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Research Article

Preliminary Physicochemical screening of bioactive based nanocarrier system

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ABSTRACT

The roles of oxidative stress (OS) in neurodegenerative diseases, the manipulation of ROS levels may represent a treatment option to slow down neurodegeneration and alleviate associated symptoms. Many compounds with antioxidant properties have been examined for their potential to attenuate neurodegenerative symptoms. The drawbacks of oral administration of antioxidants are limited entry across the blood-brain barrier. Most molecules suffer from problems of solubility, bioavailability or the ability to cross the blood brain barrier and hence nanomedicine needs to be explored. The excipient components (Carrier oil, surfactant and co-surfactant) were selected on the basis of their miscibility in alpha-pinene. The miscibility of the drug was assessed in 25 carrier oil, 5 surfactant and 6 Co-surfactant. Total 250 self-nanoemulsifying nanoformulations (SENF) compositions were prepared from the selected excipients and were preliminary screened on the basis of the number of flask inversion, emulsification time and stability up to 72 hours. Three SNEDDS formulation compositions finally selected for the development and optimization for excipient concentration as these combinations was found to possessed no. of flask inversion and emulsification time.

Keywords: Self-nanoemulsifying nanoformulations, Bioavailability, Alpha-pinene, oxidative stress, blood-brain barrier

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INTRODUCTION

Neurobiology nanotechnology consists of nano sized macromolecular structures ranging from 1-1000nm containing biological active material. It offers controlled release of the active material with reduced drug toxicity, enhanced bioavailability, enhanced therapeutic efficacy and targeted bio-distribution ¹. The use of different polymers reveals possible toxic effects due to the toxic monomers; polymerization and residues of organic solvent. The toxicological aspects of the materials incorporated in the nanoformulation have to be kept in mind ². The green approach for the nanoformulation connects the nanotechnology with the plant derived Phyto-medicines, thus eliminating the toxicity problems associated with the polymers ³. The biofabrication of bioactive as nanoformulation could result in some prominent outcomes i.e. Circulation of bio-composite in the targeted region of brain capillaries that favours the concentration gradient and ambient availability to combat the functional loss of dopaminergic neurons. Endocytosis of the bio-composite by the endothelial cells, release of the bio actives within these cells and diffusion in the brain. Transcytosis of the bio-

composite through the endothelial cells, its deposition in the cerebral compartment and final release of the bioactive to their targeted sites. Optimum use of surfactants, like poloxamer 407 and polysorbate 80, which can inhibit the P-glycoprotein (P-gp) efflux system in the BBB, which can prevent or inhibits the expulsion of the drug from the brain ⁴. Based on these above possibilities the formulations will be optimized in accordance with the factors like its safety and efficacy. Nanomedicine may be used as a solution to the limitations of orally administered non-engineered bioactive. Functionalized nanosystems may overcome several bioavailability limits of active molecules and increase their effectiveness in the brain.

Bioactive compounds with considerable anti-inflammatory and antioxidant activity when administered as nutritional values have play important role in preventing ARNs, however its efficacy and potency is drastically affected due to its poor pharmacokinetic profile and inadequate bioavailability ^{5,6}. For quite a long time, we have different routes of drug administration like topical, oral, inhalational, parenteral etc. however oral route is the major preferred and conventional route and is well accepted and acknowledged

technique for drug delivery. Beside these, oral route has several issues, mostly related to pre-systemic metabolism and poor bioavailability for the most part on account of altered pH conditions and the breakdown of the administered drugs due to proteolytic action along GI tract ⁷. For an effective gut absorption, it is essential for the bioactive loaded nanotherapeutics to efficiently cross the enterocytes plasma layer. Gut absorption of therapeutics predominantly follow passive diffusion by means of bilayer lipid membrane, however for which it is fundamental that the bioactives must possessed a balanced lipophilicity/hydrophilicity ⁸. After being absorbed, the effective bio-distribution of bioactive takes place all through the body. Although the redistribution can be ruined by various deterrents but due to hydrophobicity of bioactives they are frequently bound to plasma proteins that probably be distributed in blood ⁹. Due to hydrophobicity, as an outcome, the bioactives might be either accumulated in

adipocytes cells or being metabolised rapidly by the liver into hydrophilic component leads to be evacuated either through urine or bile.

Because of the poor solubility, permeability or even the biotransformation insufficiency that occurs when bioactives enter in systemic circulation, it is not possible for bioactive loaded nanotherapeutics to cross BBB and reach the brain. Regardless of whether a bioactive for treatment for ARNs can be systemically and sufficiently absorbed and distributed to reach the brain, despite these it needs to design in state of art-based methodology which overcome BBB for effective delivery. Consequently, one of the difficulties these days is to develop nanocarrier that can convey bioactives specifically to the brain conquering the biological boundaries faced by bioactives ¹⁰. The present study was aimed to develop bioactive loaded self-emulsifying nanosystem due to its promising advantages (Figure 1) and was preliminary screened for their physicochemical characterization ^{11,12}

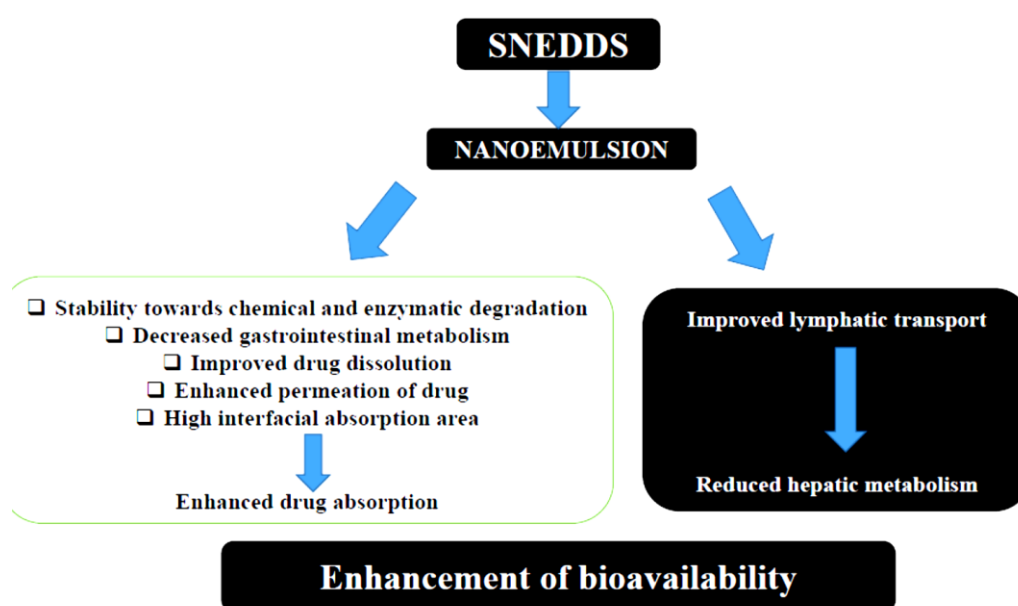


Figure 1: Advantages of self-emulsifying drug delivery system

MATERIAL AND METHODS

Miscibility of alpha pinene in different excipient system

Screening of oil

The miscibility of alpha pinene (ALP) in different oils was determined by adding it in 1:1 ratio of the oils separately in 2ml capacity of Eppendorf tube, and mixed using a vortex shaker. The mixture vials were then kept at 25 ± 1.0 °C in an isothermal shaker (Nirmal International, Delhi, India) for 72 h to reach equilibrium. The equilibrated samples were removed from shaker and centrifuged at 3500 rpm for 10 min (Scientific microprocessor-based ultracentrifuge). The resultant mixture was visualized for turbidity and phase separation.

Screening of surfactant

The miscibility of alpha pinene in surfactants (Triton X-100, Span-85, Tween-20, Tween-80 and Span-20) was determined by adding it in 1:1 ratio separately in 2ml capacity of eppendorf tube, and mixed using a vortex shaker. The mixture vials were then kept at 25 ± 1.0 °C in an

isothermal shaker (Nirmal International, Delhi, India) for 72 h to reach equilibrium. The equilibrated samples were removed from shaker and centrifuged at 3500 rpm for 10 min (Scientific microprocessor-based ultracentrifuge). The resultant mixture was visualized for turbidity and phase separation.

Screening of co-surfactant

The miscibility of alpha pinene in co-surfactants (Transcutol-P, Ethyl acetoacetate -EAA, Ethyl acetate-EA, PEG-400, Isopropyl Myristate and Isopropyl Alcohol) was determined by adding it in 1:1 ratio separately in 2ml capacity of Eppendorf tube, and mixed using a vortex shaker. The mixture vials were then kept at 25 ± 1.0 °C in an isothermal shaker (Nirmal International, Delhi, India) for 72 h to reach equilibrium. The equilibrated samples were removed from shaker and centrifuged at 3500 rpm for 10 min (Scientific microprocessor-based ultracentrifuge). The resultant mixture was visualized for turbidity and phase separation.

selection and screening of self-Nano emulsification system

Based on individual miscibility of alpha pinene in oil, surfactant and co surfactant. The components are further selected by adding selected oil, surfactant or co-surfactant in equal ratio and vortex by using vortex shaker for 10 min. The mixture was then equilibrated at room temperature for

24 h. Then resulting mixture was subjected to centrifugation at 3500 rpm for 10 min (Scientific microprocessor-based ultracentrifuge). and was characterized visually before and after dilution. The mixtures were characterized upon dilution (1:100) for percentage transmittance, number of flask inversions self-emulsification time and stability up to 72 hours for further optimization.

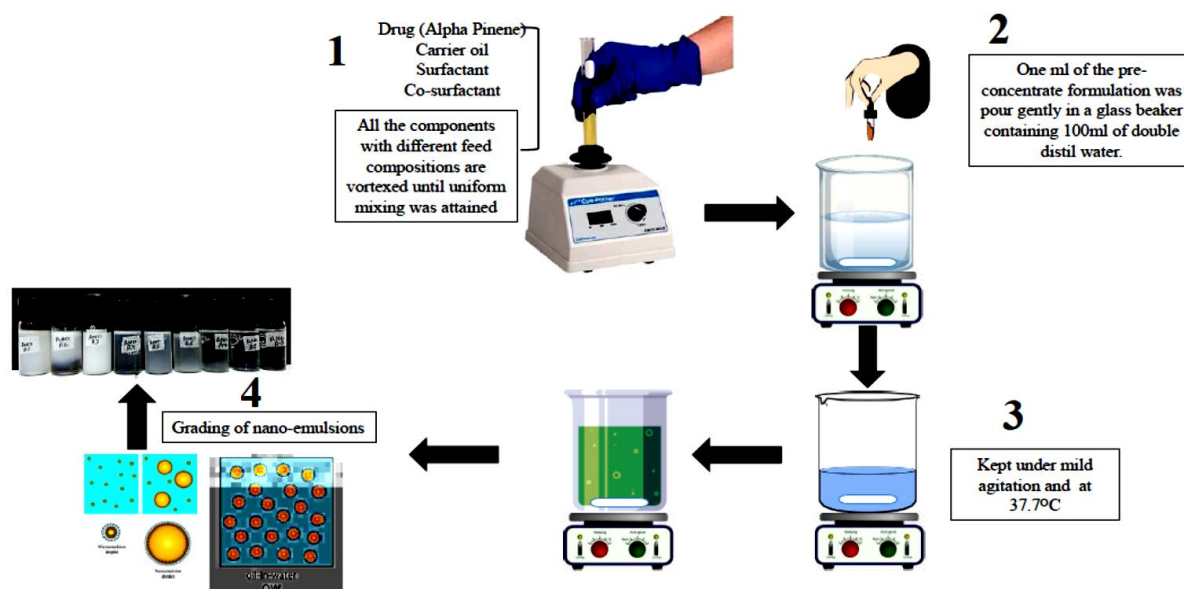


Figure 2: Set-up for preparation and characterization of nano-emulsions by the self-emulsification method

Self-Nano emulsification testing

A series of self-emulsifying systems were prepared for each of the three formulas (with varying weight percentages of oil from 8 to 78%, surfactant from 3.5 to 80% and co-surfactant (Co-S) from 0 to 53% however drug (Alpha pinene) weight

percentage was fixed at 12 % to the total formulation mixture. The mixture (0.1 ml) was gently mixed with 100 ml of distilled water in a glass beaker at room temperature (figure 2). The tendency to emulsify spontaneously and the progress of emulsion droplets spread were visually assessed using the grading criteria of ¹³⁻¹⁶ as follows:

Grade	Characteristics
Grade A:	Refers to rapidly forming (within 1 min) nanoemulsion, having a clear or bluish appearance.
Grade B:	It refers to rapidly forming (within 2 min), slightly less clear nanoemulsion, having a bluish white appearance.
Grade C:	It refers to fine milky emulsion that was formed within 2 min.
Grade D:	It is dull, grayish white emulsion having slightly oil appearance that is slow to emulsify (longer than 2 min).
Grade E:	Is a formulation, exhibiting either poor or minimal emulsification with large oil globules present on the surface (longer than 3 min).

Stability studies of the formulations:

The test was aimed to screen out the metastable formulation by assessing the phase integrity and stability of the ALP-SNEDDS by exposing it under extreme thermal variation and physical impact of centrifugal force. The thermodynamic stability testing consists of heating cooling cycles at 4 °C and 45 °C for at least 48h at each temperature, centrifugation studies at 5000 rpm for 30 min and accelerated aging freeze thaw studies between -20 °C and +25 °C for at least 48h at each temperature.

RESULT AND DISCUSSION:

The excipient components (Carrier oil, surfactant and co-surfactant) were selected on the basis of their miscibility in alpha-pinene. The miscibility of the drug was assessed in 25 carrier oil, 5 surfactant and 6 Co-surfactant (Table 1). Out of which the drug was freely miscible in 22 oils, 3 surfactant and 5 Co-surfactant. Total 250 SNEDDS compositions were prepared from the selected excipients and were preliminary screened on the basis of the number of flask inversion, emulsification time and stability up to 72 hours. The SNEDDS excipient combinations with least no. of flask inversion and emulsification time were finally 3 compositions were selected (Table 2) for the development and optimization of excipient by self-emulsification grading system.

Table 1: Miscibility of Alpha-Pinene in different excipient components

S.N.	Oil	Inference
1.	Linseed oil	Clear Transparent
2.	Olive oil	Clear Transparent
3.	Cottonseed oil	Clear Transparent
4.	Hempseed oil	Clear Transparent
5.	Castor oil	Clear Transparent
6.	Sesame oil	Clear Transparent
7.	Arachis oil	Clear Transparent
8.	Vitamin -E oil	Clear Transparent
9.	Flaxseed oil	Clear Transparent
10.	Almond oil	Clear Transparent
11.	Grapeseed oil	Clear Transparent
12.	Oleic acid	Clear Transparent
13.	Caproic acid	Clear Transparent
14.	Orange oil	Clear Transparent
15.	Eucalyptus oil	Clear Transparent
16.	Cedarwood oil	Clear Transparent
17.	Nutmeg oil	Clear Transparent
18.	Rose oil	Clear Transparent
19.	Lemongrass oil	Clear Transparent
20.	Anise oil	Clear Transparent
21.	Camphor oil	Turbid
22.	Cannola Oil	Clear Transparent
23.	Triacetin	Phase Separation
24.	Sunflower	Clear Transparent
25.	Acrysol EL-135	Clear Transparent
Surfactant		
1.	Triton X-100	Clear Transparent
2.	Span-85	Clear Transparent
3.	Tween-20	Phase Separation
4.	Tween-80	Clear Transparent
5.	Span-20	Phase Separation
Co-Surfactant		
1.	Transcutol-P	Clear Transparent
2.	Ethyl acetoacetate -EAA	Clear Transparent
3.	Ethyl acetate-EA	Clear Transparent
4.	PEG-400	Phase Separation
5.	Isopropyl Myristate	Clear Transparent
6.	Isopropyl Alcohol	Clear Transparent

Table 2: Selected stable excipient combinations with least no. of flask inversion and emulsification time.

S.L.	Surfactant/Co-Surfactant/Oil	CODE	Visual observation (before dilution)	No. flask inversions	Visual observation on dilution	Transmittance After dilution	Emulsification time (Second)
1.	Triton X-100+ Oleic acid+ Transcutol-P	OA	Clear	04	Dispersed uniformly to form a less transparent solution with blue florescence	92.5%	30
2.	Tween-80+ Anise oil+ Transcutol-P	ANO	Clear	01	Dispersed uniformly and rapidly to form a clear transparent solution.	95.2%	15
3.	Triton X-100+ Acrysol EL-135+ Transcutol-P	ACR	Clear	02	Dispersed uniformly to form a clear transparent solution.	94.8%	13

Effect of carrier oil, surfactant and co-surfactant ratio on the quality of self-emulsification in ACR-EL 135 based nanoformulations:

Based on the grading system the compositions with grade A and B were selected and were further subjected to thermodynamic stability studies. SNEDDS zones were exposed by 1:0, 1:1, 2:1, 3:1,4:1 and 1:2 mass ratio of surfactant and co-surfactant with a total 42 formulations for each composition. In case of Acrysol EL-135 (ACR) 14 formulations are of Grade A and 3 of grade B self-emulsification (Figure). It has been found that the formulations showing good self-emulsification have 8 to 38 % of ACR-EL 135. In case of 1:1 S-CoS ratio the SNEDDS zones with grade A and B self-emulsification lies in between

20 to 40 % of Triton X-100 and Transcutol-HP. However, on increasing the ratio of S-CoS by 2:1 the SNEDDS zones lies in between 33 to 53 % of Triton X-100 and 17 to 27% of Transcutol -HP with grade A self- emulsification. For 3:1 ratio the zone lies between the percentage of 37.5 to 60 % of Triton X-100 and 12.5 to 20 % of Transcutol -HP. For 1:2 ratio, formulations having 17 to 27% of Triton X-100 and 33 to 53 % of Transcutol -HP was found to show grade A self-emulsification. Conclusively it has been found that the grade of self-emulsification was improved on increasing the ratio of surfactant and co-surfactant (S-CoS) i.e., from 1:1 to 3:1. However further increase in the S-CoS ratio (4:1) does not impart any self-emulsification instead possessed grade E and D self-emulsification (Table 3).



Figure 3: Visual assessment ACR based formulation after assessment of self-emulsification grading

Effect of carrier oil, surfactant and co-surfactant ratio on the quality of self-emulsification in Anise oil (ANO) based nanoformulations:

In case of Anise oil (ANO) containing formulations the SNEDDS zones were exposed by 1:1, 2:1, 3:1 and 1:2 ratio of Tween -80 and Transcutol-HP (Table 3) with total 9 formulation out of which 6 belong to grade A and 3 to grade B self-emulsification (Figure). It has been found that the

formulations showing good self-emulsification have 8 to 28 % of Anise oil. The 2:1 ratio of S-CoS was found to exhibit three formulations, 2 with grade A and 1 with grade B self-emulsification. Further increase in the S-CoS ratio (4:1) does not impart any self-emulsification instead possessed grade C, D and E self-emulsification. The overall the self-emulsification zone exist between 23 to 60 % of Tween 80 and 15 to 53% of Transcutol-HP.



Figure 4: Visual assessment ANO based formulation after assessment of self-emulsification grading

Effect of carrier oil, surfactant and co-surfactant ratio on the quality of self-emulsification in Oelic acid (OA) based nanoformulations:

In case of Oelic acid (OA) containing formulations, the SNEDDS zones were exposed by 1:1, 2:1, 3:1, 4:1 and 1:2 ratio of Triton-X 100 and Transcutol-HP (Table 3) with total 14 formulation out of which 3 belong to grade A and 11 to grade B self-emulsification (Figure). It have been found that

the formulations showing good self-emulsification have 8 to 28 % of Oelic acid. The self-emulsification zone exists between 23 to 64 % of Triton X-100 and 12 to 53% of Transcutol-HP. It was also observed that the incorporating co-surfactant (Transcutol-HP) in all the above formulations impart a tendency of good self-emulsification because the use of surfactant alone (1:0) does not impart any good self-emulsification tendency (Grade D and E).



Figure 5: Visual assessment OA based formulation after assessment of self-emulsification grading

Table 3: Formula of α -pinene SNEDDS containing Acrysol EL-135 (ACR) as carrier oil, Triton X-100 as surfactant and Transcutol-HP as co-surfactant; Anise oil (ANO) as carrier oil, Tween 80 as surfactant and Transcutol-HP as co-surfactant and Oleic Acid (AO) as carrier oil, Triton X-100 as surfactant and Transcutol-HP as co-surfactant.

Smix (1:0) (A)	Percentage of components (w/w)				GRADE
Oil:Smix(Code)	α -pinene	Acrysol EL-135	Triton X-100	Transcutol-HP	
(9:1) ACR-A1	0.12ml (12%)	0.78ml (78%)	0.10 ml (10%)	0	E
(8:2) ACR-A2	0.12ml (12%)	0.68ml (68%)	0.20 ml (20%)	0	D
(7:3) ACR-A3	0.12ml (12%)	0.58ml (58%)	0.30 ml (30%)	0	D
(6:4) ACR-A4	0.12ml (12%)	0.48 ml (48%)	0.40 ml (40%)	0	D
(5:5) ACR-A5	0.12ml (12%)	0.38 ml (38%)	0.50 ml (50%)	0	D
(4:6)ACR- A6	0.12ml (12%)	0.28ml (28%)	0.60 ml (60%)	0	D
(3:7) ACR-A7	0.12ml (12%)	0.18 ml (18%)	0.70 ml (70%)	0	D
(2:8)ACR- A8	0.12ml (12%)	0.08 ml (8%)	0.80 ml (80%)	0	D
Smix (1:1) (B)	Percentage of components (w/w)				GRADE
(9:1) ACR-B1	0.12ml (12%)	0.78ml (78%)	0.05 ml (5%)	0.05 ml (5%)	E
(8:2) ACR-B2	0.12ml (12%)	0.68ml (68%)	0.10 ml (10%)	0.10 ml (10%)	D
(7:3) ACR-B3	0.12ml (12%)	0.58ml (58%)	0.15 ml (15%)	0.15 ml (15%)	D
(6:4) ACR-B4	0.12ml (12%)	0.48 ml (48%)	0.20 ml (20%)	0.20 ml (20%)	B
(5:5) ACR-B5	0.12ml (12%)	0.38 ml (38%)	0.25 ml (25%)	0.25 ml (25%)	B
(4:6)ACR- B6	0.12ml (12%)	0.28ml (28%)	0.30 ml (30%)	0.30 ml (30%)	A
(3:7) ACR-B7	0.12ml (12%)	0.18 ml (18%)	0.35 ml (35%)	0.35 ml (35%)	B
(2:8)ACR- B8	0.12ml (12%)	0.08 ml (8%)	0.40 ml (40%)	0.40 ml (40%)	A
Smix (2:1) (C)	Percentage of components (w/w)				GRADE
(9:1) ACR-C1	0.12ml (12%)	0.78ml (78%)	0.065 ml (6.5%)	0.035 ml (3.5%)	E
(8:2) ACR-C2	0.12ml (12%)	0.68ml (68%)	0.13 ml (13%)	0.07 ml (7%)	D
(7:3) ACR-C3	0.12ml (12%)	0.58ml (58%)	0.20 ml (20%)	0.10 ml (10%)	D
(6:4) ACR-C4	0.12ml (12%)	0.48 ml (48%)	0.265 ml (26.5%)	0.135 ml (13.5%)	D
(5:5) ACR-C5	0.12ml (12%)	0.38 ml (38%)	0.33 ml (33%)	0.17 ml (17%)	A
(4:6)ACR- C6	0.12ml (12%)	0.28ml (28%)	0.40 ml (40%)	0.20 ml (20%)	A
(3:7) ACR-C7	0.12ml (12%)	0.18 ml (18%)	0.465 ml (46.5%)	0.235 ml (23.5%)	A
(2:8)ACR- C8	0.12ml (12%)	0.08 ml (8%)	0.53 ml (53%)	0.27 ml (27%)	A
Smix (3:1) (D)	Percentage of components (w/w)				GRADE
(9:1) ACR-D1	0.12ml (12%)	0.78ml (78%)	0.075 ml (7.5%)	0.025 ml (2.5%)	E
(8:2) ACR-D2	0.12ml (12%)	0.68ml (68%)	0.15 ml (15%)	0.05 ml (5%)	E
(7:3) ACR-D3	0.12ml (12%)	0.58ml (58%)	0.225 ml (22.5%)	0.075 ml (7.5%)	E
(6:4) ACR-D4	0.12ml (12%)	0.48 ml (48%)	0.3 ml (30%)	0.1 ml (10%)	E
(5:5) ACR-D5	0.12ml (12%)	0.38 ml (38%)	0.375 ml (37.5%)	0.125 ml (12.5%)	A
(4:6)ACR- D6	0.12ml (12%)	0.28ml (28%)	0.45 ml (45%)	0.15 ml (15%)	A
(3:7) ACR-D7	0.12ml (12%)	0.18 ml (18%)	0.525 ml (52.5%)	0.175 ml (17.5%)	A
(2:8)ACR- D8	0.12ml (12%)	0.08 ml (8%)	0.60 ml (60%)	0.2 ml (20%)	A
Smix (4:1) (E)	Percentage of components (w/w)				GRADE
(9:1) ACR-E1	0.12ml (12%)	0.78ml (78%)	0.08 ml (8%)	0.02 ml (2%)	E
(8:2) ACR-E2	0.12ml (12%)	0.68ml (68%)	0.16 ml (16%)	0.04 ml (4%)	E
(7:3) ACR-E3	0.12ml (12%)	0.58ml (58%)	0.24ml (24%)	0.06ml (6%)	E

(6:4) ACR-E4	0.12ml (12%)	0.48 ml (48%)	0.32 ml (32%)	0.08 ml (8%)	D
(5:5) ACR-E5	0.12ml (12%)	0.38 ml (38%)	0.4ml (40%)	0.1 ml (10%)	D
(4:6)ACR- E6	0.12ml (12%)	0.28ml (28%)	0.48 ml (48%)	0.12 ml (12%)	D
(3:7) ACR-E7	0.12ml (12%)	0.18 ml (18%)	0.56 ml (56%)	0.14 ml (14%)	D
(2:8)ACR- E8	0.12ml (12%)	0.08 ml (8%)	0.64 ml (64%)	0.16 ml (16%)	D
Smix (1:2) (F)	Percentage of components (w/w)				GRADE
(9:1) ACR-F1	0.12ml (12%)	0.78ml (78%)	0.035 ml (3.5%)	0.065 ml (6.5%)	D
(8:2) ACR-F2	0.12ml (12%)	0.68ml (68%)	0.07 ml (7%)	0.13 ml (13%)	D
(7:3) ACR-F3	0.12ml (12%)	0.58ml (58%)	0.10 ml (10%)	0.20 ml (20%)	D
(6:4) ACR-F4	0.12ml (12%)	0.48 ml (48%)	0.135 ml (13.5%)	0.265 ml (26.5%)	D
(5:5) ACR-F5	0.12ml (12%)	0.38 ml (38%)	0.17 ml (17%)	0.33 ml (33%)	A
(4:6)ACR- F6	0.12ml (12%)	0.28ml (28%)	0.20 ml (20%)	0.40 ml (40%)	A
(3:7) ACR-F7	0.12ml (12%)	0.18 ml (18%)	0.235 ml (23.5%)	0.465 ml (46.5%)	A
(2:8)ACR- F8	0.12ml (12%)	0.08 ml (8%)	0.27 ml (27%)	0.53 ml (53%)	A
Smix (1:0) (A)	Percentage of components (w/w)				GRADE
Oil:Smix(Code)	α -pinene	Anise oil	Tween -80	Transcutol-HP	
(9:1) ANO-A1	0.12ml (12%)	0.78ml (78%)	0.10 ml (10%)	0	E
(8:2) ANO-A2	0.12ml (12%)	0.68ml (68%)	0.20 ml (20%)	0	E
(7:3) ANO-A3	0.12ml (12%)	0.58ml (58%)	0.30 ml (30%)	0	E
(6:4) ANO-A4	0.12ml (12%)	0.48 ml (48%)	0.40 ml (40%)	0	E
(5:5) ANO-A5	0.12ml (12%)	0.38 ml (38%)	0.50 ml (50%)	0	E
(4:6)ANO- A6	0.12ml (12%)	0.28ml (28%)	0.60 ml (60%)	0	E
(3:7) ANO-A7	0.12ml (12%)	0.18 ml (18%)	0.70 ml (70%)	0	E
(2:8)ANO- A8	0.12ml (12%)	0.08 ml (8%)	0.80 ml (80%)	0	E
Smix (1:1) (B)	Percentage of components (w/w)				GRADE
(9:1) ANO-B1	0.12ml (12%)	0.78ml (78%)	0.05 ml (5%)	0.05 ml (5%)	E
(8:2) ANO-B2	0.12ml (12%)	0.68ml (68%)	0.10 ml (10%)	0.10 ml (10%)	E
(7:3) ANO-B3	0.12ml (12%)	0.58ml (58%)	0.15 ml (15%)	0.15 ml (15%)	E
(6:4) ANO-B4	0.12ml (12%)	0.48 ml (48%)	0.20 ml (20%)	0.20 ml (20%)	C
(5:5) ANO-B5	0.12ml (12%)	0.38 ml (38%)	0.25 ml (25%)	0.25 ml (25%)	C
(4:6)ANO- B6	0.12ml (12%)	0.28ml (28%)	0.30 ml (30%)	0.30 ml (30%)	B
(3:7) ANO-B7	0.12ml (12%)	0.18 ml (18%)	0.35 ml (35%)	0.35 ml (35%)	C
(2:8)ANO- B8	0.12ml (12%)	0.08 ml (8%)	0.40 ml (40%)	0.40 ml (40%)	A
Smix (2:1) (C)	Percentage of components (w/w)				GRADE
(9:1) ANO-C1	0.12ml (12%)	0.78ml (78%)	0.065 ml (6.5%)	0.035 ml (3.5%)	E
(8:2) ANO-C2	0.12ml (12%)	0.68ml (68%)	0.13 ml (13%)	0.07 ml (7%)	E
(7:3) ANO-C3	0.12ml (12%)	0.58ml (58%)	0.20 ml (20%)	0.10 ml (10%)	E
(6:4) ANO-C4	0.12ml (12%)	0.48 ml (48%)	0.265 ml (26.5%)	0.135 ml (13.5%)	E
(5:5) ANO-C5	0.12ml (12%)	0.38 ml (38%)	0.33 ml (33%)	0.17 ml (17%)	C
(4:6)ANO- C6	0.12ml (12%)	0.28ml (28%)	0.40 ml (40%)	0.20 ml (20%)	B
(3:7) ANO-C7	0.12ml (12%)	0.18 ml (18%)	0.465 ml (46.5%)	0.235 ml (23.5%)	A
(2:8)ANO- C8	0.12ml (12%)	0.08 ml (8%)	0.53 ml (53%)	0.27 ml (27%)	A
Smix (3:1) (D)	Percentage of components (w/w)				GRADE

(9:1) ANO-D1	0.12ml (12%)	0.78ml (78%)	0.075 ml (7.5%)	0.025 ml (2.5%)	E
(8:2) ANO-D2	0.12ml (12%)	0.68ml (68%)	0.15 ml (15%)	0.05 ml (5%)	E
(7:3) ANO-D3	0.12ml (12%)	0.58ml (58%)	0.225 ml (22.5%)	0.075 ml (7.5%)	E
(6:4) ANO-D4	0.12ml (12%)	0.48 ml (48%)	0.3 ml (30%)	0.1 ml (10%)	C
(5:5) ANO-D5	0.12ml (12%)	0.38 ml (38%)	0.375 ml (37.5%)	0.125 ml (12.5%)	C
(4:6)ANO- D6	0.12ml (12%)	0.28ml (28%)	0.45 ml (45%)	0.15 ml (15%)	B
(3:7) ANO-D7	0.12ml (12%)	0.18 ml (18%)	0.525 ml (52.5%)	0.175 ml (17.5%)	C
(2:8)ANO- D8	0.12ml (12%)	0.08 ml (8%)	0.60 ml (60%)	0.2 ml (20%)	A
Smix (4:1) (E)	Percentage of components (w/w)				GRADE
(9:1) ANO-E1	0.12ml (12%)	0.78ml (78%)	0.08 ml (8%)	0.02 ml (2%)	E
(8:2) ANO-E2	0.12ml (12%)	0.68ml (68%)	0.16 ml (16%)	0.04 ml (4%)	D
(7:3) ANO-E3	0.12ml (12%)	0.58ml (58%)	0.24ml (24%)	0.06ml (6%)	E
(6:4) ANO-E4	0.12ml (12%)	0.48 ml (48%)	0.32 ml (32%)	0.08 ml (8%)	E
(5:5) ANO-E5	0.12ml (12%)	0.38 ml (38%)	0.4ml (40%)	0.1 ml (10%)	E
(4:6)ANO- E6	0.12ml (12%)	0.28ml (28%)	0.48 ml (48%)	0.12 ml (12%)	E
(3:7) ANO-E7	0.12ml (12%)	0.18 ml (18%)	0.56 ml (56%)	0.14 ml (14%)	E
(2:8)ANO- E8	0.12ml (12%)	0.08 ml (8%)	0.64 ml (64%)	0.16 ml (16%)	C
Smix (1:2) (F)	Percentage of components (w/w)				GRADE
(9:1) ANO-F1	0.12ml (12%)	0.78ml (78%)	0.035 ml (3.5%)	0.065 ml (6.5%)	E
(8:2) ANO-F2	0.12ml (12%)	0.68ml (68%)	0.07 ml (7%)	0.13 ml (13%)	E
(7:3) ANO-F3	0.12ml (12%)	0.58ml (58%)	0.10 ml (10%)	0.20 ml (20%)	E
(6:4) ANO-F4	0.12ml (12%)	0.48 ml (48%)	0.135 ml (13.5%)	0.265 ml (26.5%)	E
(5:5) ANO-F5	0.12ml (12%)	0.38 ml (38%)	0.17 ml (17%)	0.33 ml (33%)	E
(4:6)ANO- F6	0.12ml (12%)	0.28ml (28%)	0.20 ml (20%)	0.40 ml (40%)	C
(3:7) ANO-F7	0.12ml (12%)	0.18 ml (18%)	0.235 ml (23.5%)	0.465 ml (46.5%)	A
(2:8)ANO- F8	0.12ml (12%)	0.08 ml (8%)	0.27 ml (27%)	0.53 ml (53%)	A
Smix (1:0) (A)	Percentage of components (w/w)				GRADE
Oil:Smix(Code)	α -pinene	Oleic Acid	Triton X-100	Transcutol-HP	
(9:1) OA-A1	0.12ml (12%)	0.78ml (78%)	0.10 ml (10%)	0	E
(8:2) OA -A2	0.12ml (12%)	0.68ml (68%)	0.20 ml (20%)	0	E
(7:3) OA -A3	0.12ml (12%)	0.58ml (58%)	0.30 ml (30%)	0	E
(6:4) OA -A4	0.12ml (12%)	0.48 ml (48%)	0.40 ml (40%)	0	E
(5:5) OA -A5	0.12ml (12%)	0.38 ml (38%)	0.50 ml (50%)	0	E
(4:6) OA -A6	0.12ml (12%)	0.28ml (28%)	0.60 ml (60%)	0	E
(3:7) OA A7	0.12ml (12%)	0.18 ml (18%)	0.70 ml (70%)	0	E
(2:8) OA -A8	0.12ml (12%)	0.08 ml (8%)	0.80 ml (80%)	0	E
Smix (1:1) (B)	Percentage of components (w/w)				GRADE
(9:1) OA-B1	0.12ml (12%)	0.78ml (78%)	0.05 ml (5%)	0.05 ml (5%)	E
(8:2) OA-B2	0.12ml (12%)	0.68ml (68%)	0.10 ml (10%)	0.10 ml (10%)	E
(7:3) OA-B3	0.12ml (12%)	0.58ml (58%)	0.15 ml (15%)	0.15 ml (15%)	E
(6:4)OA-B4	0.12ml (12%)	0.48 ml (48%)	0.20 ml (20%)	0.20 ml (20%)	C
(5:5) OA-B5	0.12ml (12%)	0.38 ml (38%)	0.25 ml (25%)	0.25 ml (25%)	C
(4:6)OA- B6	0.12ml (12%)	0.28ml (28%)	0.30 ml (30%)	0.30 ml (30%)	B

(3:7) OA-B7	0.12ml (12%)	0.18 ml (18%)	0.35 ml (35%)	0.35 ml (35%)	B
(2:8)OA- B8	0.12ml (12%)	0.08 ml (8%)	0.40 ml (40%)	0.40 ml (40%)	A
Smix (2:1) (C)	Percentage of components (w/w)				GRADE
(9:1) OA-C1	0.12ml (12%)	0.78ml (78%)	0.065 ml (6.5%)	0.035 ml (3.5%)	E
(8:2) OA-C2	0.12ml (12%)	0.68ml (68%)	0.13 ml (13%)	0.07 ml (7%)	C
(7:3) OA-C3	0.12ml (12%)	0.58ml (58%)	0.20 ml (20%)	0.10 ml (10%)	C
(6:4) OA-C4	0.12ml (12%)	0.48 ml (48%)	0.265 ml (26.5%)	0.135 ml (13.5%)	C
(5:5) OA-C5	0.12ml (12%)	0.38 ml (38%)	0.33 ml (33%)	0.17 ml (17%)	C
(4:6)OA- C6	0.12ml (12%)	0.28ml (28%)	0.40 ml (40%)	0.20 ml (20%)	B
(3:7) OA-C7	0.12ml (12%)	0.18 ml (18%)	0.465 ml (46.5%)	0.235 ml (23.5%)	A
(2:8)OA- C8	0.12ml (12%)	0.08 ml (8%)	0.53 ml (53%)	0.27 ml (27%)	A
Smix (3:1) (D)	Percentage of components (w/w)				GRADE
(9:1) OA-D1	0.12ml (12%)	0.78ml (78%)	0.075 ml (7.5%)	0.025 ml (2.5%)	E
(8:2) OA-D2	0.12ml (12%)	0.68ml (68%)	0.15 ml (15%)	0.05 ml (5%)	E
(7:3) OA-D3	0.12ml (12%)	0.58ml (58%)	0.225 ml (22.5%)	0.075 ml (7.5%)	E
(6:4) OA-D4	0.12ml (12%)	0.48 ml (48%)	0.3 ml (30%)	0.1 ml (10%)	C
(5:5) OA-D5	0.12ml (12%)	0.38 ml (38%)	0.375 ml (37.5%)	0.125 ml (12.5%)	C
(4:6)OA- D6	0.12ml (12%)	0.28ml (28%)	0.45 ml (45%)	0.15 ml (15%)	B
(3:7) OA-D7	0.12ml (12%)	0.18 ml (18%)	0.525 ml (52.5%)	0.175 ml (17.5%)	B
(2:8)OA- D8	0.12ml (12%)	0.08 ml (8%)	0.60 ml (60%)	0.2 ml (20%)	B
Smix (4:1) (E)	Percentage of components (w/w)				GRADE
(9:1) OA-E1	0.12ml (12%)	0.78ml (78%)	0.08 ml (8%)	0.02 ml (2%)	E
(8:2) OA-E2	0.12ml (12%)	0.68ml (68%)	0.16 ml (16%)	0.04 ml (4%)	D
(7:3) OA-E3	0.12ml (12%)	0.58ml (58%)	0.24ml (24%)	0.06ml (6%)	E
(6:4) OA-E4	0.12ml (12%)	0.48 ml (48%)	0.32 ml (32%)	0.08 ml (8%)	E
(5:5) OA-E5	0.12ml (12%)	0.38 ml (38%)	0.4ml (40%)	0.1 ml (10%)	E
(4:6)OA- E6	0.12ml (12%)	0.28ml (28%)	0.48 ml (48%)	0.12 ml (12%)	B
(3:7) OA-E7	0.12ml (12%)	0.18 ml (18%)	0.56 ml (56%)	0.14 ml (14%)	B
(2:8)OA- E8	0.12ml (12%)	0.08 ml (8%)	0.64 ml (64%)	0.16 ml (16%)	B
Smix (1:2) (F)	Percentage of components (w/w)				GRADE
(9:1) OA-F1	0.12ml (12%)	0.78ml (78%)	0.035 ml (3.5%)	0.065 ml (6.5%)	E
(8:2) OA-F2	0.12ml (12%)	0.68ml (68%)	0.07 ml (7%)	0.13 ml (13%)	E
(7:3) OA-F3	0.12ml (12%)	0.58ml (58%)	0.10 ml (10%)	0.20 ml (20%)	E
(6:4)OA-F4	0.12ml (12%)	0.48 ml (48%)	0.135 ml (13.5%)	0.265 ml (26.5%)	E
(5:5) OA-F5	0.12ml (12%)	0.38 ml (38%)	0.17 ml (17%)	0.33 ml (33%)	E
(4:6)OA- F6	0.12ml (12%)	0.28ml (28%)	0.20 ml (20%)	0.40 ml (40%)	C
(3:7) OA-F7	0.12ml (12%)	0.18 ml (18%)	0.235 ml (23.5%)	0.465 ml (46.5%)	B
(2:8)OA- F8	0.12ml (12%)	0.08 ml (8%)	0.27 ml (27%)	0.53 ml (53%)	B

Optimization of SNEDDS formulations by thermodynamic stability and self-Nano emulsification tests.

The qualitative results of these studies are presented in Table 7, 8 and 9. Eight formulations from ACR group, five from ANO and six from OA group (Table 4) was passed the

test. These formulations were survived at all steps of thermodynamic stability tests were found to be stable and with no sign of phase separation, turbidity or drug precipitation was observed. The instability in the formulations may be due to the coagulation of the internal phase which led to phase separation and turbidity.

Table 4: Thermodynamic stability results of α -pinene SNEDDS containing Acrysol EL-135 [(ACR) as carrier oil, Triton X-100 as surfactant and Transcutol-HP as co-surfactant, Anise oil (ANO) as carrier oil, Tween 80 as surfactant and Transcutol-HP as co-surfactant and Oleic Acid (AO) as carrier oil, Triton X-100 as surfactant and Transcutol-HP as co-surfactant.

Smix (1:1) (B)	GRADE	Centrifugation study	Heating and cooling cycle	Freeze-thaw cycle	Inference
Oil:Smix(Code)					
(6:4) ACR-B4	B	Failed	Failed	Failed	Failed
(5:5) ACR-B5	B	Failed	Failed	Failed	Failed
(4:6) ACR- B6	A	Passed	Passed	Passed	Passed
(3:7) ACR-B7	B	Failed	Failed	Failed	Failed
(2:8) ACR- B8	A	Failed	Failed	Failed	Failed
Smix (2:1) (C)					
(5:5) ACR-C5	A	Passed	Passed	Passed	Passed
(4:6) ACR- C6	A	Failed	Failed	Failed	Failed
(3:7) ACR-C7	A	Failed	Failed	Failed	Failed
(2:8) ACR- C8	A	Passed	Passed	Passed	Passed
Smix (3:1) (D)					
(5:5) ACR-D5	A	Passed	Passed	Passed	Passed
(4:6) ACR- D6	A	Failed	Failed	Failed	Failed
(3:7) ACR-D7	A	Failed	Failed	Failed	Failed
(2:8) ACR- D8	A	Passed	Passed	Passed	Passed
Smix (1:2) (F)					
(5:5) ACR-F5	A	Passed	Passed	Passed	Passed
(4:6) ACR- F6	A	Failed	Failed	Failed	Failed
(3:7) ACR-F7	A	Passed	Passed	Passed	Passed
(2:8) ACR- F8	A	Passed	Passed	Passed	Passed
Smix (1:1) (B)	GRADE	Centrifugation study	Heating and cooling cycle	Freeze-thaw cycle	Inference
Oil:Smix(Code)					
(4:6) ANO- B6	B	Failed	Failed	Failed	Failed
(2:8) ANO- B8	A	Failed	Failed	Failed	Failed
Smix (2:1) (C)					
(4:6) ANO- C6	B	Passed	Passed	Passed	Passed
(3:7) ANO-C7	A	Passed	Passed	Passed	Passed
(2:8) ANO- C8	A	Passed	Passed	Passed	Passed
Smix (3:1) (D)					
(4:6) ANO- D6	B	Failed	Failed	Failed	Failed
(2:8) ANO- D8	A	Failed	Failed	Failed	Failed
Smix (1:2) (F)					
(3:7) ANO-F7	A	Passed	Passed	Passed	Passed
(2:8) ANO- F8	A	Passed	Passed	Passed	Passed
Smix (1:1) (B)	GRADE	Centrifugation study	Heating and cooling cycle	Freeze-thaw cycle	Inference
Oil:Smix(Code)					
(4:6) OA- B6	B	Failed	Failed	Failed	Failed
(3:7) OA-B7	B	Passed	Passed	Passed	Passed
(2:8) OA- B8	A	Failed	Failed	Failed	Failed
Smix (2:1) (C)					
(4:6) OA- C6	B	Failed	Failed	Failed	Failed
(3:7) OA-C7	A	Passed	Passed	Passed	Passed
(2:8) OA- C8	A	Passed	Passed	Passed	Passed
Smix (3:1) (D)					
(4:6) OA- D6	B	Failed	Failed	Failed	Failed
(3:7) OA-D7	B	Failed	Failed	Failed	Failed
(2:8) OA- D8	B	Passed	Passed	Passed	Passed
Smix (4:1) (E)					
(4:6) OA- E6	B	Failed	Failed	Failed	Failed
(3:7) OA-E7	B	Failed	Failed	Failed	Failed
(2:8) OA- E8	B	Passed	Passed	Passed	Passed
Smix (1:2) (F)					
(3:7) OA-F7	B	Failed	Failed	Failed	Failed
(2:8) OA- F8	B	Passed	Passed	Passed	Passed

Table 5: Optical clarity of α -pinene SNEDDS containing Acrysol EL-135 (ACR), Anise oil (ANO) and Oleic Acid (AO) at different dilution

Smix (1:1) (B)	Percentage of components (w/w)				Transmittance	Transmittance	Transmittance
Oil:Smix(Code)	α -pinene	Acrysol EL-135	Triton X-100	Transcutol-HP	1:50	1:100	1:1000
(4:6)ACR- B6	0.12ml (12%)	0.28ml (28%)	0.30 ml (30%)	0.30 ml (30%)	99.9	99.8	98.66
Smix (2:1) (C)							
(5:5) ACR-C5	0.12ml (12%)	0.38 ml (38%)	0.33 ml (33%)	0.17 ml (17%)	98.7	99.8	100.0
(2:8)ACR- C8	0.12ml (12%)	0.08 ml (8%)	0.53 ml (53%)	0.27 ml (27%)	99.5	98.8	99.54
Smix (3:1) (D)							
(5:5) ACR-D5	0.12ml (12%)	0.38 ml (38%)	0.375 ml (37.5%)	0.125 ml (12.5%)	98.4	99.1	99.78
(2:8)ACR- D8	0.12ml (12%)	0.08 ml (8%)	0.60 ml (60%)	0.2 ml (20%)	97.5	98.5	100.08
Smix (1:2) (F)							
(5:5) ACR-F5	0.12ml (12%)	0.38 ml (38%)	0.17 ml (17%)	0.33 ml (33%)	98.2	99.1	100.5
(3:7) ACR-F7	0.12ml (12%)	0.18 ml (18%)	0.235 ml (23.5%)	0.465 ml (46.5%)	97.5	98.2	100.6
(2:8)ACR- F8	0.12ml (12%)	0.08 ml (8%)	0.27 ml (27%)	0.53 ml (53%)	30.8	80.5	92.35
Smix (2:1) (C)	Percentage of components (w/w)				Transmittance	Transmittance	Transmittance
Oil:Smix(Code)	α -pinene	Anise oil	Tween 80	Transcutol-HP	1:50	1:100	1:1000
(4:6)ANO- C6	0.12ml (12%)	0.28ml (28%)	0.40 ml (40%)	0.20 ml (20%)	84.3	96.2	98.66
(3:7) ANO-C7	0.12ml (12%)	0.18 ml (18%)	0.465 ml (46.5%)	0.235 ml (23.5%)	88.3	96.8	98.45
(2:8)ANO- C8	0.12ml (12%)	0.08 ml (8%)	0.53 ml (53%)	0.27 ml (27%)	96.5	98.9	99.25
Smix (1:2) (F)							
(3:7) ANO-F7	0.12ml (12%)	0.18 ml (18%)	0.235 ml (23.5%)	0.465 ml (46.5%)	97.6	98.8	99.58
(2:8)ANO- F8	0.12ml (12%)	0.08 ml (8%)	0.27 ml (27%)	0.53 ml (53%)	98.6	99.2	100.1
Smix (1:1) (B)	Percentage of components (w/w)				Transmittance	Transmittance	Transmittance
Oil:Smix(Code)	α -pinene	Oleic Acid	Triton X-100	Transcutol-HP	1:50	1:100	1:1000
(3:7) OA-B7	0.12ml (12%)	0.18 ml (18%)	0.35 ml (35%)	0.35 ml (35%)	7.4	28.2	80.68
Smix (2:1) (C)							
(3:7) OA-C7	0.12ml (12%)	0.18 ml (18%)	0.465 ml (46.5%)	0.235 ml (23.5%)	8.9	35.1	80.2
(2:8)OA- C8	0.12ml (12%)	0.08 ml (8%)	0.53 ml (53%)	0.27 ml (27%)	28.6	60.8	85.2
Smix (3:1) (D)							
(2:8)OA- D8	0.12ml (12%)	0.08 ml (8%)	0.60 ml (60%)	0.2 ml (20%)	34.3	75.3	97.2
Smix (4:1) (E)							
(2:8)OA- E8	0.12ml (12%)	0.08 ml (8%)	0.64 ml (64%)	0.16 ml (16%)	36.0	78.2	97.32
Smix (1:2) (F)							
(2:8)OA- F8	0.12ml (12%)	0.08 ml (8%)	0.27 ml (27%)	0.53 ml (53%)	6.0	24.2	85.8

Transmittance was measured at 638nm using spectrophotometer

DISCUSSION:

The solubility of therapeutic drug molecules in different components is the main criteria for their selection. In this work, we have not used any solid crystalline drug molecule, solubility studies were not performed. Moreover, alpha pinene used in this study is easily miscible in oils and surfactants. Nevertheless, different components were selected on the basis of safety and nontoxicity of oil phase, surfactant and co-surfactant in this work. Based on these criteria, Anise oil, Acrysol, Oleic acid as oil phase, Triton-X100 and Tween-80 as surfactant and Tarnscutol-HP was selected as co-surfactant for the development of ALP-SNEDDS. Poor water solubility and consequent restricted absorption is a major limitation with many drugs despite their good efficacy. SNEDDS provides an opportunity for the improvement of the in vitro and in vivo performance of poorly water-soluble bioactive drugs and thus serves as an ideal carrier for the delivery of drugs¹⁷. The current study was performed to define the role of self-nanoemulsifying formulations to enhance the solubility and bioavailability of the neuroprotective drug alpha pinene. In the thermodynamic stability tests prolonged storage might cause the drug to precipitate from the nanoemulsion; seed crystals start to appear and might grow to large crystalline materials that will precipitate out at the bottom of the vessel¹⁸. Therefore, to check the stability, formulation was exposed to centrifugation study, heating and cooling cycle and freeze thawing cycle in order to eliminate the metastable ones. All the formulations containing oleic acid showed phase separation as well as turbidity, which may be due to the coagulation of the internal phase which led to phase separation^{19,20}. The stable formulations were further tested for robustness on dilution (Table 5). Uniform emulsion formation from SNEDDS is very important at different dilutions because drugs may precipitate at higher dilution in vivo which affects the drug absorption significantly^{21,22}.

The main difference between emulsions and nanoemulsions is kinetic stability, reflecting the thermodynamic stability of the two systems. SNEDDS undergoes in situ solubilization to form nanoemulsion system, and it should have stability such that it does not undergo any sort of precipitation, creaming or cracking and phase separation. However, in many cases, prolonged storage might cause the drug to precipitate from the nanoemulsion; clumps start to appear and might grow to large materials that will precipitate out at the bottom of the vessel. Therefore, to check the stability, formulation was exposed to centrifugation study, heating and cooling cycle and freeze thawing cycle in order to eliminate the metastable ones. The primary objective of thermodynamic stability tests was to remove any metastable/unstable SNEDDS because observations made during self-emulsification studies were made visually. Hence, selected SNEDDS were subjected to different thermodynamic stability tests. In the designing of colloidal drug-carrier formulations, the study of the rate at which the drug is released from the vehicle is particularly important, because it can be used as quality control data to predict in vivo behaviour, or to study the structure and release mechanism of the system²³⁻²⁴. The drug release from small droplets will be faster and more frequent than from the large oil droplets²⁵. The faster drug release of SNEDDS is attributed to the spontaneous formation of nanoemulsion due to low surface free energy at oil-water interface, which causes immediate solubilization of drug in the release medium which causes immediate solubilization of drug in the release medium²⁶. During emulsification with water, the nanoemulsion components (oil, surfactant and co-surfactant) effectively swell and decrease the globule size, which leads to increase

in surface area and decrease of surface free energy, thus eventually, increases the drug release rate.

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