Biocatalysis and Agricultural Biotechnology 18 (2019) 101025

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Synthesis of antimicrobial nanoemulsions and its effectuality for the treatment of multi-drug resistant ESKAPE pathogens

Mohd Hashim Khan, Karthikeyan Ramalingam*

School of Life Sciences, B.S. Abdur Rahman Crescent Institute of Science and Technology, GST Road, Vandalur, Chennai, 600 048, India

ARTICLE INFO

Keywords:
Nanoemulsion
ESKAPE pathogens
Drug resistant
Adherence
Biofilm
Antimicrobial

ABSTRACT

Antimicrobial nanoemulsions (NEs) are emerging as unique class disinfectant agents for the treatment of MDR pathogens. The ESKAPE pathogens are major causative agents for most of the nosocomial infections, due to drug inactivation, drug binding sites modification and biofilm formation efficiency. The efficacy of NEs and their constituents was determined by MIC, MBC, adherence assay, biofilm studies and *in vivo* toxicity evaluation with *Bombyx mori*. The droplet sizes of the nanoemulsions were measured by DLS and the measurement showed monodispersed droplet size distribution (PDI < 0.24). The results of antimicrobial activities of ten NEs formulation against eight strains of ESKAPE pathogens, the highest MIC and MBC recorded with 1, 3, 9, 10, 12, and 15. Among all ESKAPE strains, the maximum inhibitory activity was found at the dilution of 1: 196 and 1:128 against *Staphylococcus aureus* and *Enterococcus faecium* respectively. The highest adherence was observed with NE-3 (38.04%) and NE-15 (46.00%) against three strains of *S. aureus*. Among all synthesized nanoemulsions, NE-12 showed maximum (19.07, 66.04, 79.02, 86.94, 75.46 and 53.12%) efficacies against ESKAPE pathogens as anti-biofilm properties. Statistically, NEs exhibited a subsequent reduction in bacterial cell counts against all pathogens when compared with positive and negative control. Therefore we conclude with the observations that the NEs could be valuable for the improvement of promising antimicrobial agents and may be prove as a mile stone against the antibiotic-resistant pathogens.

1. Introduction

The antimicrobial drugs available in markets developed by pharmaceuticals companies are facing a challenge to cure the infectious diseases. Since most of these antimicrobial agents are not able to reach at their targeted place or they get degraded. When the drugs trying to penetrate the cell membrane or transported into the bacterial cell, drugs are partially dissolved in the medium as a consequence the effect of antimicrobial agents are greatly affected and the results arises as a major cause of drug resistant in pathogens (LiPuma et al., 2009). The emergence of resistance in ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) against resulted antibiotics, a strong demand of new alternative to overcome the problem (Billerbeck, 2007). The efficacy of nanoemulsions against multi-drug resistant pathogens is a novel search for the welfare of human being. Nanoemulsions (NEs) have wide range biocidal activities against bacteria, fungi, viruses and spores in their free-coasting or planktonic frame by disruption of their outer membranes or external layers (Hamouda and Baker, 2000) The antimicrobial actions of NEs relies on the stored energy of nano-droplets which fuses with the lipid bilayers of cell membranes of microbes by releasing their effect on targets and destabilizes the lipid membrane of the pathogens ultimately leads their killing/destruction (Hamouda and Baker, 2000). The effects of NEs are non specific, unlike that of antibiotics, hence allowing broad-spectrum action while limiting the capacity for the induction of resistance in human pathogens (Hemmila et al., 2010). These events make NEs suitable prospects for the treatment of multidrug resistant ESKAPE pathogens. In addition to this it has been reported that the use of aromatic plant essential oils particularly Mahua oil (*Madhuca longifolia*) exert an antioxidant or antimicrobial effects and have received particular attention because of their radical-scavenging properties (Barros et al., 2015 and Pandey and Agarwal, 2015).

In this present study, we synthesized ten nanoemulsions (NE-1, NE-2, NE-3, NE-7, NE-9, NE-10, NE-12, NE-13, NE-14, and NE-15) with different combinations of surfactant (Triton X-100, Brij 30, Tween 60, and Span 20), Cetylpyridinium chloride (CPC), Mahua oil and water. Dynamic light scattering (DLS) technique was used to measure the droplet size of prepared NEs and accessed *in vitro* activities of novel synthesized nanoemulsions against a panel of multidrug resistant

* Corresponding author. Tel.: +91 44 22759380, +91 7502534668.
E-mail addresses: hashimkhanamu@gmail.com (M.H. Khan), karthikeyan.sls@crescent.education, hydrophis.07@gmail.com (K. Ramalingam).

https://doi.org/10.1016/j.bcab.2019.101025
Received 29 October 2018; Received in revised form 2 January 2019; Accepted 3 January 2019
Available online 01 February 2019
1878-8181/

ESKAPE pathogens. Therefore, we evaluated the antibacterial activities of various forms of synthesized nanoemulsions containing CPC against ESKAPE pathogens, by testing their minimum inhibitory concentration (MICs), minimum bactericidal concentrations (MBCs), anti-adherent efficacy and anti-biofilm activities. The toxic effect of all synthesized NEs was tested in animal model by using *Bombyx mori*.

2. Materials and methods

2.1. Selection of ESKAPE strains and growth conditions

The strains of ESKAPE were procured from ATCC (American type culture collection) *S. aureus* (MRSA) - ATCC 3359, *K. pneumoniae*-ATCC 35657, *A. baumannii*- ATCC 19606, and *P. aeruginosa* -ATCC 27853; MTCC (Microbial type culture collection) *S. aureus*- MTCC 1430, *Enterobacter* sp.- MCC 2296; MCC (Microbial culture collection) *E. faecium*-MCC 2763 and clinical isolate obtained from Chettinad Hospital & Research Institute. Luria Britani (LB) broth (Himedia Laboratories Mumbai- India) was used for bacterial culture. The bacterial stocks have been maintained at −80 °C in LB with 50% (vol/vol) glycerol. The stock mother cultures were inoculated and grown at 37 °C in sterile fresh LB broth. For every experiment fresh inoculums were used by adjusting OD of 0.2–0.4 at 490 nm throughout the studies.

Synthesis and preparation of nanoemulsions: Total ten NEs were formulated and prepared as oil-in-water as previously reported by (Hwang et al., 2013). Briefly, the compositions of each nanoemulsion are mentioned (Table 1). All the NEs were emulsified by using a Microfluidizer- LM10 (Microfluidics Int. Corporation USA). The solutions were passed three times at moderate room temp for emulsification at 20,000 lb/in^2.

2.2. Characterization of synthesized NEs (Dynamic light scattering- DLS)

The size distribution profile of synthesized nanoemulsions was studied by intensity, volume and number of droplets of each formulated emulsion using DLS (Malvern Zetasizer Nano ZSP instrument). The size distribution of droplets was observed in different nano ranges on the basis of size distribution intensity and their percentage (Ribeiro et al., 2015). The diameters and polydispersity index (PDI) were analyzed in acquisition time 60s, at 25 °C. As a reference dispersive medium, ultrapure water with a refractive index of 1.330, viscosity of 0.8872 cP, and a dielectric constant of 78.3 was used. For DLS measurement, the solution was passed through a 0.2 ??m Polyvinylidene difluoride (PVDF) membrane. Three measurements performed by using disposable sizing cuvette for each sample and the means size of droplets were determined.

2.3. Determination of MIC and MBC against ESKAPE pathogens

To evaluate the antimicrobial activities of nanoemulsions against ESKAPE pathogens the MIC and MBC were determined by the following the Micro-dilution procedure in a 96-well plate. Briefly, 97.5 µl 2× LB and 100 µl nanoemulsions added in first well and serially diluted upto 8th well, finally 5 µl bacterial inoculums of 1–5 X 10^6 CFU/ml were added with mixed solution in each well except positive (media) control. The sterile ultra pure water (UPH_2O) with media but without bacteria and media with bacteria with the equal volume was employed as a positive and negative control respectively. Independently Mahua oil (10%) was also tested for their MIC and MBC evaluation. The Microtitter plate containing treated ESKAPE strains with different NEs and untreated as a control were incubated for 24 h at 37 °C. The MIC was determined as the highest dilutions which are showing no bacterial growth with the visible turbidity as growth (99% inhibition) of the microorganisms (Balouirin et al., 2016). After the completion of incubation period, the poor turbid and clear wells too were selected to determine the bactericidal concentrations of the nanoemulsions. For further clarification, each well were selected for 5 µl suspension drawn and drops spotted on LB agar plates in triplicate by incubating for 18 h at 37 °C. The endpoint of the active growth of microbial population or the highest dilutions of NEs that brought about (99.9%) decrease in the bacterial cell count was recorded as the MBC in terms of the dilutions.

2.4. Adherence assay

To determine the anti-adherence activity of ESKAPE pathogens on the glass surface, a bacterial adherence assay was performed on selected pathogens (*Staphylococcus aureus* (MRSA) ATCC 33591, *Staphylococcus aureus* MTCC 1430 and *Staphylococcus aureus* clinical isolate). On the basis of proficient results of MBC, NE-3 and NE-15 were selected for anti-adherence assay. Bacterial culture were grown over night containing 10^7 CFU/ml were added to glass test tubes at their MBC dilutions (NE-3, 1: 24; 1:128; 1:96; and NE-15, 1:48; 1:96; 1:64) for *S. aureus*- MRSA (ATCC 33591), *S. aureus* (MTCC 1430) and *S. aureus* (clinical isolate) respectively. The total volume of 5 ml was maintained in each test tube including positive (H_2O_2- 57.82 mg/ml) and negative

Table 1
Composition of nanoemulsions with different combinations.

Nanoemulsions	Surfactant	CPC	Tannic acid	FAC	EDTA	Mahua oil
NE-1	Brij 30, (3%)	1%	–	–	–	10%
NE-2	Brij 30, (3%)	1%	–	50 µM	50 µM	10%
NE-3	Brij 30, (3%)	1%	200 µM	–	250 µM	10%
NE-7	Span 20, (3%)	1%	–	–	250 µM	10%
NE-9	Span 20, (3%)	1%	200 µM	–	50 µM	10%
NE-10	Triton X-100, (3%)	1%	200 µM	–	50 µM	10%
NE-12	Triton X-100, (3%)	1%	–	50 µM	–	10%
NE-13	Tween 60, (3%)	1%	–	200 µM	–	10%
NE-14	Tween 60, (3%)	1%	–	–	50 µM	10%
NE-15	Tween 60, (3%)	1%	50 µM	–	250 µM	10%

Concentrations of nanoemulsions:***NE-1**: (Brij 30 = 30 mg/ml, CPC = 10 mg/ml, Mahua Oil = 100 mg/ml)***NE-2**: (Brij 30 = 30 mg/ml, CPC = 10 mg/ml, FAC = 13.09 mg/ml, EDTA = 18.61 mg/ml, Mahua Oil = 100 mg/ml)***NE-3**: (Brij 30 = 30 mg/ml, CPC = 10 mg/ml, Tannic acid = 340.24 mg/ml, EDTA = 93.06 mg/ml, Mahua Oil = 100 mg/ml)***NE-7**: (Span 20 = 30 mg/ml, CPC = 10 mg/ml, EDTA = 93.06 mg/ml, Mahua Oil = 100 mg/ml)***NE-9**: (Span 20 = 30 mg/ml, CPC = 10 mg/ml, Tannic acid = 340.24 mg/ml EDTA = 18.61 mg/ml, Mahua Oil = 100 mg/ml)***NE-10**: (Triton X-100 = 30 mg/ml, CPC = 10 mg/ml, Tannic acid = 340.24 mg/ml EDTA = 18.61 mg/ml, Mahua Oil = 100 mg/ml)***NE-12**: (Triton X-100 = 30 mg/ml, CPC = 10 mg/ml, FAC = 13.09 mg/ml, Mahua Oil = 100 mg/ml)***NE-13**: (Tween 60 = 30 mg/ml, CPC = 10 mg/ml, FAC = 52.39 mg/ml, Mahua Oil = 100 mg/ml)***NE-14**: (Tween 60 = 30 mg/ml, CPC = 10 mg/ml, EDTA = 18.61 mg/ml, Mahua Oil = 100 mg/ml)***NE-15**: (Tween 60 = 30 mg/ml, CPC = 10 mg/ml, Tannic acid = 85.06 mg/ml, EDTA = 93.06 mg/ml, Mahua Oil = 100 mg/ml).
*CPC= Cetylpyridinium chloride **FAC= Ferric ammonium citrate ***EDTA = Ethylenediaminetetraacetic acid.

(sterile water) control (Orru et al., 2010). Shaking of solutions in test tubes were done and incubated at 37 °C for 24 h in inclined position (30°). After completion of incubation period, follow the fixation process by discarding the medium supernatant and using 5 ml volume of 100% methanol per tube for 15 min. Adhering layers were remaining on fixation stairs at the glass surface followed by empting and air drying. The tubes were stained with 5 ml of 0.5% (w/v) crystal violet for 5 min. The overabundance of stain was removed by setting the tubes under running tap water in a uniform manner. The test tubes were then air dried by placing in inverted position in racks. For the quantification of stained adherent cells 5 ml of 33% (v/v) glacial acetic acid per tube was applied to remove the dye bound cells (Ramalingam et al., 2011). Three autonomous experiments were performed for each experimental condition to conclude the results. The density of the solution of bound bacteria was measured by using Uv–vis Spectrophotometer at 590 nm. The UPH_2O was used as a blank to subtract the reading from all sets of experiment.

2.5. Biofilm studies

The biofilm arrangement contemplates were executed as already depicted by (Teixeira et al., 2007; Hwang et al., 2013) with some modification briefly, A 10% (20 µl) portion of an overnight grown bacterial suspension (10^7 CFU/ml) was added to each well except blank of a 96-well plate and maintained a total of 200 µl volume with LB broth. The plates were incubated aerobically in Incubator shaker at 37 °C at 100 rpm, for 72 h. The medium containing suspended bacterial cells was changed after every 12 h intervals and an equal amount of volume of fresh LB broth was added in each well. Growth controls were acquired by incubating the microtitter plates with media and inocula but without antimicrobial agent. Blank controls were maintained by incubating plates with media without inocula. Afterward the completion of incubation period (72 h) the supernatant media was removed and all the biofilms formed by microbes were treated with four different nanoemulsions (NE-2, 7, 12 and 13) with equal volume (200 µl/well) against ESKAPE pathogens for 30 min at room temperature in aseptic condition without agitation. As a positive control, 10% (w/v) Providone-Iodine solution (Betadine- 100 mg/ml) was used. FAC (Ferric ammonium citrate) was also used to compare the antibiofilm effect without combination and with combination of NEs. Following this, NEs and Betadine was discarded and the wells were gently washed twice with sterilized UPH_2O by using wash bottle. To determine the antibiofilm efficacy of NEs, the experiments were performed in triplicate. The measurement of biofilms in microtitter plates was performed as a quantification of viable cells attached in wells. The fixation of bacterial film was done by using 100% methanol (200 µl/well) for 15 min of exposure. The evacuated plates were air dried in sterile condition and stained 0.5% (w/v) crystal violet (200 µl). The wells were washed uniformly (thrice) to rinse off excess stains by using washed bottle (Tarsons). The plates at that point were air dried; and the dye bound to the adherent cells was expelled with 33% (v/v) Glacial acetic acid (200 µl/well). The resulting biomass of dissolved biofilm was quantified by measuring the absorbance of the solubilised dye which was read as an optical density (OD) at 590 nm (Khan et al., 2018). The OD readings obtained from the three duplicate wells and the net values were expressed as average OD values, finally compared with the growth control.

2.6. In vivo toxicity evaluation of nanoemulsion in Bombyx mori

Monitored and standardized the possible adverse/side effects of concentrated NEs as toxic effect at high dose. The determination of effectiveness and cytotoxicity of NEs formulation for prevention ESKAPE pathogen infection in animal models (*Bombyx mori*). *B. mori* was obtained from the farmers at the sericulture unit in Alangayam, Tamil Nadu. It was allowed to acclimatize in the lab conditions and was fed mulberry leaves. In each experimental set, a set of 5 worms (*B. mori*) were treated with the same volume of nanoemulsions as dose of 4 units (80 µl) per individual larvae. The worms were injected with ten different NEs formulations by using 1 ml Insulin syringe and observed their activities upto 24 h at high dose (4 units). A set of worms without treatment were considered as control (Farshi et al., 2017). The percentage of worms which survived was noted after 24 h. The outcomes obtained with larvae infected by direct cuticle injection reliably connect with those of comparable mammalian examinations.

2.7. Statistical analysis

Appropriate statistical techniques were adopted using graph pad prism 7.0 and SPSS 17.0 analytical packages. The result of all the experiments were represented as mean ± SD (standard deviation) in triplicate and employed. Significant variances among the means of samples were analyzed by using paired sample *t*-test and two-way ANOVA (Row versus Column).

3. Results and discussion

3.1. Nanoemulsion formation and preparation

The synthesis of NEs was formulated by using the ingredients including edible oil Mahua (10%), surfactant (Brij 30, Span 20, Triton X-100, Tween 60) and sterile UPH_2O water. The mixing of all measured components were made and dissolved by adding partial volume of water immediately. The samples were mixed with oil by using magnetic stirrer; rest volume of water was added and heated to make them homogeneous and transparent. After heating the mixed solution, there was a distinct change in their visual aspect and hence the emulsions became optically transparent and no turbidity was remaining. The combined phase (aqueous and oil) solutions processed in an inlet of Microfluidizer to yield a course emulsions with fine droplets under high vacuumed pressure.

3.2. Dynamic light scattering (DLS)

The synthesized nanoemulsions were emulsified with the Microfluidizer and subjected to DLS for their size measurements. DLS data of synthetic formulations of NEs indicate that the droplet size varied from 78.80 to 142.00 nm with PDI values of 0.154–0.246. The size distribution histogram of DLS predicted that the mean diameter size calculated as an average of the size distribution by intensity. The peak of the graphs anticipated the mean diameter size of nanoemulsions (Mason et al., 2006). The NE-1, NE-2, NE-3, NE-7, NE-9, NE-10, NE-12, NE-13, NE-14 and NE-15 showing sharp single peak and contained particles with an average size of 142.00, 106.00, 106.00, 7.53, 106.00, 37.80, 106.00, 78.80, 91.30 and 106.00 nm respectively (Fig. 1A–J). The single peak found in DLS analysis indicated the quality of the synthesized nanoemulsion (Anna et al., 2017). High intensity distribution at lower range of droplet size indicates that the formulations were successful in their preparation in the nanometric size range. The size evaluation of all synthesized and stored NEs were repeated two times at the interval of 8 months, with no significant change in particle size makeup and distribution.

3.3. Determination of MIC and MBC against ESKAPE pathogens

The antimicrobial properties of synthesized nanoemulsions were determined by means of MIC and MBC. The MIC was recorded as the lowest concentration at which no obvious development of the test pathogens was observed. All (ten) synthesized nanoemulsions were tested for their antimicrobial activity through micro-dilution method (Almadiy et al., 2016) against ESKAPE pathogens (*E. faecium* (MCC 2763), *S. aureus* (MRSA-ATCC 33591, MTCC 1430, and Clinical isolate),

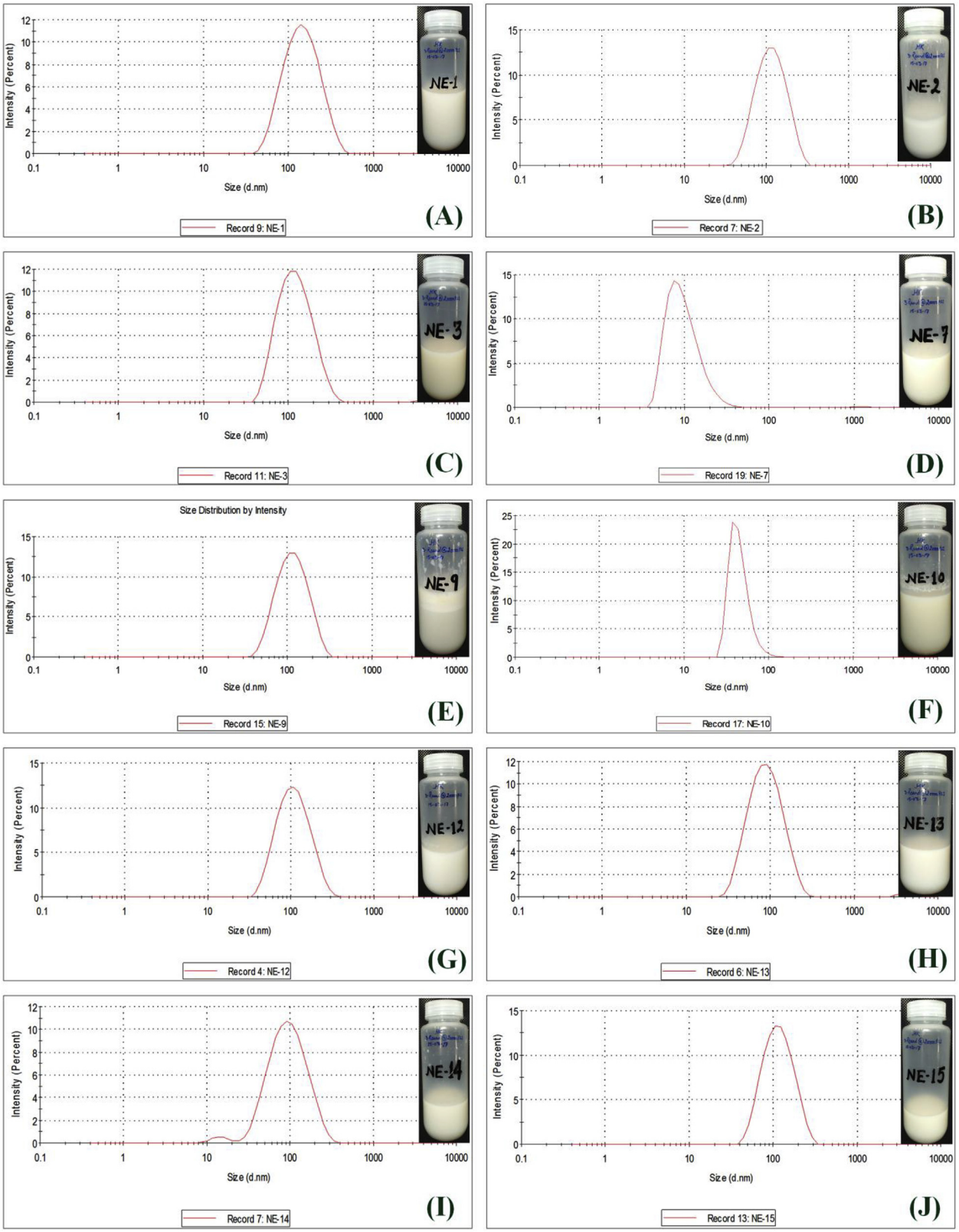

Fig. 1. Mean droplet size distribution of synthesized nanoemulsions: The ten nanoemulsions (A, B, C, D, E, F, G, H, I and J) with different detergents and Mahua oil were prepared by three rounds of emulsification at 20,000 lb/in^2 with a Microfluidizer LM-10. The average size distributions were analyzed using a Dynamic Light Scattering (DLS).

K. pneumoniae (ATCC 35657) *A. baumannii* (ATCC, 19606), *P. aeruginosa* (ATCC 27853) and *E. aerogenes* (MTCC 111). Among all NE-2, NE-7, NE-13 and NE-14 did not showed more significant antibacterial activity as measured in both MIC and MBC studies while NE-1, NE-3, NE-9, NE-10, NE-12 and NE-15 presented effective and significant antibacterial activity at high dilution ranges (MIC-1:4 to 1:196.5 and

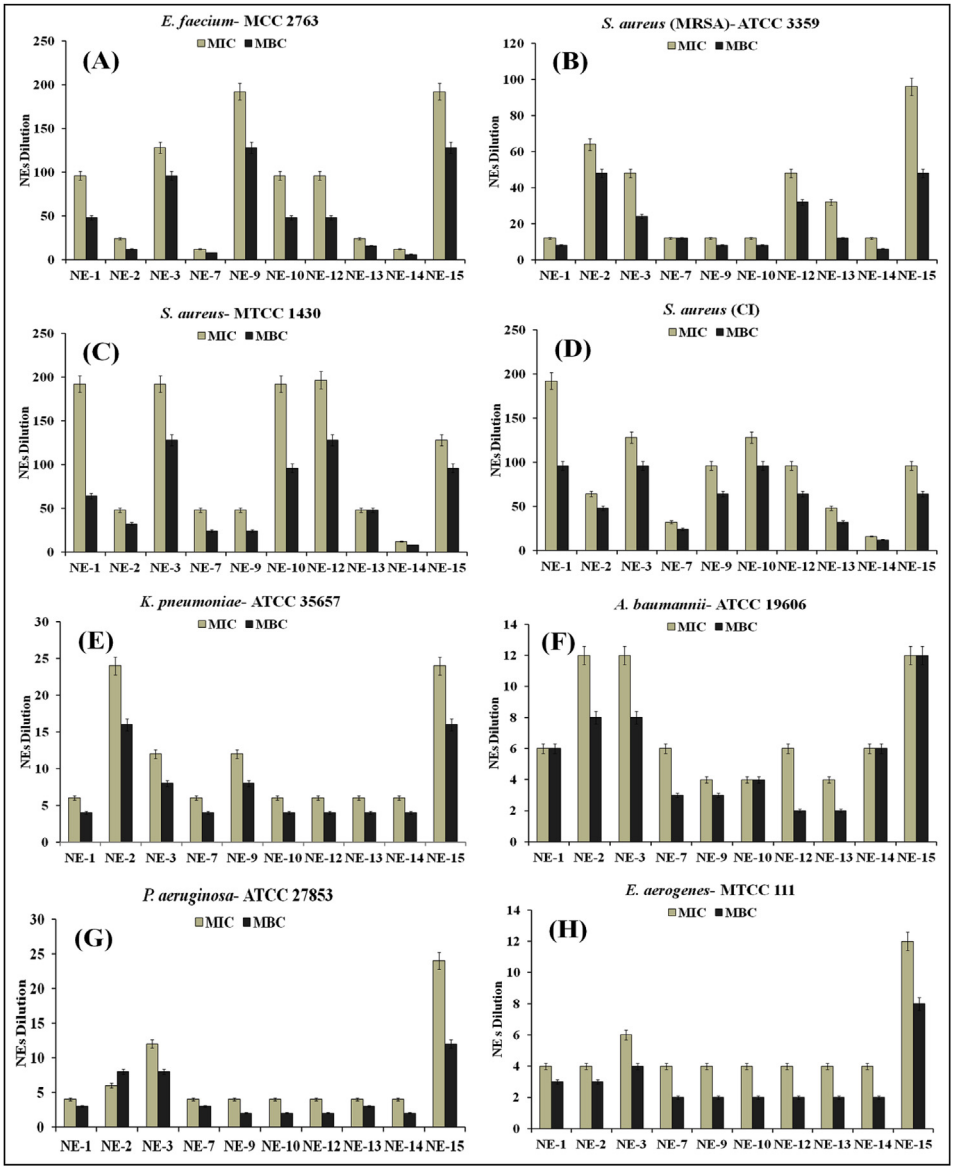

Fig. 2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of nanoemulsions (NE-1, NE-2, NE-3, NE-7, NE-9, NE-10, NE-12, NE-13, NE-14, and NE-15) against ESKAPE pathogens (A–H).

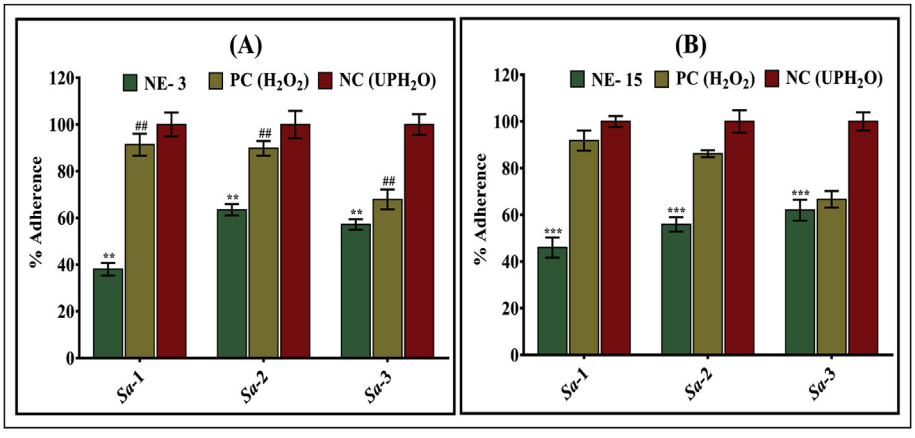

Fig. 3. Inhibitory effect of NE-3 **(A)** and NE-15 **(B)** on glass surface adherence of the strains of *Staphylococcus aureus* biofilms (NE- Nanoemulsion; PC- Hydrogen peroxide (H_2O_2); NC- negative control (sterile UPH_2O).

Note: Sa-1 = Methicillin resistant *Staphylococcus aureus* (MRSA) - ATCC 33591, Sa-2 = *S. aureus*-MTCC 1430, Sa-3 = *S. aureus*-clinical isolate.

All the data represent the mean ± SD of two independent experiments done in triplicates. The significant differences are indicated by $^{**}p < 0.05$, $^{***}p < 0.005$ nanoemulsions (NE) Vs control (NC), $^{\#\#}p < 0.05$, $^{\#\#\#}p < 0.005$ denotes a statistically significant difference compared with Hydrogen peroxide (H_2O_2) Vs control (NC).

MBC- 1:2 to 1:128) against all strains of ESKAPE pathogens in both the MIC and MBC assays (Fig. 2 A- H). Individually, Mahua oil (10%) alone against all the strains of ESKAPE pathogens not exhibited any significant growth inhibition. The presence of Mahua oil in nanoemulsions can boost the antimicrobial activities and may work as catalysis at nanoscale in the form of nanodroplets. Based on the resulted

efficacy of NEs, selected synthesized NEs were tested for further validation and additional studies on more virulent drug resistant pathogens.

3.4. Anti-adherence effect of NEs on ESKAPE strains

Based on best aftereffects of MIC and MBC as higher inhibitory impact on strains of *Staphylococcus aureus* from ESKAPE pathogens, NE-3 and 15 were chosen for hostile to ant-adherence test. The inhibitory influence of the nanoemulsions on adherence of cells to the glass surfaces of test tubes was inspected *in vitro* by using three strains of developing cells of *S. aureus* (MRSA) - ATCC 33591, *S. aureus*- MTCC 1430 and *S. aureus*-clinical isolate). The outcomes of our results stated that the adherence of MRSA- ATCC 33591, *S. aureus*- MTCC 1430 and *S. aureus*-clinical isolate, on the exposure of NE-3 was found 38.04, 63.44 and 58.42% (Fig. 3 A) and in case of NE-15, it was observed as 46.00, 55.92 and 56.23% (Fig. 3 B) adherence effect respectively, when compared with positive (H_2O_2) and negative (sterile UPH_2O) control. The level of attachment of all three strains of *S. aureus* on glass surface of test tubes was significantly reduced in NEs treated groups (Ramalingam and Lee, 2018). Among all the strains, the highest elimination was found in *S. aureus* (MRSA)-ATCC 33591 with due to the exposure of both NEs. The anti-adherence behaviour of nanoemulsions suggested that these formulations could be useful for the development of potential antimicrobials.

3.5. Effect of synthesized NEs on biofilm inhibition

The formation of biofilms is complex bacterial populations and the strategy for the estimation of microbial sustainability as well as the progression of the disease that resist the action of antibiotics. Due to the secretion of different surface molecules and virulence factors, associations of antibiotic resistant gene in bacteria promote to form a matrix (Saising et al., 2012). In this order synthesized NEs were tested to estimate the antibiofilm activities formed by ESKAPE pathogens. The metabolic activities of biofilms integrity were measured by post treatment (Ramalingam et al., 2012). Initially all the nanoemulsion performed for the biofilm assay, based on the preliminary biofilm assay we restricted to NE 2, 7, 12 and 13, because all four NE showed more effectiveness against biofilm, this may due to NE 2, 12 and 13 addition of antibiofilm agent (Ferric ammonium citrate, 50 μM–200 μM) as a key component of NEs (Wang et al., 2008). NE-7 also showed anti-biofilm properties in the preliminary assays due to its very tiny size of the nanodroplet (7.53 nm) compare other all the nanoemulsion. The NE-2, markedly inhibited the biofilm (Fig. 4 A) formed by *S. aureus* (MRSA) (54.68%) and the independent with equal concentration of FAC showed 4.98% inhibition, NE-7 showed more inhibition of biofilm (Fig. 4 B) against *E. aerogenes* (54.48%), NE-12 exhibited highest inhibition of biofilm (Fig. 4 C) on *E. faecium* (19.07%) and FAC showed only 3.93% inhibition, NE-13 expressed maximum biofilm inhibition (Fig. 4 D) of *E. aerogenes* (54.26%) and the same concentration of FAC inhibited only 2.59% among all ESKAPE pathogens when compared with the negative control (sterile UPH_2O). While the inhibition of biofilm by the exposure of betadine solution (positive control) was recorded as (*S. aureus* MRSA) 79.48%, (*E. aerogenes*) 82.61%, (*E. faecium*) 30.33%, and (*E. aerogenes*) 47.94% when compared with the negative control (Oduwole et al., 2010). Overall in our biofilm inhibition investigation, NE-12 found more effective against all ESKAPE pathogens in comparison of rest synthesized NEs. Betadine solution is a complex of polyvinyl pyrrilidine and triiodine ions, used as an antiseptic in wound cleaning and surgery to destruct the bacterial intact populations (Hoekstra et al., 2017).

The antibiofilm activities of NEs in the present study were found higher than that of betadine, which is presently available in the market as antiseptic antimicrobial and antibiofilm agent. The synthesized NEs are able to disrupt the integrity of biofilm and lowering the metabolic activities of pathogens deep inside the biofilm instead the application of the betadine solution without leaving the side effect. The efficacy of NEs in disrupting existing biofilms indicates the antibiofilm potential and can be concluded as an antibiofilm agent.

3.6. In vivo toxicity of nanoemulsion in Bombyx mori

NEs are generally stabilized by a moderately extensive measure of surfactants to create clear dispersions. In this study, we evaluated the cytotoxicity effect of concentrated (without dilution) nanoemulsion for all the formulation (Islam et al., 2017). The effects of the surfactant composition and co-solvent ratio on droplet size and on dispersion showed that the synthesized NEs are steady nanoemulsions. The toxic effects of the nanoemulsion at higher dose (4 unit/*B. mori*) were evaluated and observed as a Live/dead individual up to 20 h (Fig. 5 A). It was observed that, when the dose of NEs was given to the individuals, they expressed some laziness and also showed slow movement on mulberry leaves with eating habitat upto 10 min. After all individuals started eating and showed normal behaviour in their motion (Kaito et al., 2002). The activities and count (Live/Dead) of *B. mori* were observed upto 20 h at the intervals of 1, 2, 4, 8, 12, 16 and 20 h. Hundred percentage individuals were live up to the 8 h with the exposure of all NEs. With the exposure of nanoemulsions (NE-1, 2, 3, 7, 9, 10, 12, 13,

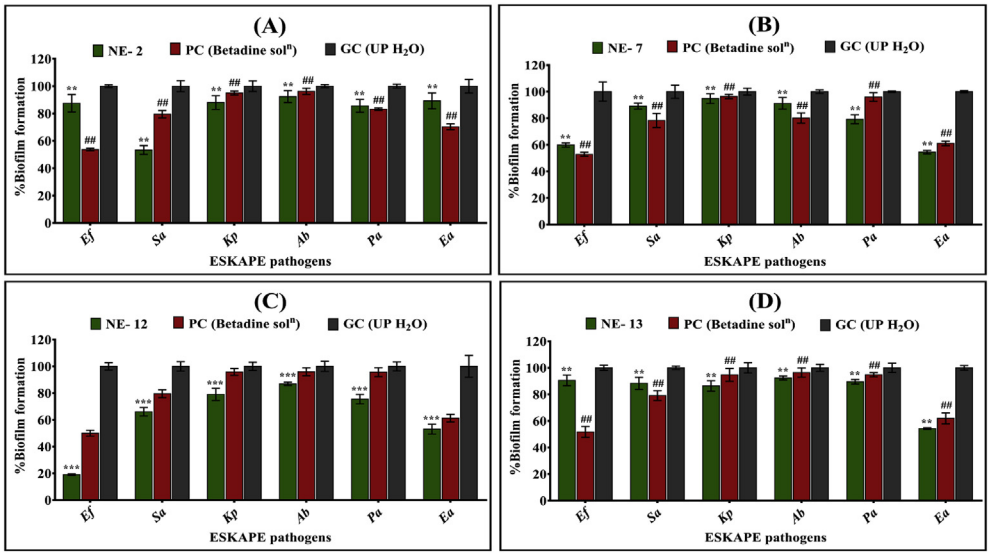

Fig. 4. Antibiofilm effects of NE-2 **(A)**, NE-7 **(B)**, NE-12 **(C)**, and NE-13 **(D)** on growth of ESKAPE pathogen's intact films (NE-Nanoemulsion; PC- positive control (Betadine solution), GC- growth control (sterile UPH_2O).

Note: (Ef = *E. faecium* (MCC 2763), Sa = *S. aureus*-MRSA (ATCC 33591), Kp = *K. pneumoniae* (ATCC 35657), Ab = *A. baumannii* (ATCC, 19606), Pa = *P. aeruginosa* (ATCC 27853) and EA = *E. aerogenes* (MTCC 111). All the data represent the mean ± SD of two independent experiments done in triplicates. The significant differences are indicated by $^{**}p < 0.05$, $^{***}p < 0.005$ nanoemulsions (NE) Vs control (GC), $^{\#\#}p < 0.05$, $^{\#\#\#}p < 0.005$ denotes a statistically significant difference compared with betadine (PC) Vs control (GC).

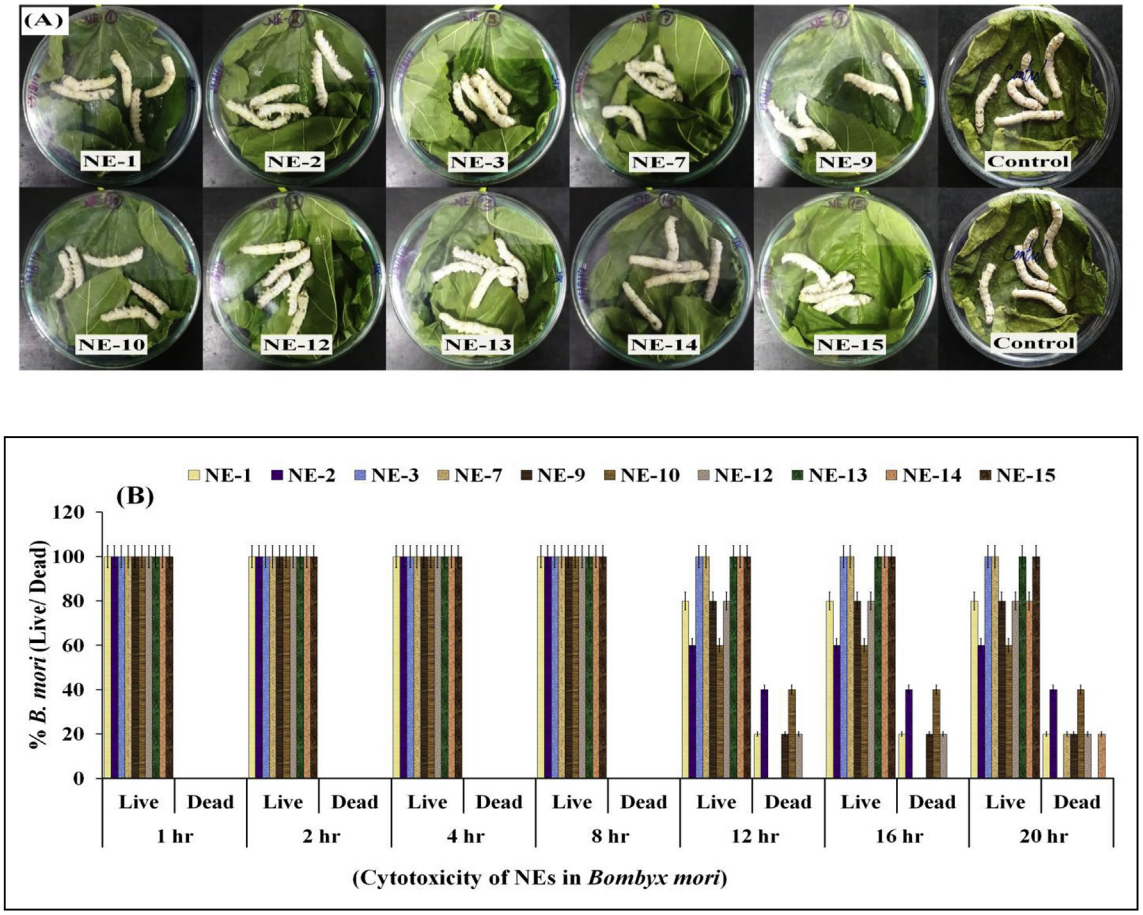

Fig. 5. *In vivo* toxicity estimation of NEs-1, NEs-2, NEs-3, NEs-7, NEs-9, NEs-10, NEs-12, NEs-13, NEs-14 and NEs-15 in *Bombyx mori.* counted as a LIVE/DEAD at different time intervals (1, 2, 4, 8, 12, 16, 20 and 24 h) of incubation.

14 and 15) at 12 and 16 h, the individuals were counted as percentage of live/dead as (80/20, 60/40, 100/0, 100/00, 80/20, 60/40, 80/20, 100/00, 100/00 and 100/00) while at the completion of 20 h incubation counted as (80/20, 60/40, 100/00, 80/20, 80/20, 60/40, 80/20, 100/00, 80/20 and 100/00) respectively (Fig. 5 B). Based on these results it is concluded that most of the formulated NEs showing biocompatibility in animal model (*B. mori*) and may be used in drug delivery and as an alternative of antimicrobial agents for the treatment of multidrug resistant human pathogens (Hamamoto et al., 2004).

4. Conclusion

The appropriate formulations and synthesis of nanoemulsions offers the several ways of mode of action to develop the antimicrobial route to attack on multidrug resistant pathogens leading to rid off chronic and nosocomial infections (Alkhatib et al., 2013). The properties of NEs make them unique such as in increasing bioavailability, controlled drug release, solubilization of non polar compounds and protecting ability of labile drugs (Jaiswal et al., 2015). In present study, all the NEs were successfully prepared using synthetic and natural additives through passing under high pressure (20000 psi) by using Microfluidizer LM-10. The droplet sizes of the synthesized NEs were evaluated through DLS technique with the values under PDI < 0.24 and access their stability factor. Screening of NEs for toxicity effect on *B. mori* validate for their biocompatibility in animal model. The results of our study supported a potential role for NEs as an antimicrobial treatment for multi drug resistant human pathogens. Here, we compared the bacteriostatic and bactericidal characteristics of ten synthesized nanoemulsion against the strains of ESKAPE pathogens. NE-3 and 15 effectively inhibited adherences of the strains of *S. aureus* to glass surfaces and NE-2, 7, 12 and 13 exhibited the significant disruption of biofilm formed by ESKAPE pathogens. The resultant NEs as anti-adherence, biocompatible, anti-biofilm broker and morphological disruptive agents evoke that these NEs could be alternative for the improvement of promising antimicrobial agents against ESKAPE pathogens. Therefore, nanoemulsions could be providing a unique way to improve the activity of sudden/partially soluble drugs and efficiency of oil-soluble agents by expanding their effects on targeted sites.

Conflicts of interest

Authors have no conflict to report.

Acknowledgements

This work was supported by the DST Science and Engineering Research Board (SERB), India [Grant number- SERB/LS-267/2014] for providing fellowship and funds to perform this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101025.

References

Alkhatib, M.H., Aly, M.M., Bagabas, S., 2013. Antibacterial activity and Mechanism of action of lipid nanoemulsions against *Staphylococcus aureus*. J. Pure Appl. Microbiol.

7, 259–267.
Almadiy, A.A., Nenaah, G.E., Al Assiuty, B.A., Moussa, E.A., Mira, N.M., 2016. Chemical composition and antibacterial activity of essential oils and major fractions of four Achillea species and their nanoemulsions against foodborne bacteria. LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 69, 529–537.
Anna, E.M.F.M., Oliveiraa Duartea, J.L., Cruza, R.A.S., da-Conceicaoc¸, E.C., Carvalhoa, J.C.T., Fernandesa, C.P., 2017. Utilization of dynamic light scattering to evaluate Pterodon emarginatus oleoresin-based nanoemulsion formation by non-heating and solvent-free method. Revis. Brasile. Farmaco. 27, 401–406. https://doi.org/10.1016/j.bjp.2016.11.005.
Balouirin, M., Sadiki, M., Ibnsouda, S.K., 2016. Methods for *in vitro* evaluating antimicrobial activity: a review. J. Pharm. Anal. 6, 71–79.
Barros, A.S., Morais, S.M., Ferreira, P.A.T., Vieira, I.G.P., Craveiro, A.A., Fontenelle, R.O.S., Menezes, J.E.S. A., Silva, F.W.F., Sousa, H.A., 2015. Chemical composition and functional properties of essential oils from Mentha species. Ind. Crop. Prod. 76, 557–564.
Billerbeck, V.G., 2007. Essential oils and bacteria resistant to antibiotics. Phytotherapy 5, 249–253.
Farshi, P., Tabibiazar, M., Ghorbani, M., Hamishehkar, H., 2017. Evaluation of antioxidant activity and cytotoxicity of cumin seed oil nanoemulsion stabilized by sodium caseinate- Guar Gum. Pharmaceut. Sci. 23, 293–300.
Hamamoto, H., Kurokawa, K., Kaito, C., Kamura, K., Razanajatovo, I.M., Kusuhara, H., Santa, T., Sekimizu, K., 2004. Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. Antimicrob. Agents Chemother. 48, 774–779.
Hamouda, T., Baker, J.R.J., 2000. Antimicrobial mechanism of action of surfactant lipid preparations in enteric Gram-negative bacilli. J. Appl. Microbiol. 89, 397–403.
Hemmila, M.R., Mattar, A., Taddonio, M.A., Arbabi, S., Hamouda, T., Ward, P.A., Wang, S.C., Baker, J.R.J., 2010. Topical nanoemulsion therapy reduces bacterial wound infection and inflammation after burn injury. Surgery 148, 499–509.
Hoekstra, M.J., Westgate, S., 029*-J, Mueller, S., 2017. Povidone-iodine ointment demonstrates *in vitro* efficacy against biofilm formation. Int. Wound J. 14, 172–179.
Hwang, Y.Y., Ramalingam, K., Bienek, D.R., Lee, V., You, T., Alvarez, R., 2013. Antimicrobial activity of nanoemulsion in combination with Cetylpyridinium chloride in multidrug-resistant *Acinetobacter baumannii*. Antimicrob. Agents Chemother. 57, 3568–3575.
Islam, M.T., Streck, L., Alencar, M.V.O.B., Silva, S.W.C., Machado, K.C., Machado, K.C., Júnior, A.L.G., Paz, M.F.C.J., Mata, A.M.O.F., Sousa, J.M.C., Junior, J.S.C., Rolim, H.M.L., Silva-Junior, A.A., Melo-Cavalcante, A.A.C., 2017. Evaluation of toxic, cytotoxic and 394 genotoxic effects of phytol and its nanoemulsion. Chemosphere 177, 93–101.
Jaiswal, M., Dudhe, R., Sharma, P.K., 2015. Nanoemulsion: an advanced mode of drug delivery system. 3 Biotech 5, 123–127.
Kaito, C., Akimitsu, N., Watanabe, H., Sekimizu, K., 2002. Silkworm larvae as an animal model of bacterial infection pathogenic to humans. Microb. Pathog. 32, 183–190.
Khan, M.H., Unnikrishnan, S., Ramalingam, K., 2018. Bactericidal potential of silver-tolerant bacteria derived silver nanoparticles against Multi Drug Resistant ESKAPE pathogens. Biocat. Agri. Biotechnol (BCAB939). https://doi.org/10.1016/j.bcab.2018.12.004 (Article(in press).
LiPuma, J.J., Rathinavelu, S., Foster, B.K., Keoleian, J.C., Makidon, P.E., Kalikin 1, L.M., Baker, J.R., 2009. *In vitro* activities of a novel nanoemulsion against burkholderia and other multidrug-resistant cystic fibrosis-associated bacterial species. Antimicrob. Agents Chemother. 249–255.
Mason, T.G., Wilking, J.N., Meleson, K., Chang, C.B., Graves, S.M., 2006. Nanoemulsions: formation, structure, and physical properties. J. Phys. Condens. Matter 18, 635–666. https://doi.org/10.1088/0953-8984/18/41/R0.
Oduwole, K.O., Glynn, A.A., Molony, D.C., Murray, D., Rowe, S., Holland, L.M., McCormack, D.J., O'Gara, J.P., 2010. Anti-biofilm activity of sub-inhibitory povidone iodine concentrations against *Staphylococcus epidermidis* and *Staphylococcus aureus*. J. Orthop. Res. 28, 1252–1256.
Orru, G., Nero, S.D., Tuveri, E., Ciusa, M.L., Pilia, F., Erriu, M., Orrù, G., Liciardi, M., Piras, V., Denotti, G., 2010. Evaluation of antimicrobial- antibiofilm activity of a hydrogen peroxide decontaminating system used in dental unit water lines. Open Dent. J. 28, 140–146.
Pandey, B., Agarwal, S., 2015. Study of antimicrobial activity of *Madhuca longifolia* and its against various microorganisms. J. Env. Sc. Tox. Food Technol. 1 (2), 10–15.
Ramalingam, K., Amaechi, B.T., Rawls, H.R., Lee, V.A., 2011. Antimicrobial activity of nanoemulsion on cariogenic *Streptococcus mutans*. Arch. Oral Biol. 56, 437–445.
Ramalingam, K., Amaechi, B.T., Ralph, R.H., Lee, V.A., 2012. Antimicrobial activity of nanoemulsion on cariogenic planktonic and biofilm organisms. Arch. Oral Biol. 57, 15–22.
Ramalingam, K., Lee, V.A., 2018. Antibiofilm activity of an EDTA-containing nanoemulsion on multidrug-resistant *Acinetobacter baumannii*. Artif. Cells Nanomed. Biotechnol. 2, 1–7.
Ribeiro, R.C., Barreto, S.M., Ostrosky, E.A., Rocha-Filho, P.A., Verissimo, L.M., Ferrari, M., 2015. Production and characterization of cosmetic nanoemulsions containing *Opuntia ficus-indica* (L.) mill extract as moisturizing agent. Molecules 20 (2), 2492–2509.
Saising, J., Dube, L., Ziebandt, A.K., Voravuthikunchai, S.P., Nega, M., Gotz, F., 2012. Activity of gallidermin on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Antimicrob. Agents Chemother. 56, 5804–5810.
Teixeira, P.C., Leite, G.M., Domingues, R.J., Silva, J., Gibbs, P.A., Ferreira, J.P., 2007. Antimicrobial effects of a microemulsion and a nanoemulsion on enteric and other pathogens and biofilms. Int. J. Food Microbiol. 118, 15–19.
Wang, E.W., Agostini, G., Olomu, O., Runco, D., Jung, J.Y., Chole, R.A., 2008. Gentian violet and ferric ammonium citrate disrupt *Pseudomonas aeruginosa* biofilms. Laryngoscope 118, 2050–2056.