RESEARCH PAPER

Design and Development of Natural Vitamin-E Enriched Nanoemulsion Fabricated With Natural and Synthetic Surfactants for the Management of Melasma

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ABSTRACT

BACKGROUND: Melasma is a chronic hyperpigmentation disorder frequently linked to UV exposure, hormonal shifts, and genetic factors. While traditional topical therapies exist, synthetic surfactants often induce skin irritation, driving the demand for biocompatible alternatives.

OBJECTIVE: This study aimed to design and optimize a natural Vitamin E (Tocotrienol)-based nanoemulsion to improve delivery efficacy while minimizing skin irritation.

METHODS: Two nanoemulsion prototypes were developed via the spontaneous emulsification method, utilizing either the natural alkyl polyglucoside, Decyl Glucoside, or the synthetic polysorbate, Tween 80, as stabilizers. Characterization was performed using Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM), and DPPH antioxidant assays.

RESULTS: The Decyl Glucoside formulation exhibited superior physicochemical properties, with a smaller droplet size (11.58 ± 0.773 nm) and higher kinetic stability (zeta potential: -33.5 ± 0.323 mV) compared to the Tween 80 system (18.55 ± 0.816 nm; -23.6 ± 0.767 mV). The invitro drug release studies using a Franz diffusion apparatus demonstrated a sustained release profile (95% over 12 hours). *Ex vivo* permeation studies stated that the formulation with Decyl Glucoside formulation achieved significantly higher skin permeation (82%) than the synthetic alternative (75%). Furthermore, *in vitro* assays in murine melanoma cells revealed enhanced tyrosinase inhibition for the natural surfactant-based system.

CONCLUSION: These findings suggest that incorporating natural surfactants like Decyl Glucoside into nanoemulsions not only improves formulation stability and skin penetration but also enhances bioactivity and safety, offering a promising sustainable approach for cosmetic melasma treatments without compromising natural skin barrier.

Keywords: *Tocotrienols; Decyl glucoside; Emulsion; Tyrosinase inhibition; Formulation; Skin permeation.*

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INTRODUCTION

Melasma, formerly termed "chloasma," is an acquired pigmentary disorder primarily affecting the facial region (1). The condition is multifactorial, with key etiological drivers including ultraviolet (UV) exposure, hormonal fluctuations, genetic predisposition (particularly among first-degree relatives), specific cosmetic ingredients, autoimmune disorders, pregnancy, and certain pharmacological agents (2). Due to its high prevalence during gestation, it is frequently colloquially labeled the "mask of pregnancy." Clinically, melasma manifests as symmetrical, well-defined, yet irregularly bordered macules and patches ranging from brown to grey (3).

Epidemiological data suggest a 1% prevalence in the general population, which escalates significantly to 9–50% within high-risk groups (4). This variation is attributed to geographic location, UV intensity, and the prevalence of darker phototypes within specific ethnic populations (5). The condition exhibits a strong female predilection, with a reported female-to-male ratio of approximately 9:1 (6). While not life-threatening, melasma exerts a profound impact on the emotional and psychological well-being of affected individuals (6).

The Mechanism of Melanogenesis

The skin's pigmentation is governed by melanin, produced through the complex biochemical process of

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melanogenesis within melanocytes (8, 9, 10). This process is primarily regulated by the enzyme **tyrosinase**, which catalyzes the initial rate-limiting steps: the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and the subsequent oxidation of L-DOPA to L-dopaquinone (11). Consequently, the inhibition of tyrosinase is a primary therapeutic target for mitigating melanin accumulation (12, 3).

Limitations of Current Therapies

Current management of melasma is often recalcitrant to treatment, typically requiring a multimodal approach including UV/visible light protection, depigmenting agents, and aggressive interventions such as laser therapy, chemical peels, or cryotherapy (7). However, conventional treatments can yield significant adverse effects. For instance, prolonged use of hydroquinone is associated with risks of localized toxicity, paradoxical depigmentation, vitiligo-like hypochromia, and exogenous ochronosis (15, 16). Thus, there is an urgent need for safer, more effective whitening agents (17).

Nanotechnology and Tocotrienols

To achieve clinical efficacy, topical formulations must bypass the stratum corneum (SC) barrier and ensure controlled penetration into the melanocyte-containing epidermal layers (13). Nanotechnology offers a robust solution by optimizing drug delivery and modulating permeation profiles through controlled release (14).

Tocotrienols, a subfamily of Vitamin E, have emerged as potent antioxidant candidates for this purpose (18). Research indicates that tocotrienols and their isomers effectively downregulate the expression of tyrosinase (TYR) and tyrosinase-related protein (TYRP/TRP) genes, thereby reducing cellular melanin content (19, 18).

Study Objectives: Nanoemulsion Stabilization

This study aims to develop and stabilize a tocotrienol-based nanoemulsion for melasma treatment. To prevent droplet aggregation and ensure stability, surfactants and co-surfactants are essential (20). We compared two prototypes:

1. **Natural Surfactant:** Decyl Glucoside, a plant-derived, non-ionic surfactant (21, 22).
2. **Synthetic Surfactant:** Tween 80.

Decyl glucoside offers several advantages, including superior biodegradability, skin mildness, and a lower environmental footprint compared to synthetic alternatives like Tween 80, which may cause skin sensitivity (21, 23, 24). The developed formulations were characterized based on morphology, physicochemical stability, *in vitro* skin permeation, antioxidant capacity, tyrosinase inhibition, and cytotoxicity.

MATERIALS:

Natural Vitamin-E (Tocotrienols) and Medium Chain Triglycerides (MCT) were obtained as a complimentary sample from Orah Nutrichem Pvt. Ltd., Pimpri Chinchwad, Maharashtra. Tween 80 was procured from Loba Chemia Pvt. Ltd., Mumbai, India and Decyl glucoside from InnoVision Cosmochem Solutions, New Delhi, India as a gift sample. Biogod was procured from Godrej Chemicals, India. All the other chemicals used in the current study were of analytical grade.

METHODOLOGY:

HPLC-UV Analysis

The UV spectrophotometric method was used to determine the maximum wavelength of Vitamin-E. (1) HPLC spectrophotometric analytical method development for estimation of Vitamin-E was initiated with the following specifications: The HPLC Agilent-1100 series with Detector-UV VIS Dual Absorbance Detector Agilent-2487. The Hypersil ODS (OctaDecylSilane) C-18 column (150 mm × 4.6 mm having 5.0 µm particle size equilibrated with a mobile phase consisting of acetonitrile to water (95:5, v/v)) was used. There was no need of pH adjustment of mobile phase. The flow rate was maintained at 0.8 ml/min, and column was set to room temperature. Eluents were detected using a UV-VIS Dual Absorbance Detector at maxima wavelength i.e. 295.0 nm.(2) The analytical method developed was then validated as per ICH Q1A (R2) for following parameters: linearity and range, accuracy, precision, robustness, LOD, LOQ, system suitability studies.(3)

Screening of surfactants and co-surfactants

Miscibility studies were carried out for the pre-screening of oils, surfactants and co-surfactants. Vitamin E was mixed with various oils, surfactants and co-surfactant in ratio of (1:1) and vortexed. They were kept for 72 hours on orbital shaker and incompatibility was noted. The solutions that showed miscibility were selected for further studies.(4)

Emulsification Ability

Emulsification ability is the potential of surfactants and cosurfactants to emulsify with oil. Oil and S-mix (Surfactant to co-surfactant ratio) were taken in a test tube in the ratio 1:1 and homogenized at 45-60°C. 50 mg of this mixture was diluted to 50 ml with water in a volumetric flask. Kept standing for 2 hours. % Transmittance was determined at 638.2 nm with water as blank.(5)

Construction of Pseudo Ternary Phase Diagram

Based on the results of miscibility and emulsification studies, specific oils, surfactants, and co-surfactants were combined in varying proportions to form a nanoemulsion. To define the optimal concentration ranges for these components, **pseudo-ternary phase diagrams** were constructed using the water titration method.

The surfactant to co-surfactant ratio (Smix) was investigated at weight ratios of 1:1, 1:2, and 2:1. Mixtures of oil and (Smix) were prepared in weight ratios (% w/w) ranging from 9:1 to 1:9 in separate test tubes. Distilled water was then added to these mixtures in 0.1 ml increments; following each addition, the samples were vortexed for 2 to 3 minutes and visually inspected for turbidity or phase separation.

The volume of water required to induce turbidity was recorded, and the precise percentages of all incorporated components were calculated. These data points were used to plot the pseudo-ternary phase diagrams using **Chemix School 12.00 software**. From these diagrams, the clear and stable nanoemulsion zones were identified, providing a template for the successful formulation of stable nanoemulsions. (30)

Preparation of Nanoemulsion

The formulation which showed maximum nanoemulsion region with minimum surfactant and maximum water content was selected. Spontaneous Emulsification method was adopted for the preparation of nanoemulsion batches. (6) The spontaneous emulsification method is advantageous due to its simplicity, cost-effectiveness, and ability to produce nanoemulsion with small particle sizes, high stability, and uniformity without requiring high-energy input. (7) The surfactant to the co-surfactant mixture (S-mix) was separately prepared by dissolving the required amounts of surfactant (Tween-80) or (Decyl Glucoside) and co-surfactant (Biogod) in aqueous phase. The active drug Natural Vitamin-E (tocotrienol) was added to the MCT oil under continuous stirring on a vortex mixer. Both the phases were heated at 75℃. The aqueous phase was added to the oil- phase containing the drug and mixed on a magnetic stirrer for about 10–15 min until clear emulsion was obtained. These emulsions were further optimized and then subjected to thermodynamic studies.(6)

Statistical Optimization of the Formulation Parameters

Optimization of nanoemulsion was carried out using Design Expert software. Design Expert is a piece of software designed to help with the design and interpretation of multi-factor experiments. It helps us design an experiment to see how a property such as particle size varies with changes in the processing conditions (32) - e.g. changes in surfactant concentration. Here we are using factorial design. It is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. (8) The factors are evaluated at various levels. 3^2 factorial design was developed. A total of 9 formulations of Natural Vitamin-E nanoemulsion were prepared by varying two independent variables Drug: oil concentration and surfactant: cosurfactant concentration. Its effect was observed on the dependable variables such as particle size and zeta potential.(9)

Physical Characterization of Nanoemulsion

Visual Observation: Nanoemulsion batches were observed visually for clarity, transparency and phase separation.

Percent transmittance: The Percent transmittance of the batches was examined by UV spectrophotometry at 638.2 nm, taking distilled water as a blank. The formulations were checked for their clarity by making appropriate dilutions with water and their transmittance was checked.(10)

pH: pH of the nanoemulsion batches was determined by using a digital calibrated pH meter. 1g of the nanoemulsion was diluted with 10ml milli-pore water. The pH of the formulation was determined in triplicates.(4)

Fourier Transform Infrared Spectroscopy (FT-IR) Studies

FTIR studies were performed for Vitamin-E using Shimatzu IR Affinity-IS Fourier Transform Infrared Spectrophotometer.(11) A small amount of drug was placed on flat plate prism and the spectrum was recorded in the scanning range of 4000-400 $cm^{-1.}$ The graphs were identified, and interpretation for each peak was obtained at a specific wavenumber. The drug excipient interaction was also recorded by mixing the excipients.

Globule size and Polydispersity index: The Globule size and Polydispersity index of prepared nanoemulsion batches were determined by Malvern Zetasizer ZS90. The average globule size is based on the principle of photon correlation spectroscopy, which analyzes fluctuations in light scattering due to the Brownian movement of the particles. The Polydispersity Index (PDI) can range from 0 to 1, where 0 represents a monodisperse system and 1 represents a polydisperse particle system. The test samples were diluted in a ratio of 1:100 using purified water, and measurements were made at a 90°C angle at 25°C in triplicate.(12)

Zeta Potential: Particle charge determines the physical stability of the nanoemulsion. Particle charge is quantified as zeta potential value which is measured via electrophoretic mobility of particles in an electrical field. It helps predict the dispersion stability of the sample, which depends on the physicochemical properties of the drug, polymer, vehicle, presence of electrolytes, and their adsorption. Zeta potential of optimized formulation was measured using Malvern Zetasizer ZS90. The test samples were diluted in a ratio of 1:1000 using millipore water to obtain Zeta potential values in triplicates.(13)

Viscosity: Viscosity of nanoemulsion was determined by 64 Spindle at 10rpm using Brookfield Viscometer (Brookfield Engineering Laboratories).(4)

Transmission Electron Microscopy (TEM):

Transmission Electron Microscopy was used to determine the morphology, size and structure of the nanoemulsion batches.(14)

Drug content: Drug content of the prepared nanoemulsion batches were examined by taking 1g of nanoemulsion and diluting with ethanol, 100 times which was further analyzed by HPLC Spectrophotometer.(10)

Thermodynamic Stability of Nanoemulsion

Centrifugation Test

The selected nanoemulsion batches were subjected to centrifugation at 1500 rpm for 30 minutes. The

formulations not showing phase separation were taken for heating and cooling cycle.(15)

Heat and Cool Cycle
Formulations were kept between refrigerator temperature (5 °C ± 3°C) and 45°C for 48 hours each. Six such cycles were repeated. Stable formulations were further subjected to freeze thaw cycle test.(15)

Freeze thaw Cycle
Formulations were kept at -20°C for 24 hours. Formulations were removed and kept at room temperature for 24 hours. 3 cycles were repeated. Physically stable nanoemulsions returned to their original form within 2-3 minutes.(15)

Comparative Antioxidant Activity using DPPH Reagent
Total free radical scavenging capacity of the Vitamin-E and other antioxidant samples were estimated using the stable DPPH radical, which has an absorption maximum at 517 nm. Comparative antioxidant activity was performed using two solvents ethanol and ethyl acetate. The following formula was used to compute the percentage of antioxidants or radical scavenging activity:

% of antioxidant activity= [(Ac−As) ÷Ac] × 100

where: Ac—Control reaction absorbance; As—Testing specimen absorbance.(16)

Invitro Drug Release Study
To analyze the quantity of drug released the optimized formulations were subjected to diffusion study using dialysis bag. It was done using a Franz diffusion cell of 22 ml which has the donor compartment and the receptor compartment. The receptor compartment was filled with Phosphate buffer (PBS) pH 5.5 + Ethanol in 1:1 ratio. The temperature of the cell was maintained at 37°C ± 0.5°C with constant stirring at 300 rpm. 1 gram of formulation was placed in each donor compartment and covered. Aliquots were withdrawn at regular intervals (1, 2, 3, 4, 5, 6, 7, 8, 9, 10,11, 12 hours). The aliquots were further diluted with the acetonitrile and water mixture in (95:5) ratio. The amount of drug release was analyzed using HPLC Spectrophotometry. The receptor compartment was replenished with fresh medium to maintain sink conditions after each aliquot. Percent cumulative release was plotted as function of time. The study was carried out in triplicates and their mean values were reported.(17) To investigate the mechanism of drug release from the nanoemulsion, the release data was subjected to kinetic analysis. The invitro release studies were fitted into different kinetic models, such as zero order, first order, Higuchi and Korsmeyer-Pepppas.(18)

Ex-vivo skin permeation studies
The optimized formulations were subjected to an ex-vivo diffusion study on Franz diffusion apparatus using porcine ear skin as the membrane. Freshly excised porcine ear skin was obtained. Thin layers of skin samples were excised from the porcine ear. The skin samples were placed onto the Franz diffusion cell between the receptor and donor compartments, with the stratum corneum facing the donor compartment. The receptor compartment was filled with a release medium consisting of phosphate buffer pH 5.5+ ethanol (1:1). The temperature was maintained at 37°C ± 0.5°C with constant stirring at 200 rpm and allowed a pre-incubation time of 20 minutes. Formulation aliquots of 1 mL were collected at predetermined time intervals (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 14 hours) from the receptor compartment, filtered, and analyzed for drug content by HPLC. Fresh media were replenished at each sampling time point to maintain sink conditions. The study was performed for a duration of 14 h. After completion of the permeation study, the percent cumulative drug release was plotted as a function of time.(19)

Ex-vivo drug retention studies
Ex-vivo drug retention studies were carried out using tape stripping method. Tape stripping involves sequentially removing layers of the Stratum Corneum (SC) by repeatedly applying and removing adhesive tapes. In this study, materials used included Transpore 3M tape, porcine skin, nanoemulsion of vitamin E, ethanol, and acetonitrile. The porcine ear was soaked in Tyrode solution for over two hours, the SC was carefully removed with a scalpel, and the skin was flattened to equal thickness. (20) The nanoemulsion was uniformly applied on the skin and kept in a diffusion cell for 12 hours. After this period, tapes were placed on the skin, with the first few tapes discarded to remove excess formulation. Each tape was then dissolved in a mixture of ethanol and acetonitrile, and the drug content was evaluated using HPLC. The penetration of the drug was determined by calculating and plotting the area under the curve (AUC) against the number of tapes stripped.(21)

In-Vitro Efficacy Studies

Cell Viability Assay:
The MTT Assay was performed using the B16F10 mouse skin melanoma cell line to assess Vitamin E's cytotoxic effects. This colorimetric method measures cell proliferation and cytotoxicity by converting the yellow tetrazolium dye MTT into formazan crystals. A 200 µL cell suspension at 20,000 cells per well was taken in a 96-well format and incubated for 24 hours. Various concentrations of Vitamin E (0.5, 1, 1.5, 2.0, and 2.5) were then added, and the plate was incubated for another 24 hours at 37°C in 5% CO2. After removing the media, MTT reagent was added to a final concentration of 0.5 mg/mL, and the plate was incubated in the dark for 3 hours. Following this, the MTT reagent was discarded, and 100 µL of Dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm to calculate cell viability.(22)

% cell viability=Abs of treated cells/Abs of Untreated cells x 100

Tyrosinase Inhibition Assay:

An assay for tyrosinase inhibition was performed using the B16F10 mouse skin melanoma cell line to evaluate cellular tyrosinase activity. A 1000 µl cell suspension at 50,000 cells per well was plated in a 12-well plate without the test agent and allowed to grow for 48 hours. Test compounds and Kojic Acid were then added and incubated for 24 hours. After incubation, the media was removed, and the cells were rinsed with PBS. They were lysed in 250 µl of 50 mM sodium phosphate buffer (pH 6.8) with Triton-X 100 and PMSF for 30 minutes. The lysate was centrifuged, and the supernatant was collected for tyrosinase activity analysis or stored at -80°C. For analysis, 100 µl of supernatant was mixed with 100 µl of 5 mM L-DOPA in a 96-well plate, incubated for 2 hours at 37°C, and absorbance was measured at 475 nm. The cellular tyrosinase activity was calculated using the formula:

% Cellular Tyrosinase activity = (Absorbance of treated cells / Absorbance of untreated cells) x 100.(23)

Skin Irritation Test: Hen's Egg Test- Chorioallantonic Membrane (HET-CAM) Study:

The Hen's Egg Test Chorioallantoic Membrane (HET-CAM) method was employed to evaluate the irritant potential of the prepared formulations. For the test, 8th day-old, incubated hen's eggs were used. Initially, the egg shell around the air chamber was removed and the inner membrane was moisturized. In sequence, the membrane was carefully removed and 0.3 ml of formulations were directly applied on the chorioallantoic membrane (CAM). For 300 seconds, reactions on the CAM surface were monitored. Every endpoint's appearance time (haemorrhage, vascular lysis, and coagulation) was tracked and documented in seconds. The following formula was used to determine a prospective irritancy (PI) score based on the amount of time needed for the endpoints to develop:

PI={[(301-h) *5] + [(301-v) *7] + [(301-c) *9]} / 300

Where, h= appearance time in seconds of hemorrhage

v= appearance time in seconds of vasoconstriction

c= appearance time in seconds of coagulation

RESULTS AND DISCUSSION

HPLC UV Analysis

The linearity curve for Tocotrienol and ethanol was established with concentrations ranging from 100 to 500 µg/ml. The linear regression equation was determined to be and it had a high correlation coefficient (R2) of 0.99. The linearity curve is depicted in Fig 1A and Fig 1B and Table I.

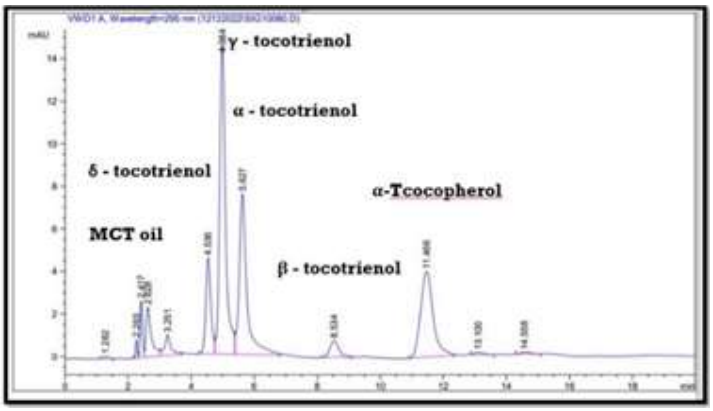

Figure 1a: Chromatogram of Natural Vitamin-E

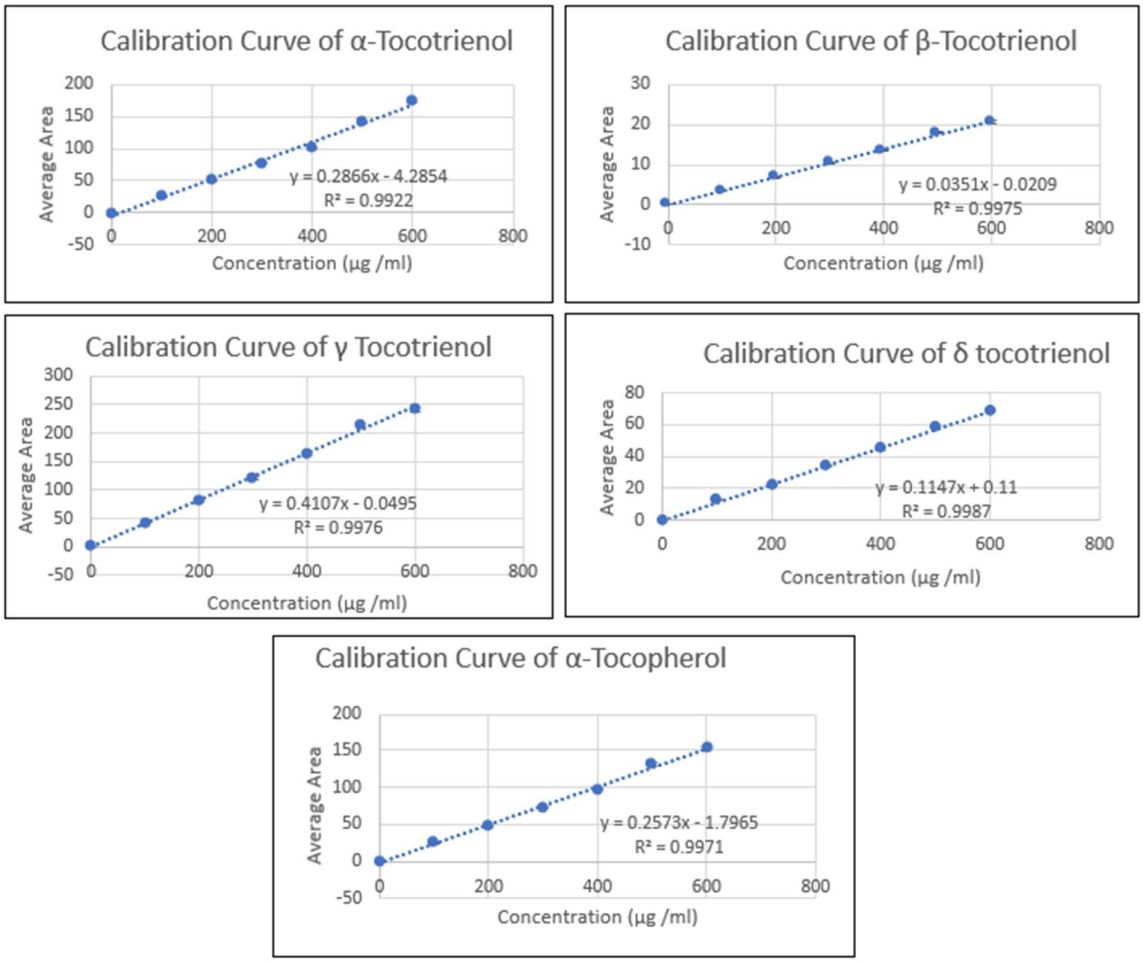

Figure 1b: Calibration Curves of the Isomers of Natural Vitamin-E

Table I. Linearity of Vitamin-E

Sr. no.	Concentration (ug/ml)	Peak area (mean ± SD) (n=3) Alfa T3	%RSD of Alpha tocotrienol	Peak area (mean ± SD) (n=3) Beta T3	%RSD of Beta tocotrienol	Peak area (mean ± SD) (n=3) Gamma T3	%RSD of Gamma tocotrienol	Peak area (mean ± SD) (n=3) Delta T3	%RSD of Delta tocotrienol	Peak area (mean ± SD) (n=3) Alfa Tocopherol	%RSD of Alpha tocopherol
1	100	26.489 ± 0.418	1.50	3.677 ±0.053	0.00	46.139 ± 0.000	1.26	12.957±0.192	1.59	26.300 ± 0.328	1.45
2	200	51.520 ± 0.687	1.63	6.933 ± 0.085	1.15	82.076 ± 0.935	1.34	22.894 ± 0.369	1.33	48.513 ± 0.644	1.24
3	300	77.952 ± 0.830	1.63	10.1764 ± 0.183	1.69	122.262 ± 2.057	1.43	33.982 ± 0.556	1.06	73.018 ± 1.033	1.78
4	400	102.028 ± 1.516	1.42	13.715 ± 0.200	1.55	165.498 ± 2.536	1.33	45.356 ± 0.639	1.50	98.025 ± 1.301	1.56
5	500	143.072 ± 1.469	1.07	18.182 ± 0.134	1.52	215.590 ± 3.255	1.37	59.345 ± 0.632	1.03	132.158 ± 1.799	0.73
6	600	174.021± 1.335	1.48	21.545± 0.235	1.23	241.254 ±2.22	1.72	69.214±0.525	1.23	154±1.665	1.46

Formulation Development of Vitamin E Nanoemulsion

Screening of oils, surfactants and co-surfactants by miscibility Studies:

All the surfactants and co-surfactants were pre-screened via miscibility test. Where Natural Vitamin-E was mixed with various surfactants and co-surfactant in ratio of (1:1) for 24 hours for any phase separation and discolouration indicating immiscibility and incompatibility. Miscibility studies were carried out for pre- screening of oils, surfactants and co-surfactants which would be required for formulation of nanoemulsion, miscibility studies provide array of two non-compatible and immiscible fluids that further would be subjected to further transmittance and water uptake study. The results are displayed in Table II, III, and IV. All the surfactants like that were screened were miscible with Natural Vitamin-E. Co-surfactants like Kollisolv 300, Transcutol P and Transcutol CG shows miscibility with Natural Vitamin-E. Most of the oils except oleic acid shows excellent miscibility.

Table II Miscibility Studies for Surfactants

Surfactants	HLB	Miscibility
Tween 20	16.7	Miscible
Tween 80	15	Miscible
Span 20	8.6	Miscible
Span 80	4.3	Miscible
Kolliphor EL	12- 14	Miscible
Kolliphor RH 40	14-16	Miscible
Coffee Oil	10-12	Miscible
Unitop 100x	6.5	Miscible
Decyl Glucoside	12	Miscible

Table III Miscibility Studies for Co-Surfactants

Co-surfactants	HLB	Miscibility
PEG- 400	13	Immiscible
PEG- 600	11.7	Immiscible
Kollisolv -300	15	Miscible
Transcutol P	4.2	Miscible
Transcutol CG	5	Miscible
Biogod	10-13	Miscible

Table IV Miscibility Studies for Oils

Oil	**HLB**	**Miscibility**
Capmul	5.5-6	Miscible
Captex	6.7-7	Miscible
Oleic acid	1	Immiscible
MCT	2.5	Miscible
Olive oil	7	Miscible

Emulsification Ability:

The main aim was to check which combination might give a transparent emulsion. Oil and Smix were taken in a test tube in the ratio 1:1 and homogenized at 45- 60℃. 10 mg of this mixture was diluted with 10 ml of water in a volumetric flask. Kept standing for 2 hours and % transmittance was noted at 638.2 nm with water as blank. The results are depicted in Table V.

Table V Emulsification Ability

Smix + Oil	**Percent Transmittance**
Tween 20 + Transcutol P+ oil	91.66
Tween 80 + Transcutol P+ oil	25.44
Span 20 + Transcutol P+ oil	82.64
Span 80 + Transcutol P+ oil	93.42
Kolliphor RH 40 + Transcutol P+ oil	56.25
Kolliphor EL + Transcutol P+ oil	52.85
Coffee oil + Transcutol P+ oil	79.65
Kolliphor RH 40+ Tween 80+ Transcutol CG + Oil	94.34

Tween 20 + PEG 300+ oil	69.40
Tween 80 + PEG 300 + oil	41.83
Span 20 + PEG 300+ oil	69.56
Span 80 + PEG 300+ oil	58.46
Kolliphor RH 40 + PEG 300+ oil	48.56
Kolliphor EL + PEG 300+ oil	42.67
Coffee oil + PEG 300+ oil	75.65
Tween 80 + Biogod + oil	95.76

The combinations showing more than 94% transmittance were selected for construction of pseudo ternary phase diagram. Since the combination of Kolliphor RH40, Tween 80 and Transcutol and the combination of Tween 80 and Biogod shows better emulsification ability they were further selected for the construction of pseudoternary phase diagrams.

Construction of Pseudoternary phase diagram:

The aim for the construction of pseudo ternary phase diagram was to find out the nanoemulsion region using blends of oil, surfactant and cosurfactant. Water intake capacity of Natural Vitamin-E in different ratios of Tween 80 and Transcutol, and Kolliphor RH40 and Tween 80 and Biogod were tried in different ratio from 1:9 to 9:1. A primary mix of constant 10 % of oil and 90 % of s-mix was prepared of total 100 mg equivalent (10 mg oil 90 mg s mix) of 90% s-mix different ratio of surfactant to co-surfactant were experimented as water was added each quantity of s-mix and oil would be decrease by amount in equal ratios respectively. Subsequent formation of pseudo-ternary phase diagrams of respective surfactants and co-surfactants were observed using Chemix School Software 12.0. The results are depicted in the Fig 2 and 3 and Table VI.

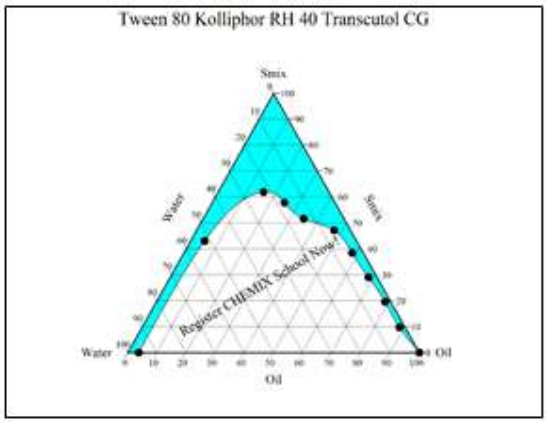

Figure 2: Pseudo Ternary Phase Diagram of Tween 80 Kolliphor Rh and Transcutol Cg

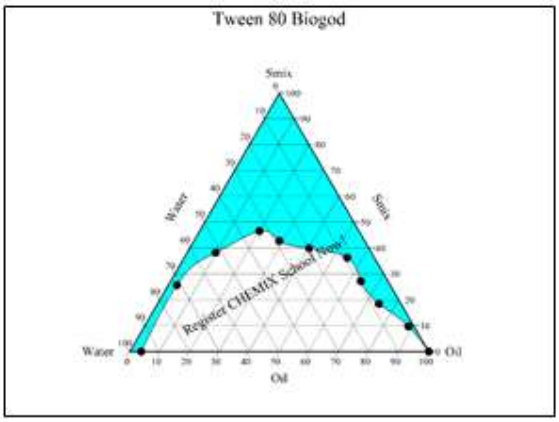

Figure 3: Pseudo Ternary Diagram of Tween 80 and Biogod

Table VI Percentage of Water Uptake

Smix: Oil	Percentage of water uptake of nanoemulsion of Tween 80 Kolliphor Rh and Transcutol CG	Percentage of water uptake of nanoemulsion of Tween 80 and Biogod
9: 1	52.15	69.69
8: 2	22.48	16.66
7: 3	17.35	9.09
6: 4	13.79	5.66
5: 5	5.66	2.91
4: 6	3.84	2.91
3: 7	2.91	1.96
2: 8	1.96	1.96
1: 9	1.96	0.99

Since a larger area for emulsion was observed with surfactants Tween 80 and biogod. This combination was selected as a surfactant for formulation development.

Preparation of Nanoemulsion

The nanoemulsion batches were prepared using Spontaneous emulsification method as shown in fig 4. Preliminary trial batches for optimization of oil and s-mix concentration range were prepared and they were further optimized using design of experiment software to determine the most stable batch of nanoemulsion.

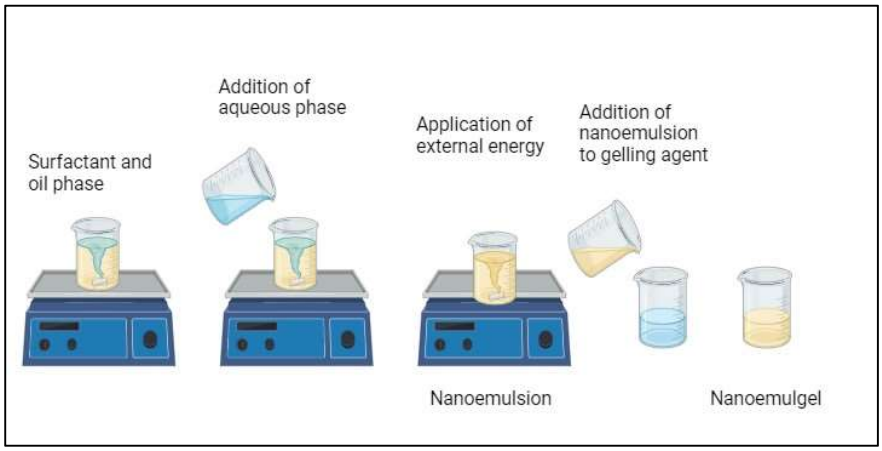

Figure 4: Formulation Development of Nanoemulsion by Spontaneous Emulsification Method

Table VII Formula Table for Nanoemulsion of Tween 80 and Biogod

Ingredients	F1	F2	F3	F4	F5
Natural Vitamin E	1%	1%	1%	1%	1%
MCT Oil	1%	1%	1%	1%	1%
Tween 80	2%	5%	7.5%	10%	14.9%
Biogod	-	0.1%	0.1%	0.1%	0.1%
Water	q.s.	q.s.	q.s.	q.s.	q.s.

Table VIII Formula Table for Preparation of Nanoemulsion of Decyl Glucoside and Biogod

Ingredients	F1	F2	F3	F4	F5
Natural Vitamin E	1%	1%	1%	1%	1%
MCT Oil	1%	1%	1%	1%	1%
Decyl Glucoside	10%	15%	25%	30%	35%
Biogod	-	0.1%	0.1%	0.1%	0.1%
Water	q.s.	q.s.	q.s.	q.s.	q.s.

Since F5 formulation with 2% oil phase and 15% S-mix (Tween 80: Biogod) and F5 formulation with 2% oil phase and 35% S-mix (Decyl Glucoside: Biogod) showed clear nanoemulsion, these formulations were selected for further optimization of the surfactants and cosurfactants to get a stable nanoemulsion as depicted in Table VII and VIII.

Statistical Optimization of the Formulation Parameters

The impact of two independent formulation variables on the properties of the nanoemulsion was examined using a 3^2 factorial design. Smix ratio (X1) and MCT oil content (X2) were the independent variables, and each was examined at three levels: low (-1), medium (0), and high (+1). Particle size (Y1) and zeta potential were the responses assessed (Y2). Using statistical software, nine experimental runs were conducted for every formulation system (Design Expert).

Several mathematical models, such as linear, quadratic, and interaction models, were fitted to the experimental data. The linear model demonstrated the best fit for both answers with substantial model statistics, according to statistical analysis. The results are displayed in the Table no. IX, X, XI, and XII.

Table IX Optimization Parameters

	High (+1)	**Medium (0)**	**Low (-1)**
Tween 80: Biogod	14.9%: 0.1%	10.9%: 0.1%	7.9%: 0.1%
MCT oil	2%	1%	0.5%

Table X Optimization Nanoemulsion of Tween 80 and Biogod

Batch	**Factor 1 (X1) Tween 80: Biogod**	**Factor 2 (X2) MCT oil**	**Response 1 (Particle Size) (Y1)**	**Response 2 (Zeta potential) (Y2)**
A1	+1	+1	14nm	-0.01
A2	0	0	12nm	-0.24
A3	-1	-1	13nm	-7.46
A4	+1	0	18nm	-25
A5	0	-1	10nm	-13.3
A6	-1	+1	12nm	-11.9
A7	+1	-1	40nm	-13.9
A8	0	+1	10nm	-17.1
A9	-1	0	26nm	-21.4

Table XI Optimization Parameters for Nanoemulsion of Decyl Glucoside and Biogod

	High (+1)	**Medium (0)**	**Low (-1)**
Decyl Glucoside: Biogod	40%: 0.1%	35%: 0.1%	30%: 0.1%
MCT oil	2%	1%	0.5%

Table XII Optimization Table for Preparation of Nanoemulsion of Decyl Glucoside and Biogod

Batch B	**Factor 1 (X1) Decyl Glucoside: Biogod**	**Factor 2 (X2) MCT oil**	**Response 1 (Particle Size) (Y1)**	**Response 2 (Zeta potential) (Y2)**
B1	+1	+1	10.57	-35.7
B2	0	0	8.44	-36.1
B3	-1	-1	9.12	-8.71
B4	+1	0	11.06	-32.6
A4	0	-1	11.58nm	-33.5
B6	-1	+1	8.074nm	-31.6
B7	+1	-1	9.08nm	-28.1
B8	0	+1	12.45nm	-8.71
B9	-1	0	8.44nm	-36.1

Effect of Formulation Variables on Particle Size

The Smix ratio and MCT oil content had a substantial impact on the particle size of Vitamin E nanoemulsion. According to the statistical analysis, Smix concentration had a negative impact on particle size, which means that droplet size decreased as surfactant concentration increased. Higher surfactant concentrations allow the interfacial tension between the oil and aqueous phase to decrease, which results in the creation of smaller droplets and improved stability of the nanoemulsion.

On the other hand, a modest increase in particle size was seen when the concentration of MCT oil was increased. This could be because more surfactant was needed for stabilisation due to the higher dispersed phase volume.

The model was statistically significant ($p < 0.05$), according to the ANOVA findings, suggesting that the chosen variables had a substantial impact on the nanoemulsion's particle size. The contour plots (Fig. 5 and Fig. 7) clearly demonstrate the relationship between Smix ratio, oil concentration, and particle size.

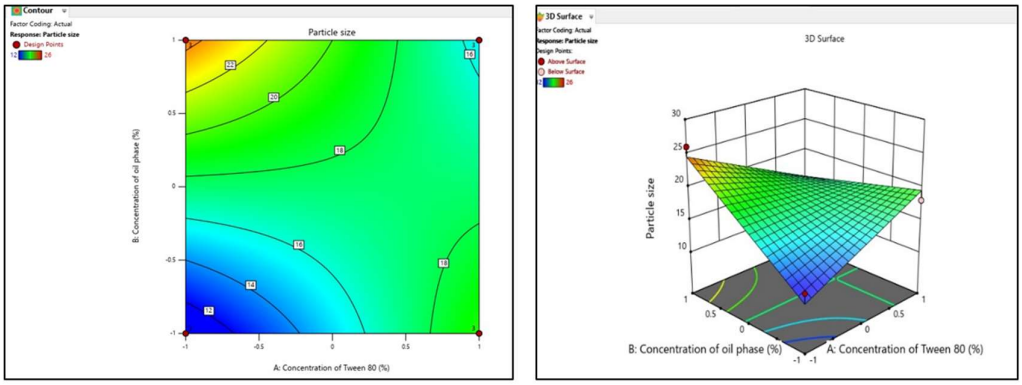

Figure 5: Contour Plot for Particle Size of Batch A4 Nanoemulsion

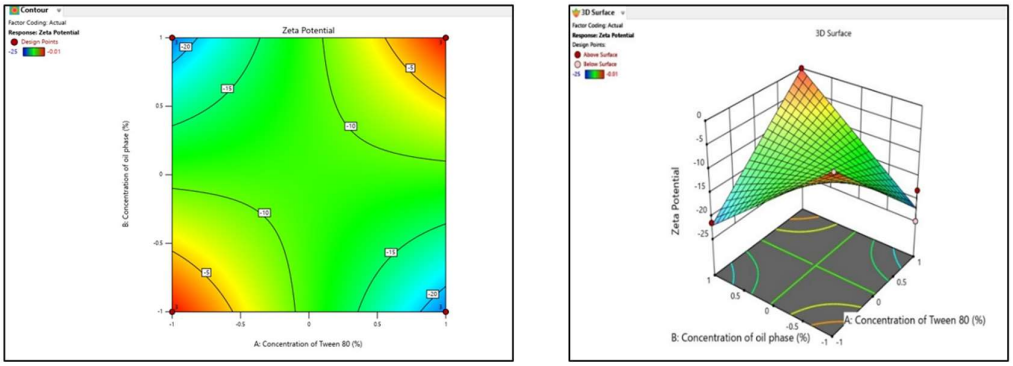

Figure 6: Contour Plot for Zeta Potential of Batch A4 Nanoemulsion

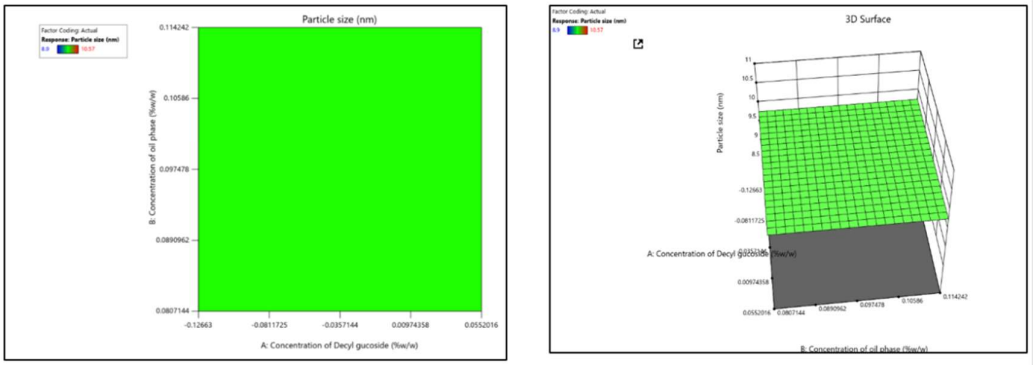

Figure 7: Contour Plot for Particle Size of Batch B5 Nanoemulsion

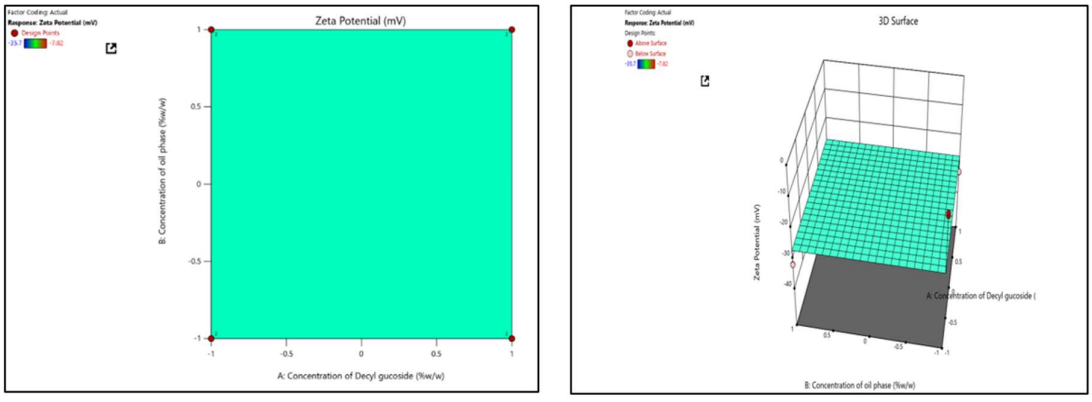

Figure 8: Contour Plot for Zeta Potential of Batch B5 Nanoemulsion

Effect of Formulation Variables on Zeta Potential
The stability and surface charge of the nanoemulsion system are represented by the zeta potential. According to statistical analysis, increasing the Smix concentration led to an increase in the zeta potential's magnitude (more negative values), suggesting that the droplets' electrostatic stability had improved.

Similarly, raising the concentration of MCT oil led to a modest rise in the zeta potential magnitude, indicating enhanced stability as a result of stronger interfacial layer development surrounding the oil droplets.

The response surface and contour plots (Figs. 6 and 8) show how the oil concentration and Smix ratio together affect the zeta potential. The impact of the formulation factors on zeta potential was confirmed by the statistical analysis, which showed that the model was significant with $p < 0.05$.

Selection of Optimized Formulations
Based on the desirability criteria of minimum particle size and maximum magnitude of zeta potential, two optimized batches were selected.

For the formulation containing Tween 80: Biogod, batch A4 containing 1% MCT oil and 15% Smix exhibited a particle size of 18 nm and zeta potential of –25 mV, indicating good nanoemulsion stability.

For the formulation containing Decyl Glucoside: Biogod, batch B5 containing 0.5% MCT oil and 35% Smix showed an average particle size of 11.58 nm and zeta potential of –33.5 mV, demonstrating excellent stability.

Therefore, batches A4 and B5 were considered the optimized nanoemulsion formulations and were selected for further characterization and stability studies.

Physical Characterization of Nanoemulsion

Visual Observation:
Nanoemulsion batches A4 and B5 were found to be clear and yellow with no phase separation.

Percent transmittance:
The Percent transmittance of the batches A4 and B5 were examined by UV spectrophotometry at 638.2 nm, taking distilled water as a blank.

pH:
The pH of the formulation for batch A4 and batch B5 was found to be between the range of 5.68 and 5.75 respectively. It was determined in triplicates.

Drug-Excipient interaction by FTIR
The FTIR spectra peaks of Vitamin-E was obtained and was compared to that of Tocopherol due to structural similarity. The FTIR spectra of tocotrienol contains a O-H Stretching with wave number 3561.62 cm^{-1}, C-H Stretching of alkanes which corresponds to a wavenumber of 2924.13 cm^{-1}, C-O-C Stretching of wavelength 1105.23 cm^{-1} which corresponds to an ester bond, C=C Stretching Alkene with a wavelength of 1460.14 cm^{-1} and C=C Aromatic Stretching with a wavelength of 1741.75 cm^{-1} . (24) We found that the spectra of Vitamin-E were comparable to that of Tocopherol spectra which was reported in literature. All the characteristic peaks of Vitamin E were also identified in FTIR spectra of physical mixture and formulation. Thus, FTIR results indicated that Vitamin E and selected excipients were compatible. Further, all retained peaks of Vitamin E suggested that original form of drug was unaltered during the preparation of nanoemulsion and possess good stability in this carrier system. FTIR showed no formation of new peaks, thus removing the possibility of chemical interaction between the drug and excipients. Hence, the results of FTIR spectroscopy indicated that Vitamin E was compatible with the excipients and have good stability in a nanoemulsion formulation. See fig 9.

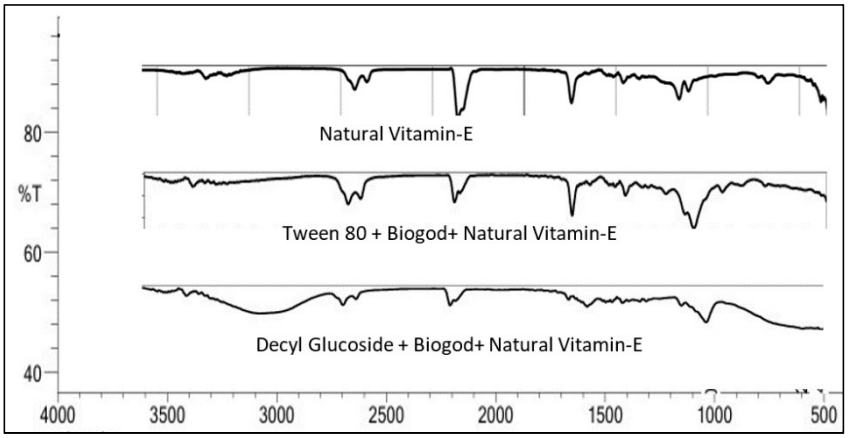

Figure 9: Ftir Of Natural Vitamin-E; Tween 80, Biogod And Natural Vitamin-E; And Decyl Glucoside, Biogod and Natural Vitamin-E

Globule Size and Polydispersity Index:
The Globule size and Polydispersity index of prepared nanoemulsion were determined by Malvern Zetasizer ZS90. The average globule size for Batch A4 was found to be 18.55nm with the PDI of 0.235 and for batch B5 it was found to be 11.58 with PDI of 0.321 as depicted in the fig 10. The Polydispersity Index (PDI) can range from 0 to 1, where 0 represents a monodisperse system and 1 represents a polydisperse particle system. (25)

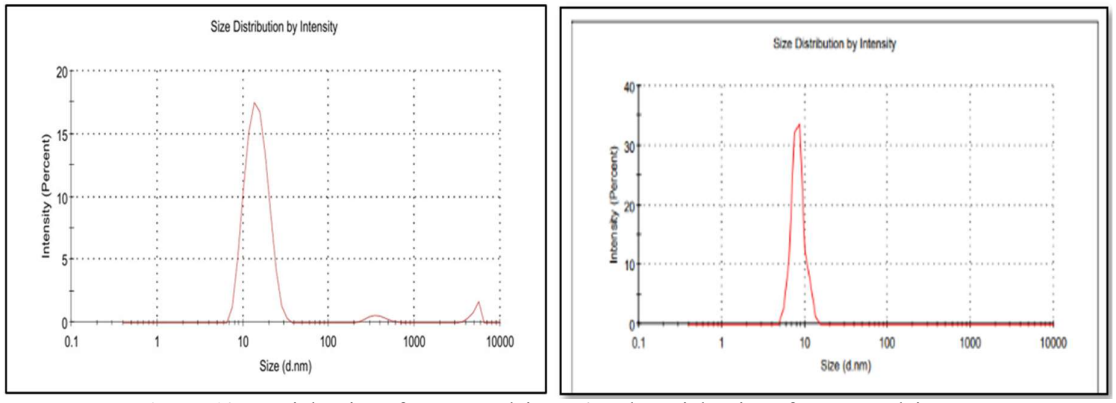

Figure 10: Particle Size of Nanoemulsion A4 and Particle Size of Nanoemulsion B5

Zeta Potential:
Zeta potential of optimized formulation was measured using Malvern Zetasizer ZS90. The zeta potential of the batch A4 was found to be -23.6mV which indicates moderate stability, that of batch B5 was found to be -33.5mV which indicates that the emulsion has great stability as shown in the fig11. The zeta potential should generally range above +30 or -30mV.(26)

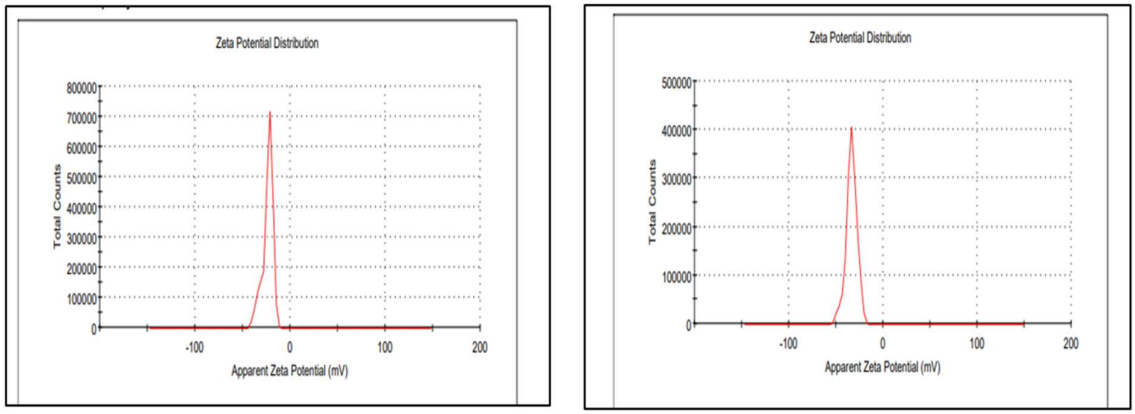

Figure 11: Zeta Potential of Nanoemulsion A4 and Zeta Potential of Nanoemulsion B5

Viscosity:
Viscosity of nanoemulsion was determined by 64 Spindle at 10rpm using Brookfield Viscometer (Brookfield Engineering Laboratories). The viscosity of batch A4 was found to be 225.6cP, for batch B5 was found to be 539cP.

Transmission Electron Microscopy (TEM):
Transmission Electron Microscopy was measured for the finalized batches. It was found to be 50nm for batch A4 and 33nm for batch B5 as depicted in fig12.

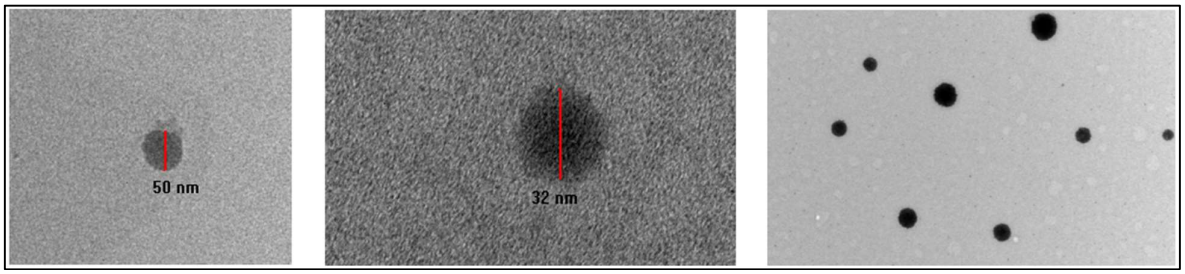

Figure 12A: Tem Images of Nanoemulsion A4

Figure 12B: Tem Images of Nanoemulsion B5

Figure 12C: Tem Images of Nanoemulsion

Drug Content

The drug assay for all the nanoemulsion was found to be in the range of 97-100 % for both the nanoemulsion batches.

Table XIII Characterization of Vitamin- E Loaded Nanoemulsion

Sr. No.	Tests	Observation Batch A4	Observation Batch B5
1	Visual observation	Clear transparent and yellow	Clear transparent and yellow
2	pH	5.68± 0.012	5.75± 0.023
3	% Transmittance	99.89± 0.008%	99.88± 0.004%
4	Globule size	18.55±0.816nm	11.58±0.773nm
5	PDI	0.235± 0.008	0.321± 0.007
6	Zeta Potential	-23.6± 0.767mV	-33.5± 0.323mV
7	Dilution test with water (10, 50, 100, 250 times)	Clear and stable	Clear and stable
8	Viscosity (Spindle No. 64) AT 10 rpm	225.6± 0.04cP	539± 0.98cP
9	Drug Assay		
	Alpha Tocotrienol	98.23± 0.021	98.256± 0.12
	Beta Tocotrienol	96.25± 0.022	96.565± 0.32
	Gamma Tocotrienol	98.47± 0.045	98.54± 0.54
	Delta Tocotrienol	99.58± 0.435	99.214± 0.54
	Alpha Tocopherol	98.256± 0.43	97.25± 0.45

Thermodynamic Stability

The formulation batches with the maximum nanoemulsion area, were subjected to the thermodynamic stability tests such as heating–cooling cycle, freeze–thaw cycle, and centrifugation. Instability like phase separation, turbidity, creaming, or cracking was observed in some of the batches of nanoemulsion. Thermodynamic stability confers long shelf life to the nanoemulsion as compared to ordinary emulsions. Table XIV shows the thermodynamic stability of these formulations.

Table XIV Thermodynamic Stability of Nanoemulsion

Batch No.	Centrifugation Cycle	Heat Cool Cycle	Freeze Thaw Cycle
A4	Clear & Stable	Clear & Stable	Clear & Stable
B1	Clear & Stable	Phase Separation	-
B3	Clear & Stable	Phase Separation	-
B4	Clear & Stable	Clear & Stable	Clear & Stable
B5	Clear & Stable	Clear & Stable	Clear & Stable
B7	Clear & Stable	Clear & Stable	Clear & Stable

Antioxidant Activity

The DPPH assay, in which the DPPH radical is reduced by antioxidants, was performed to assess the antioxidant activity of the Vitamin E nanoemulsion of batch A4 and B5 and they were compared to the standard antioxidant such as BHT. Results are depicted in fig13 and expressed as a DPPH inhibition percentage. BHT was used as a positive control at a concentration range of 0- 10mg/mL (same concentration as Vitamin E nanoemulsion) DPPH inhibition at the lowest concentration of 2mg/ml was

found to be 90.48%, 89.58% and 93.92% for batch A4, batch B5 and BHT respectively are depicted in the Table XV. From the graph shown in fig 13, we can conclude that the nanoemulsion of batch A4 shows excellent anti-oxidant activity compared to batch B5 and is comparable to the antioxidant activity of BHT which is a potent antioxidant suggesting that nanoemulsion of batch A4 could be able to inhibit tyrosinase better than nanoemulsion of batch B5.

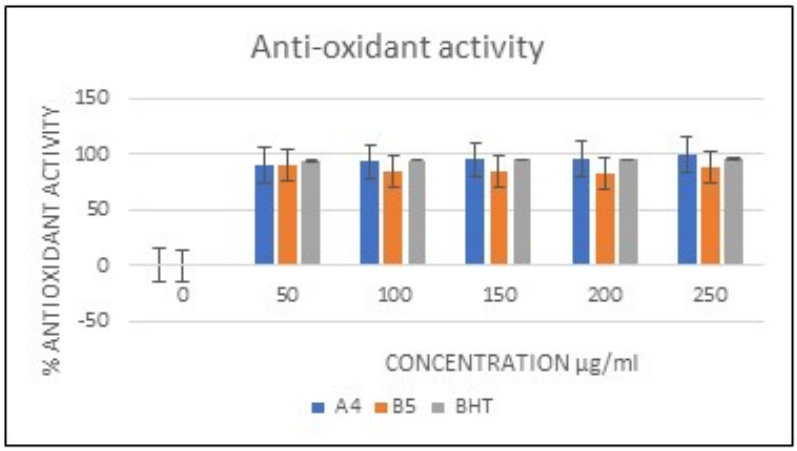

Figure 13: Antioxidant Activity of Nanoemulsion Batch A4, B5 and BHT

Table XV Comparative Antioxidant Activity of Vitamin- E Loaded Nanoemulsion and Bht

Concentration (µ g/ml)	Percent Anti-oxidant Activity		
	A4 Nanoemulsion	B5 nanoemulsion	BHT
0	0	0	0
50	90.48	89.58	93.92
100	92.98	84.68	94.70
150	94.88	84.56	95.09
200	95.83	82.48	95.09
250	98.81	87.99	95.88

Invitro Drug Release Study

To analyze the quantity of drug released the optimized formulations were subjected to diffusion study using dialysis bag. The receptor compartment was filled with Phosphate buffer (PBS) pH 5.5 + Ethanol in 1:1 ratio at 37°C ± 0.5°C with constant stirring at 300 rpm.

It was observed that maximum drug release from nanoemulsion was achieved within 12h. The release profile of vitamin E from A4 formulation is shown in fig 14 and Table XVI. And the release of vitamin E from B5 formulation is given in the fig 15 and Table XVII.

Table XVI Invitro Drug Release Study of Batch A4 Nanoemulsion

	% Cumulative release				
Time in hours	Delta Tocotrienol	Gamma Tocotrienol	Alpha Tocotrienol	Beta Tocotrienol	Alpha Tocopherol
0	0	0	0	0	0
0.5	0	1.961±1.12	0	0	0
1	21.94±0.11	15.849±1.15	21.363±0.43	0	6.614±0.24
2	26.743±0.26	25.126±0.74	23.601±0.32	17.196±1.07	14.835±0.06
3	31.838±0.21	40.492±2.66	28.355±0.72	27.408±2.08	18.133±0.23
4	36.132±0.05	45.744±2.08	33.809±0.06	33.145±0.95	23.276±0.41
5	46.683±0.00	54.014±2.06	39.468±0.70	38.203±1.25	30.268±0.63
6	53.749±0.01	60.650±2.22	53.330±0.65	43.314±0.79	34.322±1.08
7	61.179±0.00	67.73±2.27	60.581±0.43	47.922±1.17	40.363±0.58
8	66.402±0.00	72.448±0.89	67.143±0.41	54.479±1.93	46.393±0.73
9	74.506±0.01	79.199±0.89	71.679±0.32	65.569±0.29	51.108±0.18

10	85.662±0.03	85.064±0.89	82.306±0.08	76.022±1.92	63.916±0.42
11	90.951±0.02	90.946±0.89	94.961±1.31	87.557±0.29	73.809±0.47
12	99.001±0.34	97.485±0.89	99.833±0.44	96.167±0.88	93.886±0.15

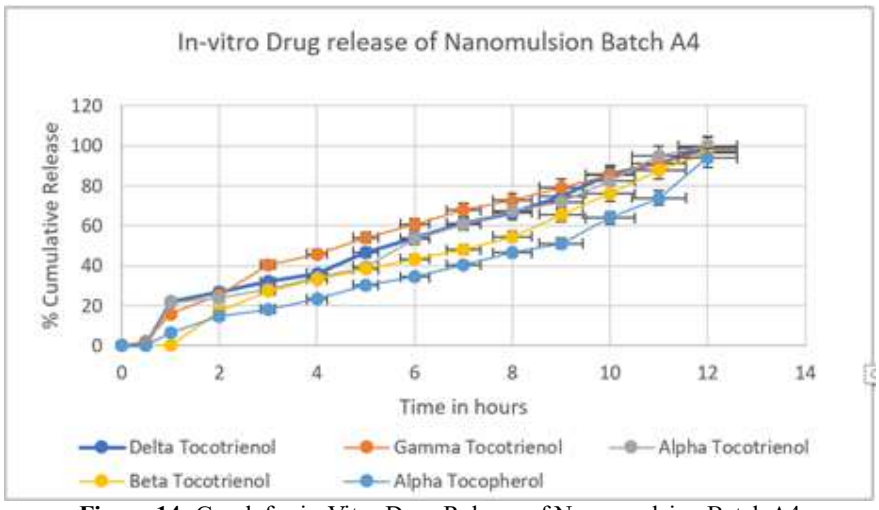

Figure 14: Graph for in-Vitro Drug Release of Nanoemulsion Batch A4

Table XVII Invitro Drug Release Study of Batch B5 Nanoemulsion

Time in hours	% Cumulative release				
	Delta Tocotrienol	Gamma Tocotrienol	Alpha Tocotrienol	Beta Tocotrienol	Alpha Tocopherol
0	0	0	0	0	0
0.5	0	0	0	0	0
1	0	0.053±0.0	21.056±0.43	0	0
2	2.474±0.36	2.154±0.08	23.374±0.32	15.441±0.34	0
3	5.318±0.79	2.880±0.39	27.849±0.72	25.935±0.43	8.298±0.32
4	14.827±1.17	6.704±0.65	33.851±0.06	30.473±0.12	14.108±0.32
5	17.347±1.23	11.377±0.36	38.970±0.70	34.320±0.34	19.306±0.78
6	24.045±1.35	18.191±1.48	52.870±0.65	40.253±0.56	36.897±0.45
7	29.772±1.49	25.814±0.83	60.274±0.43	45.095±1.23	46.883±0.34
8	34.176±1.53	43.760±1.15	66.85±0.41	52.615±0.87	59.402±1.34
9	40.704±1.61	50.613±1.47	71.453±0.32	65.774±0.76	71.447±0.67
10	56.402±1.76	56.999±1.75	82.359±0.08	73.160±0.76	86.052±0.56
11	67.482±1.83	65.689±1.51	93.269±1.31	85.852±0.67	93.560±0.34
12	88.480±1.95	89.151±1.57	98.769±0.44	95.543±0.76	95.554±0.67

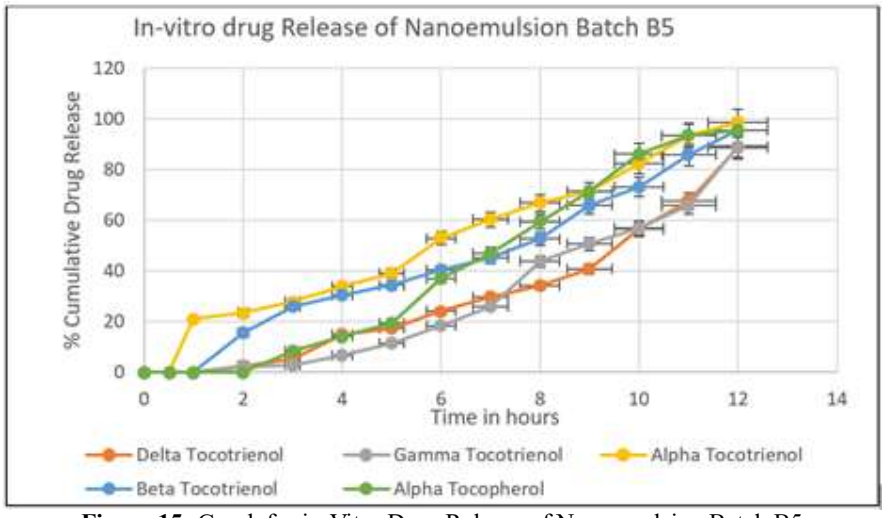

Figure 15: Graph for in-Vitro Drug Release of Nanoemulsion Batch B5

It was observed from the results that the 99.00, 97.48, 99.83, 96.16, 93.88% of delta, gamma, alpha, beta tocotrienol and alpha tocopherol respectively, from nanoemulsion batch A4 were released in 12 hours whereas nanoemulsion of B5 formulation gave a sustained release with 88.480, 89.151, 98.769, 95.54, 95.554% of delta, gamma, alpha, beta tocotrienol and alpha tocopherol respectively in 14 hrs.

Kinetic Modeling and Mechanism of Arug Release

The correlation coefficient (R2) of the zero-order model was found to be 0.9692, 0.9829, 0.9634, 0.9842, and 0.9813 of delta, gamma, alpha, beta tocotrienol and alpha tocopherol respectively which was higher when compared with the Peppas plot and Higuchi's plot for final selected optimized batch A4 nanoemulsion and that for batch B5 nanoemulsion was found to be 0.9057, 0.9838, 0.9594, 0.9831 and 0.9174 of delta, gamma, alpha, beta tocotrienol and alpha tocopherol respectively . Hence the release of drug from the preparation followed zero order kinetics for both the batches batch A4 and batch B5 of nanoemulsion which indicates controlled release for extended period of time and it is independent of the drug concentration.

Ex-vivo Skin Permeation Studies

From the ex vivo permeation studies, the drug permeability was found 75% for batch A4 nanoemulsion and 82% for batch B5 nanoemulsion within 14 h. The results are shown in Table XVIII and XIX and Figs 14 and 15 respectively.

Ex-vivo Drug Retention Studies

To investigate the amount of drug retained on the skin Tape Stripping method was employed. The first four tapes showed the maximum concentration of the drug because of excess left over of the drug which was not able to penetrate. Later the strips till 15 showed decrease in the peak area that reduced considerably in case of both nanoemulsion batch A4 and nanoemulsion batch B5. Thus, proving that the drug had penetrated through the stratum corneum layer of the skin into the epidermal layers of the skin where the drug is supposed to show the efficacy. The drug content reduced to negligible amount by the time the 15th tape is stripped which is seen by reduction in the peak area.

Table XVIII Ex-Vivo Drug Release of Nanoemulsion Batch A4

Time in hours	% Cumulative release				
	Delta Tocotrienol	Gamma Tocotrienol	Alpha Tocotrienol	Beta Tocotrienol	Alpha Tocopherol
0	0	0	0	0	0
0.5	0	0	0	0	0
1	0.422±0.12	0.053±	6.579±0.0	0	0
2	0.441±0.23	0.055±0.0	6.878±0.0	0.262±0.0	3.072±0.0
3	0.921±1.23	0.058±0.0	7.177±0.0	0.274±0.0	3.212±0.0
4	4.038±1.95	1.899±1.2	8.746±0.17	0.286±0.0	6.506±0.29
5	7.837±1.94	2.882±0.87	9.982±0.43	1.984±0.45	14.289±0.52
6	14.539±1.27	7.032±0.76	15.937±1.07	3.570±0.53	19.642±1.40
7	22.044±1.69	11.781±1.23	22.343±0.29	19.998±0.93	27.488±1.59
8	27.796±1.10	16.371±1.34	29.211±1.06	31.492±1.59	33.396±0.22
9	34.831±1.77	24.244±0.89	33.161±1.11	40.086±0.45	38.465±0.91

10	41.368±0.91	41.055±1.34	42.118±0.94	44.337±1.35	48.982±1.03
11	52.778±1.23	45.870±0.98	50.884±0.97	51.658±1.55	57.889±0.90
12	55.550±1.83	56.397±0.67	60.631±0.07	66.104±0.18	67.359±0.59
14	74.940±0.57	75.625±0.73	75.472±0.43	74.372±0.78	75.903±1.30

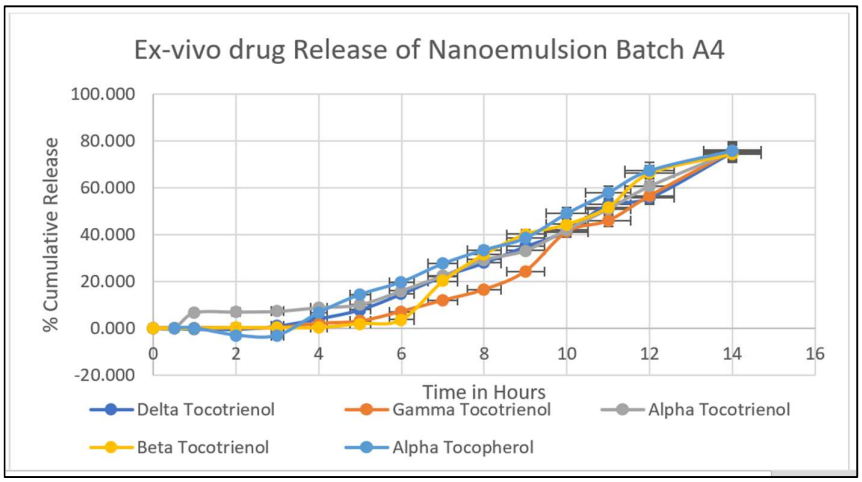

Figure 16: Graph for Ex-Vivo Drug Release of Nanoemulsion Batch A4

Table XIX Ex-Vivo Drug Release of Nanoemulsion Batch B5

Time in hours	% Cumulative release				
	Delta Tocotrienol	Gamma Tocotrienol	Alpha Tocotrienol	Beta Tocotrienol	Alpha Tocopherol
0	0	0	0	0	0
0.5	0.211±0.0	0.053±0.0	6.579±0.0	0	1.536±0.0
1	0.432±0.0	0.053±0.0	6.878±0.0	0.262±0.0	3.142±1.17
2	1.525±0.30	0.055±0.0	8.359±0.0	1.454±0.0	5.918±0.10
3	6.533±0.01	1.912±0.28	9.826±0.04	3.102±0.09	14.431±1.03
4	12.534±0.01	3.515±0.55	12.990±0.87	21.813±0.08	17.345±0.00
5	18.996±0.28	6.240±0.80	15.509±0.07	30.664±1.77	21.981±0.20
6	20.608±0.29	12.143±1.08	18.109±0.06	35.151±0.67	28.138±0.36
7	28.771±0.11	17.563±1.24	26.094±0.15	37.974±0.40	31.201±0.25
8	32.129±0.24	26.022±1.42	35.644±0.02	41.353±0.77	35.636±0.04
9	37.218±0.35	38.622±1.59	46.944±0.03	44.908±0.27	41.527±0.05
10	47.899±0.04	45.488±1.66	54.987±0.02	51.299±0.07	53.092±0.29
11	62.661±0.07	54.057±1.73	67.585±0.06	67.441±0.98	60.511±0.19
12	64.144±0.23	66.778±1.82	77.891±0.37	76.469±0.75	79.889±0.48
14	82.553±0.04	80.980±1.91	82.787±0.16	81.954±0.61	82.000±0.59

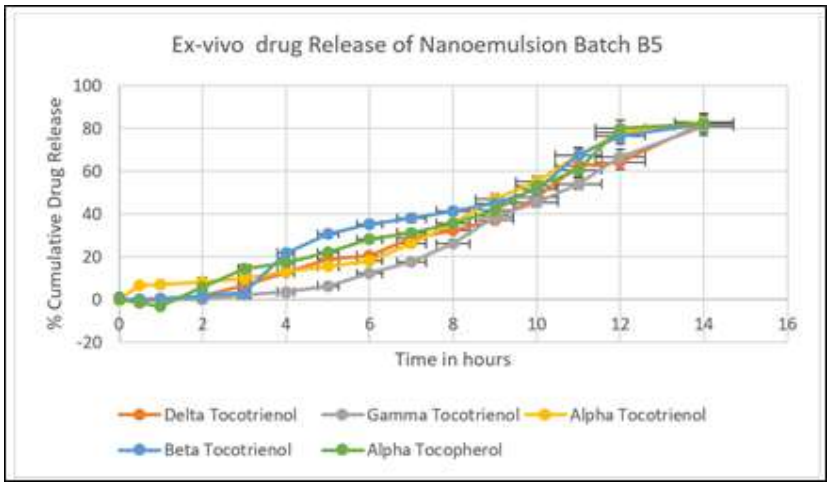

Figure 17: Graph for Ex-Vivo Drug Release of Nanoemulsion Batch B5

It was observed that 1 gram of the formulation containing 1% i.e. 10 mg Vitamin-E was applied to the skin. The cumulative release of nanoemulsion A4 was found to be 75%, i.e., 7.5 mg of the drug permeated through the skin, while only 25%, i.e., 2.5 mg, was present topically. In Case of Nanoemulsion B5 the cumulative drug release was found to be 82%, i.e., 8.2 mg of the drug permeated through the skin, while only 18% i.e., 1.8 mg of the drug was present topically. Thus, the developed formulation was able to permeate without any hindrance of the skin layer. Further, it was noted that about 2.3 mg was recovered from the topical stratum corneum layers, and 0.20 mg accounted for the loss in case of nanoemulsion A4. For nanoemulsion B5, 1.7 mg of drug was recovered from the topical stratum corneum layers, and 0.1mg accounted for loss. It can be concluded that the drug penetration of nanoemulsion B5 was better than the drug penetration of nanoemulsion A4. See fig 16.

In-Vitro Efficacy Studies

Cell viability assay

The Observations in statistical data of MTT cytotoxicity study as shown in table no. 20 suggested that against B16F10 cell lines, given test compound labeled as Vitamin-E showed moderate cytotoxic potential properties with the 68% cell viability at 2.5% dose and Kojic acid were nontoxic till the maximum dose of 2.5% respectively after the incubation period of 24 hours respectively.

Table XX The % Cell Viability Values of the B16f10 Cell Lines

MTT ASSAY-SUMMARY-Vitamin-E		**MTT ASSAY-SUMMARY-Kojic acid**	
Condition	**% cell viability**	**Condition**	**% cell viability**
Untreated	100.00	Untreated	100.00
Vitamin-E 0.5%	98.87	Kojic Acid 0.5%	98.96
Vitamin-E 1%	96.95	Kojic Acid1%	97.76
Vitamin-E 1.5%	89.17	Kojic Acid 1.5%	96.46
Vitamin-E 2%	76.34	Kojic Acid 2%	94.31
Vitamin-E 2.5%	68.33	Kojic Acid 2.5%	91.09

Cellular Tyrosinase Activity

The Observations in Statistical data of Cellular Tyrosinase activity study as shown in the table XXI and fig 18 suggests that in B16F10 cell lines, Placebo Nanoemulsion A4 Placebo Nanoemulsion B5 caused moderate inhibition of Tyrosinase enzyme with the cellular tyrosinase activity of 94.57% and 94.29% and the formulations of Vitamin E loaded nanoemulsion A4 and nanoemulsion B5 caused significant inhibition of Cellular Tyrosinase activity B16F10 cells of 77.24% and 67.37%. From the observations in fig 17 we can conclude that the nanoemulsion batch B5 has better cellular tyrosinase activity than batch A4 nanoemulsion.

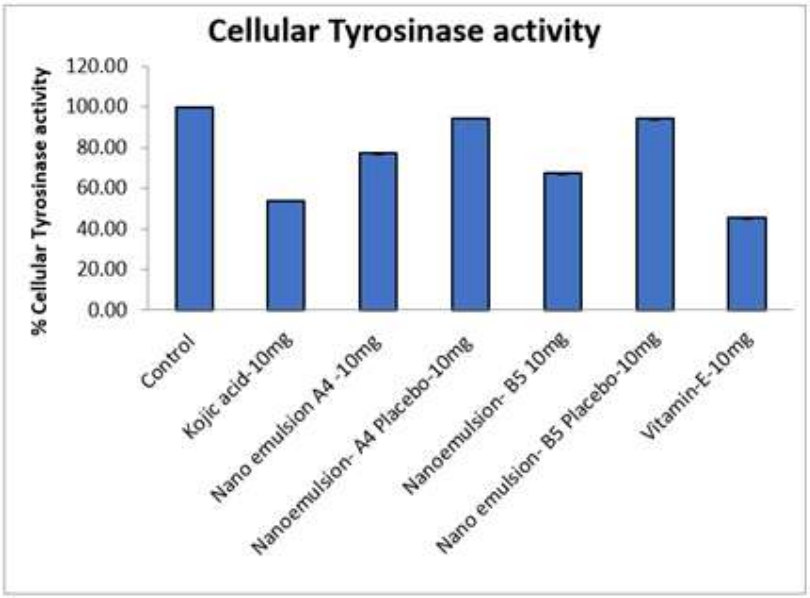

Figure 18: Graph with the % Cellular Tyrosinase Inhibitory Effect

Table XXI Table Showed the % Cellular Tyrosinase Activity

Culture condition	% Cellular Tyrosinase activity
Control	100.00
Kojic acid-10mg	53.84
Nanoemulsion A4-10mg	77.24
Nanoemulsion- A4 Placebo-10mg	94.57
Nanoemulsion B5 -10mg	67.37
Nanoemulsion B5 Placebo-10mg	94.29

Skin Irritation Test: Hen's Egg Test- Chorioallantonic Membrane (HET-CAM) Study:

As mentioned in Table No. 22 and 23 and Figure No.19, the nanoemulsion batches were found to be non-irritating and non-sensitizing in nature compared to the positive control, which has irritation potential. This suggests that encapsulation of these drugs in nanocarriers, such as Nanoemulsion, could be an effective way to reduce skin irritation caused by pure API.

Table XXII Reference Table for Het Cam Irritation Study

Photochemical change	Score	Irritation level
No visible hemorrhage	IS<0.9	Non-irritating
Only visible membrane hemorrhage	1.0 < IS <4.9	Mildly irritating
Visible membrane discoloration/hemorrhage, Structures are covered partially	5.0< IS < 8,9	Moderately irritating
Visible membrane discoloration/ hemorrhage, structures are entirely covered	9.0< IS<21.0	Severely irritating

Table XXIII Het Cam Irritation Study of the Developed Formulations

Fig	Group	Score	Inference
A	Negative control	0	Non-Irritating
B	Positive control	7.7	Moderately Irritating
C	Vitamin-E	0	Non-Irritating
D	Nanoemulsion- A4(placebo)	0	Non-Irritating
E	Nanoemulsion A4	0	Non-Irritating
F	Nanoemulsion B5 (placebo)	0.1	Non-Irritating
G	Nanoemulsion B5	0.1	Non-Irritating

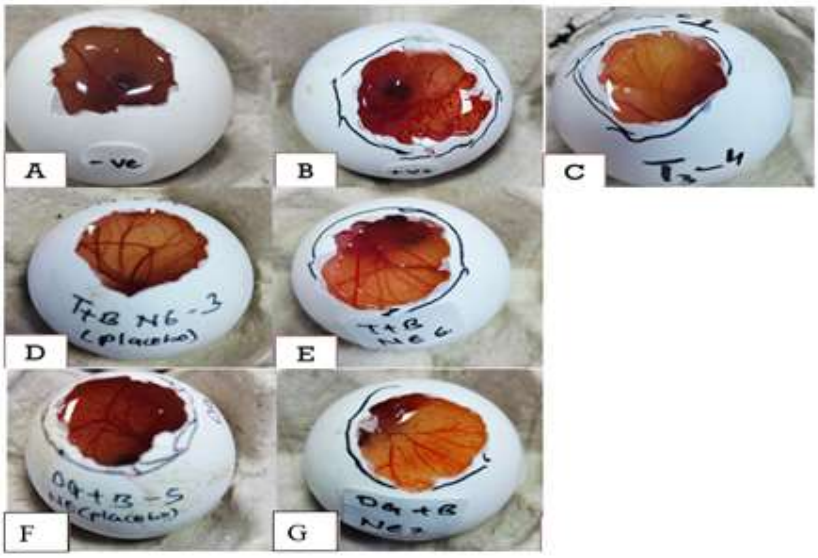

Figure 19: Images of the Het Cam Irritation Study

CONCLUSION

Vitamin-E-loaded nanoemulsion were successfully formulated and evaluated using the spontaneous emulsification method. The oil and S-mix percentages were optimized through the construction of pseudo-ternary phase diagrams, employing design of experiment software. The thermodynamic stability of the nanoemulsion were assessed using centrifugation, freeze-thaw, and heat-cool cycles. Key characteristics such as particle size, zeta potential, drug content, and viscosity were measured for the developed formulations. The release profile demonstrated that the formulation could sustain drug release for up to 14 hours. From the above studies we can conclude that both the nanoemulsion batches the one with Decyl glucoside and the other with Tween 80 shows excellent physicochemical properties and thermodynamic stability.

The in vitro and ex vivo drug release and permeation studies demonstrated that batch B5, containing Decyl Glucoside a natural surfactant exhibited sustained drug release and enhanced permeation compared to batch A4, which included synthetic surfactants like Tween 80. This indicates that Decyl Glucoside could serve as a promising alternative to synthetic surfactants in drug delivery systems, particularly for nanoemulsion. Batch B5 not only improved drug permeation but also showed superior tyrosinase inhibition and better cell viability, making it highly effective for treating conditions like melasma.

As a natural surfactant, Decyl Glucoside is biodegradable, eco-friendly, and extremely mild, which makes it ideal for sensitive skin and suitable for sustainable formulations. With increasing consumer demand for clean, natural, and environmentally responsible products, Decyl Glucoside stands out as a preferred ingredient in skincare formulations. Its ability to enhance drug delivery while being gentle on the skin suggests that natural surfactants, like Decyl Glucoside, offer significant advantages in developing effective, eco-conscious drug delivery systems for dermatological treatments like melasma.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of Data and Materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.