Supporting information for publication

Zinc Oxide nanoparticles dispersed in ionic liquids show high antimicrobial efficacy to skin-specific bacteria

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1. Materials and methods:

1.1 Materials:

Trypticase soy broth, media components, crystal violet, Propidium Iodide (PI) stain, Zinquin ethyl ester dye, all the media components and other chemicals were obtained from Sigma Aldrich.

1.2. Methods

1.2.1 Zinquin assay

Dried powder of ZnO nanoparticles was dispersed in Phosphate buffer saline (PBS) or in IL-1 and IL-2 at a concentration of 40 µg/ml. The solution was sonicated for 10 minutes (pulse on: 50 seconds and pulse off: 10 seconds cycle) for proper dispersion. Next, the solution was centrifuged at 8000 r.p.m at different time points (0,2,4,8 hrs). 100 µl of supernatant was mixed with 25µM of zinquin ethyl ester dye (Ex 364 nm/Em 385 nm) in order to quantitate Zn^{2+} in the solution. Fluorescence reading was captured in a Tecan plate reader.

1.2.2 Bacterial Live/Dead assay

Bacterial viability test was performed as per the procedure mentioned in the Baclight™ Live/Dead assay kit (Thermo Fisher Scientific). In brief, 30 mL culture of *S. epidermidis* was grown to late log phase in LB media. Treatment was done with 105 µg/ml of ZnO+PBS, ZnO+IL2 (105 µg/ml ZnO in 10mM IL2), 10 mM of IL2 and 100% alcohol. The mixture was concentrated at 10,000 × g for 10–15 minutes. Supernatant was removed and resuspended in the pellet in 2 mL of 0.85% NaCl. To this 1.5 µl of PI was added and mixed thoroughly. The suspension was incubated in the dark for 15 minutes. The stained samples were analyzed through both Flow cytometer FACS-Accuri™ (Becton Dickinson, USA) using BD Accuri™ software and fluorescence microscope Leica TCS SP8. Average of three independent experiments was considered.

1.2.3 Biofilm inhibition assay

10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism (*Staphyloccus epidermidis*) from overnight culture on nutrient agar. The broth was incubated at 37^0C for 24 hours. The culture was further diluted 1:100 with fresh medium. 96 wells flat bottom tissue culture plates were filled with 0.2 ml of diluted cultures individually. Treatment was done with 105 µg/ml of ZnO+PBS, ZnO+IL2 (105 µg/ml ZnO in 10mM IL2) and 10 mM of IL2. Cultures which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with deionized water and plates were dried properly. Optical densities (OD) of stained adherent Cultures were obtained with a micro ELISA autoreader at wave length 570 nm.

1.2.4 Bacterial viability in co-culture experiment:

10,000 HaCaT cells were seeded in 96 well plate and on 80-90% confluency, it was co-cultured with *Staphylococcus epidermidis* with 0.1 OD. After 24 hours of incubation, treatments of ZnO+IL1(40 µg/ml of ZnO, 10mM of IL1), ZnO+IL2 (40 µg/ml of ZnO, 10mM of IL2), ZnO+PBS, IL1 (10mM) and IL2 (10mM) were given. Media containing the bacterial cells was taken from the wells after 12 hours of incubation and washed with PBS. Then 1.5 µl Propidium iodide solution (50 µg/ml) was added to the suspension of bacterial cells and incubated for 15 min in dark. Later the number of live and dead bacteria in the suspension was identified using Accuri C6 Flow cytometer.

1.2.5 Cellular viability through MTT assay:

10,000 HeLa/ CHO-K1 obtained from NCCS (National Centre for Cell Science, Pune) India were plated in 96 well plates and 5% CO_2 at 37 °C. After 24 h, the medium was replaced with 100 µL of Opti-MEM medium containing 40µg/ml of ZnO+IL(10mM) or ZnO+PBS solutions and incubated for 4 h. After that cellular viability was measured using the same protocol as mentioned in Section 2.7 of the Main text.

2. Results:

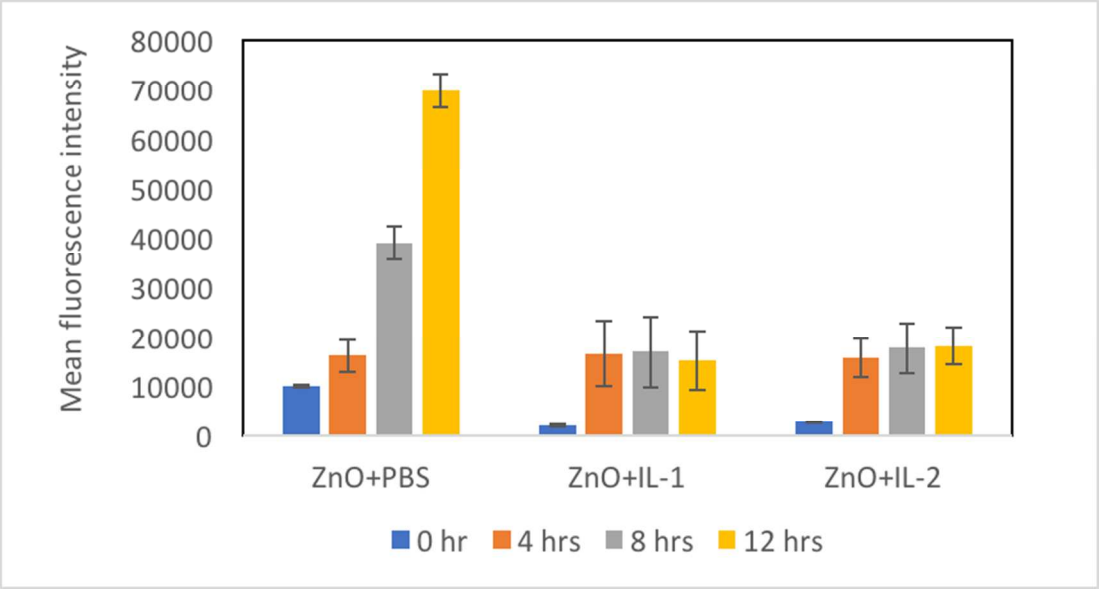


Figure S1: Zn^{2+} dissolution levels of ZnO in PBS and ionic liquids (40µg/ml ZnO concentration) quantitated through a UV excitable zinquin ethyl ester (25µM) and analyzed through fluorescence plate reader.

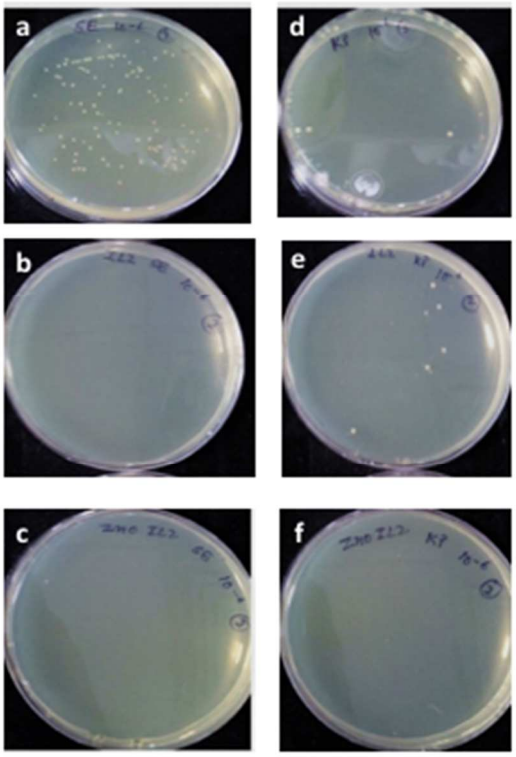


Figure S2: Macroscopic photographs of treatments of (a) no treatment control cells, (b) IL2 and (c) ZnO+IL2 on *S. epidermidis* (SE) and (d) no treatment control cells, (e) IL2 and (f) ZnO+IL2 on *Klebsiella pneumoniae* (KP) where concentration of IL is 10mM and ZnO at a concentration is 105μg/ml, has been represented. For Materials and Methods, see section 2.6 of main manuscript.

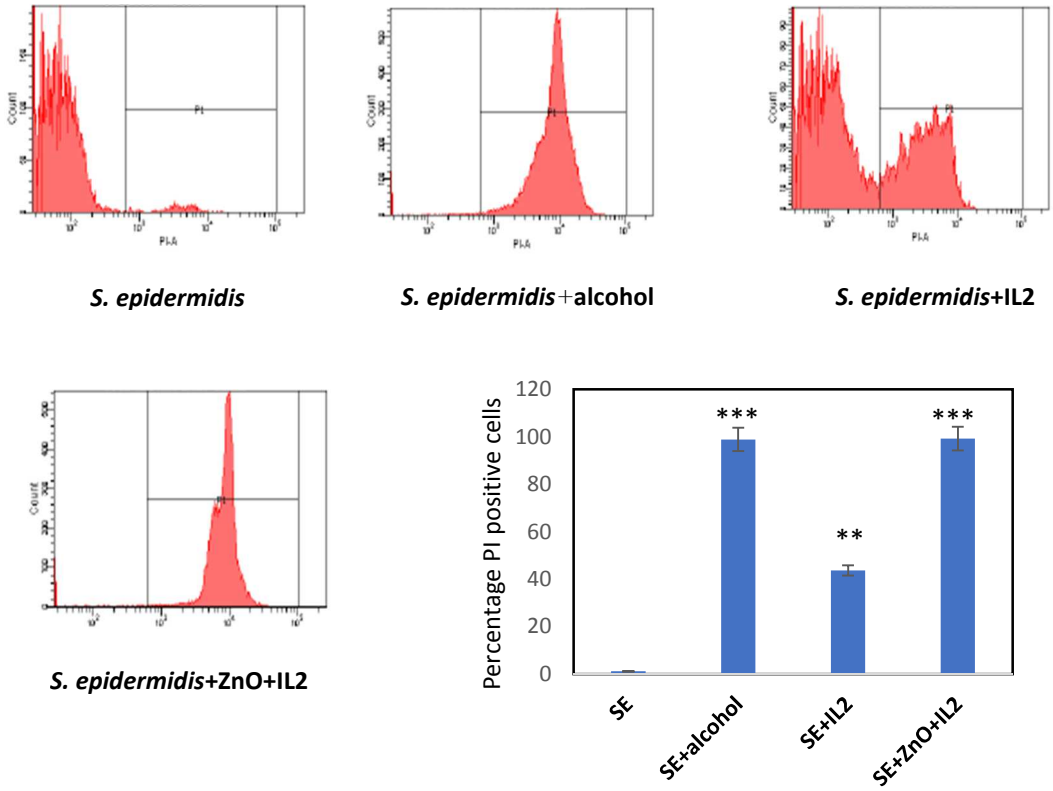


Figure S3: Qualitative and quantitative representation of PI stained (dead bacteria) with different treatments. Clearly, *S. epidermidis* (SE) when treated with alcohol (positive control) and ZnO+IL2 at a concentration of 105 µg/ml showed maximum bacterial killing of ~90%. The shift was clearly visible in the count vs. FL2-A intensity plots from flow cytometry. Error bars represent standard error across the mean of 3 independent readings.

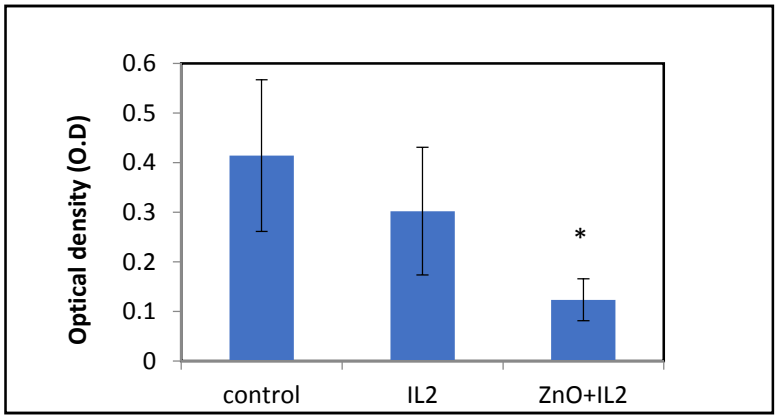
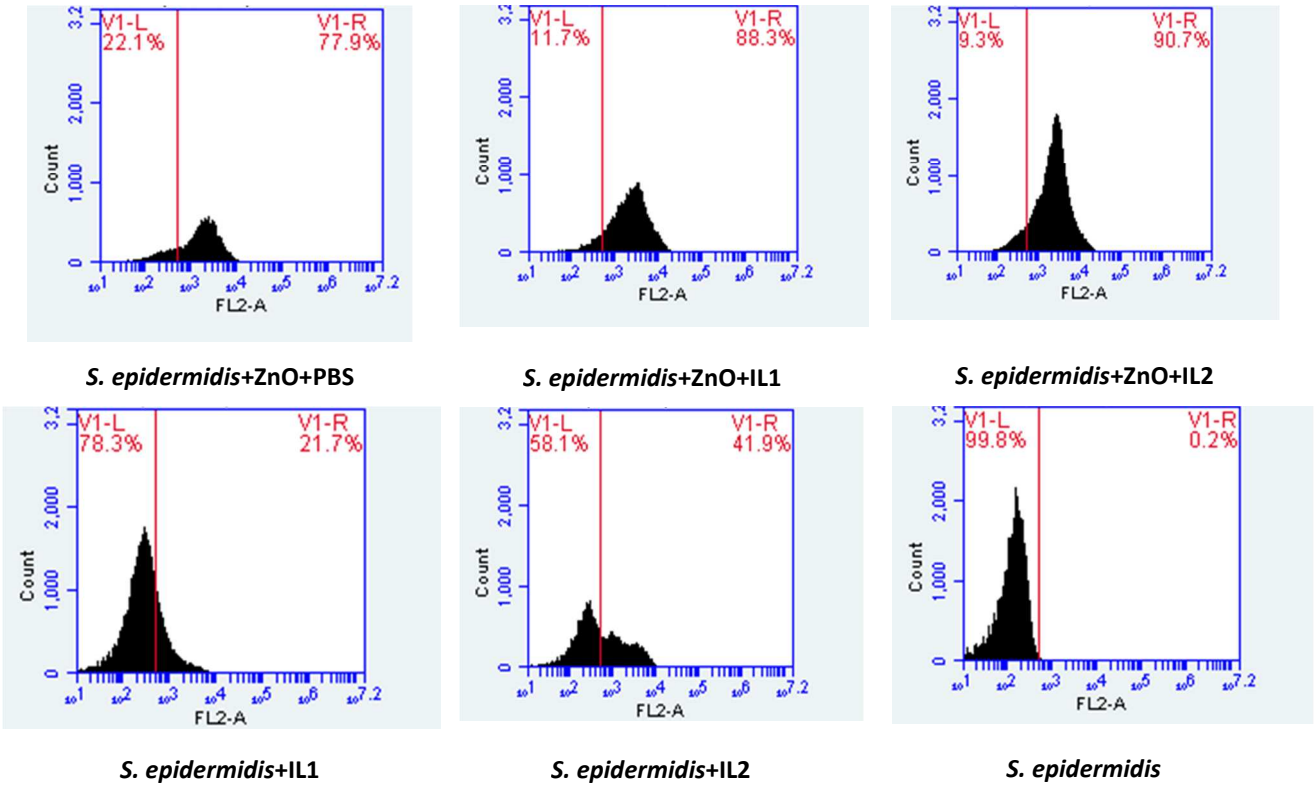


Figure S4: Quantitation of optical density of crystal violet dye adhered to *S. epidermidis* (SE) cells forming biofilm in the presence of IL2 and ZnO+IL2 at a concentration of 105 μg/ml. It has been clearly observed that the dye optical density drops with IL2 and more significantly with ZnO+IL2 treatment. Thus, biofilm formation by *S. epidermidis* was hampered by ZnO dispersed in imidazolium based ionic liquid.



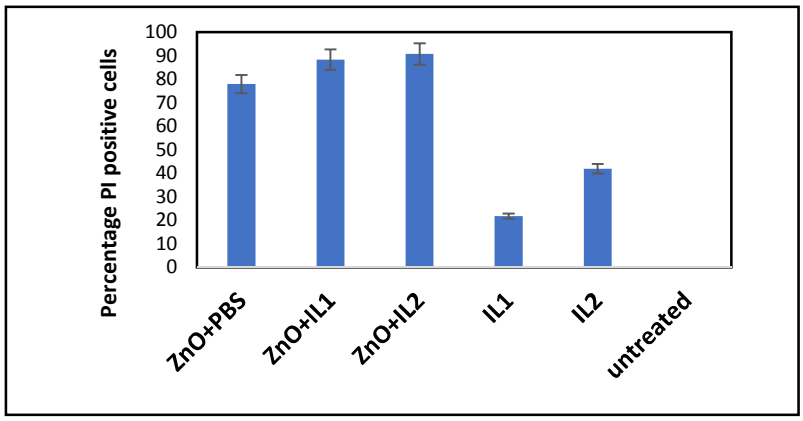


Figure S5: Qualitative and quantitative representation of PI stained (dead bacteria) in a co-culture condition with HaCaT cells. Clearly, *S. epidermidis* when treated with ZnO+PBS and ZnO+IL2 at a concentration of 40 µg/ml in co-culture condition showed maximum bacterial killing of ~90% with ZnO+IL2 treatment. Error bars are representative of standard error across the mean of 3 independent readings.

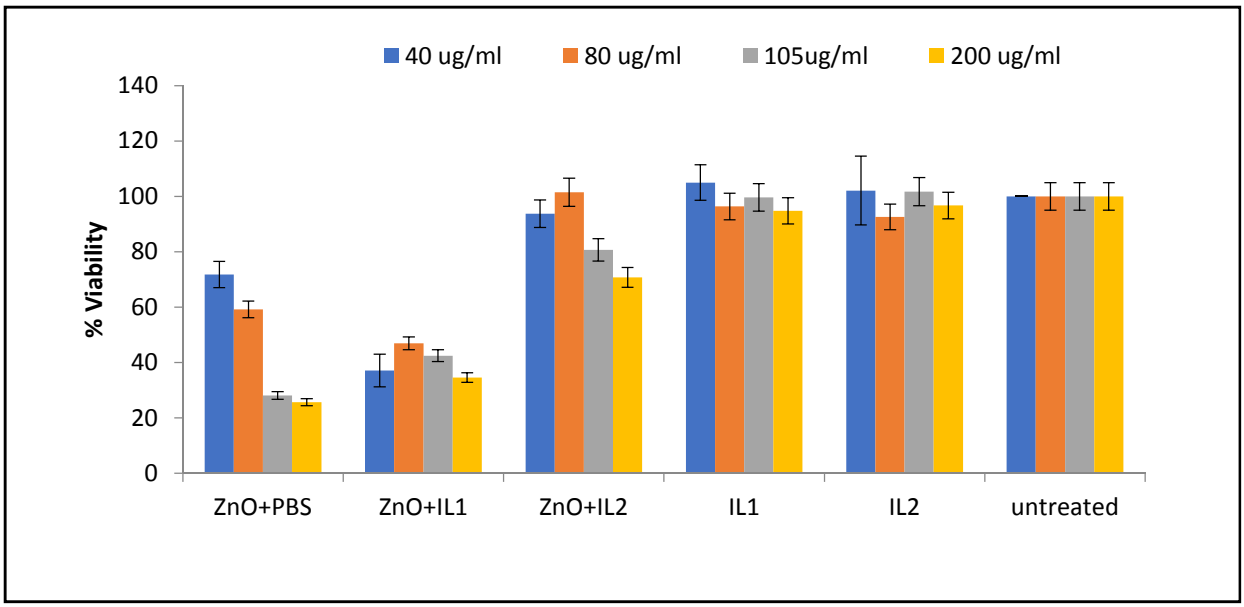


Figure S6: a) Biocompatibility of ZnO+PBS, ZnO+IL1, ZnO+IL2, IL1, IL2 with keratinocyte cells (HaCaT) was determined through MTT assay at concentration of 40,80,105 and 200µg/ml. Bars represent the average of 3 independent readings. Error bars represent standard error with respect to mean across 3 biological replicates.

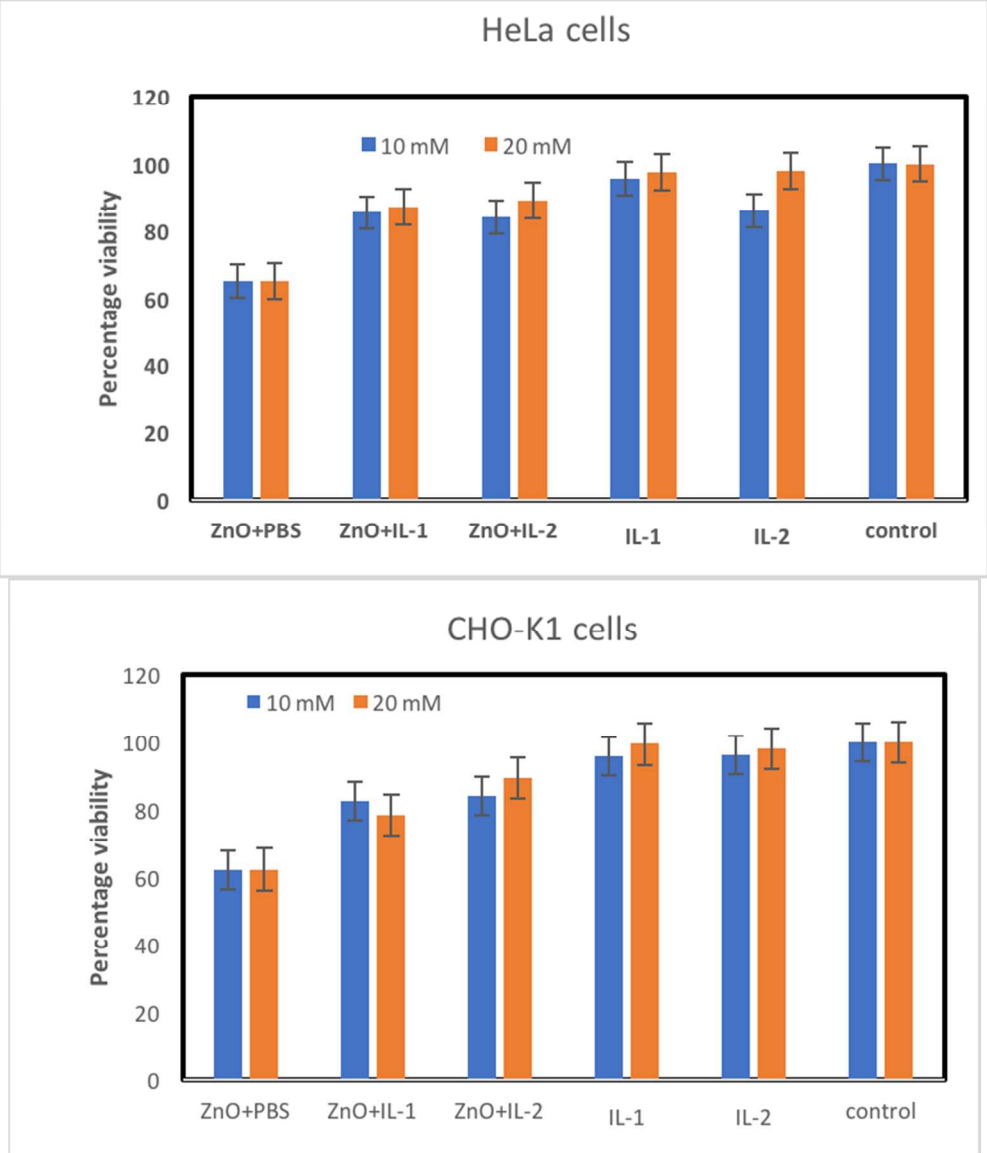


Figure S7: Percentage viable cells after administration of 40 µg/ml ZnO nanoparticles in PBS, IL-1 or IL-2 and only IL-1 and IL-2, as measured after 24 hrs in HeLa and CHO-K1 cells. Data expressed as mean±SD, n=3.

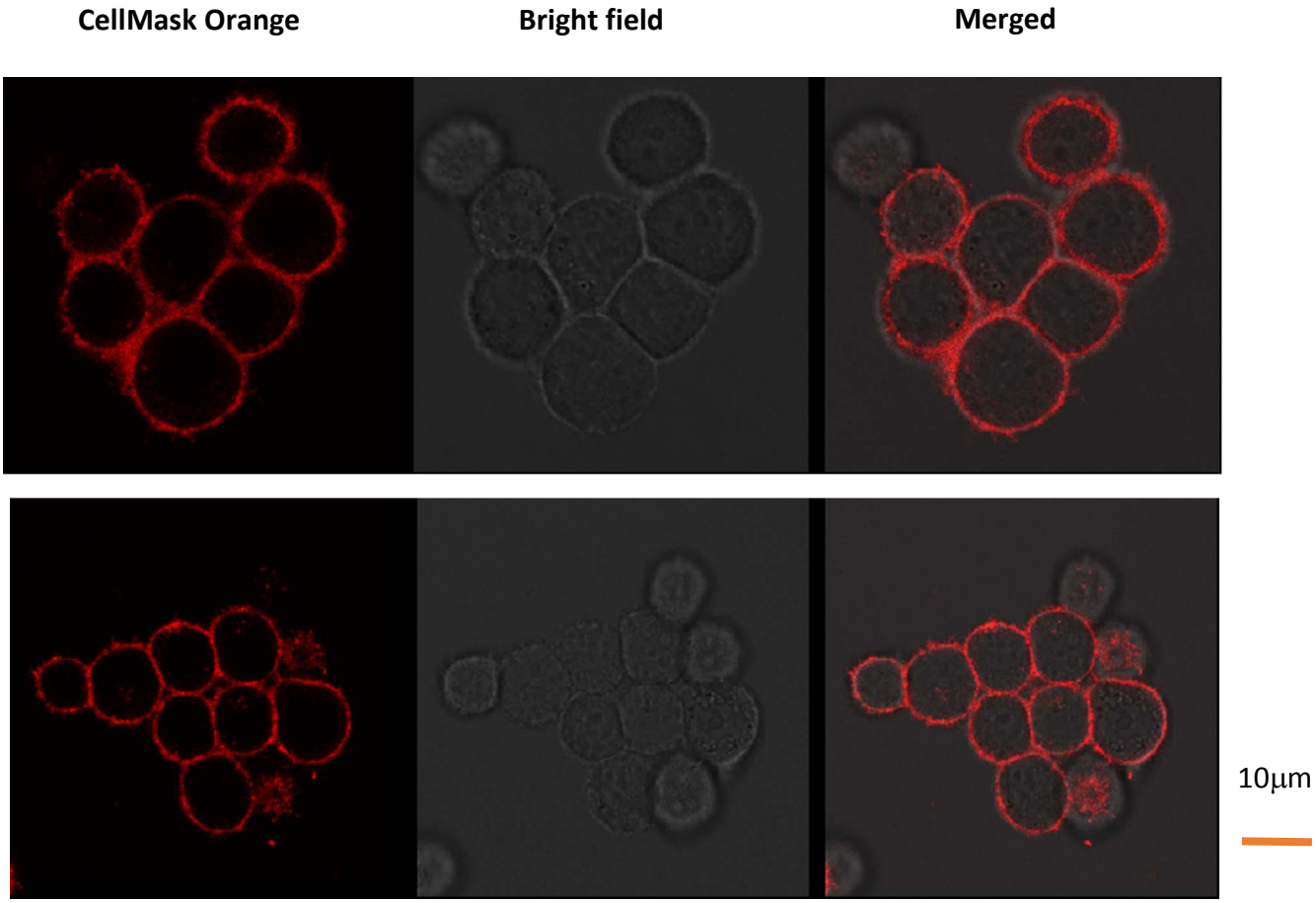


Figure S8: Biocompatibility of PBS (negative control), 500 μM H_2O_2 (Positive control) with keratinocyte cells (HaCaT) was determined through cell membrane staining (confocal microscopy). Red on microscopic images indicate the cell membrane of HaCaT cells. Scale bar= 10 μm