Supporting Information

Membrane Targeted Multifunctional Cationic Nanoparticles Conjugated Fusogenic Nanoemulsion (CFusoN): Promoting Membrane Depolarization and Lipid Solubilization to Accelerate the Killing of *Staphylococcus aureus*

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Materials

Lemongrass oil (LGO) and tea tree oil (TTO) were purchased from Ethnocure Products Pvt Ltd., India. Propylene glycol (PG), cetyltrimethylammonium bromide (CTAB), polyoxyethylene sorbitan monooleate (Tween 80), silver nitrate, sodium borohydride, sodium hydroxide, cetylpyridinium chloride (CPC), Nile red, rhodamine B (RhoB), 3,5-dipropylthiacarbocyanine iodide [$DiSC_3(5)$], N-phenyl-1-naphthylamine (NPN), 3,3-diethyloxacarbocyanine iodide [$DiOC_2(3)$], Triton X and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were procured from Sigma Aldrich, India. A bacterial Live/Dead cell viability assay kit containing SYTO 9 and propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay kit and a mammalian cell viability assay kit containing calcein AM and ethidium homodimer-I (EH-I) were purchased from Invitrogen. Penicillin, nutrient broth, agar powder, Mueller Hinton agar (MHA) and all other ingredients were purchased from Himedia, India. L929 cells were procured from NCCS, Pune, India.

Microorganisms

Staphylococcus aureus (MTCC 3160) was bought from the Microbial Type Culture Collection (MTCC), CSIR-Institute of Microbial Technology (IMTech), Chandigarh, India. The microorganism was cultured in nutrient broth (0.3 % beef extract, and 0.5 % peptone) and sub-cultured in nutrient agar slants (0.3 % beef extract, and 0.5 % peptone, and 1.2 % agar) to maintain their viability.

Synthesis of oil in water nanoemulsion (NE)

The aqueous phase utilized for the synthesis of nanoemulsion was 6 % (w/w) Tween 80, 4 % (w/w) and Propylene glycol (PG) dispersed in deionized water. 1 % LGO and 1 % TTO (w/w) mixed in equal mass ratios were utilized as the lipidic phase in the nanoemulsion synthesis.[1] Briefly, Tween 80 and Propylene Glycol were dissolved in water followed by addition of the essential oils in their respective mass ratios. The entire mixture had undergone high speed sonication. The final mixture was stirred with deionized water for 24 h at 30 °C. The nanoemulsion was synthesized in a batch of 50 ml and was stored for further functionalization and characterization.

Characterization

The UV-vis spectra of NE, CNP and NECNP were recorded in the range 200-800 nm on JASCO-V730 UV-vis spectrophotometer, while the FTIR spectra were collected on PerkinElmer FTIR spectrometer in the range of 4000–400 cm^{-1}. The NE, CNP and NECNP were further analyzed using ^{1}H-NMR. Samples for ^{1}H-NMR analysis with or without essential oils or CNP were prepared following the same protocol in D_2O as the aqueous phase. The samples were transferred to the NMR tubes, dispersed in 0.6 ml D_2O and their ^{1}H spectra were recorded on a 600 MHz NMR (Bruker Ultrashield). Chemical shifts of samples were determined relative to the residual solvent peak of D_2O at δ_H 4.790. Further, the HRTEM image of NECNP was recorded on HRTEM (The Tecnai G2 20 S-TWIN (FEI)) instrument operating at an accelerating voltage of 200 kv. Prior to that, the NECNP was dropped on a copper grid and held for 1 min. The excess samples were wiped out by a filter paper. Subsequently, 10 µl of 1% phosphotungstic acid was added on the TEM grid as a negative stain, incubated for 30 sec and excess stain was removed.[2, 3] The sample for AFM was prepared by placing a single drop of NECNP on freshly cleaved mica sheet and image was taken in ambient condition using a multimode AFM.[4] (Agilent Technologies Pico5500) The samples for FESEM image were prepared by drop casting the solution on the glass coverslips and images were recorded on Nova Nano FESEM 450 (FEI) having a resolution of 1.6 nm at 1 kv (TLD-SE) & <1 nm at 15 kv (TLD-SE). The hydrodynamic size distribution and surface charge of NECNP was recorded by DLS and ζ potential analysis and the data was recorded on Zetasizer (Malvern Zetasizer, UK) equipped with a 633 nm HeNe laser.[4] CLSM (Leica TCS SP8) was also utilized to view the fluorescent image of NECNP. RhoB (50 µg/ml) was loaded in the NECNP solution during the synthesis of the NE. Subsequently, RhoB loaded NECNP was dropped on a glass slide followed by placing a coverslip on top of the sample and image was recorded under CLSM at an excitation and emission of 510 nm and 650 nm, respectively.[5]

Bacterial sample preparation for AFM and HRTEM analysis

To study the effect of NECNP on the alteration and damage of the bacterial cells, the AFM and HRTEM was used. The bacterial cells were washed 3 times with PBS (pH 7.2) to decant the media and then treated with NECNP for 4 h at 37°C. PBS (pH 7.2) treatment was considered as the negative control. After the treatment, both the PBS and NECNP treated cells were dropped on the mica sheet for air drying to visualize under AFM. The cantilever with long tips with aspect ratio 4:1 and spring constant 40 N/m and resonance frequency of 250 kHz were utilized. Scanning was initiated with the area of 100 µm x 100 µm, and then the scan area of imaging got gradually reduced to 3.6 µm x 3.6 µm. The image analysis software was used to get the size, 3D images, height and roughness of the samples. The bacterial sample for HRTEM analysis was prepared by drop casting the PBS and NECNP treated cells on the copper grid.

Analysis of Membrane Fatty Acid Composition

The bacterial lipids and fatty acid profile after NECNP treatment were proceeded with hydrolysis (**A**) and then esterification reaction (**B**).

(A) Hydrolysis: The extracts were taken in 2.0 M ethanolic KOH (1.0 ml) and heated at 60°C in stirring condition for 1.5 h. The reaction mixture was cooled at room temperature and 1.0 M HCl (2.0 ml) was added dropwise to acidify at pH 1-2. It was further extracted with n-hexane (1.0 ml×3), organic layers were combined, dried over anhydrous sodium sulphate and concentrated to obtain brown oily liquid.

(B) Esterification: The obtained hydrolyzed product was further subjected to methylation in HPLC grade methanol (2.0 ml) added with catalytic amount of concentrated sulphuric acid and kept for 3 h stirring at 60°C. Then, methanol in the reaction mixture was concentrated, added with 1.0 ml water and extracted with ethyl acetate (1.0 ml×3). The ethyl acetate layers were pooled and concentrated to obtain brownish oil. The derivatized sample was further subjected to gas chromatograph-mass spectrometer (GC-MS) analysis.[6] (Agilent 8890 gas chromatograph coupled with Agilent 7010B mass spectrometer)

Results

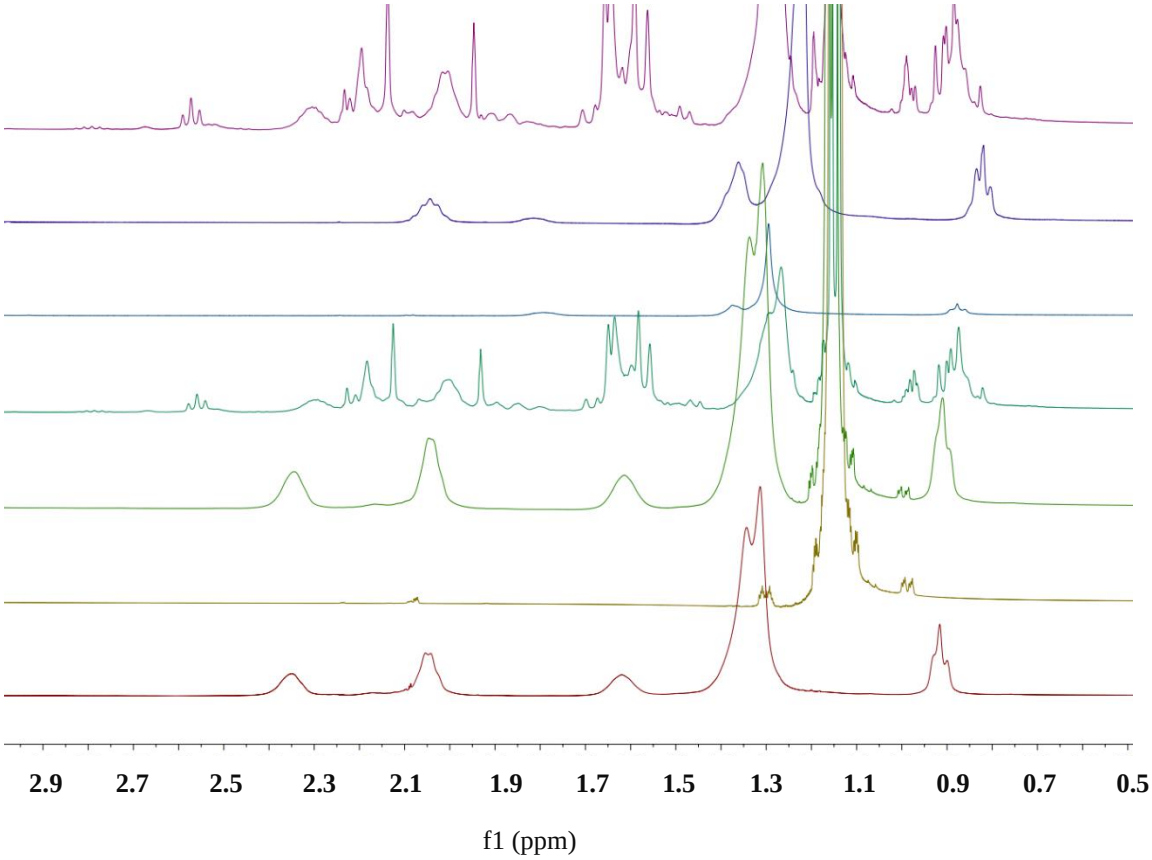

Fig. S1A Expansion of NMR spectrum (0.5-2.9 ppm).

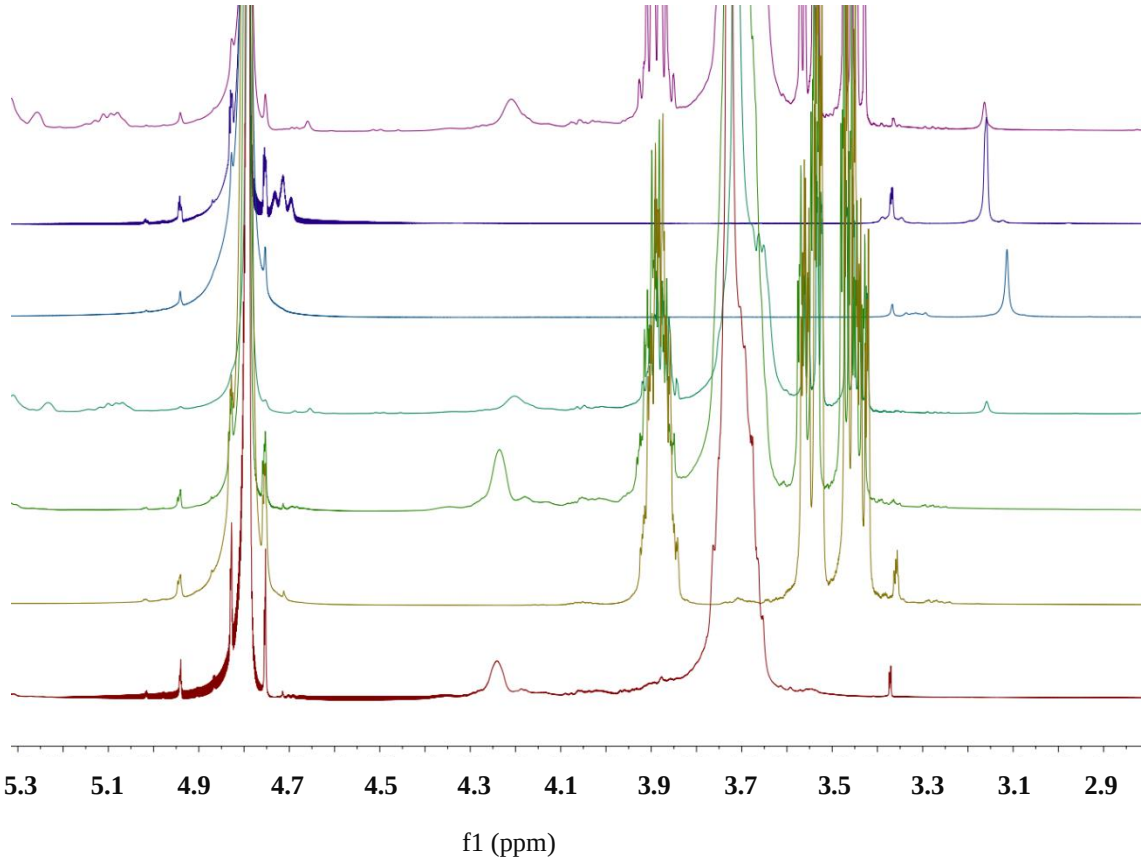

Fig. S1B Expansion of NMR spectrum (2.9-5.3 ppm).

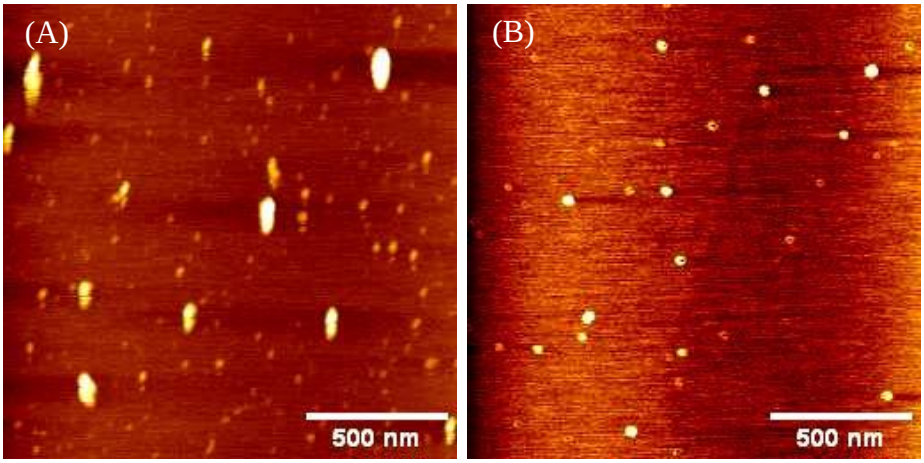

Fig. S2 AFM images of NE (A) and CNP (B).

The as prepared **CFusoN** showed good stability upto 90 days with yellowish brown colour as depicted in **Fig. S3A, inset**. The results in **Fig. S3A** showed that NECNP depicted a significant sharp peak at around 410 nm without much shifting for the period of 90 days. The spectrum of NECNP at Day 1 showed a sharp peak at 412 nm while, at Day 90 it exhibited a peak at 416 nm with minimum reduction in intensity. The droplet size is also considered as an important parameters of stability study. The droplet size (**Fig. S3B)** and ζ potential **(Fig. S3C**) of NECNP at Day 1 was found to be 98.1 ± 4.3 nm and 24.4 ± 2.9 mV, respectively while, at Day 90 exhibited 120.5 ± 6.6 nm and 22.1 ± 2.3 mV, respectively. This minimum difference in their UV-vis spectrum, droplet size and surface charge may be due to the stabilization of NECNP. The antibacterial efficacy (**Fig. S3D**) of NECNP against *S. aureus* at Day 1 and Day 90 was 23.7 ± 1.5 mm and 21 ± 1 mm, respectively also supported the other stability results.

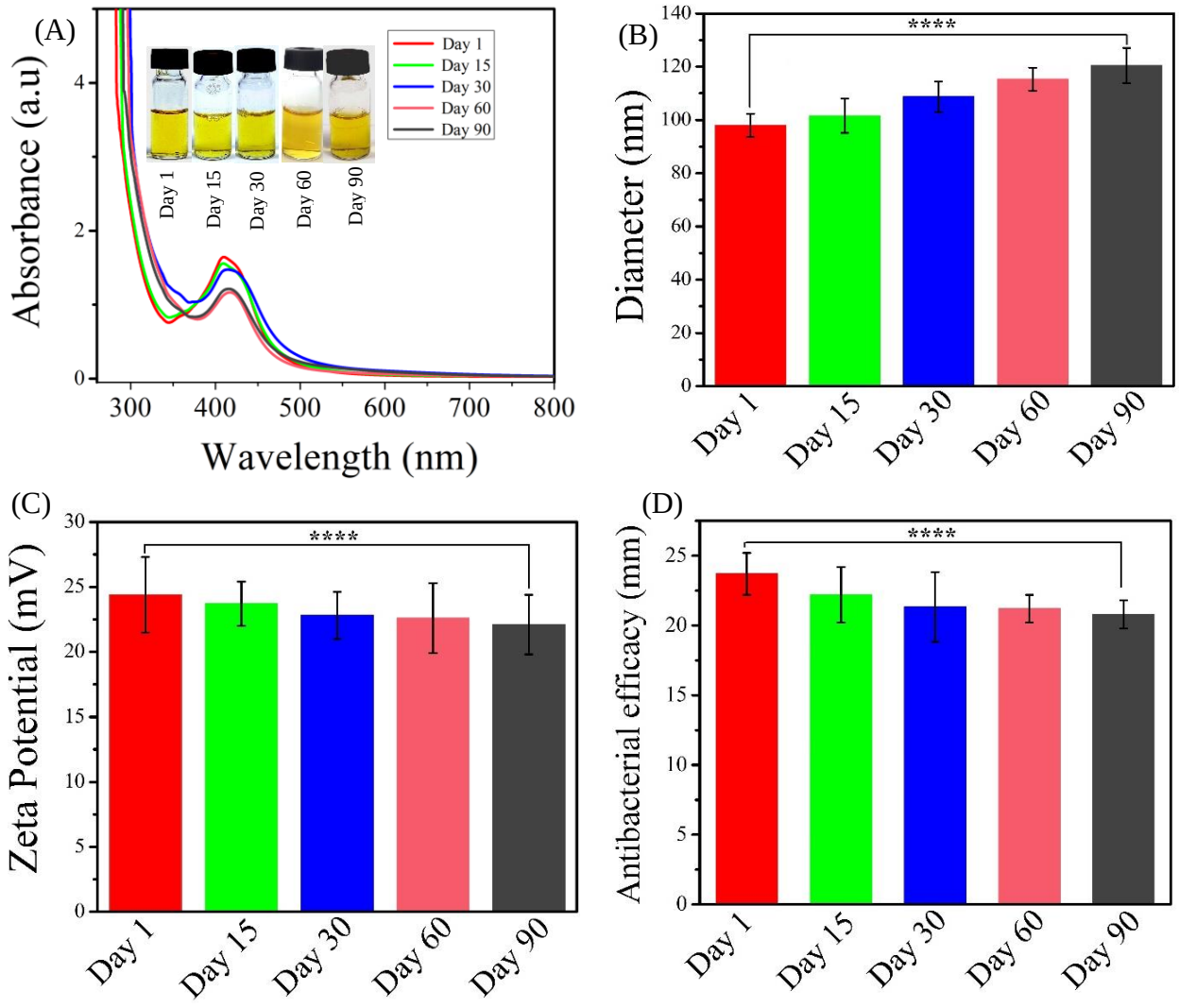

Fig. S3 UV-vis spectrum (A), hydrodynamic diameter (B), zeta potential (C) and antibacterial efficacy (D) of NECNP upto 90 days. Data demonstrated an average of three independent experiments, error bar is shown by ± SD; significant differences are denoted by ****p value < 0.05.

The antibacterial efficacy of NE, CNP and NECNP against *S. aureus* was investigated by the Kirby Bauer method. After overnight incubation of *S. aureus* with NE, CNP and NECNP, there appeared a visible zone of inhibition after the treatments. The result (**Fig. S4A**) showed that NECNP formed the most distant clear zone around the well of 23.7 ± 1.5 mm as compared to individual NE and CNP, which showed 16.7 ± 0.6 and 19 ± 1 mm, respectively. The NECNP demonstrated an increase in the zone diameter due to

the synergistic activity of NE and CNP. The CPC treated bacterial cells also exhibited strong zone of inhibition similar to the NECNP consequently, AgNPs also inhibited the bacterial growth. In the case of the PBS and Tween 80 treated bacterial cells, there was no zone of inhibition as expected. On the other hand, penicillin showed visible zones of inhibition (**Fig. S4B**).

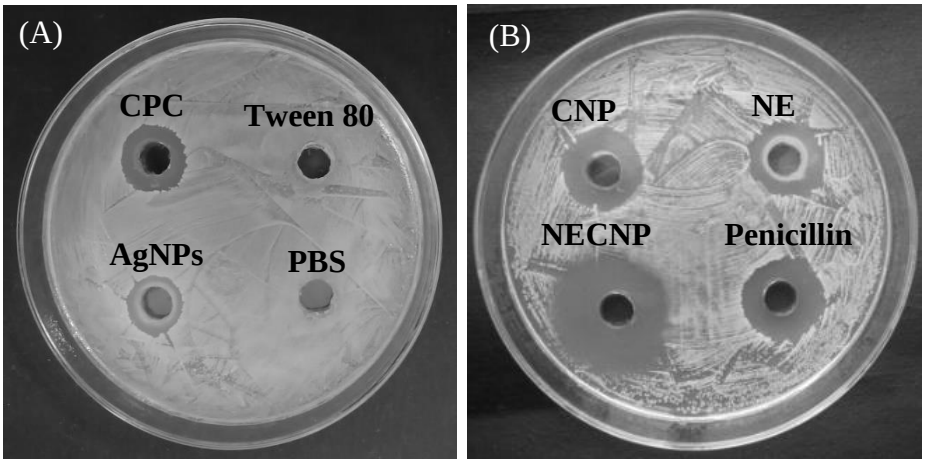

Fig. S4 Zone of inhibition of AgNPs, Tween 80, CPC and NC (A) and NE, CNP, and NECNP (B).

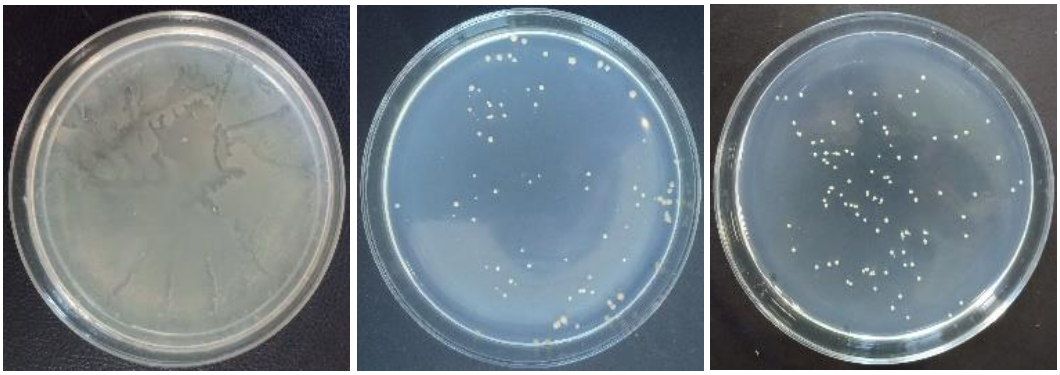

Fig. S5. Time dependent antibacterial activity of NECNP by plate count method.

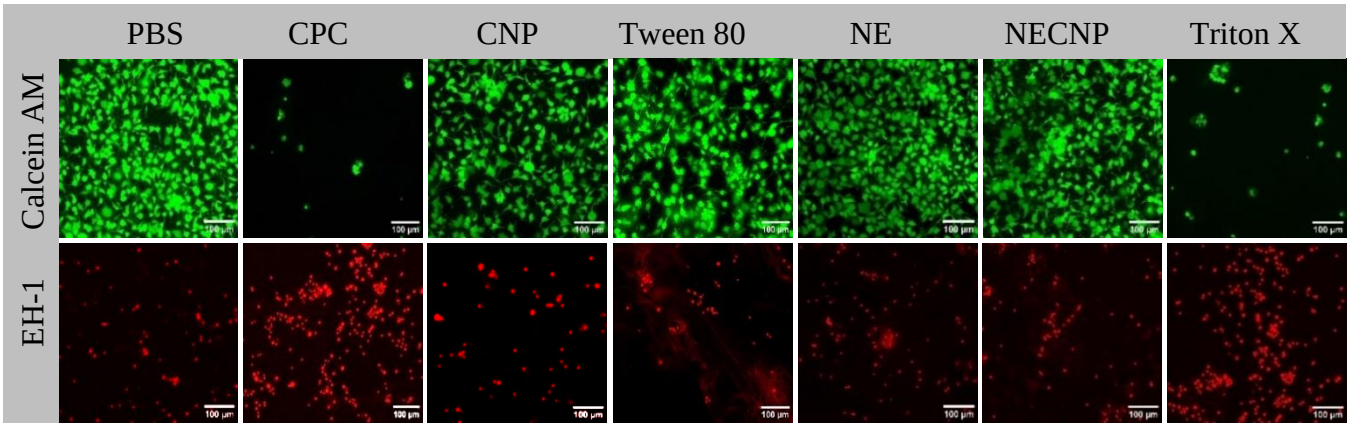

Fig. S6 Live/Dead cell viability assay following treatment of the L929 fibroblast cells with CPC (Second panel), CNP (Third panel), Tween 80 (Fourth panel), NE (Fifth panel) and NECNP (Sixth panel) and calcein AM and EH-1 utilized for the fluorescent staining of the cells following treatment. Scale bar, 100 μm. PBS (pH 7.2) (First panel) and 0.1 % Triton X (Seventh panel) was considered as the negative and positive control.

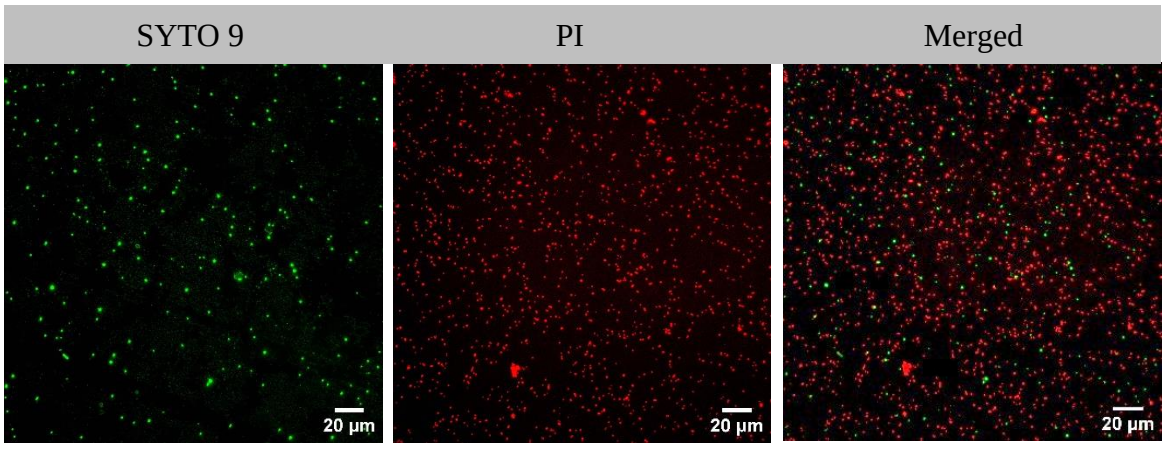

Fig. S7 Cell viability assay of *S. aureus* after the treatment with Penicillin.

Table S1. Size of *S. aureus* with PBS and NECNP treatment according to DLS and AFM		
Sample	DLS	AFM
PBS	0.9 ± 0.1 μm	1.1 ± 0.05 μm
NECNP	0.6 ± 0.1 μm	0.8 ± 0.10 μm

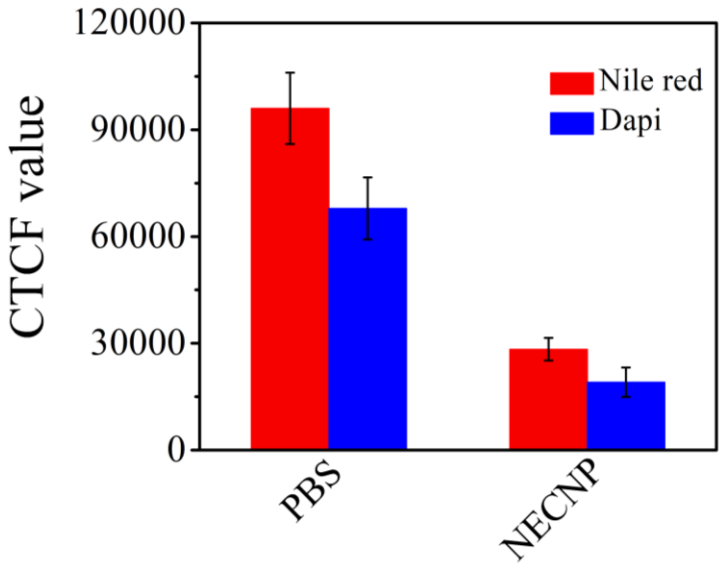

Fig. S8 Quantification of Nile red and DAPI fluorescence in *S. aureus* with PBS (50 mM, pH 7.2) and NECNP (20 μl/ml) treatment measured by calculating the CTCF values of Nile red and DAPI.

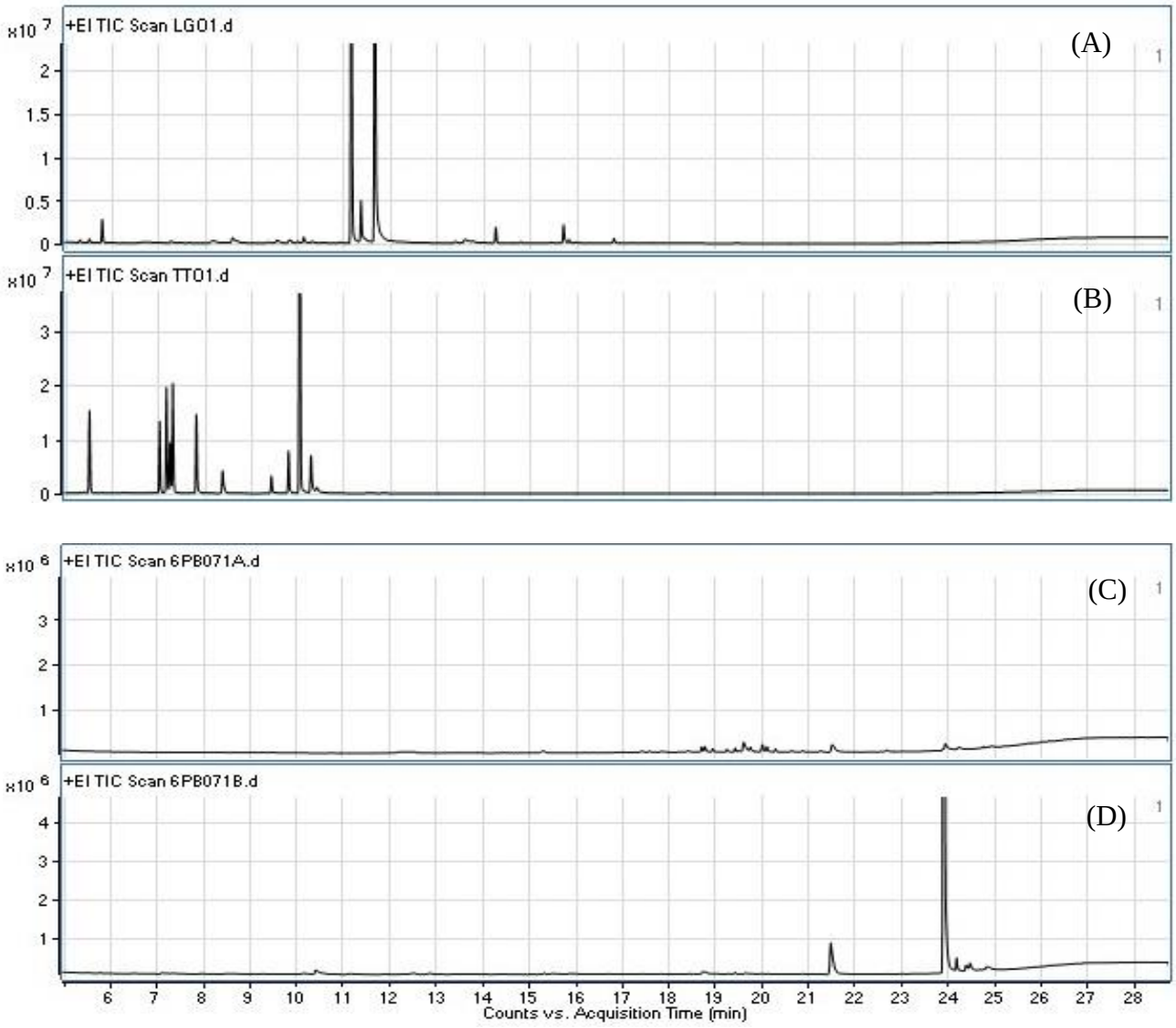

Fig. S9 Stack plot of the GC-MS chromatograms (R_t 5.00-28.75 min) for (A) lemongrass oil, (B) tea tree oil, (C) *S. aureus* cell membrane extract without treatment and with (D) NECNP treatment.

Table S2. List of retention time, chemical identity, retention indices (calculated and literature value), obtained molecular ion, calculated mass and relative abundance of the identified peaks in the GC-MS chromatogram of treated culture extract (hydrolyzed/esterified)

Sl. No.	R_t (min)	Chemical identity	RI (calc)	RI (lit)	$[M]^+$	Mass (calc)	Relative %
1	21.48	Palmitic acid, methyl ester	1923	1926	270.1	270.2	3.50
2	23.91	11-Octadecenoic acid, methyl ester	2113	2115	296.1	296.3	94.07
3	24.18	Stearic acid, methyl ester	2137	2128	298.1	298.3	0.53
4	24.39	Octadecadienoic acid isomer, methyl ester	2157	-	294.0	294.2	0.32
5	24.47	Octadecadienoic acid isomer, methyl ester	2163	-	294.1	294.2	0.41

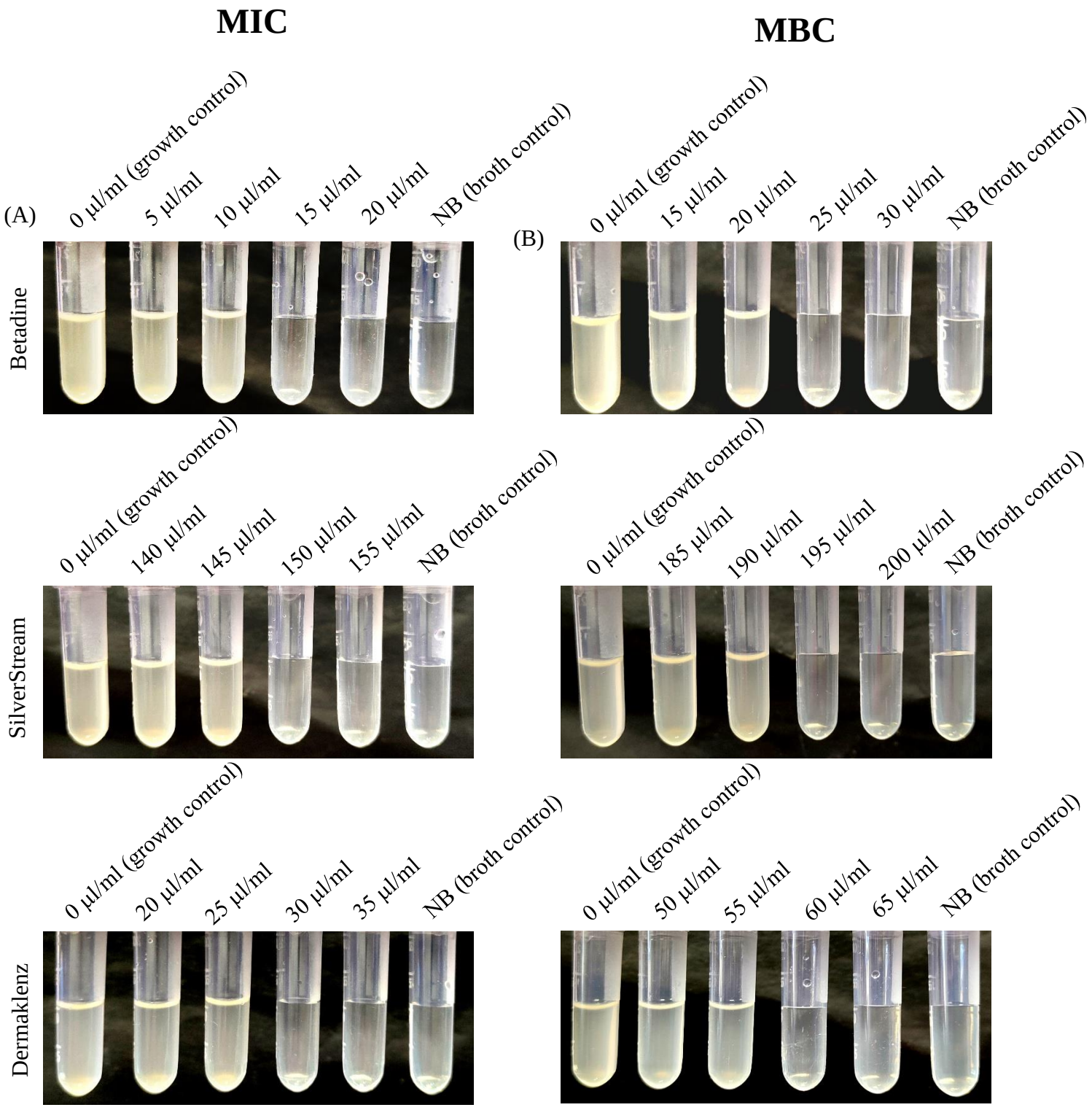

Fig. S10 MIC (A) and MBC (B) values of Betadine, SilverStream Solution and Dermaklenz.

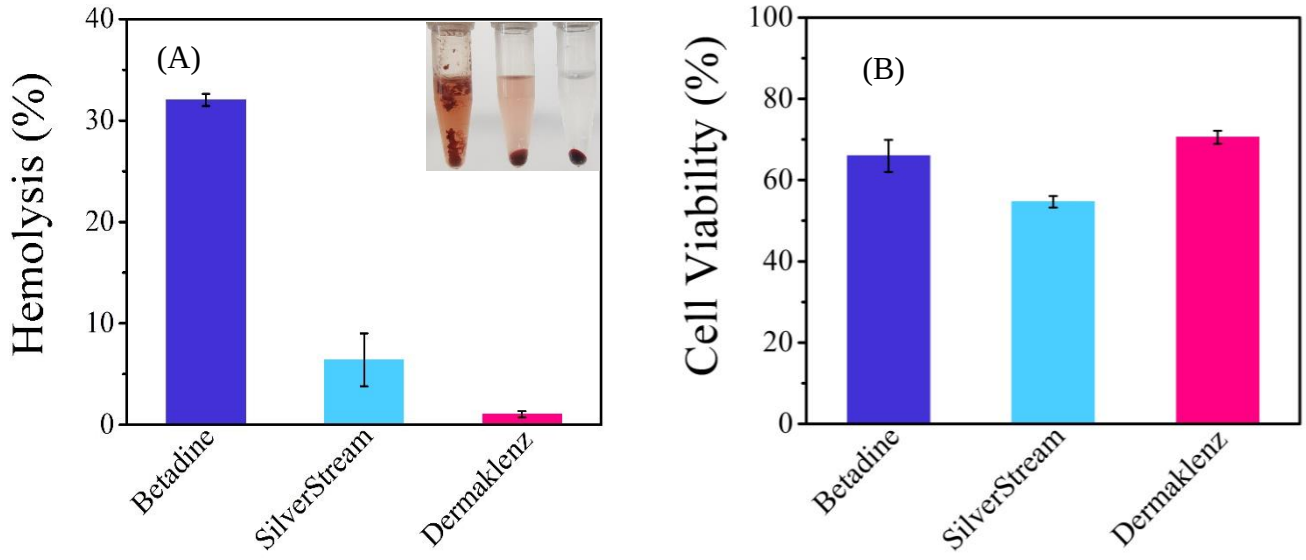

Fig. S11 Hemolysis percentage (A) and Cell viability percentage (B) of Betadine, SilverStream solution and Dermaklenz.

References

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