

## Supplementary Information

for

### Optimization of Surfactant and Co-Surfactant aided Pine Oil Nanoemulsion by Isothermal Low-Energy Methods for Anticholinesterase activity

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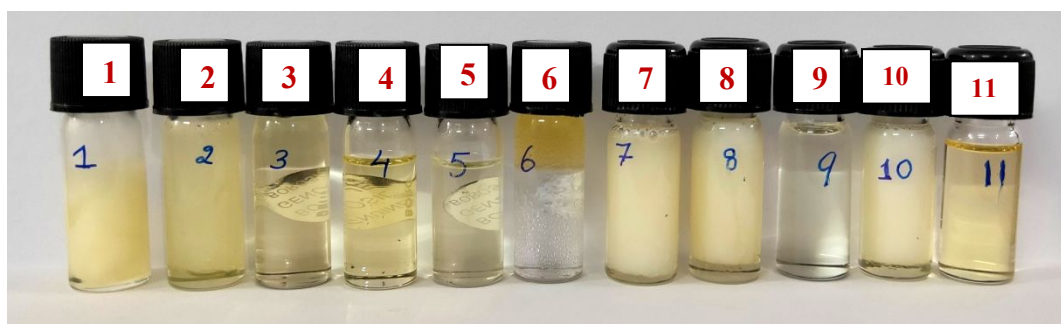
#### Miscibility studies

Pine oil when added in equivalent ratio of 1:1 with Tween 60 and 80 provides clear solution. But when same equivalent ratio added to Tween 20 and 40 then translucent solution was observed. Clear transparent solutions observed with ethanol and methanol when equivalent ratio of pine oil was added. With water, biphasic observation was when equivalent ratio of pine oil added. Polyethylene glycol (PEG) series was also assessed for miscibility studies in which PEG 200, 300 and 600 were observed with translucent appearance when mixed with pine oil, except PEG 400.

**Table S1:** Miscibility data of Pine oil with various solvents and observation

S. No.	Solvent	Observation
1	Tween 20	Translucent
2	Tween 40	Translucent
3	Tween 60	Transparent
4	Tween 80	Transparent
5	Ethanol	Transparent
6	Water	Biphasic
7	Polyethylene glycol 200	Translucent
8	Polyethylene glycol 300	Translucent
9	Polyethylene glycol 400	Transparent

10	Polyethylene glycol 600	Translucent
11	Methanol	Transparent



On the basis of miscibility data, we inferred that Tween 20 and 40 were not appropriate surfactants for the nanoemulsion formulation by using spontaneous emulsification process. Selection of Tween 60 and 80 was done for further emulsification using titration method, while ethanol and PEG 400 as cosurfactant as per miscibility studies.

### Emulsion preparation

Pseudoternary phase diagrams were analysed to determine nanoemulsion region using spontaneous emulsification process. Surfactant mixture of Tween 60 with ethanol and PEG 400, while Tween 80 with ethanol and PEG 400 in varying ratios were prepared. Table S2 summarizes the ratio of surfactant mixture employed for preparation of pseudoternary phase diagram.

**Table S2:** Pseudoternary phase diagram code and ratio of surfactant and co-surfactant used

S. No.	PPD Code	Surfactant	Co-surfactant	Ratio
1.	PPD 1	Tween 60	Ethanol	1:1
2.	PPD 2	Tween 60	Ethanol	1:2
3.	PPD 3	Tween 60	Ethanol	2:1
4.	PPD 4	Tween 60	PEG 400	1:1
5.	PPD 5	Tween 60	PEG 400	1:2
6.	PPD 6	Tween 60	PEG 400	2:1
7.	PPD 7	Tween 80	Ethanol	1:1
8.	PPD 8	Tween 80	Ethanol	1:2
9.	PPD 9	Tween 80	Ethanol	2:1
10.	PPD 10	Tween 80	PEG 400	1:1
11.	PPD 11	Tween 80	PEG 400	1:2
12.	PPD 12	Tween 80	PEG 400	2:1

In a vial a set amount of pine oil, surfactant mixture was added and followed by volume of water left to make total 100%. Table S3 summarizes the code provided to observation after final amount of water was added.

**Table S3:** Code for observation after addition of final amount of water

Code	Observation
1	Nanoemulsion
2	Turbidity
3	Biphasic

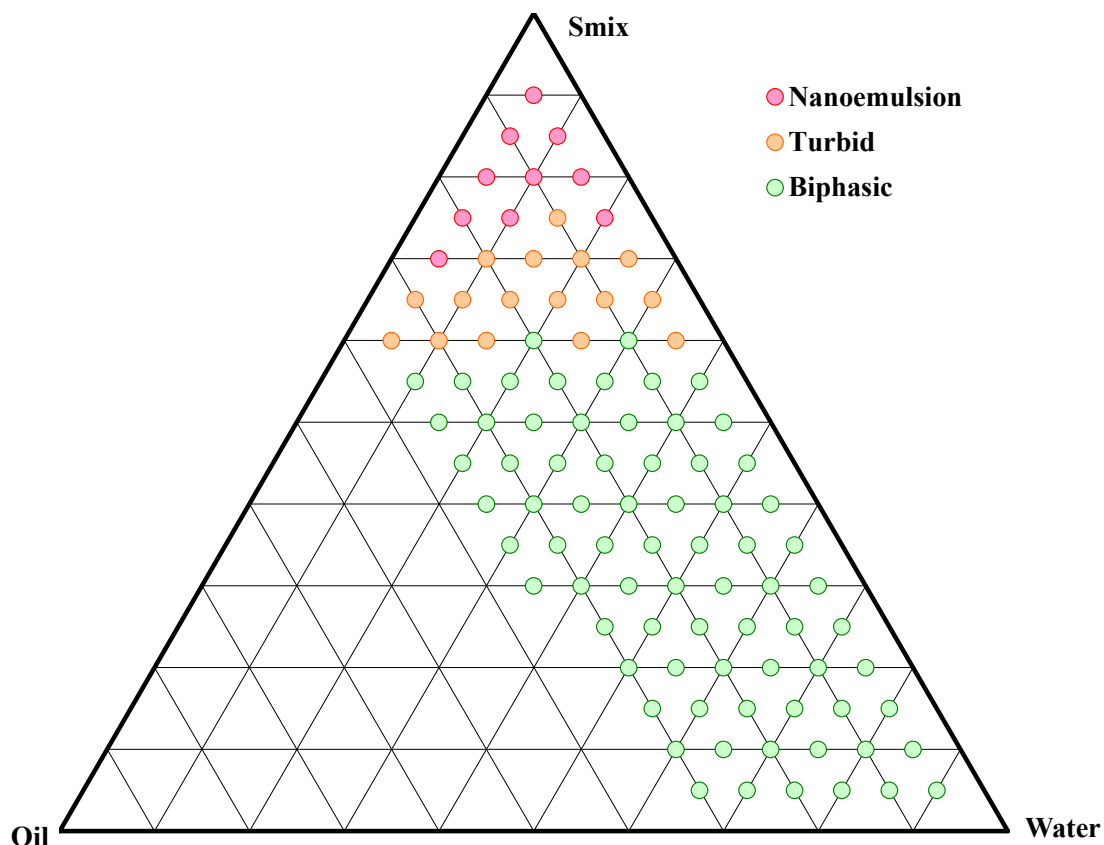
With Tween 60 and PEG 400 as well as Ethanol not much promising results were obtained and in pseudoternary phase diagram not much nanoemulsion regions were obtained.

**Table S4:** Trials of varying concentration of Smix (Tween 80: PEG 400 :: 1:1) with varying concentration of water

Oil (%v/v)	Water (%v/v)	Smix (%v/v)	Observation
5	5	90	1
5	10	85	1
5	15	80	1
5	20	75	1
5	25	70	2
5	30	65	2
5	35	60	2
5	40	55	3
5	45	50	3
5	50	45	3
5	55	40	3
5	60	35	3
5	65	30	3
5	70	25	3
5	75	20	3
5	80	15	3
5	85	10	3
5	90	5	3
10	5	85	1
10	10	80	1
10	15	75	2
10	20	70	2
10	25	65	2
10	30	60	3
10	35	55	3
10	40	50	3
10	45	45	3
10	50	40	3
10	55	35	3
10	60	30	3
10	65	25	3

10	70	20	3
10	75	15	3
10	80	10	3
10	85	5	3
15	5	80	1
15	10	75	1
15	15	70	2
15	20	65	2
15	25	60	2
15	30	55	3
15	35	50	3
15	40	45	3
15	45	40	3
15	50	35	3
15	55	30	3
15	60	25	3
15	65	20	3
15	70	15	3
15	75	10	3
15	80	5	3
20	5	75	1
20	10	70	2
20	15	65	2
20	20	60	3
20	25	55	3
20	30	50	3
20	35	45	3
20	40	40	3
20	45	35	3
20	50	30	3
20	55	25	3
20	60	20	3
20	65	15	3
20	70	10	3
20	75	5	3
25	5	70	1
25	10	65	2
25	15	60	2
25	20	55	3
25	25	50	3
25	30	45	3
25	35	40	3
25	40	35	3

25	45	30	3
25	50	25	3
25	55	20	3
25	60	15	3
25	65	10	3
25	70	5	3
30	5	65	2
30	10	60	2
30	15	55	3
30	20	50	3
30	25	45	3
30	30	40	3
30	35	35	3
30	40	30	3
30	45	25	3
30	50	20	3
30	55	15	3
30	60	10	3
30	65	5	3
35	5	60	2
35	10	55	3
35	15	50	3
35	20	45	3
35	25	40	3
35	30	35	3
35	35	30	3
35	40	25	3
35	45	20	3
35	50	15	3
35	55	10	3
35	60	5	3



**Figure S1:** Pseudoternary phase diagram of PPD 10 representing nanoemulsion, turbid and biphasic region of obtained points

From figure S1, it was analysed that very few nanoemulsion regions were observed. High concentration of surfactant mixture was required to obtain nanoemulsion region. So from PPD 10, it was observed that Tween 80 and PEG 400 in ratio 1:1 was not acceptable for further studies. To counterfeit these trials further PPD 11 and PPD 12 were obtained by varying ratio of Tween 80 and PEG 400 with 1:2 and vice versa.

**Table S5:** Trials of varying concentration of Smix (Tween 80: PEG 400 :: 1:2) with varying concentration of water

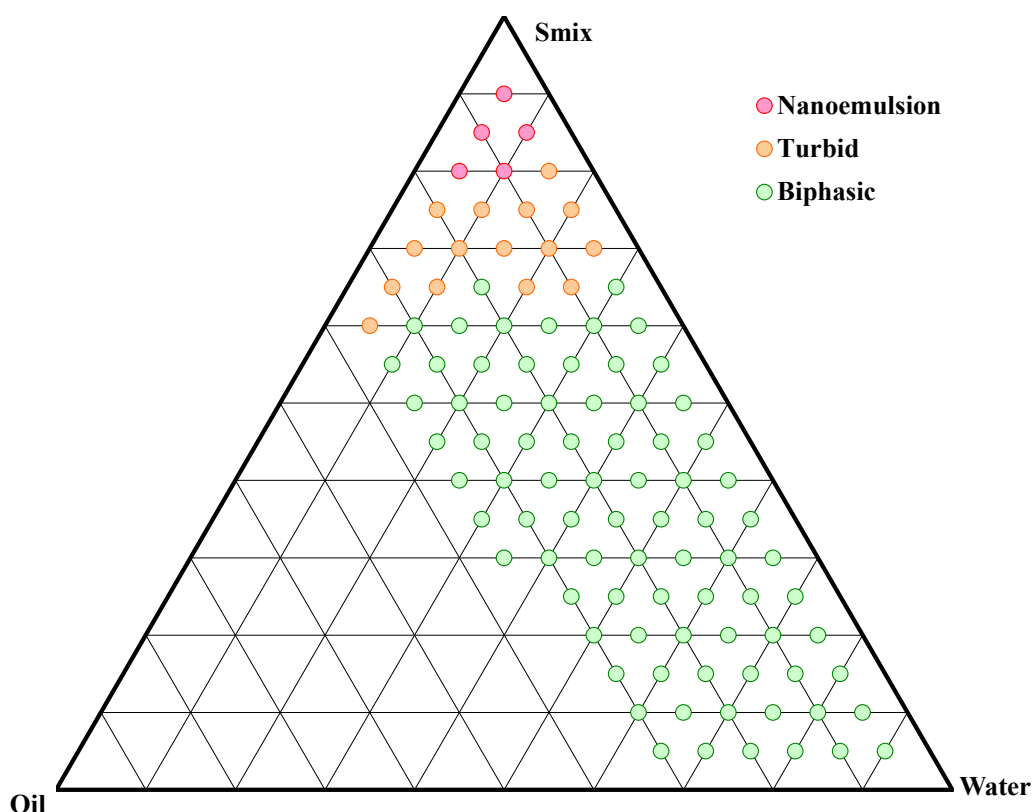
Oil (%v/v)	Water (%v/v)	Smix (%v/v)	Observation
5	5	90	1
5	10	85	1
5	15	80	2
5	20	75	2
5	25	70	2
5	30	65	3
5	35	60	3
5	40	55	3
5	45	50	3
5	50	45	3

5	55	40	3
5	60	35	3
5	65	30	3
5	70	25	3
5	75	20	3
5	80	15	3
5	85	10	3
5	90	5	3
10	5	85	1
10	10	80	1
10	15	75	2
10	20	70	2
10	25	65	2
10	30	60	3
10	35	55	3
10	40	50	3
10	45	45	3
10	50	40	3
10	55	35	3
10	60	30	3
10	65	25	3
10	70	20	3
10	75	15	3
10	80	10	3
10	85	5	3
15	5	80	1
15	10	75	2
15	15	70	2
15	20	65	2
15	25	60	3
15	30	55	3
15	35	50	3
15	40	45	3
15	45	40	3
15	50	35	3
15	55	30	3
15	60	25	3
15	65	20	3
15	70	15	3
15	75	10	3
15	80	5	3
20	5	75	2
20	10	70	2

20	15	65	3
20	20	60	3
20	25	55	3
20	30	50	3
20	35	45	3
20	40	40	3
20	45	35	3
20	50	30	3
20	55	25	3
20	60	20	3
20	65	15	3
20	70	10	3
20	75	5	3
25	5	70	2
25	10	65	2
25	15	60	3
25	20	55	3
25	25	50	3
25	30	45	3
25	35	40	3
25	40	35	3
25	45	30	3
25	50	25	3
25	55	20	3
25	60	15	3
25	65	10	3
25	70	5	3
30	5	65	2
30	10	60	3
30	15	55	3
30	20	50	3
30	25	45	3
30	30	40	3
30	35	35	3
30	40	30	3
30	45	25	3
30	50	20	3
30	55	15	3
30	60	10	3
30	65	5	3
35	5	60	2
35	10	55	3
35	15	50	3



35	20	45	3
35	25	40	3
35	30	35	3
35	35	30	3
35	40	25	3
35	45	20	3
35	50	15	3
35	55	10	3
35	60	5	3



**Figure S2:** Pseudoternary phase diagram of PPD 11 representing nanoemulsion, turbid and biphasic region of obtained points

From figure S2, it was analysed that very few nanoemulsion regions were observed. High concentration of surfactant mixture was required to obtain nanoemulsion region. So from PPD 11, it was observed that Tween 80 and PEG 400 in ratio 1:2 was not acceptable for further studies.

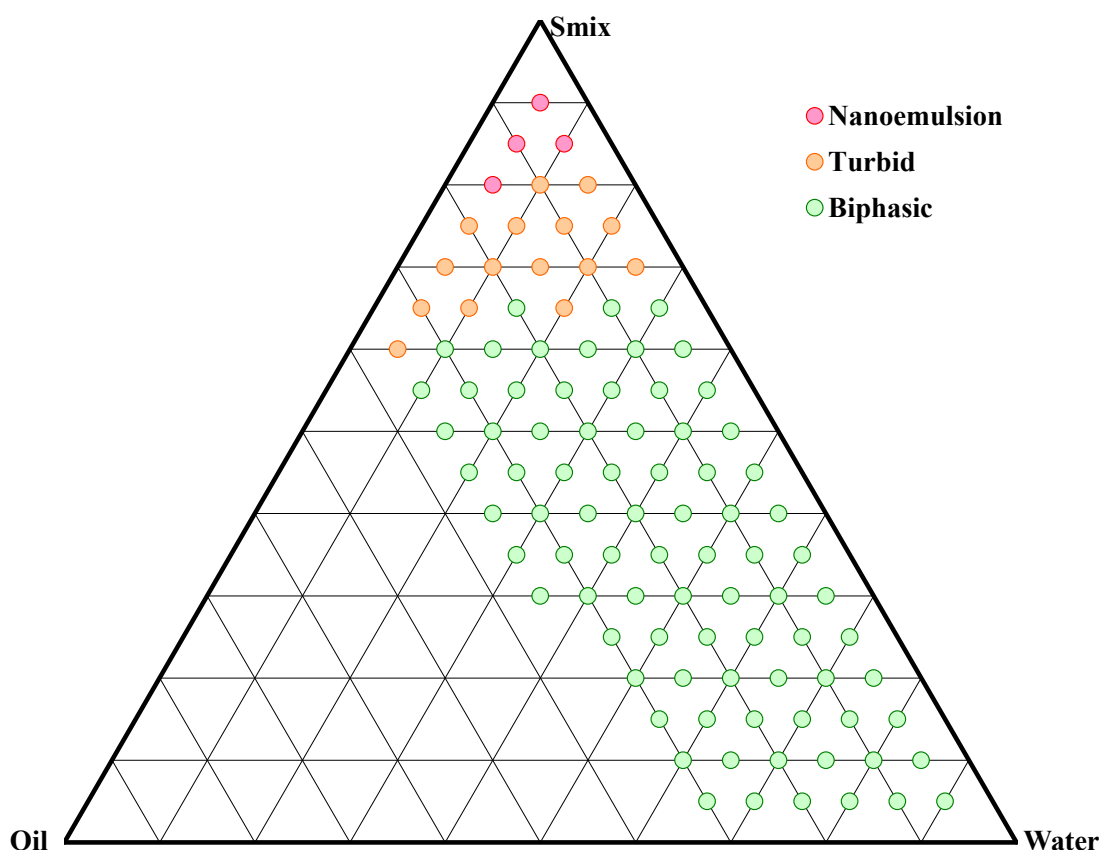
**Table S6:** Trials of varying concentration of Smix (Tween 80: PEG 400 :: 2:1) with varying concentration of water

Oil (%v/v)	Water (%v/v)	Smix (%v/v)	Observation
5	5	90	1
5	10	85	1

5	15	80	2
5	20	75	2
5	25	70	2
5	30	65	3
5	35	60	3
5	40	55	3
5	45	50	3
5	50	45	3
5	55	40	3
5	60	35	3
5	65	30	3
5	70	25	3
5	75	20	3
5	80	15	3
5	85	10	3
5	90	5	3
10	5	85	1
10	10	80	2
10	15	75	2
10	20	70	2
10	25	65	3
10	30	60	3
10	35	55	3
10	40	50	3
10	45	45	3
10	50	40	3
10	55	35	3
10	60	30	3
10	65	25	3
10	70	20	3
10	75	15	3
10	80	10	3
10	85	5	3
15	5	80	1
15	10	75	2
15	15	70	2
15	20	65	2
15	25	60	3
15	30	55	3
15	35	50	3
15	40	45	3
15	45	40	3
15	50	35	3
15	55	30	3
15	60	25	3
15	65	20	3
15	70	15	3
15	75	10	3

15	80	5	3
20	5	75	2
20	10	70	2
20	15	65	3
20	20	60	3
20	25	55	3
20	30	50	3
20	35	45	3
20	40	40	3
20	45	35	3
20	50	30	3
20	55	25	3
20	60	20	3
20	65	15	3
20	70	10	3
20	75	5	3
25	5	70	2
25	10	65	2
25	15	60	3
25	20	55	3
25	25	50	3
25	30	45	3
25	35	40	3
25	40	35	3
25	45	30	3
25	50	25	3
25	55	20	3
25	60	15	3
25	65	10	3
25	70	5	3
30	5	65	2
30	10	60	3
30	15	55	3
30	20	50	3
30	25	45	3
30	30	40	3
30	35	35	3
30	40	30	3
30	45	25	3
30	50	20	3
30	55	15	3
30	60	10	3
30	65	5	3
35	5	60	2
35	10	55	3
35	15	50	3
35	20	45	3
35	25	40	3

35	30	35	3
35	35	30	3
35	40	25	3
35	45	20	3
35	50	15	3
35	55	10	3
35	60	5	3



**Figure S3:** Pseudoternary phase diagram of PPD 12 representing nanoemulsion, turbid and biphasic region of obtained points

From figure S3, it was analysed that very few nanoemulsion regions were observed. High concentration of surfactant mixture was required to obtain nanoemulsion region. So from PPD 12, it was observed that Tween 80 and PEG 400 in ratio 2:1 was not acceptable for further studies.

Promising results were not obtained from pseudoternary phase diagram of PPD 10, 11 and 12. So there was need to change the co-surfactant system. Ethanol was used instead of PEG 400. By using ethanol as cosurfactant PPD 7, 8 and 9 was obtained and discussed as below.

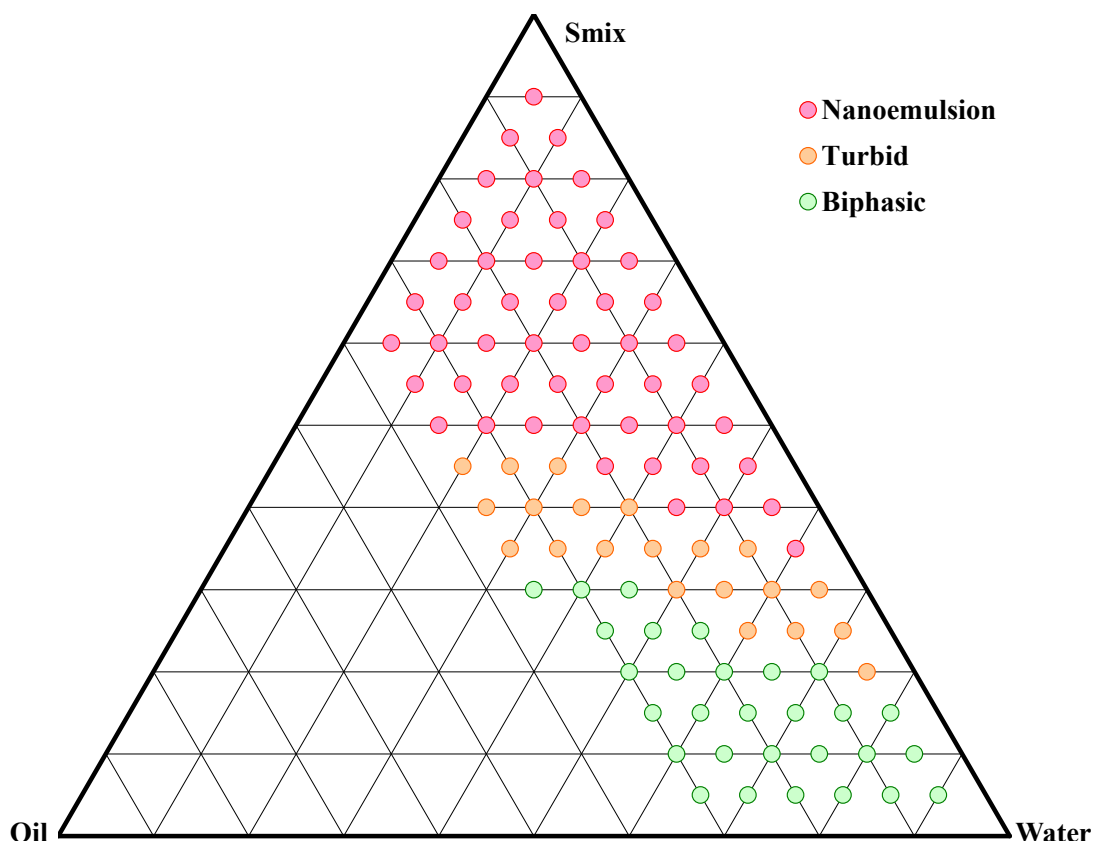
**Table S7:** Trials of varying concentration of Smix (Tween 80: Ethanol :: 1:1) with varying concentration of water

Oil (%v/v)	Water (%v/v)	Smix (%v/v)	Observation
5	5	90	1

5	10	85	1
5	15	80	1
5	20	75	1
5	25	70	1
5	30	65	1
5	35	60	1
5	40	55	1
5	45	50	1
5	50	45	1
5	55	40	1
5	60	35	1
5	65	30	2
5	70	25	2
5	75	20	2
5	80	15	3
5	85	10	3
5	90	5	3
10	5	85	1
10	10	80	1
10	15	75	1
10	20	70	1
10	25	65	1
10	30	60	1
10	35	55	1
10	40	50	1
10	45	45	1
10	50	40	1
10	55	35	2
10	60	30	2
10	65	25	2
10	70	20	3
10	75	15	3
10	80	10	3
10	85	5	3
15	5	80	1
15	10	75	1
15	15	70	1
15	20	65	1
15	25	60	1
15	30	55	1
15	35	50	1
15	40	45	1
15	45	40	1
15	50	35	2
15	55	30	2
15	60	25	2
15	65	20	3
15	70	15	3
15	75	10	3
15	80	5	3
20	5	75	1
20	10	70	1

20	15	65	1
20	20	60	1
20	25	55	1
20	30	50	1
20	35	45	1
20	40	40	2
20	45	35	2
20	50	30	2
20	55	25	3
20	60	20	3
20	65	15	3
20	70	10	3
20	75	5	3
25	5	70	1
25	10	65	1
25	15	60	1
25	20	55	1
25	25	50	1
25	30	45	2
25	35	40	2
25	40	35	2
25	45	30	3
25	50	25	3
25	55	20	3
25	60	15	3
25	65	10	3
25	70	5	3
30	5	65	1
30	10	60	1
30	15	55	1
30	20	50	1
30	25	45	2
30	30	40	2
30	35	35	2
30	40	30	3
30	45	25	3
30	50	20	3
30	55	15	3
30	60	10	3
30	65	5	3
35	5	60	1
35	10	55	1
35	15	50	1
35	20	45	2
35	25	40	2
35	30	35	2
35	35	30	3
35	40	25	3
35	45	20	3
35	50	15	3

35	55	10	3
35	60	5	3



**Figure S4:** Pseudoternary phase diagram of PPD 7 representing nanoemulsion, turbid and biphasic region of obtained points

As per PPD 7, promising results were obtained but to assess further effect of surfactant and co surfactant ratio on the nanoemulsion regions, PPD 8 and 9 were evaluated by varying the concentration of Tween 80 and ethanol.

**Table S8:** Trials of varying concentration of Smix (Tween 80: Ethanol :: 1:2) with varying concentration of water

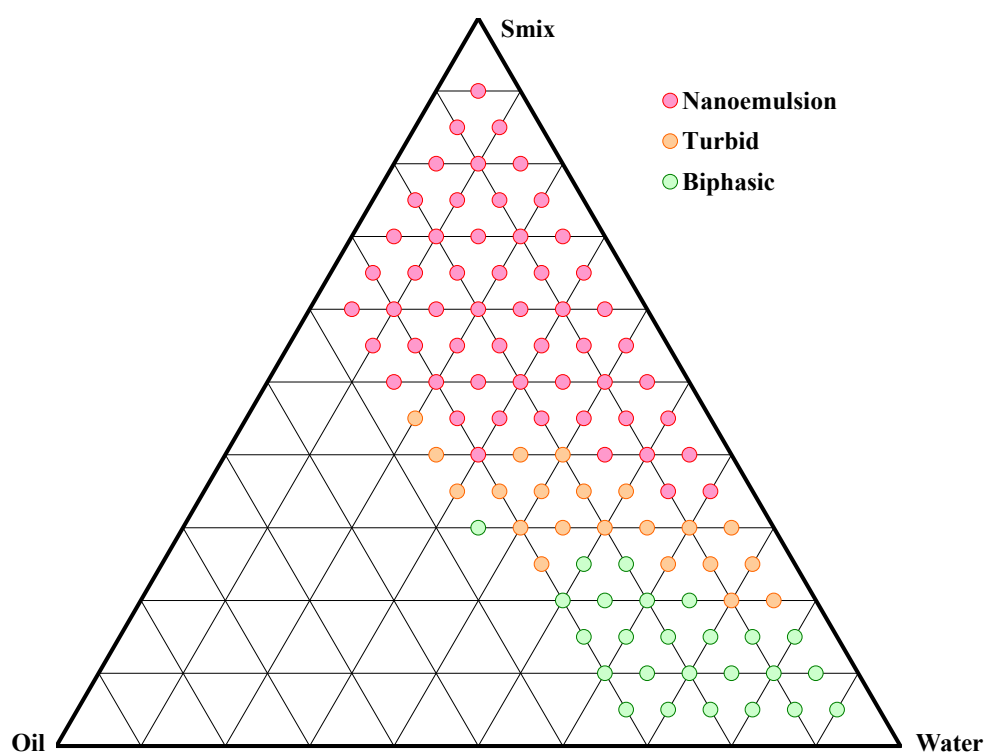
Oil (%v/v)	Water (%v/v)	Smix (%v/v)	Observation
5	5	90	1
5	10	85	1
5	15	80	1
5	20	75	1
5	25	70	1
5	30	65	1
5	35	60	1
5	40	55	1
5	45	50	1

5	50	45	1
5	55	40	1
5	60	35	1
5	65	30	2
5	70	25	2
5	75	20	2
5	80	15	3
5	85	10	3
5	90	5	3
10	5	85	1
10	10	80	1
10	15	75	1
10	20	70	1
10	25	65	1
10	30	60	1
10	35	55	1
10	40	50	1
10	45	45	1
10	50	40	1
10	55	35	1
10	60	30	2
10	65	25	2
10	70	20	2
10	75	15	3
10	80	10	3
10	85	5	3
15	5	80	1
15	10	75	1
15	15	70	1
15	20	65	1
15	25	60	1
15	30	55	1
15	35	50	1
15	40	45	1
15	45	40	1
15	50	35	2
15	55	30	2
15	60	25	2
15	65	20	3
15	70	15	3
15	75	10	3
15	80	5	3
20	5	75	1



20	10	70	1
20	15	65	1
20	20	60	1
20	25	55	1
20	30	50	1
20	35	45	1
20	40	40	2
20	45	35	2
20	50	30	2
20	55	25	3
20	60	20	3
20	65	15	3
20	70	10	3
20	75	5	3
25	5	70	1
25	10	65	1
25	15	60	1
25	20	55	1
25	25	50	1
25	30	45	1
25	35	40	2
25	40	35	2
25	45	30	2
25	50	25	3
25	55	20	3
25	60	15	3
25	65	10	3
25	70	5	3
30	5	65	1
30	10	60	1
30	15	55	1
30	20	50	1
30	25	45	1
30	30	40	1
30	35	35	2
30	40	30	2
30	45	25	2
30	50	20	3
30	55	15	3
30	60	10	3
30	65	5	3
35	5	60	1
35	10	55	1

35	15	50	1
35	20	45	2
35	25	40	2
35	30	35	2
35	35	30	3
35	40	25	3
35	45	20	3
35	50	15	3
35	55	10	3
35	60	5	3



**Figure S5:** Pseudoternary phase diagram of PPD 8 representing nanoemulsion, turbid and biphasic region of obtained points

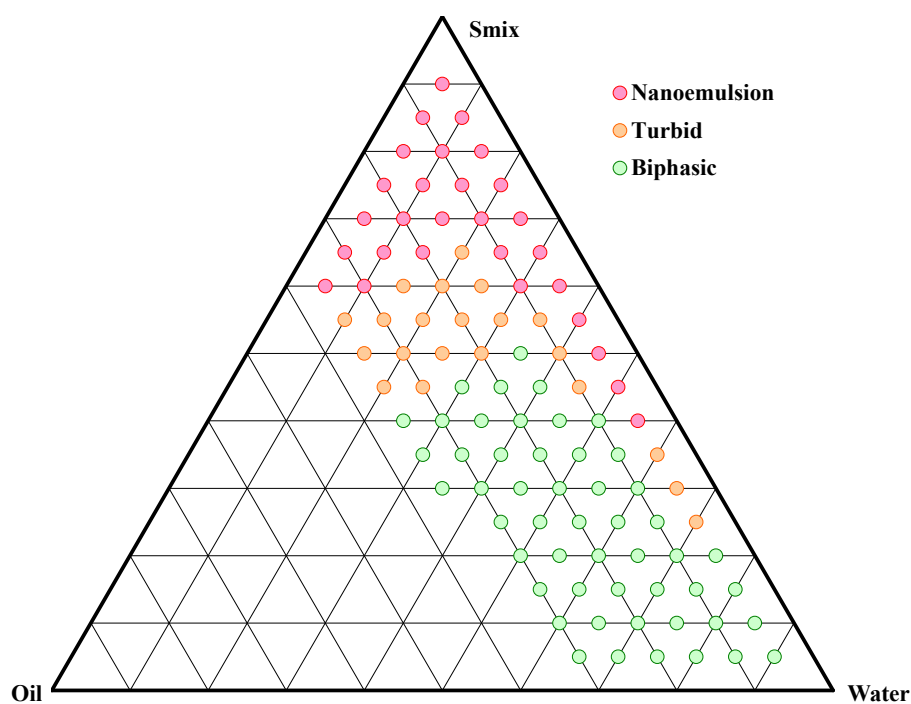
**Table S9:** Trials of varying concentration of Smix (Tween 80: Ethanol :: 2:1) with varying concentration of water

Oil (%v/v)	Water (%v/v)	Smix (%v/v)	Observation
5	5	90	1
5	10	85	1
5	15	80	1
5	20	75	1
5	25	70	1
5	30	65	1

5	35	60	1
5	40	55	1
5	45	50	1
5	50	45	1
5	55	40	1
5	60	35	2
5	65	30	2
5	70	25	2
5	75	20	3
5	80	15	3
5	85	10	3
5	90	5	3
10	5	85	1
10	10	80	1
10	15	75	1
10	20	70	1
10	25	65	1
10	30	60	1
10	35	55	2
10	40	50	2
10	45	45	2
10	50	40	3
10	55	35	3
10	60	30	3
10	65	25	3
10	70	20	3
10	75	15	3
10	80	10	3
10	85	5	3
15	5	80	1
15	10	75	1
15	15	70	1
15	20	65	2
15	25	60	2
15	30	55	2
15	35	50	3
15	40	45	3
15	45	40	3
15	50	35	3
15	55	30	3
15	60	25	3
15	65	20	3
15	70	15	3

15	75	10	3
15	80	5	3
20	5	75	1
20	10	70	1
20	15	65	1
20	20	60	2
20	25	55	2
20	30	50	2
20	35	45	3
20	40	40	3
20	45	35	3
20	50	30	3
20	55	25	3
20	60	20	3
20	65	15	3
20	70	10	3
20	75	5	3
25	5	70	1
25	10	65	1
25	15	60	2
25	20	55	2
25	25	50	2
25	30	45	3
25	35	40	3
25	40	35	3
25	45	30	3
25	50	25	3
25	55	20	3
25	60	15	3
25	65	10	3
25	70	5	3
30	5	65	1
30	10	60	1
30	15	55	2
30	20	50	2
30	25	45	2
30	30	40	3
30	35	35	3
30	40	30	3
30	45	25	3
30	50	20	3
30	55	15	3
30	60	10	3

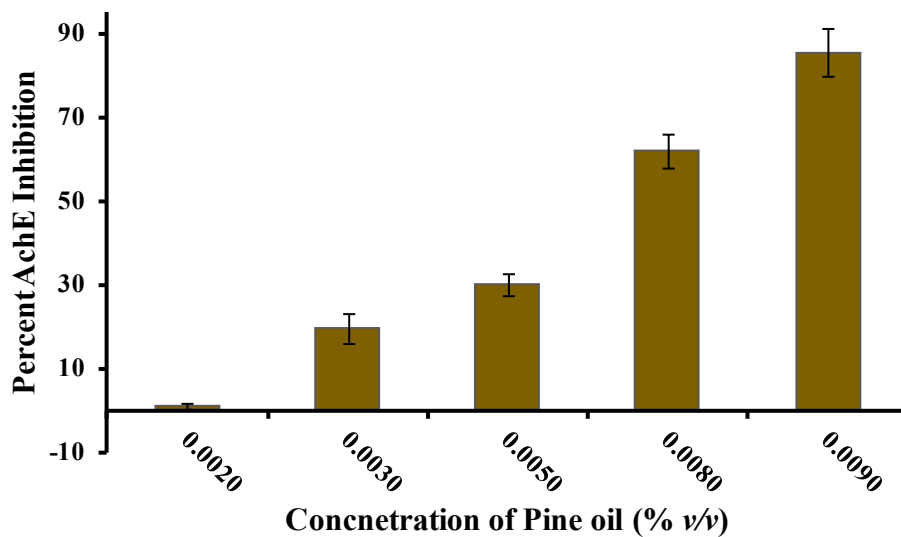
30	65	5	3
35	5	60	1
35	10	55	2
35	15	50	2
35	20	45	2
35	25	40	3
35	30	35	3
35	35	30	3
35	40	25	3
35	45	20	3
35	50	15	3
35	55	10	3
35	60	5	3



**Figure S6:** Pseudoternary phase diagram of PPD 9 representing nanoemulsion, turbid and biphasic region of obtained points

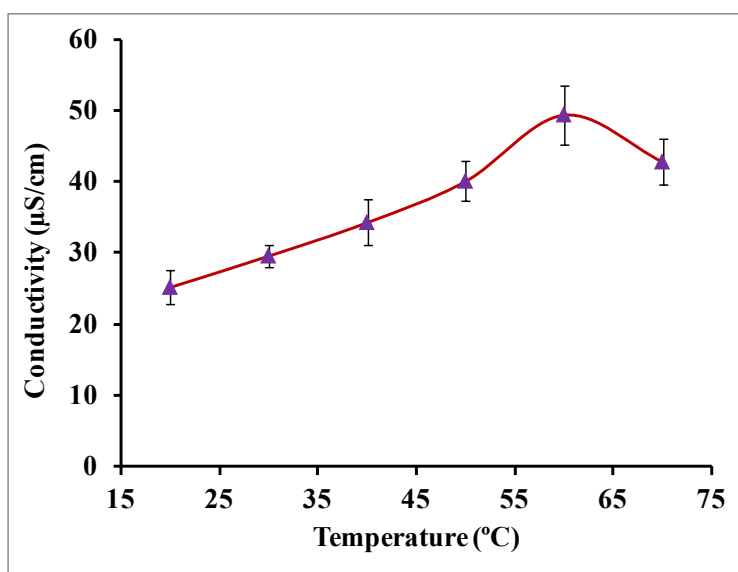
***In- vitro* anticholinesterase (AChE) activity**

*In vitro* AChE studies with Pine oil (active compound) has been demonstrated in Figure S7



**Figure S7:** Represent the AChE inhibition activity in comparison to alone Pine oil

**Conductivity studies**



**Figure S8:** Conductance study of nanoemulsion at 20°, 30°, 40°, 50°, 60° and 70°C