



Formulation and Evaluation of Herbal Emulgel and Clinical Assessment in the Management of Inflammation in Knee Osteoarthritis

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Abstract

Objective: This study was aimed to formulate and to evaluate a nanoemulgel along with preliminarily assess the clinical response, for the treatment of inflammation associated with knee osteoarthritis.

Methods: Nanoemulsions containing herbal oils and camphor as penetration enhancers were formulated using Low Energy Emulsification technique. The formulation was optimized with a Box-Behnken design to evaluate variables like droplet size, entrapment efficiency, % drug release. The optimum NE formulation was selected using Design-Expert software and analysed through TEM and *ex vivo* permeation study. The pH, Viscosity, Spreadability and *In-vitro* release study of nanoemulgel has been evaluated and assessed for its anti-inflammatory activity.

Results: This optimized formulation (OPZ-EG; including 6ml of eucalyptus oil, 1ml of sesame oil and 1.8:1ratio Smix for 100ml formulation) achieved a %EE=99.58%, VS=202nm, PDI= 0.400, ZP=-26.3mV, DR=81.34% for 12hr. The FTIR study verified that the drug and the other chemical compounds did not interact. By *ex-vivo* study J and Kp value was 2.36mg/cm²/hr and 0.787cm/hr respectively. Within the spectrum of skin pH, the formulation's pH was studied at 6.3 to 6.71. Prepared emulgel shows optimum viscosity and spreadability with % drug release 80.04% at 12 hrs. Hence, optimized nanoemulgel were studied for anti-inflammatory activity by using suitable model.

Conclusion: Nanoemulsions are lipoidal drug delivery systems with droplet size less than 300nm can effectively enhance skin penetration and provide anti-inflammatory action for KOA. The formulated emulgel showed significant improvement in the managing joint pain, swelling and ROM of joint. The statistical results show significant improvement, indicating its potential for further clinical study.

Keywords: Nanoemulgel; Knee Osteoarthritis; Nanoemulsion; Anti-inflammatory

Abbreviations

OA: Osteoarthritis; KOA: Knee Osteoarthritis; %: Percentage; % v/v: Percentage Volume by Volume; % w/v: Percentage Weight by Volume; µg: Micro-gram; cm: Centi-meter; cm⁻¹: Per centi-meter; cm³: Cubic-centimeter; cps: Centipoise; Fig: Figure; FTIR: Fourier Transform Infrared Spectroscopy; g: Gram; h: Hours; kg: Kilogram; M: Molarity; m³: Cubic-meter; mg: Milli-gram; µg/ml: Micro-gram Per Milli Liter; min: Minutes; ml: Milli-liter; mol: Mole; nm: Nano-meter; °C: Degree Celsius; °F: Degree Fahrenheit; pH: Negative Log of Hydrogen Ion Concentration; rpm: Rotation Per Minute; gm.cm/sec: Gram Centimeter Per Second; SD: Standard Deviation; Sec: Seconds; USP: United State Pharmacopeia; UV: Ultra Violet; Wt: Weight; λ max: Absorption Maxima; ROM: Range of Motion; CDR: Cumulative Drug Release; MDF: Multiple Dilution Factor; CLA: Cumulative Loss Adjustment; % CDR: % Cumulative Drug Release; PDI: Poly Dispersity Index; PEG: Polyethylene Glycol; BHA: Butylated Hydroxy Anisole; TEM: Transmission electron Microscopy; NEs: Nanoemulsions; VS: Vesicle Size; % EE: % Entrapment Efficiency; DR: Drug Release; OPZ: Optimized; EG: Emulgel; NE: Nanoemulsion; 2FI: Two factor Interaction; J: Flux; Co: Initial Concentration of Drug; Kp: Permeability Constant; NEG: Nanoemulgel; BT: Before Treatment; AT: After Treatment; gms: Grams; gm: Gram.

Introduction

Emulsion is defined as a biphasic liquid dosage form in which one immiscible liquid is uniformly dispersed in another liquid as a fine droplet. Emulsions can be categorized as w/o and o/w. Based on the size and shape of the droplets the emulsions divided into three types: macro emulsion (0.5-100nm), microemulsion(10-100nm) and nanoemulsion(100-1000nm) [1].

A nanoemulsion is a mixture of colloidal particulates complex consisting of two or more immiscible liquids distributed as tiny droplets size in the range of 20 to 500 nm. These nanoemulsion have been classified into two categories, o/w nanoemulsions consist of a few oil droplets scattered in a liquid phase, whereas w/o nanoemulsions comprise tiny water droplets scattered in an oil phase. Using a nanoemulsion to distribute a medicine can boost its effectiveness while reducing its overall dose and adverse effects [2]. The high energy and low energy methods are used for formulation of nanoemulsion drug delivery systems [3].

Knee osteoarthritis also referred as synovial joints are impacted by osteoarthritis often known as degenerative joint disease. Based on its etiology it is categorized as either primary or secondary. Primary KOA is a complicated pathophysiology that includes several variables, including heredity, age, body, weight, sex, inflammation, metabolism and immunity. The three main causes of secondary KOA are genetic damage, congenital articular abnormalities and trauma. Active alterations brought on by an imbalance between articular tissue damage and healing, rather than passive degenerative. The gradual deterioration and loss of articular cartilage which leads to whole joint, including the bone below the cartilage (in the knee), peripheral ligaments and synovium which experiences structural and functional changes [4].

Compared to rheumatoid arthritis (RA) and other joint diseases, osteoarthritis (OA) is one which is prevalent, expensive and destructive types of the joint illness. The prevalence and occurrence of KOA is higher in women, regardless of demographic variables including age, race or genetics. Extensive literature review shows that the incidence of arthritis rises with age is higher in urban areas than in rural ones. KOA is higher in women around 51% than in men about 30.09% and is more prevalent in those with higher body mass index (BMI), due to postmenopausal in women [5].

KOA can be treated with pharmacologic, non-pharmacologic, surgical, complementary and other alternative methods. To attain optimal results, patients usually a mix of these therapy choices. The goals of treatment are to enhance functional status and alleviate symptoms, and the start of treatment is determined by the severity and length of symptoms. Options for treatment should be unique to each patient [6].

Eucalyptol can inhibit the production and synthesis of TNF-α, interleukin-1β (IL-1β), leukotriene B₄, and thromboxane B₂ in human blood monocytes. It showed that eucalyptol is a potent cytokine inhibitor, making it suitable for long-term treatment of inflammation of the airways in bronchial asthma and other steroid-sensitive disorders [7]. Sesame oil possesses analgesic and anti-inflammatory properties and has been shown to reduce knee pain and improve joint function following topical application in patients with knee osteoarthritis [8].

Overweight and obesity are recognized to raise the risk of osteoarthritis (OA) by raising the mechanical stress on lower limb

joints, but it has also been demonstrated that they are responsible for a higher incidence of hand OA, highlighting the systemic effects which indicate the need for more investigation. Through the activation of receptors, the maintenance of articular cartilage homeostasis is significantly influenced by the mild loading of knee joints during regular everyday activities. However, abnormally high mechanical loads cause normal joint morphology to alter and cartilage homeostasis to be affected, which causes and speeds up the development of knee osteoarthritis [9].

The formation of NEs occurs when two immiscible liquids, usually water and oil dispersed together without exhibiting distinct phase boundaries because of their insoluble nature. Because of its improved drug solubility, faster drug loading capacity, prolonged thermodynamic stability and capacity for enhancing penetration without irritating the skin, the system can be utilized for topical use. A promising technique for administering pharmaceuticals that seeks to increase the efficacy of lipophilic agents is nanoemulgel [10].

NEG is a combination of gel and nano emulsion preparation. Low viscosity in NEs might lead to poor spreadability and retention time. This can be addressed, however, by generating NEG and adding a suitable gelling agent. It enhances stability and enables drugs to be administered with rapid and controlled release. Compared to other nano-carrier systems, it also offers benefits such as high capacity for drug loading, enhanced penetration, improved diffusion and reduced skin irritation [11].

Materials and Methods

Materials

The suppliers of eucalyptus oil (Laboratory grade), PEG-400(Extra pure) and We purchased Sesame oil (Laboratory grade) from Swadeshi Vasthu Bhandar, Bagalkot and Camphor (Laboratory grade, $\geq 98.5\%$ purity), Butylated hydroxy anisole (BHA) (Laboratory grade, $\geq 98.5\%$ purity), from Nice Chemicals Pvt. Ltd. Kochi, Kerala. Furthermore, Tween 80 and triethanolamine (Laboratory grade, $\geq 99.0\%$ purity) from Moly Chem, Mumbai and Carbopol 934 provided from Loba Chemie Pvt. Ltd.

Methods

Determination of $\lambda_{\max}$ of camphor

The $\lambda_{\max}$ was determined by using UV-Visible spectrophotometer in the range of 200-400nm.

Preparation of calibration curve $\lambda_{\max}$ of camphor

In a 100ml volumetric flask, accurately weighed 100mg of camphor was taken and is been dissolved in 5ml of methanol. Then the volume was made up to 100ml with 6.4 pH Phosphate buffer and it is referred as stock I solution. Then from the above stock solution, 10ml was pipette out and transferred in to another 100ml volumetric flask and volume was made up to the 100ml mark with Phosphate buffer. This is referred as stock II solution, from the above stock II solution appropriate aliquots of sample were withdrawn and transferred to different volumetric flasks of 10ml and volume was made up to the mark with buffer to produce 10,20,40,60,80 and 100 μ g/ml concentrations. The standard calibration graph of concentration v/s absorbance was developed by using 292 nm wavelength. Slope, intercept and regression coefficients were reported [12].

Method of preparation of nanoemulsion

Nanoemulsions were prepared by using a low-energy emulsification method. The aqueous medium containing distilled water was stirred using propylene glycol were SD Fine Chem Ltd, Mumbai. lab stirrer. The mixture of oils, camphor, tween 80 and PEG 400 was added continuously drop-wise to the aqueous medium at 3000 rpm up to 60 minutes. the resulting non emulsion was stored at room temperature in transparent glass containers [13].

Construction of pseudo ternary phase diagram

Phase diagram was plotted using CHEMIX ternary plot software (CHEMIX School 7). To identify the component's concentration within the current range by constructing the pseudo ternary diagrams utilizing a water titration approach at room temperature. Oil, surfactant, and cosurfactant (Smix) mixed with weight ratios of 1:1 and 2:1 respectively. For each phase diagram, the oil and specific Smix ratio (Tween 80: PEG 400) were mixed thoroughly in different weight ratios (1:9 to 9:1) in different glass vials. Each stable combination passed through a gradual water titration. The physical state transitions from transparent to turbid and vice versa were visually seen and recorded on the three components of the ternary phase diagram, each axis indicates the Water, Oil and the mixture of surfactant and cosurfactant [14].

Experimental design

The formulation factors for nanoemulsions were statistically optimized using a 3-level, three factor Box Behnken design with

the aim to maximize drug release, entrapment efficiency as well as in range of droplet size as mentioned in table 1. Design Expert® software (version 12.0.1.0, Stat-Ease Inc Minneapolis, MN, USA) was used to create and assess the experimental design. As we reported in table 2, total 15 experimental runs were obtained with 12 representing the mid-points of each edge with multidimensional

cube and the remaining 3 were replicated of the cube's center point. The 3 factors such as Eucalyptus oil (X1), Sesame oil (Span 60) (X2), and Smix (X3) are identified as independent variables and droplet size (Y1: VS), cumulative percentage drug released from Nanoemulsions (Y2: % DR at 12h) and entrapment efficiency (Y3: EE%) were identified as the dependent variables [15].

Factor	Level of Variables		
	Low (-1)	Medium (0)	High (+1)
Independent Variables			
X ₁ : Eucalyptus oil (% v/v)	2	4	6
X ₂ : Sesame oil (% v/v)	1	2	3
X ₃ : Smix (ratio)	1:1	2:1	3:1
Dependent Variables	Constraints		
Y ₁ Droplet Size (nm)	In range		
Y ₂ : Drug Release (%)	Maximize		
Y ₃ : Entrapment Efficiency (%)	Maximize		

Table 1: Behnken design parameters and constraints of Nes.

Batch code (100ml)	Runs	Ingredients					
		Eucalyptus oil (% v/v)	Sesame oil (% v/v)	Camphor (% w/v)	Tween 80	PEG 400	Distilled water (ml)
					Smix (ratio)		
NE-1	1	6	2	5	3	1	100
NE-2	2	6	1	5	2	1	100
NE-3	3	6	2	5	1	1	100
NE-4	4	2	3	5	2	1	100
NE-5	5	2	1	5	2	1	100
NE-6	6	4	1	5	3	1	100
NE-7	7	4	2	5	2	1	100
NE-8	8	4	1	5	1	1	100
NE-9	9	2	2	5	1	1	100
NE-10	10	2	2	5	3	1	100
NE-11	11	4	3	5	1	1	100
NE-12	12	4	3	5	3	1	100
NE-13	13	4	2	5	2	1	100
NE-14	14	4	2	5	2	1	100
NE-15	15	6	3	5	2	1	100

Table 2: Formulation composition of Nanoemulsion Box Behnken design.

Characterization of nanoemulsion

Compatibility studies

A 5mg of sample was put on the top of the machines transparent crystal glass and it was gripped until a click was heard. The Spectra were captured at a resolution of 4cm^{-1} between 4000 and 400cm^{-1} [16].

Droplet size and zeta potential

Using a Malvern Zeta Sizer apparatus, the dynamic scattering of light in which the nanoemulsion droplets were determined to figure out their size. Prior to analysis each sample was diluted 1:100 with distilled water. The measurement was carried out at 25°C and 90° for scattering angle. Each sample was evaluated and the mean value was reported [17].

Entrapment efficiency

The amount of drug that was trapped in the formed NE was measured using the UV spectrophotometric technique. After centrifuging a 2ml aliquot of optimized NE by using high speed centrifuge machine equipped with fixed angle CRotor for 30 minutes at 3500rpm, the liquid supernatant was collected. It was diluted with 10ml of distilled water and UV spectroscopy was used to measure the drug's concentration. The following formula was used to determine the %EE: [18].

$$EE(\%) = \frac{C_t - C_s}{C_t}$$

where, C_t = total drug concentration

C_s = concentration of drug in the supernatant

In-vitro diffusion study

The diffusion cell was used to measure the amount of drug to be release for the developed NE formulation. The diffusing membrane had been soaked in medium for 24 hours, 2ml of the formulation was added to one end of the diffusion tube in which the diffusing membrane has been tied. The entire assembly was fixed so that the diffusion medium surface was only 1-2mm below the lower end of the NE containing diffusion tube, which was placed in 150 ml beaker containing 100ml of the 6.4 pH phosphate buffer at room temperature. The samples were taken out at various times and diluted up to 10ml with fresh 6.4 pH phosphate buffer. More dilution

could be done to get in the beer's range. The sample followed by measurement at 292nm using a UV-Vis spectroscopy [19].

Transmission electron microscopy

The morphology of the enhanced nanoemulsion was investigated using transmission electron microscopy and staining that is negative. On Copper TEM grids, a drop of non-emulsion was applied and allowed to stick to the carbon substrate for a minute. 1% (w/v) sodium phosphotungstate solution was used to negatively stain the sample, and filter paper was used to completely remove any remaining stain. The samples were allowed to dry at room temperature. Ultimately, the adversely stained sample was seen at 80kV [20].

Optimization of final formulation

The formulation prepared based on Box-Behnken design and this design was used for evaluating the various response variables. Multiple regression analysis was used to look at the response variables in order to find the relation among the factors used and the response values generated. The optimized formula identified from the above optimization technique was formulated and evaluated to check the validity and reproducibility of evolved model.

$$\%Error = \frac{\text{Experimental value} - \text{Predicted value}}{\text{Predicted value}} \times 100$$

Method of preparation of nano emulgel

As shown in table 3, weigh accurate quantity of Carbapol-934 and soak it in formulation, kept it aside for 24hr for complete swelling. Butylated hydroxyl anisole was dissolved in propylene glycol. Carbapol-934 was combined with dissolved content of propylene glycol until it was uniform. It was carried out by using a lab stirrer set to 800 rpm for 10 minutes. The gel's pH has been adjusted by adding a small amount of triethanolamine and gently stirred with the glass rod to form a homogeneous mixture.

Evaluation of nanoemulgel

pH determination

The digital pH meter was used to measure the pH at room temperature. After dispersing 2 gms of gel in 20ml of pure water. Then the pH was determined [21].

Homogeneity

The prepared gel was examined visually for its homogeneity. They were assessed for aggregate presence and appearance [22].

Spreadability

Glass slides and wooden blocks were used to determine the spreadability. A 20 gms weight was placed on glass slides as a load to compress the excess sample to a uniform thickness for 5mins in order to determine spreadability. 2gms of sample was used to perform this test. Spreadability was measured by determining how long it took to separate the two slides or how long it took for the upper glass slide to move across the lower plate until both plates detached. Spreadability was calculated by using the formula [23]:

$$S = \frac{M \times L}{T}$$

Where, M = Weight tied to upper slide,

L= Length moved on glass slide

T = Time taken to separate the slide completely from each other

Viscosity

The prepared gel's viscosity was measured using a Brookfield viscometer at 100rpm by using spindle number 64 and the readings were recorded [24].

In-vitro release study

2gms of the gel formulation was placed in glass cylinder which act as a donor chamber. The cylinder was suspended so that the membrane barely touched the surface of a beaker (receptor chamber) filled with 50ml of diffusion medium. The receptor chamber was maintained at $37 \pm 2^\circ\text{C}$ and stirred at 70rpm using magnetic stirrer up to 6 hours. Every hour about 4ml of the sample were taken out and replaced with an equivalent volume of freshly prepared diffusion media. A UV Spectrophotometer was used to evaluate the aliquots at 292 nm [25].

Ex-vivo permeation study

The study was carried out in accordance with OECD rules. A sacrificed rat was taken from the animal house of HSK College of Pharmacy, Bagalkote. Its skin was surgically removed after the hairs on its abdomen were trimmed. Alcohol and distilled water were used to rinse the removed skin. To hydrate the membrane, it was immersed in phosphate buffer for at least 30 to 60 minutes. The donor and receptor compartment of Franz diffusion cell apparatus were utilized. The receiver compartment held the dissolution medium, which included phosphate buffer (pH 6.8) and methanol (10% v/v) which was continuously stirred using a magnetic stirrer

at 110 rpm for 24 hours. The optimum formulation, approximately 0.3 gm of nanoemulgel was placed in the donor compartment. Samples were taken every hour and added with the same volume of dissolution media while the temperature was maintained at 37°C . A UV Spectrophotometer with the wavelength of 292nm was used to evaluate the samples. Additionally, estimates of several skin permeation parameters were calculated, including the permeability coefficient (Kp) and Drug flux(J) [26].

Clinical assessment of gel

Single Arm Open Labelled Clinical Study.

Study Criteria:

- **Inclusion criteria**
 - Subjects diagnosed with Knee Osteoarthritis.
 - Patients of age group 25-80 years.
- **Exclusion criteria**
 - Patients of age <25 and >80 are excluded.
 - Any other systemic chronic disorders which affect the study.
- **Other Criteria**
 - Single-arm design
 - No control/placebo group
 - Open-label design
 - Non-randomized
 - Small sample size (30 subjects)
 - No a priori power calculation
 - Investigator-assessed grading scale

Study procedure

Anti-inflammatory activity study for formulated nano emulgel was done in prospective observational study for out-patient of Shri Kalidas Ayurvedic Medical College and Hospital, Badami. The institutional Ethics Committee's prior consent for research involving human subjects has been obtained and recorded. The 30 subjects who knee osteoarthritis of age 25-80 years were included of either sex in the study after obtaining the written consent from the subjects and were treated. The subjects were instructed for the procedure of application of gel thrice a day. On each visit of study center the containers of medication containing oils of nano emulgel

was assessed for treatment. Subject's analysis of inflammation was made by the investigator for parameters like joint pain, swelling, tenderness, crepitus and ROM of joints. Each of these parameters were graded absent, mild, moderate and severe. The scores of the five assessed parameters were added together to obtain the total clinical score for present study were collected from subject's case report. Progress that the obtained final results were statistically processed using the student paired t-test [27].

Results and Discussion

Determination of $\lambda_{\max}$ of Camphor: Standard Stock Was Scanned between 400-200 nm and $\lambda_{\max}$ of Camphor was found to be 292 nm as shown in figure 1.

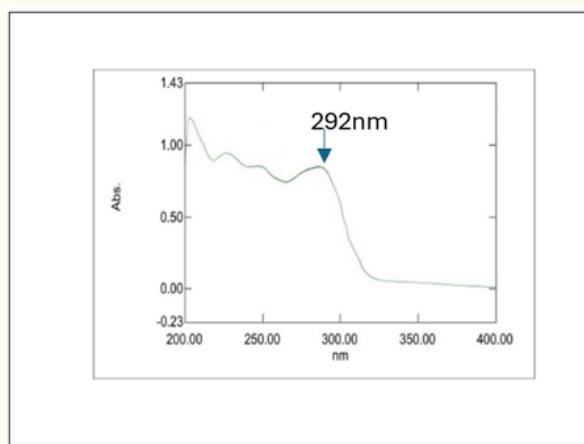


Figure 1: Determination of $\lambda_{\max}$ of Camphor.

Preparation of Calibration Curve of Camphor: Calibration Curve of Camphor was constructed with slope of 0.009, intercept 0.021 and regression value 0.997 as shown in figure 2.

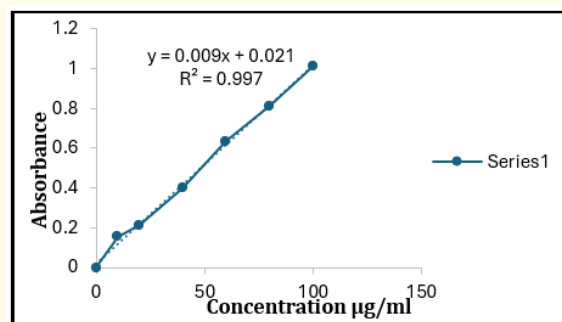


Figure 2: Preparation of Calibration Curve $\lambda_{\max}$ of Camphor.

Development of pseudo ternary diagram:

The water titration method was used to create the pseudo ternary diagram. Oil, surfactant, and cosurfactant (Smix) in each group were mixed at weight ratios of 1:1 and 2:1. For each phase diagram, the oil (Eucalyptus oil and Sesame oil) and appropriate Smix ratio (Tween 80: PEG 400) were mixed completely in varying weight ratios (1:9 to 9:1) in different glass vials. Water was gradually added to each isotropic combination. The change in physical state from transparent to turbid and vice versa which was experienced visually. Phase diagram was generated using CHEMIX ternary plot program. The figures are displayed in the figure 3a and 3b.

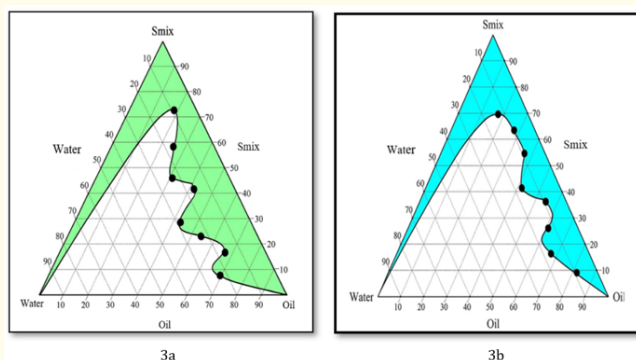


Figure 3: 3a & b are the pseudo ternary diagrams of Smix ratio of 1:1 & 2:1 respectively.

Experimental design

A three-level, three-factor Box Behnken design was adopted for the optimization of Nanoemulsions using Design Expert® software (version 12.0.1.0). Particle size (Y1:PS), cumulative percentage of drug release over 12 hours (Y2: DR12hr), and entrapment efficiency (Y3:EE%) were the three dependent variables that were examined at three levels such as low (-1), medium (0), and high (+1). The independent variables were concentration of Eucalyptus oil (X1), Sesame oil (X2), and Smix (X3) as illustrated in table 4. As response or dependent variables, the droplet size (Y1:PS), cumulative percentage of drug release over 12 hours (Y2: DR12hr), and entrapment efficiency (Y3:EE%) of all the manufactured formulations according to the design were examined. Several mathematical models, such as quadratic and two factor interaction (2FI), were simultaneously fitted to the observed data because these two models are more appropriate for best fit

to the experimental data based on ANOVA, with significant model terms, a non-significant lack-of-fit, and high R^2 values. The 2FI model was selected because the interaction between formulation variables significantly influenced the response. To comprehend the link between the independent and dependent or response variables, the Design Expert® program produced 2D-contour plots and 3D-response surface plots. Ultimately, the optimization of nanoemulsions was done using numerical and graphical optimization techniques to achieve the desired formulation parameters, including droplet size (Y1:PS), cumulative percentage

drug release over 12 hours (Y2: DR12hr), and entrapment efficiency (Y3:EE%). Using the overlay plot and desirability function in the design space, the approach was carried out. In the overlay plot, a flag indicated the optimization's location. Further, the validation of the devised approach was carried out by creating the optimized formulation and analysing the droplet size (Y1:PS), Cumulative % drug released from NE's over 12hr (Y2: DR12hr) and Entrapment efficiency (Y3:EE %). The expected and observed answers were compared to determine the percentage.

Batch code	Runs	Factor 1 Eucalyptus oil (% v/v)	Factor 2 Sesame oil (% v/v)	Factor 3 Smix (ratio)	Response 1 Particle size (nm)	Response 2 Drug release (%)	Response 3 Entrapment efficiency (%)
EG-1	1	6	2	3:1	244.41	80.12	98.25
EG-2	2	6	1	2:1	212.73	81.6	101.28
EG-3	3	6	2	1:1	245.76	82.3	98.25
EG-4	4	2	3	2:1	306.15	65.83	83.49
EG-5	5	2	1	2:1	412.1	67.95	61.14
EG-6	6	4	1	3:1	214.46	65.79	79.38
EG-7	7	4	2	2:1	192.3	73.15	87.22
EG-8	8	4	1	1:1	220.54	72.81	82.25
EG-9	9	2	2	1:1	347.82	70.71	75.58
EG-10	10	2	2	3:1	349.52	66.42	73.68
EG-11	11	4	3	1:1	225	65.39	90.5
EG-12	12	4	3	3:1	238.51	66.16	91.2
EG-13	13	4	2	2:1	196.2	73.33	86.6
EG-14	14	4	2	2:1	195.4	73.6	85.9
EG-15	15	6	3	2:1	304.52	78.19	99.18

Table 4: Trial as per Box Behnken design along with response.

Filling of data into the model

Using Design Expert® software, to find out the most appropriate model of all the observed responses for 15 runs were simultaneously fitted into multiple mathematical models. High values of multiple correlation coefficients(R^2), Adjusted R^2 and Projected R^2 values and small values of standard deviation (SD), Coefficients of variation (%CV) and predicted residual sum of the square (PRESS) were used for determining the best fit model. The

best fit model for droplet size and drug release has a quadratic model and entrapment efficiency has a two-factor interaction(2FI), for fitting into various models, regression analysis results were obtained for the responses Y1(droplet size), Y2(% drug release) and Y3(Entrapment efficiency).

Droplet size

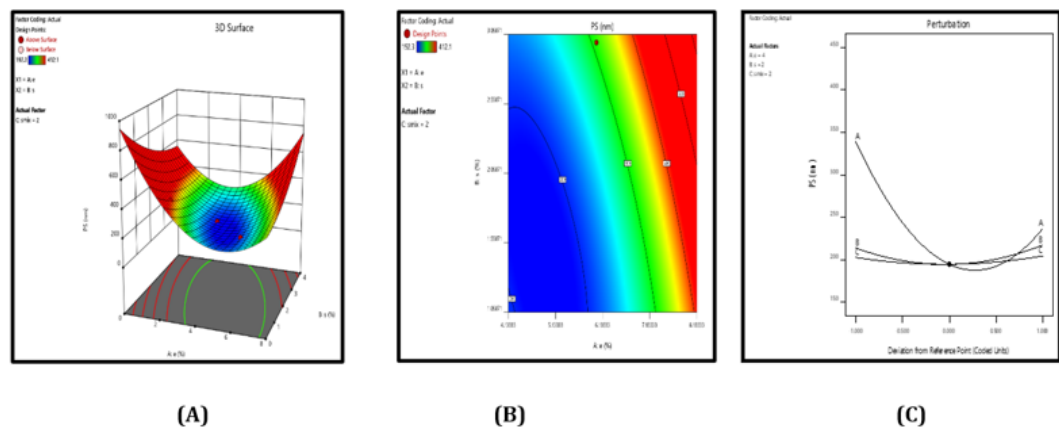


Figure 4: 3D Response surface (A), 2D Contour plot (B) and Perturbation plots (C) showing the effect of eucalyptus oil, sesame oil and Smix on Droplet Size of Nanoemulsion.

Drug release

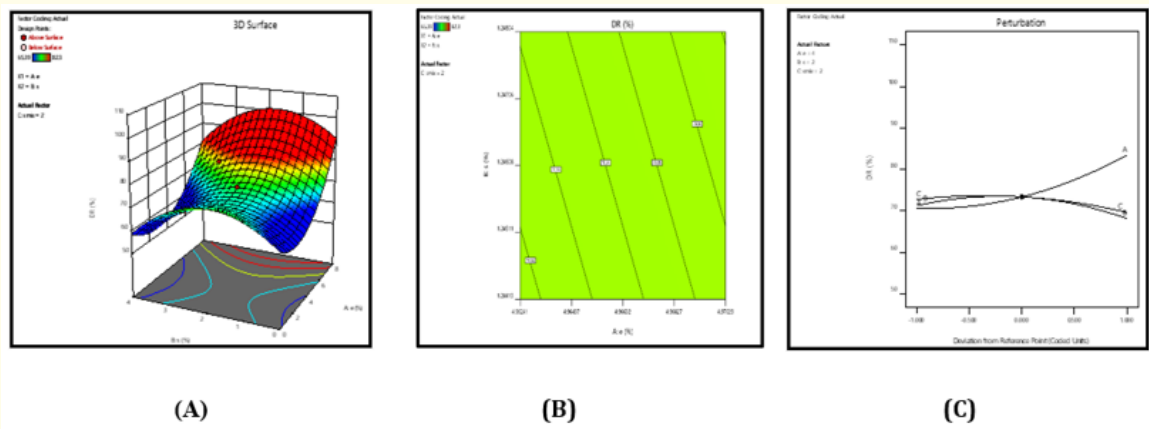


Figure 5: 3D Response surface (A), 2D Contour plot (B) and Perturbation plots (C) showing the effect of eucalyptus oil, sesame oil and Smix on Drug release of Nanoemulsion.

Entrapment Efficiency

Characterization of nanoemulsion

Compatibility study

Compatibility studies were conducted using FTIR spectroscopy. There is no contact between the drug and formulation components

because the physical mixing of camphor and excipients shows that the drug was compatible with them.

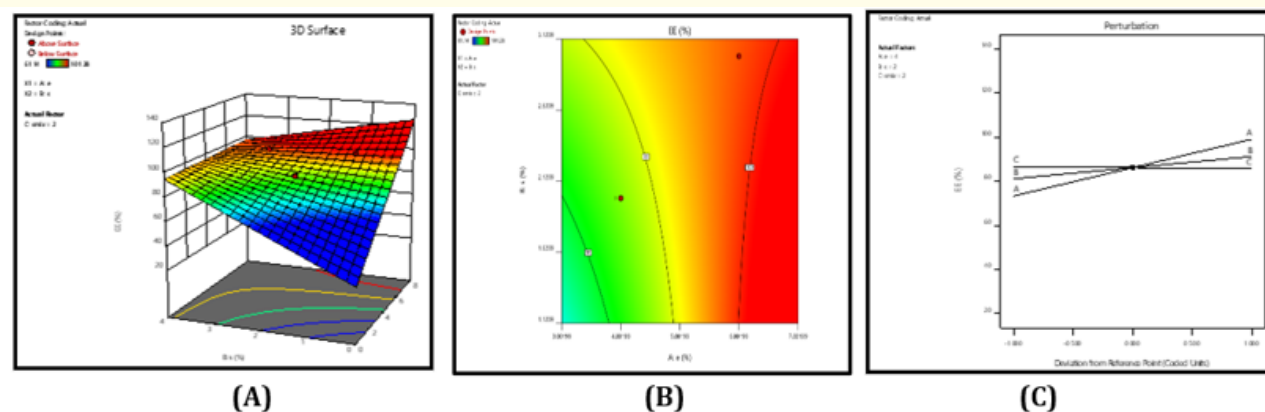


Figure 6: 3D Response surface (A), 2D Contour plot (B) and Perturbation plots (C) showing the effect of eucalyptus oil, sesame oil and Smix on Entrapment Efficiency of Nanoemulsion.

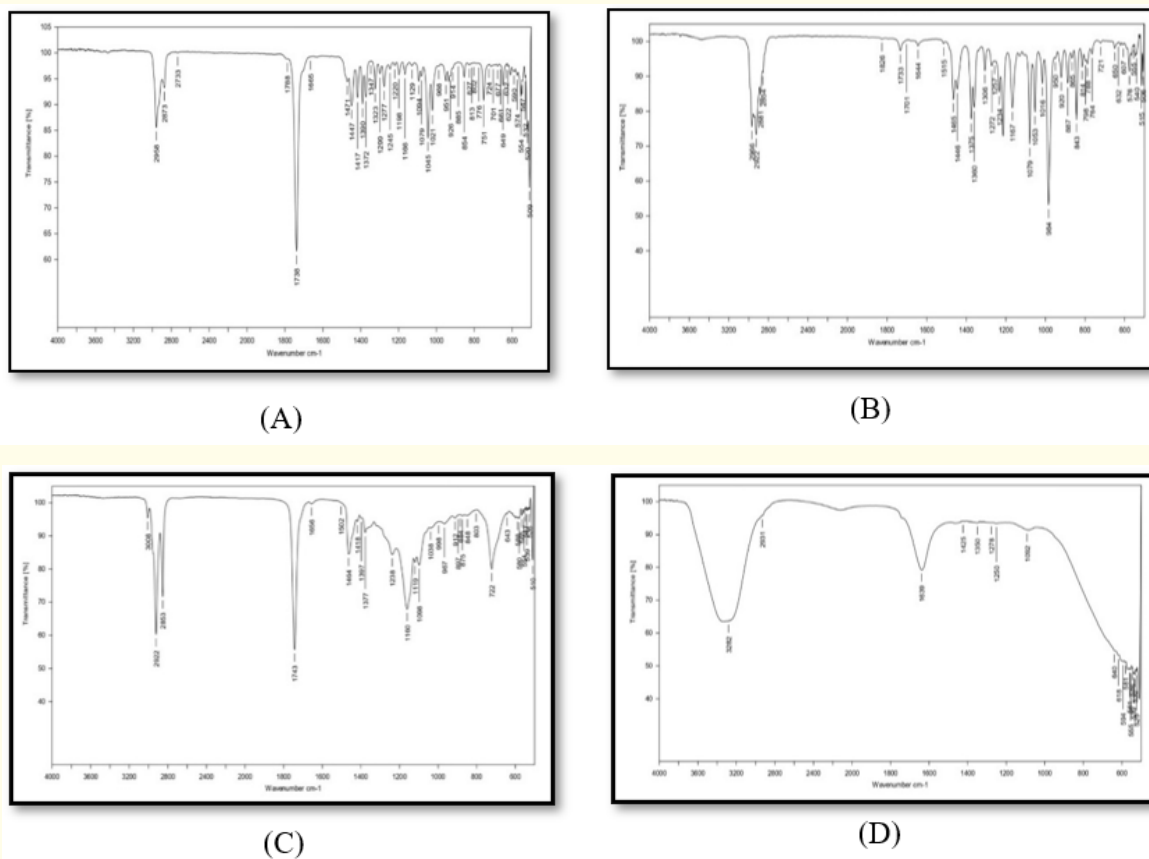


Figure 7: FTIR Spectra (A) Camphor (B) Eucalyptus oil (C) Sesame oil & (D) Optimized Formulation.

Droplet size, PDI, zeta potential and entrapment efficiency

As displayed in table 5, the generated emulgel formulations' droplet sizes varied from 192.30 to 412.10 nm, suggesting the generation of nanosized droplets appropriate for topical medication administration. Smaller droplets can enhance skin penetration and improve drug deposition at the target site. Among all formulations, EG-7 displayed the smallest droplet size (192.30 nm) with the lowest PDI (0.270), suggesting a generally homogenous particle size distribution, which is generally associated with better physical stability. In contrast, formulations with higher PDI values

indicate greater particle size variability, which may increase the likelihood of aggregation during storage and reduce formulation stability. The zeta potential values ranged from -18.3 to -23.8 mV, showing moderate electrostatic stability of the droplets that helps minimize droplet aggregation. EG-2 had the highest entrapment efficiency, followed by EG-15 and EG-1, indicating effective drug encapsulation. Entrapment efficiency ranged from 59.58% to 101.28%. Overall, EG-2 was the best formulation for additional testing since it showed the best combination of small droplet size, acceptable PDI, sufficient zeta potential, and the highest entrapment efficiency.

Sl. No.	Formulation Code	Droplet size (nm)	PDI	Zeta Potential (mV)	Entrapment Efficiency (%)
1	EG-1	244.41	0.582	-22.8	98.25
2	EG -2	212.73	0.611	-21.7	101.28
3	EG -3	245.76	0.858	-23.3	98.25
4	EG -4	306.15	0.438	-22.1	83.49
5	EG -5	412.10	0.456	-23.8	59.58
6	EG -6	214.46	0.415	-18.3	79.38
7	EG -7	192.30	0.270	-19.1	87.22
8	EG -8	220.54	0.358	-20.6	79.38
9	EG -9	347.82	0.484	-21.4	78.58
10	EG -10	349.52	0.456	-20.5	73.68
11	EG -11	225.00	0.680	-21.5	90.5
12	EG -12	238.51	0.700	-22.1	91.2
13	EG -13	196.20	0.379	-20.9	86.6
14	EG -14	195.40	0.461	-22.2	85.9
15	EG -15	304.52	0.464	-19.1	99.18

Table 5: Droplet size, PDI, Zeta Potential & Entrapment efficiency of formulation EG-1 to EG-15.

Characterization of optimized nanoemulsion

The Optimized nanoemulsions are by trading off different response variables in order to achieve the intended goals, minimize particle size, maximize drug release after 12 hours and maximize entrapment efficiency, the formulation was found using numerical, optimization and desirability function analysis. The optimum formulation consists of Eucalyptus oil, Sesame oil, Smix and Camphor which exhibited a droplet size is 202nm, *in-vitro* release study at 12hr is 81.34% and entrapment efficiency is 99.58% predicted by Box-Behnken reported in table 6.

Sl. No.	Parameters	Formulation OPZ-NE
1.	Droplet Size	202 nm
2.	PDI	0.400
3.	Zeta Potential	-26.3 mV
4.	Entrapment Efficiency	99.58%

Table 6: Droplet Size, PDI, zeta potential & entrapment efficiency results of Optimized nanoemulsion.

Transmission electron microscopy (TEM)

TEM analysis of the enhanced formulation OPZ-NE exhibited non aggregating nanoemulsion droplets with restricted size distribution and a mainly spherical shape, as illustrated in figure

8. The picture clearly demonstrates that the dimension of the droplets shown in the micrograph was in nanoscale and consistent with the measurements made using Malvern zeta sizer.

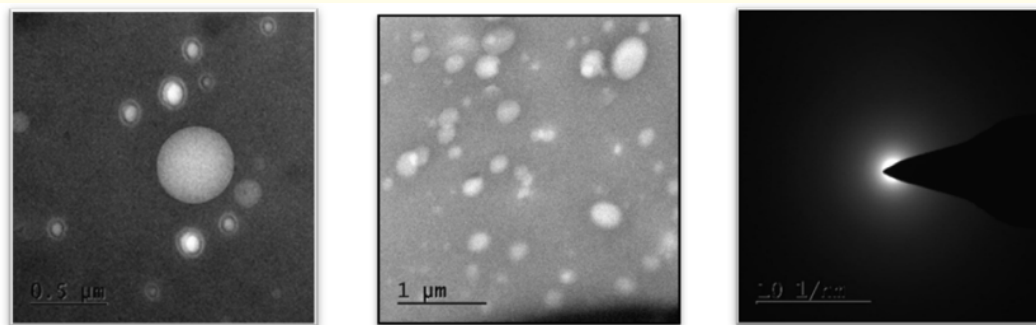


Figure 8: TEM images of Optimized nanoemulsion.

In-vitro release study for optimized nanoemulsion

The optimized nanoemulgel exhibited a sustained drug release profile, with approximately 15% drug release in the first hour and about 81% after 13 h. The initial release may be due to surface-associated drug, while the sustained release is attributed to the diffusion of the encapsulated drug through the vesicular system and gel matrix. This release pattern is comparable to previously reported nanoemulgel formulations for osteoarthritis, which typically show 70–90% drug release over 12–24 h, indicating effective drug encapsulation and controlled release suitable for prolonged topical therapy. The figure.9 displayed a graphical representation.

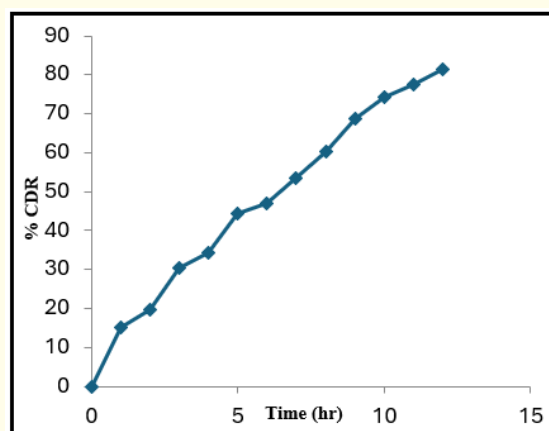
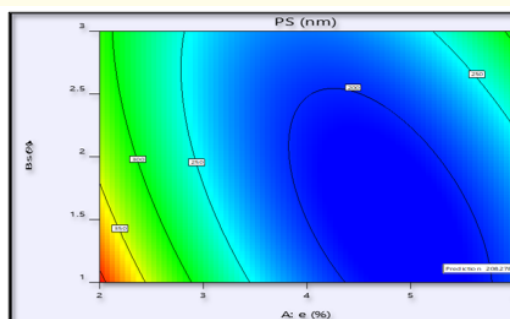
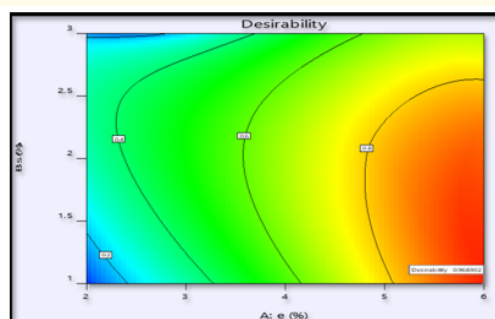


Figure 9: Graphical representation of % *in-vitro* release results of optimized nanoemulsion.

Desirability and overlay plot



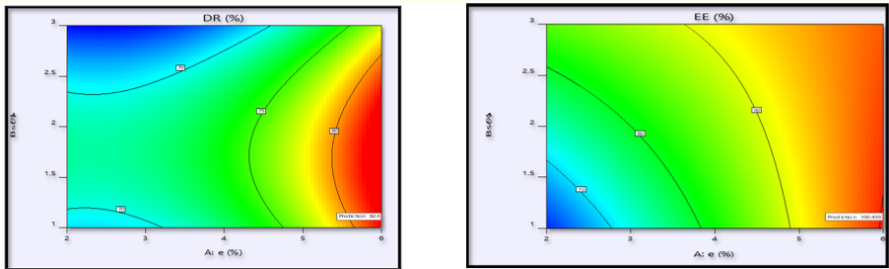


Figure 10: Desirability of Droplet size, drug release and entrapment efficiency.

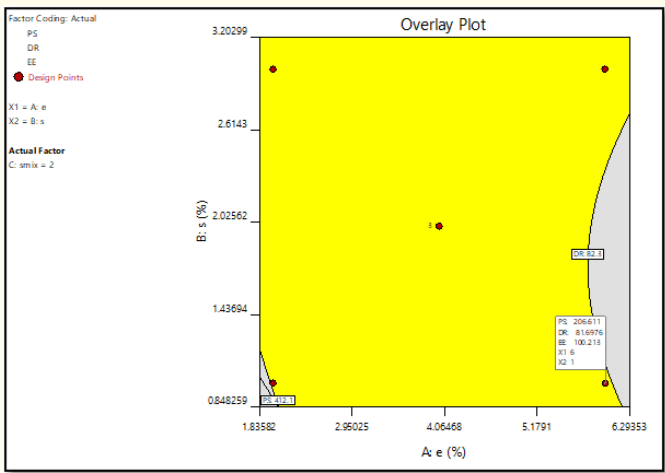


Figure 11: The overlay plot displaying the optimized nanoemulsion formulation in the design space and their predicted responses.

Characterization of optimized nanoemulgel

pH Determination

The formulated herbal emulgel pH was determined to ensure the stability and compatibility with the skin pH. The pH of the gel formulation was ranges from 6.38 to 6.71 which falls within the normal skin pH range. The formulation exhibited no appreciable change in pH during the one-month preliminary stability assessment, indicating no potential for skin irritation. Visual examination also showed no visible particle aggregation, and the formulation remained homogeneous.

Homogeneity

The formulated gel was filled in the container and visually inspected. There was no particle aggregation development and the formulation was found to be homogeneous.

Spreadability

The spreadability studies of gel formulation data are shown in table 7. The spreadability values were ranges from 21.47 to 24.09 gm.cm/sec. The spreadability data showed that a small amount of shear may rapidly spread the gel. After the application at the site of action, the gel was found to be easily spreadable.

Sl. No.	Viscosity (Cps)	Spreadability
1.	11598	22.91

Table 7: Viscosity and Spreadability results of optimized Emulgel.

Viscosity

The formulated emulgel was subjected to viscosity studies and data are presented in the table 7. The viscosity value in the range of 11498cps to 11635cps. The prepared gel formulation showed good viscosity and enabling them to retain at the site of application for an extended period. The optimized formulation shows an optimum viscosity; thus, the gel will not drip from the site of application.

In-vitro release study of optimized nanoemulgel

In-vitro drug release study of OPZ-EG was found to be 80.04 % for 12 hours and its graphical representation of % cumulative drug release was shown in figure 12.

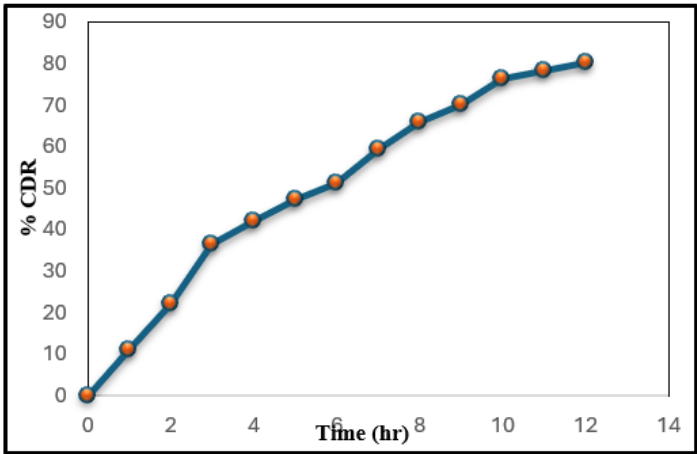


Figure 12: Graphical representation of % in-vitro release results of optimized nanoemulgel.

Ex-vivo permeation study

The improved nanoemulgel ex-vivo permeation profile is shown in figure 13. Overtime, the formulation showed a consistent increase in drug penetration. Superior release was demonstrated by optimized nanoemulgel, which may be explained by their reduced viscosity and smaller size. Higher flux values result from maintaining a larger quantity of free drug. The below table 8 displayed the flux(J) and permeability constant (Kp). Therefore, it was discovered that the drug flow and permeability constant values for the improved nanoemulgel formulation were both substantial.

Sl. No.	Flux (mg/cm ² /hr)	Permeability Coefficient (cm/hr)
1.	2.363	0.787

Table 8: Permeation parameters of Optimized nanoemulgel.

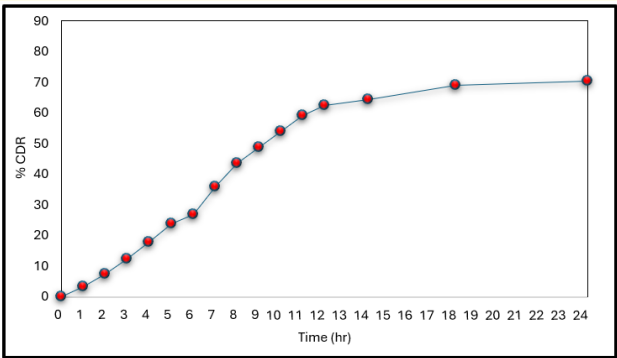


Figure 13: Graphical representation of % release study of optimized nanoemulgel at the end of 24 hr for permeation study.

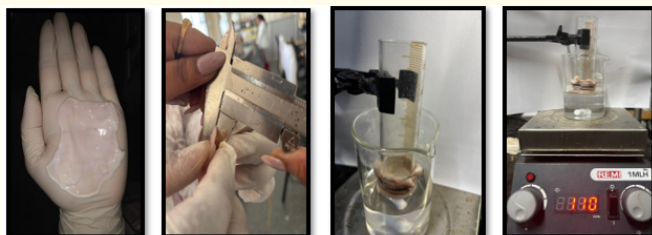


Figure 14: Ex-vivo permeation study.

Single arm open label clinical study

Distribution of subjects based on age

Among 30 subjects the maximum number of subjects belonged to an age group of 41-50 years, i.e., 15 subjects (50%) while 8 subjects (26.66%) belonged to the age group of 31-40 years, 4 subjects (13.33%) belonged to the age group of 51-60 years and 3 subjects (10%) belonged to the age group of 21-30 years respectively.

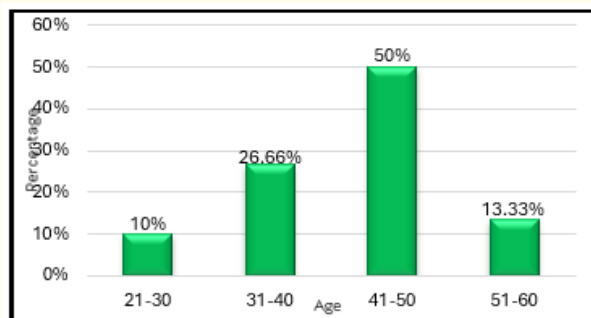


Figure 15: Graph showing distribution of subjects based on Age.

Distribution of subjects based on Sex

Among 30 subjects, 16 subjects (53.33%) are female and remaining 14 subjects (46.66%) are men. Women are disproportionately affected by knee osteoarthritis; women are having 55-60% higher risk of developing knee osteoarthritis as compared to men. Hormonal fluctuations contribute to increased osteoarthritis risk in postmenopausal women.

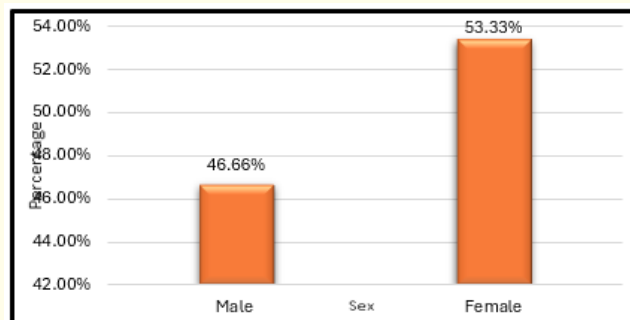
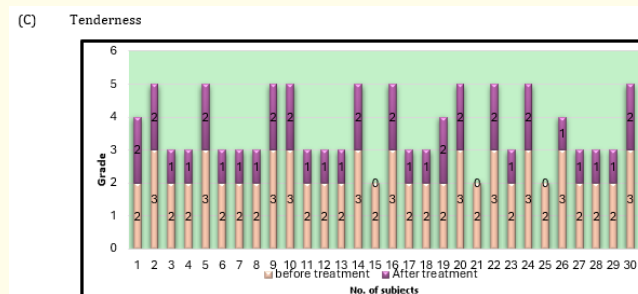
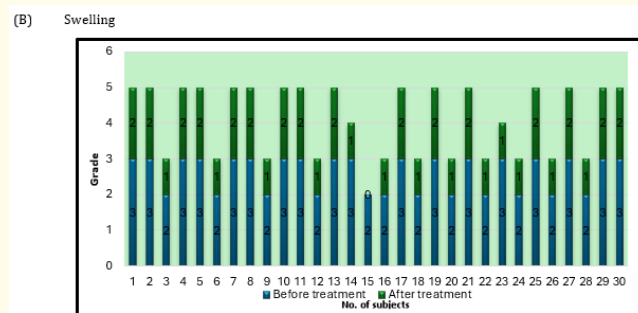
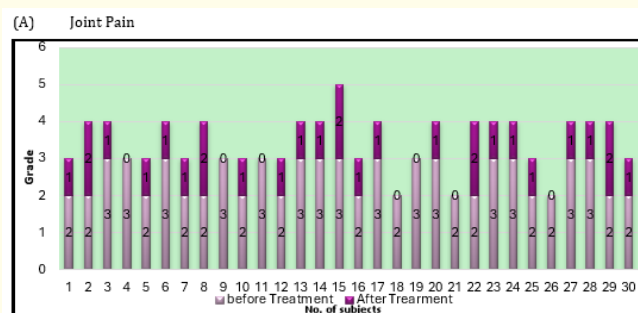


Figure 16: Graph showing distribution of subjects based on Sex.



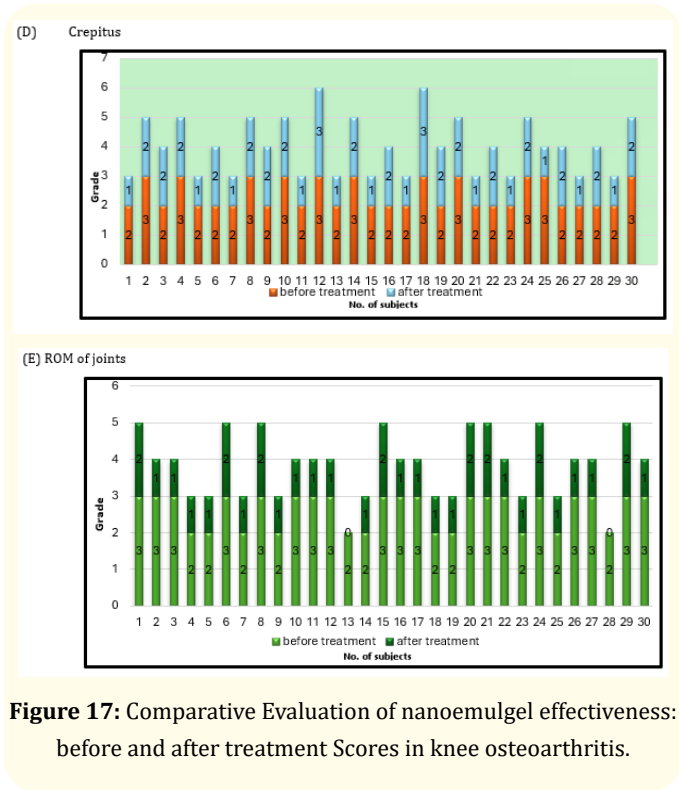


Figure 17: Comparative Evaluation of nanoemulgel effectiveness: before and after treatment Scores in knee osteoarthritis.

Analysis of statistics: for the paired “t” test

Paired ‘t’ test conducted and results were expressed in table 9 using graph pad software

- **Pain:** The statistical analysis showed a remarkable decrease in mean joint pain scores, from 2.50 before treatment to 0.93 after treatment, respectively. Statistically preliminary improvement was observed.
- **Swelling:** The statistical analysis showed a remarkable decrease in mean swelling scores, from 2.60 before treatment to 1.50 after treatment, respectively. This reduction was statistically improved.
- **Tenderness:** The statistical analysis showed a remarkable decrease in mean tenderness scores, from 2.37 before treatment to 1.33 after treatment, respectively. This reduction was statistically improved.
- **Crepitus:** The statistical analysis revealed that the mean score of crepitus during extension which was 2.40 before the treatment and was reduced to 1.67 after the treatment respectively. By conventional criteria, this difference is considered to be statistically preliminary improved.

Sl. No	Parameters	Mean		Difference in mean	% of Relief	SD	t-value	p-value	Remarks
		BT	AT						
01	Joint Pain	2.50	0.93	1.57	62.8%	0.50	12.45	<0.001	Moderate Improvement
02	Swelling	2.60	1.50	1.10	42.30%	0.71	7.80	<0.001	Mild Improvement
03	Tenderness	2.37	1.33	1.03	43.88%	0.50	5.33	<0.001	Mild Improvement
04	Crepitus	2.40	1.67	0.73	30.41%	0.51	8.32	<0.001	Mild Improvement
05	ROM of Joint	2.63	1.20	1.43	54.37%	0.51	8.32	<0.001	Moderate Improvement

Table 9: Statistical results of Effect on joint pain, swelling, tenderness, crepitus and ROM of Joints.

- **ROM of joints:** The statistical analysis revealed that the mean score of ROM of joint during extension which was 2.63 before the treatment and was reduced to 1.20 after the treatment respectively. Statistically, preliminary improvement was observed.

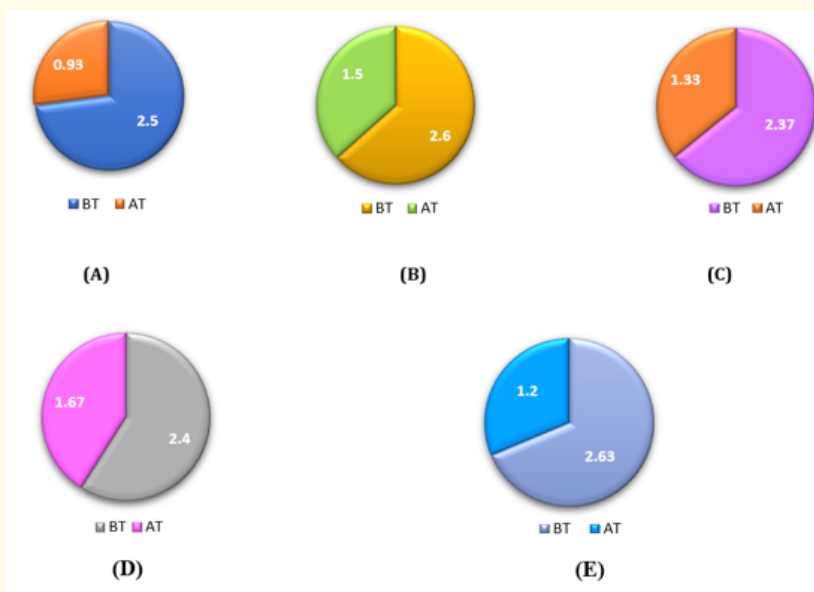


Figure 18: Improvement of parameters within Subjects (n=30) A) Joint Pain B) Swelling C) Tenderness D) Crepitus and E) ROM of joints.

Conclusion

The present study was inspired by the traditional use of eucalyptus oil, sesame oil, and camphor for their anti-inflammatory and analgesic properties. Herbal nanoemulgel was successfully developed to enhance the topical delivery of these natural ingredients. The optimized formulation exhibited favourable physicochemical characteristics and showed improved skin penetration, indicating its potential to enhance the local delivery of the active components. Preliminary clinical observations and patient feedback suggested mild to moderate improvement in the symptoms of knee osteoarthritis. However, these findings should be interpreted cautiously due to the limited clinical evaluation. Overall, the developed herbal nanoemulgel demonstrates promise as a topical formulation for the management of pain and inflammation associated with knee osteoarthritis. Further large-scale clinical studies are required to establish its long-term safety and therapeutic efficacy.

Limitations

The present study was designed as a preliminary clinical evaluation to assess the efficacy and tolerability of the newly developed formulation. Therefore, a single-arm, open-label study

design was adopted to obtain initial clinical evidence regarding its therapeutic effects in the target population. This design was considered appropriate for an exploratory assessment of the formulation; however, the absence of a control group and blinding limits the ability to distinguish treatment-related effects from placebo responses, natural disease progression, or other potential confounding factors.

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Conflict of Interest

The authors declare there is no conflict of interest.

Funding Source

The authors declare there is no funding is allotted to carry out this research.

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Ethical Clearance

Institutional ethical clearance certificate has been taken from Shri Kalidas Ayurvedic Medical College and Hospital, Badami, Karnataka, for the conduct of clinical studies on 31/07/2024 with reference number SKAMCH/2025-26/253.

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