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Synthesis, Characterization, and In Vitro Drug Release and In Vitro Antibacterial Activity of O/W Nanoemulsions Loaded with Natural Eucalyptus Globulus Essential Oil

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Abstract

Due to diverse biological activities, eucalyptus oil from eucalyptus waste leaves has tremendous applications in food and pharmaceutical industries. Since it is highly unable, hydrophobic and volatile and so easily loses its anti-microbial activity. To protect this herbal product having bioactive properties, nanoemulsion technology is the best way to encapsulate it. The objective of this study was synthesis of O/W type nanoemulsions, its characterization and evaluation of biological activities. O/W type eucalyptus oil based nanoemulsions were prepared by using sodium dodecyl sulphate, tween 20. Other physiochemical parameters viz; conductivity, pH, and antibacterial activity were also studied. UV, fluorescent and FT-IR techniques were also used. Drug release and pharmokinetic studies were also conducted. Stability of nanoemulsions was studied for 50 days. As compared to tween 20, SDS based nanoemulsions were smaller is size. Nanoparticles were spherical in shape and maximum encapsulation efficiency of 58%. Nanoparticles were stable even after 50 days of storage. Both nanoemulsions exhibited antibacterial and anti-inflammatory activities. Pharmokinetic studies revealed that formulated nanoemulsions followed korsmeyer-Peppas model. All these observations indicated that nanoemulsions from eucalyptus oil have key potential to act as key therapeutic agents in pharmaceutical and food based industries.

Keywords*: Nanoemulsion, Eucalyptus oil, Tween 20, In virto antimicrobial activity.*

1. INTRODUCTION

During course of time, consumers have developed an ever-increasing interest in herbal products as alternative pharmacologically relevant **bio-molecules**. Due to having antimicrobial, antiviral, fungicidal, insecticidal and herbicidal essential oils (EOs) have emerged popularity and attracted the attention of researches due to biological activities. In food and pharma based industries, EOs represent "green technology" due to diverse biological activities like: anti-microbial and antioxidant. EOs are composed of complex mixture of various bioactive lipophilic such as terpenes, esters, alcohols and other aromatic compounds and highly volatile secondary plant metabolites [1]. Due to these diverse complex bio-structures which act synergistically, EOs possess various biological activities like antimicrobial, antiviral, fungicidal, insecticidal and herbicidal. Therefore, over the time a great interest has been paid to essential oils that may be used as therapeutic medicines [2].

Eucalyptus oil from *Eucalyptus globules,* member of Myrtaceae family, is widely used herbal agent in alternative and complementary medical practice [2]. Major source of Eucalyptus oil is leaves which are generally considered as biological waste in

agricultural fields and empty plots. Obviously, Eucalyptus oil has great potential value, but hydrophobicity, unstable with light and volatile nature limits its biological applications. Therefore, alternative delivery system is prerequisite for making this oil more stable.

Recently, encapsulation of EOs has been method of choice, to enhance its efficiency, stability and utilization [3]. Nanoemulsions, also known as sub-micron emulsion, mini-emulsions, ultra-emuslions sized colloidal systems which are thermo-dynamically and kinetically stable dispersions. In pharmaceutical sector, it is the most concerned delivery system due to solubilizing water-insoluble drugs and increased bioavailability of drugs. Nanoemulsions intensify bioavailability by dispersing drugs into small particles to greatly increase its solubility and permeability. Herbal drugs encapsulated into nanoemulsions droplet is free from air, light and hard environment; hence as delivery system emulsion not only improve bioavailability but also protect them from oxidation and hydrolysis. In recent times, nanoemulsions have been extensively studied and offer substantial potential as functional ingredient in cosmetic, pharmaceutical, food and agriculture products [4]. Nanoemulsions most recently are classified into following categories such as oil-in-water (O/W), water-in-oil (W/O), transparent/translucent and colloidal dispersions [5]. Nanoemulsions are effective to deliver hydrophobic bioactive compounds having aroma and flavors with increased surface area and enhanced bioavailability, incorporate both lipophilic and hydrophilic drugs and increased rate of absorption. Therefore, the lipophilic bioactive components can be included in oil phase of nanoemulsion and aqueous medium by using emulsifier like Tween 20 and SDS [4]. Tween-20 is nonionic, biocompatible, and non-toxic surfactants and ideally used as emulsion surfactant in wide range of food, cosmetic and pharmaceutical products [6]. Eucalyptus oil is one among hundreds of different types of essential oils and due to presence of 1-8 cineole (eucalyptol), Eucalyptus oil possess various activities like antimicrobial, insecticide and expectorant. It is also insoluble in water at neutral pH, hence nanoemulsion technology may be a good method to encapsulate, solubilize and protect this bioactive compound. It can be used as oil soluble part of O/W nanoemulsions. This system can increase the concentration of bioactive compounds like eucalyptol in microorganism rich areas. Hence the aim of the present study was to form nanoemulsions of eucalyptus oil and characterize by UV-VIS, FT-IR and fluorescent techniques and investigation of antimicrobial activity.

2. EXPERIMENTAL

2.1. Extraction of Eucalyptus Essential Oil

The green leaves of *Eucalyptus globules* were collected growing under natural conditions, from vicinity of Lyallpur Khalsa College, Jalandhar, located at 71^0 - 31^0 east latitude and 30^0 -33^0 north longitude . Steam-distillation method was used for extracting of essential oil. Extraction was performed using Clevenger-type apparatus. About 20 g of crushed green leaves were distilled at a working temperature of 100°C for 2 hr with addition of 100 ml of double-distilled water. Extraction of oil continued until no more oil was retrieved. The oil was collected from Clevenger apparatus and stored at laboratory condition (25°C). The oil obtained was used without further purification or filtration.

2.2. Preparation of Nanoemulsion

Nanoemulsions of *Eucalyptus globules* essential oil were prepared by method given [4] (Table 1). Schematic representation to formation of nanoemulsions is shown in Fig

1. Briefly, two nanoemulsion formulation (F1 and F2) were prepared. For F1, Tween 20 (2v/v%) were dissolved in double distilled water at room temperature and allowed to shaken with magnetic stirrer for 15 min to get homogenous solution. Then eucalyptus essential oil (3 v/v%) was added drop wise to the above said mixture and shaken with magnetic stirrer for 25 minutes. For F2, SDS (0.25 w/w%) was added to mixture containing Tween 20 (2v/v%) and distilled water. The mixture was allowed to stir with magnetic type stirrer for 10 minutes. Similarly, essential oil was added drop wise to the mixture for 25 min with continue stirring.

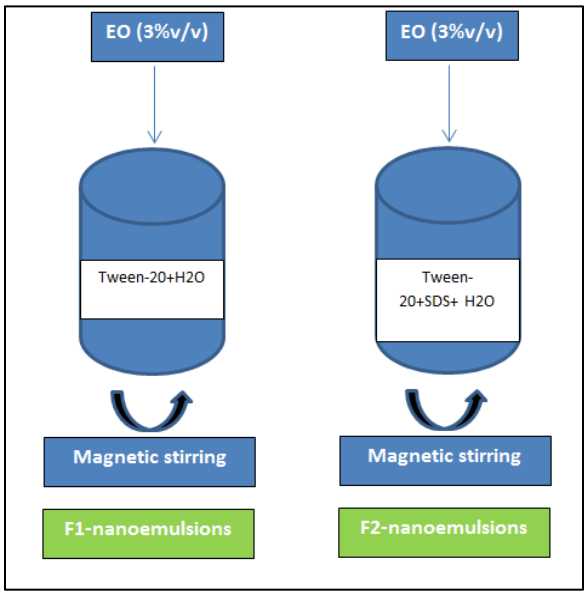

Figure 1*. Schematic representation of formation of nano-formulations.*

Table 1*. Composition of nanoemulsion components***.**

Sample name	Essential oil (%v/v)	Tween 20 (%v/v)	SDS (%w/v)
F1	3	2	-
F2	3	2	0.25

2.3. Characterization

Both F1 and F2 formulations were characterized for various physiochemical parameters such as morphology, pH, particle size, % transmittance, dye test, drug content and drug release. Nanoemulsions were also tested for in storage stability.

2.4. Size and Morphology

Size and morphology of the formulated nanoemulsions was observed with the help of the microscope (Debro DM RL) having 5 MP 1 / 2.5 APTINA CMOS sensor. The size of the nanoemulsion was noted with the help of MicroView software with inbuilt calibration tools.

2.5. Turbidity and % Transmittance

Turbidity and % transmittance were measured using UV-VIS spectrophotometer (Labtronics). All measurements were recorded three times for each sample and the average values were reported. One ml nano-formulations were diluted 100 times using double distilled water and analyzed at 600 nm against water as blank.

2.6. PH and Conductivity

The pH values of the nanoemulsions were measured by immersing the pH electrode into the undiluted emulsion using pH meter (Labtronics). Conductivity of the nanoemulsions formulations was measured by using conductometer (Labtronics). All the values were determined at room temperature.

2.7. Dilution test

Dilution test was performed to observed phase inversion of F1 and F2 nano-emulsions. For this 1 ml of F1 and F2 **nano-formulations** were diluted with 10 ml of water in the test tubes and observed for phase inversion.

2.8. Dye Solubility Test (O/W Test)

Few drops of methylene blue dye was added to 1 ml of nanoemulsion in an eppendorf tube and mixed gently. The formulation was observed under microscope.

2.9. Drug Content

Essential oil content in F1 and F2 nanoemulsions were conducted by solubilizing 1 ml of formulation in 5 ml of methanol. Formulations were kept in shaking incubator (Remi, India) (50 rpm, 37°C) for 30 min. After incubation, supernatant was collected and analyzed using UV spectrophotometer at 210 nm against methanol as blank. Free essential oil was determined by measuring non-incorporated essential oil present in the aqueous phase. Essential oil concentration was determined by establishing linearity of the calibration curve at five concentration levels within the range of 10-100 mg. Least square regression analysis was done for the data ($y = 0.82x + 0.76$, $R^2 = 0.987$). The incorporation efficiency (IE%) of essential oil into nanoemulsions was calculated from following equations: IE(%)= [(TEOC-FEOC)/ThEOC]x 100, where TEOC is total essential oil concentration, FEOC is free essential oil concentration, ThEOC is theoretical essential oil concentration.

2.10. Stability of Nanoemulsions

Stability of F1 and F2 nanoemulsions was determined keeping samples at 4°C, -20°C and RT for 30 days and analyzed for any change in droplet size using microscope. Thermal cycling was performed to establish physical stability by subjecting the F1 and F2 formulations to stress conditions of freeze thaw cycling (12 h at 4°C and 12 h at room temperature 37°C for period of one week) and measuring the change in the particle size. Effect of centrifugation on F1 and F2 formulations was studied to evaluate potential metastable conditions. F1 and F2 formulations were centrifuged at 2000 rpm for 30 min and visually observed for phase separation or creaming.

2.11. In Vitro Release Assay

In vitro essential oil release study was carried out using the dialysis technique. Two ml of F1 and F2 nanoemulsions (containing about 60mg, 350 mM of essential oil) was mixed with 1 ml of phosphate buffer saline (PBS, 50mM, pH 6.4) simulating gastric and intestinal conditions in a dialysis bag (Sigma, MW 1000). The bag was placed in glass beaker having 60 ml of PBS at 37°C with continuous string (100 rpm). Five ml of aliquots were taken out of the dissolution medium at pre-determined time intervals, replaced with fresh PBS solutions to maintain the total volume unchanged and analyzed for essential oil release by spectrophotometry UV at 210 nm. The release profile was expressed as % essential oil release.

2.12. Drug Release Kinetics

Release kinetics of essential oil from nanoemulsions were explained by using four different kinetic models, that is , zero order, first order, Higuchi model and Korsmeyer-Peppas model following Bohrey et al [7]. The zero order rate Eq. (2) explains the systems where the rate of drug release does not depend on its concentration. The first order Eq. (3) explains the release from the system where rate of drug release is concentration dependent. Higuchi model described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion Eq. (4). Korsmeyer model derived a simple mathematical relationship which described the drug release from a polymeric system Eq. (5). C=kot (2), where, C is the concentration of drug at time t, t is the time and k0 is zero-order rate constant expressed in units of concentration/time.

$LogC0-LogC=k1t/2.303$ (3), where, C0 is the initial concentration of drug and k1 is the first order rate constant.

$C=KHt\sqrt{}$ t (4), where, KH is the constant reflecting the design variables of the system.

$M^t/M^\infty=KKP^{tn}$ (5), where M^t/M^∞ is the fraction of drug released at time t, KKP is the rate constant and n is the release exponent.

2.13. UV-VIS, FT-IR and Fluorescence Spectroscopy Analysis

UV-spectrophotometric analysis of F1 and F2 formulations was conducted using UV-VIS spectrophotometer (Labtronics) with slit width of 2nm, using a 10-mm cell at room temperature and were examined in the wavelength ranging from 200-400 nm. The peak values of the UV-VIS were recorded. FT-IR was used to identify functional groups. A small amount of nano-formulations was taken in the sample cup of a diffuse reflectance accessory. IR spectrum was obtained using FT-IR infrared spectrophotometer (Perkin Elmer, USA spectrophotometer). The sample was scanned from 4000 to 400 $cm^{-1.}$ The peak values of the FTIR were recorded. The fluorescence spectrum of formulations was measured on Perkin Elmer Spectro-photometer (FL6500). The excitation wavelength was 380 nm and fluorescence emission spectrum was recorded over wavelength range from 400-700 nm. All experiments were done at room temperature (~30ºC).

2.14. Anti-Inflammatory Activity (Protein Denaturation)

Protein denaturation essay was done according to the protocol described by Sharma et al [8] with some modification. The reaction mixture consisted of 0.4mL of 1% BSA, 4.78 mL of phosphate buffered saline (PBS, 50 mM, pH 6.4) and F1 and F2 formulations. The reaction mixture was incubated in water bath at 37°C for 15 min. After that reaction mixture was heated at 70°C for 5 min. The reaction mixture was immediately cool down. After cooling, 1 mL of mixture was subjected to fluorescent spectroscopy analysis on Perkin Elmer Spectrophotometer (FL6500). The excitation wavelength was 280 nm and fluorescence emission spectrum was recorded over wavelength range from 300-400 nm. All experiments were done at room temperature (~30ºC).

2.15. In Vitro Antibacterial Activity of O/W Nanoemulsions

In vitro antibacterial activity of the *Eucalyptus globules* essential oil and nanoemulsions was carried out by agar disc diffusion method against test organism (gram-negative bacteria *Escherichia coli,* MTCC 40 and *Streptomyces aureus* MTCC 3160). A swab of bacteria suspension was spread on to the petri plates having Luria Broth. Sterile paper discs (6 mm in diameter) impregnated with *Eucalyptus globules* essential oil nanoemulsions were placed on culture plates. Nanoemulsions without essential oil were taken as negative control while standard vancomycin (30 mg) discs were served as positive control. The plates were incubated at 37^0 C for 24 hours. After incubation, the EO diffuses into the luria agar plates and prevent growth of the test microorganism. Antibacterial activity was observed as the zone of inhibition around the discs. The experiment was repeated three times.

2.16. Statistical Analysis

MS Excel software was used to determine P values by student t test. Values of $P< 0.05$ were significant.

3. RESULTS AND DISCUSSION

3.1. Physiochemical Studies

Eucalyptus essential oil contains natural secondary metabolites that are water insoluble, unstable under normal conditions and possess low bioavailability, so it should be incorporated into O/W nanoemulsions in order to increase its pharmokinetic profile. In light of this possibility, two different nanoemulsions formulations (F1 and F2) were prepared and their main - parameters analyzed. Physiochemical and morpho-logical of F1 and F2 formulated nano-emulsions are shown in Table 2 and Figure 2. Nanoemulsions droplets are circular with smooth margin. Particles were uniformly distributed, discrete and non-aggregated. Mean diameter of nanoemulsions prepared with tween-20 was about 200 nm. Addition of SDS decreased emulsion diameter significantly. In comparison to F1 formul-ation, the equivalent nanoemulsions prepared with mixture of tween 20 and SDS had much smaller droplets with mean diameter of 80 nm. It can be due to difference in molecular weight of surfactants (MW SDS<MW tween 20). The results of our study are supported by Moradi et al, [9]. It was also observed that the number of nanoemulsions was more in the case of nanoemulsions formulated with SDS (F2) than nanoemulsions formulated without SDS (F1) (Figure 2). Our observations are supported by Silva et al, [10]. It was argued that small molecule emulsifier like SDS can form small particles under same process conditions. It can be assigned to differences in adsorption rate and interfacial properties like charge, thickness, permeability and environmental responsiveness [13]. Qian and McClements [4] also cited that emulsifiers with higher adsorption rate and smaller interfacial forces lead to smaller particle sizes. Dye solubility confirmed that nanoemulsion formed was O/W types. Dilution test indicated that both F1 and F2 O/W nanoemulsion did not shown any sign of phase inversion or any kind of precipitation thus confirmed the formulated nanoemulsions were stable.

Table 2*. Physio-chemical parameters of nanoemulsions (mean±S.D, n=3).*

Parameters	Formulations	
	F1	F2
Structure type	O/W	O/W
Mean Size (nm)	200	80
%T	94	99
pH	2.5	3.5
Conductivity (µs/cm)	250	1200

F1 and F2 nanoemulsions were characterized by measuring their physiochemical properties like pH and conductivity at room temperature (Table 2). Conductivity is the ability of any molecule to transfer electricity between two points. To check the stability and the nature of formulation, electrical conductivity of the nanoemulsions was determined [11]. It provides information about nanoemulsions continuous phase and phase inversion phenomenon [7]. As shown in Table 2, conductivity of F1 nanoemulsions prepared with Tween 20 was 250. However, when surfactants were blended with SDS, increased conductivity of nanoemulsions was observed. This is quite feasible as conductivity is directly linked to the amount of ions. Conductivity increased as the amount of ions increased. Turbidity of the

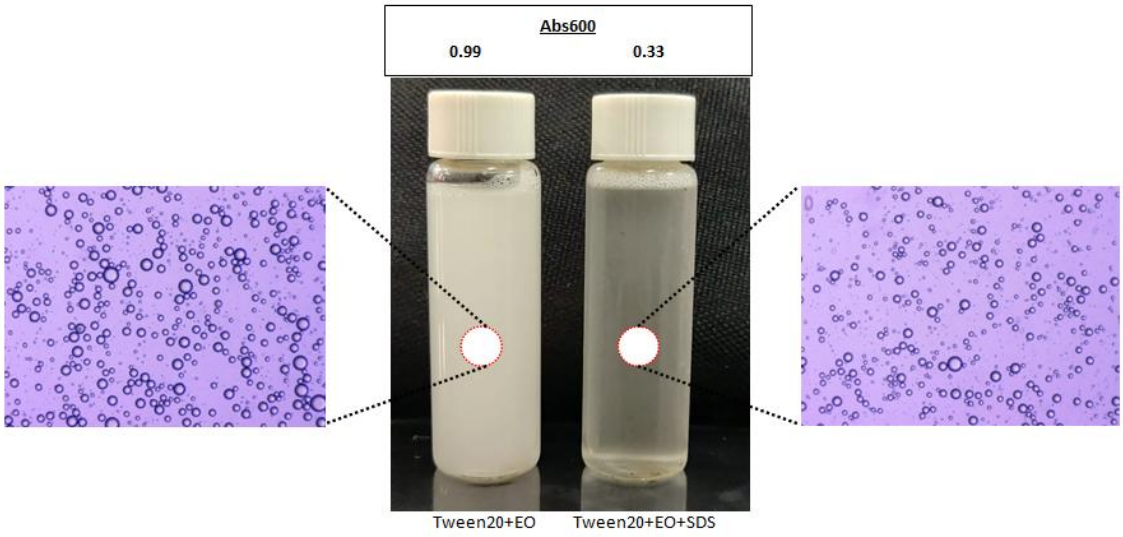

***Figure** 2. Photo-images of nanoemulsions containing essential oil.*

formulated nanoemulsions was noted at 600 nm with UV-VIS spectrophotometer. It determines qualitative and quantitative analyses of samples [12]. Photo-images of nanoemulsions prepared are shown in Figure 2. As it can be seen that emulsions prepared with Tween 20 are turbid, while those prepared with both surfactants are transparent. This is because of lower diameter leading to more transparent solutions. UV-VIS evaluates amount of light absorbed by the bioactive sample by measuring light intensity. The results of UV-VIS analysis is presented in Figure 3. As per absorbance (Abs600) results, addition of SDS decreased Abs at 600 and increased transparency. % Transmittance of F1 nanoemulsion was about 94% while those of F2 formulations were 99%. Tomaszewsk et al [13] cited that optical properties of nanoemulsion are very sensitive to size, shape and concentration changes.

3.2. Characterization using UV-VIS, Fluorescent and FT-IR Spectroscopy

Spectroscopic techniques have become important analytic tools for qualitative and quantitative analysis of bio active components from plants. UV-VIS, fluorescent and FT-IR spectroscopy methods together or separate can be used in this sense as conventional methods [13]. Therefore, in the present study, UV-VIS and FT-IR techniques are employed to characterize formulated nanoemulsions. The qualitative UV Spectroscopy profile of essential oil revealed different sharp peaks from 200-250 nm with absorbance of 2.0-2.5 (Figure 3) , indicating the accumulation of secondary metabolites. In UV profile, occurrence of peaks at 200-250 nm reveals the presents of flavonoids, and phenolics in essential oil. Our earlier study by using phytochemical assays and GC-FID on pure eucalyptus essential oil also validated the presence of phytochemicals in oil [14]. Notably, F1 and F2 nanoemulsion drastically increased absorbance. Enhanced absorption of nanoemulsions formulation may be attributed to large surface area due to small particle size of nanoemulsions.

Table 3*. FT-IR analysis of pure oil.*

Peak Number	X (cm-1)	Y (%T)	Vibration mode	Functional grp	Intensity
1	2921.01	74.35	C-H stretching	alkene	Medium
2	1457.23	87.44	C-H bending	Alkane	High
3	1375.66	84.23	S=O stretching	Sulphonamide	High
4	1364.04	85.89	S=O stretching	Sulphonamide	High
5	1214.68	84.59	C-O stretching	Ester	High
6	1166.81	88.08	C-O stretching	Aliphatic ether	High
7	1079.67	83.68	C-O stretching	Primary alcohol	High
8	1053.53	87.65	C-O stretching	Primary alcohol	High
9	985.26	77.68	C=C bending	Alkene	Medium
10	880.27	85.57	C-H bending	1,2,4 trisubstituted	High
11	843.08	87.19	C=C bending	Alkene	High
12	787.59	89.34	C-H bending	1,3 disubstituted	High

The florescent emission spectra of pure essential oil and F1/F2 nanoemulsions are shown in Figure 4. As illustrated in Figure 3, with pure oil, one minor peak in green fluorescent region (GF, 500nm) region was detected. Surprisingly, when pure oil was entrapped into nanoemulsions (F1 and F2), a sharp increase in fluorescent intensity was observed. This observation was in consonance with report of Boni et al [15]. It effect may ascribed to scattering of light by spherical nano droplets. The droplets act as optical resonator signal amplifying the florescent signal. F2 nanoemulsion showed more intensity than F1 nanoemulsions. Two clear peaks near red fluorescent region (RF, 400 nm) region (fingerprints) were detected. Based on the spectra it was suggested that at least two different florescent substances possibly presented in the eucalyptus essential oil. It was reported that candidates for GF emission (lambda near 500 nm) are, flavonoids, terpenoids and flavins [16,19]. Another set of bioactive compounds, as evidenced by minor peak, were also detected in red-florescent region (RF) may be due to accumulation of other fluorescent compounds like anthocyanins, phenolics, alkalioids and aromatic benzenoids.

Fourier Transform Infrared Spectrophotometer (FT-IR) is perhaps most powerful, rapid, non destructive method to determine the nanoemulsions of plant extracts or powders and for detecting types of bonds and functional groups present in the extracts [17]. The IR spectrum region 3000-600 cm-1, (fingerprint) contains absorption bands that characterize the molecular structures by vibrations of the spectrum like deformation, combining, harmonic bands. By interpreting the IR absorption spectrum, the chemical bonds in a compound can be determined. FT-IR peak values profile and functional groups are tabulated in Figure 4B and Table 3. Essential oil showed the presence of bio active compounds. In FT-IR spectrum gave a broad peak at 2921 cm-1 which indicated the presence of C-H stretching due to alkenes. It showed peaks at about 1457 cm-1 attributed to C-H bending due to alkanes. The peak around 1214 and 1079 were due to esters and alcohols. There is out of plane bending vibration peaks at 3500 cm^{-1}. One common peak near 1500 cm^{-1} due to C-H bending for alkanes groups was detected in pure oil and both F1 and F2 formulations. FT-IR spectra illustrated that essential oil encapsulated in

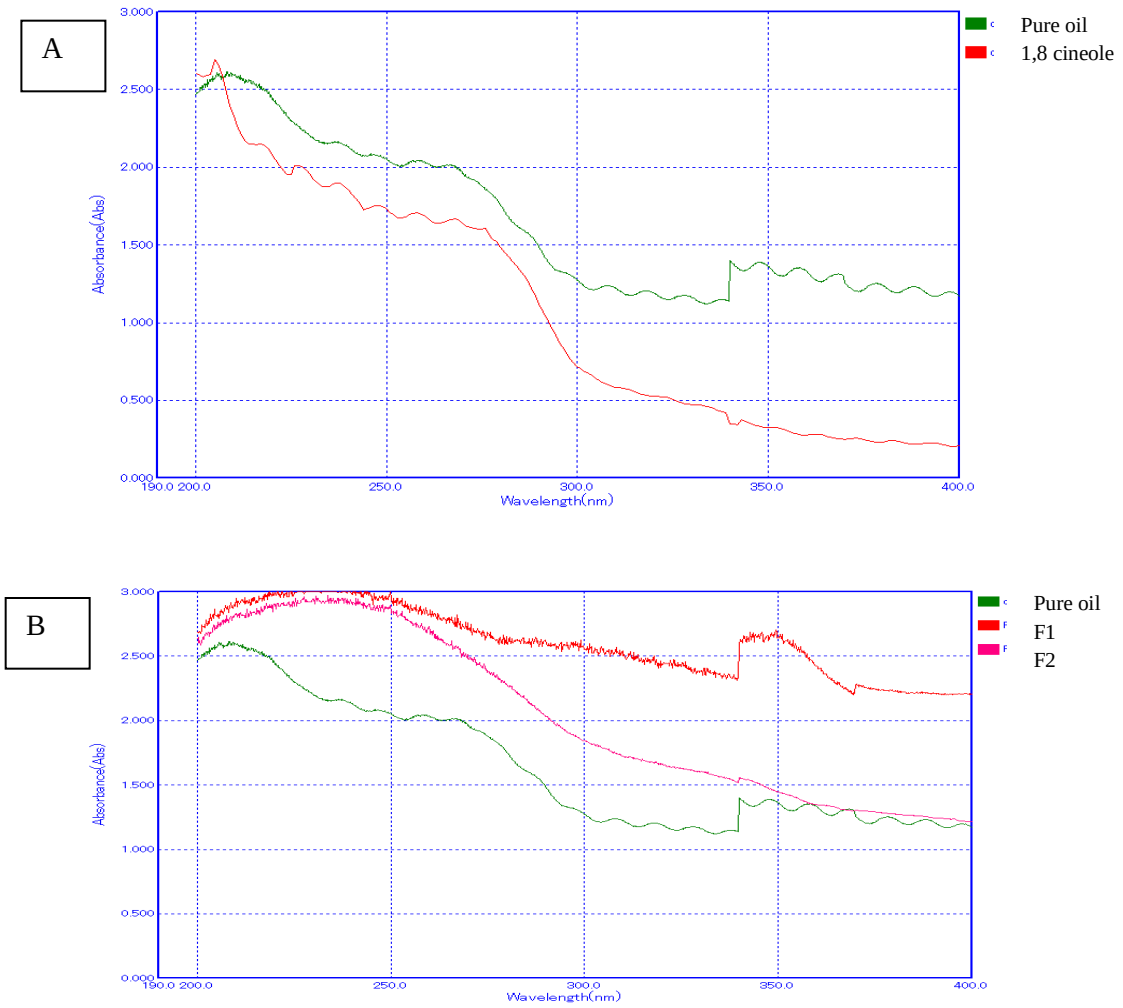

***Figure** 3. **(A)**: UV-VIS spectra of eucalyptus essential oil and pure 1,8 cineole (eucalyptol). **(B):** UV-VIS spectra of nanoemulsions and pure essential oil.*

nanoemulsions droplets. FT-IR spectrum confirmed the presence of aromatic compounds, alcohols, phenols, alkanes, alkynes, and amines in plant extracts [18]. All these compounds belong to the secondary plant metabolites as per researcher explanations [18]. The presence of above said secondary metabolites could be reason for its various medicinal properties of eucalyptus essential oil.

3.3. Storage Stability

One of the major factors in preparation of nanoemulsions is their stability over the time. For using as anti-microbial or anti-bacterial agents in delivery system, long term stability is prerequisite. Stability studies were conducted to assess stability of F1 and F2 based nanoemulsions under various environmental stresses like factors. Thermodynamic studies were conducted by storing the nanoemulsions for under dispersed and freeze dried conditions. No substantial change in size of nanoemulsion was observed (Figure 5). The formulation was further chosen for centrifugation studies. No sign of creaming phase separation or cracking was observed. For determining the long term stability and shelf life of nanoemulsions, accelerated stability studies were performed at three different temperatures for 60 days. Based on the

results, in case of F1 nanoemulsions, particle size did not changed much at 4°C and -20°C, but particle cracking was observed at 37°C (Figure 6). While in F2 nanoemulsions, although no substantial change in size was observed at 4°C and -20°C, however, Ostwald repining was observed at 37°C. Ostwald repining is the conversion of small droplets to larger ones due to diffusion of oil molecules through intervening aqueous phase [20]. Therefore, low temperature was needed for protection of nanoemulsions to prevent particle growth and agglomeration, flocculation, coalescence, phase separation or Ostwald repining. The formulated nanoemulsions were physically stable over the 60 days months of storage which makes them interesting candidate for the practical applications.

3.4. Anti-Protein Denaturation Activity

Protein denaturation is the process in which native proteins lose their tertiary and secondary structures. Protein denaturation is well established cause of tissue inflammation which is associated with indications like redness, pain, heat, swelling and loss of function at that area of tissues [12]. The major reason behind loss of protein functions is the disruption of hydrogen, hydrophobic and disulphide bonds in protein structures. Hence it was deduced that compounds which are able to prevent these changes and inhibit heat induced protein denaturation have potential to be used as therapeutic anti-inflammatory drugs. In the present study protein denaturation inhibitory study of essential oil was investigated by tryptophan fluorescent spectroscopy technique [18]. So as a part of investigation on mechanism of the anti-inflammatory activity, ability of formulated nanoemulsions to inhibit protein denaturation was studied. Typical fluorescence spectra for denatured BSA and F1 and F2 nanoemulsions are shown in Figure 7. F1 formulation exhibited a minor decrease in inhibition of protein denaturation, as fluorescence intensity decreased marginally after the addition of F1 nanoemulsions to BSA. However, after the addition of F2 nanoemulsions to BSA, a marked decrease in fluorescence intensity was observed. These results clearly indicate the potential of eucalyptus essential oil based nanoemulsions as an anti-inflammatory agent. The anti-denaturation activity may be ascribed to complex mixtures having multiple compounds which may interact synergistically. The present findings were in corroboration with earlier studies reported on bioactive components from plants [12].

3.5. Antibacterial Properties of Nano-emulsions

Eucalyptol (1,8 cineole), owing to lipophilic in nature, has the capability to penetrate plasma cell membrane of bacteria. It leads to breakdown of cell membrane integrity followed by leakage of cellular components and loss of ions. All these changes affect many cellular activities like energy production (membrane-coupled), membrane transport, and other metabolic regulatory functions and eventually cell death [20]. The antibacterial activity of nanoemulsions was measured against gram-negative bacteria *Escherichia coli* and *Streptomyces aureus*. Both F1 and F2 formulations showed considerable anti-bacterial activity (Figure 8A,B). It may be attributed to easier access of essential oil to the bacterial cells. The antibacterial activity was in according to earlier reports from researchers [3]. Nanoemulsions can keep essential oils from evaporation and decomposition during heat, sunlight and pressure [20]. Literature revealed that the antibacterial properties of nanoemulsions highly depend upon the formulation of the nanoemulsions [9].

3.6. Drug Release Study and Kinetics

In-vitro drug diffusion studies of nanoemulsions were carried out using dialysis bag method. The data of percentage essential oil release formulation was shown in Figure 9. It was found that *in- vitro* drug release from F1 formulation was about 25 % while F2 nanoemulsions led to an increase in dissolution rate with maximum essential oil release of 50% in 24h. In order to completely understand the mechanism of essential oil release, kinetics of essential oil from F1 and F2 nanoemulsions are described using four kinetic models such as : such as : % drug release vs. time (zero order kinetic model); log % drug remaining vs time (first order kinetic model); % drug release vs square root of time (Higuchi model); log % drug release vs log time (Korsmeyer–Peppas model). These model were used to evaluate the kinetic and mechanism of essential oil release from nanoemulsions. All Plots are shown in Fig. 10, 11. The model that best fits the release of essential oil is based on correlation coefficient (R) values in various models. The model that gives high 'R^2' value is considered to be the best fit for essential oil release. On the basis of best fit with the highest correlation (R^2) value it is concluded that in the optimized F1 and F2 formulation of nanoparticles follow the Korsmeyer-Peppas model with release exponent values n = 0.38 and 0.37 respectively. The magnitude of the release exponent 'n' indicates the release mechanism is non Fickian diffusion.

4. CONCLUSION

EO based nanoemulsions have obtained greater interest as pharmaceutical, drug delivery, food products and cosmetic formulations. Nanoemulsions of *Eucalyptus globulus* essential oil and different surfactants were prepared and investigated in this study. Various physiochemical properties of formulated nanoemulsions such as pH, size and conductivity were measured after preparation and after 3 months of storage in order to check the stability of the nanoemulsions at room temperature. The physical stability was good and suitable for 50 days at low temperature with not much phase separation or particle growth. Eucalyptus essential oil showed substantial antibacterial activity. The prepared eucalyptus oil based nanoemulsion could be a cost effective method with apt potential in an array of applications in variety of fields like food, cosmetic and pharmaceutical in future.

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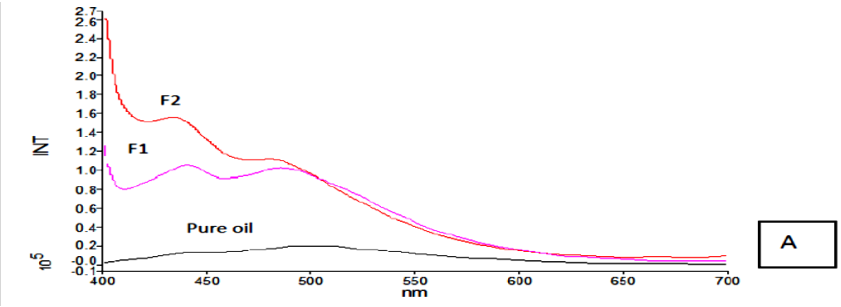
AUTHORS' CONTRIBUTIONS

ADS: Designed the problem and written MS.

IJK: analysis antimicrobial analysis.

NS: analysis of samples by FT-IR and Fluorescent.



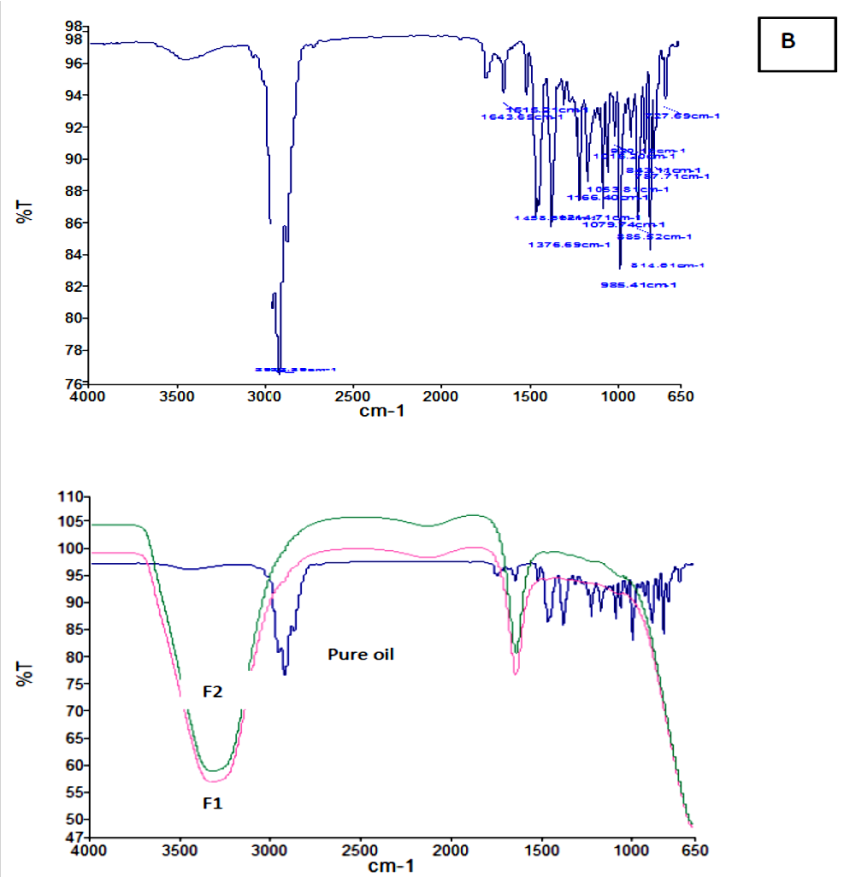


***Figure** 4. Fluorescent spectral analysis (A), FT-IR analysis of Nanoemulsions (B).*

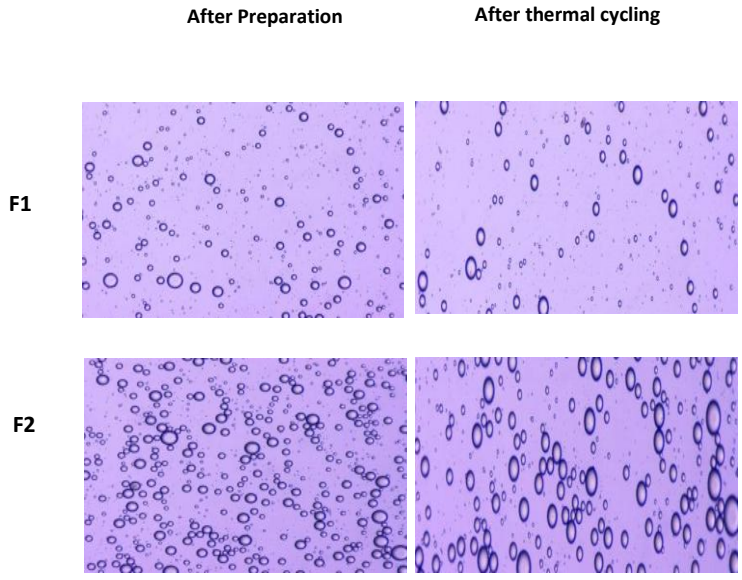


***Figure** 5. Thermodynamic studies of F1 and F2 nanoemulsions.*

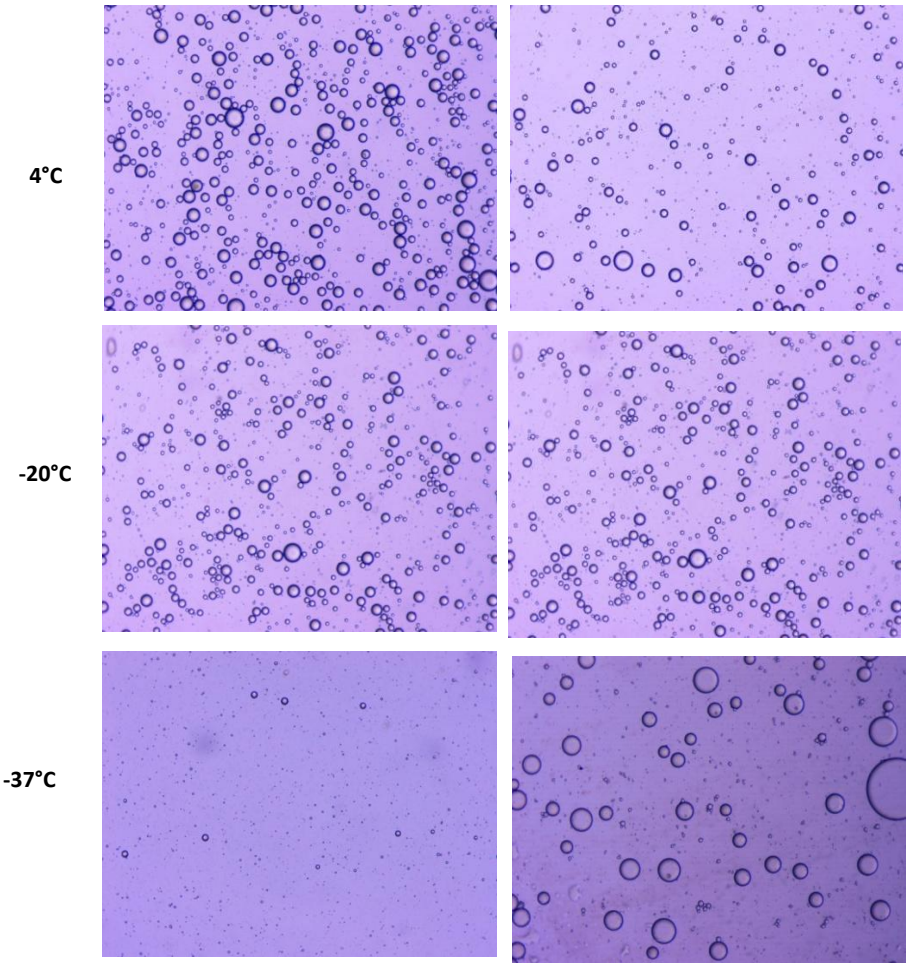


***Figure** 6. Storage stability of nanoemulsions after 50 days at different temperatures.*

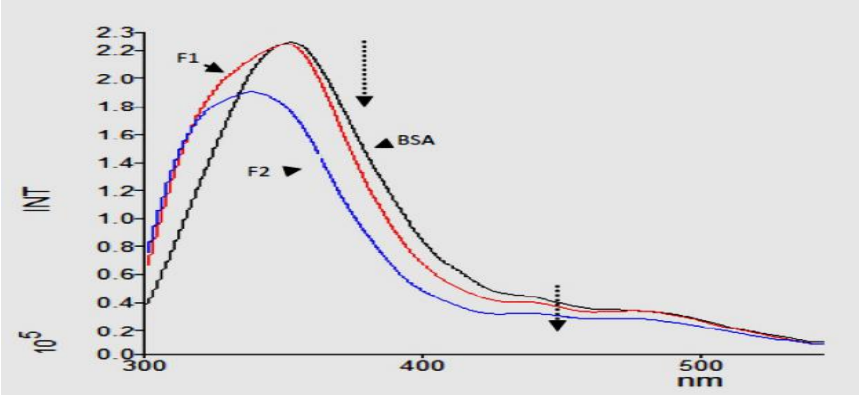


***Figure** 7. Protein denaturation (tryptophan fluorescent spectroscopy) analysis of nanoemulsions. F1: Tween 20+oil, F2: Tween 20=oil+sds*

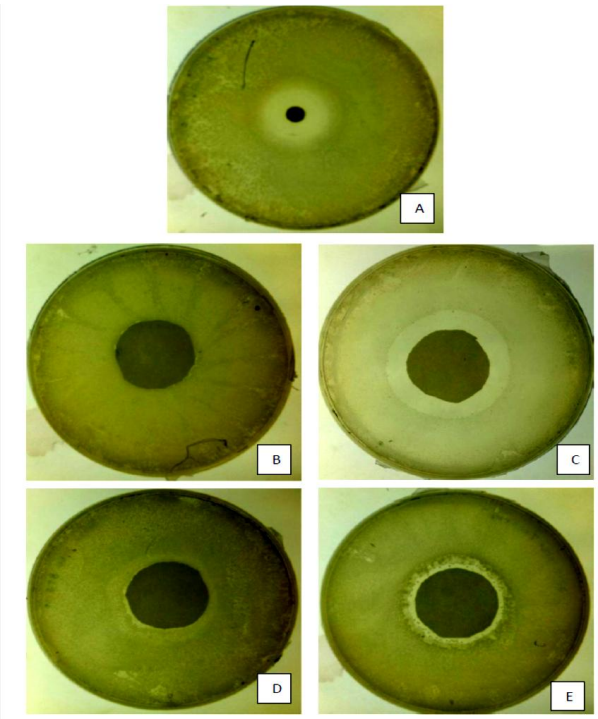


Figure 8A. *Antibacterial property of Eucalyptus globulus essential oil and formulated nanoemulsion against S. aurius (MTCC 3160) A = Vancomycin 30 Mg (Positive control), B= Blank (Tween 20), C= Tween 20 + Oil (100 µL), D= Blank (Tween 20+SDS), E= Tween 20 + SDS+Oil (100 µL).*

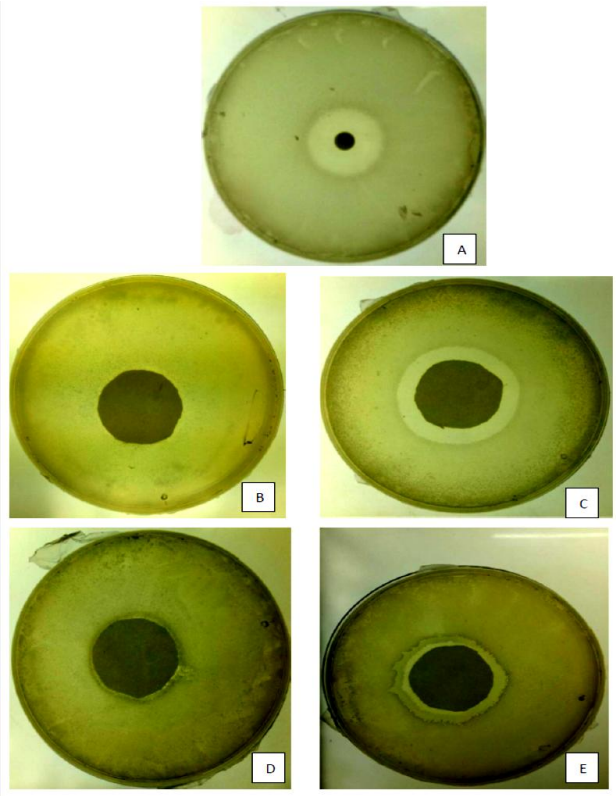


Figure 8B. *Antibacterial property of Eucalyptus globulus essential oil and formulated nanoemulsion against E. coli (MTCC 40) A = Vancomycin 30 Mg (Positive control), B= Blank (Tween 20), C= Tween 20 + Oil (100 µL), D= Blank (Tween 20+SDS), E= Tween 20 + SDS+Oil (100 µL)*

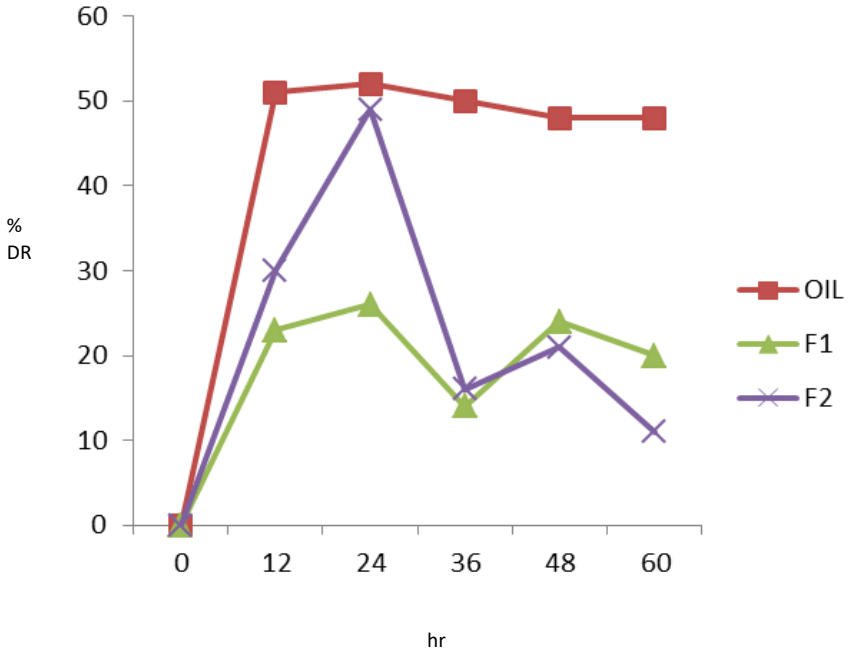
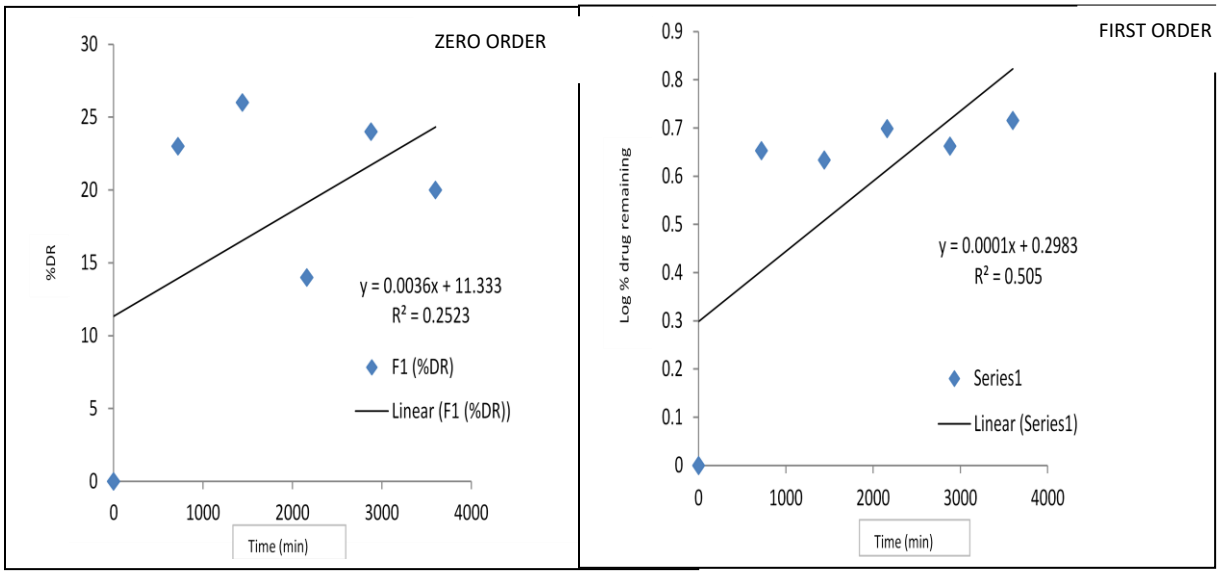


Figure *9. In vitro release profile of nanoemulsions and pure oil. %DR: drug release.*



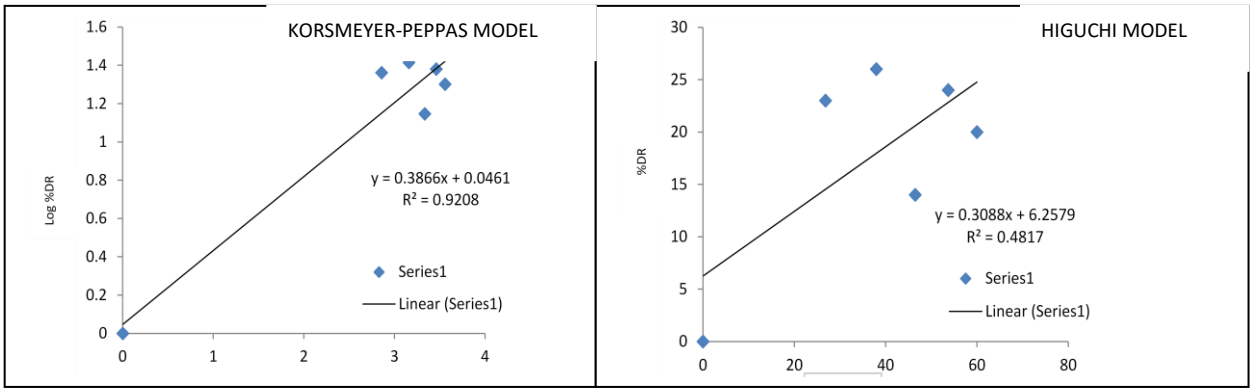


Figure 10. *Drug release kinetics of F1 nanoemulsions.*

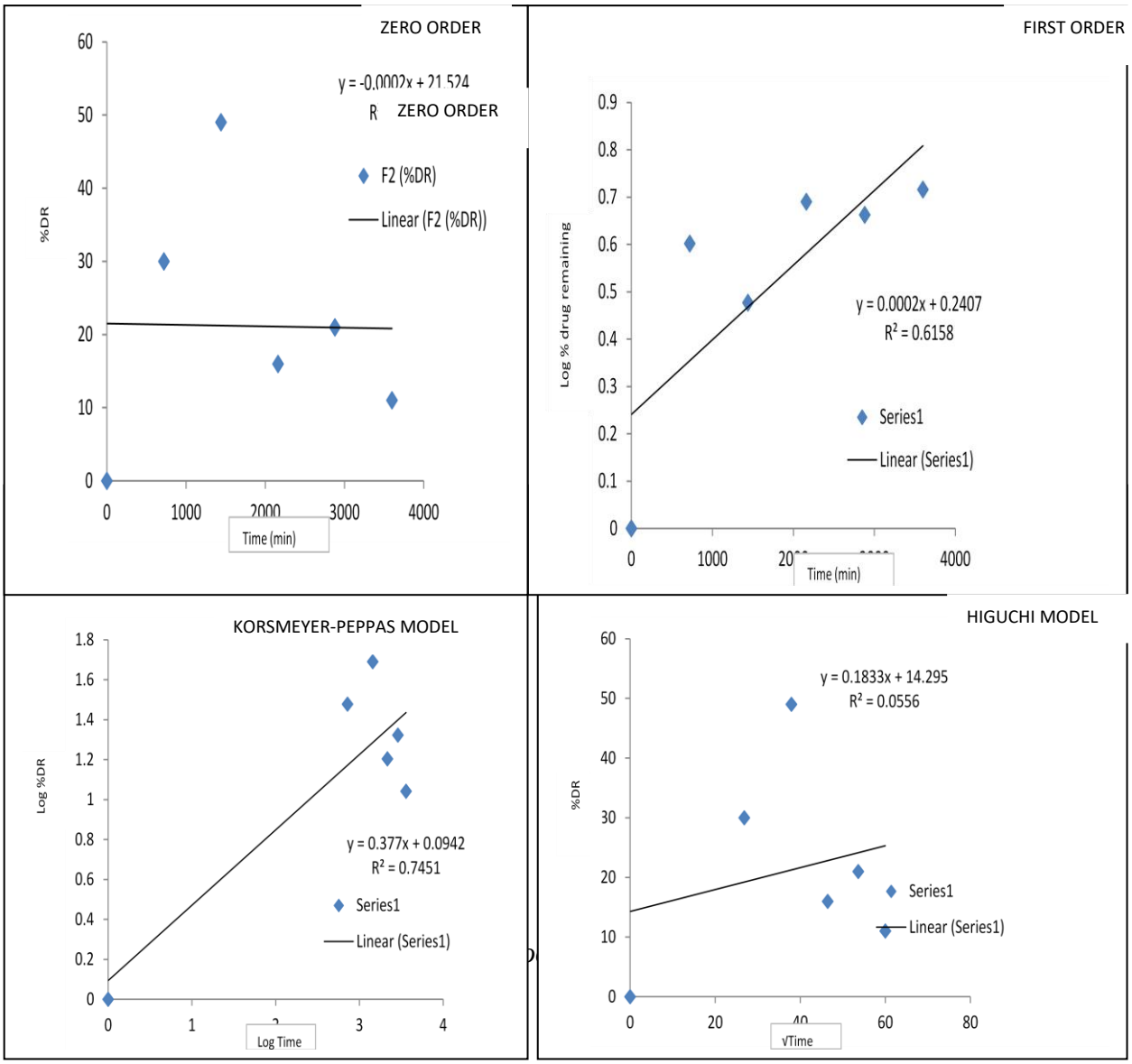


Figure 11. *Drug release kinetics of F1 nanoemulsions.*

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