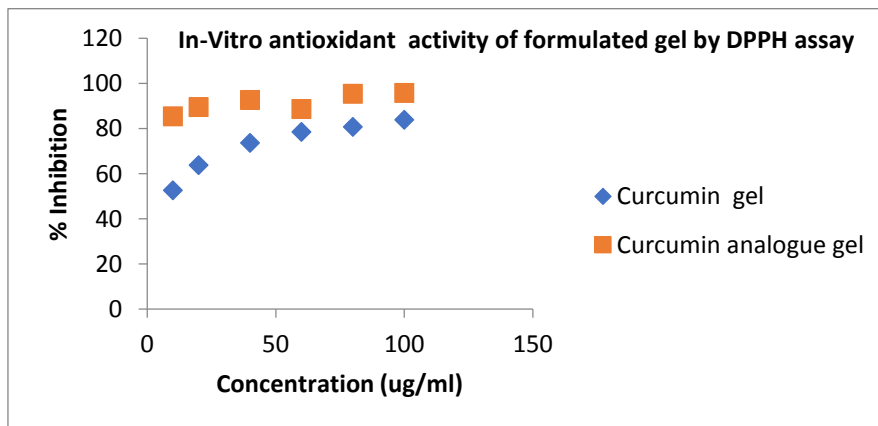**Fig. 5. Calibration curve phosphate buffer**

Fig. 6. Overlay plot for anti-oxidant activity of curcumin & curcumin analogue gel

Table 4. Stability study of drug

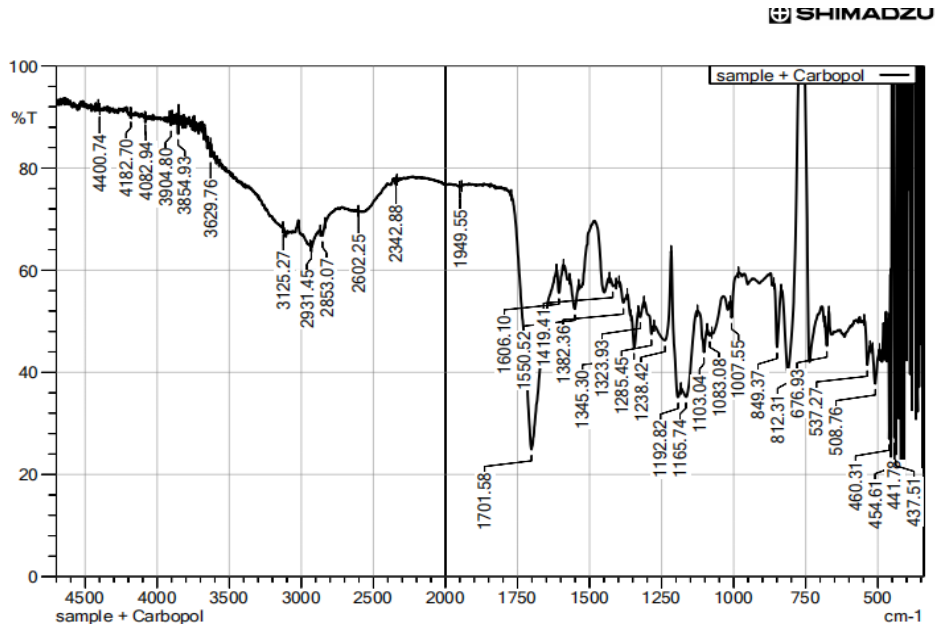| pH	0 hrs	1 hrs	2 hrs	3 hrs	4 hrs	5 hrs	24 hrs
1.2	0.425	0.416	0.429	0.417	0.418	0.398	0.391
2.1	0.398	0.381	0.384	0.379	0.371	0.362	0.331
4.5	0.309	0.313	0.321	0.353	0.388	0.385	0.380
5.5	0.312	0.317	0.328	0.319	0.349	0.345	0.340
6.8	0.306	0.280	0.273	0.254	0.250	0.280	0.301
7.4	0.315	0.281	0.293	0.308	0.331	0.366	0.392
water	0.221	0.213	0.211	0.178	0.154	0.144	0.135

Fig. 7. FTIR spectra of drug with Carbopol 934

Fig. 8. FTIR spectra of drug with PLGA

Table 5. Selection of solvent

Sr. No	Solvent used	Solubility	Result
1.	PEG-400	Very soluble	Gelation with Carbopol 934
2.	Propylene glycol	Slightly soluble	No gelation
3.	Glycerin	Insoluble	No gelation

Preliminary screening of excipients

Selection of a gelling agent: Carbopol 934 was screened in different concentrations based on the physical properties of the gel, consistency, and appearance. The concentration of selected polymers was decided upon its gel-forming capacity. The dispersion property of these gelling agents was checked by mixing them into a suitable non-aqueous solvent. Based on the gelation property, the concentration was selected for further studies.

Selection of solvent: Curcumin analogue found to be moisture sensitive drug; hence the solvent of choice for curcumin analogue gel could be a hydrophilic nonaqueous agent. Following three popular solvents mostly used for the formulation of topical gels were considered, Carbopol 934 was soaked in propylene glycol, PEG 400, and glycerine. The different gel bases were obtained and observed for physical appearance, consistency, homogeneity, and spreadability. The results of the gelling capacity of all the combination are given in the Table 5.

Part B-

Formulation and Development

Preparation of curcumin analogue loaded non-aqueous gel (CA-NP): The gel was prepared using a simple dispersion method. As mentioned in the preliminary study, Carbopol 934 was used as a gelling agent. Weighed quantity of polymer was dispersed in 4 ml of PEG-400; it was mixed gently and kept overnight for soaking. Drug (10mg) was dissolved in 2 ml of PEG-400 with the help of magnetic stirrer, preservatives: methylparaben (0.05%) and propylparaben (0.02%) were added to this solution. Resulting clear solution was then added to the soaked Carbopol and stirred using magnetic stirrer for sufficient time (30 min). The volume was made up to quantity sufficient to 10 gm. amount using PEG -400. It was again mixed using a magnetic stirrer to get a uniform gel-like consistency. The prepared gel was stood until all the entrapped air

was no more visible. The formulated gel was then filled into the wide-mouth bottle and labelled accordingly. Similarly, the gel containing a nano-particle was prepared.

Formulation and optimization of curcumin analogue loaded non-aqueous gel: To optimize the concentration of polymer five different formulations with varied concentrations of each polymer were prepared. The composition of the above formulations is summarized in Table 6.

The concentration of carbopol ranging from 1-4% was used and best formulation was selected based upon physical appearance, consistency, and spreadability. Better gelling ability was obtained with 1.5% and 2.5% but desired consistency was not obtained, liquefaction was observed. The gel containing 3% Carbopol showed desired consistency as a smooth gel with no liquefaction was seen. Gels of 3.5 and 4% polymer concentration showed thick consistency and sticky. Therefore 3% was selected as the optimized concentration of Carbopol 934 (Table 7).

Evaluation of curcumin analogue loaded topical gel formulation: All developed formulations showed good homogeneity, clarity with the absence of lumps. The pH of the formulated gels was measured using a digital calomel glass electrode (pH meter) pH was found to be 4.5 that suits the acidic pH of the skin. The gel was also evaluated of its spreadability, consistency, viscosity and drug content. Results are summarized in Table 8.

Curcumin analogue loaded nanoparticles and curcumin analogue loaded nanoparticles as Topical *Gel*.

Preparation of curcumin analogue loaded PLGA nanoparticles: Curcumin analogue loaded PLGA nanoparticles were formulated using the solvent evaporation method which resulted in the formation of discrete nanoparticles in the size range of 200 to 800 nm.

Table 6. Optimization of gel composition

Batches	Drug (mg)	Carbopol 934 (%)	Propyl Paraben (%)	Methyl Paraben (%)	PEG-400
C1	10	1	0.05	0.02	Qs
C2	10	1.5	0.05	0.02	Qs
C3	10	2	0.05	0.02	Qs
C4	10	2.5	0.05	0.02	Qs
C5	10	3	0.05	0.02	Qs
C6	10	3.5	0.05	0.02	Qs
C7	10	4	0.05	0.02	Qs

Table 7. Preliminary evaluations results of formulated gels (C1-C7)

Sr. no	Batch	Viscosity (cps)	pH	Spreadability (gm.cm/sec)	Physical appearance
1	C1	1400	4.52	65.12	Yellow and clear
2	C2	1702	4.57	58.39	Yellow and clear
3	C3	2254	4.68	46.21	Yellow and clear
4	C4	3152	4.64	40.02	Yellow and clear
5	C5	3642	4.72	34.20	Yellow and clear
6	C6	4123	4.73	31.18	Yellow and clear
7	C7	4300	4.82	29.02	Yellow and clear

Table 8. Results of selected formulations of carbopol with curcumin analogue and CA-NP

Parameter	Curcumin analogue	Nanoparticle loaded gel
Physical appearance	Yellow and clear	Yellow and clear
Consistency	Viscous	Viscous
Viscosity	3642	3700
pH	4.72	4.68
Spreadability	34.20	32.03
Assay	100.6%	87.52%

Application of experimental design for optimization of nanoparticles using 2^3 Factorial design: Based on the results from random trial and error, a 2^3 full factorial design was employed to study the effect of independent variables on dependent variables **like stirring time, the concentration of surfactant, and the ratio of drug: polymer** on percentage entrapment efficiency and particle size. Sonication time was kept constant i.e., 30 min. Entrapment efficiency and particle size were performed and the results were evaluated (Table 9).

Optimization of factorial design batches

Effect of formulation variable on particle size

It was studied by one way ANOVA. In the case of particle size, the polynomial equation in terms of coded factors was obtained as,

$Y_1 = 449.98 + 8.45 \cdot x_1 + 17.83 \cdot x_2 + 5.27 \cdot x_3 + 63.05 \cdot x_1 x_2 - 78.10 \cdot x_1 x_3 - 156.63 \cdot x_2 x_3$

Equation 4: ANNOVA equation

R^2 was found to be 1 which implies that 100 of the variation in the responses were attributed to independent variables (Table 10).

Effect of formulation variable on entrapment efficiency

In the case of particle size, the polynomial equation in terms of coded factors was obtained as,

$Y_2 = 45.77 + 2.05 \cdot x_1 + 2.67 \cdot x_2 - 2.3 \cdot x_3 + 12.03 \cdot x_1 x_2 - 3.00 \cdot x_1 x_3 + 13.05 \cdot x_2 x_3$

Equation 5: ANNOVA equation

R^2 was found to be 0.9994% which implies that 99.94 of the variation in the responses were attributed to independent variables (Table 11).

Results of the factors affecting particle size and entrapment efficiency are also represented in Figs. 11-14.

Selection of optimized batch: The batch with the highest desirability i.e the probability was performed and was evaluated for particle size and entrapment efficiency, eight batches were provided by Design Expert 13 software (Tables 12, 13).

Table 9. Outline and observed responses for 2^3 factorial design

Batch	X_1	X_2	X_3	Particle size (nm)	% Entrapment efficiency
F1	600	0.5	2:1	726.4	40.25
F2	800	1	2:1	309.5	69.82
F3	800	0.5	2:1	461.7	15.22
F4	600	0.5	1:1	247.1	65.89
F5	800	1	1:1	769.1	55.23
F6	600	1	1:1	469.2	20.15
F7	800	0.5	1:1	293.4	51.01
F8	600	1	2:1	323.4	48.55

Table 10. Effect of formulation variables on particle size

Source	Sum of Squares	df	Mean Square	F Value	P value	
Model	2.802E+05	6	46697.68	47650.70	0.0035	**significant**
A-Stirring rate	571.22	1	571.22	582.88	0.0264	
B-Conc. of surfactant	2541.85	1	2541.85	2593.72	0.0125	
C-ratio of Drug: polymer	222.61	1	222.61	227.15	0.0422	
AB	31802.42	1	31802.42	32451.45	0.0035	
AC	48796.88	1	48796.88	49792.73	0.0029	
BC	1.963E+05	1	1.963E+05	2.003E+05	0.0014	
Residual	0.9800	1	0.9800			
Cor.Total	2.802E+05	7				

Table 11. Effect of formulation variables on entrapment efficiency

Source	Sum of Squares	df	Mean Square	F value	P value	
Model	2726.38	6	454.40	271.37	0.0464	**significant**
A-Stirring rate	33.78	1	33.78	20.18	0.1395	
B-Con of surfactant	57.14	1	57.14	34.12	0.1079	
C-ratio of Drug: polymer	42.50	1	42.50	25.38	0.1247	
AB	1158.25	1	1158.25	691.72	0.0242	
AC	71.76	1	71.76	42.86	0.0965	
BC	1362.94		1362.94	813.96	0.0223	
Residual	1.67	1	1.67			
Cor. Total	2728.05	7				

Table 12. Selection of optimised batch

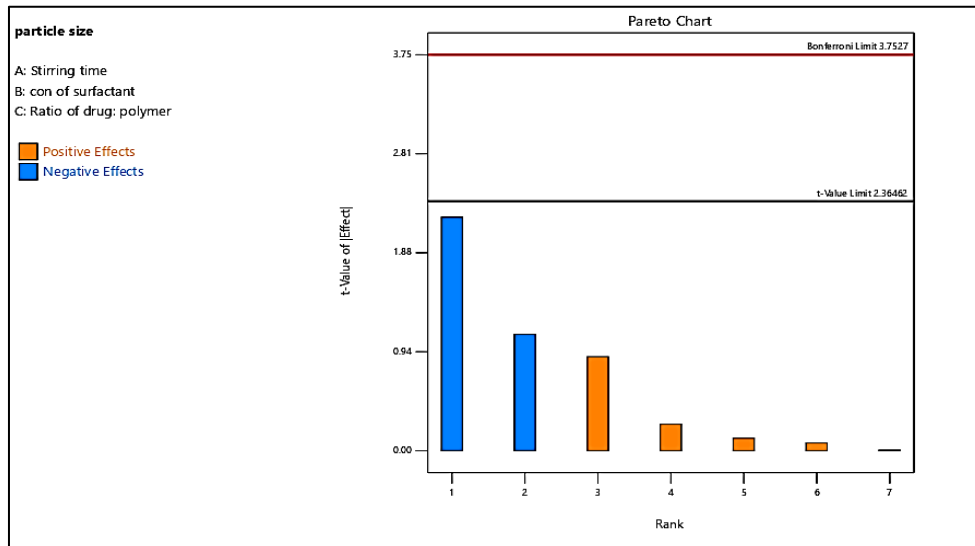| Number	Stirring rate	Con. of surfactant	The ratio of the drug: polymer	Particle size	Entrapment	Desirability	
1	**800.000**	**0.996**	**2:1**	**311.098**	**69.820**	**0.966**	**Selected**
2	799.011	0.997	2:1	310.877	69.820	0.965	
3	798.043	0.998	2:1	310.651	69.820	0.965	
4	797.317	0.999	2:1	310.478	69.820	0.964	
5	800.000	0.990	2:1	312.809	69.193	0.959	
6	800.000	0.988	2:1	313.464	68.953	0.957	
7	799.999	0.974	2:1	317.786	67.369	0.941	
8	765.848	1.000	2:1	312.105	66.489	0.909	

Fig. 9. Pareto chart showing positive and negative factors affecting particle size
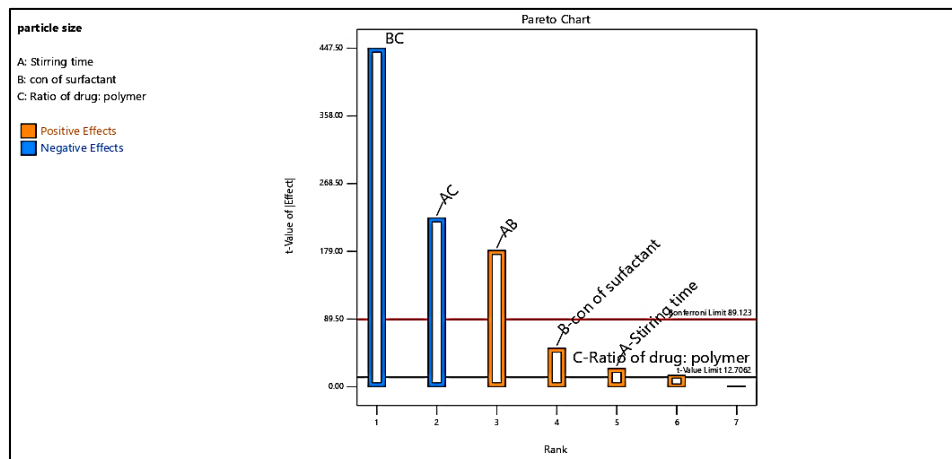

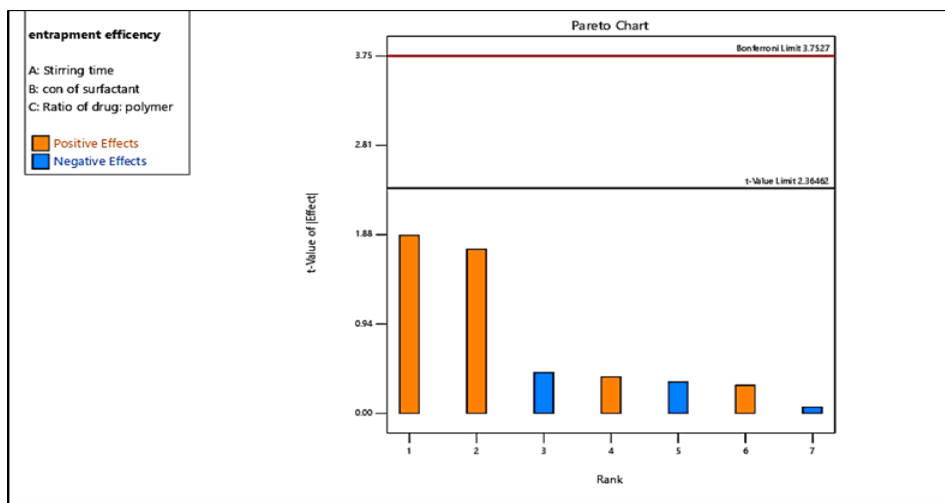**Fig. 10. Pareto chart showing significant factors affecting particle size**

Fig. 11. Pareto chart Showing positive and negative factors affecting entrapment efficiency
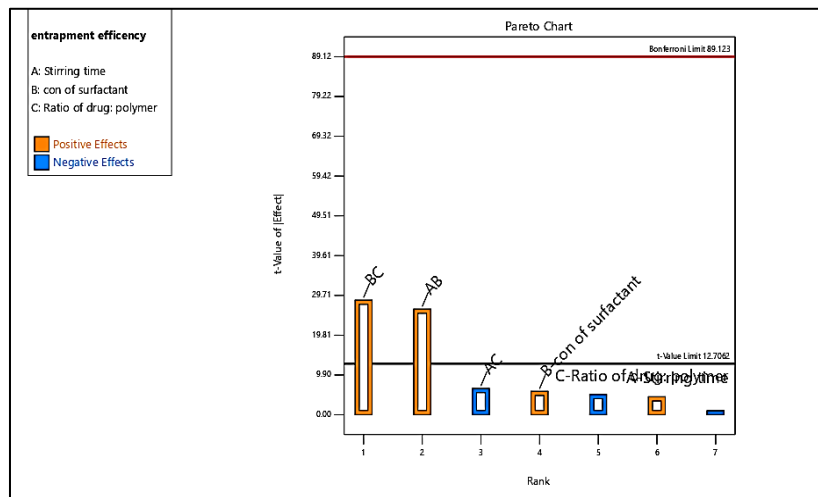
297

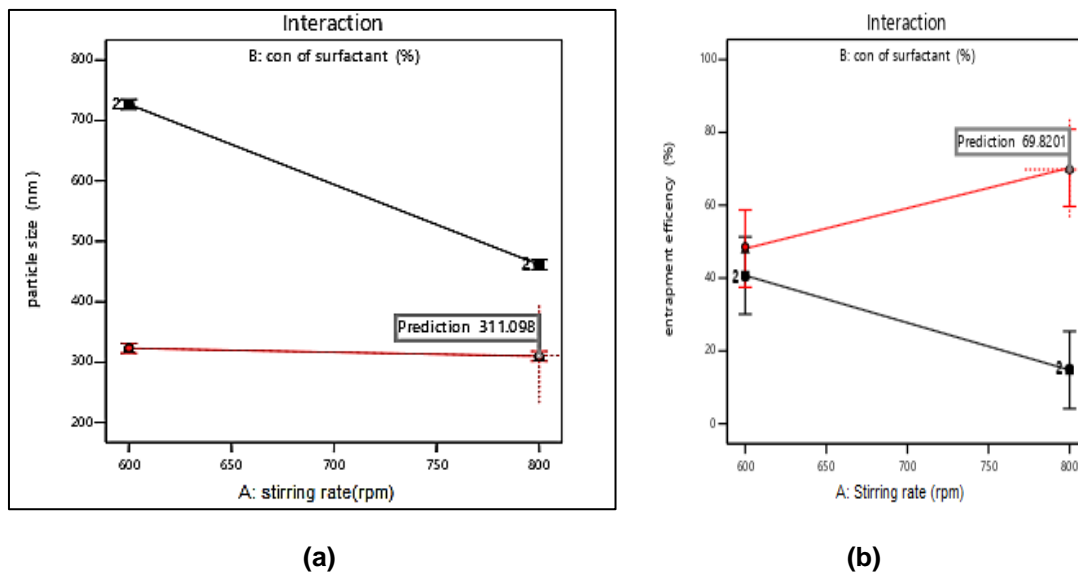**Fig. 12. Pareto chart showing significant factors affecting entrapment efficiency**

| (a) | (b) |

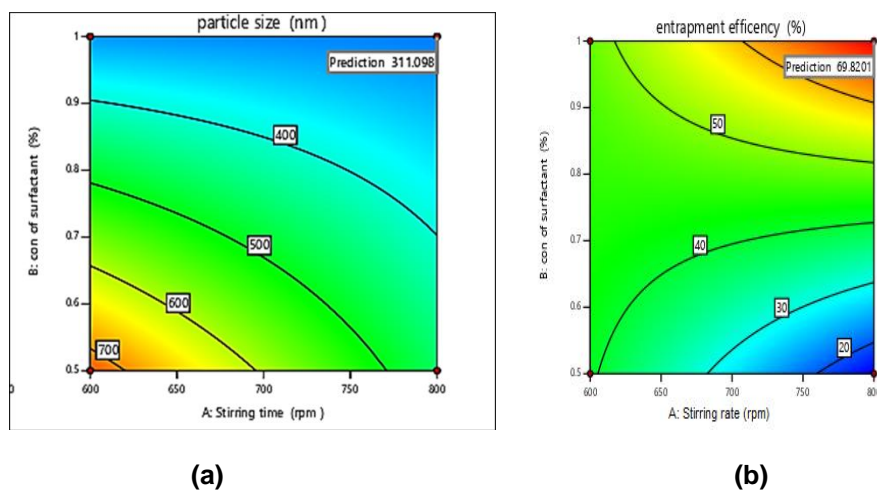**Fig. 13. Interaction plot showing factors affecting (a) Particle Size (b) Entrapment Efficiency**

| (a) | (b) |

Fig. 14. Contour plot for (a) Particle size (b) Entrapment Efficiency

Table 13. Results of predicted batch and challenge batch

BATCH NO.	Stirring rate	Con. of surfactant	The ratio of the drug: polymer	Particle size	% entrapment efficiency
Predicted	800	0.996	2:1	311.098	68.82
Actual	800	1	2:1	309.5	65.22

There is no significant difference in % entrapment efficiency and particle size of predicted batch and actual batch. This indicates that the speed time, number of cycles, and ratio of drug: polymer is optimized. This optimized batch was further selected and studied for drug release profile.

Evaluation of optimized nanoparticle batch

Particle Size and Polydispersity Index: The most important parameter, which needs to be monitored during nanoparticle formulation, is the particle size and size distribution of nanoparticles. The size and size distribution determines theirs *in vivo* or *ex vivo* performance. The mean particle size was found to be **309.5nm** for the optimized batch and the polydispersity index was found to be **0.554.** The low polydispersity index indicates particle size uniformity within the formulation (Fig. 15).

Zeta Potential: Another most important factor which needs to be evaluated during nanoparticle formulation is the zeta potential, which tells about the particle stability of the formulation. Particles with higher magnitude zeta potential exhibit increased stability due to a larger electrostatic repulsion between particles. The zeta potential for the optimized batch was observed at **+45.8mV** (Fig. 16).
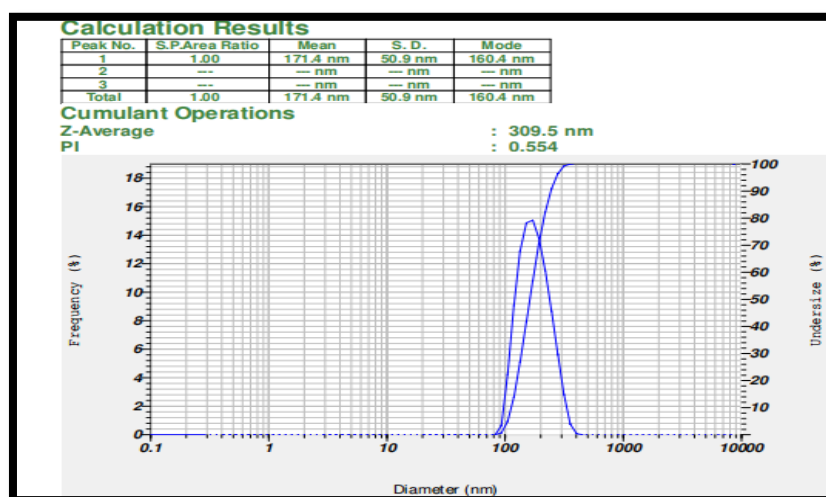
Surface morphology study using SEM: SEM was performed on the optimized curcumin analogue loaded nanoparticles. The results of SEM confirm the spherical structure of nanoparticles (Fig. 17).

***In -vitro* release diffusion studies:** In vitro release studies showed that the developed curcumin Analogue gel and CA-NP gel are capable to release the drug for 5 hours. Table 15 shows the results.

Table 14. Particle size and size distribution of the optimized batch

Batch	Particle size	PI
Optimized batch	309.5	0.554

Details of permeation studies for topical gel: The result of *in vitro* permeation studies of curcumin loaded gel and Curcumin loaded nanoparticle gel across cellulose membrane are shown in Table 16. The amount of curcumin released from both gel formulation shows a linear relationship with square root of time (r >0.9). The cumulative amounts permeated at each time interval for both formulation is shown in Table 16. The result suggests that curcumin loaded topical gel had a highest percentage of drug permeated after 5 hrs.

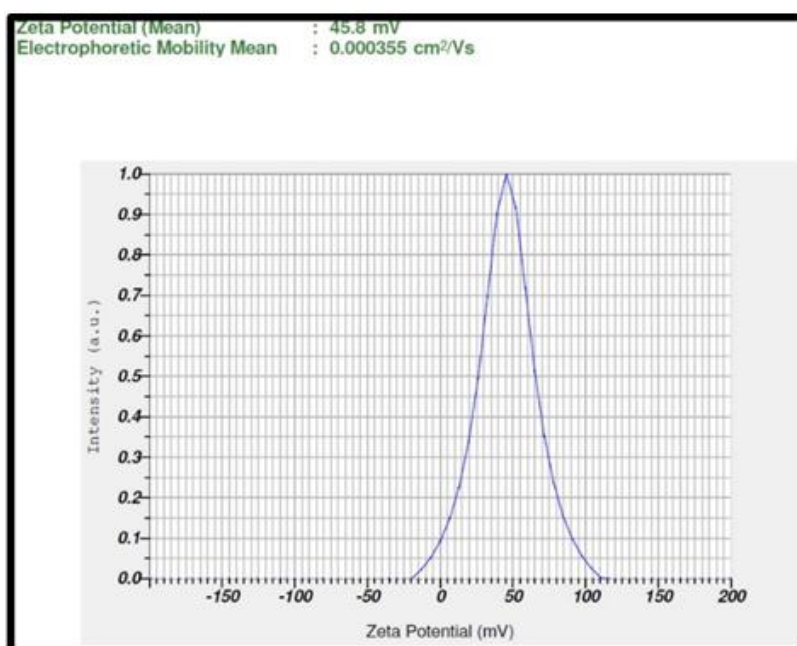**Fig. 15. Particle size distribution of optimized batch**

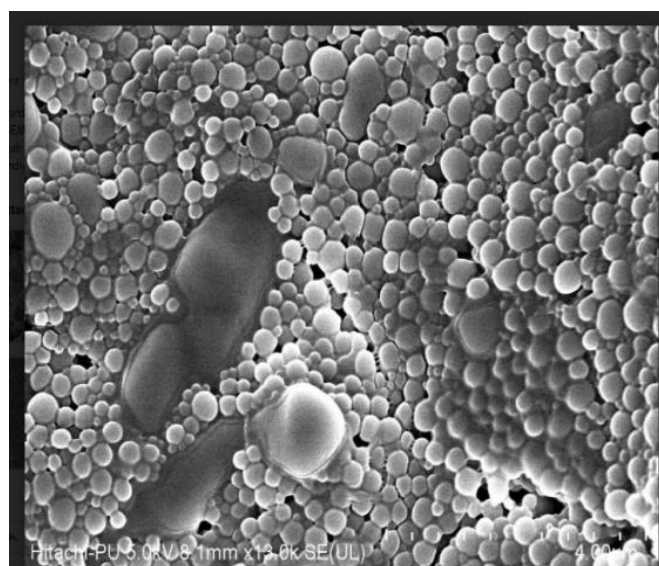**Fig.16. Zeta potential of optimized batch**

Fig 17. SEM Images of CA-NP

Table 15. *In-vitro* release

No.	Time (in min)	% Release	
		Curcumin gel	CA-NP gel
1	0	1	0
2	30	17.25	5
3	60	20.85	11.25
4	120	31.23	22
5	180	40.23	33
6	240	51.23	42
7	300	61.21	47.36

Table 16. Permeation studies

Topical gel	% drug release (ug cm^{-2} hr^{-1}) at 5 hrs.	P (cm hr^{-1})	k	r
Curcumin gel	61.21	0.024	146.53	0.9999
CA-NP gel	47.36	0.004	-428.71	0.9477

Table 17. Stability study data of formulations

Parameters evaluated	Initial	1st month	2nd month	3rd month	6th month
Physical appearance	Yellow and clear	No change	No change	No change	No change
Viscosity	3642	3642	3642	3637	3500
pH	4.72	4.72	4.68	4.71	4.70
Spreadability (gm.cm/sec)	34.20	34.18	34.20	34.16	34.15
Assay % w/w	100.60%	100.58	100.60	100.56	100.01

Stability studies: Stability studies of all prepared batches were performed by storing at $40^{o}C \pm 2\,C$ and 75% RH and room temperature. An optimized formulation was found to be stable over a specified period of testing. Stability studies results are summarized in the Table 16.

4. CONCLUSION

The current study aims to develop curcumin analogue gel loaded novel topical delivery systems for the treatment of skin cancer that would exhibit improved efficacy, stability and reduced toxicity over the existing anticancer products. After extensive literature survey approaches of nanocarriers with potential advantages over conventional anti-cancer therapy, curcumin analogue loaded nanoparticles (CA-NP) as the topical gel was found suitable and hence formulated.

A. Curcumin analogue loaded non-aqueous gel as topical gel

The drug is moisture sensitive so the novel approach was to formulate anhydrous non-aqueous gel. Simple dispersion method was used for the formulation of topical gel. The gel was developed for the improvement of topical applicability of anhydrous gel to localized active pharmaceutical ingredient site of action.

Formulating topical gel containing curcumin analogue offers a suitable practical approach to target localized delivery of the drug for skin cancer. The gel was prepared with successful incorporation in carbopol gel matrix made with simple dispersion method using different concentrations of Carbopol 934. Carbopol 934 was used for the preparation of non-aqueous topical gel. Different concentrations like 1%, 1.5% 2% 2.5% and 3% of Carbopol-934 were tried for development of non-aqueous gel. Depending upon the appearance and consistency of the gel, the concentration of 1.5% was finalized for the development of non-aqueous gel formulation.

All the parameters such as physical appearance, drug content (100.60%), pH (4.72), viscosity (3642cps), spreadability (34.20 gm/cm sec^{-1}) were studied and found within the acceptable range.

The formulations exhibited satisfactory antioxidant activity which can be related to the anticancer potential of the formulated topical gel. The formulated gel was found to be stable under specified conditions over 6 months.

B. Curcumin analogue nanoparticle (CA-NP) loaded non-aqueous gel as topical gel

The present study aimed to formulate CA-NP for topical delivery which can sustain the release of the drug and limits its elimination by enhancing the residence time at the topical surface of affected skin. For the preparation of CA-NP solvent evaporation method was used. PLGA was used as a polymer for the encapsulation of curcumin analogue. Based on the results obtained from preliminary trials method was finalized. Nanoparticle preparations were formulated considering various factors that affect the particle size and entrapment efficiency using factorial design. After selection of factors, nanoparticles were formulated using varying Drug: polymer ratio, stirring rate, and concentration of surfactant during optimization studies with two levels of three-factor design. Few batches were selected based on entrapment efficiency (60-75%) and mean particle size 250-

320 nm) and batch with the highest desirability was selected for incorporation in topical gel formulations.

FUTURE SCOPE

This project work can be made more superior by focusing on following areas:

1. Experimenting with selection of another polymer and surfactant.
2. Exposing activity of synthesized analogue to other cells.
3. New dosage form such as GRDD as it found stable at gastric pH during preformulation studies.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

ACKNOWLDEGEMENT

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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