Levofloxacin loaded clove essential oil nanoscale emulsion as an efficient system against *Pseudomonas aeruginosa* biofilm

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Abstract

Biofilm formation by bacteria has become one of the major reasons for escalation of antimicrobial resistance. The present research focused on the development, optimization and evaluation of antibiofilm potential of clove oil nanoscale emulsion (o/w) loaded with levofloxacin (LFX-NE) against *Pseudomonas aeruginosa* biofilm. The optimized NE was selected through the construction of pseudo-ternary phase diagrams and was found to be stable. The particle size and drug content of LFX-NE was found to be 18.84 ± 0.5 nm and 99.2 ± 0.5 %, respectively. It demonstrated high antibacterial activity with 16-fold and 8-fold reduction in MIC of LFX against *P. aeruginosa* and *Escherichia coli* and *Klebsiella pneumoniae*, respectively. LFX-NE was able to eradicate pre-formed biofilm of *P. aeruginosa* as evidenced by field emission scanning electron microscopy and confocal laser scanning microscopy. Antiquorum sensing study revealed inhibition of quorum sensing activity. LFX-NE was found to be non-irritant and cytocompatible as confirmed by HET-CAM test and safety study in normal human epidermal keratinocytes (NHEK) cell line. This study demonstrates the potential of LFX-NE in possible treatment of non-healing wounds inflicted with bacterial biofilm.

Keywords: Antibiofilm, clove oil, levofloxacin, HET-CAM, antimicrobial resistance

1. Introduction

The advent of antibiotics ushered mankind into an era of prosperity and higher life expectancy. It made possible to conduct complex surgeries with very low mortality rate. But even the antibiotics could not be saved from the human tendency to abuse. Overuse and misuse of antibiotics led to antimicrobial resistance (AMR) which at present has taken a shape of global epidemic. It has been estimated that by 2050 antibiotic resistant infections will account for more than 10 million deaths annually [1]. One of the major contributing factors in development and progression of AMR is biofilm formation by bacteria. Biofilms are sessile communities of bacteria which can form and adhere firmly on any abiotic or biotic surfaces. Biofilms are composed of extracellular polymeric substances (EPS), composed of polysaccharides, proteins and extracellular DNAs which forms a matrix enclosing the bacteria within it and making them over 1000 times resistant to antibiotics [2]. Biofilms are implicated in diverse range of diseases such as wounds, otitis media, cystic fibrosis and osteomyelitis where they grow on tissues and implanted devices [3]. Antiphagocytic property of biofilms, make leukocytes ineffective against them, thereby hampering the host immune response to the infection [4]. *Pseudomonas aeruginosa* has been implicated in variety of infections owing to its ability to establish chronic infections by the means of biofilm formation [5]. It is second most common pathogen after *Staphylococcus aureus* found in chronic wounds [6]. Systemic treatment with antibiotics is unable to clear the wound infection due to the presence of biofilm. Moreover, higher concentrations of antibiotics are required to kill the bacteria present within the biofilm leading to systemic side effects such as hepatotoxicity, changes in heart rhythm etc [7, 8]. Hence, topical treatment of chronic wound with an antibiotic aided by the use of an antibiofilm agent can prove to be an efficient therapy. Levofloxacin (LFX), a third generation fluoroquinolone was selected for this study as it has wide spectrum of activity against gram negative and gram positive bacteria, and it is well prescribed for skin and skin structure infections [9]. Slow growing and non-growing bacteria have been reported to be susceptible to fluoroquinolones [10] and LFX has been shown to have a killing effect on *P. aeruginosa* biofilms *in vitro*.

Esseential oils are secondary metabolites produced by aromatic plants and recently, they have garnered enormous attention due to their reported antibacterial and antibiofilm activities [11, 12]. Clove oil, an essential oil with gamut of medicinal properties is derived from different parts of

clove tree (Syzygium aromaticum). Eugenol is the major component of clove oil and has been reported to possess antibiofilm activity against biofilms of *S. aureus*, *Escherichia coli* and *P. aeruginosa* [13-15]. It has also been known to produce anti-inflammatory effect by preventing neutrophil chemotaxis and prostaglandin production. Other constituents of clove oil include eugenyl acetate, caryophyllene, pinene, flavonoids such as quercetin and phenolic acids such as ellagic acid, ferulic acid [16]. A study by Hemaiswarya and Doble [17] demonstrated synergistic effect of eugenol with various antibiotics against five gram negative bacteria. Thus, reduction in antibiotic dose required for treatment can be achieved leading to decrease in side-effects. To this effect and circumvent hydrophobic nature of clove oil, we aimed to use nanoscale emulsion (NE) system for its encapsulation by emulsification and to bring together antibacterial and antibiofilm properties of LFX and clove oil, respectively. The term nanoscale emulsion is an umbrella term for emulsions which have globule size in nanometer range which includes both micro and nano-emulsions. Particularly, here the nanoscale emulsion system refers to microemulsion since they have been prepared spontaneously by using low-energy method and the globule size obtained for the LFX loaded NE was found to be < 20 nm. Therefore, to accurately reflect the nano character, the term nanoscale emulsion has been used here. Moreover, the term microemulsion is a misnomer and there has been, and there still is, confusion in the literature with regard to the correct use of the terms microemulsion and nano-emulsion.

Rationale for this design was that clove oil will act both as an oil phase for NE and an antibiofilm agent. NEs are transparent, isotropic and thermodynamically stable colloidal dispersions and can be formed spontaneously without the input of any external energy. NEs are stabilized by surfactant layer around the oil droplets, thereby preventing them from coalescing. The advantages of NEs include easy scale up, increased drug solubility, reduction of side-effects [18, 19]. Morever, NEs can protect EOs from degradation by light, air and temperature and preserve their volatility [20].

The present work encompasses development and characterization of LFX loaded clove oil nanoscale emulsion, followed by detailed evaluation of antibacterial and antibiofilm activity of the system. Cytocompatibility of the developed formulation was also assessed by carrying out HET-CAM and cell line studies. This work can be instrumental in providing a basis for potential treatment of biofilm-infected chronic wounds.

2. Materials and Methods

2.1 Materials

Levofloxacin (LFX) was procured ex-gratia from Pinnacle Life Science Pvt. Ltd, Baddi, Himachal Pradesh, India. Clove oil was purchased from Katyani Exports, New Delhi, India. HPLC grade acetonitrile (ACN) was purchased from Merck specialities Pvt. Ltd. (Mumbai, India). Tween 80 and PEG-300 were procured from M/s Fisher Scientific, India. Microbial culture media were obtained from Himedia Laboratories Pvt. Ltd, Mumbai, India. All other materials used were of analytical grade. Normal human epidermal keratinocytes (NHEK) were purchased from Clonetics™, Lonza, Switzerland.

2.2 Bacterial strains

Standard strains of *Klebsiella pneumoniae* B5055, *Pseudomonas aeruginosa* PAO1, *Escherichia coli* 25922 and methicillin resistant *Staphylococcus aureus* (MRSA) 43300 used in present study, were available in the microbiology laboratory. Throughout the period of study bacterial cultures were maintained on the nutrient agar slants.

2.3 Ultra High Performance Liquid Chromatography (UHPLC) assay

The UHPLC analysis of LFX was performed on Shimadzu, Prominence UFLC. LFX was analyzed using a ShiSeido® reverse phase C18 (250 ×4.6 mm, 5 μm particle size) column and the mobile phase constituted of water:ACN in ratio (80:20, v/v) with 0.3% triethylamine and pH adjusted to 3.3 with o-phosphoric acid. The mobile phase was degassed and passed through 0.22 μm membrane filter before pumping into the HPLC system and flow rate was set at 1 ml/min. The column temperature was set at 25º C. The injection volume of the sample was 20 μl and the eluent was analyzed at 295 nm using photo-diode array detector. The standard plot was calibrated in the concentration range of 1–50 μg/ml [21].

2.4 Equilibrium solubility study

Solubility of LFX was determined in various oils (Clove oil, lemongrass oil, eucalyptus oil, sandalwood oil and oleic acid), surfactants (Tween 80, Poloxamer 407, Tween 20, Span 80) and co-surfactants (PEG-300, PEG-600, PG, IPA). Excess amount of drug was added to 1 ml each, of all the excipients in tightly closed vials and allowed to solubilize at 37 ± 0.5 °C for 72 h under the influence of continuous stirring in a water bath shaker. Then the contents of vials were centrifuged at 3000 rpm for 15 min and supernatants (diluted suitably with methanol) were quantified for the

amount of LFX solubilized via UHPLC at 295 nm, after filteration through a 0.45 µm membrane [22].

2.5 Surfactant miscibility study

Selection of appropriate surfactant or surfactant mixture (S_{mix}) is vital for developing thermodynamically and kinetically stable nanoscale emulsion. Thus, the miscibility of clove oil and surfactants (Tween 80, Poloxamer 407, Tween 20, Span 80, Brij 35) was determined by mixing them on a vortex mixer in 1:1 ratio for 5 min and kept for 1 h at room temperature. Surfactants rendering a clear and transparent binary mixture were classified as miscible while those that gave opaque mixtures were classified as immiscible with clove oil [23].

2.6 Psuedoternary phase diagram and preparation of LFX loaded o/w nanoscale emulsion

Pseudoternary phase diagrams were constructed using an aqueous titration method. Based on the solubility study for LFX, Tween 80 and PEG-300 were chosen as surfactant and co-surfactant, respectively whereas clove oil constituted the oil phase. Three phase diagrams were prepared using Tween 80 and Tween 80: PEG-300 (S_{mix}) in ratios 1:1 and 2:1. From constructed pseudoternary phase diagrams, the optimized LFX loaded nanoscale emulsion (LFX-NE) was prepared [23]. Briefly, LFX (1% w/v; 200 mg) was solubilized in clove oil (6% v/v; 1.2 ml) under continuous magnetic stirring at 300 rpm, followed by addition of S_{mix} [2:1] (25% v/v; 5 ml) and distilled water (69% v/v; 13.8 ml) dropwise to form 20 ml of clear and transparent LFX-NE which formed spontaneously. It was allowed to equilibrate at room temperature.

2.7 *In vitro* characterization studies

2.7.1 Thermodynamic stability studies

LFX-loaded nanoscale emulsion was evaluated for thermodynamic stability by subjecting to centrifugation test, freeze-thaw cycle and heating–cooling cycle [22]. Physical stability evaluation was done by continuous monitoring of NE for any phase separation and turbidity.

2.7.1.1 Centrifugation Studies

LFX-loaded nanoscale emulsion was studied for phase separation by subjecting the samples to centrifugation at 5000 rpm for 30 min using REMI R-8C centrifuge system.

2.7.1.2 Freeze-Thaw Cycle

Selected samples of LFX loaded nanoscale emulsion were subjected to three freeze-thaw cycles by storing the samples at − 20 °C for 24 h in deep freezer and subsequent thawing at room temperature. The samples were observed for any phase separation and drug precipitation.

2.7.1.3 Heating–Cooling Cycle

LFX loaded nanoscale emulsion was subjected to three cycles of heating-cooling by alternating between 4 °C and 40 °C for 24 h each. Samples without any phase separation, turbidity, or drug precipitation were deemed to be thermodynamically stable.

2.7.2 Short-term stability studies

Short-term stability studies for optimized formulation (LFX-NE) were carried out for 3 months at 25 ± 0.5º C and 40 ± 0.5º C. The samples were analyzed for particle size, PdI, zeta potential and visual appearance at intervals of 0, 1 and 3 months.

2.7.3 Particle size, polydispersity index (PDI) and zeta potential

Particle size, PDI and zeta potential of optimized formulation (LFX-NE) were measured by using Malvern ZS90 zetasizer (Malvern Instruments, UK), wherein light scattering was monitored at 25ºC at 90º angle. The NE was diluted 10-fold in distilled water prior to the analysis. Measurement was carried out in triplicate.

2.7.4 High-Resolution Transmission Electron Microscopy (HRTEM)

For HRTEM analysis, LFX-NE was diluted 100 times and a drop was placed over Formvar-coated copper grid. The excess fluid was drained using Whatman filter paper and sample was stained negatively with 0.5% w/v phosphotungstic acid for 5 min at room temperature. The excess acid was removed using Whatman filter paper and the grid was allowed to dry for 5 min. Subsequently, the grid was examined under HRTEM (JEOL, JEM 2100 Plus, Tokyo, Japan) operating at 80 kV [23].

2.7.5 % Drug content

% Drug content of LFX-NE was analyzed by using UFLC at 295 nm by appropriately diluting 1 ml of NE in methanol and injecting the sample after filtering through 0.45 µm nylon filter.

2.7.6 % Transmittance and pH

Percent transmittance of LFX-NE was determined using UV-visible spectrophotometer (UV 3000+, LabIndia, Mumbai, India) at 650 nm using distilled water as blank. pH of the formulation was measured at room temperature using a pH meter (CyberScan pH 51, Eutech Instruments).

2.7.7 Viscosity

The viscosity of the optimized NE was determined using Rheolab QC rotational rheometer (Anton Paar, Germany). The sample (2 mL) was filled in the sample holder, and shear rate (1-130 s^{-1}) was applied to the sample, and corresponding viscosity was recorded using Rheoplus instrument software at constant temperature of 25 °C.

2.7.8 Fourier Transform Infrared Spectroscopy (FTIR) analysis

To ascertain any physico-chemical interactions between the drug (LFX) and excipients, FTIR study was carried out. The FTIR spectra was recorded on PE-1600 (PerkinElmer, USA) spectrophotometer in the spectral region of 4000-400 cm^{-1} using KBr disc method.

2.8 *In vitro* drug release study

The *in vitro* release study of optimized nanoscale emulsion (LFX-NE) was carried out in phosphate buffered saline (PBS) pH-7.4 using semi-permeable dialysis membrane (Himedia; MWCO: 12 kDa) on Franz diffusion cell assembly at 32 ± 0.5 °C. The process was conducted for the LFX-NE and LFX in free form (free LFX solution) in order to compare their *in vitro* release behaviour. Free drug solution of LFX was prepared in double distilled water. Before conducting the experiment, the membrane was submerged in the acceptor fluid (PBS-7.4) for 24 h. The dialysis membrane was clamped between the donor and the receptor compartment, and LFX-NE/free-LFX containing equivalent to 10 mg of drug was applied to it. The receptor compartment was filled with 25 ml of PBS-7.4 and stirred at 100 rpm using magnetic stirrer. 1 mL aliquot of samples were withdrawn at regular time intervals from the sampling arm, and replaced with fresh PBS to maintain the sink conditions. The study was carried out for a period of 360 min (6 h). The samples were then analyzed via HPLC at λ_{max} 295 nm. Further, drug release curves of the optimized formulation and free drug were constructed by plotting a mean percent drug released against time period with standard deviations (SD) [24].

2.9 Microbiological studies

2.9.1 Agar well diffusion assay

Well diffusion assay was carried out to evaluate the antibacterial potential of LFX-NE against a wide array of pathogens. 100 µl of log phase culture (10^5–10^6 CFU/ml) of *K. pneumoniae*, *P. aeruginosa*, *E. coli*, and *S. aureus* (MRSA) were spread plated on different nutrient agar plates. With the help of 5 mm sterile gel puncher, wells were punched in the plates and 50 µl of LFX-NE (containing 10 mg/ml of LFX), blank NE, LFX and CO were filled in each well. Afterwards, the plates were incubated at 37°C overnight and next day, zone of inhibition was measured, and diameters of the zones were reported in the mean of triplicates.

2.9.2 Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

MIC and MBC of LFX-NE was determined by broth micro-dilution method as put forward by National Committee for Clinical Laboratory Standards. Briefly, a broad range of dilutions (0.1-256 µg/ml) of LFX, LFX-NE and blank NE were prepared. 100 µl of each dilution was incubated with 100 µl of log culture (10^5–10^6 CFU/ml) of *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *S. aureus* (MRSA) in a 96 well microtitre plate for 18–24 h at 37°C. The minimum concentration which exhibited no visible turbidity was designated as MIC of LFX-NE against the tested pathogen. For the estimation of MBC, separate nutrient agar plates were spreaded with contents from each well and incubated overnight at 37°C. Next day, the lowest concentration showing no growth on nutrient agar plate was termed as MBC of LFX-NE.

2.9.3 Establishment and processing of *P. aeruginosa* biofilm

Establishment of *P. aeruginosa* PAO1 biofilm was performed in 96-well microtiter plate up to 7 days as described previously by Wagner *et al* [25]. Briefly, each well of the plate was filled with 100 µl of *P. aeruginosa* culture (OD_{600} = 0.5) equivalent to 10^8 CFU/ml of *P. aeruginosa*, whereas sterile nutrient broth served as control and incubated at 37ºC overnight. Post-24 h incubation, broth containing planktonic cells was removed from the wells and wells were washed thrice with PBS (pH 7.4). Then, fresh sterile fresh nutrient broth was added into each well and further incubated at 37 °C. In order to establish the biofilm, same procedure was carried out upto 7^{th} day. The biofilm formation was examined by using viable cell counting. For viable cell counting after each 24 h,

selected wells were aspirated to remove planktonic cells, washed thrice with PBS, biofilm scraped and suspended in 100 µl of PBS. Appropriate dilution of scraped cells from each well were plated on nutrient agar plates and viable count estimated after overnight incubation at 37ºC.

2.9.4 Effect of nanoscale emulsion on *P. aeruginosa* biofilm

Antibiofilm efficacy of the LFX-NE on *P. aeruginosa* biofilm was evaluated using MIC and MBC concentrations. 200 µl of LFX-NE, LFX and blank NE diluted in nutrient media broth were added to selected wells, whereas control wells were filled with 200 µl of sterile PBS upto 7 days. Microtiter plates were re-incubated for 6 h at 37ºC. Only selected wells in each plate were processed for viable count where as in the rest of wells the media was replaced and plates incubated further upto 7 days. Biofilm disruption was recorded in the form of bacterial count as compared to control wells [26].

2.9.5 FESEM analysis of biofilm

For FESEM analysis, *P. aeruginosa* biofilm was grown on glass cover slip using batch culture model in 12 well plates under aseptic conditions. 500 µl of nutrient broth and 500 µl of bacterial culture of *P. aeruginosa* (OD_{600} = 0.5) and a sterile cover slip were added to each well of the plate. Addition of this, 1000 µl of nutrient broth to last well served as sterility control. Microtiter plate was incubated at 37ºC and media was replaced with fresh media each day upto 7 days. To ascertain the antibiofilm efficacy of NE on the integrity of *P. aeruginosa* biofilm, LFX-NE at its MIC and MBC, blank NE were added to the selected wells and in case of control well, 200 µl sterile water was added on the peak day of biofilm formation. The plates were incubated again at 37ºC for 6 h. Following this, the biofilms on fourth day (which is the peak day of biofilm formation) were fixed with 2.5% gluteraldehyde, dehyrated and coated with ion sputter, and subjected to FESEM (Hitachi SU8010, Japan) in order to evaluate the structure of biofilm [27].

2.9.6 Confocal Laser Scanning Microscopy (CLSM) evaluation

Biofilm was established on coverslips as described in previous section. After overnight incubation, media from the well was removed and cover slip gently washed with normal saline thrice to remove free bacterial cells. Cover slips harboring biofilm were treated with LFX-NE at its MIC and MBC, blank NE for 6 h at 37º C on peak day (4^{th} day) and control sample was treated similarly with sterile PBS. For CLSM analysis, coverslips from 4^{th} day biofilm, were stained with Live/Dead

Baclight kit (Propidium iodide and SYTO9, Thermo scientific, USA), fixed onto glass slide and observed under confocal laser scanning microscope (SCLM, Nikon Ti Eclipse).

2.9.7 Quorum sensing inhibition (QSI) assay

The 24 h biofilm of *P. aeruginosa* PAO1 was treated with sub-minimum inhibitory concentrations of LFX-NE, blank NE and LFX. After 24 h, the amount of AHL (Acyl homoserinelactone) was determined. The catalytic efficiency of the developed system was evalutaed by calculating the change in the amount of AHLs present in the biofilm. Quorum sensing signal molecules (AHLs) produced by *P. aeruginosa* PAO1 were extracted from the culture supernatant by following standard procedure. Extracted AHLs were quantified in the culture supernatant based on its β-galactosidase activity. The culture of bio-reporter *E. coli* MG4 was diluted 1:1 in Z-buffer and assayed for β-galactosidase activity by using O-nitrophenyl-D-galactopyranoside (ONPG) as a substrate [28].

2.10 Hen egg chorioallantoic membrane (HET-CAM) irritation study

HET-CAM test is performed to assess the irritation/toxicity potential of any compound or formulation. Nine freshly fertilized hen eggs were procured from Central Poultry Development Organization, Industrial Area, Phase I, Chandigarh, India on day 0. Eggs were incubated for 9 days at 37 ± 0.5º C and 40 ± 5º C. Eggs were placed horizontally in tray during incubation period to allow the development of embryo away from CAM surface. Eggs were rotated 3 times a day to ensure viability and embryo development. Eggs were taken out on 9^{th} day and 2 ml of albumin was taken out by using syringe from small hole created on the narrow end of shell without harming the developed embryo. Eggs were sealed using paraffin film and again kept for incubation. Eggs were taken out 10^{th} day and egg shell was removed carefully from pricked and sealed site upto 4-5 cm diameter without damaging the vessels inside. Chorioallantoic membrane was exposed by carefully clearing inner membrane using forceps. 0.1 ml of LFX-NE, 0.1M NaOH (positive control) and 0.9% NaCl (negative control) were added directly onto CAM surface and scoring was taken for each egg by observing the changes such as lysis, haemorrhage and coagulation for 5 minutes. Scoring was performed to get the combined score as given in supplementary Table 2. Photographs were taken to show vascular changes in CAM [29].

2.11 *In vitro* safety study in Normal Human Epidermal Keratinocytes (NHEK) cell lines

NHEK cells were cultured in KGM™ Gold keratinocyte growth medium BulletKit™ (Clonetics™, Lonza, Switzerland) containing 0.5 ml of gentamycin-amphotericin, 0.5 ml of hydrocortisone, 0.5 ml of transferrin, 0.25 ml of epinephrine, 2 ml of bovine pituitary extract, 0.5 ml of insulin and 0.5 ml of human epidermal growth factor per 500 ml of basal medium and cells were maintained at 37 ± 0.5º C in a humidified incubator with 5% CO_2. Cells were plated in 75-cm^2 flasks and allowed to grow to ~ 80% confluency before the safety study experiment.

The cell viability for LFX, LFX-NE and blank NE was evaluated in NHEK cells in a 96-well plate using Presto blue assay. NHEK cells were seeded in 96-well plate at density of 5 x 10^3 cells/well and incubated at 37 ± 0.5º C in 5% CO_2 for 24 h. After the incubation, the culture medium was removed from the plate and replenished with 90 µL of medium per well containing various concentrations (1, 5, 10, 20, 50 and 100 µg/ml) of LFX-NE, LFX and blank NE and re-incubated for 24 h. Each concentration of formulations was repeated in three wells. Following incubation period of 24 h, 10 µL of PrestoBlue™ (Invitrogen, USA) was added to each well and incubated for a period of 1 h, following which the fluorescence was recorded using a microplate reader (Tecan, Männedorf, Switzerland), at the excitation and emission wavelengths of 535 and 612 nm, respectively. Growth inhibition by the formulations at different concentrations was calculated as % cell viability, taking the viability of the control cells as 100% [30].

2.12 Statistical analysis

Data was expressed as mean ± SD. For comparison between two groups, student's *t*-test was used whereas, for comparing more than two groups, one-way ANOVA was used. Results with level ($p < 0.01$) were considered statistically significant. Statistical analysis were carried out using GraphPad Prism 5.0.

3. Results and Discussion

3.1 Solubility study of LFX

Solubility of LFX in various oils and surfactants/co-surfactants was determined to choose the formulation excipients with maximum solubilizing capacity. Prevention of drug precipitation is an essential aspect to be kept in mind while formulation of nanoscale emulsions [29]. Hence, maintaining high solubilization of drug is of an utmost importance. LFX exhibited exceptionally high solubility of 205.1 ± 2.81 mg/ml in clove oil whereas Tween 80 surfactant showed highest

solubility of 20.3 ± 1.25 mg/ml for LFX. Among all co-surfactants screened, highest amount of LFX (17.10 ± 1.52 mg/ml) was solubilized by PEG-300. Moreover, in addition to serving as high solubilizing oil phase of NE, clove oil was also selected for its antibacterial and antibiofilm activity as reported in various studies [14, 15, 31].

3.2 Surfactant miscibility study

Clove oil demonstrated high miscibility with Tween 20, Tween 80, Brij 35 and Poloxamer 407. Uniform transparent solutions were obtained with these surfactants, whereas mixtures of clove oil with Span 80 and Span 20 showed phase separation. This can be attributed to absence of polyoxyethylene groups in sorbitan esters which can interact with –OH groups of eugenol [32]. Additionally, for preparation of o/w NE, surfactant with HLB >10 is deemed fit for the emulsification of oil. Tween 80 having HLB value of 15 was able to reduce the interfacial tension and free energy required for NE formation [33]. Although, Tween 20, Brij 35 and Poloxamer 407 have HLB values >15, Tween 80 was selected as surfactant based on the solubility study of LFX.

3.3 Pseudoternary phase diagram and preparation of Nanoscale emulsion

There are various reports of nanoscale emulsion systems (microemulsion and nanoemulsion) in the literature, but the prediction of type of oil and surfactant to be selected for a specific application is a challenge for the formulation scientist, as there are marked differences from case to case. For instance, HLB requirement of surfactants for various oils are different and phase behaviour could be affected in different ways by different drugs. Therefore, construction of pseudoternary phase diagram is very crucial for the preparation of nanoscale emulsions.

After performing the solubility study, components displaying highest drug solubility were used to construct pseudoternary phase diagrams. Three phase diagrams were constructed using Tween 80, and S_{mix} [Tween 80:PEG-300 (1:1) and Tween 80:PEG-300 (2:1)] as shown in (Fig. 1). The largest nanoscale emulsion area was obtained using Tween 80 as surfactant, followed by S_{mix} ratio of 2:1 and least area with S_{mix} ratio of 1:1. Although region of NE formation was found to be maximum with Tween 80, PEG-300 was added as a co-surfactant to evaluate the effect of its addition on NE formation. When used in ratio of 1:1, the area of NE region decreased in comparison to when Tween 80 alone was used. However, when S_{mix} ratio of 2:1 was used, there was increase in the area in comparison to S_{mix} ratio of 1:1.

The concentration of clove oil to be used in the preparation of NE was fixed at 6% v/v in order to completely solubilize the required amount of LFX (1% w/v) in NE. The concentration of S_{mix} [Tween 80:PEG-300 (2:1)] selected for the formulation of NE was 25% v/v. The reason for the selection of S_{mix} ratio of 2:1 rather than using only the surfactant Tween 80 was that minimum 25% v/v concentration of Tween 80 was required for the emulsification of 6% v/v oil. On the contrary, 25% v/v concentration of S_{mix} [Tween 80:PEG-300 (2:1)] was able to emulsify 6% v/v of clove oil. Thus, the actual concentration of Tween 80 in the NE was reduced to 16.6% v/v. PEG-300 is a FDA approved biocompatible polymer which is practically non-toxic. Although, inclusion of PEG-300 lead to minor reduction in the monophasic region as compared to use of Twenn 80 alone, it managed to reduce the concentration of Tween 80 required for emulsification of clove oil. Moreover, it is desirable to have as low a concentration of surfactant as possible to have reduced irritation/toxicity potential. For *in vitro* characterization studies, 3 NEs were selected from each phase diagram based on the lowest concentration of surfactant/S_{mix} required to emulsify the oil phase.

3.4 *In vitro* characterization

3.4.1 Thermodynamic stability

The stress tests including heating-cooling cycles, centrifugation and freeze-thaw cyles showed that all the formulated NEs covering the entire region of the phase diagrams were found to be physically stable. They exhibited no visual signs of phase separation, drug precipitation, creaming or cracking.

3.4.2 Short-term stability studies

The stability studies for LFX-NE were performed at 25 ± 0.5º C and 40 ± 0.5º C for a period of 3 months. Supplementary Table 4 shows results for the stability studies. Also, supplementary Fig. 2 depicts particle size distribution graphs for LFX-NE after 3 months of stability testing. There were no significant changes in the particle size, PdI, zeta potential and visual appearance of the LFX-NE after 3 months of testing. Thus, the optimized formulation LFX-NE can be considered a stable system.

3.4.3 Particle size, PDI and zeta potential

The NEs were homogeneous, transparent and slightly yellowish in colour. The droplet size and PDI ranged between 18-22 nm and 0.140-0.206, respectively, demonstrating narrow size

distribution. LFX-NE showed lowest particle size of 18.84 nm as compared to 21.89 nm of LFX-NE-2 (Table 1). This can be attributed to addition of PEG-300 as co-surfactant which aids in reduction of interfacial tension and provides fluidity to surfactant film [34]. In particular context of biofilm and bacteria, particle size is a vital factor determining the antibacterial efficacy. Such a low droplet size facilitates enhanced contact of NE droplets around the bacterial cell due to increased surface area [29]. The zeta potential of developed NEs ranged from -3.44 to -5.72 mV. The negative charge ensures sufficient electrostatic repulsion between the droplets, thus keeping the NE stable. This minimizes chances of coagulation of the system when exposed to biological environment and during the storage (Table 1).

3.4.4 Transmission electron microscopy (TEM)

TEM analysis revealed spherical globules as seen in photomicrographs (Fig. 2A-B). The oil droplets were found to be randomly dispersed and no agglomeration was observed.

3.4.5 % Drug content and viscosity

The drug content of NEs ranged from 95.5-99.9 %. The viscosity of NEs was in the range of 175-206 mPa.s upon the application of shear rate from 1-100 s^{-1} (Table 1). NEs exhibited pseudoplastic flow (shear thinning) i.e. visocity decreased as the shear rate increased (supplementary Fig. 1).

3.4.6 % Transmittance and pH

Percent transmittance of NE is indicative of optical clarity. The NEs were found to be optically clear with transmittance values ranging from 96.1- 99.2 %. pH of the NEs was found be compatible with skin and ranged between 6.2-6.6 (Table 1).

3.4.7 FTIR study

FTIR spectrum of LFX, CO, Tween 80, blank NE and LFX-NE is shown in (Fig. 3). The spectrum of LFX showed the following characteristic peaks: 3266.13 cm^{-1} (carboxylic acid; O-H stretching), 2931.98 cm^{-1} (CH_3; C-H stretching), 3081 cm^{-1} (aromatic; C-H stretching), 799.82 cm^{-1} (aromatic; C-H bending), 1723 cm^{-1} (C=O stretching), 1618.94 cm-1 (aromatic; C=C stretching), 1294.37 cm^{-1} (amines; C-N stretching) and 1086.18 cm^{-1} (C-F stretching). The FTIR spectrum of LFX-NE did not exhibit characteristic peaks of LFX and was found to be similar to that of blank NE, indicating complete solubilization and absence of larger precipitates of the drug within the NE. Other researchers also reported similar kind of results [35, 36].

3.5 *In vitro* drug release study

The percent drug release from LFX-NE and free LFX solution was observed for 6 h. The *in vitro* drug release profile curves are shown in Fig. 2C. The LFX-NE demonstrated a biphasic release pattern of drug i.e., initial burst release followed by sustained release. LFX-NE showed initial burst drug release of 62.83 ± 2.10 % in 15 min and then complete sustained release of 99.13 ± 2.71% of drug after 360 min (6 h). As, the nanoscale emulsion exhibited low viscosity, initial high burst release of LFX was observed. This can be controlled by incorporation of the NE into suitable secondary carrier such as hydrogel, which will impart thickness to the system. In contrast, 42.70 ± 3.10 % of drug was released from free LFX solution in 15 min. However, even after 6 h only 52.74 ± 2.94 % of drug was released from the solution. These results suggest that the diffusion of free LFX through the membrane is very poor in comparison to LFX-NE and permeation of LFX was significantly enhanced when incorporated into the NE. This enhancement can be attributed to presence of clove oil and nanometric size of the formulation. Clove oil has been reported to be a permeation enhancer [37]. Our results are in agreement with those observed by Volpe *et al.* [24] where similar release profiles were observed for ciprofloxacin loaded microemulsion.

3.6 Microbiological studies

3.6.1 Agar well diffusion assay

Well diffusion assay revealed antibacterial nature of LFX-NE against both gram negative and gram positive pathogens. Supplementary Table 1 shows diameters of the zones of inhibition (ZOI) of LFX, clove oil, blank NE and LFX-NE. LFX-NE exhibited highest ZOI against *P. aeruginosa* with mean diameter of 58.9 ± 0.3 mm followed by mean diameter of 55 ± 0.5 mm for *E. coli and* 51.5 ± 0.7 mm for *K. pneumoniae* ($p < 0.01$). *S. aureus* (MRSA) showed low sensitivity to LFX-NE as compared to other pathogens with mean diameter of the ZOI to be 35.3 ± 0.3 mm. Clove oil demonstrated little antibacterial activity against all pathogens whereas levofloxacin aqueous solution showed appreciable antibacterial activity. Incorporation of LFX into clove oil nanoscale emulsion enhanced its antibacterial effect substantially as evidenced by large diameters of ZOIs in comparison to pure drug and blank NE.

3.6.2 Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

MIC and MBC values for LFX solution, blank NE and LFX-NE are shown in Table 3. Loading of LFX into clove oil nanoscale emulsion, significantly reduced its MIC by 16-fold against *P. aeruginosa* while for *E. coli* and *K. pneumoniae* there was 8-fold reduction in MIC ($p < 0.01$). However, LFX-NE inhibited the growth of *S. aureus* (MRSA) at higher concentration (4 µg/ml) in comparison to other pathogens tested. There was a 2-fold difference between MIC and MBC of LFX. There was also significant reduction in the MBC of LFX when present in the Nanoscale emulsion. Blank NE exhibited higher MIC and MBC values against tested pathogens. The enhancement in MIC and MBC values of LFX upon emulsification with clove oil can be attributed to nanoscale of the formulation leading to increase in the surface area of oil droplets and combined bactericidal effect of the drug with clove oil. Eugenol, a major component of clove oil, has been reported to possess bactericidal effects against wide range of pathogens. Moreover, Al-Adham *et al.* [38] demonstrated membrane disrupting activity of microemulsions against *P. aeruginosa* PAO1. Rapid reduction (5 log) was observed in *P. aeruginosa* titre over a period of 45 s. LFX-NE exhibited highest bactericidal activity against *P. aeruginosa*. Hence, further experiments were carried out on *P. aeruginosa* biofilms.

3.6.3 Establishment and Processing of *P.aeruginosa* biofilm

The establishment, processing and monitoring of *P. aeruginosa* biofilm was performed for a period of 7 days. Biofilm formation was confirmed via viable cell counting method with biofilm growth peaking on 4^{th} day as maximum bacterial count of 11.48 ± 0.25 log10 CFU/ml was observed. After 4^{th} day a gradual decline in bacterial count was observed upto 7^{th} day of incubation (Fig. 4A).

3.6.4 Effect of nanoscale emulsion on *P.aeruginosa* biofilm

P. aeruginosa biofilm was established on a 96 well microtitre plate upto 7 days and treated with blank NE and LFX-NE (MIC and MBC concentration) on each day. As shown in (Fig. 4B), significant reduction ($p < 0.01$) in bacterial load of young biofilm of *P. aeruginosa* was observed following treatment with LFX-NE. On 4^{th} day i.e. peak day of biofilm, a significant reduction ($p < 0.01$) was observed from 11 ± 0.05 log10 CFU/ml for control biofilm to 6.64 ± 0.21 log10 CFU/ml and 5.44 ± 0.13 log10 CFU/ml for LFX-NE treated biofilm at its MIC and MBC, respectively. The susceptibility of mature biofilm towards LFX-NE was also seen with significant reduction ($p < 0.01$) in bacterial counts in biofilms treated on days 5, 6 and 7 in comparison to control biofilm. She *et al.* [39] reported LFX to inhibit biofilm formation by *P. aeruginosa* PAO1

strain at its MIC i.e. 2 µg/ml. However, it was unable to completely eradicate pre-formed biofilm even at a higher concentration of 16 µg/ml. Therefore, incorporation of LFX in clove oil nanoscale emulsion enhanced its antibacterial activity as evidenced by ~5 log10 reduction in bacterial count at its MIC of 0.125 µg/ml.

3.6.5 FESEM analysis of biofilm

P. aeruginosa biofilm was established on glass coverslip and treated with blank NE, LFX-NE at its MIC and MBC on 4th day i.e. the peak day. FESEM photomicrographs revealed 3-dimensional structure of the control biofilm with dense aggregates of bacterial cells embedded within the matrix. Water channels were also visible clearly (Fig. 5. A-B). Biofilm treated with blank NE showed minor disruption of biofilm matrix (Fig. 5. C-D). Whereas, LFX-NE at its MIC, demonstrated severe disruption of biofilm architecture with gross morphological changes in the bacterial cells (Fig. 5. E-F). The shrinkage of cytoplasmic contents is clearly visible. However, at its MBC, LFX-NE treated biofilm showed grave disruption as compared to LFX-NE at its MIC with elongation of bacterial cells in addition to shrinkage of cytoplasmic contents (Fig. 5. G-H). Our results are in line with those reported by She *et al.* [39] where elongation of cells was observed in presence of LFX. Al-Adham *et al.* [40] also demonstrated antibiofilm nature of microemulsion with upto 3 log reduction in bacterial cell viability 4 h post treatment of *P. aeruginosa* biofilm grown for over 72 h. In another study by Husain *et al.* [31] SEM analysis revealed poor biofilm formation by *P. aeruginosa* when treated with clove oil in comparison to untreated biofilm.

3.6.6 Confocal Laser Scanning Microscopy (CLSM) evaluation

Confocal laser scanning microscopy was used to determine the effect of NE treatment on bacterial cell viability in 4 day old *P. aeruginosa* biofilm. Control biofilm exhibited tightly packed live adherent cells, well integrated within the biofilm (Fig. 5-I). Biofilm treated with blank NE showed few number of dead cells among the population (Fig. 5-J). LFX-NE at its MIC, showed biofilm disruption with majority of dead cells whereas, at its MBC, LFX-NE treatment made biofilm lose its structural integrity completely with no live cells present (Fig. 5 K-L). This confirms high efficacy of LFX-NE in pre-formed biofilm eradicaton.

3.6.7 Quorum sensing inhibition (QSI) assay

To determine the anti-QS activity of the developed NE, LasR-dependent lasB promoter and reporter gene fusion system (lasB:lacZ) was used for measuring β-galactosidase activity in cells. The quantitative estimation of AHLs from the biofilm of *P. aeruginosa* was done by colorimetric method aided by the use of bioreporter strain of *E. coli* MG4 for detection of AHLs. For determining the antiquorum sensing activity of LFX-NE, sub-MICs were used to demonstrate that the reported activity was not merely due to the killing of bacteria but was purely due to the antiquorum sensing property of the system. The amount of AHLs in the control biofilm was found to be 529.42 ± 16.2 MU, which reduced to 491.14 ± 18.15 MU upon treatment with LFX solution. Whereas upon treatment with blank NE and LFX-NE, it decreased to 372.61 ± 15.34 MU and 342. 21 ± 18.27 MU ($p < 0.01$), respectively. This reflects toward the efficiency of the developed system in quenching the AHLs. Husain *et al.* [31] reported the anti-QS activity of clove oil in *P. aeruginosa* PAO1 and found it to reduce the AHL levels by 56%. Zhou *et al*. [41] also showed that eugenol, the major component of clove oil, reduced β-galactosidase activity upto 75% in *E. coli* MG4/pKDT17. This demonstrated las-controlled transcription is directly inhibited by clove oil. Moreover, expression of various virulence related genes is known to be regulated by las system. Hence, QS inhibition by clove oil significantly decreased virulence factors of *P. aeruginosa* such as elastase, pyocyanin. Since, our developed formulation contains clove oil, it can be inferred that anti-QS activity of the LFX-NE can be attributed to presence of clove oil.

3.7 Hen egg chorioallantoic membrane (HET-CAM) irritation study

The objective of this study was to assess irritation potential, if any, of the developed formulation and its components when exposed to vasculature of chorioallantoic membrane. The inflammatory symptoms and irritancy level were classified in terms of scoring as shown in supplementary Table 3. LFX-NE was compared with 0.9% (w/v) NaCl (negative control) and 0.1 N NaOH (positive control). It was seen that in comparison with positive control where within 30 seconds, lysis of blood vessels occured, 0.9% (w/v) NaCl and LFX-NE were found to be non-irritant. No hemorrhage or coagulation was observed with LFX-NE as shown in (Fig. 6A). From the results it can be concluded that the developed formulation is non-irritant and can be used for topical application safely. Our results our in line with study by Mehanna *et al*. [42] where LFX loaded limonene based self-nanoemulsifying system was found to be non-toxic and non-irritant in HET-CAM study.

3.8 *In vitro* safety study in Normal Human Epidermal Keratinocytes (NHEK) cell lines

The purpose of this study was to assess the safety of LFX loaded NE for its potential use in topical delivery. The safety studies of blank NE, LFX-NE and pure drug were carried out using PrestoBlue assay on normal human epidermal keratinocytes cell line. The percent cell viability with respect to increasing concentrations of blank NE, LFX-NE and pure drug from 1 μg/mL to 100 μg/mL are shown in (Fig. 6B). Low toxicity is associated with higher value of percent cell viability (≤10%, high toxicity; 11–40%, moderate toxicity; 41–70%, low toxicity and ≥ 70%, without toxicity). The results showed that the LFX, blank NE and LFX-NE were found to be non toxic upto 50 μg/mL concentration with 74.75 ± 1.57%, 80.94 ± 1.23% and 81.85 ± 1.63% ($p < 0.01$) cell viability, respectively. Further, at highest concentration of 100 μg/mL, percent cell viability for pure drug was found to be 60.25 ± 1.81% whereas, for blank NE and LFX-NE it was found to be reduced to 69.56 ± 1.86% and 68.85 ± 1.35%, respectively, indicating very low toxicity. However, incorporation of LFX into the nanoscale emulsion reduced its toxicity at the concentration of 100 μg/mL. Prashar *et al.* [43] determined clove oil to be cytotoxic in range of 0.03-0.25 % v/v in human dermal fibroblasts and human dermal endothelial cell lines. However, it can be observed from our results that upon nanoemulsification of clove oil, its cytotoxicity has been ameliorated substantially. Hence, this study showed that LFX-NE can be safely used for topical applications.

4 Conclusion and future perspective

Development of resistance to antibiotics by the formation of biofilm by bacteria has caused a major blow to the treatment of infections, especially wound infections. Biofilms act as a safety shied for the bacteria and protects them from the host immune cells and bactericidal action of antibiotics. The present study aimed at the formulation of nanoscale emulsion loaded with antibiotic levofloxacin. On the basis of the solubility of drug and reported antibacterial and antibiofilm activity of clove oil, it was selected as oil phase of the NE. The pseudoternary phase diagrams showed large nanoscale emulsion area. The developed NEs were found to possess extremely small nanometric size and were thermodynamically stable. FTIR study revealed complete solubilization of LFX within the NE. LFX-NE demonstrated excellent antibacterial activity with 8-16 fold reduction in the MIC of LFX against variety of test pathogens. This will lead to reduction of dose of LFX required for the treatment of wound infections. It also showed antiquorum sensing activity

with severe disruption of mature biofilm of *P. aeruginosa*. This demonstrates the combined effect of clove oil and LFX when incorporated in nanoscale emulsion system. Similar synergistic antibacterial profiles were obtained by Mehanna *et al.* [42] when LFX was incorporated into limonene based self-nanoemulsifying system, highlighting the role of nanoemulsification in enhancing the activity of antibiotics. LFX-NE was found to be non-irritant and biocompatible with Hen's chorioallantoic membrane, and non-cytotoxic in normal human epidermal keratinocytes at a concentration upto 100-fold higher than the MBC. In conclusion, LFX-NE can serve as an excellent system for the eradication of mature biofilms of *P. aeruginosa*. Further research will be focused on development and characterization of suitable carrier for LFX-NE and evaluation of LFX-NE in validated animal model for non-healing wound infections harbouring biofilms.

Declarations

Author contribution

Karan Razdan: Concenptualization, Methodology, Investigation, Writing-original draft. **Vijay. S. Gondil:** Investigation, Writing-review. **Sanjay Chhibber, Kamalinder. K. Singh:** Supervision, Writing-Review. **V.R. Sinha:** Concenptualization, Supervision, Writing-Review & Editing.

Conflict of Interest

Authors declare no conflict of interests

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Figure Legends

Fig. 1: Pseudoternary phase diagrams. (A) Tween 80 used as surfactant; (B) S_{mix} is Tween 80:PEG-300 [1:1] and (C) S_{mix} is Tween 80:PEG-300 [2:1]

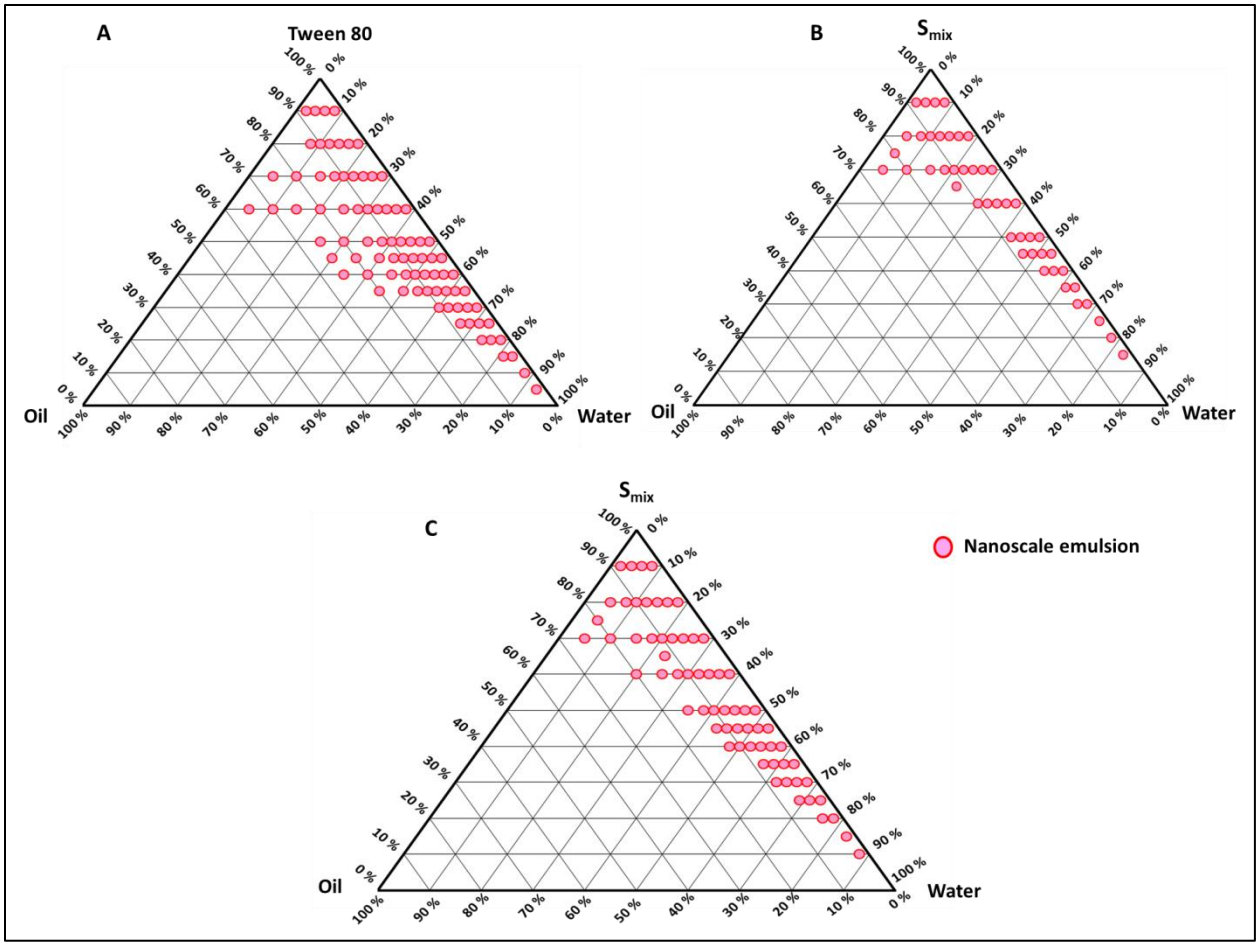
Fig. 2: HRTEM image of LFX-NE at magnification of (A) 30000x and (B) 50000x, and (C) Drug release profile curve of LFX-NE in comparison to free drug solution

Fig. 3: FTIR spectra of (A) LFX, (B) Tween 80 (C) PEG-300 (D) Blank NE and (E) LFX-NE

Fig. 4: (A) Graph showing the kinetics of *P. aeruginosa* PAO1 biofilm as determined by viable cell count and (B) Bacterial count in *P. aeruginosa* biofilm established for seven days and treated with LFX-NE on each day. * $p < 0.01$ in comparison to control biofilm and blank NE.

Fig. 5: FE-SEM images of *P.aeruginosa* bioflim on 4^{th} day: (A-B) control biofilm and treated with Plain NE (C-D); LFX-NE at MIC (E-F) and LFX-NE at MBC (G-H). The biofilm was observed at 5-22 k magnification. *P. aeruginosa* biofilm stained with LIVE/DEAD Baclight kit (I) control biofilm and treated with (J) Plain NE; (K) LFX-NE at MIC and (L) LFX-NE at MBC. The biofilm was observed under CLSM at x400. Yellow arrows show water channels. Red arrows demonstrate biofilm disruption and morphological changes in bacterial cells.

Fig. 6: (A) HET-CAM images. (B) Cell viability assay for LFX-NE on NHEK cells



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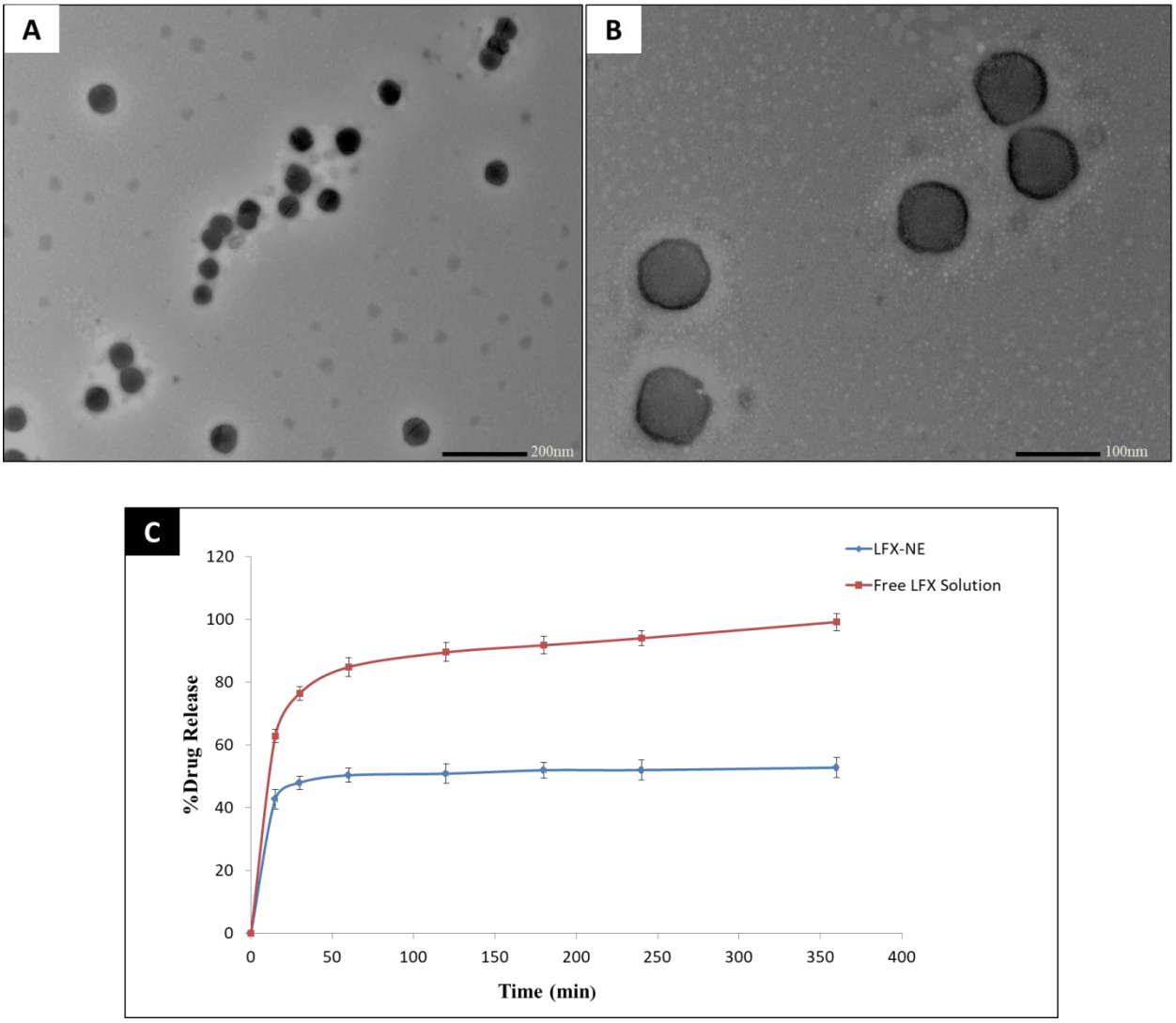

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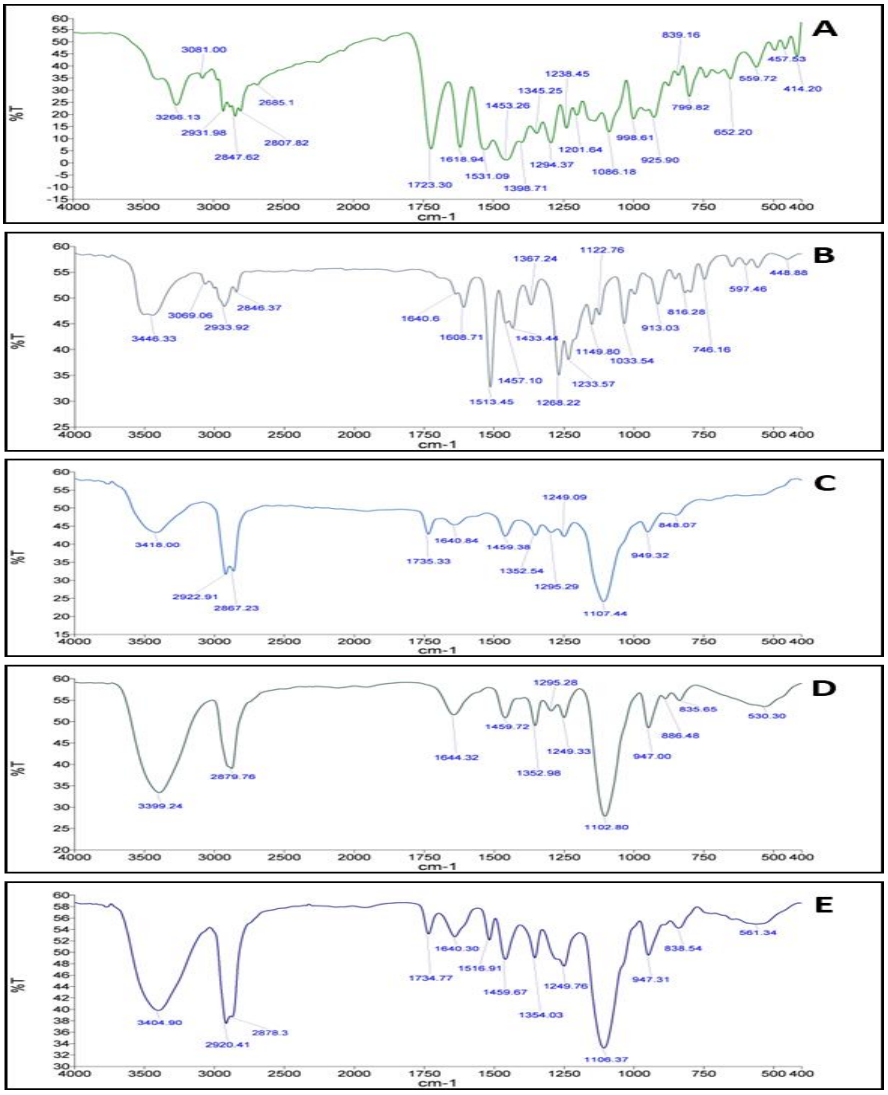

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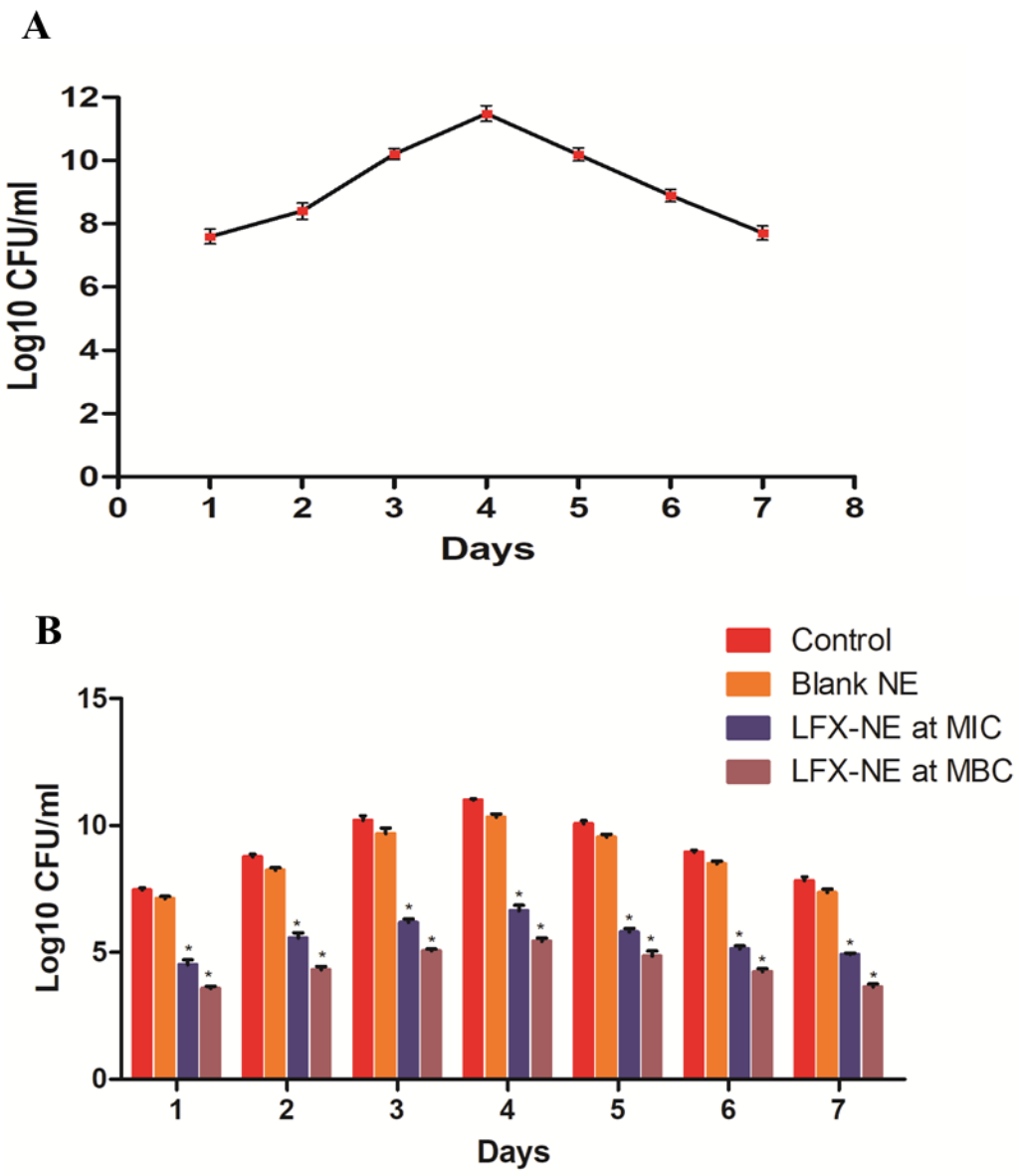

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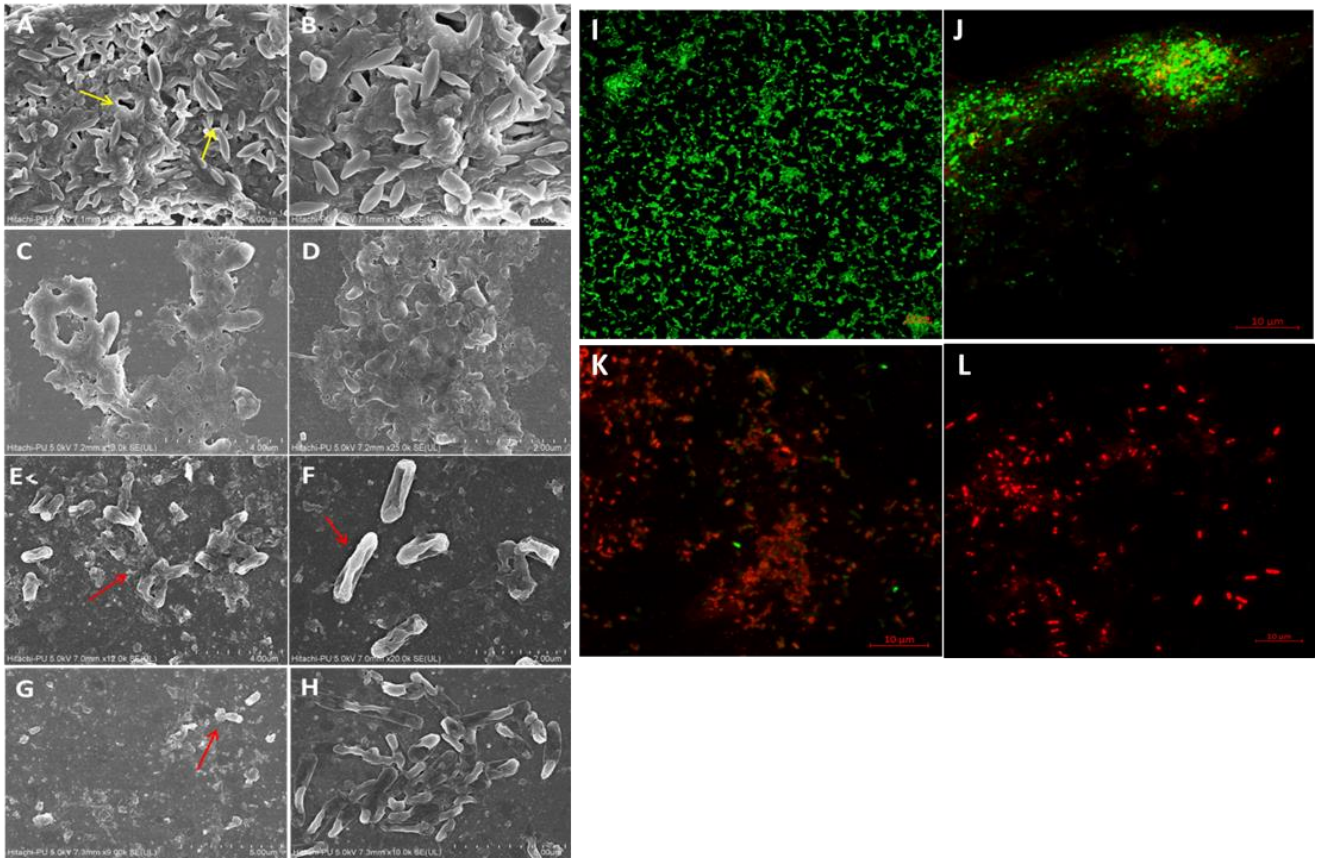

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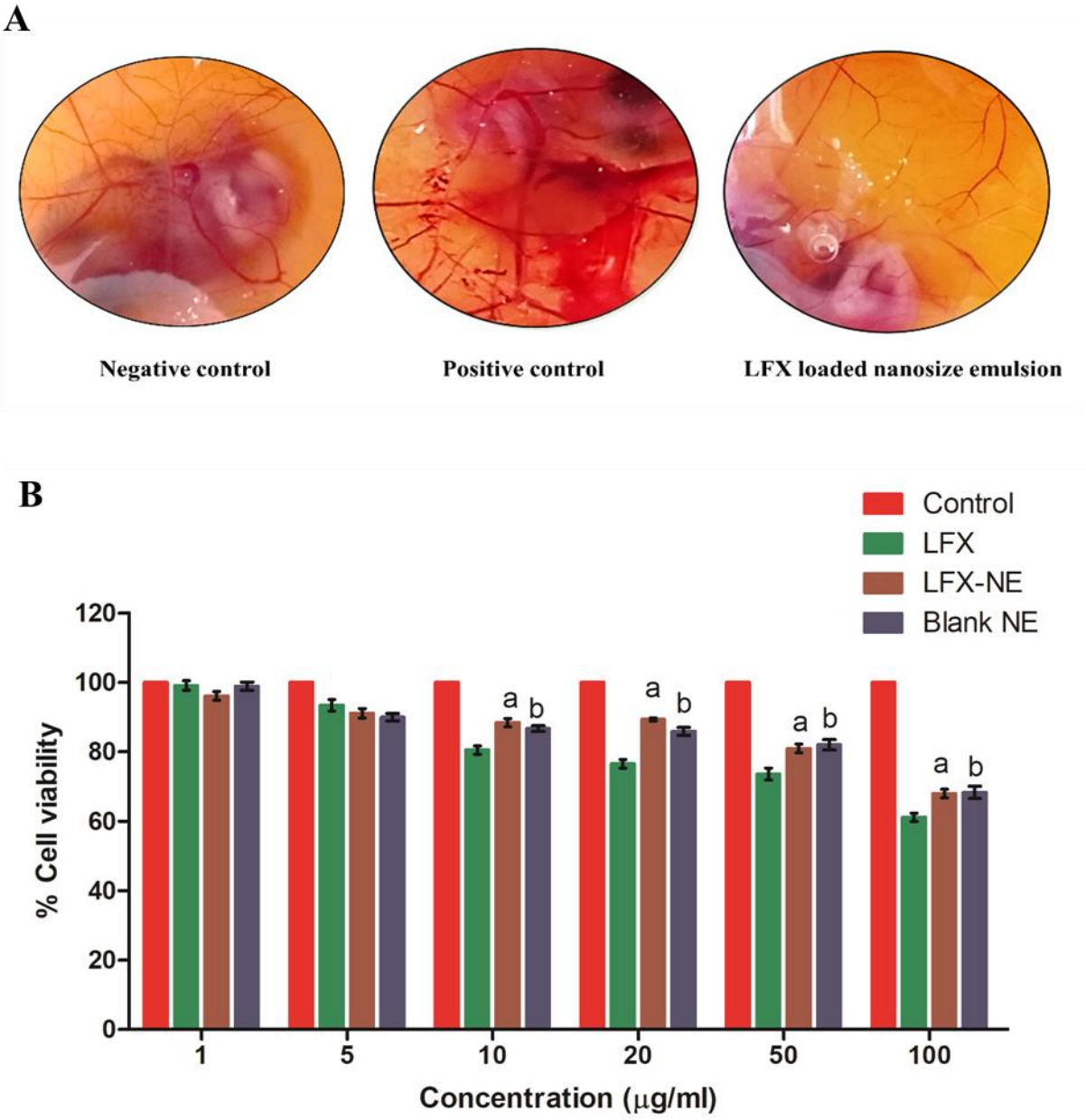

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Table legends

Table 1. Composition and characterization parameters of levofloxacin loaded nanoscale emulsion

Table 2: MIC and MBC of LFX, LFX-NE and blank NE against a range of pathogens. * $p < 0.01$ in comparison to pure levofloxacin solution

Table 1. Composition and characterization parameters of levofloxacin loaded nanoscale emulsion

Nanosize emulsion	Clove oil (v/v)	Surfactant/Co-surfactant (v/v)	LFX (w/v)	Water (v/v)	Particle size (nm)	PDI	Zeta potential (mV)	% Drug content	pH	% Transmittance	Viscosity (mPa.s)
Blank NE	6%	T80:PEG300 (2:1) (25%)	-	69	11.64 ± 0.68	0.077	- 3.95 ± 0.2	-	6.30 ± 0.15	99.1 ± 0.3	198 ± 4
LFX-NE	6%	T80:PEG300 (2:1) (25%)	1	69	18.84 ± 0.5	0.140	-4.05 ± 0.8	99.9 ± 0.1 %	6.2 ± 0.17	99.2 ± 0.5	206 ± 5
LFX-NE-1	6%	T80:PEG300 (1:1) (40%)	1	54	21.67 ± 1.0	0.160	-3.44 ± 1.3	98.9 ± 0.08 %	6.4 ± 0.12	98.5 ± 0.3	276 ± 8
LFX-NE-2	6%	Tween 80 (25%)	1	69	21.89 ± 2.1	0.206	-5.72 ± 1.7	95.5 ± 0.18 %	6.6 ± 0.19	96.1 ± 0.2	175 ± 3

Table 2: MIC and MBC of LFX, LFX-NE and blank NE against a range of pathogens. * $p < 0.01$ in comparison to pure levofloxacin solution

Strain	MIC of LFX (µg/ml)	MIC of blank NE (µg/ml)	MIC of LFX-NE (µg/ml)	MBC of LFX (µg/ml)	MBC of blank NE (µg/ml)	MBC of LFX-NE (µg/ml)
E. coli	2	64	0.25 *	4	128	0.5
K. pneumoniae	2	64	0.25 *	4	128	0.5
***S. aureus* (MRSA)**	4	32	2 *	8	64	4
P. aeruginosa	2	32	0.125 *	4	64	0.25

Supplementary Figure Legends

Supplementary Fig. 1: Viscosity graph of selected batches of LFX-NE

Supplementary Fig. 2: Particle size distribution graphs for LFX-NE by intesity (A) at 25 ± 0.5ºC (B) at 40ºC 25 ± 0.5ºC and, by number (C) at 25ºC 25 ± 0.5ºC (D) at 40ºC 25 ± 0.5ºC after 3 months of storage

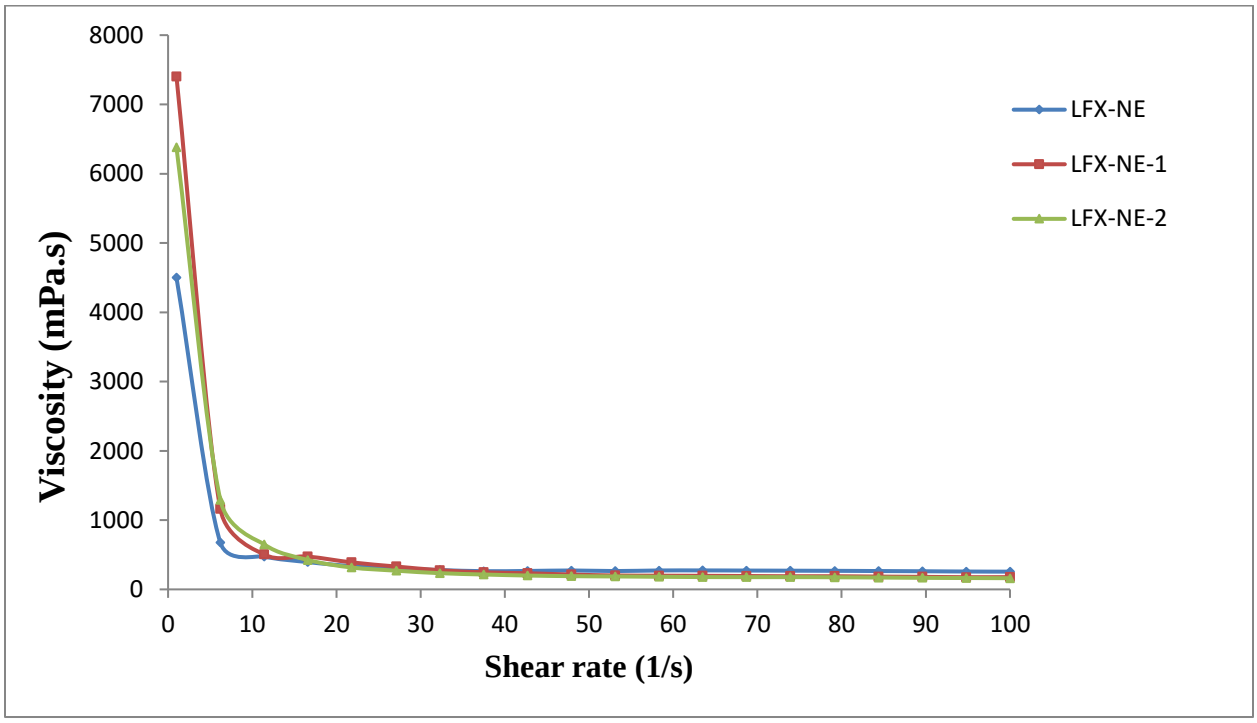
Supplementary Table Legends

Supplementary Table 1: Zone of inhibition (mm) diameters determined using agar well diffusion assay. * $p < 0.01$ in comparison to pure levofloxacin solution

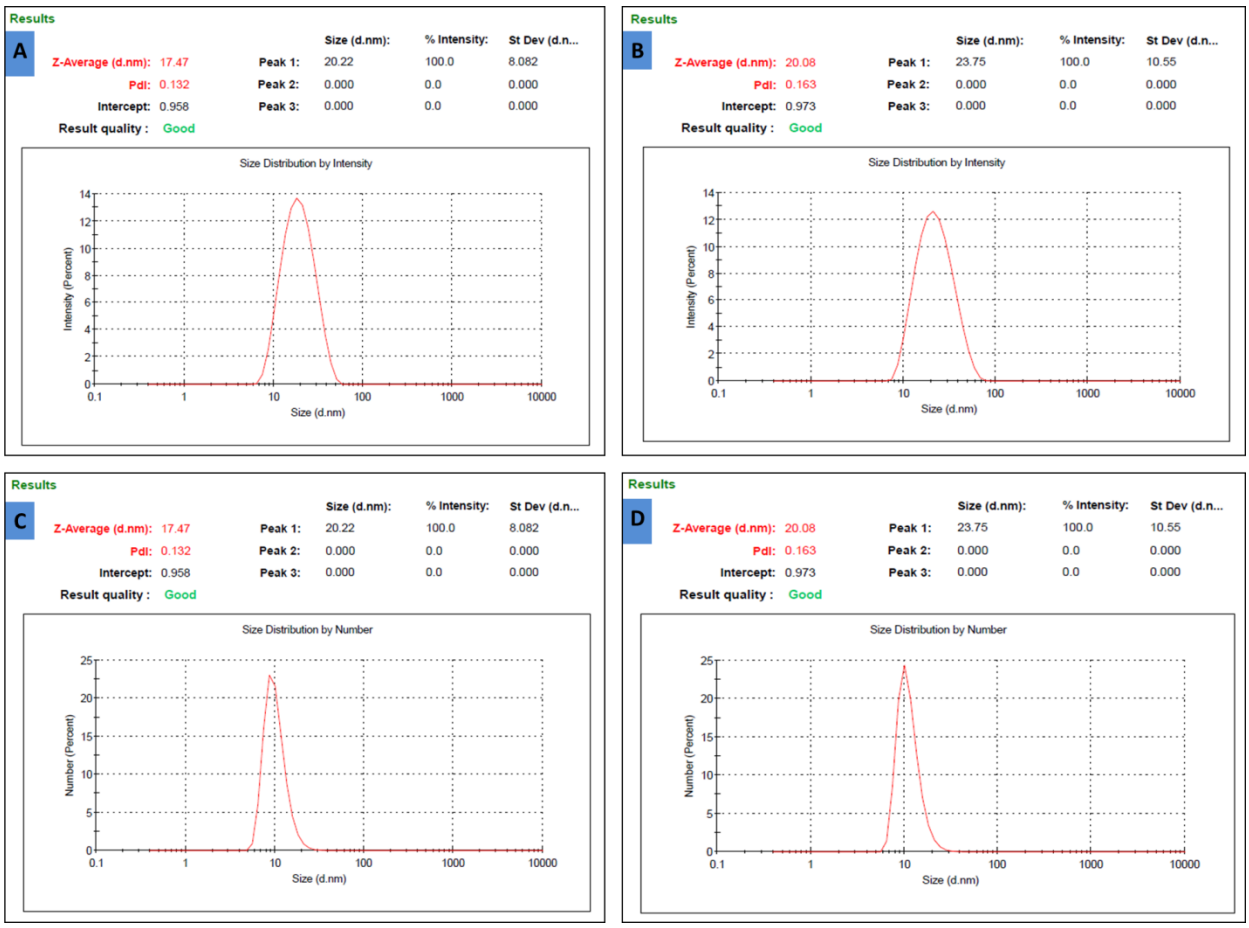
Supplementary Table 2: Scoring system for HET-CAM

Supplementary Table 3: Description of irritation score and level of different test substances on chorioallantoic membrane

Supplementary Table 4: Stability data for LFX-NE



Supplementary Fig. 1: Viscosity graph of selected batches of LFX-NE



Supplementary Fig. 2: Particle size distribution graphs for LFX-NE by intesity (A) at 25 ± 0.5ºC (B) at 40ºC 25 ± 0.5ºC and, by number (C) at 25ºC 25 ± 0.5ºC (D) at 40ºC 25 ± 0.5ºC after 3 months of storage

Supplementary Table 1. Zone of inhibition (mm) diameters determined using agar well diffusion assay. * $p < 0.01$ in comparison to pure levofloxacin solution

Bacteria	Zone of inhibition (mm)			
	LFX	**Clove oil**	**LFX-NE**	**Blank NE**
E. coli	46 ± 1	20.8 ± 1.1	55 ± 0.5 *	21 ± 0.2
K. pneumoniae	43.2 ± 0.8	19.1 ± 0.4	51.5 ± 0.7 *	22 ± 0.5
S. aureus (MRSA)	25 ± 0.5	13.7 ± 0.9	35.3 ± 0.3 *	19.2 ± 0.8
P. aeruginosa	48.8 ± 0.21	18.2 ± 0.8	58.9 ± 0.3 *	27.5 ± 0.5

Supplementary Table 2: Scoring system for HET-CAM

End points	Score at various time points		
	0.5 min	**2 min**	**5 min**
Lysis	5	3	1
Haemorrhage	7	5	3
Coagulation	9	7	5

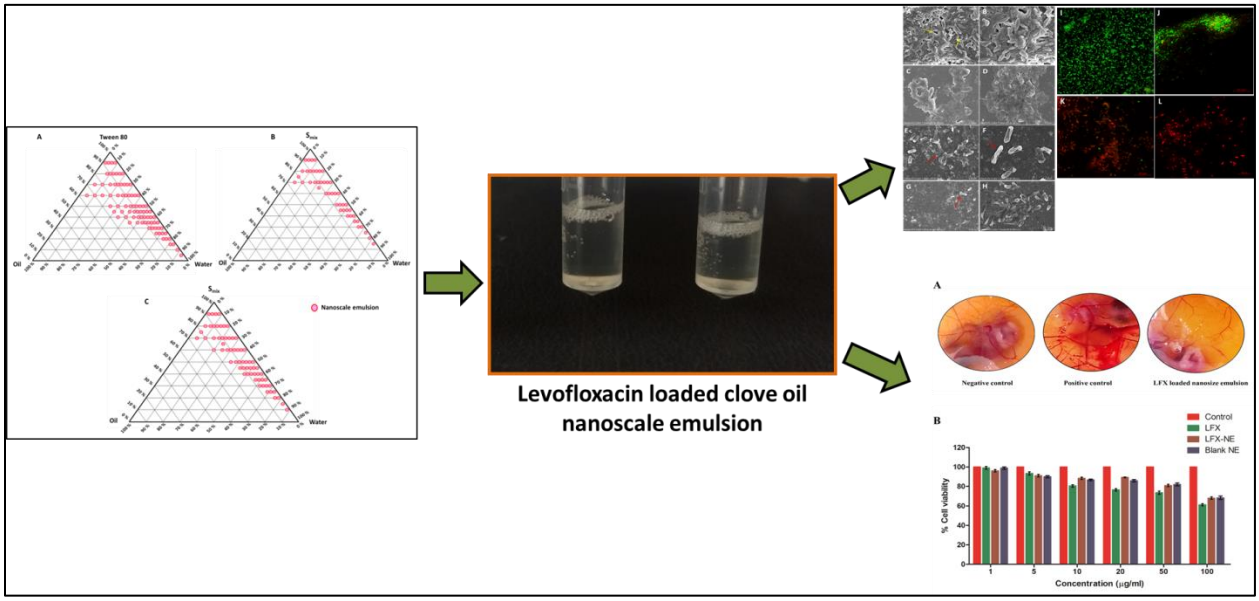
Supplementary Table 3: Description of irritation score and level of different test substances on chorioallantoic membrane

Treatment	Parameters	Time (min)			Total Score	Inference
		0.5	2	5		
0.1 N NaOH (Positive Control)	Lysis	5	0	0	13	Severe irritant
	Haemorrhage	0	5	3		
	Coagulation	0	0	0		
0.9% NaCl (Negative Control)	Lysis	0	0	0	0	Non-irritant
	Haemorrhage	0	0	0		
	Coagulation	0	0	0		
LFX-NE	Lysis	0	0	0	0	Non-irritant
	Haemorrhage	0	0	0		
	Coagulation	0	0	0		

Supplementary Table 4: Stability data for LFX-NE

Months	Evaluation parameters at 25 ± 0.5º C			Evaluation parameters at 40 ± 0.5º C			Visual appearance
	Particle size (nm)	PdI	Zeta potential (mV)	Particle size (nm)	PdI	Zeta potential (mV)	
0	18.84 ± 0.50	0.140	-4.05 ± 0.80	18.84 ± 0.50	0.140	-4.05 ± 0.8	Clear and Transparent
1	18.05 ± 0.24	0.138	-4.87 ± 0.41	18.79 ± 0.43	0.148	-4.57 ± 0.33	Clear and Transparent
3	17.47 ± 0.45	0.132	-6.14 ± 0.26	20.08 ± 0.24	0.163	-5.35 ± 0.26	Clear and Transparent

Graphical abstract



Highlights

- Levofloxacin was loaded into clove oil nanoscale emulsion (LFX-NE).
- LFX-NE showed nanometric size and excellent thermodynamic stability.
- LFX-NE eradicated mature *Pseudomonas aeruginosa* biofilm after 6 h treatment.
- LFX-NE was found to be cytocompatible with normal human epithelial keratinocytes.
- LFX-NE has great potential for treatment of biofilm harbouring non-healing wounds.