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FORMULATION, OPTIMIZATION AND EVALUATION OF RESVERATROL LOADED NANOEMULSION USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT: Phytochemicals are intensively addressed for clinical uses, and nanostructured phytochemical delivery vehicles are on the limelight. A viable potential treatment for neurological disorders is resveratrol. In the current investigation, we sought to create a nanoemulsion containing resveratrol utilising the response surface methodology. Using Neem oil, Pluronic-f 68, and Tween 80, the effects of the formulation and probe sonication processing parameters were analysed even during nanoemulsification process. As process factors, the duration of probe sonication has been preferred. The concentrations of oil and surfactant, along with their intensity, were revealed have a significant influence on globule size. The optimum nanoemulsion formulation constituted 2.6442 mg/ml of resveratrol. The responses picked were globule size, PDI, and zeta potential. The optimised RES-nanoemulsion had a globule size of 179 3.4 nm, a PDI of 0.175, a zeta potential of 29.9 2.1 mV, and an entrapment efficiency of RES-NEs of 96.47 2.3%. The in vitro drug release in pH 6.8 phosphate buffer displayed the nanoemulsion formulation's superiority. The results indicate that resveratrol nanoemulsion can be blended with neem oil to improve the bioavailability of this poorly water soluble drugs. When the optimized batch has been subjected to an accelerated stability study at different temperature and relative high humidity over a three-month period, no significant variations were observed.

INTRODUCTION: Phytoconstituents are secondary metabolites that have been investigated for a number of medical applications. A wide variety of phytochemicals with varying chemical structures have been researched and observed for their therapeutic activities. Polyphenols, vitamins, tannins, alkaloids, glycosides, and other substances are among them.

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Polyphenols have been identified as having health-promoting properties [1]. Polyphenols are also beneficial to brain functions and act as neuroprotectants [2]. They play several roles in the brain other than being antioxidants. Among them are neuroinflammation inhibition and memory, learning, and cognitive ability enhancement [3].

As a result, these polyphenols have the potential to substantially contribute to the treatment of neurodegenerative diseases [4]. Resveratrol, a naturally polyphenolic antioxidant found in grape skin, red wine, and berries, appears to have anti-inflammatory, anticancer, and neuroprotective properties. Resveratrol, a naturally polyphenolic antioxidant found in grape skin, red wine, and

berries, appears to have anti-inflammatory, anticancer, and neuroprotective properties [5,6]. Resveratrol, in particular, has been shown to prevent activated macrophages from releasing proinflammatory mediators [7-9]. Resveratrol can cross the blood–brain barrier with a detectable amount in the brain but in very low concentrations since the bioavailability of oral resveratrol is poor [10]. Because of its neuroprotective properties, resveratrol has recently gained popularity. Preclinical research suggests that resveratrol has a similar effect to caloric restriction, and that treatment with resveratrol reduces age-related cognitive deterioration and Alzheimer's disease-like pathologies in animals [11].

Despite its enormous medicinal benefits, resveratrol's solubility and bioavailability limit its therapeutic applications. The cis-form is less stable and less bioactive than the trans-form. Because of its low water solubility, resveratrol is classified as Biopharmaceutics Classification System (BCS) Class II. Resveratrol dissolves in water at around 30 mg/L [12, 13]. Furthermore, it is quickly eliminated from the body due to first-pass metabolism, resulting in low resveratrol bioavailability. As a result, a number of strategies for increasing resveratrol bioavailability have been developed [14,15]. Extensive research has been conducted on novel drug delivery systems. Surprisingly, nanostructured delivery systems have proven to be extremely effective in dealing with many of the issues. Some of them include polymeric nanoparticles, silica nanoparticles, inorganic nanoparticles, nanoemulsions, etc. Furthermore, these techniques worked well for a variety of delivery methods, including oral, intravenous, nasal, and topical [12, 14, 16].

When ingested through dietary sources, however, resveratrol clinical efficacy results vary between studies and appear to be complicated by metabolite pharmacological properties, which also vary across different disease states [17]. The poor bioavailability, water solubility, and chemical instability of resveratrol may also contribute to the lacklustre clinical data [18]. Encapsulating the drug in a nanoemulsion is one way to increase the bioavailability and chemical stability of poorly water soluble drugs like resveratrol. Nanoemulsions are kinetically stable emulsions with a low surfactant concentration (10% w/v) that are usually generated using high energy techniques such as microfluidization or sonication. Resveratrol has recently been found in oil in water nanoemulsions, according to several groups [18-25]. These novel drug delivery systems have an emulsified water and oil system that has a range of 20 to 1000 nm [26]. Major benefits of nanoemulsion include improved bioavailability and simple blood-brain barrier crossing [27]. It has been shown to be a successful way to increase the bioavailability of resveratrol by encapsulating it in nanoemulsions. Additionally, it has been demonstrated that nanoemulsions shield resveratrol from chemical deterioration and stop it from isomerizing into inactive Z-resveratrol [28].

The oil phase in the current study was chosen to be neem oil. Because of its low toxicity in in vivo studies, neem oil, a deep yellow extract from Azadirachta indica A. Juss. (Meliaceae) seeds, is widely used in India and other South Eastern countries as a multipurpose medicinal product for a variety of diseases, as a pesticide/insecticide, and in cosmetics [29, 30] both Subapriya and ``Nagini (2005). In this study, Pluronic F 68 and Tween 80 (1:1) were investigated as surfactants, and we attempted to prepare nanoemulsions from Neem seed oil. Because nonionic surfactants are reputed to be less sensitive to pH, we used Tween 20 as a nonionic surfactant [29]. Additionally, the formulation's stability as a nanoemulsion was investigated. Similar to choosing the right oil and excipients, the nanoemulsion formulation process is crucial. The process of nanoemulsification has been developed using both high energy and low energy methods [31].

Oil globule size can be reduced to nanometers using high energy methods that use high mechanical energy. Among high energy techniques, probe sonication is the most appealing strategy. In comparison to other techniques, it requires less energy input and can even create nanoemulsions without the use of a surfactant. The globule size and stability of nanoemulsions are also influenced by probe sonication time in addition to surfactant and oil concentrations [32].

MATERIALS AND METHODS: Materials Resveratrol was bought from Kshipra Biotech Pvt. Ltd, Indore (India). Both Tween 80 and Pluronic F

68 were purchased from Mumbai-based companies: Loba chemicals Pvt. Ltd. for Tween 80, and Research Lab fine chem industries for Pluronic F 68. From Moly chem in Mumbai, olive oil was purchased. Neem oil and clove oil were bought from Mumbai's Research Lab Fine Chem Industries. From Merck Specialities Pvt. Ltd. in Worli, Mumbai, India, ethanol was purchased. All of the other reagents were readily available and of high analytical quality.

Selection of Oil Phase: The solubility of resveratrol in three distinct oils was investigated. The study's main topics were neem oil, clove oil, and olive oil. A vortex mixer was used to combine extra resveratrol with 2 mL of oil in each of five 5 mL stoppered vials (J. S. Enterprises, New Delhi, India). The samples were then shaken for 72 hours to bring the samples' equilibrium temperature up to 25±2 °C. The samples were then centrifuged at 15000 rpm for 20 minutes, and the supernatant has been dispersed in an ethanol solution. A 0.45 m filter was used to filter out the supernatant. A UV-vis spectrophotometer (Shimadzu 1900) set to 306 nm was used to measure the concentration of resveratrol in the supernatant.

Experimental Design: To create nanoemulsions, two nonionic surfactants, Pluronic F-68 and Tween 80, were dissolved in water and combined with neem oil as the non-aqueous phase. Simple magnetic stirring was used to create coarse emulsions by combining neem oil with various amounts of aqueous phases that contain surfactants. In neem oil, resveratrol (26 mg/mL) was dissolved. The oil phase percentage ranged from 10% to 30%. Surfactant concentrations in this study ranged from 1% to 5%. **Table 1** contains a list of the parameters and levels that were selected. Two steps were used to create the emulsions. A coarse emulsion was produced by adding the oil phase to the surfactant solution drop by drop while magnetic stirring. The mixture was then emulsified using a probe sonicator with a 3 mm stepped microtip in a pulsatile manner (50 s sonication with 10 s break) at an amplitude of 60% with a constant frequency of 20 kHz.50 Hz, changing time as shown in **Table 2.** The independent variables A (% of oil phase), B (surfactant concentration), and C (time) were examined for their effects on the emulsion globule size using the response surface methodology. Resveratrol-dissolved oil Smix and water were combined to create the nanoemulsions used in this study, which were then probe-sonicated. As a result, there won't be any loss and the entire drug will be present. The trial runs were produced using Design Expert (Version 7). ANOVA was used to analyse the data with a 0.05 level of significance.

TABLE 1: INDEPENDENT FACTORS AND THE SELECTED LEVELS

Factor code	Factor	Lower level	Higher level
A	Oil phase	10%	30%
B	Surfactant concentration	1%	5%
C	Time	5 min	10 min

TABLE 2: COMPOSITION OF DIFFERENT NANOEMULSION FORMULATIONS SELECTED FOR THE STUDY DESIGN

Run	A	B	C
	Oil %	Surf con	Time (min)
1	20	3	5
2	20	3	7.50
3	20	3	3.30
4	10	5	10
5	20	3	7.50
6	30	3	7.50
7	10	5	5
8	30	5	10
9	30	5	5
10	20	5	7.50
11	30	1	5
12	20	3	11.70
13	10	1	5
14	30	1	10
15	10	1	10

Characterization of Nanoemulsion:

Globule size and PDI Determination: Utilizing a Zeta Sizer Nano (ZS90, Malvern Instruments Limited, UK) that operates on a Dynamic Light Scattering basis and analyses fluctuations in the intensity of light scattering brought on by the preparation of nanoemulsions, the nanoemulsions' globule size and size distribution have been determined. Kinetics of brownian particles. The formulation was diluted with distilled water and filtered through a 0.22 mm membrane filter to eliminate experimental errors and multiscattering phenomena. At 25 °C and a 90 ° scattering angle, light scattering was observed [33].

Zeta Potential Determination: The zeta potential of the formulation (0.1 ml) was measured using a zeta sizer Nano (Nano ZS90, Malvern Ltd., UK) after it had been diluted 100 times with double-distilled water [34].

Entrapment Efficiency: In order to determine the effectiveness of entrapment, the amount of free drug (unentrapped) in an aqueous medium was measured (EE percent). This is important because it has an impact on how the drug molecule releases. The nanoemulsion formulations were ultracentrifuged at 15,000 rpm for 10 min using an Optima "MAX-XP" ultracentrifuge to separate the aqueous phase. After the drug and nanoemulsion formulations are separated, the amount of drug entrapped per unit weight of nanostructures is calculated.

EE (%) = Weight of total drug in formulation - Weight of drug in aqueous phase / Weight of total drug in formulation × 100

FTIR: Fourier transformed infrared spectra of resveratrol was taken by using the KBr disk method. The FTIR spectra of resveratrol, neem oil, and nanoemulsion are collected using a Nicolet iS10 FT-IR spectrometer (8400S Shimadzu, Japan) in the 4000 to 400 cm^{-1} range.

Surface Morphology by HR-TEM: HR-TEM imaging of optimised RES-NE has been performed to observe particle shape and size. Transmission electron microscopy (HR-TEM) imaging was used to confirm the size measurement results and analyse the morphological characteristics of the NEs. (Icon Labs pvt. Ltd. Icon House, Ground floor, Plot no. 52, service Industrial Area, Sector-6, Sanpada, Navi Mumbai) was applied. A drop of diluted sample was placed (100 times) on a 200-mesh film grid and dried at room temperature.

pH Measurement: Using a Mettler Toledo pH metre, the pH of the prepared nanoemulsion formulation was assessed (Mettler Toledo Inc., Columbus, Ohio). Before each measurement, a standard buffer solution between pH 4.0 and 7.0 was used to standardise the pH metre.

Drug Content: 10 mL RES-NE was prepared which contained 26 mg of RES, 1mL from this formulation was taken out and diluted 10 times with ethanol. The concentration of RES in nanoemulsion has been determined using a UV visible spectrophotometer at 306 nm. The percentage drug content indicates how much RES is actually present in the formulation overall.

***In-vitro* Drug Release Study:** The Franz diffusion cell was used to conduct *in-vitro* percent drug release studies. Filtration were accomplished using a cellophane membrane. Before being mounted on the cell, the membrane was dipped in phosphate buffer for 12 hours. The donor compartment was filled with nanoemulsion formulation, while the receiver compartment was filled with diffusion medium phosphate buffer pH 7.4.

A magnetic stirrer was used to agitate the contents of the cell at 37°C. Serial sampling was carried out at intervals of 0.5, 1, 2, 3, 4, 5, and 6 hours. To maintain a consistent volume, a new phosphate buffer was introduced to the receptor compartment. In-vitro, the resveratrol concentrations in the samples were measured spectrophotometrically at 306 nm release research.

Stability: The storage stability of developed RES-NEs was identified by examining the change in globule size, PDI, zeta potential, and entrapment efficiency after 3 months of room temperature storage.

RESULT & DISCUSSION:

Selection of Oil Phase: Neem oil, clove oil, and olive oil were tested to see if they could be used as the organic phase in the nanoemulsion. **Fig. 1** shows the solubility of resveratrol in different oils.

The results show that resveratrol solubility in neem oil is much higher ($p < 0.001$) than in olive and clove oils. Meanwhile, resveratrol solubility was similar ($p > 0.05$) in olive oil and clove oil.

For further optimization and formulation tests, neem oil with a resveratrol solubility of 26.23 ± 2.11 mg/ml was used.

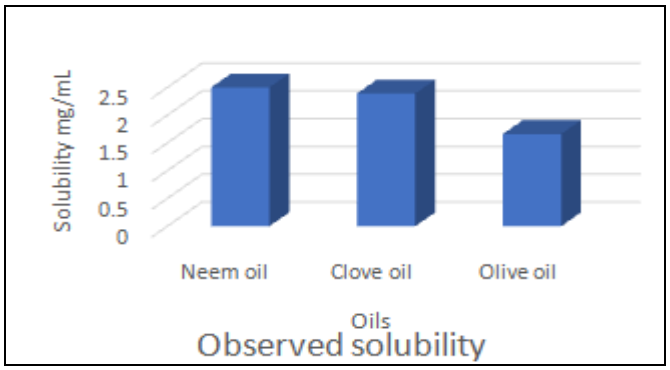


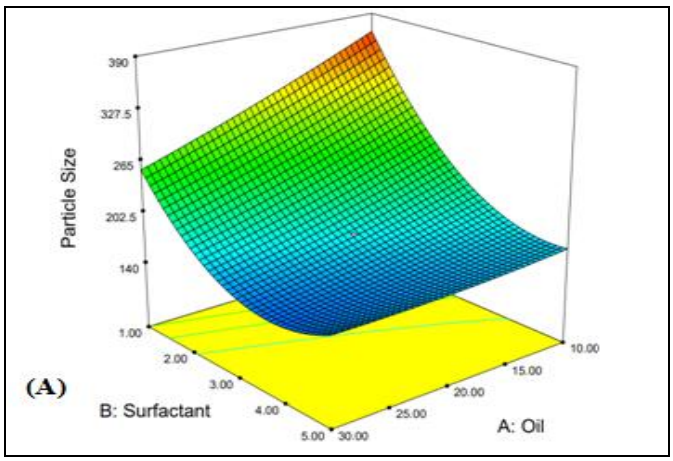
FIG. 1: SOLUBILITY OF RESVERATROL IN DIFFERENT OILS

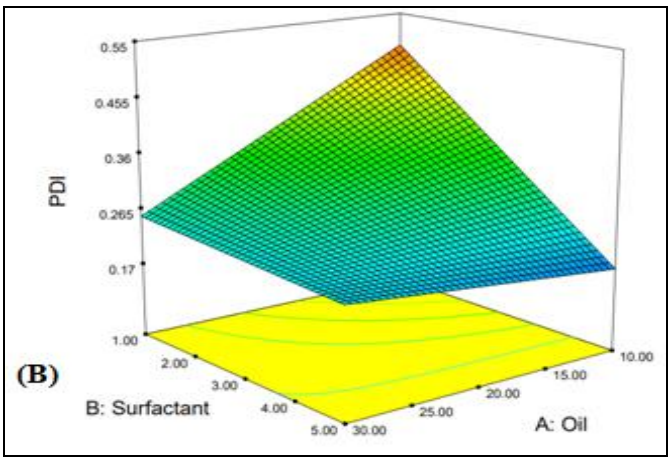
Effect of Independent Variables on Nano-emulsification: The effect of oil and surfactant concentrations, as well as the time of the probe sonication process, on droplet size, polydispersity index, and zeta potential of a nanoemulsion formulation was investigated in this study. The results of several nanoemulsification trials proposed by the software for the study are shown in **Table 3.**

TABLE 3: EXPERIMENTAL RUNS OF NANOEMULSIONS AND THEIR OBSERVED RESPONSES

Run	*X1*(mL)	*X2*(mL)	X_3 (min)	*PSY1*	*ZP Y2*	*PDI Y3*	*EE*(%)Y4
F1	20.00	3.00	5.00	385.9	-23.7	0.542	79.43
F2	36.82	3.00	7.50	168.5	-21.7	0.27	83.72
F3	20.00	3.00	3.30	158.6	-22.3	0.207	92.11
F4	10.00	5.00	10.00	153.8	-27.4	0.135	96.32
F5	20.00	3.00	7.50	180.2	-19.3	0.202	91.96
F6	3.18	3.00	7.50	184.3	-24	0.319	81.62
F7	10.00	5.00	5.00	265	-20.2	0.343	94.64
F8	30.00	5.00	10.00	115.8	-27.3	0.283	95.71
F9	30.00	5.00	5.00	209.7	-16.6	0.185	95.77
F10	20.00	5.00	7.50	181.2	-26.5	0.176	96.47
F11	30.00	1.00	5.00	160.2	-27.6	0.193	89.12
F12	20.00	3.00	11.70	148	-21.9	0.195	93.7
F13	10.00	1.00	5.00	376.2	-28.2	0.519	84.45
F14	30.00	1.00	10.00	304.5	-28.8	0.25	78.21
F15	10.00	1.00	10.00	385.6	-14	0.498	91.43

Independent Variables- X1= Oil; X2= S-mix, X3= Time & Dependent Variables – Y1= PS (Particle size), Y2= ZP (Zeta Potential); Y3= Polydispersity index (PDI); Y4 = Entrapment Efficiency.





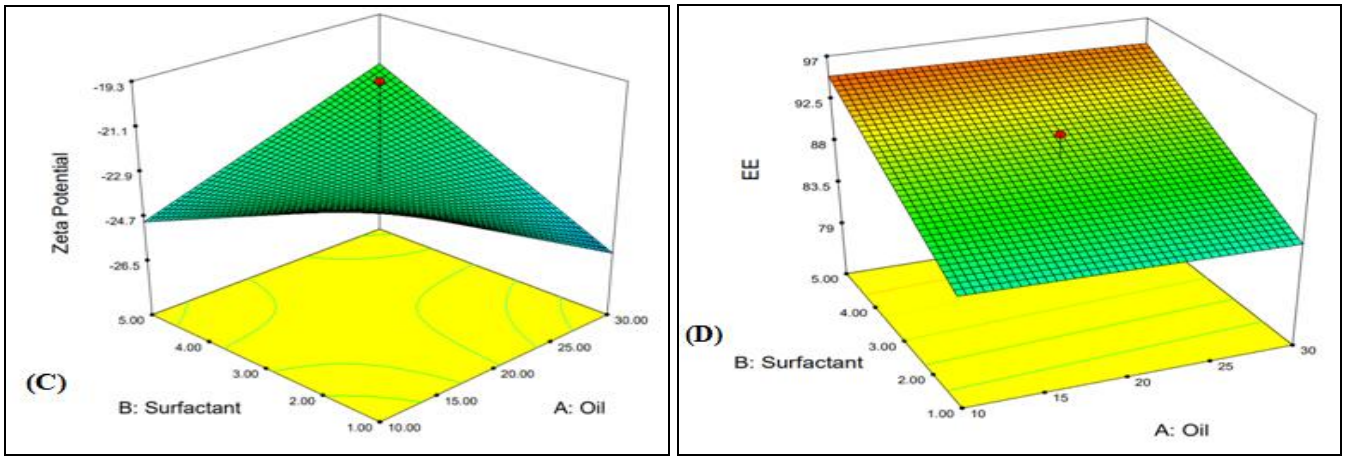


FIG. 2: RESPONSE SURFACE PLOTS FOR VARIOUS INDEPENDENT FACTORS: (A) SURFACTANT CONC. VERSUS OIL CONC. OVER THE PARTICLE SIZE. (B) SURFACTANT CONC. VERSUS OIL CONC. OVER THE PDI (C) SURFACTANT CONC. VERSUS OIL CONC. OVER THE ZETA POTENTIAL (D) SURFACTANT CONC. VERSUS OIL CONC. OVER THE ENTRAPMENT EFFICIENCY

Globule size and Polydispersity Index Determination: The globule size and polydispersity index of a nanoemulsion were determined using dynamic light scattering with the Zetasizer Nano (ZS90, Malvern instruments Limited, UK). The size of the particles of NEs is significant because it influences the rate and extent of drug release and also permeation. The larger interfacial surface area for drug absorption is provided by the smaller droplet size. It's also possible that the smaller droplet size facilitates for a faster release rate. It has also been reported that the nanoemulsion droplets' smaller particle size may lead to faster absorption and improved bioavailability. PDI also determines the width of the particle size distribution. If the PDI is less than 0.1, the particle population may be homogeneous, so while high PDI values indicate a wide size distribution. The particle size and polydispersity index (PDI) of the fabricated batches ranged from 115.8 to 385.9 nm and 0.135 to 0.542, respectively. The optimised RES-NE particle size and polydispersity index (PDI) were determined to be 179.4 nm and 0.175, respectively.

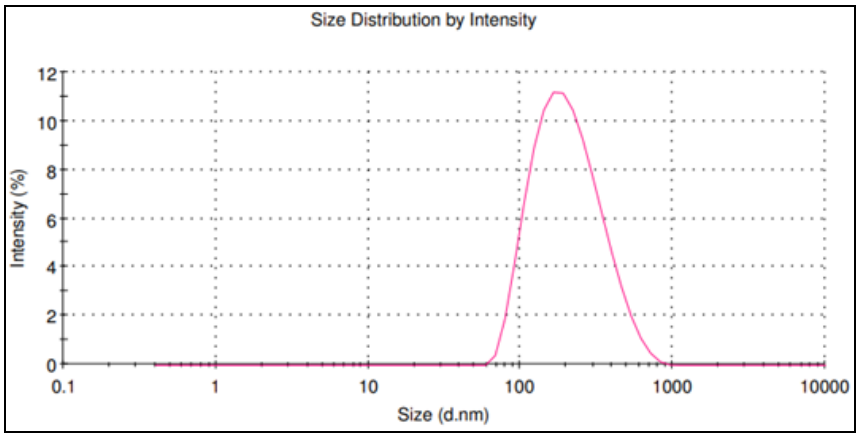


FIG. 3: GLOBULE SIZE OF OPTIMIZED RES LOADED NANOEMULSION FORMULATION

Zeta Potential Determination: The optimised RES-zeta-potential NE's was found to be -29.9mV, as shown in **Fig. 3**. Because of the negatively charged Tween 80, it has negative surface charges. Furthermore, both the surfactant (Tween 80) and the co-surfactant (Pluronic F-68) were negatively charged. It is now accepted that zeta potentials less than -30 mV are optimal and less than (-60) mV are required for complete electrostatic stabilisation. Electrostatic surfactants are known to give 3 16 17 21 26 27 nanoparticles a favourable electrical potential, resulting in a higher zeta potential. However, higher concentrations of these surfactants reduce the zeta potential of nanoparticles due to a reduction in the thickness of the diffuse layer. As a result, it has been proposed that electrostatic

surfactants be combined with stearic surfactants to give nanoemulsions practical electrical properties. When a absolute value of zeta potential is greater than 30 mV, particles can be dispersed stably due to electric repulsion between them. We found NE zeta potential values ranging from -14 to -29.9 mV. This implies that the RES-NEs prepared by solvent evaporation followed by probe sonication emulsification are physically stable.

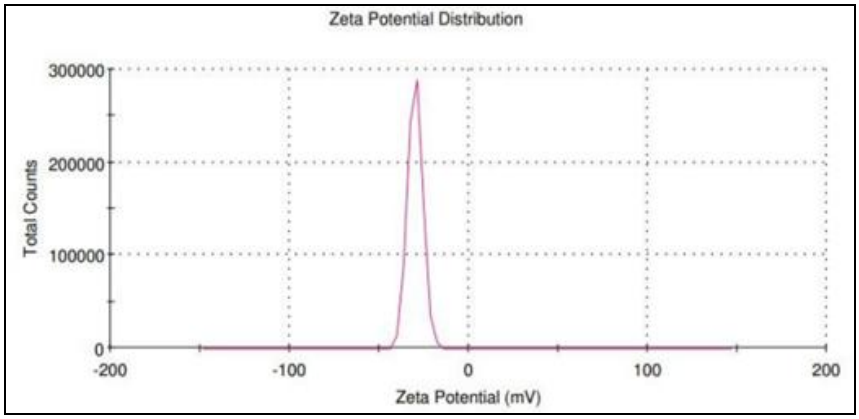


FIG. 4: ZETA POTENTIAL OF OPTIMIZED RES LOADED NANOEMULSION FORMULATION

Entrapment Efficiency: By varying the oil and Surfactant concentrations, 15 different batches of RES-NEs were prepared. The amount of drug used in each batch was the same. Across all batches, the entrapment efficiency ranges from 78.21 to 96.47%. The highly lipophilic nature of the drug RES and its high solubility in Pluronic F-68 may account for such a high value of entrapment efficiency. **Table 3** depicts all of the values.

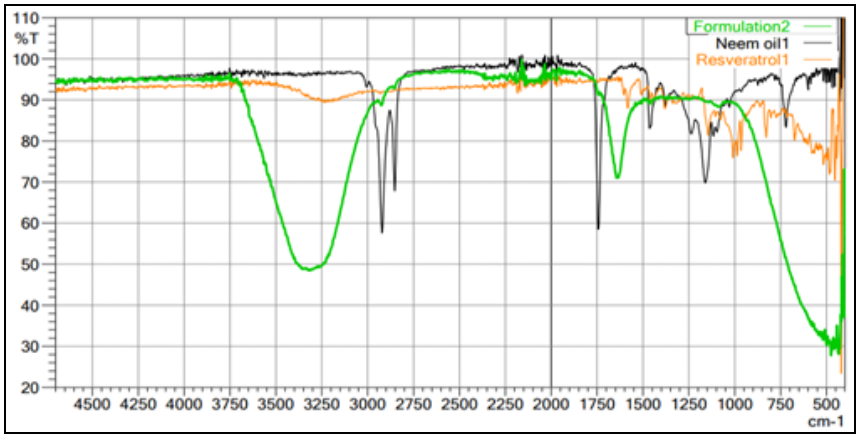


FIG. 5: OVERLAY FTIR SPECTRA OF RESVERATROL, NEEM OIL, AND NANOEMULSION

Surface Morphology by HR-TEM: The result of TEM image of optimized as shown in **Fig. 6** indicated that the particles presented uniformly nano-sized spherical shape.

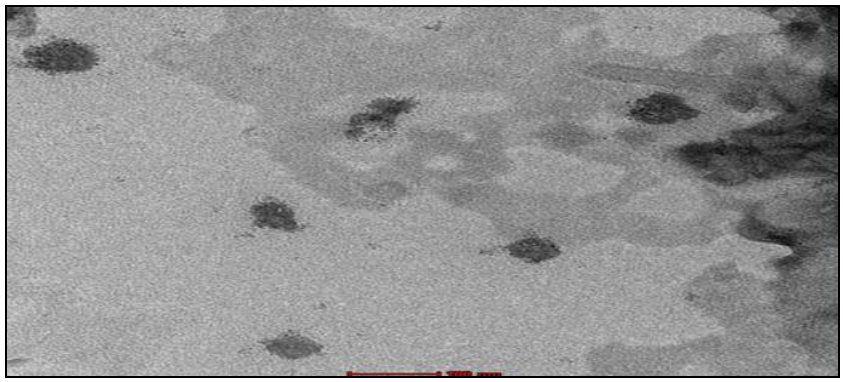


FIG. 6: HR-TEM IMAGE OF OPTIMIZED RES-NE FORMULATION

pH Measurement: The pH of the optimised nanoemulsion was evaluated in triplicate using a digital pH metre, and it ranged from 6.8±0.589 to 7.2±0.428, which is required for oral drug delivery.

Viscosity Measurement: The viscosity of optimized nanoemulsion (F) was found 0.0159 ± 0.003 Pas (mean ± SD, n=3). They are Non-Newtonian Liquids (viscosity decreases when the shear rate or shear stress increases) having Low viscosity values ensure easy handling and packing. Therefore, the viscosity of RES-NE showed pseudo-plastic behaviour.

Drug Content: The optimised batch had a drug content of 85.92% ± 0.896 (mean SD, n=3). The optimal concentration of oil, surfactant, and co-surfactant was required for maximum drug loading in the formulation to achieve maximum drug content in the optimised batch. All formulation variables had a statistically significant effect on drug content.

***In-vitro* Drug Release:** A Franz diffusion cell was used for the RES-NE *in-vitro* release study. A nano emulsion containing 125 mg of the drug was used in the study. The nature of the surfactant influences drug release from nanoemulsion. The % CDR value for optimized batch after carrying out the entire in-vitro study was found to be 95.97±1.27. The release profile of RES from optimized nanoemulsion formulation through the franz diffusion cell at PBS (pH 7.4) showed in **Fig. 7** and **Table 4.**

TABLE 4: *IN-VITRO* RELEASE PROFILE OF OPTIMIZED BATCH

Sr. no.	Time(h)	%CDR
1	0	0
2	0.5	4.00234
3	1	16.1032
4	2	30.1995
5	3	46.4084
6	4	66.0798
7	5	87.277
8	6	95.9741

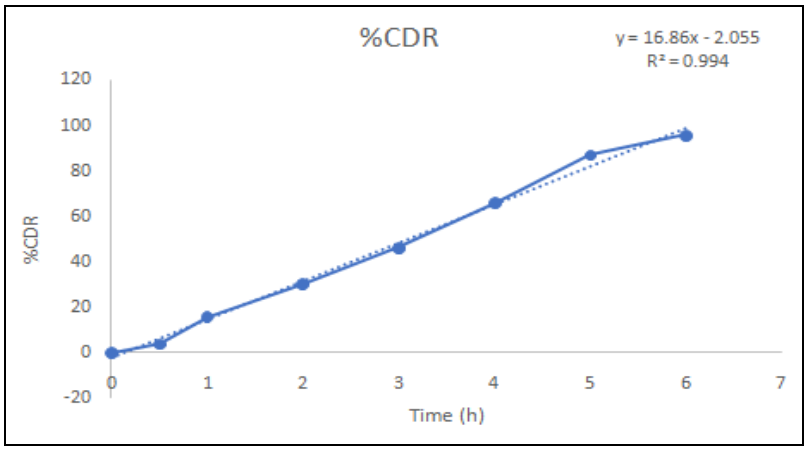


FIG. 7: % DRUG RELEASE OF OPTIMIZED RES- NANOEMULSION

Stability Studies on Optimized Nanoemulsion: The storage stability of the nanoemulsion formulation at room temperature was found to be satisfactory, as evidenced by globule size, PDI, and zeta potential values. Even after 3 months of storage at room temperature, there was no significant difference in the values of the studied parameters, indicating that the optimised formulation was stable for a long time.

TABLE 5: STABILITY STUDIES ON OPTIMIZED NANOEMULSION

Stability parameter	Test period			
	0 month	1 month	2 months	3 months
PS(nm)	179.4± 0.01	181.2±1.03	191.3±0.63	198.2±0.89
ZP(mV)	-29.9±0.03	-31±0.55	-28.4±0.01	-26.1±0.24
PDI	0.175± 0.97	0.196±0.03	0.209±0.22	0.249±0.23
EE(%)	96.47± 1.8	94.30±0.15	93.75±0.89	91.43±0.74

CONCLUSION: Considering the need of Resveratrol, it was successfully incorporated into NEs by hot probe sonication method. The RES nanoemulsion was created using the central

composite Design y = 16.86x - 2.0557 R² = 0.9944 -20 0 20 40 60 80 100 120 0 1 2 3 4 5 6 7 % CDR Time (h) % CDR 9 response surface methodology, which involved fitting a quadratic model to the response data. An optimised formulation was selected based on globule size, zeta potential, and PDI. The drug release studies were precise and followed a first order model with an abnormal non-Ficknian release mechanism. Accelerated stability study proved the stability of developed RES- NEs.

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CONFLICTS OF INTERESTS: Nil

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