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FORMULATION AND EVALUATION OF CHLORELLA PYRENOIDOSA LOADED SOLID LIPID NANOPARTICULATE CREAM

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ABSTRACT

Chlorella pyrenoidosa(CP), a nutraceutical has poor aqueous solubility resulting in restricted bioaccessibility. Literature survey reveals that β carotene a natural antioxidant found in the CP exhibits improved lipid solubility and thus formulating a lipid based delivery system of it would be advantageous for drug targeting in the treatment of cancer. The objective of the present investigation was therefore to formulate and evaluate *Chlorella pyrenoidosa* loaded solid lipid nanoparticle (SLN) cream for breast cancer. The SLN were formulated by hot high shear homogenization and ultrasonication and evaluated for Fourier transform Infra Red Spectroscopy (FTIR), in vitro drug release (%DR), particle size (PS), zeta potential (ZP), Scanning Electron Microscopy (SEM). FTIR confirmed absence of drug-excipients interactions, %DR was 77.122 ± 0.043 %, particle size was 292.2 nm, zeta potential was -22.9 mV and SEM revealed spherical shape of the particles. The CP loaded SLN were incorporated into cream base and evaluated for % drug content, FTIR, viscosity, spreadability, in vitro drug release respectively. The % drug content of CP loaded cream was 0.172 ± 0.002 mg/100mg, viscosity, spreadability and in vitro drug release were 4.6 pascals, 22.64 gm/cm/s, 57% respectively. The anticancer activity of *Chlorella pyrenoidosa* loaded topical cream were evaluated on human cancer cell lines, MCF-7, with positive anticancer activity with 50% inhibition at 526.67 ± 1.1 (μ g/ml). Thus it can be inferred that *Chlorella pyrenoidosa* loaded solid lipid nanoparticles cream can be used topically for breast cancer.

INTRODUCTION

An evolution has taken place with functional foods containing nutrients since food is being linked to cure for diseases. New emerging technologies such as particle size reduction to nanoparticles and nanotechnology offer a solution for delivering these nutraceuticals by improving their stability and bioavailability. Due to the increased attention towards natural products available from marine or fresh water sources, especially algae products, having natural antioxidants (beneficial in cancer) and which are safe for consumption by humans, but have poor solubility a 'nano' mode of delivery can be adopted to improve efficacy of supplements. *Chlorella pyrenoidosa* dietary supplement is one such promising agent. Few researches in Japan suggest that Chlorella- Green algae could be effective in fighting major life style diseases. It is thought to boost the immune system and help fight against infection. It is also claimed that it has an anticancer activity. β carotene is a natural antioxidant found in the algae *Chlorella pyrenoidosa*, that has been reported to exhibit improved lipid solubility. CP is insoluble in water. It releases the constituents within its cell wall matrix on digestion. Literature survey reveals that β carotene a natural antioxidant found in the CP exhibits improved lipid solubility and thus formulating a lipid based delivery system of CP would be advantageous for drug targeting in the treatment of cancer.

Cancer is a class of disease characterized by out-of-control cell growth and is ultimately the result of cells that uncontrollably grow and do not die. More than any other cancer, breast cancer is an emotional disease, which for women is a depressive condition, often demanding mutilation, followed by aggressive treatment, a long period of painful illness, of hope, recurrence and death. It is obvious that breast cancer requires an alternative approach to the historical treatment options of surgery, chemotherapy and radiation. Topical administration can serve an alternative approach. The literature survey reveals very few work has been conducted on topical preparation for breast cancer. Topical administration of drugs is still a challenge in pharmaceuticals and drug delivery due to the difficulties in controlling and determining the exact amount of drug that reaches the different skin layers. The objective of the present investigation was therefore to formulate and evaluate (CP) loaded solid lipid nanoparticle cream for breast cancer.

MATERIALS AND METHODS

Materials:

Chlorella pyrenoidosa and Cetomacrogol 1000 was obtained as gift from Sun Chlorella corporation Japan, β Carotene (marker) purchased from Sigma Aldrich, Germany, Dynasan 118 (Solid lipid), L- α - Phosphatidylcholine (Co- surfactant and lipid stabilizer) was purchased from Sigma Aldrich, Germany. Poloxomer 188 (surfactant), Cetostearyl Alcohol, Glycerine and parabens was purchased from Reasearch Lab., Fine Chem Industries, Mumbai.

Methods:**1. Preformulation Studies****2. Organoleptic Characteristics**

CP, β carotene all the other excipients were evaluated for organoleptic properties like colour, odour and appearance.

3. Acute oral toxicity studies in mice (LD 50) ⁽¹³⁾

The Acute oral toxicity studies were carried out as per OECD Guidelines 423. Fifteen Swiss albino female mice, healthy, nulliparous, non-pregnant, weighing in the range 20 ± 2 gm were divided in three groups (i.e. five mice per group) were taken under housing condition.

The mice were acclimatized for 5 days, fasting 4 hours prior to dosing the animals CP suspension was prepared in honey and sterile water (1:2:2). Observation period was maintained of 14 days. The initial body weight, weekly and 14th day body weights were recorded. Mortality, observation for gross CNS effect and any general symptoms such as ill health and behavioural changes were also observed. At the end of 14th day the animals were humanely sacrificed and vital organs such as heart, liver and kidney were dissected and histopathological observations were recorded.

4. Analytical Method to confirm the presence of β Carotene (Marker) in the Drug***Thin layer Chromatography (TLC) ⁽¹⁴⁻¹⁶⁾***

Thin layer chromatography was carried out to determine the presence of the marker β carotene in CP sample obtained from Sun Chlorella when compared to the pure β carotene obtained from Sigma Aldrich, Germany.

a) Development of Mobile Phase

Development of mobile phase was based on trial and error method. Depending upon the polarity of the compound various combinations and ratios of polar and nonpolar solvents were used for the development of mobile phase. In each mobile phase, TLC plate carrying spot of β Carotene (Marker) and CP was run and then R_f values were compared.

b) TLC Analysis:

1 μ g/ml solution of β Carotene (Marker) was prepared in ethanol. CP was triturated in ethanol in order to solubilise the contents. The supernatant was filtered and stored in tightly closed vial. TLC plates precoated silica gel aluminium plate 60 F254 with 250 μ m thickness; (E. MERCK, Darmstadt, Germany) of 10cm x 1cm and 10cm x 3cm were used. TLC plates were activated in oven at 60°C for 10 min prior to chromatography. Chamber saturation time with 10 ml mobile phase was 10 min at room temperature ($30 \pm 1^\circ$ C) and RH ($60\% \pm 5$). Spots of β carotene and the CP ethanol solution were applied at 2cm above from the end of the plate to be dipped in the

mobile phase on individual activated plates of 10cm x 1cm. The plates were then carefully placed into the saturated chamber containing the mobile phase and allowed to run till a particular distance on the plate. The plates were then carefully removed, air dried and observed visually. Yellow coloured spots referred to β Carotene of all the spots obtained for the CP sample. The Rf values were measured for both in each tried mobile phase and compared. The mobile phase that exhibited good agreement between the Rf values of both the samples were further selected and TLC separation was carried out again in those mobile phases using the activated 10cm x 3cm plates. This time both the spots were applied on the same plate separated by 1cm distance in order to avoid the mixing. The plates then placed in the saturated chambers of the selected mobile phases and allowed to run till a particular distance after which they were removed, air dried and observed. The Rf values of these spots were calculated using the following equation:

$$RfValue = \frac{\text{Distance compound has moved from origin}}{\text{Distance of solvent front from origin}}$$

5. Solubility studies ⁽¹⁷⁾

Excess amount of CP was added separately to 50ml of various solvents such as DW, methanol, ethanol, different buffers pH 1.2, pH 6.8, pH 7.4, DMSO, hexane, cyclohexane and chloroform in volumetric flask and kept on the (Whirlmatic Shaker) for 24 hours and then observed visually. Solubility of pure β carotene (marker) in these solvents was checked in the similar manner.

a) *Determination of solubility of β Carotene (marker) in Phosphate Buffer Solution (PBS) pH 7.4 and Ethanol*

Accurately weighed 10 mg of β Carotene was added to 10 cleaned and dried volumetric flasks each of 10 ml capacity. Phosphate Buffer pH 7.4-ethanol solvent system was added in the ratio 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 and the samples were shaken for 1 hour on orbital linear shaker. The solutions were checked visually for their clarity. Triplicate readings were taken and average was calculated.

6. Spectroscopic Studies ⁽¹⁷⁻¹⁸⁾

a) **Determination of λ max of β Carotene (marker) in Ethanol and PBS pH 7.4: Ethanol (3:1) system.**

The ultraviolet absorption spectrum of a solution of β carotene in the two solvent systems was obtained by scanning for maximum absorbance between 200 to 600 nm on Double-beam UV spectrophotometer J V-530 Jasco Corporation, Tokyo, Japan.

b) Calibration curve of β Carotene (marker) in Ethanol and PBS pH 7.4: ethanol system⁽¹⁹⁾

The calibration curve of β carotene was constructed by measuring its absorbance on UV-VIS (double beam 2000 Spectrophotometer, Jasco Corporation, Tokyo, Japan) in the range 0.1 $\mu\text{g/ml}$ to 1 $\mu\text{g/ml}$ solution at the λ max 450 nm and 452 nm respectively.

7. Drug content (β Carotene Content in CP)

Accurately weighed 100 mg of the CP was added to 10 ml of ethanol and was centrifuged at 1200 rpm for 10 min using a cooling centrifuge (REMI, Mumbai, India) followed by filtering the contents from tube using 0.45 μm millipore filter and taking absorbance at 450 nm to analyse β carotene content.

8. Compatibility Studies⁽¹⁷⁾

CP-excipients interaction was studied by FTIR spectroscopy. The spectra were recorded for pure CP and excipients mixture using FTIR Spectrophotometer (Model No. FTIR4100 Jasco Corporation Tokyo, Japan).

9. Factorial Batches

The SLNs of CP were prepared by hot high shear homogenization and ultrasonication using Dynasan 118 (solid lipid), Poloxamer 188 (surfactant) and L- α - Phosphatidylcholine (co-surfactant). The selection of solid lipid, surfactant and co-surfactant ratio was based on emulsification property and done by plotting pseudoternary phase diagram. Accurately weighed CP (3%) was placed in beaker containing molten solid lipid (2.5%) at 75°C. Surfactant and co-surfactant (Smix km = 3:1, 6.5%) were added subsequently, followed by making up the volume upto q.s. 100 ml with double distilled water also maintained at 75°C. The components were homogenized at 75°C using Ultra Turrex IKA T25, Remi Motors Ltd, RM-12C Mumbai at 3000 rpm for 30 mins to get microparticulate dispersion. This was subjected to ultrasonication using Probe Sonicator, PS 150, Orchid Scientifics and Innovates India Pvt. Ltd. to form SLNs by varying the intensity and time of sonication as given in Table I. The batches were passed through a 0.45 μm Millipore filter and further evaluated for in vitro drug release.

10. Evaluation of Factorial Batches⁽²¹⁻²³⁾

All the nine batches were evaluated for the % in vitro drug release. A modified dialysis method was used to evaluate the in vitro release of SLNs of CP. A dialysis bag (cellophane membrane, molecular weight cut off 10,000–12,000, Hi-Media, India) was soaked overnight in the dissolution medium. To the pre-swollen dialysis bag, 2 ml of SLN formulation was placed (corresponding to 0.108 mg of β carotene) and both the ends of bag were tied to prevent any leakage. Later, dialysis bags were carefully placed in the volumetric flasks containing the

dissolution medium of 100 ml of PBS pH 7.4 – ethanol system (7:3), which was stirred continuously at 100 rpm using Magnetic stirrer TH-100 Whirlmatic, Mega, 6 stations, Spectra lab, India and maintained at 37°C for 12 hrs. The receptor compartment was closed to prevent evaporation of the dissolution medium. At selected time intervals, aliquots were withdrawn and assayed spectrophotometrically for β carotene at 452 nm.

11. Formulation of Optimized Batch

The effect of method variables on the responses were statically evaluated by applying one-way ANOVA at 0.05 levels, using the commercially available software package Design-Expert version 8.0.7.1 in order to optimize the formulation parameters. On the basis of the results obtained optimized batch was formulated in the similar manner as the factorial batches with 100% Sonication Intensity and 90 min Sonication Time. Optimized batch was evaluated for appearance, % EE, *Invitro* drug release, particle size, zeta potential, SEM, DSC, PXRD, FTIR and in vitro cytotoxicity assessment (Anticancer cell line studies).

12. Evaluation of Optimised batch

Various parameters were evaluated for optimised batch which include appearance, % Entrapment Efficiency, Cumulative % Drug release, Particle Size Analysis, Zeta Potential, Scanning Electron Microscopy, Particle X-Ray Diffraction, Differential Scanning Calorimetry, and Stability Studies.

a) *Appearance*

The optimized formulation was evaluated for its final appearance.

b) *In vitro* release (Cumulative % Drug Release)

It was carried out in same manner as that of the factorial batches.

c) *Particle Size Analysis* ⁽²¹⁾

Mean particle size and size distribution of optimised batch of SLN was determined by dynamic light scattering using Zetasizer Ver. 6.34 (Malvern instrument Ltd., Malvern, UK) at 25°C.

d) *Zeta Potential* ⁽²¹⁾

Charge on drug loaded droplet surface was determined using Zetasizer Ver. 6.34 (Malvern Instruments Ltd., Malvern, UK). Analysis time was kept for 60s and average ZP, charge and mobility of optimized batches of polymeric nanoparticles was determined at 25 °C

e) *Scanning Electron Microscopy*

SEM analysis for surface morphology of the prepared formulation was carried out using scanning electron microscope (SEM, JSM- 6330 TF, Jeol, Tokyo, Japan). The samples were vacuum-dried and sputter-coated with platinum with accelerating voltage of 8 kV for 90 s.

f) Differential Scanning Calorimetry⁽²⁴⁾

The melting behavior of pure CP, Dynasan 118, physical mixture of CP and excipients optimized SLN batch was studied using DCS SII Nanotechnology (SIEKO), model/ series: DSC 6220. Accurately weighed 10 mg of sample and alumina filled in aluminium pan was placed in sample and control compartment of furnace. Samples were heated from 30 to 300°C increase at 10 C / min under constant flushing with nitrogen gas, flow rate is adjusted to 50 ml/min.

g) Particle X-Ray Diffraction⁽²⁴⁾

X-ray scattering measurements were carried out with a Philips PAN analytical expert PRO X-ray diffractometer 1780. A Cu Ka α radiation source (1.542 Å) was used; the scanning rate was 5°C/min. The sample was analysed between 2 and 50° (2 θ). The voltage and current were used 30 kV and 30 mA, respectively. X-ray diffraction measurements were carried out on pure CP, Dynasan 118, physical mixture of CP and excipients and optimized SLN batch.

h) Fourier Transform Infrared Spectroscopy (FTIR) of Chlorella Pyrenoidosa (Drug)

Optimized CP loaded SLNs was subjected to FTIR studies (Model No. FTIR-4100 Jasco Corporation Tokyo, Japan) for the purpose of characterization.

i) Anti Cancer - Cell Line Studies (In- Vitro Assay)⁽²⁶⁻²⁸⁾

An anticancer activity of CP and its SLNs was performed to determine its cytotoxicity on two human cancer cell lines, MCF-7 (Breast adenocarcinoma) and SiHa (squamous cell carcinoma; Cervix). The cytotoxicity of the crude drug (solubilised by sonication in neat DMSO) and its SLN formulation against the MCF-7 cells and SiHa cells was determined using the colorimetric MTT assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)].

j) In vitro cytotoxicity assessment

Two human cancer cell lines MCF 7 (Breast adenocarcinoma), SiHa (squamous cell carcinoma; Cervix), were obtained from National Animal Cell Repository at National Center for Cell Science, Pune. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 2mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin and maintained at 37°C in humidified 5% CO₂ atmosphere. For cytotoxicity evaluation, cells were trypsinized and the cell suspension containing 1 x 10⁴ cells were seeded into each well of 96 well microtitration plates. The plates were incubated at 37°C in humidified 5% CO₂ atmosphere for 24 hrs. to allow the adherence of cells prior to administration of various drug samples for testing.

h) Drug application

After 24 hrs. of incubation, cells were treated with 6.3 µg/ml – 1000 µg/ml concentrations of CP solution in DMSO and its SLN formulation in multiple wells of microtest plates. To evaluate possible effect of DMSO on cell viability, cells were also treated with similar concentrations of DMSO. Equal amount of PBS was added to wells which served as control. After addition of all test samples, plates were incubated in 5% CO₂ atmosphere for next 48 hr. The cytotoxic effect was analysed using MTT assay.

i) MTT assay

10 µl of 5 µg/ml MTT was added to all the wells of the test plates and plates were incubated in dark for 5 - 6 hrs. About 100 µl of DMSO and 25 µl of glycine buffer were then added to dissolve the formazan crystals resulting from the reduction of the tetrazolium salt by the metabolically active cells. The absorbance was measured at 540 nm using a micro-plate reader (BIO-RAD, Model 680). Since the absorbance directly correlates with the number of viable cells, cell survival is measured as absorbance (OD) of the mean of the replicate wells compared to that of control. IC₅₀ values, defined as the concentration of the drug that killed 50% of cells in comparison with the untreated cultures, were estimated by plotting OD readings versus the drug concentrations. Assays were repeated three times to confirm the results.

13. 3² Factorial formulation.

The effect of the formulation variables on the responses was evaluated by 3² factorial designs for optimization of the cream. It contained two independent variables at three levels +1, 0 and -1. The different independent variables include: (homogenization time and homogenization speed). The selection of the optimized batch out of the nine batches was on the basis of responses like viscosity and % drug release (Q₂).

14. Evaluation of 3² Factorial Batches

Evaluations of all the Factorial batches F₁ to F₉ was done for viscosity and In-vitro % drug release (Cumulative % Drug Release).

a) Viscosity

Viscosities measure the flow characteristics of a topical formulation. Changes in the viscosity of the product are indicative of changes in the stability or effectiveness of the product. Brookfield digital viscometer was used to measure the viscosity of prepared cream formulations. The spindle no. 6 was rotated at 10 rpm. The reading, near to 100 % torque was noted. Samples were measured at 37 ± 1 °C.

b) *In-vitro* Drug Diffusion Study

In-vitro drug release studies on Factorial batches were performed by using a modified Franz diffusion cell with a receptor compartment capacity of 15 ml. The synthetic cellophane membrane was mounted between the donor and receptor compartment of the diffusion cell. The formulated cream was weight up to 1g and placed over the drug release membrane and the receptor compartment of the diffusion cell was filled with phosphate buffer pH 7.4 and ethanol (7:3). The whole assembly was fixed on a magnetic stirrer, and the solution in the receptor compartment was constantly and continuously stirred using magnetic beads at 400rpm; the temperature was maintained at 37 ± 0.50 °C. The samples of 1 mL were withdrawn at time interval of 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450 and 480 minutes and analyzed for drug content spectrophotometrically at 450 nm against blank. The receptor phase was replenished with an equal volume of phosphate buffer and ethanol(7:3) at each time of sample withdrawal. The cumulative amounts of drug diffused from cream were plotted against time.

15. Formulation of Optimized Batch

The effect of method variables on the responses were statically evaluated by applying one-way ANOVA at 0.05 levels, using the commercially available software package Design-Expert version 8.0.7.1 in order to optimize the formulation parameters. On the basis of the results obtained optimized batch was formulated in the similar manner as the factorial batches with 45 min homogenization time and 1500 homogenization speed. Optimized batch was evaluated for appearance, viscosity, pH, spreadability, *In vitro* drug release, and *in vitro* cytotoxicity assessment (Anticancer cell line studies).

16. Evaluation of optimized batch**a) *Appearance***

Appearance was observed visually of optimised batch i.e. F9.

b) *Viscosity*

Brookfield digital viscometer was used to measure the viscosity of prepared cream formulations (F9). The spindle no. 6 was rotated at 10 rpm. The reading, near to 100 % torque was noted. Samples were measured at 37 ± 1 °C.

c) *Measurement of pH*

The pH of F9 formulation is due to its ingredient present in it, and not due to the addition of pH adjusting agent. The pH values of optimised formulation were found in the range 5 to 6.5. Hence the formulations lie in the normal pH range of the skin and would not produce any skin irritation.

d) Spreadability

Spreadability of the F9 formulation was determined by an apparatus, which was suitably modified in the laboratory and used for the study. It consists of a wooden block and provided with a pulley at one end. A rectangular ground glass plate was fixed on the wooden block. Excess of cream (about 2gm) under study was placed on this ground plate, and then the cream was sandwiched between this plate and another glass plate having the dimensions of the ground plate attached with a hook. A 300gm weight was placed on the top of the two plates for 5 minutes to expel air and to provide a uniform film of the cream between the plates. Excess of the cream was scrapped off from the edges. The top plate was then subjected to a pull of 30gm with the help of a string attached to the hook and the time (in seconds) required by the top plate to cover a distance of 10 cm was noted. The spreadability was calculated using the formula

$$S = ML/T$$

S = Spreadability

M = Weight tied to upper glass slide l = length of glass slide T = Time taken in second

e) *In-vitro* Drug Diffusion Study

In-vitro drug release studies of F9 Batch were performed by using a modified Franz diffusion cell with a receptor compartment capacity of 15 ml. The synthetic cellophane membrane was mounted between the donor and receptor compartment of the diffusion cell. The formulated cream was weight up to 1 g and placed over the drug release membrane and the receptor compartment of the diffusion cell was filled with phosphate buffer pH 7.4 and ethanol (7:3). The whole assembly was fixed on a magnetic stirrer, and the solution in the receptor compartment was constantly and continuously stirred using magnetic beads at 400 RPM; the temperature was maintained at 37 ± 0.50 °C. The samples of 1 mL were withdrawn at time interval of 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450 and 480 minutes and analyzed for drug content spectrophotometrically at 450 nm against blank. The receptor phase was replenished with an equal volume of phosphate buffer and ethanol (7:3) at each time of sample withdrawal. The cumulative amounts of drug diffused from cream were plotted against time.

f) Anticancer cell line study

3-(4,5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E. Merck Ltd., Mumbai, India.

MCF-7 (Breast carcinoma) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

For Cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

RESULT AND DISCUSSION

In the present work an attempt was made to explore the anticancer potential of CP, to improve its bioaccessibility in general and β Carotene in particular by formulating it as a Solid Lipid Nanoparticulate and thus incorporate it into cream base. CP, β Carotene and excipients were evaluated for organoleptic characteristics as depicted in Table I.

The Acute oral toxicity studies were carried out as per OECD Guidelines 423. Observation included absence of significant alteration in body weight, mortality, gross changes in behaviour or general body conditions in test group mice as compared to control group. The histopathology examination of heart, liver and kidney were performed and the results have been shown in Figure 1(a) and 1(b) for the control and test groups. The images indicate normal histological observations of Heart, Liver and kidneys in control as well as the test groups and an absence of any evidence of toxic injury in heart, liver and kidney. The results also depict that GHS Category- 5 (Globally Harmonised System-mg/kg) i.e. LD - 50 in mice is more than 5000mg/kg of supplied sample of CP and that the safe dose of CP is in the range of 2000 mg – 5000mg/ kg.

The TLC was performed to determine the presence of the β carotene (marker) in the CP sample. The Rfvalue for CP ethanol solution as well as β carotene (marker) were compared. It was observed that the Rfvalue obtained for the yellow spot on the CP sample plate (0.74) was closer to that of the marker (0.76), in the presence of mobile phase consisting of Petroleum Ether, Hexane and Ethanol in the ratio 2:1:1 respectively.

λ_{max} of β Carotene in Ethanol and PBS pH 7.4 -Ethanol System (7:3) was determined by scanning between 300-600 nm in UV spectrophotometer. The maximum absorbance was observed at 450 nm in ethanol and 452 nm in PBS pH 7.4 - Ethanol System (7:3). The visual

solubility study of CP and β Carotene was performed in different solvents and buffer systems such as DW, methanol, ethanol, different buffers pH 1.2, pH 6.8, pH 7.4, DMSO, hexane, cyclohexane and chloroform respectively. The results have been presented in the Table II. The solubility of β Carotene was observed in the solvent system PBS pH 7.4 and Ethanol in different ratios. The ratio of 7:3 to 1:9 exhibited an excellent visual solubility of β Carotene. 7:3 ratio was selected to minimize the concentration of organic solvent during dissolution studies. The content of β Carotene in CP was calculated and was found to be $0.172 \pm 0.002 \text{ mg}/100 \text{ mg}$ which is in close proximity with the value reported in the literature (0.1808 mg).

The compatibility between CP and the selected lipid, surfactant, co-surfactant, cetomacrogol 1000, cetostearyl alcohol, liquid parafins and parabens were evaluated by FTIR peak matching method. FTIR spectra are mainly used to determine if there is any interaction between the drug and any of the excipients used. The existence of an interaction is detected by the disappearance of an important functional groups. As depicted in Figure 3(a) and 3(b),3(c) the FTIR spectrum of drug CP was characterized by bands at 931 cm^{-1} (-C-O, -C-S, -C-N bonds present), 1223.61 cm^{-1} (Alkene and Aromatic ring present), $1660.41, 1853.26 \text{ cm}^{-1}$ (Carbonyl group of Aldehyde, Ketone, Acid, Ester present), $2364.3 - 2794.66 \text{ cm}^{-1}$ (-C=N, -C=C, -C-H bonds present) and $3661.19 - 3171.36 \text{ cm}^{-1}$ (Alcoholic -OH, -NH bonds present). After careful inspection of the spectra of the physical mixture of CP with Dynasan 118, Poloxomer 188, L- α -Phosphatidylcholine, cetomacrogol 1000 and cetostearyl alcohol the absorption peak between $3661.19 - 3171.36 \text{ cm}^{-1}$ due to presence of Alcoholic -OH, -NH bonds were found to be at lower intensity by the presence of these ingredients, however the activity of the whole compound as well as the activity of characteristic groups of drug were unaffected. This finding confirms an absence of any interaction between CP with any of the excipients.

SLN of CP were successfully formulated by employing hot high shear homogenization and ultrasonication technique using triglycerides such as Dynasan 118 as the solid lipid and Poloxomer 188 and L- α Phosphatidylcholine as surfactant phase. Of all the factorial batches evaluated for in vitro drug release, F1 batch which showed the highest % release of $77.122 \pm 0.043 \%$. The optimized batch prepared was further evaluated using various parameters like appearance, In vitro drug release, particle size, zeta potential, SEM, DSC, PXRD, FTIR and in vitro cytotoxicity assessment (Anticancer cell line studies). The optimized batch showed 8 β carotene release of more than 50 % within 2 hours and $68.47 \pm 0.062 \%$ at the end of 48 hours from SLN; higher as compared to its release from CP suspension. Photon correlation spectroscopy (PCS) is a technique used to determine the mean particle size diameter (mean PCS

diameter)/ Z average and the width of the particle size distribution expressed as Polydispersity Index (PDI). The measurement using PCS is based on the light scattering phenomena in which the statistical intensity fluctuations of the scattered light from the particles in the measuring cell are measured and the Particle Size Distribution is represented as Intensity. The data indicates that 68.8% nanoparticles were of 598.1 nm and that 31.2% nanoparticles exhibited 151.8 nm size. The mean particle size diameter/Z-average was 292.2 nm size. PDI shows the particle size distribution. PDI of 0.321 was obtained for optimized colloidal nanoparticle dispersion – Solid Lipid Nanoparticles. Mean particle size and size distribution of optimised batch of SLN was determined by dynamic light scattering using Zetasizer Ver. 6.34 (Malvern instrument Ltd., Malvern, UK) at room temperature. Charge on drug loaded droplet surface was determined using Zetasizer Ver. 6.34 (Malvern Instruments Ltd., Malvern, UK). Analysis time was kept for 60s and average ZP, charge and mobility of optimized batches of polymeric nanoparticles was determined. The measurement was performed at 25°C. In colloidal systems according to electric double layer theory, there is a net balance of attractive as well as repulsive forces. Zeta potential imparts positive or negative charge on surface of colloidal particle and one of these two forces may play its role. Zeta potential for Nanoparticles like SLN, Nanoemulsion, Nanostructured Liquid Carrier etc. should possess zeta potential either in positive or in negative side but more than 20 mV that is either $> +20$ mV or > -20 mV. Then it is considered as a deflocculated system. If it is between $+20$ to -20 mV, there are chances for instability of system, because attractive forces increase as compared to repulsion forces. The Zeta Potential Optimized batch of CP loaded SLN was found to be -22.1 mV. Therefore, it can be inferred that the colloidal dispersion possess negative charge on the surface. To obtain more information about the particle size and the shape of freeze dried SLN SEM analysis was performed. The micrograph revealed spherical SLN with smooth surface as seen in Figure 5. DSC, PXRD and FTIR graphs exhibit the entrapment of CP in SLN as depicted in the Figures 6(a), 6(b) and 6(c) respectively

Preparation of trial batches of CP loaded SLN cream

Out of three batches batch **A** was found to be stable compare to **B** and batch **C** was rejected due to phase separation the one which was found the best was selected and factorial is applied on it, as depicted in table III. Thin layer chromatography was performed to determine the presence of the β carotene (marker) in the CP sample. The Rf value for CP ethanol solution as well as β carotene (marker) were compared. It was observed that the Rf value obtained for the yellow spot on the CP sample plate (0.68) and in cream (0.66) was closer to that of the marker (0.71), in the presence of mobile phase consisting of Petroleum Ether, Hexane and Ethanol in the ratio 2:1:1 respectively.

Brookfield digital viscometer was used to measure the viscosity of prepared cream formulations 2.5 pascals. The pH of formulation is due to its ingredient present in it, and not due to the addition of pH adjusting agent. The pH values of all formulations were found in the range 5 to 6.5. Hence all the formulations lie in the normal pH range of the skin and would not produce any skin irritation. In-vitro Drug Diffusion study, for it was 34%

Evaluations of all the Factorial batches F_1 to F_9 was done for viscosity and In-vitro % drug release (cumulative % drug release). Viscosity, Brookfield digital viscometer was used to measure the viscosity of prepared cream formulations. The spindle no.6 was rotated at 10rpm. The reading, near to 100% torque was noted. Samples were measured at $37 \pm 1^\circ\text{C}$. Batch F_9 was found to have maximum % Drug release i.e. with increase in viscosity there is an increase in % Drug release as depicted in fig 7. In-vitro drug release studies were performed by using a modified Franz diffusion cell with a receptor compartment capacity of 15ml and depicted in fig 8. Optimization by Design – Expert, the effect of method variables on the responses were statically evaluated by using the commercially available software package Design-Expert version 8.0.7.1 in order to optimize the formulation parameters. On the basis of the results obtained optimized batch was formulated in the similar manner as the factorial batches with 45 mins. homogenization Time and 1500 rpm homogenisation speed. Evaluations of optimised batch F_9 was performed for viscosity, pH, In-vitro % drug release and anticancer activity study which showed a positive effect thus confirming its anticancer activity as depicted in fig 9, 10, 11, 12, and 13

CONCLUSION

From the present study it is concluded that CP loaded SLN cream is a very good carrier for sustaining the drug release for breast cancer. The studies stability of solid lipid nanoparticle in cream base are in progress.

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Table:**Table I: Organoleptic Properties of *Chlorella pyrenoidosa*, β Carotene (Marker) and Excipients**

Sr.no	Chemical Name	Colour	Odour	Appearance
1	<i>Chlorella pyrenoidosa</i>	Dark Green	Pleasant	Dark Greenish to black powder
2	Beta-Carotene	Dark Red	No odour	Dark red coloured powder
3	Dyansyl 118	White	No odour	White Free Flowing Granules
4	Poloxamer 188	White	No odour	White Flakes
5	L- α - Phosphatidyl Choline	Yellowish	Characteristic	Yellowish Free Flowing Granules
6	Cetomacrogol 1000	Creamish	No odour	Cream coloured pellets.
7	Cetostearyl Alcohol	White	No odour	White pellets
8	Glycerin	Colourless	No odour	Colourless liquid
9	Liquid Paraffin	Colourless	No odour	Colourless liquid
10	Parabens	White	No odour	White free flowing powder

Table II: Visual Solubility Studies of *Chlorella pyrenoidosa* and β Carotene

Sr. No.	Solvents	Observation	
		<i>Chlorella pyrenoidosa</i>	β Carotene
1.	Distilled Water	Slightly Soluble	Practically Insoluble
2.	Methanol	Slightly Soluble	Slightly Soluble
3.	Ethanol	Slightly Soluble	Soluble
4.	PBS pH 1.2	Slightly Soluble	Slightly Soluble
5.	PBS pH 6.8	Slightly Soluble	Slightly Soluble
6.	PBS pH 7.4	Slightly Soluble	Slightly Soluble
7.	DMSO	Slightly Soluble	Slightly Soluble
8.	Hexane	Slightly Soluble	Soluble
9.	Cyclohexane	Slightly Soluble	Soluble
10.	Chloroform	Slightly Soluble	Slightly Soluble

Table no III: Trial Batches for O/W Cream of SLN of *Chlorella Pyrenoidosa*

Ingredients	Cream A	Cream B	Cream C
Drug	3%	3%	3%
Oily phase			
Cetosteryl alcohol(w/w)	7%	-	-
Cetomacrogol 1000(w/w)	2.5%	2%	2.5%
Liquid Paraffin(w/w)	10%	-	10%
Stearic acid(w/w)	-	8.5%	-
Glyceryl Monostearate(w/w)	-	9%	-
Bees wax(w/w)	-	-	6%
Water phase			
Methyl paraben(w/w)	0.18%	0.2%	0.2%
Propyl paraben(w/w)	0.02%	-	-
Glycerine(w/w)	3%	3%	3%
Disodium EDTA(w/w)	-	0.05%	0.05%
Water Q.S to(w/w)	100%	100%	100%

Table IV: 3² Factorial Batches

Factorial Batch	Coded value		Actual value	
	X ₁	X ₂	Homogenization speed (rpm)	Homogenization Time(min)
F ₁	-1	-1	500	15
F ₂	0	-1	1000	15
F ₃	+1	-1	1500	15
F ₄	-1	0	500	30
F ₅	0	0	1000	30
F ₆	+1	0	1500	30
F ₇	-1	+1	500	45
F ₈	0	+1	1000	45
F ₉	+1	+1	1500	45

Table V: Responses of 3² Factorial Batches

Batches	Appearance	Cum %Drug Release	Viscosities (pascals)	pH	Spreadability (gm.cm/s)
F ₁	Light Greenish	50.11	1.57	5.9	11.21
F ₂	Light Greenish	52.13	2.8	6.1	13.11
F ₃	Light Greenish	51.43	2.2	6	13.50
F ₄	Light Greenish	49.19	1.4	6.1	14.90
F ₅	Light Greenish	53.23	2.32	6.2	15.28
F ₆	Light Greenish	49.14	1.7	6	15.70
F ₇	Light Greenish	54.04	2.94	6.4	15.64
F ₈	Light Greenish	49.95	1.14	6.2	22.79
F ₉	Light Greenish	57.01	4.631	6.5	22.98

Table VI: Cytotoxic Properties of Test Drugs Against MCF-7 Cell Line

Sr. No	Name of test sample	Test conc. (µg/ml)	% Cytotoxicity	CTC ₅₀ (µg/ml)
1.	RR 1757	1000	75.53±1.2	526.67±1.1
		500	48.28±0.5	
		250	13.05±1.5	
		125	10.52±1.9	
		62.5	5.05±0.4	

Figures**Figure 1(a): Histopathology Images of Heart, Liver and Kidney of Control Group**

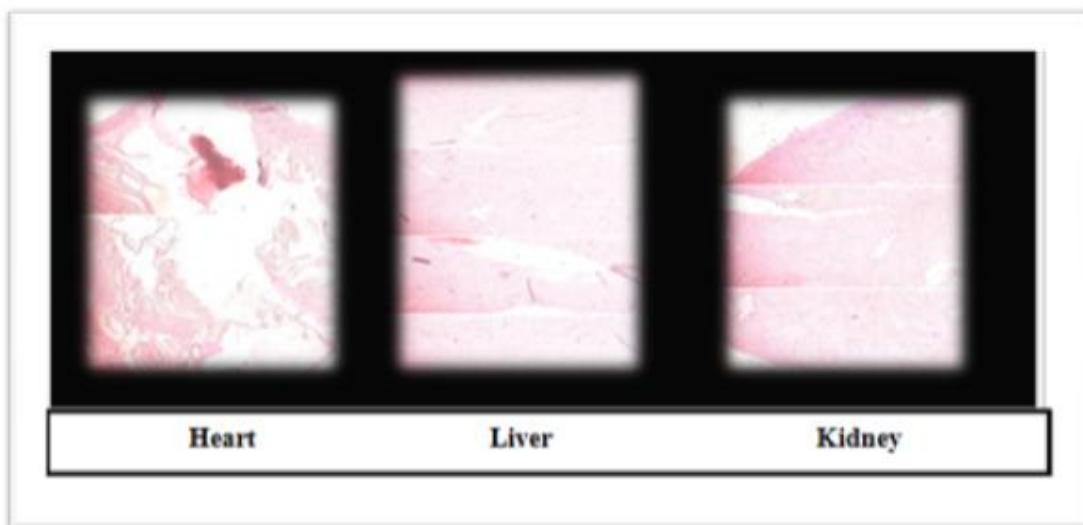


Figure 1(b): Histopathology Images of Heart, Liver and Kidney of Test Group

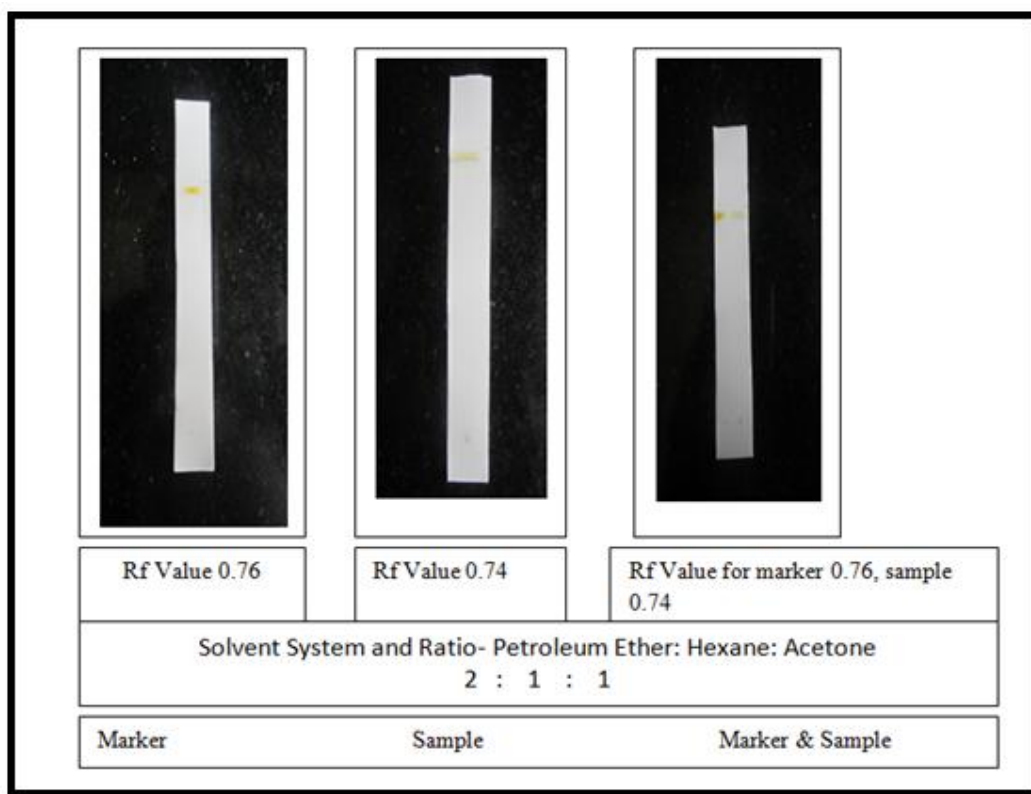


Figure 2: TLC of β Carotene (marker) and *Chlorella pyrenoidosato* determine the presence of β carotene (marker) in the drug.

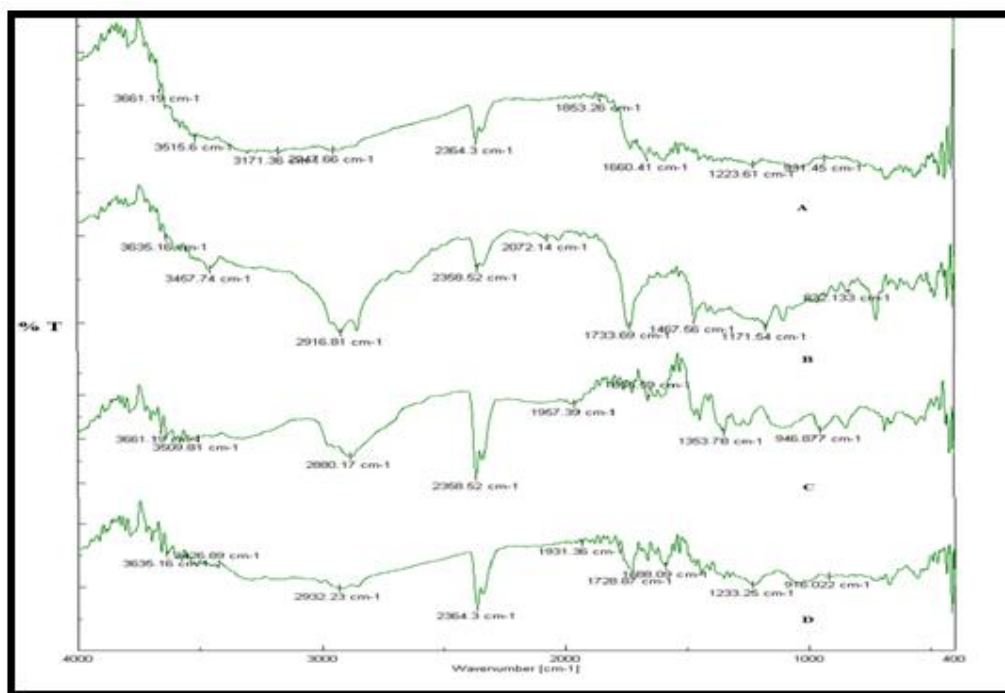


Figure 3(a) : Drug and excipient Compatibility –Initial (A= *Chlorella pyrenoidosa* B= Drug +Dynasan 118, C=Drug +Poloxomer 188, D= Drug + L- α -Phosphatidylcholine)

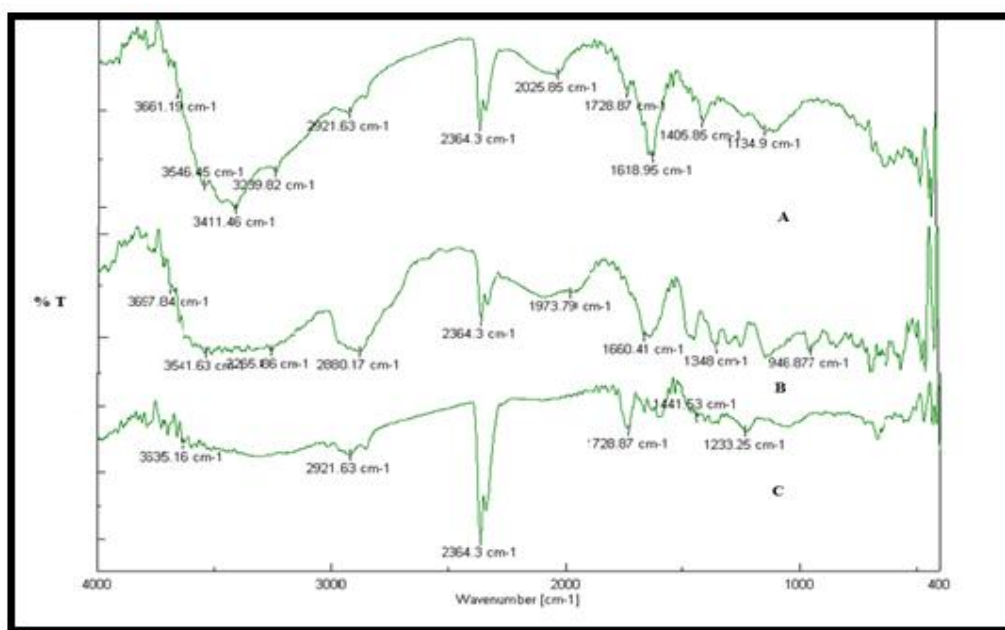


Figure 3(b): Drug and excipient Compatibility - After 1 month (A= Drug +Dynasan 118, B=Drug +Poloxomer 188, C= Drug + L- α -Phosphatidylcholine)

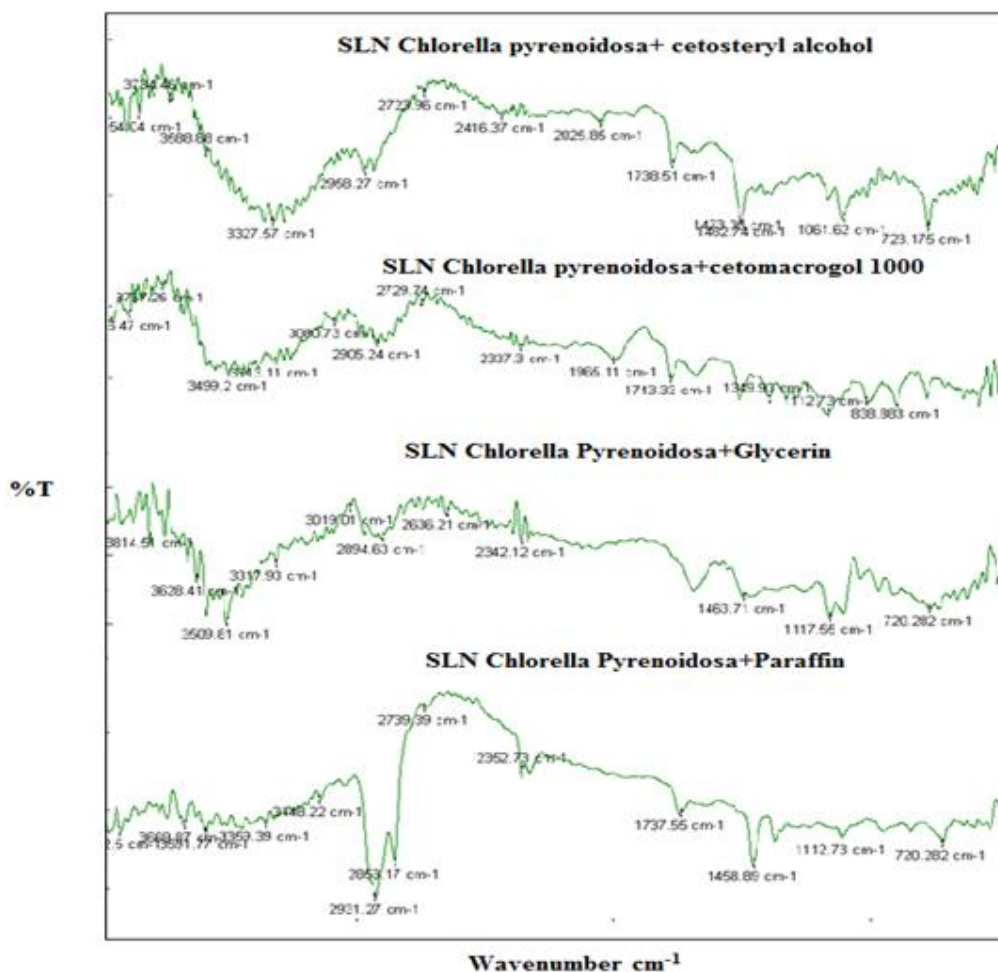


Figure 3(c): Drug and Excipients Compatibility Studies

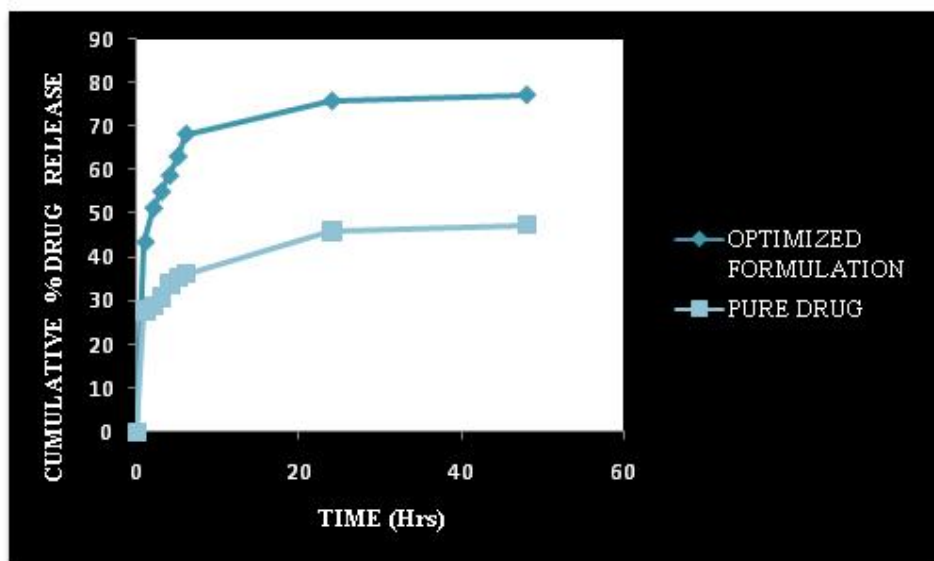


Figure 4: Cumulative % Drug Release of Optimized batchand Pure Drug CP

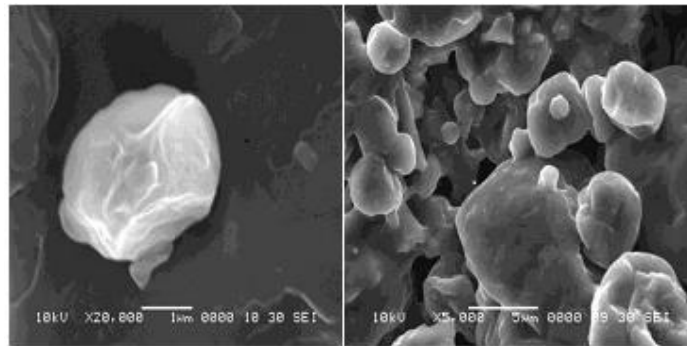


Figure 5: Scanning Electron Microscopy of Optimized SLN

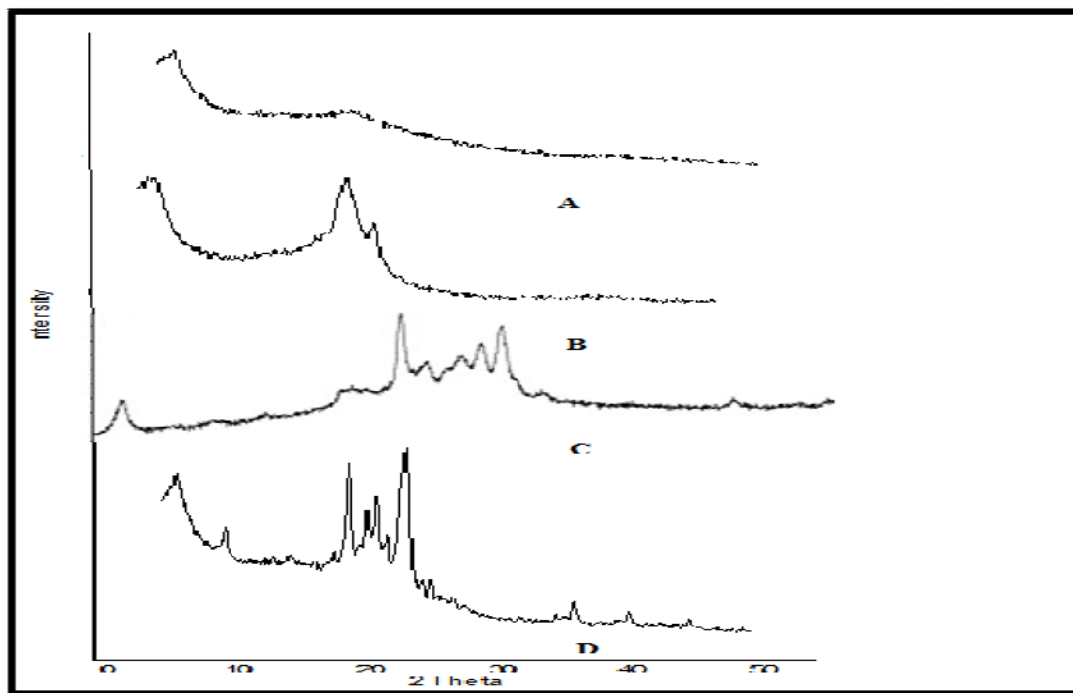


Figure 6(b): X-ray diffractograms of pure drug *Chlorella Pyrenoidosa* (curve A), Dynasan 118 (curve B), physical mixture of drug and excipients (curve C), Drug loaded SLN (curve D).

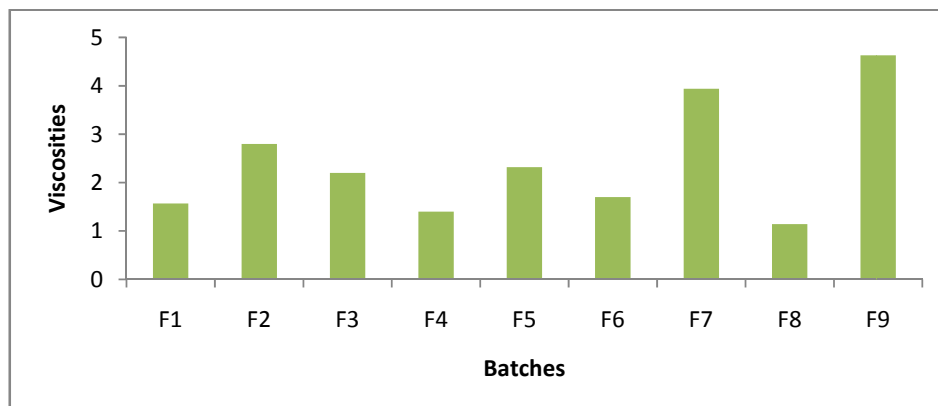


Figure 7: Viscosities of Formulation.

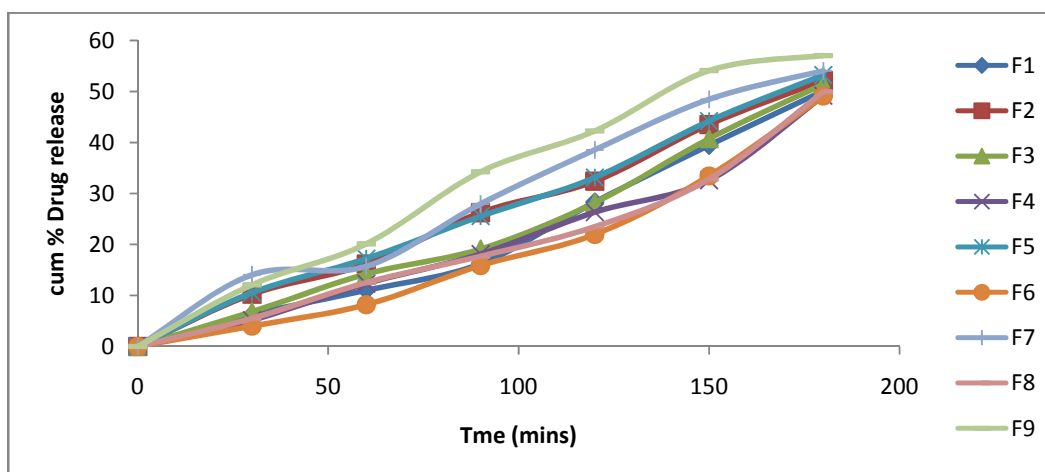


Figure 8: *In vitro* % cumulative drug release from CP loaded SLN cream batches.

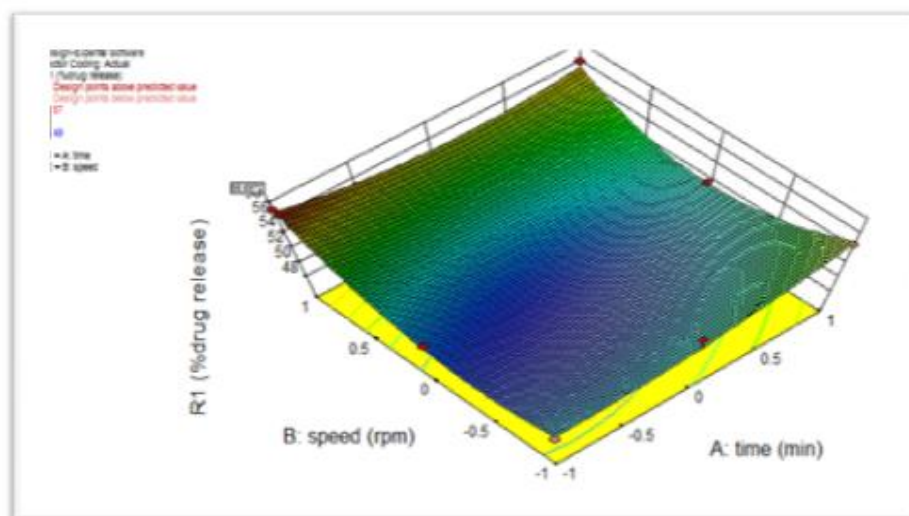


Figure No 9: Response Surface Plot Showing Effect of Formulation Variables on %Drug Release (Y_1) of CP SLN Loaded Cream.

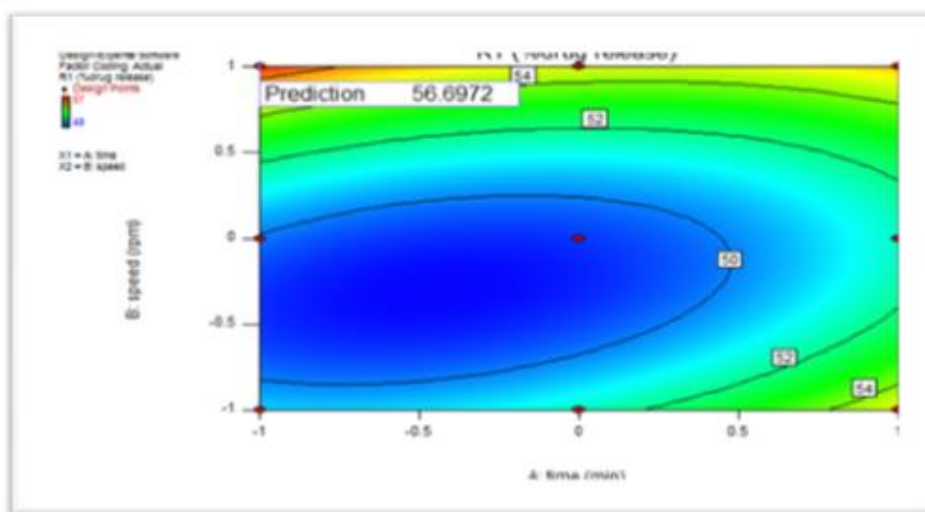


Figure No.10: Contour Plot Showing Effect of Formulation Variables on %Drug Release (Y_1) of CP SLN Loaded Cream.

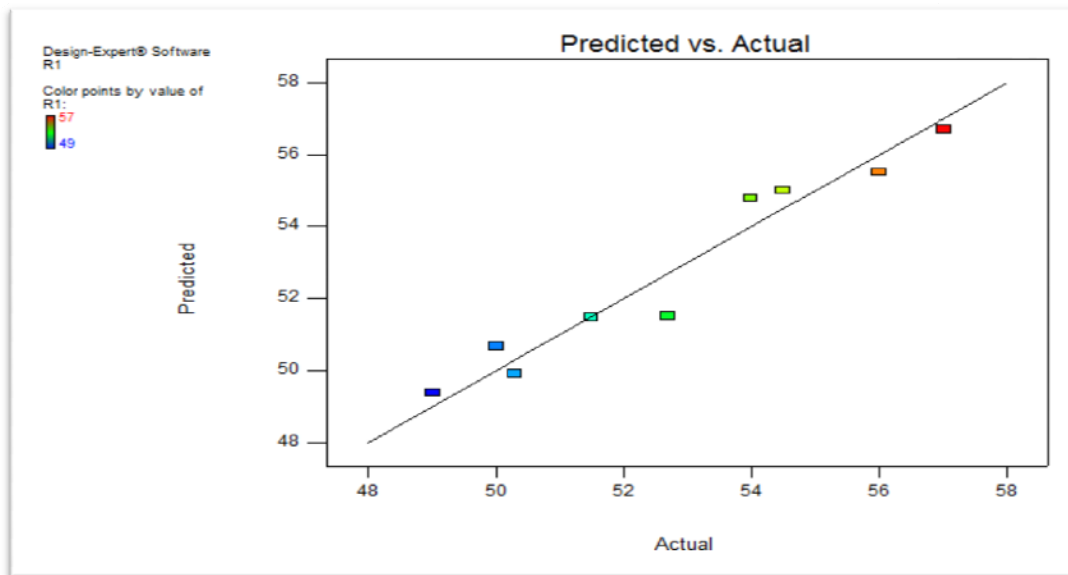


Figure No.11: Correlation Between Actual and Predicted Values for %Drug Release (Y_1) of CP SLN Loaded cream.

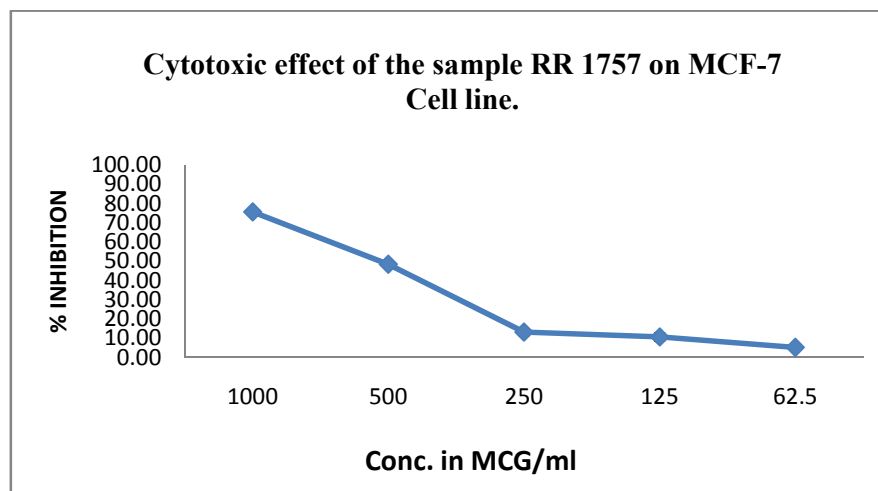
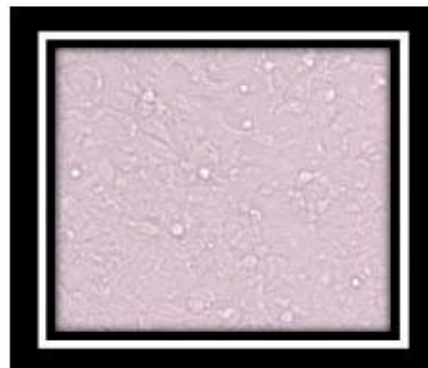
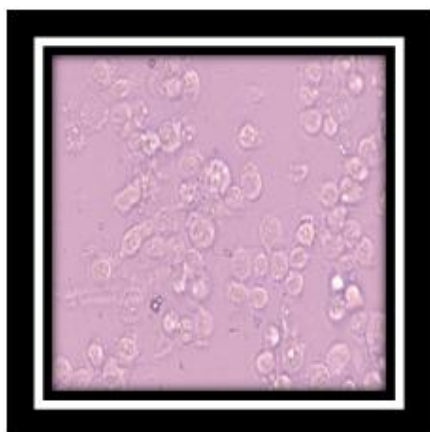


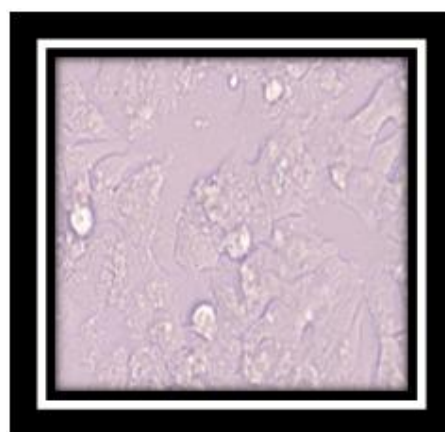
Figure 12: Anticancer Cell Line Study of Optimized Batch



MCF-7 Control



RR 1757 – 1000C



RR 1757 – 500C

Figure 13 :Images of Anticancer Cell Line Studyof Optimized Batch.