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FORMULATION AND PHYSICAL EVALUATION OF GLUCOCORTICOID LOADED TEA TREE OIL NANOEMULSION: A SUMMARIZED TECHNICAL NOTE

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ABSTRACT: Clobetasol propionate (CP) has anti-inflammatory, immunomodulatory, and anti-proliferative activity. The current work aimed to prepare and evaluate the nanoemulsions of CP loaded tea tree oil (TTO) would be stable nanoemulsion. Clinical use of CP is restricted to some extent due to its poor permeability across the skin. So, to increase its permeation across the skin, microemulsion based formulations were prepared and characterized. Microemulsions were prepared by aqueous phase titration method, using TTO, tween 20 transcutol P, and distilled water as the oil phase, surfactant, co-surfactant, and aqueous phase, respectively. Selected formulations were subjected to physical stability studies. Malven Zeta seizer did particle size distribution of nanoemulsion. In surface studies of the optimized formulation were done by Transmission Electron Microscopy. The nanoemulsion (A2) was a colloidal dispersion having average droplet size ranging from 100 to 200 nm. The nanoemulsion (A2) formulation exhibited a narrow size distribution (PI = 0.131). The optimized formulation exhibited viscosity 28.30 ± 1.91 mP, refractive index 1.403, pH 6.15, and conductivity 10^{-4} s cm^{-1}. The optimized formulation was found that the formulation A2 consisting of 15% oil phase, 35% (S_{mix} 1:1) and 50% distilled water exhibited 42.52% of cumulative amount of drug permeated (µg/cm^2) after 24 h and highest amount of drug deposited in skin 31.06 (µg/cm^2). **Conclusion:** The developed CP loaded TTO nanoemulsion was stable and good deposition of CP in the deeper layer of skin which may result in sustainable and good anti-inflammatory activity for the treatment of psoriasis.

INTRODUCTION: Psoriasis is a common dermatological condition affecting 2% of the population. Psoriasis is chronic; T-lymphocyte mediated autoimmune inflammatory disorder that affects the skin, joints, and tendons and characterized by erythematous scaly plaques [1].

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In 1998 National Psoriasis Foundation Survey found that 10% of 18-34-year-old patients and 7% of 35-54-year-old patients with severe psoriasis had considered suicide. This disease is both physically and psychologically disturbing.

Moreover, the treatment of psoriasis requires continuous treatment [2]. The cost of medication and visit to doctors amount to enormous financial losses. Corticosteroids, such as Clobetasol propionate have been a long mainstay in topical therapy of mild to moderate psoriasis [3, 4]. There are so many drugs that are used topically for the treatment of psoriasis.

But corticosteroids are the mainstay for the topical use as it has good clinical efficacy and minimum side effect when it is applied to the maximum body surface areas. Clobetasol propionate: It exerts immunosuppressive, antiproliferative, and anti-inflammatory action and is effective in the treatment of psoriasis [5]. The corticosteroid as an ointment has low permeation. The problem of low permeation can be overcome by formulating microparticulate drug delivery systems like nano-emulsions. In recent years they have shown good potential as drug delivery vehicle due to their high penetration ability through the stratum corneum, high solubilizing power, transparency, good stability, longer shelf life and ease of manufacturing.

Most of the topical preparation used currently, they contain chemical penetration enhancers to increase drug availability to the dipper site of the skin. However, the problem faced with the use of these chemical enhancers in topical preparation may lead to irritation, redness on the skin, stinging or itching sensation to the skin when applied for a long term. Therefore, naturally derived tea tree oil (TTO) selected for the current work which acts as a carrier for the drug for nanoemulsion as well as a penetration enhancer for the formulation [6]. This oil has the potential to reduce inflammation. The antiseptic activity of TTO is also sometimes may become beneficial because psoriasis may be trigger due to some microbial infection [7].

One of the most novel formulations for the increase of skin permeation of drugs is nanoemulsion. The added advantages of nanoemulsions regarding low irritation to skin, strong permeation ability of the drug to the dipper site of skin, and high drug loading capacity lead to superior over the other dosages forms for topical application [8]. The objective of the current work was to test the hypothesis that the loading of corticosteroid like Clobetasol propionate in tea tree oil nanoemulsion formulation is an enhancement and sustaining of corticosteroid delivery that can lead to better anti-psoriatic activity. It was anticipated that nanoemulsion would result in the release of drug at an appropriate rate to maintain suitable drug levels required for therapeutic efficacy in the dermal layer. The permeation through the transdermal route is always a difficult problem, so it is imperative to design a formulation which can enhance the permeation of drug through the skin. Thus, the present study aimed to develop a topical nanoemulsion formulation for CP, a poorly water-soluble drug. It is also anticipated that developed formulations will offer the following advantages:-

- Sustained activity hence, improvement in overall therapy of psoriasis.
- Better patient compliance and good tolerability after prolonged treatment in elderly patients.
- Suitable for effective therapy overnight.
- Easy termination of medication leading to better patient compliance.

MATERIALS AND METHODS:

Materials: Drug sample, *i.e.*, Clobetasol propionate (CP) was gifted as a gift sample from Ranbaxy Research Laboratory (Gurgaon, India). TTO was gifted as a gift sample from Natural Aroma Products Pvt. Ltd., New Delhi, India. PEG 200, PEG 400, tween 40, tween 20, tween 60, and ethanol were procured from Merck (Merck, India). Plurololeique, transcutol P, labrafac, and labrasol were obtained as gift sample on request from Gattefosse (India). CMC, HPMC, sodium alginate, carbopol 934, and salicylic acid were gifted from S. D. Fine Chemicals, India. All other chemicals were of generally recognized as safe categories.

Formulation & Development of Nanoemulsion:

Screening of Excipients: An important criterion for the screening of components for high loading of drug and more stability in nanoemulsions is the solubility/miscibility of the drug in oil, surfactant, and co-surfactant. For determination of solubility of CP in tea tree oil, an excess amount of CP was added to each 5 ml capacity stoppered vial and mixed using a vortex mixer (Nickel-Electro Ltd., Oldmixon Crescent, UK). The mixture vial was then kept at 37 °C ± 1 °C in an isothermal shaker Nirmal International, New Delhi, India) for 72 h to get to equilibrium.

The equilibrated samples were removed from the shaker and centrifuged at 3000 rpm for 15 min. The supernatant was taken and filtered through a 0.45 µm membrane filter. The concentration of CP was determined in tea tree oil by UV spectrophotometer (Shimadzu, Kyoto, Japan) at 241 nm.

For a selection of surfactant and co-surfactant, miscibility of tea tree oil was done with some surfactants like tween 20, tween 80, labrasol and tween 60 and co-surfactants like ethanol, transcutol P, plurololequie, PEG 200 and PEG 400, in 1:1 ratio (oil: surfactant/ co-surfactant). Observations were done visually for miscibility. The mixtures which were clear/ transparent in the 1:1 ratio were considered for further studies [9].

Phase Studies and Construction of Pseudo-Ternary Phase Diagram: By solubility/miscibility studies, tea tree oil was selected as the oil phase, tween-80 as a surfactant and transcutol P as a co-surfactant. Double distilled water was used as an aqueous phase to avoid surface active impurities. Surfactant and co-surfactant were mixed (S_{mix} in different weight ratios (1:0, 1:1, 2:1, 3:1, 4:1, 5:1) with an increasing amount of surfactant concerning co-surfactant. Sixteen different combinations of oil and S_{mix} (1:9,1:8, 1:7, 1:6, 1:5 1:4, 1:3.5, 1:3, 3:7, 1:2, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1) were made so that maximum ratio could be covered for the study to delineate the boundaries of the phases formed precisely in the phase diagrams. For the determination of the existing zone of the nanoemulsion, pseudo-ternary phase diagrams were constructed using the aqueous phase titration method. Slow titration with the aqueous phase was done for each weight ratio of oil and S_{mix}, and visual observations were made for transparent and easily flowable oil-in-water (o/w) nanoemulsions. The physical state of nanoemulsion was marked on a pseudo-three-component phase diagram with one axis representing the aqueous phase, the second representing oil and the third representing a mixture of surfactant and co-surfactant at a fixed weight ratio (S_{mix} ratio) [10].

Selection of Formulations: Among the pseudo-ternary phase diagrams showing maximum nanoemulsion area, some formulations were selected covering the entire range of nanoemulsion occurrence in the phase diagrams with minimum surfactant and maximum water concentration. Exactly 0.05% w/w of CP, which was kept constant in all the selected formulations, was added to the oil phase during the formulation of nanoemulsions. Selected formulations were subjected to various physical stability tests [11].

Formulation of Placebo Nanoemulsions: From the pseudoternary phase diagrams showing maximum nanoemulsion area, some nanoemulsions with different formulas were selected. The almost entire range of nanoemulsion occurrence in the phase diagrams was covered, and different oil compositions with minimum surfactant and maximum water concentration showing nanoemulsion existence were selected.

The oil phase was taken, required amount of surfactant was added, and water was added dropwise drop till a clear and transparent liquid was obtained. The prepared nanoemulsions were tightly sealed and stored at ambient temperature.

Thermodynamic Stability Testing of Placebo Nanoemulsions: To find out the stable nanoemulsion and to discard the unstable nanoemulsions, the placebo nanoemulsions were subjected to following thermodynamic stability studies.

Freeze-Thaw Cycle: Placebo nanoemulsions were kept in the deep freezer (at -20 °C) for 24h. After 24h the nanoemulsions were removed and kept at room temperature. The thermodynamically stable nano-emulsions returned to their original form within 2-3 min. 2-3 such cycles were repeated [12].

Centrifugation Studies: Placebo nanoemulsions after the freeze-thaw cycle was subjected to centrifugation studies where they were made to undergo centrifugation for 30 min at 5,000 rpm in a centrifuge. The stable formulations did not show any phase separation or turbidity [13].

Heating Cooling Cycle: Six cycles between refrigerator temperature (4 °C) and 40 °C with storage of 48h were performed. Those formulations which were stable at this temperature, subjected to study further.

Formulation of Drug Loaded Nanoemulsion: For the preparation of drug-loaded nanoemulsions, 0.5 mg of Clobetasol propionate was dissolved in the oil phase. The required amount of surfactant was added, and water was added dropwise drop till a clear and transparent liquid (Nanoemulsion) was obtained. The prepared nanoemulsions were tightly sealed and stored at ambient temperature [14].

Thermodynamic Stability Testing of Drug Loaded Nanoemulsions: To check that the nanoemulsions were stable after loading with the drug, the drug-loaded nanoemulsions were subjected to freeze-thaw cycle, heating cooling cycle and centrifugation studies. Some formulation becomes unstable after loading the drug into placebo formulation. Various aspects like phase separation, turbidity, *etc*. at room temperature were observed.

Evaluation of Nanoemulsions:

***In-vitro* Skin Permeation Studies:** To evaluate the effect of nanoemulsion vehicle on skin permeation, various nanoemulsion formulations were selected from the phase diagrams.

Fabrication of Diffusion Cell: Franz diffusion cells were fabricated from the local fabricator for the permeation studies. These have a water jacket for maintaining the temperature of assembly at 37 ± 0.5 °C. It consisted of two half cells, the top part known as the donor compartment and the other part (body) known as the receiver compartment. The area of Franz diffusion cell between two half cells was 7.461 $cm^{2,}$ and the capacity of the receiver compartment was 40 ml. The diffusion cell was maintained at 37 °C using a re-circulating water bath, and the solution in the receptor chambers was stirred continuously at 200 rpm with a magnetic stirrer. Skin sample along with the formulation was sandwiched between the two compartments with stratum corneum facing the donor compartment.

Selection of Animal Model: A fairly good number of species have been reported as animal models that can be used for skin permeation studies either *in-vitro* or *in-vivo, e.g.*, mouse, rabbit, rat, guinea pig, pig, hairless rat, dog, cat, horse, squirrel monkey, rhesus monkey, chimpanzee, *etc*. Human cadaver skin also can be used for studying skin permeation [15]. In the present work due to easy availability, skins of male Albino Wistar rats were used with the permission of Animal Ethical Committee.

Procedure for Skin Permeation Studies:

Preparation of Skin for Permeation Studies: Male Wistar albino rats (200-250 g) were obtained from the central animal facility of the university was used for the preparation of skin. Cares of the rats were by the Institutional guidelines. The rats were killed by cervical dislocation. The skin was excised from the abdominal region, stored at -21 °C and used within a week. Dorsal hair was removed with an electric clipper, and subcutaneous fat was removed by using isopropyl alcohol. Finally, the skin was washed with distilled water and observed physically for any damage. The skin was wrapped in aluminum foil and kept overnight at 4 °C.

Stabilization of the Skin: The skin was cut to appropriate size and mounted between the two half cells with stratum corneum facing the donor compartment. The receptor media consisting of phosphate buffer (pH 7.4) was first sonicated for 30 min to remove any dissolved gases and then filled into the receptor compartment and magnetically stirred at 200 rpm for proper mixing. The diffusion cell was thermostated at 37 ± 0.5 °C. The buffer solution was replaced after every half an hour to stabilize the skin. The skin was stabilized for 4-5 h. After stabilization, UV spectrum was taken, and skin was considered stabilized when no UV absorption bands were visible.

Permeation Studies: The receptor cell was filled with the media and 0.5 mg of Clobetasol propionate in nanoemulsion was applied on the skin surface in the donor compartment. The receptor media was maintained at 37 ± 0.5 °C & magnetically stirred at 200 rpm for proper mixing. After application of the test formulation on the donor side, 1 ml of aliquot was collected from the receiver cell at designated time intervals (*viz.* 15, 30 mint and 1, 2, 3, 4, 5 , 6, 8, 10, 12, and 24 h) for 24h period and replaced immediately with the same volume of fresh media maintained at 37 ± 0.5 °C. After appropriate dilutions, the samples were filtered using 0.45 μm membrane filter, and the amount of drug in the receptor media was analyzed by using UV method [16].

Data Analysis: The cumulative amount of Clobetasol propionate permeated through the albino rat skin (Q, $\mu g/cm^2$) was plotted as a function of time (h). The drug flux (permeation rate) at the steady state (Js, $\mu g/cm^2/h$) and lag time were calculated from the slope and intercept of the straight line obtained by plotting the amount of clobetasol propionate permeated *vs.* time in steady state condition.

Permeability coefficient (kp) was calculated by dividing the flux by initial drug concentration (Co) in the donor portion of the cell. The result obtained from potential nanoemulsion formulation was analyzed for the significance using a paired t-test.

Determination of Drug Deposition in Skin: At the end of the test, the formulation remaining on the skin was removed, cleaned with cotton soaked in a 0.05% sodium lauryl sulphate and washed with distilled water. The skin was then weighed, cut into small pieces and sonicated for 15 min with methanol to extract the Clobetasol propionate content. The resulting solution was then centrifuged, and their drug content (µg/cm^2 of skin) was determined by UV analysis [17].

Characterization of Nanoemulsion:

Viscosity: The viscosity of the nanoemulsion was determined using Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) using spindle # CPE40 at 25 ± 0.3 °C.

Refractive Index: Refractive index (RI) of nanoemulsion formulation was determined using an Abbes type refractometer (Precision Standard Testing Equipment Corporation, India).

pH: Measurement of pH of the samples was made by using the pH-meter (Cyberscan, Eutech Instrument, Singapore). The pH-meter was calibrated before each measurement of pH of the nanoemulsion. The pH-reading was recorded after transferring the samples into a beaker, and the pH-meter probe was immersed into it. The pH of the freshly prepared formulations was measured and used to compare the changes in pH of the formulations after specific time intervals at different temperatures.

Conductivity: The conductivity was measured using a conductometer (Cyberscan, Eutech Instrument, Singapore). The sample (2 g) was transferred into a beaker, and the conductometer probe was immersed into it. The conductivity reading in µs was recorded.

The conductivity of freshly prepared formulations was measured and compared with changes in conductivity of the formulations after specified time intervals at different temperatures.

Particle Size Analysis: The average size and polydispersity index of the nanoemulsion droplets were determined by Photon Correlation Spectroscopy (Nano ZS90, Malvern Instrument, U.K.), which are based on the principle of dynamic light scattering? Dynamic light scattering (DLS) is a technique for measuring the particle size of colloidal suspensions. In DLS, the sample is illuminated with a laser beam, and the intensity of the resulting scattered light produced by the particles fluctuates at a rate that is dependent upon the size of the particles. Analysis of these intensity fluctuations yields the diffusion coefficient of the particles and hence the particle size. The measurements were performed using a He-Ne laser at 633nm by using Avalanche photodiode detector. The cell was used standard and small volume disposable polystyrene cuvette. All measurement were carried out at 25 °C.

Transmission Electron Microscopy: Transmission Electron Microscope (TEM) was employed for the microscopic evaluation of optimized formulations using TEM CM-10 (Philips, Netherlands). For evaluation, a drop of nano-emulsion was applied on the carbon-coated grid with 2% phospho-tungstic acid (PTA), and it was left for 30 sec. The dried coated grid was taken on a slide and after placing the coverslip, observed under TEM operated at 60-80 KV at different magnifications (1550x, 2150x, 4600x, 21500x and 44000x). The nanoemulsions appeared dark, and the surroundings were bright a "positive" image was seen using TEM [18, 19].

RESULTS AND DISCUSSION:

Formulation & Development of Nanoemulsion:

Screening of Excipients: The excipients selected needed to be pharmaceutically acceptable, non-irritating, and non-sensitizing to the skin and to fall into the GRAS (generally regarded as safe) category. Higher solubility of the drug in the oil phase was another important criterion, as it would help the nanoemulsion to maintain the drug in solubilized form. Safety is a major determining factor in choosing a surfactant, as a large number of surfactants may cause skin irritation. To that non-ionic surfactants are considered to be less toxic than ionic surfactants. An important criterion for selection of the surfactants is that the required hydrophilic-lipophilic balance (HLB) value to form the o/w nanoemulsion be greater than 10.

The right blend of low and high HLB surfactants leads to the formation of a stable nanoemulsion formulation. The presence of co-surfactant decreases the bending stress of interface and allows the interfacial film sufficient flexibility to take up different curvatures required to form nanoemulsion over a wide range of composition.

Drug loading per formulation is a very critical design factor in the development of nanoemulsion systems for poorly soluble drugs, which is dependent on the drug solubility in the oil phase. The solubility of CP in tea tree oil was found to be 20.90 mg/mL which is very good for topical delivery since the dose of CP is very less.

TABLE 1: MISCIBILITY OF TEA TREE OIL WITH SURFACTANTS AND CO-SURFACTANTS

Miscibility of Tea Tree oil				
S. no.	**With surfactant (1:1)**	**Observation**	**With co-surfactant**	**Observation**
1	Tween 20	Clear	Ethanol	Turbid
2	Tween 20	Turbid	Transcutol P	Clear
3	Lecithin	Turbid	PEG 200	Turbid
4	Unitop 100	Turbid	Pleurololeique	Turbid

But due to the presence of another fatty acid in tea tree oil, emulsification of oil is very difficult. For getting good nanoemulsion region in the ternary phase diagram, the miscibility of oil with surfactant and co-surfactant is important. Therefore, the miscibility of oil was performed with different surfactants and co-surfactants **Table 1**. Another important criterion is the selection of surfactant with proper HLB value. Hydrophilic surfactant and co-surfactant are considered to prefer the interface and to lower the necessary energy to form the nanoemulsions, consequently improving the stability. For example, the required HLB value to form o/w nanoemulsion is greater than 10.

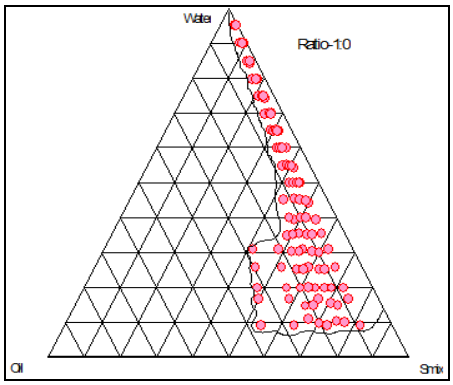
So, selection of surfactant and co-surfactant with appropriate HLB value is necessary. The miscibility of tea tree oil was found to be highest with tween 20 in case of surfactant and transcutol P in case of co-surfactant in 1:1 ratio. Apart from this, tween 20 has high HLB value which can provide good emulsification to the tea tree oil. transcutol P is a very good solubilizing agent who can provide better penetration to the lipophilic drug such as CP by increasing the solubility to the drug in the lipophilic domain of the stratum corneum. So, for the development of pseudo-ternary phase diagram tea tree oil was selected as an oil phase, tween 20 as a surfactant and transcutol P as a co-surfactant.

Phase Studies and Construction of Pseudo Ternary Phase Diagrams: Constructing a phase diagram is one of the primary steps and makes a backbone for the nanoemulsion drug delivery system, particularly when the aim is to accurately delineate a phase boundary [20]. Observations are made carefully to separate metastable systems from phase boundary, although the free energy required to form an emulsion is very low, the formation is thermodynamically spontaneous.

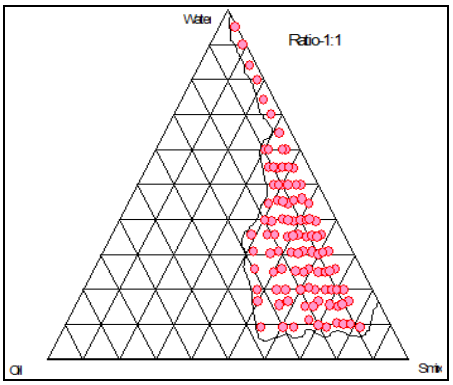
The relationship between the phase behavior of a mixture and its composition can be selected with the aid of a phase diagram. Tea tree oil, tween 20 (surfactants) and transcutol P (co-surfactant), were used to study the phase diagrams in detail. The systems were observed for visual clarity and flowability characteristics. Those who did not show a change in the meniscus after tilting to an angle of 90° were classified as nanoemulsion gels a metastable system, and it was not selected. After taking an observation, pseudo-ternary phase diagrams were constructed based on the observations marked during titration. Phase diagrams were constructed separately for each ratio of S_{mix} prepared so that o/w nanoemulsion regions could be identified. In the phase diagrams **Fig. 1(A-G)** only o/w nanoemulsion region is shown. After building the backbone of the nanoemulsion delivery system, different formulations were selected at a different point from the phase diagram justifying the drug dose.

The construction of pseudoternary phase diagrams was started using surfactant, *i.e.* Tween 20 alone (1:0). It was found that the region of nanoemulsion existence was very less and most of the region was composed of emulsions. Now with surfactant tween 20, cosurfactant transcutol P was also incorporated in the ratio 1:1 and pseudoternary phase diagrams were constructed. It was found that the region of

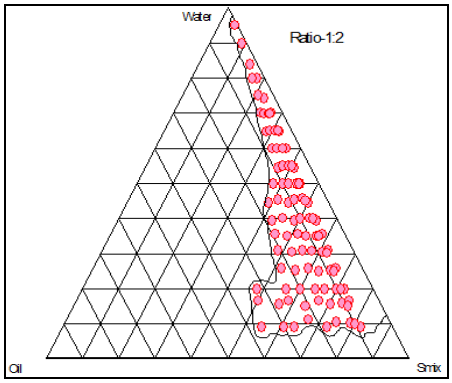
nanoemulsion existence increased greatly. Increase in the concentration of co-surfactant (1:2), resulted in an even larger area of nanoemulsion existence, along with some emulsion, gels or nanoemulsion gels area. Increasing co-surfactant concentration further from 1:2 to 1:3, and 1:4 resulted in the reduction of the nanoemulsion existence area and more area was composed of emulsion and gels.



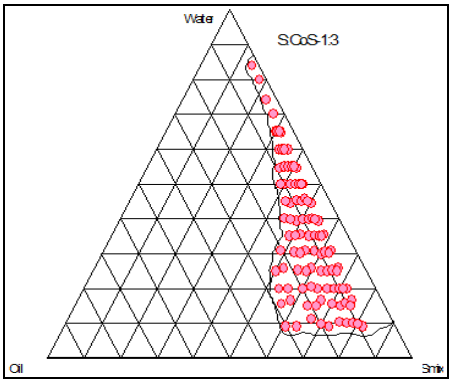
(A): Phase diagram of S_{mix} 1:0



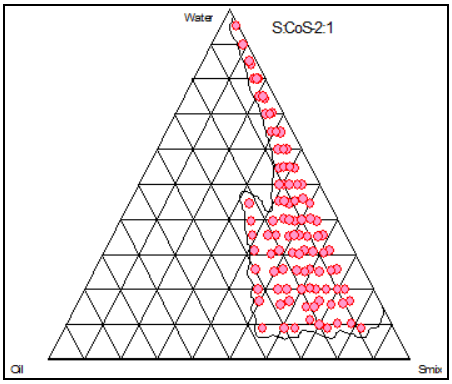
(B): Phase diagram of S_{mix} 1:1



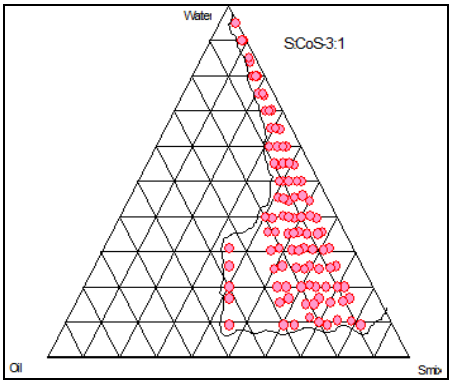
(C): Phase diagram of S_{mix} 1:2



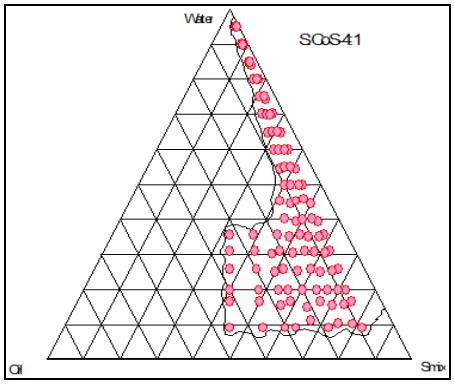
(D): Phase diagram of S_{mix} 1: 3



(E): Phase diagram of S_{mix} 2:1



(F): Phase diagram of S_{mix} 3:1



(G): Phase diagram of S_{mix} 4:1

FIG. 1(A-G): PSEUDOTERNARY PHASE DIAGRAM OF GROUP 1 INDICATING O/W NANOEMULSION REGION USING TEA TREE OIL, TWEEN 20 (SURFACTANT), TRANSCUTOL P (CO-SURFACTANT)

The existence of a nanoemulsion region whether large or small depends on the capability of that particular surfactant or surfactant mixture to solubilize the oil phase. The extent of solubilization results in a greater area with more of the clear, homogenous solution. It was seen that when the surfactant (Tween 20) was used alone, oil phase was solubilized to a lesser extent implying that surfactant alone was not able to reduce the interfacial tension of the oil droplets to a sufficiently low level & thus was not able to reduce the free energy of the system to ultra-low level desired to produce nanoemulsions. When a co-surfactant was added, the interfacial tension was reduced to a much low level, and very small free energy was achieved which helped in larger nanoemulsion area existence in the phase diagram. With a further increase in co-surfactant from 1:1 to

1:2, a further drop in interfacial tension and free energy was achieved resulting in a maximum area of nanoemulsion formation. With a further increase in co-surfactant concentration (1:3), the interfacial tension of interfacial film increased as compared to above, and more of gel area and less of the nanoemulsion area was observed.

Selection of Nanoemulsion: From each phase diagram constructed, different formulations were selected from nanoemulsion region so that drug could be incorporated into the oil phase.

Formulation of Placebo Nanoemulsions: Following criteria was used for the selection of different formulation from phase diagrams:

- ✓ The dose of Clobetasol propionate frequently used is 0.05% w/v topically.
- ✓ The oil concentration should be such that it dissolves the drug easily.
- ✓ From each phase diagram different concentration of oil, which solubilized the drug was selected.
- ✓ For each percentage of oil selected, that formula was taken from the phase diagram, which used the minimum concentration of S_{mix} for its nanoemulsion formation.
- ✓ The emphasis for the selection of formulations was given on the minimum concentration of the S_{mix}, from the phase diagram and **Table 2** and **3**.

TABLE 2: SELECTED FORMULATIONS FROM PSEUDOTERNARY PHASE DIAGRAM OF S_{mix} RATIO 1:1, 1:2, 1:3

S_{mix} Ratio (S: CoS)	Code	Percentage v/v of different components in the formulation		
		Oil	S_{mix}	Aqueous phase
1:1	1	10	30	60
(Fig. 1B)	2	10	37	53
	3	15	35	50
	4	15	40	45
	5	20	40	45
	6	20	45	35
	7	25	39	36
	8	25	45	30
1:2	9	10	31	59
(Fig. 1C)	10	10	40	50
	11	15	40	45
	12	15	45	40
	13	20	38	42
	14	20	40	45
	15	25	36	39
	16	25	40	35
1:3	17	10	37	53
(Fig. 1D)	18	10	40	50
	19	15	45	40
	20	15	50	35
	21	20	48	32
	22	20	50	30

TABLE 3: SELECTED FORMULATIONS FROM PSEUDOTERNARY PHASE DIAGRAM OF S_{mix} RATIO 2:1, 3:1 & 4:1

S_{mix} Ratio (S: CoS)	Code	Percentage v/v of different components in the formulation		
		Oil	S_{mix}	Aqueous phase
2:1	23	10	35	55
(Fig. 1E)	24	10	41	49
	25	15	42	43
	26	15	45	40
	27	20	40	40
	28	20	46	34
	29	25	38	37
	30	25	40	35
3:1	31	10	40	50
(Fig. 1F)	32	10	45	45
	33	15	44	43
	34	15	45	40
	35	20	40	40
	36	20	45	35

	37	25	40	35
	38	25	38	38
4:1	39	10	40	50
(Fig. 1G)	40	10	45	45
	41	15	45	40
	42	15	50	35
	43	20	46	34
	44	20	50	30
	45	25	40	35
	46	25	45	30

Thermodynamic Stability Testing of Placebo Nanoemulsions: The thermodynamic stability testing was done to ascertain that the prepared nanoemulsions were stable when subjected to freeze-thaw cycle (to check stability at low temperature), centrifugation studies (to check stability at high shear) and heating-cooling cycle (to check the stability at higher temperature).

Formulation of Drug Loaded Nanoemulsion: The drug stock solutions in oil mixture were prepared in such a way that 0.05% w/v dose is present in each formulation complying the oil percentage for each formula as shown selected from the phase diagram. This was prepared by dissolving the following weighed amount of drug which complied the 10%, 15%, 20%, and 25% oil compositions respectively in the formulae. The drug stock table is shown in **Table 4**. As per saturation solubility studies of Clobetasol propionate in tea tree oil, around 20.90 ± 1.05 mg of drug was solubilized per ml of mixture. The concentration, 10% (100 µl) of oil in 1 ml of the formulation is just able to solubilize 0.5 mg of Clobetasol propionate. Therefore, 10% was selected as the least oil concentration to be taken for one ml formulation from the phase diagram. The drug stock solutions in oil mixture were prepared in such a way that 0.5 mg dose is present in each formulation complying the oil percentage for each formula as shown in **Table 4**.

TABLE 4: PREPARATION OF DRUG STOCK FOR EACH FORMULA SELECTED IN PHASE DIAGRAM

S. no.	Oil percentage in formulations	Amount of drug (mg)	The volume of oil mix (ml)	Final concentration (mg/mL)
1	10%	10	2	5 mg/mL
2	15%	6.66	2	3.33 mg/mL
3	20%	7.5	2	2.5 mg/mL
4	25%	4	2	2 mg/mL

Thermodynamic Stability Testing of Drug Loaded Nanoemulsion: When the drug-loaded nanoemulsions were subjected to thermodynamic stability testing some formulations became unstable **Table 5** and **6**. The composition of stable nanoemulsion formulation after drug loading is given in **Table 7**. The oil used: tea tree oil, the surfactant used: tween 20, co-surfactant used: transcutol P, external phase: distilled water.

TABLE 5: THERMODYNAMIC STABILITY TESTS OF DIFFERENT SELECTED FORMULATIONS FROM S_{mix} RATIO 1:1, 1:2, 1:3

S_{mix} Ratio (S: CoS)	S. No.	Percentage v/v of different components in formulations			Observations based on the thermodynamic stability studies			Inference
		Oil	S_{mix}	Water	H/C	Cent	Freez	
1:1	1	10	30	60	√	√	√	Passed
(Fig. 1B)	2	10	37	53	√	√	X	Failed
	3	15	35	50	√	√	√	Passed
	4	15	40	45	√	√	√	Passed
	5	20	40	45	√	√	√	Passed
	6	20	45	35	√	√	√	Passed
	7	25	39	36	√	√	√	Passed
	8	25	45	30	√	√	√	Passed
	9	10	31	59	X	√	√	Failed
	10	10	40	50	√	√	√	Passed

	11	15	40	45	√	√	√	Passed
1:2	12	15	45	40	√	√	X	Failed
(Fig. 1C)	13	20	38	42	√	√	√	Passed
	14	20	40	45	√	√	√	Passed
	15	25	36	39	-	X	X	Failed
	16	25	40	35	-	X	√	Failed
1:3	17	10	37	53	√	√	√	Passed
(Fig. 1)	18	10	40	50	√	√	√	Passed
	19	15	45	40	√	√	√	Passed
	20	15	50	35	√	√	√	Passed
	21	20	48	32	√	√	X	Failed
	22	20	50	30	√	√	√	Passed

H/C = Heating-cooling cycle, Cent = Centrifugation, Freez = Freeze-thaw cycle. The oil used: Tea Tree oil Surfactant used: Tween 20, Co-surfactant used: Transcutol P, External phase: Distilled water

TABLE 6: THERMODYNAMIC STABILITY TESTS OF DIFFERENT SELECTED FORMULATIONS FROM S_{mix} RATIO 2:1, 3:1 & 4:1

S_{mix} Ratio (S: CoS)	S. No.	Percentage v/v of different components in formulations			Observations based on the thermodynamic stability studies			Inference
		Oil	S_{mix}	Water	H/C	Cent	Freeze	
	23	10	35	55	√	√	√	Passed
2:1	24	10	41	49	√	√	√	Passed
(Fig. 1E)	25	15	42	43	√	√	√	Passed
	26	15	45	40	√	√	√	Passed
	27	20	40	40	√	√	√	Passed
	28	20	46	34	√	√	√	Passed
	29	25	38	37	√	√	√	Passed
	30	25	40	35	√	√	√	Passed
3:1	31	10	40	50	√	√	√	Passed
(Fig. 1F)	32	10	45	45	-	X	-	Failed
	33	15	44	43	-	X	-	Failed
	34	15	45	40	√	√	√	Passed
	35	20	40	40	-	√	X	Passed
	36	20	45	35	√	√	√	Passed
	37	25	40	35	-	X	-	Failed
	38	25	38	38	√	√	√	Passed
4:1	39	10	40	50	√	√	√	Passed
(Fig. 1G)	40	10	45	45	-	X	-	Failed
	41	15	45	40	√	√	√	Passed
	42	15	50	35	-	X	-	Failed
	43	20	46	34	-	X	-	Failed
	44	20	50	30	-	X	-	Failed
	45	25	40	35	√	√	X	Failed
	46	25	45	30	√	√	√	Passed

H/C = Heating-cooling cycle, Cent = Centrifugation, Freeze = Freeze-thaw cycle

TABLE 7: COMPOSITION OF STABLE NANOEMULSIONS FORMULATION AFTER DRUG LOADING SELECTED FOR PERMEATION STUDIES

Nanoemulsion code no.	Tea tree oil volume in (µl)	Tween 20 + Transcutol P (S_{mix}) volume in (µl)	Distilled water volume in (µl)	Drug (mg)	Volume of nanoemulsion (ml)
A1	100	300 (S_{mix} 1:1)	600	0.5mg	1
A2	150	350 (S_{mix} 1:1)	500	0.5mg	1
A3	200	400 (S_{mix} 2:1)	400	0.5mg	1
A4	150	450 (S_{mix} 1:3)	400	0.5mg	1
A5	200	450 (S_{mix} 3:1)	350	0.5mg	1
A6	250	450 (S_{mix} 4:1)	300	0.5mg	1

DISCUSSION: Nanoemulsions are thermo-dynamically stable systems and are formed at a particular concentration of oil, surfactant, and water, with no phase separation, creaming or cracking. It's

thermostability which differentiates from emulsions that have kinetic stability and will eventually phase separate. Thus, the selected formulations were subjected to different thermodynamic stability by using heating-cooling cycle, centrifugation and freeze-thaw cycle stress tests. Those formulations, which passed thermodynamic stability tests, were taken for further studies.

Evaluation of Nanoemulsions:

***In-vitro* Skin Permeation Studies:** The permeation ability of the various nanoemulsions was evaluated using the *in-vitro* permeation experiments. The drug flux (permeation rate) at the steady state (Js, µg/cm^2/h) and lag time were calculated from the slope and intercept of the straight line obtained by plotting the amount of Clobetasol propionate permeated *vs.* time in steady state condition. Permeability coefficient (kp) was calculated by dividing the flux by initial drug concentration (Co) in the donor portion of the cell. Drug disposition in the skin was also determined [21].

- The cumulative amount of drug permeated (µg) = Concentration (µg/ml) × volume of receptor cell × Dilution factor
- The cumulative amount of drug permeated (µg/cm^2) = Cumulative amount of drug permeated (µg)/Area of the skin
- Permeation medium = Mixture of 0.04 M phosphate buffer (pH 7.4) and 2-propanol (70:30)
- Flux (µg/cm^2/h) = Slope of steady state of the cumulative amount of drug permeated (µg/cm2) verses time
- Permeability coefficient (kp) was calculated by dividing the flux by initial drug concentration (Co) in the donor portion of the cell.
- Here area of modified Franz diffusion cell = 7.461 cm^2
- The volume of modified Franz diffusion cell = 40 ml
- Clobetasol propionate in donor compartment = 500 µg
- Salicylic acid in donor compartment = 50000µg
- Dilution factor for Clobetasol propionate = 1
- Dilution factor for salicylic acid = 4
- Drug deposition was calculated according to the procedure given in the experimental part

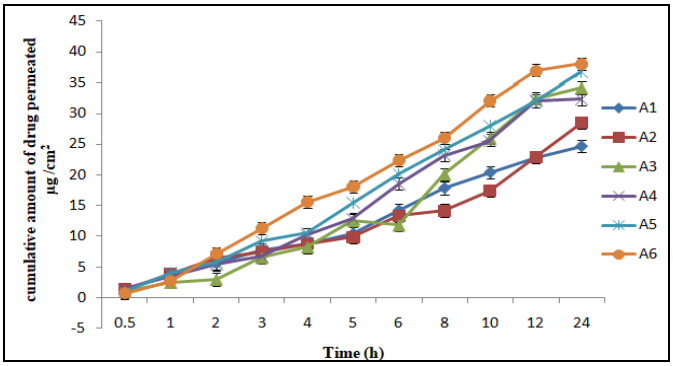


FIG. 2: *IN-VITRO* SKIN PERMEATION OF CLOBETASOL PROPIONATE FROM NANOEMULSION IN PHOSPHATE BUFFER (pH 7.4)

For the evaluation of nanoemulsions, various formulations from the phase diagrams with the maximum area were selected for *in-vitro* skin permeation studies. In these formulations, the content of the oil phase was varied as 10, 15, 20 & 25% while the content of surfactant was varied from 38% to 50%. The effect of the content of oil and surfactant mixture on the skin permeation of Clobetasol propionate was evaluated at (pH 7.4). On the basis of permeation studies of different formulation shown in **Fig. 2** it was found that the formulation A2 consisting of 15% oil phase, 35% (S_{mix} 1:1) and 50% distilled water exhibited 42.52% of cumulative amount of drug permeated (µg/cm^2) after 24 h and highest amount of drug deposited in skin 31.06 (µg/cm^2). On the basis highest cumulative % permeated and drug deposition in formulation code A2 was selected for the further studies.

Characterization of Nanoemulsion Formulations: Refractive index of nanoemulsion was determined using an Abbes type refractometer (Nirmal International, New Delhi, India) at 25 ± 0.5 °C. The apparent pH of the formulation was measured by pH meter in triplicate at 25 ± 1 ºC.

TABLE 8: VISCOSITY

Formulation code	Viscosity (mP) ± SD (n=3)
A2	28.30 ± 1.91 mP

TABLE 9: REFRACTIVE INDEX AND pH

Formulation code	Refractive index	pH
A2	1.403	6.15

Conductivity: The specific conductivity of nanoemulsion A2 was found to 10^{-4} s cm^{-1}.

Droplet Size and Size Distribution of Nanoemulsion Formulation (A2): The average size and polydispersity index of the nanoemulsion droplets were determined by photon correlation spectroscopy (Nano ZS90, Malvern Instrument, U.K.). The droplets size of all nanoemulsions ranged from 50-250 nm. The peak is shown in **Fig. 3** that all the nanoemulsions had narrow size distribution.

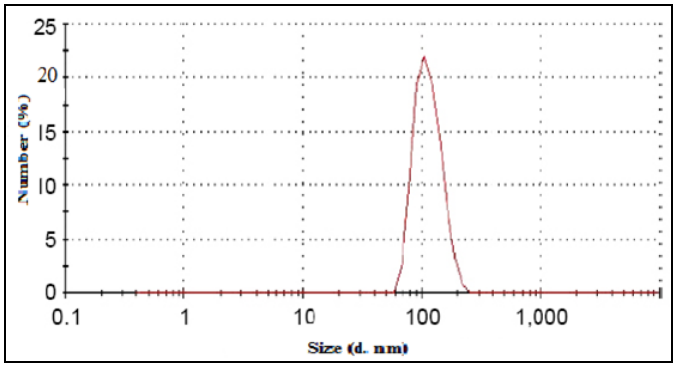


FIG. 3: DROPLET SIZE DISTRIBUTION OF NANOEMULSION FORMULATION (A2)

Transmission Electron Microscopy: When TEM was performed for optimized formulation it was finally concluded that the particles were spherical and finely distributed with a micro range of particles. Due to spherical shape and micro-size, the permeation of Clobetasol propionate was high **Fig. 4**.

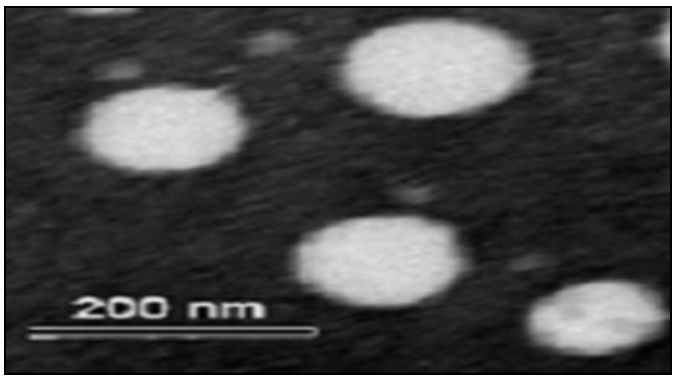


FIG. 4: TEM PHOTOGRAPH OF PARTICLE SIZE OF NANOEMULSION (A2) DRUG LOADED CLOBETASOL PROPIONATE

In this work, optimized drug loaded nanoemulsion (A2) was characterized by characteristics parameters of the nanoemulsion. The nanoemulsion (A2) was a colloidal dispersion having average droplet size ranging from 100 to 200 nm. A value of polydispersity index (PI), which is a measure of uniformity of droplet size within the formulation, was also calculated. The nanoemulsion (A2) formulation exhibited a narrow size distribution (PI=0.131).

The results of dynamic light scattering (DLS) measurements were in agreement with the droplet size measured by TEM **Fig. 4**. As a result of viscosity measurement, Viscosity of the nano-emulsion (A2) formulation was very low as expected for o/w emulsion was (28.30 ± 1.91mP). The low viscosity may be due to the presence of low amount of tween 20 (a fatty acid polyhydric alcohol ester having high intrinsic viscosity) compared to isopropyl alcohol (short chain alcohol having low intrinsic viscosity) and also the low concentration of oil. Refractive index is the net value of the components of nanoemulsion and indicates the isotropic nature of the formulation. The mean value of the refractive index for the formulation (A2) was found to be 1.403.

CONCLUSION: It is confirmed that the developed CP loaded TTO nanoemulsion was physically stable and good deposition of CP in the skin at the deeper layer which may result in sustainable and good anti-inflammatory activity for the treatment of psoriasis.

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