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BIOAVALIBILITY ENHANCEMENT OF RESVERATROL USING SELF NANOEMULSIFYING DRUG DELIVERY SYSTEM DEVELOPED APPLYING CENTRAL COMPOSITE DESIGN

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Keywords:

Self nanoemulsifying drug delivery system (SNEDDS), Resveratrol, Solubility, Stability, Surfactants

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ABSTRACT: The objective of current study was to develop self-nanoemulsifying drug delivery systems using long-chain triglycerides of resveratrol to enhance its bioavailability. Solubility studies performed in different lipids, surfactants and cosurfactants. Phase diagrams constructed to select areas of nanoemulsion. SNEDDS formulation optimized using 3^3 central composite design (CCD) considering lipid (X_1), surfactant (X_2) and co surfactant (X_3) as critical variables and optimized formulation was located using overlay plot. The nanometer size and high values of zeta potential depicted non-coalescent nature of SNEDDS. Resulted SNEDDS formulation improved *in vitro* release followed by Hixson Crowell model with higher regression R^2 value 0. 929. Thermodynamic stability studies ascertained stable formulation. Mean droplet size in selected nanocarrier was found to be 83.29 nm. The nanocarriers showed enhanced bioavailability in albino rabbits when compared to pure drug. The novel approach developed by selecting optimum blends of lipids, surfactants and cosurfactant using central composite design.

INTRODUCTION: Self-nanoemulsifying delivery system is lipid based nanocarrier system [1]. SNEDDS as novel technology have immense potential in oral bioavailability enhancement of lipophilic drugs [2, 3]. Potential advantages of SNEDDS formulation include capability of bypassing hepatic portal route and promote lymphatic transport of lipophilic molecules by reducing degradation *via* cytochrome - P450 enzymes present in the gut enterocytes [4, 5, 6] and overcome enterohepatic recirculation [1].

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SNEDDS have been currently investigated for its advantages, providing a large interfacial area for partitioning the drug between lipid and GI fluid [7]. Studies have shown that long-chain triglycerides (LCTs) used in SNEDDS formulation, drastically influence the type of absorption pathway and subsequent transportation of drug [8, 9]. The LCTs are transported *via* the intestinal lymphatics system, bypassing hepatic first-pass metabolism [1, 6, 10].

The LCTs are likely to augment the lymphatic transport of a lipophilic drug substance leading to enhanced oral bioavailability [10]. Resveratrol (RVT), chosen in the present study is BCS class II molecule with poor water-solubility and high permeability (log P of 3.1) [11]. Resveratrol (trans-3, 5, 4'-trihydroxystilbene; RSV) occurs in nature as polyphenol and isolated from more than 70% plants

species including foods [12]. Resveratrol molecule found to be highly soluble in organic solvents and very stable in acidic pH ranges [13]. Resveratrol is off-white powder having melting point 253-255 ºC [14] solubility in water is 3 mg/100 mL [15], inorganic solvents is 65 mg/ml and in phosphate buffer at pH 7.2 is 100 µg/mL, UV maxima observed at 306 nm [15]. Researchers are consistently reporting that this molecule is therapeutically active**,** but only if its concentration is maintained in blood as well as plasma for longer duration [14].

Besides this, it undergoes rapid first-pass metabolism by CYP3A4 in the liver and suffers enterohepatic recirculation [16, 17], eventually leads to reduction in the drug bioavailable fraction (almost zero) in humans and animals [11]. Under the circumstances, various formulation approaches of RVT have been reported, such as liposome [18], solid dispersions [19], b-cyclodextrins inclusion complex [20], microspheres [21], suspensions**,** but all with limited fruitful results.

The current work endeavors to design an optimized (OPT) SNEDDS formulation of RVT, resulting in enhanced solubility, release kinetics and bioavailability. It is anticipated that this study not only offers a good example of augmenting the oral bioavailability of RVT using SNEDDS but also provides a promising oral formulation of RVT for clinical application.

MATERIAL AND METHODS:

Materials: Resveratrol purchased from "Avans Cure Pharmaceutical Company, India", Ethanol Absolute purchased from "Changshu Yanguan Chemicals, China", Methanol, purchased from "Thermo Fischer Scientific India Pvt. Ltd., Mumbai", Disodium Hydrogen orthophosphate, Potassium Dihydrogen orthophosphate, Sodium hydroxide bought from "Thomas baker (chemicals) Pvt. Ltd., Mumbai, India", n-Octanol, Ethyl Oleate, Oleic acid, PEG 200, PEG 400 were purchased from SDFCL, Mumbai, Myritol purchased from "BASF, Mumbai, India", Tween 20, Tween 60, Tween 80 purchased from " Molychem, Mumbai, India", Kolliphor RH 40, Kolliphor HS 15, Kolliphor ELP, PEG 300 purchased from "BASF, Germany", Iso Propyl Alcohol, Glycerol were bought from "Avantor Performance Materials Limited, Haryana, India", Propylene Glycol obtained from "Qualikems Fine Pvt. Ltd., Vadodara, India", Labrafil M 2125 was obtained from "Gattefosse, Germany".

Methods:

Solubility of Resveratrol in Lipids, Surfactant, Co - Surfactants: Shake flask method [22] was used for solubility studies of resveratrol in various lipids (Labrafil M 2125, Ethyl Oleate, Oleic acid), surfactants (Kolliphor HS15, Kolliphor ELP, Kolliphor RH 40 and Tween 80) and co surfactants (Ethanol, PEG 200, PEG 400, PEG 300, n-butanol and Propylene Glycol). An excess amount of resveratrol was added in 2 ml of lipophilie in stoppered vials and mixed on vortex shaker (Remi CM 101, International Mumbai, and India). These vials were then kept at 25 °C for 24 h in water bath shaker (Nirmal International, Delhi, India). The resulting samples were centrifuged at 2000 rpm for 15 min (Remi, International Mumbai, and India). The supernatant was filtered through 0.22 µm filter. The concentration of resveratrol was then quantified using HPLC (Shimadzu, Kyoto, Japan) instrument equipped with two LC-10 ATVP pumps, SPD-10AVP UV-Vis detector.

Combinations of Oil, Surfactant and Co-Surfactant Selection: Further screening of lipid, surfactant and cosurfactant combinations was done by transmittance and ease of emulsification. The ease of emulsification was judged by number flask inversion method [22] required to yield homogeneous emulsion of lipid: surfactant (1:1 w/w) and lipid: surfactant: co surfactant (3:2:1 w/w). The % transmittance was evaluated at 629 nm by using UV spectrophotometer.

Construction of Ternary Phase Diagram: The presence of Self-emulsifying fields in formulation that might self-emulsify under dilution and gentle agitation was identified from ternary phase diagrams of systems containing lipids, surfactants and co surfactants. Phase diagrams were constructed by titration method [23]. Titration of lipid: surfactant: co-surfactant (S_{mix}) in different w/w ratios (0.5:9.5, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, 9.5:0.5 w/w) was done with water at ambient temperature. After each aliquot addition of aqueous phase, physical state of mixture was marked on ternary phase diagram using Chemmix software. The phase diagrams so constructed to

delineate the boundaries of various phases, *i.e.* nano / microemulsion, emulsion [9]. The samples which produced clear or slightly bluish colour, were considered as nanoemulsions.

Optimization of SNEDDS Formulation using Central Composite Design (CCD): Once the self-emulsifying region was identified based on the formation of maximal nanoemulsion region in the ternary-phase diagram, the desired component ratios of SNEDDS were selected for drug incorporation. For the preparation of SNEDDS, Labrafil M 2125 as lipid (X_1) and Kolliphor ELP as surfactant (X_2) and Ethanol as cosurfactant (X_3) were chosen and finally selected as the three critical influential factors for further formulation optimization work [24].

A central composite design (CCD) was employed using Statistical® version 10.0 (Stat software, Inclusive United States America) and version 8 Design Expert® (Statistical-Ease, Minneapolis, United States America) for the optimization of SNEDDS [25] by varying its three independent variables X_1 (lipid), X_2 (surfactant), X_3 (cosurfactant) in minimum and maximum range selected from nanoemulsion region (ternary plots). Based on experimental plan 20 formulations of 2 g each with six central points (run order F1, F3, F6, F13, F14 and F17) were prepared by mixing various portions of lipid, surfactant and co-surfactant as recommended by the experimental design **Table 1**.

After adding all the components in glass vial, the mixture was vortexed (Remi CM 101, International Mumbai, and India) for few minutes and then homogenized at 45 ºC for 5 min. After homogenization, the mixture was sonicated in bath sonicate (Remi CM, International Mumbai, and India) by occasionally vortexing until resveratrol completely dissolved. Formulations prepared were further subjected to evaluation and characterization to select best formulation. Each formulation was assessed for response Y (dependent variable) *i.e.* drug content. The optimization goal was to maximize Y (>99%). The results were analyzed using Statistical® version 10.0 (Stat soft, Inc. USA) and version 8 Design Expert® v (Statistics-Ease, Minneapolis, United States of America). A best fitted quadratic equation was built for the response.

$$Y = \beta_0 + \beta_1.X_1 + \beta_2.X_2 + \beta_3.X_3 + \beta_4.X_4.X_2 + \beta_5.X_1.X_3 - \beta_6.X_2.X_3 - \beta_7.X_1^2 + \beta_8.X_2^2 + \beta_9.X_3^2$$

Overlay plot obtained by superimposing various response variables were considered to locate OPT formulation. The observed and predicted responses were critically compared and the percentage bias (*i.e.* error prediction) was calculated regarding responses observed.

Evaluation and Characterization of Optimized Formulation:

Percentage Resveratrol Content: The percent resveratrol dissolved in all 20 formulations were quantified using HPLC (Shimadzu, Kyoto, Japan) instrument equipped with two LC-10 ATVP pumps, SPD - 10AVP UV-Vis detector. Formulations having 99 to 100% dissolved resveratrol were evaluated further for transparency.

Transparency and Resveratrol Precipitation: To determine the transparency 1 g of optimized formulation was diluted up to 5 ml with water and observed visually for the transparency and resveratrol precipitation. Formulation that remain transparent and shown no signs of resveratrol precipitation was further evaluated for stability.

Thermodynamic Stability Studies: The optimized formulation selected based on percentage resveratrol content dissolved, transparency and resveratrol precipitation were further subjected to thermodynamic studies to assess stability [9, 26].

Heating and Cooling Cycle: The optimized formulation was subjected to six heating and cooling cycles between 4 °C and 40 °C while storing for not less than 48 h. The formulation was observed for physical instability such as phase separation, creaming, or cracking.

Freeze Thaw Cycle: Freeze thaw cycle involved three cycles at temperature between -21 °C and +25°C while storing for not less than 48 h. The formulation was observed for physical instability such as phase separation, creaming, or cracking.

Cloud Point Measurement: The optimized formulation was diluted with distilled water in ratio 1:100 w/v. The diluted formulation was placed in a water bath and their temperature was gradually increased.

Cloud point was spectrophotometrically examined. The temperature at which sudden change in cloudiness appeared was considered as cloud point [2].

Centrifugation Study: The optimized formulation was centrifuged at 18000 rpm for 30 min. (Remi CM, International Mumbai, and India). The formulation was observed for physical instability such as phase separation, creaming, or cracking.

Dispersibility Studies: The optimized formulation was added to 200 ml and 900 ml distilled water in standard USP II apparatus (lab India, Delhi, India) at temperature 37 ºC [27]. Formulation was observed visually for clarity till 24 h.

Emulsification Time: The emulsification time of formulations was estimated using USP II apparatus (lab India, Delhi, India). Each formulation (835 mg) containing 20 mg resveratrol was added dropwise to 200 ml and 900 ml distilled water maintained at 37 °C. Gentle agitations was provided by standard dissolution paddle rotating at 50 rpm. The emulsification time was assessed visually.

Percentage Transmittance: The optical clarity of SNEDDS formulation was measured spectro-scopically. Transmittance percentage was observed using Shimadzu UV spectrophotometer. 835 mg formulation containing 20 mg resveratrol was diluted 100 times with distilled water and analyzed at 306 nm using distilled water as blank.

Determination of Zeta Potential: Zeta potential of optimized formulation was measured by photon correlation spectroscopy using Zetasizer (ZS nano, Malvern Instruments, United Kindom) with 4.0 mW He-Ne red laser (633 nm) which measures potential range from -120 to 120V. SNEDDS formulations (835 mg) were diluted 100 times using distilled water and analyzed for zeta potential measurement at 25 °C.

Viscosity: The viscosity of optimized SNEDDS formulations was determined by Brookfield viscometer using spindle C-62 at 25 °C. 600 ml of formulation was used for viscosity determination. Controlled stress rate was observed to get data about its flow behavior with change in spindle speed (rpm).

Droplet Size Analysis: Optimized SNEDDS formulation was mixed gently with distill water by inverting flask method. The droplet size of globules was evaluated by light scattering technique with Zetasizer (Nano ZS, Malvern Instruments, UK) equipped with 4.0 mW He-Ne red laser, 632.9 nm at temperature 25 °C and refractive index 1438 or adjustments which needed.

Transmission Electron Microscopic Analysis: The morphology of the optimized formulation was observed using TEM [28, 29]. Optimized SNEDDS formulation was diluted with distilled water in ratio 1:200 and mixed by shaking. A drop of diluted SNEDDS was applied to a 300-mesh copper grid and was left for 1 min. After this a drop of phosphotungstic acid (PTA) 2% w/v was applied to grid kept inverted for 10s. Excess of PTA was removed by absorbing on filter paper and grid was analyzed using JEM 2100F operated at 200 KV operated with AMT image capture instrument.

***In vitro* Resveratrol Release Study:** Dissolution studies were carried out for optimized formulation, pure drug, marketed preparation in triplicate, employing USP Apparatus 2 (Labindia, Mumbai, India) with 900 ml of 1.2 pH simulated gastric fluid containing 0.3% (w/v) sodium lauryl sulphate (SLS), stirred at 50rpm at a temperature of 37 ± 0.5ºC [1]. At predetermined time intervals, an aliquot (1 ml each) of the sample was collected, filtered, and analyzed for the content of RVT by the HPLC method [17]. An equivalent volume (1 ml) of fresh dissolution medium was added to compensate for the loss due to sampling.

***In vivo* Bioavailability Study in Rabbits:** The animal study protocol IAEC/NIET/2016/01/10 was reviewed and approved by NIET, Greater Noida, CPCSEA Reg. no.: 1845/PO/Re/S/16/CPCSEA. White New Zealand rabbits weighing 2.1 ± 0.13 Kg were procured from central drug research institute, Lucknow. Before commencing experiment, rabbits were fasted overnight. Zero hour fasting blood samples were withdrawn early in the morning. In the first phase, oral suspension of resveratrol (20 mg/ml/kg) was administered through feeding tube followed by rinsing with 10 ml of water. After a washout period of 14 days, during second phase group received marketed preparation.

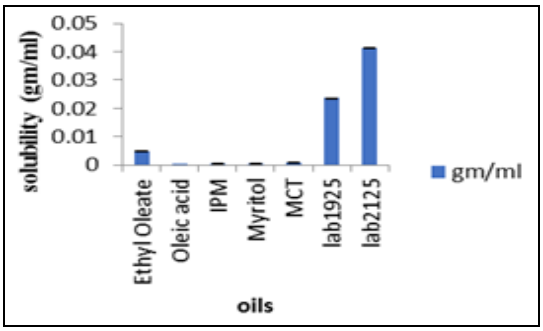
Again, after a washout period of 14 days, during third phase group received SNEDDS formulation. Blood samples (0.5 ml) withdrawn from marginal ear vein as shown in **Fig. 1** were collected at pre-set intervals of 0.05, 0.1, 0.3, 0.5, 0.7, 1, 2, 4, 6 h. respectively.

FIG. 1: BLOOD SAMPLES WITHDRAWN FROM MARGINAL EAR VEIN

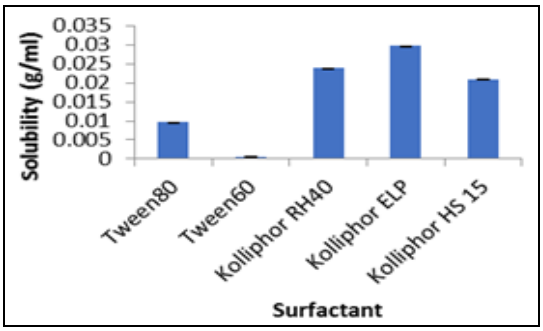
The blood samples were collected in a vial containing anticoagulant (0.4 ml of 2.5% sodium citrate), samples were centrifuged at 2500 rpm for 4 min and the plasma samples separated were stored at -20 °C. Same procedure repeated once again after one month. The amounts of resveratrol in the samples were estimated using HPLC [30].

RESULT:

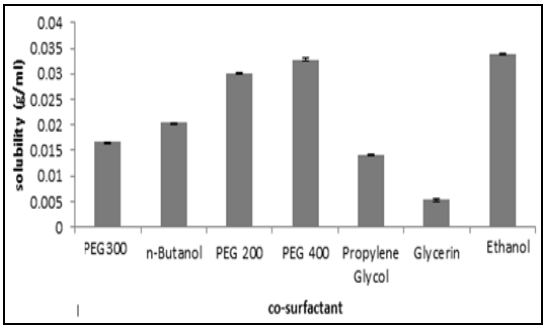
Solubility of Resveratrol in Lipids, Surfactant, Co-Surfactants: Solubility studies were carried out to investigate the maximum soluble fraction of resveratrol in different lipids, surfactants and co-surfactants. The maximum solubility of resveratrol was observed in Labrafil M 2125 (0.04142 ± 0.000017g/ml), while the minimum was found in oleic acid (0.00002 ± 0.000015 g/ml) **Fig. 2A**. Higher solubility in lipids lead to lower requirement of surfactant and co surfactants, which reduce its toxic effects. Likewise, among the surfactants, the maximum solubility of RVT was observed in Kolliphor ELP (0.02972 ± 0.16 g/ml) and minimum solubility was in Tween 60 (0.00063 ± 0.08 g/ml) **Fig. 2B** and in cosurfactant maximum solubility was observed in Ethanol and PEG 400 (0.03383 ± 0.000222 g/ml) and minimum solubility was found in Glycerin (0.00527 ± 0.000231 g/ml) **Fig. 2C**.



(A) Solubility of drug in various oils



(B) Solubility of drug in various surfactants



(C) Solubility of drug in various co-surfactants

FIG. 2: SOLUBILITY OF DRUG IN VARIOUS (A) OILS (B) SURFACTANTS (C) CO-SURFACTANTS

Combinations of Oil, Surfactant and Co-Surfactant Selection: The ease of emulsification judged and % transmittance was evaluated at 629 nm by using UV spectrophotometer. From the observations three combinations Labrafil M 2125: Kolliphor HS15: PEG 200, Labrafil M 2125: Kolliphor ELP: Ethanol, Labrafil M 2125: Kolliphor RH40: PEG 400 were selected having more than 90% transmittance even after 24 h and minimum number of inversions for emulsification *i.e.* less than 10.

Phase Diagrams: Ternary phase diagrams were constructed to identify the self-nanoemulsifying zone as they give clear picture of concentrations of components that can yield spontaneous emulsion. A simple ternary phase comprises of lipid, surfactant, cosurfactant at each corner in the phase diagram represent 100% of particular component. Ternary phase diagrams were constructed for all combinations selected on basis of ease of emulsification and % transparency **Fig. 3A, 3B, 3C**. It was observed from ternary phase diagram that nanoemulsion region for SNEDDS was prominent with Kolliphor ELP and ethanol whereas minimal with Kolliphor RH 40 and PEG 400 combination. Therefore, Kolliphor ELP and ethanol selected for SNEDDS formulation. The isotropic clear regions were identified by optical observations after formation of mono-layers around the emulsion droplets which reduces interfacial tension and minimizing the destabilizing effect because of gain in emulsion entropy. Phase diagrams with different concentration(w/w) ratios of lipid: S_{mix} (0.5:9.5, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, 9.5:0.5 w/w) were prepared by titrating the mixture with aqueous phase at an increment of 9 - 95%. After each aliquot addition of aqueous phase, physical state of mixture was marked on ternary phase diagram using chemmix software where one axis corresponds to lipid, one symbolize surfactant and cosurfactant (S_{mix}) and third symbolize water (yielded nanoemulsion and emulsion regions. The results obtained showed that apart from HLB value other factors such as structure and relative length of hydrophobic chains of surfactants also influence on nanoemulsion region.

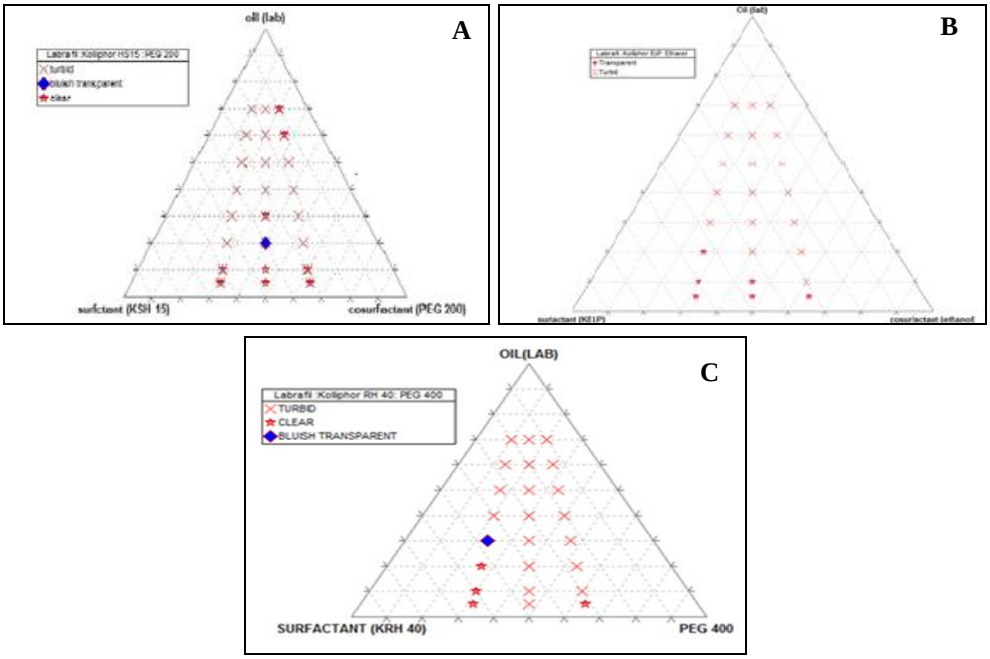


FIG. 3: PHASE DIAGRAMS INVOLVING (A) LABRAFIL M 2125: KOLLIPHOR HS15 (1:1) AND PEG 200 AS COSURFACTANT, (B) LABRAFIL M 2125: KOLLIPHOR ELP (1:2) AND ETHANOL AS COSURFACTANT, (C) LABRAFIL M 2125: KOLLIPHOR RH40 (2:1) AND PEG 400 AS COSURFACTANT

Response Surface Analyses: Percent drug content was taken as response variable and determined experimentally and predicted by composite design as shown in **Table 1**.

The response surface plots were constructed to facilitate the understanding of contribution of formulation variables and their interactions. The plot **Fig. 4A** shows that curvilinear increase in drug content from lower to higher levels of lipid. With an increase in surfactant concentrations, however, a relatively curvilinear decrease in drug content trend was observed till intermediate levels and then sharp linear increase in drug content was observed. With an increase in co-surfactant concentrations, however, a sharp increase in drug content trend was observed **Fig. 4B.**

As illustrated in **Fig. 4C,** sharp linear increase in drug content was observed with increase in levels of surfactant as well as cosurfactant.

TABLE 1: OIL: LABRAFIL M2125, SURFACTANT: KOLLIPHOR ELP, CO-SURFACTANT: ETHANOL AND FORMULATIONS CONTAINING 20 mg DRUG FORMULATED USING CENTRAL COMPOSITE DESIGN. EXPERIMENTAL PERCENT DRUG CONTENT DISSOLVED, PREDICTED PERCENT DRUG CONTENT DISSOLVED

	Independent Variables				Dependent Variables		
S. no.	**Formulation code**	**Oil %**	**Surfactant %**	**Co-surfactant %**	**Experimental % drug content**	**Predicted % drug content**	**Residual**
		(X_1)	**(X_2)**	**(X_3)**	**(Y)**		
1	F1	13.1265	56.5945	28.2725	89.57 ± 0.58	90.8007	-1.2307
2	F2	4.88	51.136	31.056	95.68 ± 0.53	86.12331	9.55668
3	F3	13.1265	56.5945	28.2725	82.75 ± 0.19	90.8007	-8.0507
4	F4	13.1265	65.77457	28.2725	81.578 ± 0.29	81.0905	0.487498
5	F5	4.88	51.136	25.489	99.7 ± 0.52	86.12331	13.57669
6	F6	13.1265	56.5945	28.2725	90.84 ± 1.54	90.8007	0.039297
7	F7	13.1265	56.5945	32.95377	100.59 ± 0.58	90.8007	9.789297
8	F8	4.88	62.053	31.056	15.89 ± 0.21	44.88337	-28.9934
9	F9	21.373	62.053	31.056	89.5 ± 0.55	93.81549	-4.31549
10	F10	13.1265	56.5945	23.59123	78 ± 0.61	90.8007	-12.8007
11	F11	-0.7424	56.5945	28.2725	12.1 ± 0.4	30.27936	-18.1794
12	F12	13.1265	47.41443	28.2725	90.89 ± 0.56	100.5109	-9.6209
13	F13	13.1265	56.5945	28.2725	91.94 ± 0.61	90.8007	1.139297
14	F14	13.1265	56.5945	28.2725	89.51 ± 0.69	90.8007	-1.2907
15	F15	21.373	51.136	31.056	80.48 ± 0.88	75.67043	4.809573
16	F16	21.373	51.136	25.489	73.97 ± 0.65	75.67043	-1.70043
17	F17	13.1265	56.5945	28.2725	89.4 ± 0.85	90.8007	-1.4007
18	F18	26.9954	56.5945	28.2725	68.27 ± 0.94	62.63641	5.633591
19	F19	4.88	62.053	25.489	88.51 ± 0.49	44.88337	43.62663
20	F20	21.373	62.053	25.489	92.74 ± 0.4	93.81549	-1.07549

The optimized formulation F5 and F7 **Fig. 5** was selected by ‘‘trading off’’ various response variables *i.e.* maximization of drug content (*i.e.* indicating drug transport potential across GI tract).

% Drug Content: >90.8007%

Finally, the prognosis of optimized formulation was conducted using overlay plots, drawn using the Design Expert software (M/s Stat-Ease, MN) [17]**.** Overlay plot obtained by superimposing various response variables to locate OPT formulation **Fig. 4D**. Observed and predicted responses were compared critically and the percentage bias (*i.e.* prediction error) was calculated regarding responses observed and formulation exhibited % Drug Content: >90.8007%. Formulations F7 (containing Labrafil M 2125: 13.1265 mg, Kolliphor ELP: 56.5945 mg, Ethanol: 32.95377) was found to fulfill maximal criteria for optimal performance. A best fitted quadratic equation obtained for the response.

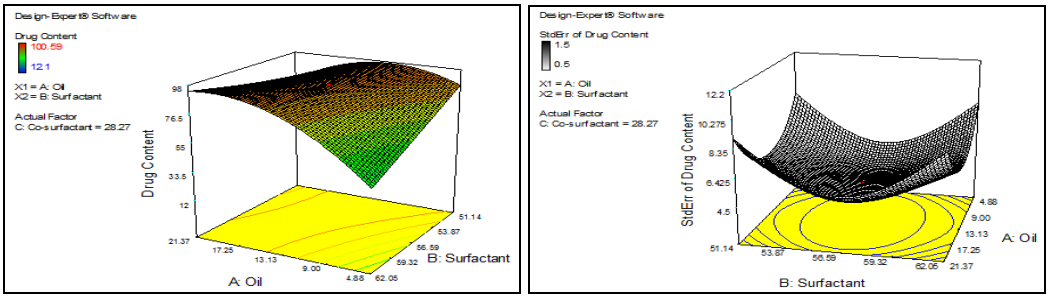
$$\% \text{ Drug Content} = 88.721 + 9.61\ X_1 - 5.773\ X_2 - 2.590\ X_3 + 14.846\ X_1 X_2 + 9.988\ X_1 X_3 - 9.793\ X_2 X_3 - 15.425\ X_1^2 + 0.855\ X_2^2 + 1.937\ X_3^2$$

The results were further analyzed using Statistical® version 10.0 (Stat software, Inclusive United States America) and version 8 Design Expert® (Statistical-Ease, Minneapolis, United States America). ANOVA results showed significant results having p value < 0.05.

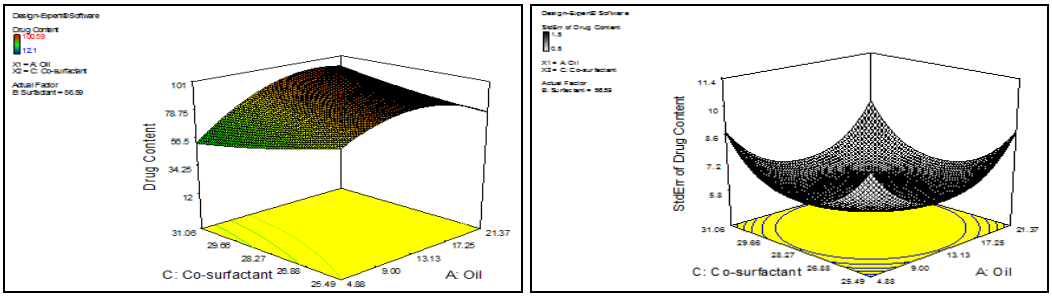
Characterization of Optimized (OPT) Formulation:

Transparency and Resveratrol Precipitation: 1 g of optimized formulations diluted up to 5 ml with water remained transparent and shown no signs of resveratrol precipitation.

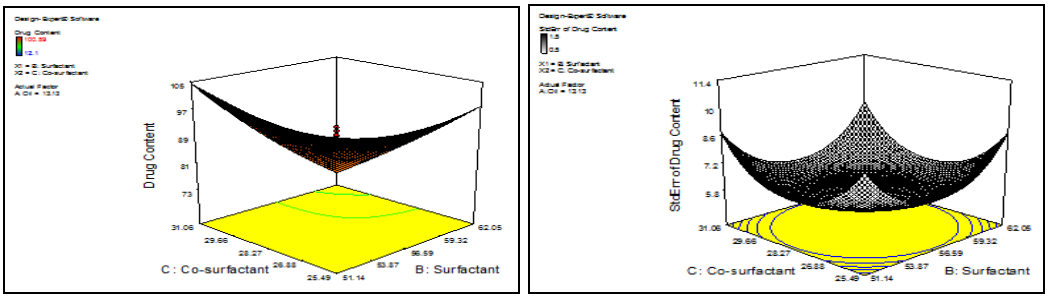
Thermodynamic Stability Studies: SNEDDS system undergo in situ solublization to form nanoemulsion and it must be stable, it doesn’t undergo precipitation, creaming, or cracking. Therefore, to check stability, optimized formulation was exposed to centrifugation study, heating and cooling cycle, freeze thawing cycles and cloud point, observations are **Table 2.** The optimized formulation did not show any signs of precipitation, creaming, or cracking thereby establishing the kinetic stability of the system.



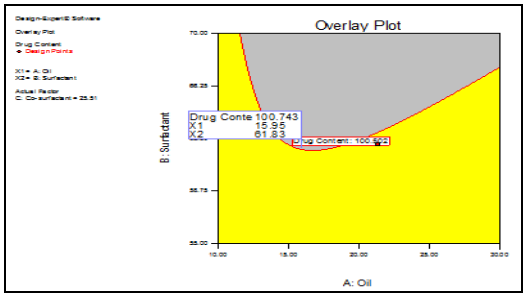
(A) 3D Surface Response Graph between Oil (X_1), Surfactant (X_2) with % Drug Content (Y)



(B) 3D Surface Response Graph between Oil (X_1), Co-Surfactant (X_3) with % Drug Content (Y)



(C) 3D Surface Response Graph between Surfactant (X_2), Co-Surfactant (X_3) with % Drug Content (Y)



(D) Overlay Plot

FIG. 4: 3D VIEW USING CENTRAL COMPOSITE DESIGN (A) 3D SURFACE RESPONSE GRAPH BETWEEN OIL (X_1), SURFACTANT (X_2) WITH % DRUG CONTENT (Y), (B) 3D SURFACE RESPONSE GRAPH BETWEEN OIL (X_1), CO-SURFACTANT (X_3) WITH % DRUG CONTENT (Y), (C) 3D SURFACE RESPONSE GRAPH BETWEEN SURFACTANT (X_2), CO-SURFACTANT (X_3) WITH % DRUG CONTENT (Y), (D) OVERLAY PLOT

Dispersibility Studies: SNEDDS systems are released in the lumen of gastrointestinal tract; it disperses to form fine emulsion with aid of GI fluid. Thus, it is important that formed nano-emulsion doesn't undergo precipitation following phase separation with infinite dilution in GI fluids. So dispersibility studies were performed in 200 ml and 900 ml medium. Optimized formulation (F7) passed dispersibility test after dilution and remained to be in nanoemulsion zone **Table 2**.

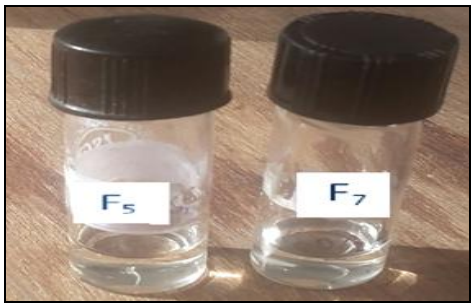


FIG. 5: BEST FORMULATIONS OBTAINED AFTER APPLYING CENTRAL COMPOSITE DESIGN

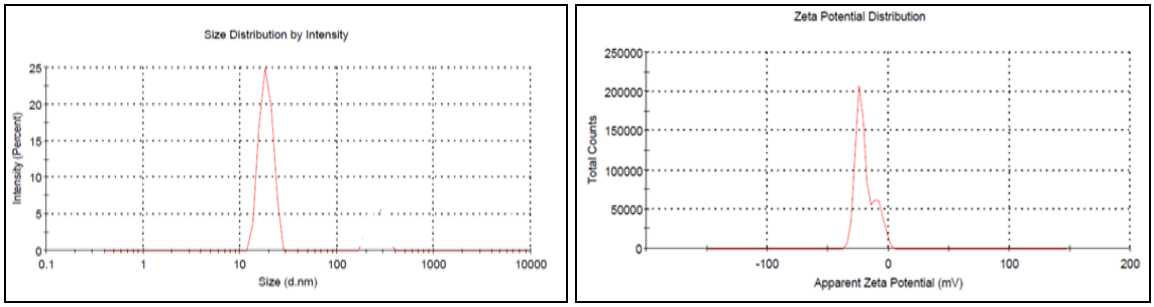
TABLE 2: CENTRIFUGATION STUDY, HEATING AND COOLING CYCLE, FREEZE THAW CYCLE AND CLOUD POINT, DISPERSIBILITY OF SNEDDS FORMULATION IN 200 ml AND 900 ml DILUTION MEDIUM AND EMULSIFICATION TIME, ZETA POTENTIAL, PARTICLE SIZE OF OPTIMIZED FORMULATIONS

Code	Centrifugation	Heating and cooling cycle	Freeze thaw cycle	Cloud Point	Emulsification time	Appearance in 200 ml		Appearance in 900 ml	
						After dilution	After 24 h	After dilution	After 24 h
F7	Homogenous, no phase separation	Homogenous, no phase separation	Homogenous, no phase separation	58.62 - 60.32 ± 0.89	Within 3sec.	Homoge-nous, Clear transparent	Homoge-nous, Clear transparent	Homoge-nous, Clear transparent	Homoge-nous, Clear transparent

Emulsification Time: Emulsification time of optimized formulation F7 was found to be 3 seconds **Table 2**.

Percentage Transmittance: The percentage transmittance of optimized formulations (F7) was determined and value was closer to 100%.

Determination of Zeta Potential and Droplet Size: The zeta potential of optimized Formulations (F7) was found to be -21.3 mV zeta potential **Fig. 6B** and globule size was found to be 83.25nm **Fig. 6A.**



A: Graph of Particle Size of F7 Formulation **B: Graph of Zeta Potential of F7 Formulation**

FIG. 6: GRAPH OF PARTICLE SIZE ANALYSIS AND ZETA POTENTIAL OF F7 FORMULATION

Transmission electron microscopic analysis: Transmission electron microscopic (TEM) images of formulations F7 after 24 h of post dilution in distilled water **Fig. 7**. It was observed that spherical micelles had no signs of coalescence even after 24 h of post dilution. The nanoemulsion droplets emerged as dark and the surroundings were observed inferring the stability of formed nanoemulsion. Closer images reveal that each globule is surrounded by thick layer. This indicates formation of monolayer around the emulsion droplets, reducing the interfacial energy. Image shows the electron microscopic image, depicting the morphology of the reconstituted optimized formulation, all the globules were of uniform shape, with globule size of most of them was less than 100 nm. The figure clearly illustrates that there are no indications of coalescence, so physical stability of the formulation was enhanced.

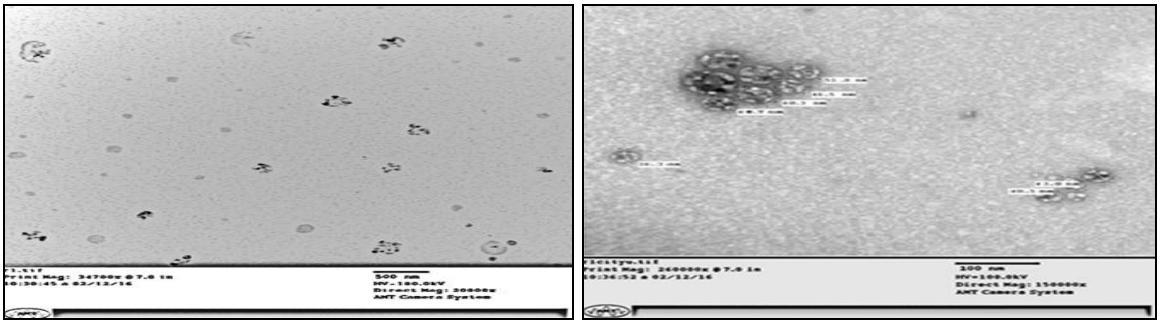
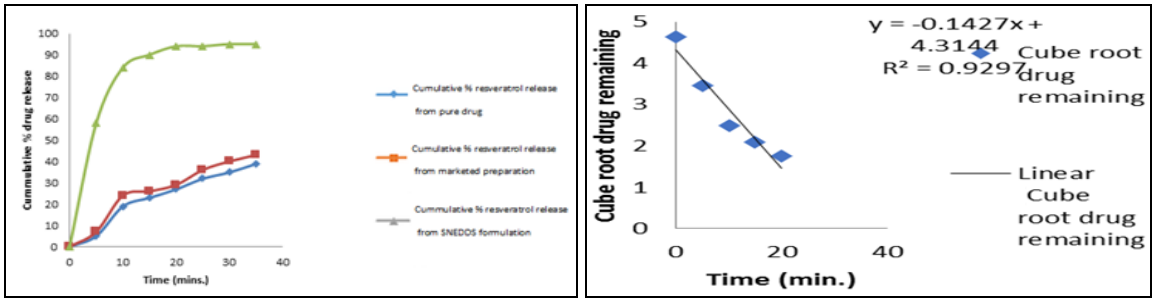


FIG. 7: TEM IMAGES OF F7 FORMULATION AFTER 24 h POST DILUTION IN DISTILLED WATER

Viscosity: Viscosity studies are necessary for SNEDDS to characterize the system physically and to control its stability. The viscosity of optimized formulations F7 was observed to be 78.52 cps and when formulation diluted 100 times it was observed to be 1.74 cps.

***In vitro* Resveratrol Release Study:** Resveratrol release from SNEDDS formulation F7 was compared with pure resveratrol and marketed resveratrol powder formulation. Release was observed to be significantly improved in SNEDDS formulation **Fig. 8A**. Drug dissolution was nearly completed within 30 min in case of the optimized formulation, as compared with that of the pure drug and marketed preparation where drug release was less than 50% in 30 min. Release kinetics followed Hixson Crowell model as shown in **Fig. 8B**.



A **B**

FIG. 8: (A) COMPARATIVE RESULTS OF PERCENT CUMULATIVE RELEASE FROM PLAIN RESVERATROL, SNEDDS FORMULATION F7 AND MARKETED FORMULATION (B) HIXSON-CROWELL ORDER GRAPH OF FORMULATION F7

***In vivo* Bioavailability Study in Rabbits:** The drug plasma concentration after the administration oral suspension of resveratrol, marketed preparation, SNEDDS formulation is shown in **Fig. 9**.

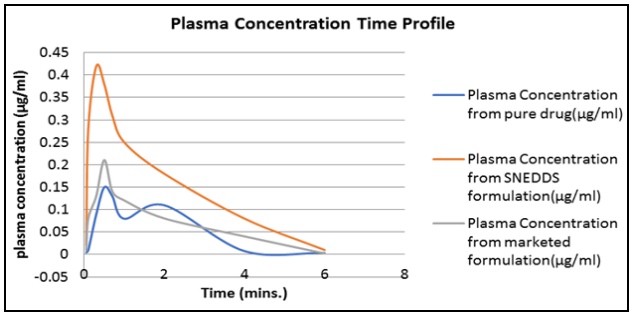


FIG. 9: COMPARATIVE RESULTS OF PLASMA FROM PURE RESVERATROL SUSPENSION, SNEDDS FORMULATION F7 AND MARKETED FORMULATION

It was observed maximum drug concentration (C_{max}) reached for SNEDDS formulation was 0.42 ± 0.86 µg/mL in t_{max} 30 min as compared to pure drug suspensión C_{max} 0.15 ± 0.43 µg/mL in t_{max} 50 min, marketed preparation C_{max} 0.21 ± 0.43 µg/mL in t_{max} 50 min respectively.

The disposition kinetic parameters are also listed in **Table 3**. The values of biological AUC, AUMC and MRT for resveratrol were calculated. There was statistically significant difference among the different formulations with respect to the estimated AUC with pure oral suspension it was found to be 30.68 h*mg/L as compared to marketed formulation 38.7225 h*mg/L and SNEDDS formulation 84.55 h*mg/L. SNEDDS formulation had maximum area under the curve. Mean residence time was also found to be maximum 2.0716 h for SNEDDS formulation.

TABLE 3: PHARMACOKINETIC PARAMETERS AUC, AUMC, MRT CALCULATED

Time (h)	Plasma Conc. from pure drug	AUC pure drug	AUMC pure drug	Plasma Conc. from SNEDDS	AUC SNEDD	AUMC SNEDDS	Plasma Conc. from marketed formulation	AUC marketed product	AUMC marketed product
0.05	0.005 ± 0.56	0.000375	0.0000312	0.02 ± 0.36	0.0075	0.00035	0.009 ± 0.56	0.002225	0.000211
0.1	0.01 ± 0.87	0.01	0.0028	0.28 ± 0.31	0.07	0.0085	0.08 ± 0.32	0.021	0.0047
0.3	0.09 ± 0.12	0.024	0.0102	0.42 ± 0.86	0.08	0.0262	0.13 ± 0.14	0.034	0.0144
0.5	0.15 ± 0.43	0.028	0.0166	0.38 ± 0.64	0.069	0.0484	0.21 ± 0.43	0.035	0.0203
0.7	0.13 ± 0.88	0.0315	0.02565	0.31 ± 0.71	0.084	0.0921	0.14 ± 0.88	0.039	0.0327
1	0.08 ± 0.11	0.095	0.15	0.25 ± 0.41	0.215	0.42	0.12 ± 0.16	0.1	0.14
2	0.11 ± 0.26	0.118	0.252	0.18 ± 0.83	0.26	0.88	0.08 ± 0.32	0.12	0.32
4	0.008 ± 0.12	0.012	0.056	0.08 ± 0.91	0.09	0.402	0.04 ± 0.43	0.042	0.172
6	0.004 ± 0.32	-0.012	-0.072	0.01 ± 0.23	-0.03	-0.126	0.002 ± 0.24	-0.006	-0.036
	0.3068	.44128		0.8455	1.75155		0.387225	0.668311	
Mean Residence Time		MRT = AUMC/AUC = 1.4383			MRT = AUMC/AUC = 2.0716			MRT = AUMC/AUC = 1.7259	

DISCUSSION: The current work objective was to develop optimized formulation of resveratrol. SNEDDS are the formulations which immediately forms nanoemulsion when comes in contact to GIT fluids. The spontaneous development of nano-emulsion presents the drug in dissolved form. The nano size droplet formed provides large interfacial surface area for drug absorption. In addition, the specific nanoemulsion promote intestinal transport of drug through lymphatic transport of system [31].

Solubility studies were performed to find out the maximum solubility of resveratrol in different lipids, surfactants and co-surfactants. Results obtained clearly indicate that resveratrol was having maximum solubility in Labrafil M 2125 which was selected as the lipid excipient for formulating SNEDDS. Labrafil M 2125 is a known as bioavailability improving agent which enhances oral bioavailability by inhibiting enzyme CYP3A4 which is an enterocyte drug-metabolizing enzyme. Lipid phase is one of the crucial components of SNEDDS formulations, as it is the agent which solubilize lipophilic drugs and increase fraction of dissolved lipophilic drug which is absorbed through intestinal lymphatic system, hence absorption is enhanced through gastrointestinal tract, which depends on the nature of the triglycerides used for formulation.

Non-ionic surfactants are known to have less toxicity than ionic surfactants. Therefore, usually accepted for oral formulations [2]. In addition, they are known to produce reversible changes in mucosa of intestine, thus lead to change permeability of lipophilic drugs [32]. Therefore, Kolliphor ELP was selected as surfactant as resveratrol showed maximum solubility, whereas ethanol was selected as cosurfactant in the formulation of SNEDDS with an objective to enhance drug-loading efficiency. To ensure continuous conversion of SNEDDS formulation to nanoemulsion in GI tract, the construction of ternary plots is must [9]. Literature clearly reports that HLB value of surfactant lower interfacial energy, which leads to formation of a stable emulsion. The surfactant added in SNEDDS formulation, *i.e.* Kolliphor ELP, has HLB value of 15. Thus, HLB values and ternary plots obtained indicated the synergistic effect in reducing interfacial tension and formation of stable nanoemulsion.

The central composite design is the most efficient design to generate optimized formulation. The high values of R^2 shown by the polynomial equation proved high statistical validity (p value < 0.05) fitting to the experimental data. The linearity of the correlation between predicted and observed responses (p value < 0.05), and nearly uniform scattering of the residual plots indicate highly postulated model.

The optimized formulation was found to fulfill maximal criteria for optimal performance. The said formulation exhibited % Drug Content: >90.8007 %. The optimized formulation had rapid emulsification rate (3s). Rapid emulsification rate can be correlated with lower lipid and higher cosurfactant content requirement, which further result in lower viscosity [3].

The SNEDDS systems are released in lumen of the gastrointestinal tract, it disperses to form a fine micro or nano emulsion with aid of GI fluids. Thus, it is important that formed nanoemulsion doesn't undergo precipitation following phase separation on dilution.

Thermodynamic stability of SNEDDS formulation shows kinetic stability. Formulation must exhibit stability to prevent creaming, precipitation, or cracking. Optimized SNEDDS formulation subjected to centrifugation, heating and cooling cycle, cloud point determination. To avoid an irreversible phase separation due to dehydration of SNEDDS formulation, which may affect drug absorption the cloud point should be above body temperature (*i.e.* 37 ºC). Hence, cloud point 58.62-60.32 ± 0.89 ºC of SNEDDS formulation indicate high stability of the optimized SNEDDS with no risk of phase separation.

As SNEDDS formulation in gastrointestinal track must meet patient acceptability, therefore % transmittance closer to 100% indicates globule size in nanometer range. The nano globule size resulted in low emulsification time which in turn will increase absorption through lymphatic system and subsequently improve efficacy of drugs. Zeta potential 21.3 mV indicates high repulsive forces, thus it rules out the possibility of coalescence [33].

The nanoemulsion droplets emerged as dark and the surroundings were observed inferring the

stability of formed nanoemulsion. Closer images in TEM reveal that each globule is surrounded by thick layer. This indicates formation of monolayer around the emulsion droplets with reduced interfacial energy. Significantly high dissolution rate of SNEDDS formulation could be attributed to small globule size of 83.25 nm, high percentage transmittance (99.5 ± 0.87), negative zeta potential and *in situ* solubilization as confirmed by TEM studies, which provided large surface area for release of drug and thus permitting faster rate of drug release.

In vivo studies clearly indicated higher bioavailability from SNEDDS formulation. During analysis plasma levels in SNEDDS formulation were observed to be much higher as compared to marketed preparation and resveratrol suspension which could be attributed to small globule size. Significant difference in area under the curve was observed as well as mean residence time also increased for SNEDDS formulation.

CONCLUSION: The novel approach was developed for SNEDDS formulations by selecting optimum blends of lipids, surfactants and cosurfactant using systematic ‘‘DoE’’ methodology of central composite design. SNEDDS formulation were found to have high dissolution rate as compared to pure drug and marketed preparation which could be attributed to nano globule size, high percentage transmittance and high zeta potential and in situ solubilization, of developed SNEDDS formulation, which in turn provide large surface area for release of drug. The spontaneous development of nanoemulsion presents the drug in dissolved form. The nano size droplet formed provides large interfacial surface area for drug absorption. In addition, the specific nanoemulsion will promote intestinal transport of drug through lymphatic transport of system.

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CONFLICT OF INTEREST: Nil

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