

Synthesis and Characterization of *Citrus limonum* Essential Oil Based Nanoemulsion and Its Enhanced Antioxidant Activity with Stability for Transdermal Application

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Abstract

Lemon oil (LO), also known as *Citrus limonum* is a highly volatile essential oil (EO) with potential therapeutic properties like anti-oxidative, anti-proliferative, anti-fungal and anti-cancerous. However, the efficacy of LO is limited due to its physiological factors such as high volatility, poor stability (particularly sensitive to sunlight) and quick degradability upon exposure. To overcome these challenges, we formulated lemon oil loaded nanoemulsion system (LO-NE) (oil-in-water), using aqueous titration method. The formulation comprised of lemon oil (LO), Tween 80 and ethanol as oil, surfactant and co-surfactant phases respectively. The existence zone of NE was established by constructing pseudo-ternary phase diagrams using different concentrations of LO, surfactant and co-surfactant (S_{mix}). The quantitative estimation of LO was performed using a high throughput gas chromatography, revealing the presence of various compounds like Limonene, Alpha-Pinene and Linalyl acetate followed by the estimation of total phenolics and flavonoid content. The characterization of LO-NE indicated the particle size of 60 ± 2.5 nm along with the polydispersity index of 0.125 and zeta potential of -14.9 mV. The size range of the NE particles dispersed in the colloidal system was further verified by TEM micrograph which shows size range between 46.2 - 104.7 nm. All the anti-oxidant assays outcomes exhibited the higher activity of LO-NE in comparison to LO alone with lower IC_{50} values. The release kinetics statistical data showed that LO-NE had a sustained release and followed the Higuchi's model

in comparison to burst release of LO alone. Lastly, the stability analysis of the optimised formulation (LO-NE) and LO was estimated through antioxidant assay and subjecting them for thermodynamic stability after 6 months. The results attained, showed higher stability and anti-oxidant capability of LO-NE than LO alone. The study suggested that formulated nanoemulsion can be effectively used as a highly efficacious biologically active alternative nanoformulation against many transdermal disorders.

Keywords

Lemon Oil, Surfactants, Particle Size Analysis, Ternary Phase Diagrams, Release Kinetics, Thermodynamic Stability

1. Introduction

The therapeutic management of skin infections and disorders is one such continuous challenge globally that is not resolved completely until now and needs more targeted and efficacious drug delivery designs. In this context, the control or prevention of microbial infiltrations along with other skin disorders can be efficiently managed by the dual medicinal activity of the essential oils (EO). EO comprises of concentrated compounds extracted from leaves, twigs, fruits and roots of plants exhibiting many folds higher medicinal properties than the plant extracts or the pure compounds. They are commonly used in aromatherapy from last several decades and have a special mention in traditional medicinal practices where it is been equally used in cosmetics, therapeutics and even in spiritualism too. They are constituted by blends of ketones, aldehydes, oxides of terpenes, phenols, esters, alcohol and ethers which are associated in producing characteristic aroma and potential in alleviating stress; and augmenting attention, memory, sedation, arousal, sleep and immune system. Almost all the EOs are listed under the class of highly volatile compounds, exceptionally rich in secondary metabolites (terpenoids, flavonoids, nitrogen and sulphur containing alkaloids) [1]. Similarly, *Citrus limonum* (Lemon oil-LO), an EO with immense therapeutic benefits for dermal disorders is extracted from lemon peels of *Rotaceae* through cold press extraction technique. It is composed of Limonene (up to 90%) and citral (3% - 5%); others are coumarins (bergamotone and limettine), and flavones (diosmine and limotricine) extensively used to treat cough, sprains, fungal infections, superficial wounds, etc. [2]. It exhibits important medicinal properties like anti-inflammatory, anti-nociceptive, anti-cancer, antioxidant, anti-fungal and antimutagenic. Also, citrus flavonoids are reported to be effective cytostatic anticancer agents [3] [4]. But despite of its potential therapeutic properties, its efficacy is restrained due to its physiological structural arrangements [5]. Further, it possesses very high content of hydrocarbons (terpenes-monoterpenes, diterpenes and sesquiterpenes), oxygenated compounds (alcohols and carbonyls) that makes it highly volatile and other phytoconstituents like 1 - 8 cineole are

reported to cause severe inflammation, redness and itching, if applied directly [6]. Additionally, its stability in light and normal temperature is also questionable and causes dermatitis if there is exposure to sunlight after applying on skin [7]. Therefore, its direct application on skin is not recommended even after being an excellent choice for curbing infections. Pertaining to all the concerns listed and to improve the overall efficacy, bioavailability, residence time and stability of LO, an effective and stable nanocarrier system was required [8]. Since last few decades, various research works have displayed the evidences for NE system being the most suited carriers of active EOs. Also, they have been seen as the nanocarrier system with deeper penetration through the dermal layers, higher formulation stability, longer shelf life, enhanced drug solubility, and balanced pH, making them most preferred choice in pharmaceutical industries for transdermal application. Furthermore, it was also reported that NE on conjugating with EO produces a synergistic effect and broad-spectrum biocidal activity which is far much higher than the EO itself. Hence, in the present study, we have fabricated a LO based oil-in-water (O/W) nanoemulsions (NE) system for enhanced therapeutic efficiency and stability of LO through transdermal route [9]. Here we have taken *Citrus limonum* pure EO as an oil phase, tween -80 as surfactant and ethanol as co surfactant to develop and optimise the NE formulation for transdermal application.

2. Materials and Methodology

Citrus limonum essential oil was provided by Aesthetic company, Saharanpur, Uttar Pradesh, India as a gift sample. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), azino-bis reagent (3-ethylbenzothiazoline-6-sulfonic acid), Folin-Ciocalteu reagent, Griess reagent were obtained from Sigma Aldrich (USA). All other chemicals used were of analytical grade.

2.1. Quantitative Analysis of Lemon Oil

To determine the total quantity of phytochemicals (terpenes and oxygenated compounds) present in the LO, quantitative analysis was done using Gas chromatography-mass spectrometry (GC-MS). GC-MS was performed on Agilent Technology (Thailand) 6890 series gas chromatography (GC) system equipped with 5973 mass spectrometry (MS) detector and 7683 series auto-injector. The compounds were separated on HP5 column (30 m × 0.25 mm, 0.25 µm filter). The column temperature was 70°C for 2 min, and 130°C at 30°C/min and changes the gradient to 230°C with 10°C/min and held at 230°C for 6 min with the total run time of 20 min. An electron ionization (EI) system with ionization energy 70 eV was used for detection. The temperature of ion source was set at 230°C, detector voltage was 2 kV and the interface temperature were 250°C. The mass spectrum was acquired in full scan mode at a rate of 0.98 scan/sec (mass range of 20 - 800 amu). The measurement was performed in duplicate for each sample with solvent delay for 2 min [10] [11].

2.2. Fabrication and Optimization of LO-NE

The fabrication and optimization of varied excipients in colloidal solution was performed by Spontaneous Emulsification method. For the preparation of LO-NE, LO was selected as an oil phase, Tween 80 and Ethanol as surfactant and co-surfactant, respectively with distilled water as an aqueous phase. Further, these excipients were blended together in 7 different S_{mix} (surfactant and cosurfactant) ratios ranging from 1:0 to 1:6 and 16 varied combinations of oil: S_{mix} (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3.5, 1:3, 3:7, 1:2, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1) ratios. Thereafter, these prepared NE samples were kept on shaker at 37°C for 72 h to achieve equilibrium and then centrifuged at 2800 g for 15 min and analyzed for clear titration zones with no phase separation in the colloidal solution. Finally, the formulations were measured spectrophotometrically for the presence of flavonoid and phenolic content and the combinations ratios were plotted on ternary phase Triplot software [12] [13].

2.3. Total Phenolic Content (TPC) Estimation

Phenolics are considered amongst the class of most effective free radical scavengers that can interact with the biological systems and play role in anti-inflammatory, antimicrobial, anti-carcinogenic and antioxidant activities [14]. The TPC was performed by the Folin-Ciocalteu method with Gallic acid (GA) as a standard. Briefly, LO was dissolved in deionized water (10% v/v) and 0.5 mL of Folin-Ciocalteu (1 N) was added followed by vortexing and incubation for 5 min at 37°C. Thereafter, 0.12 mL of 5% sodium bicarbonate was added to all the tubes and the reaction mixture was incubated for 40 min in dark, then absorbance was recorded at 725 nm subsequently. The mean of three replications were recorded and results were expressed as mg of Gallic acid equivalents (GAE) per mL of LO [15] [16].

2.4. Total Flavonoid Content (TFC) Estimation

Flavonoids are a strong antioxidant and show a radical scavenging activity and appear to lower the risk of chronic diseases. The TFC of LO was determined by aluminium chloride method against Quercetin standard (1 mg/mL). LO was dissolved in deionized water (10% v/v) followed by addition of 150 μL of 5% sodium nitrite and the solution was vortexed for 5 min. 150 μL of 10% aluminum chloride was added and incubated for 5 min. Lastly, 2 mL of 4% sodium hydroxide was added and absorbance was measured at 510 nm. The samples were prepared in triplicate and the same procedure was repeated for the standard solution. The TFC was expressed as Quercetin equivalents in per mL of LO [17].

2.5. Ternary Phase Studies

Ternary Phase diagrams were constructed to study the interfacial tension between oil and water, stabilized by adding surfactant and co-surfactant in to the colloidal system. Pseudo-ternary phase diagrams of the prepared LO-NE with clear titration zones and different surfactant/cosurfactant ratios were plotted by

using Triplot 4.1.2 software tool. Consequently, the existence zones representing the physical state of NE were determined by plotting the pseudo ternary phase diagram with three axis, one presenting the oil, another showing a constant S_{mix} ratio and the third one representing the aqueous phase. It provides the insight into the behavior and compositional structure of the prepared NE system [18] [19].

2.6. Thermodynamic Stability Studies

The prepared NE formulations were analyzed for stability by subjecting them to varied alternative temperature and stress conditions. Firstly, they were incubated at 4°C for 3 days followed by 45°C for another 3 days, repeating the same cycle for 3 times. Thereafter, NEs were centrifuged at 2800 g for 30 min and the formulations that cleared the previous steps were exposed to freeze-thaw cycles by storing them at -20°C (3 days) followed by 25°C (3 days). Lastly, all those NEs that sustained through all the tests were dispersed in water (2 mL) and checked for any creaming, coalescence and phase separations issues, if any. The ones that cleared all the tests and remained stable were further taken for characterization [20].

2.7. Rheological Parameters

The rheological parameters that were studied for the optimized formulation (LO-NE) included *pH*, density, conductivity and viscosity. *pH* is the logarithmic concentration of hydrogen ion in the dispersed sample and the hydrogen bond interaction between the NE particles and their viscosity were found to be a key factor in influencing the process of agglomeration between them. The *pH* was measured using a *pH* meter (Thermo Orion, 420A+) and viscosity was determined by Brookfield viscometer. Similarly, electrical conductivity is known as a measure of electrical charge present on the dispersed particles in relation to dispersion medium, which was further analyzed by the conductometer (CM 180, Elico, India) at the frequency of 1 Hz [21].

2.8. Characterization of Optimized LO-NE

NE can be characterized by two ways—determination of physical properties (like shape, size, and dispersity) and chemical characteristics (like the presence of chemical bonding of ligands and other conjugated molecules, zeta potential). The techniques that were employed to characterize physical and chemical aspects of the NE were: Transmission electron microscopy, Particle size analysis; and Fourier transform infrared spectroscopy.

2.8.1. Particle Size, Poly Dispersity Index and Zeta Potential Analysis

Particle size analysis (PSA) is an integral tool for characterizing the size and homogeneity of the dispersed droplets in the NE. Likewise, Zeta potential (ZP) is a measure of effective electric charge present on the surface of the nanoformulation and quantifies the charge stability and degree of electrostatic repulsion between the particles in the colloidal solution. The average PSA and poly dispersity

index (PDI) of LO-NE gives information about the size of the particle and their homogeneity was analyzed on a Photon Correlation Spectroscopy (Malvern Zetasizer 3000 HS) by diluting the test samples in ratio of 1:50 with water [23].

2.8.2. Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is a high magnification measurement technique that images the transmission of electron beam through a sample. TEM is used to determine the morphological and structural framework of the colloidal suspension. The morphology of optimized NE was characterized by TEM analysis using TECHNAI 200 kV TEM (Fei, Electron Optics) [24]. The sample was prepared by diluting 100 times with water and placing it on a copper TEM grid with LO-NE colloidal solution and 2% Phosphotungstic acid. The slide was observed on an electron microscope [24] and staining it.

2.8.3. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The FTIR measurements were done to identify the functional groups present on the surface of LO and LO-NE nanoformulation (IR-810, JASCO, Tokyo) at Punjab University, Chandigarh, Punjab, India. The test samples (LO and LO-NE) were exposed to different wavelengths of IR spectra and the instrument measures the wavelengths that were absorbed. The samples were mixed with potassium bromide (KBr) to make pellets for scanning in the copper grid and the frequency ranges were measured from wave numbers 400 cm^{-1} to 4000 cm^{-1} [25].

2.9. In Vitro Antioxidant Assay

2.9.1. DPPH Assay

The capacity of LO and LO-NE to donate hydrogen to reduce the 2,2-diphenyl-1-picrylhydrazyl radical to 2,2-diphenyl-1-picrylhydrazyl helps to estimate the radical scavenging activity of the sample which is colorimetrically measured by observing the color change from purple to pale yellow color solution upon intensity of reaction [25]. Different concentrations of LO and LO-NE (2 - 10 $\mu\text{L}/\text{mL}$) were taken and 5 mL of 0.1 mM DPPH solution was added and incubated for 20 min at 27°C . Absorbance was taken at 517 nm and ascorbic acid (AA) was used as a positive control. The minimum concentration of LO needed to scavenge 50% of DPPH solution (inhibitory concentration; IC50) was then further calculated from the plotted graph. The ability to scavenge DPPH was calculated using:

$$\text{Scavenging activity}(\%) = \frac{I_0 - I_1}{I_0} \times 100$$

where, I_0 and I_1 are the absorbance of control and test samples, respectively [24] [25].

2.9.2. ABTS Assay

The reaction between ABTS reagent (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and potassium persulfate helps in direct formation of the ABTS radical of blue/green chromophore that plays a vital role in determining the sca-

venging activity. Upon the addition of the sample, radical cation reduces the ABTS (de-colored solution) which is detected spectrophotometrically. Different concentrations of LO and LO-NE (2 - 10 $\mu\text{L}/\text{mL}$) were taken and 1 mL of ABTS solution (7 mM) with 2.45 mM potassium per-sulphate was added in these test samples followed by incubation of 12 - 16 h at 37°C and absorbance was measured at 734 nm [26]. AA was used as a positive control. Then, the concentration of LO sample required to scavenge 50% of ABTS solution (IC_{50}) was calculated from the graph. The ability to scavenge ABTS was calculated using:

$$\text{inhibition}(\%) = \frac{I_0 - I_1}{I_0} \times 100$$

where, I_0 and I_1 are the absorbance of control and test samples, respectively [27].

2.9.3. Hydrogen Peroxide Scavenging Assay

Hydrogen peroxide (H_2O_2) is a mild oxidant and is present in the phagosomes of the cells. Oxidation of H_2O_2 by phytochemicals decreases the absorbance which in turn helps in the estimation of the scavenging activity. Different concentrations of LO and LO-NE (2 - 10 $\mu\text{L}/\text{mL}$) were taken and 600 μL of H_2O_2 (2 mM) was added [28]. The tubes were filled with phosphate buffers (0.2 M, pH -7.4) to make up a constant volume of 0.4 mL and incubated for 10 min and the absorbance was measured at 230 nm. The hydrogen peroxide scavenging activity of both the formulations was estimated and compared with AA which was a reference compound, by using the following equation:

$$\text{H}_2\text{O}_2 \text{ activity}(\%) = \frac{I_C - I_T}{I_C} \times 100$$

where, I_C and I_T are the absorbance of control and test samples, respectively [29].

2.9.4. Superoxide Radical Scavenging Assay

The assay is based on the ability to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Different concentrations of LO and LO-NE (2 - 10 $\mu\text{L}/\text{mL}$) were taken followed by the addition of 3 mL of reaction mixture, consisted of 20 μg riboflavin, 12 mM EDTA and 0.1 mg Nitro blue tetrazolium (NBT) salt in 50 mM sodium-phosphate buffer (pH 7.6) [30]. The tube was illuminated for 90 sec and absorbance was measured at 590 nm. Superoxide radical scavenging activity was calculated by-

$$\text{Inhibition}(\%) = \frac{I_C - I_T}{I_C} \times 100$$

where, I_C and I_T are the absorbance of control and test samples, respectively [31].

2.10. In Vitro Release Kinetics

In order to assess the release of LO from the prepared NE system, Franz diffusion cell facilitated release kinetics study was performed using pre-treated dialysis membrane (D9652, Sigma Aldrich, Singapore) and the receiver compartment was filled with phosphate buffer saline (PBS, 20 mL, pH 7.4). The formulations

were added through the donor membrane and 2 mL of test samples (LO and LO-NE) were taken out after every 30 min followed by addition of equal volume of PBS to maintain the equilibrium. The experiment was conducted for 12 hours and the measure of active phytoconstituents was done by GC-MS analysis as explained earlier [32].

2.11. Stability Studies

The stability of LO and LO-NE was tested by the method described by Basheer *et al.*, 2013. Herein, we tested the stability of LO and LO-NE at the highest concentration (10 µl/mL) in terms of their ability to scavenge DPPH radical after storing the samples (LO, LO-NE) at 37°C for 6 months and then comparing with the fresh prepared samples [33] [34].

2.12. Statistical Analysis

All the data is expressed as mean \pm standard deviation and tested using one-way ANOVA. The values were considered significant at $p < 0.01$.

3. Results and Discussion

3.1. Quantitative Analysis of Lemon Oil by GC-MS

LO is known to possess a high content of terpenes including monoterpenes, di-terpenes, sesquiterpenes and oxygenated compounds such as carbonyl, alcohols and phenols, all of them reported to be highly volatile of varied phytoconstituents. Therefore, to determine the total quantity present in the standard extract of LO A high throughput GC-MS method for EO analysis [35] was used. The predominant compound in LO was Limonene (Retention time, RT 17.66 min), accounting for the peak area of 40.93% followed by Alpha-Pinene and Linalyl acetate with the intensity of 29.24% (RT-12.98 min) and 6.05% (RT-28.71 min) as represented in **Figure 1**. **Table 1** summarizes the compound name, RT and chemical structures of all the identified compounds in LO found in this study.

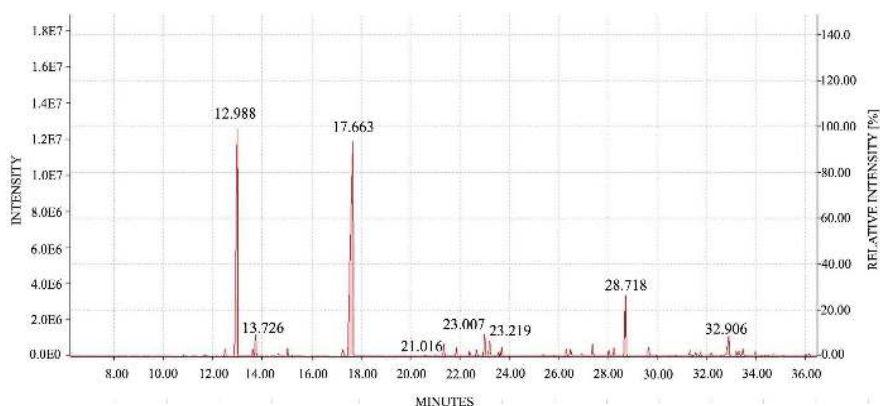
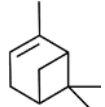
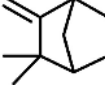
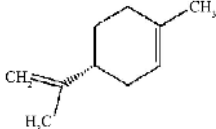
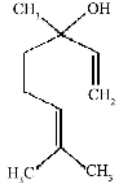
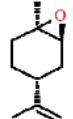
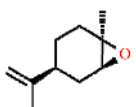
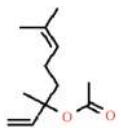
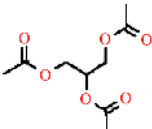


Figure 1. Representing the gas chromatography-mass spectrometry chromatogram of pure LO.

Table 1. Summary of composition of LO.

Compound identified	Chemical structure	Phytocompound	RT (min)
Alpha Pinene		Terpene	12.98
Camphene		Bicyclic monoterpene	13.72
D-Limonene		Cyclic monoterpene	17.66
Linalool		Monoterpeneoid	21.01
Cis-Limonene Oxide		Cyclic monoterpene	23.00
Trans-Limonene Oxide		Cyclic monoterpene	23.21
Linalyl Acetate		Acyclic monoterpeneoid	28.71
Triacetin		Triglyceride	32.90

3.2. Ternary Phase Studies

Phase diagrams (**Figures 2(a)-(e)**) were constructed individually for varied S_{mix} ratios (1:1, 1:2, 1:3, 1:4 and 1:5) to attain O/W ME regions (**Table 2**). It was observed from the graphs that as the contents of surfactant were increased in the S_{mix} ratio, there was the decline in isotropic region and it shifted more towards S_{mix} axis (**Figure 2**). Further, this micelle behaviour and the degree of solubilisation of the optimised NE indicated the homogenous colloidal solution.

3.3. Total Phenolic and Flavonoid Estimation

The total phenolics content in LO was 70 ± 2.5 GAE/ml of sample and 20 ± 1.56 mg Quercetin equivalent/mL of sample.

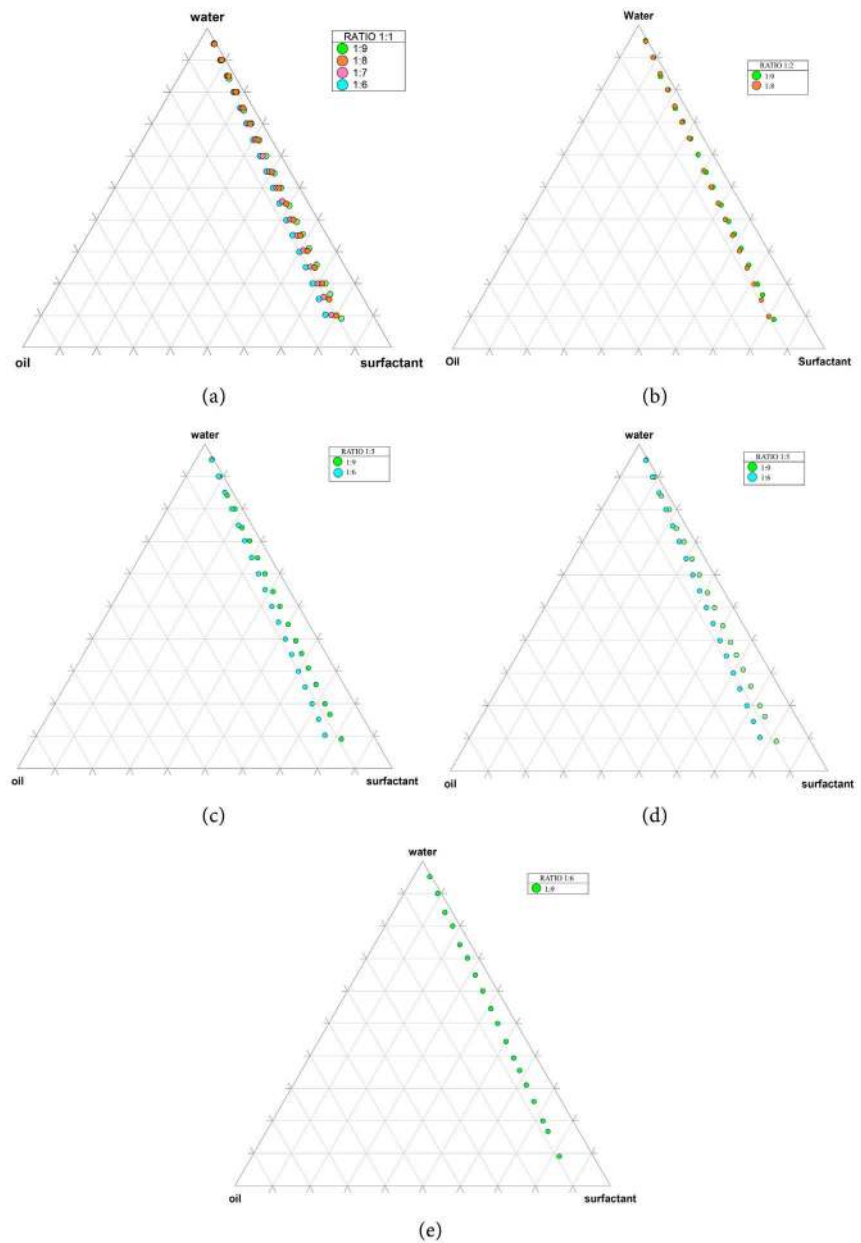


Figure 2. Pseudoternary phase diagrams indicating O/W nanoemulsion using Lemon oil (Oil), tween 80 (surfactant) and ethanol (co-surfactant) using S_{mix} ratio of (a) 1:1, (b) 1:2, (c) 1:3, (d) 1:4, (e) 1:5.

Table 2. Summary of various excipient combinations with their S_{mix} ratios and attained clear titration ratios.

S. No.	Combinations	S_{mix} ratio	EO: S_{mix} ratio
1	LO + Tween 80 + Ethanol	1:1	1:9, 1:6, 1:7, 1:8
2	LO + Tween 80 + Ethanol	1:2	1:9, 1:8
3	LO + Tween 80 + Ethanol	1:3	1:9, 1:6
4	LO + Tween 80 + Ethanol	1:4	1:9
5	LO + Tween 80 + Ethanol	1:5	1:9, 1:6
6	LO + Tween 80 + Ethanol	1:6	1:9

3.4. Thermodynamic Stability Study

The thermodynamic stability testing of all the five combinations of LO-NE was done to analyse their physiological stability [36]. They were subjected for heating-cooling cycle followed by centrifugation tests and then freeze thaw cycle, lastly dispersibility test and during the entire tenure of testing time the samples were checked for constantly any kind of creaming, cracking, coagulation, phase separation or turbidity issues [37] [38]. After completing all the cycles of thermodynamic stability testing the most suitable formulation preparation (S_{mix} ratio: 1:1 and LO: S_{mix} ratio: 1:9) was selected as final optimized formulation based on its visual inspection and categorization as grade “A”. This selected formulation was then taken further for all other characterization tests to study more about its structural and biological properties [39].

3.5. Rheological Parameters

The pH for LO and LO-NE was found to be 3.5 ± 0.5 and 5.3 ± 0.27 respectively (Table 3), which suggested that the formulation is much more suitable for transdermal application and safe as per the prescribed GRAS (Generally Regarded as Safe) limits, avoiding skin irritation. Consequently, the density of LO (1.6 ± 0.4 g/mL) was recorded slightly higher than LO-NE (1.05 ± 0.2 g/mL), much closer to the density of water (1 g/ml) making it safe and suitable to impregnate the dermal layers and reach much deeper layers through transcellular route. Then the conductivity of LO-NE (452.8 ± 0.6 mS/cm) was found to be closer to the conductivity of deionized water, sustaining an ideal action potential inside the biological system and enhancing the permeation of LO-NE. Lastly, the viscosity of LO-NE was observed to be 35 ± 0.2 cps regarded as safe for dermal usage [40] [41].

3.6. Characterization of LO-NE

3.6.1. Particle Size, Poly Dispersity Index and Zeta Potential Analysis

The PSA results obtained for LO-NE was found to be 59.06 ± 1.16 nm with PDI score of 0.125 (Figure 3) exhibiting homogenous colloidal solution with NE particles existing in less than 100 nm range enabling the smoother transition of LO-NE delivery through dermal routes. ZP measures to be -14.9 mV suggesting that there is higher degree of stability in the electro-kinetic effect and surface charge present on the particle [42].

3.6.2. Transmission Electron Microscopy

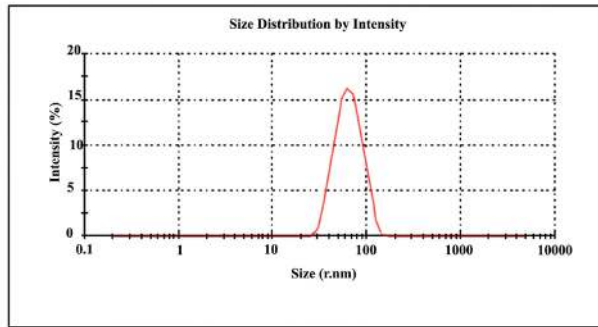
The TEM micrograph showed the uniformly distributed spherical structure morphology of the optimized nanoparticles (LO-NE) with the size range of 46.2 - 104.7 nm which is in accordance with the average particle size of 60 ± 2.5 nm obtained from zeta sizer (Figure 4).

Table 3. Rheological parameters of LO and LO-NE.

Sample	pH	Density (g/ml)	Conductivity (mS/cm)	Viscosity (cP)
LO	3.5 ± 0.5	1.6 ± 0.4	440 ± 0.4	50 ± 0.6
LO-NE	5.3 ± 0.27	1.05 ± 0.2	452.8 ± 0.6	35 ± 0.2

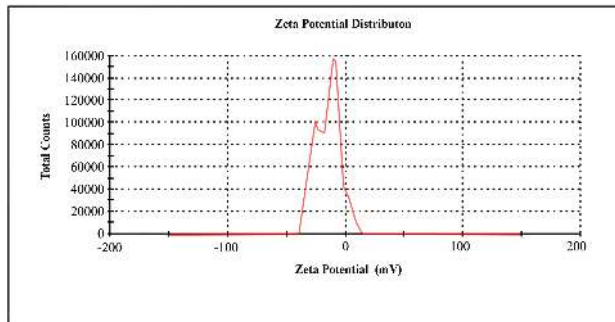
RESULTS

Z-Average (r.nm) : 59.06	Size (r.nm) :	% Intensity	Width (r.nm):
Pdl : 0.125	Peak 1: 66.330	100.0	21.730
Intercept : 0.945	Peak 2: 0.000	0.0	0.000
Result quality : Good	Peak 3: 0.000	0.0	0.000



(a)

Zeta Potential (mV) : -14.9	Mean (mV)	Area (%)	width (mV)
Zeta Deviation : 10.3	Peak 1: -9.43	63.7	6.80
Conductivity (mS/cm) : 0.148	Peak 2: -25.5	36.3	4.95
Results quality : Good	Peak 3: 00.00	0.0	0.00



(b)

Figure 3. (a) Particle size analysis and (b) zeta potential of the optimized LO-NE.

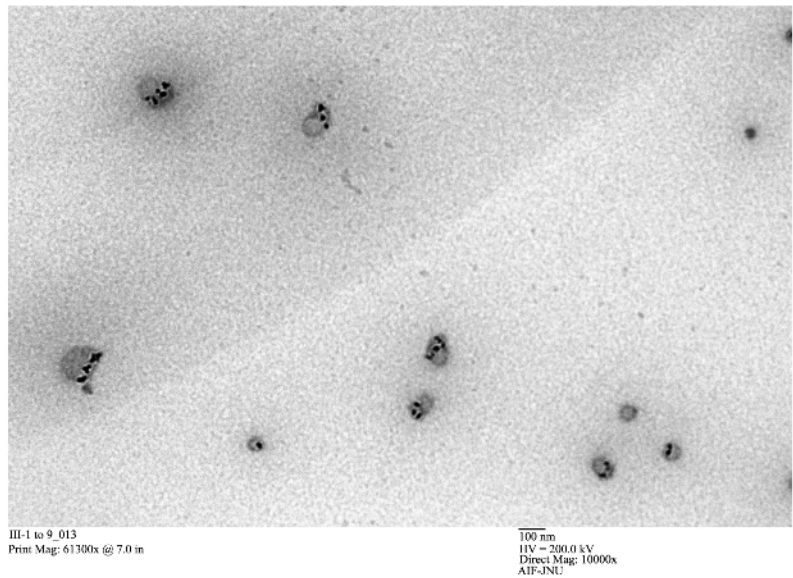


Figure 4. Micrograph showing Transmission Electron Microscopy (TEM) analysis of LO-NE at 100 nm magnification.

3.6.3. Fourier-Transform Infrared Spectroscopy (FTIR)

The FTIR peaks of LO and LO-NE consists of Linalool, D-Limonene, Triacetin, α -Pinene and trans-Limonene corresponding to O-H stretch, C-H stretch, C=O stretch, C=C stretch and C-O stretch at 3441 cm^{-1} , 2915 cm^{-1} , 1745 cm^{-1} , 1645 cm^{-1} and 1218 cm^{-1} respectively. The O-H stretch of Linalool shows a strong and broad peak in the range of $3550 - 3200\text{ cm}^{-1}$, whereas LO-NE revealed the same peak at 3367 cm^{-1} . The peak shift towards lower wavenumber can be attributed to increased mass of molecule. The mass of vibrating molecule is inversely proportional to the frequency of vibration as higher the mass of the molecule, less will be the vibration frequency and lower will be its wavenumber. Similarly, this pattern was observed in C-H stretch of D-Limonene in the range of $3333 - 3267\text{ cm}^{-1}$, the peaks appeared to be strong and sharp and in case of LO-NE this stretch was observed in increased wavenumber 2921 cm^{-1} . The C=O stretch of Triacetin in LO was observed with a strong peak in range of $1750 - 1735\text{ cm}^{-1}$ and LO-NE exhibited same peak at 1725 cm^{-1} . Furthermore, C=C stretch of α -Pinene and C-O stretch of trans-Limonene was seen at $1650 - 1638\text{ cm}^{-1}$ and $1310 - 1250\text{ cm}^{-1}$ corresponding to LO-NE at 1649 cm^{-1} and 1253 cm^{-1} . Therefore, we conclude that all the characteristic peak of LO-NE and LO followed the same path [43] [44] (see Figure 5).

3.7. In Vitro Antioxidant Assay

DPPH analysis is considered as one of the accurate methods for the antioxidant evaluation. This assay depicts the stable radical scavenging rate of DPPH by the presence of antioxidative compounds in the LO-NE. The IC_{50} value is the measure of the potency of a substance in inhibiting the scavenging activity by 50%. LO-NE showed IC_{50} of $4.74 \pm 0.93\text{ }\mu\text{L/mL}$ whereas for LO it was $5.98 \pm 0.65\text{ }\mu\text{L/mL}$ (Table 4). This clearly indicated that the LO-NE has higher ability to scavenge the free radicals present at a lower concentration in comparison to LO alone. The maximum antioxidative ability was observed at a concentration of $10\text{ }\mu\text{L/mL}$ where the corresponding activity was $93\% \pm 0.91\%$ in LO-NE which was much more than the LO ($75.99\% \pm 0.91\%$) (Figure 6(a)). Therefore, the order for inhibition was in the following order: LO-NE > LO > control. Similarly, in case of ABTS, the antioxidant activity increased with increase in the concentration of test samples. For LO-NE the scavenging was $97.66\% \pm 1.02\%$ whereas, for LO, it was $81.36\% \pm 1.92\%$ at $10\text{ }\mu\text{L/mL}$ (Figure 6(b)). Furthermore, the IC_{50} value was $4.53 \pm 0.59\text{ }\mu\text{L/mL}$ in case of LO-NE and $6.51 \pm 0.52\text{ }\mu\text{L/mL}$ for LO. This proved that LO-NE exhibited higher scavenging ability than LO and control. Among all the reactive oxygen species present, hydrogen peroxide is the most reactive one which has the ability to generate the radical molecule during a reaction. LO-NE has the ability to scavenge out OH^- free radicals in a dose dependent manner. The highest activity of scavenging was observed at a concentration of $10\text{ }\mu\text{L/mL}$ which corresponded to $88.72\% \pm 1.09\%$ and 92 ± 1.25 from Hydrogen peroxide and Superoxide radical scavenging assays, respectively (Figure 6(c))

and Figure 6(d)). Therefore, from all the antioxidant assay conducted we can conclude that the LO-NE possess higher free radical scavenging activity when compared to that of LO [45] [46].

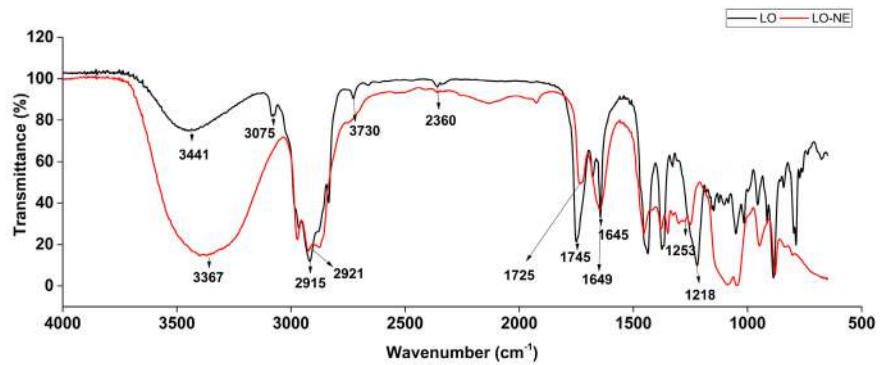


Figure 5. Graphical representation of FTIR results of LO and LO-NE.

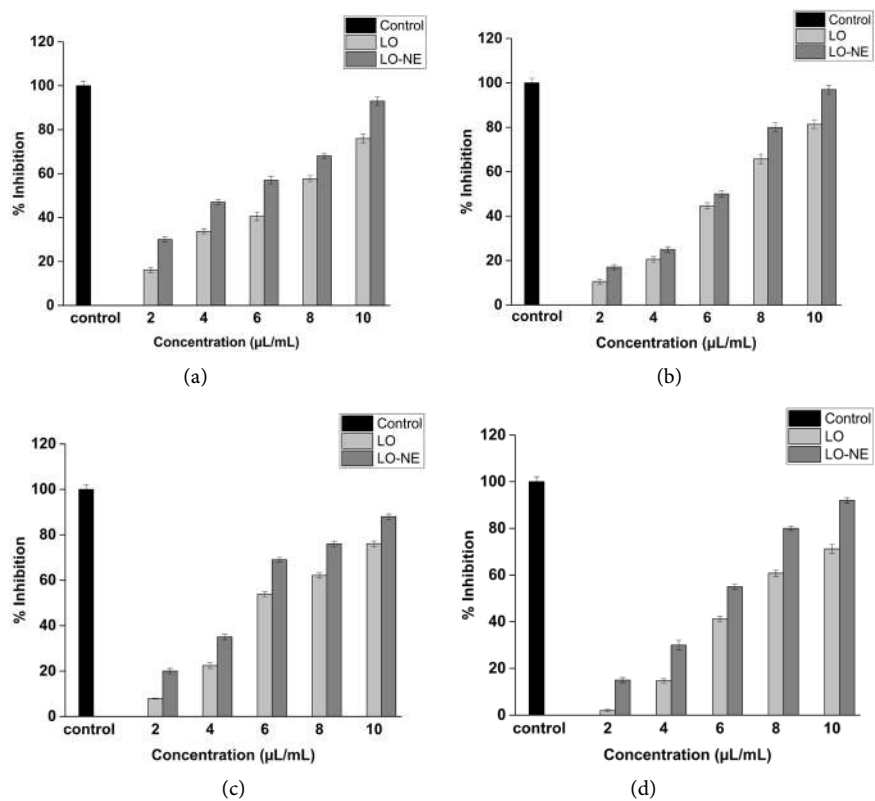


Figure 6. Graph depicting (a) DPPH; (b) ABTS; (c) Hydrogen peroxide; (d) Superoxide radical scavenging activity (%) of LO, LO-NE at different concentrations (2 - 10 µL/mL).

Table 4. IC₅₀ Values of the LO and LO-NE in ABTS and DPPH assay.

Assay	IC ₅₀ of LO (µL/mL)	IC ₅₀ of LO-NE (µL/mL)
ABTS assay	6.51 ± 0.52	4.53 ± 0.59
DPPH assay	5.98 ± 0.65	4.74 ± 0.93

3.8. In Vitro Release Kinetics

The Franz diffusion setup for evaluating compound release kinetics was run for 12 hours and the readings were recorded at the interval of every hour. The pattern of compound release showed that 80% of the drug was released in initial 6 hours and only 20% was released in the second half of the experiment. The graph of percentage cumulative compound release versus time can be studied to validate the sustained release of the compound with time (Figure 7). The pattern of release for LO and LO-NE evidently showed the improvement in release profile of LO in the form of NE in contrast to the pure extract form. The analysis of data and verification from the standard release kinetics models (Figure 8) exhibits that the data best fits with Higuchi's model, giving the highest value for correlation coefficient ($R^2 = 0.9812$ for LO-NE) (Table 5). The Higuchi's model for release kinetics analysis is considered as the most well-known equation for controlled release [47] [48].

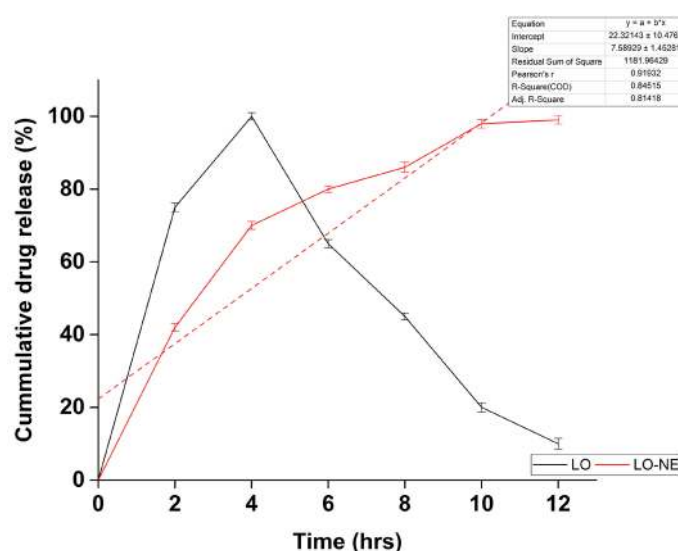
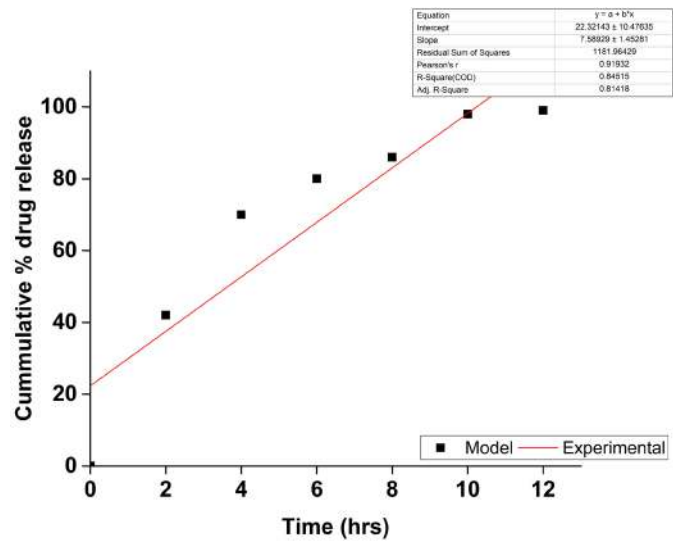


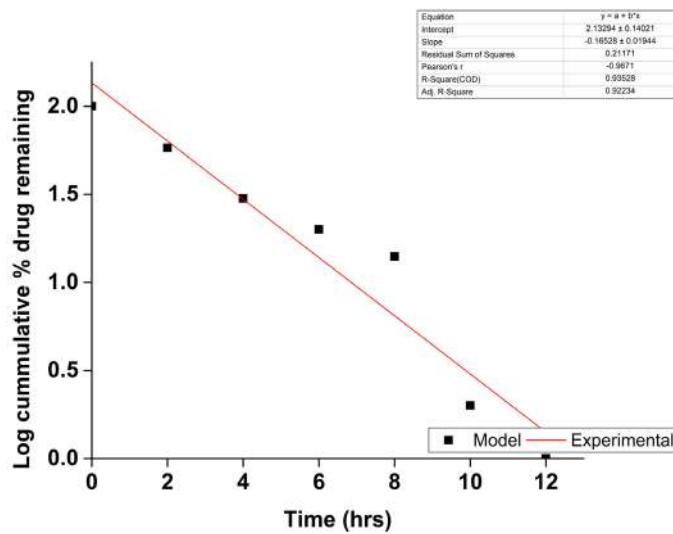
Figure 7. Comparative analysis of drug release in LO and LO-NE.

Table 5. Kinetic modelling of LO and LO-NE.

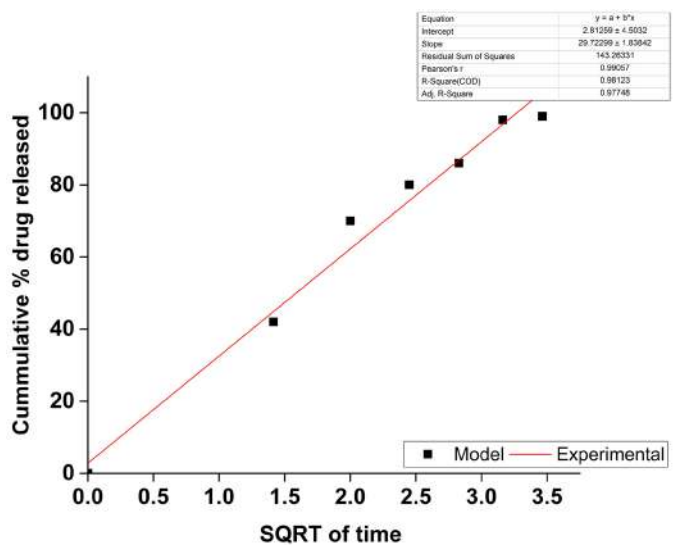
Formulation	Kinetic model	Equation	R ²
LO-NE	Zero order	$y = 7.58x + 22.32$	0.845
	First order	$y = -0.16x + 2.13$	0.933
	Higuchi's model	$y = 29.72x + 2.81$	0.981
	Hixson Crowell's model	$y = 0.30x + 0.122$	0.978
	Korsmeyer Peppas model	$y = 89.16x + 8.45$	0.979
LO	Zero order	$Y = -2.41x + 59.46$	0.079
	First order	$Y = 0.082x + 0.725$	0.0613
	Higuchi's model	$Y = 0.131x + 44.71$	2E-05
	Hixson Crowell's model	$Y = -0.104x + 1.89$	0.0876
	Korsmeyer Peppas model	$Y = -5.433x + 48.61$	0.0034



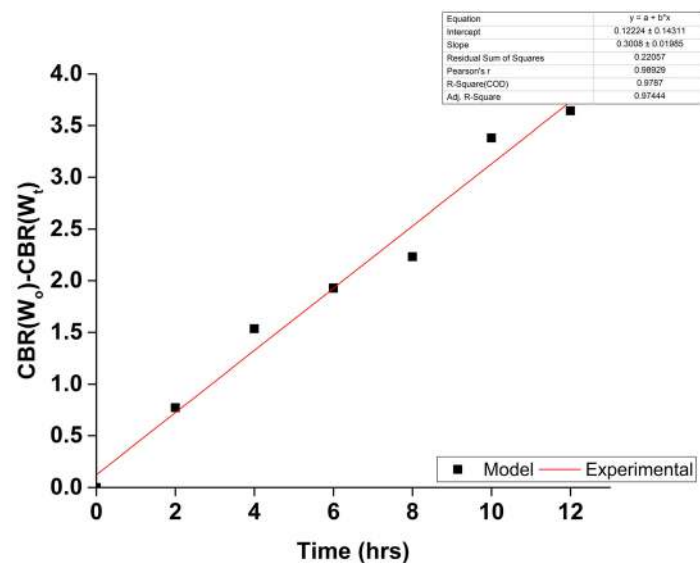
(a)



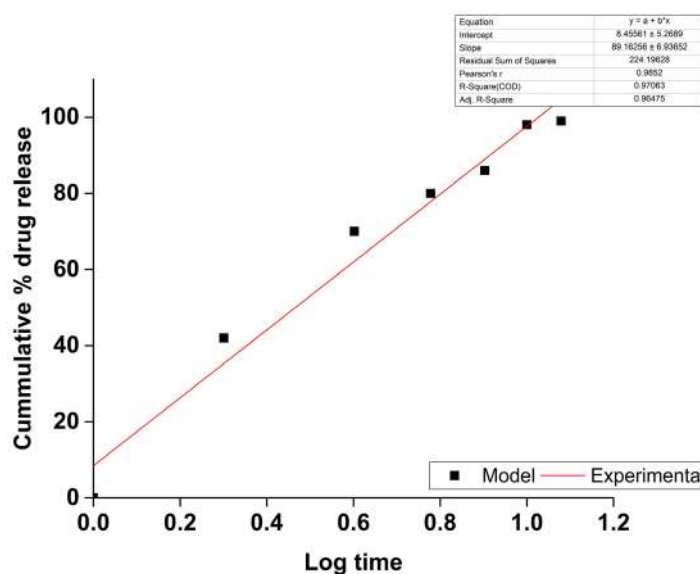
(b)



(c)



(d)



(e)

Figure 8. Kinetic drug release analysis of LO-NE using (a) Zero order; (b) First order; (c) Higuchi's; (d) Hixson Crowell's and (e) Korsmeyer Peppas model.

3.9. Stability Studies

In the current study the stability of the LO-NE, LO was tested in terms of antioxidant activity after 6 months at 517 nm by DPPH assay. LO-NE was found to be stable in comparison to LO at the temperature of 37°C (Figure 9). For the 10 µL/mL LO, the antioxidant activity was $75.99 \pm 1.97\%$ which degraded within the span of 6 months and reduced to $60 \pm 2.54\%$ which can be attributed to the volatile nature of LO. On the contrary, the antioxidant activity of LO-NE was estimated to be $93 \pm 1.51\%$ initially which after 6 months with a minor degradation reduced to $85 \pm 1.61\%$. Therefore, the results suggested that, the LO-NE have higher stability percentage index than that of the pure LO alone [49].

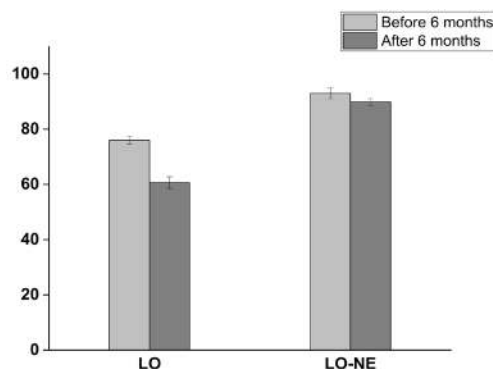


Figure 9. Stability of LO and LO-NE after 6 months using DPPH assay at 10 µl/mL.

4. Conclusion

In the present study, pertaining to all the concerns regarding the volatility and stability of LO was addressed. For this LO based nano sized, biodegradable carrier system with higher therapeutic index and shelf life was successfully designed. The GC-MS analysis results identified several phytochemicals such as Limonene, Alpha-Pinene, Linalyl acetate. Meanwhile total phenolic and flavonoids were estimated as 70 ± 2.5 GAE/mL and 20 ± 1.56 mg quercetin equivalent/mL of sample respectively for LO. Further, the antioxidant assays such as ABTS, DPPH, H_2O_2 and Superoxide Radical Scavenging activity assays showed a higher scavenging activity percentage for LO-NE in comparison to LO and control; and lower IC_{50} for LO-NE. Hence, suggesting that NE system has reduced the biochemical degradation, limited the volatilization of LO components thus, improving the bioavailability of phytochemicals and also allowing the controlled drug release. Furthermore, all the recorded physicochemical parameters were in suitable range to facilitate the transportation of LO-NE across the deeper dermal layers. FTIR scans of LO and LO-NE reflected various types of peaks, stretching and functional group presence within the particular range with carbon stretching and maximum of alkyl and alkane group present with no changes in compound properties, concluding to the fact that the properties of lemon oil were not changed. Thus, on the basis of attained results data it can be well stated that this formulation (LO-NE) can prove out to be a vital replacement as natural therapeutic medicine for transdermal application.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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