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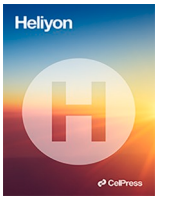

Research article

Imidazolium, pyridinium and pyrazinium based ionic liquids with octyl side chains as potential antibacterial agents against multidrug resistant uropathogenic *E. coli*

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A B S T R A C T

Urinary tract infections (UTIs) are the second most prevalent infectious disease with *E. coli* being the most common etiological agent behind these infections, affecting more than 150 million people globally each year. In recent decades, the emergence of multi-drug resistant (MDR) pathogens has rapidly escalated. To combat antimicrobial resistance (AMR), it is important to synthesize new biologically effective alternatives like ionic liquids (ILs) to control the bacterial infection and their spread. Ionic liquids are poorly coordinated organic salts characterized by melting points typically below 100 °C. The ability of ILs to form anionic and cationic interactions contributes to their versatile chemical, physical and biological attributes. In the present study, a total of 9 previously chemically synthesized and characterized ILs were used. For exploration of their antibacterial potential against the urinary tract infections (UTIs) caused by MDR Uropathogenic *E. coli* (UPEC) strains, *in vitro* and *in vivo* evaluation of ILs were performed. ILs showed pronounced zone of inhibition (ZOI), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 29.5 mm, 3.81 μM and 5.08 μM by agar disk diffusion and broth micro-dilution methods, respectively. Scanning electron microscopy results depicted substantial morphological changes in UPEC biofilm formation ascertaining antibiofilm potential of tested ILs. Moreover, ILs showed exceptional antioxidant potential depicted by DPPH assay along with low cytotoxic effect toward mammalian cell lines (NB4), red blood cells and whole blood. Furthermore, the gene expression analysis results justified the antibacterial potential of ILs showing down-regulation of fimH, uvrY and up-regulation of csrA gene in UPEC after ILs treatment. *In vivo* dermal sensitivity assessment also established their non-cytotoxic behavior. *In silico* analysis validated these results, with the majority of the compounds exhibiting moderate to good absorption.Due to remarkable antibacterial and antioxidant potential and negligible cytoxicity, it could be inferred that ILs could serve as novel antimicrobial alternative agents in the treatment of UTIs.

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1. Introduction

Urinary tract infections (UTIs) are the second most prevalent infectious condition affecting more than 150 million people worldwide each year [1]. It is most common infectious disease after respiratory tract infections [2,3]. In the United States, roughly 11.3 million community-acquired UTIs each year, costing $1.6 billion annually [4,5]. Gram-negative bacilli are the most prevalent cause of UTI, with *E. coli* accounting for more than 80 % of all acute urinary tract infections [6]. Recently, an increase in antibiotic resistance in bacteria causing UTIs has been reported and the selection of a highly efficient and effective antibiotic is the foundation for treating UTIs [7].

Moreover, the increasing resistance of pathogens against standard antimicrobial treatments is a major health concern worldwide. World Health Organization (WHO) has declared that "antimicrobial resistance (AMR) is one of the top global public health threats facing humanity" and in order to achieve sustainable development goals, AMR requires urgent multisectorial action [8]. Due to misuse and overuse of antimicrobials, pathogenic bacteria have built resistance against the antibiotics. According to WHO estimate, bacterial infections resistant to antibiotics account for atleast 700,000 deaths annually [9]. Moreover, WHO also warns that by 2050, if no action is taken against antibiotic-resistant bacteria, 10 million people will die every year [10]. To overcome the threat of AMR, it is important to hunt for alternative antimicrobial strategies and drug delivery systems to eradicate the resistant bugs and to cure UTI ailments.

The ionic liquids (ILs) have gained attention of researchers for a wide variety of applications in pharmaceutical industry for drug delivery systems as well for targeting different diseases in recent years. ILs also termed as ''Solutions for success'' are salts having melting point of up to 100 °C with extremely low volatility and high thermal stability [11,12]. Their unique characteristics have made them suitable for different biomedical applications including drug transport enhancers [13] and drug carriers [14,15], in extraction of different proteins [16], drug additives, disease diagnosis and therapy [17,18].

ILs are categorized into four different generations based on the molecular makeup and physicochemical characteristics of their molecules. ILs designed on the basis of predicted biological properties belong to the third generation of ILs which also includes pharmaceuticals [19]. Initially, the use of ILs in biosciences was challenging due to high inherent toxicity, but more recently it has been demonstrated that some hydrophobic ILs have lower inherent toxicity and consequently less of an adverse effect on human health and the environment [20].

Ionic liquids possess a wide range of applications in the pharmaceutical industry. The most significant areas of application include the use of ILs as biologically active compounds as drugs or potential drug products, the synthesis of active substances as catalysts, or in any biotechnological product, the improvement of the solubility of active substances by combinations of IL with known drugs, carriers of medicinal substances, and the optimization of the bioavailability of medicinal products [21–23].

The present study is aimed to explore the antibacterial and therapeutic potential of imidazolium and pyridinium based Ionic liquids against MDR uropathogenic *E. coli* causing UTIs. This has been achieved by performing antibiofilm assay, antioxidant and various safety profiling assays and these compounds have proved to be an important step in this direction. A skin sensitization model was established to demonstrate the antimicrobial efficacy of ILs *in vivo* (*Fig. 1*).

2. Materials and methods

The brief sketch of overall methodology adopted is represented in Fig. 2.

2.1. Sample collection and UPEC isolation

The clinical isolates of multidrug resistant uropathogenic *E. coli* (UPEC) strains obtained from different hospitals of Rawalpindi and Islamabad were used in study. For this, urine samples were collected from different diagnostic labs of Rawalpindi and Islamabad. Urine samples were procured via the mid-stream collection technique in sterile containers to avoid contamination. Samples were stored at 4 °C refrigerators until microbes were isolated.

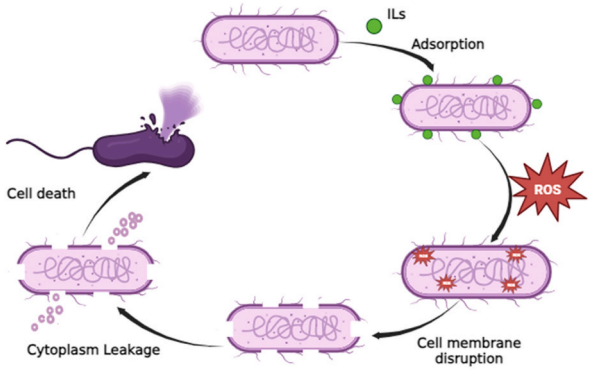

Fig. 1. Possible Mechanism of Action of ILs on *E. coli* bacterial cells.

These isolates have been confirmed as UPEC by growth in human urine and for their confirmation as *E. coli*. PCR was employed to detect the *uidA* gene, an important housekeeping gene in *E. coli*, using boiled DNA extracts obtained from the isolates. Following isolation, each UPEC sample was tested for their susceptibility pattern to multiple antibiotics using the Kirby-Bauer disc diffusion method. Among tested strains ten most multidrug resistant UPEC strains were selected for this study.

2.2. Ionic liquids

The ionic liquid compounds including 1-octyl Pyridinium Bromide [C8Py]Br, 1,3-dioctyl Imidazolium Bromide [C88Im]Br, 1-octyl Pyrazinium Bromide [C8Pyr]Br, 1-methyl-3-octyl Imidazolium Bromide [C8mim]Br, Dioctyl Succinamic Acid DOSA, 1-octyl Pyrazinium Dioctyl Succinamic acid [C8Pyr]DOSA, 1-octyl Pyridinium Dioctyl Succinamic Acid [C8Py]DOSA, 1-methyl-3-octyl Imidazolium Dioctyl Succinamic Acid [C8mim]DOSA, 1,3-dioctyl Imidazolium Dioctyl Succinamic Acid [C88im]DOSA previously synthesized [24] in School of Natural Sciences, NUST, have been used in this study (Table S1). All the ionic liquids were dense liquids at room temperature.

2.3. Solubility

The solubility of all of the Ionic liquids in different solvents with variable polarity index was performed according to the method outlined in Vogel's Textbook of Practical Organic Chemistry [25]. 'High solubility' applies to ILs (0.1 g), which were fully dissolved in 1 mL of the solvent, 'medium solubility' means that ILs were dissolved in 2 or 3 mL of the solvent, 'low solubility' applies to ILs, that did not dissolve in 3 mL of the solvent. All the solubility measures were carried out in 20 mL glass vials and thermostatted water bath MEMMERT WNB 7 at 25 °C.

2.4. Antimicrobial potential of ionic liquids

Antimicrobial tests (antibiogram and ionic liquid testing) for the microorganisms study were carried out using the Disk Diffusion method as previously described [26]. A small amount of each microbial culture was diluted in sterile 0.9 % sodium chloride solution until the turbidity was equivalent to the 0.5 McFarland standard. These suspensions were further diluted 1:10 in medium MHA and

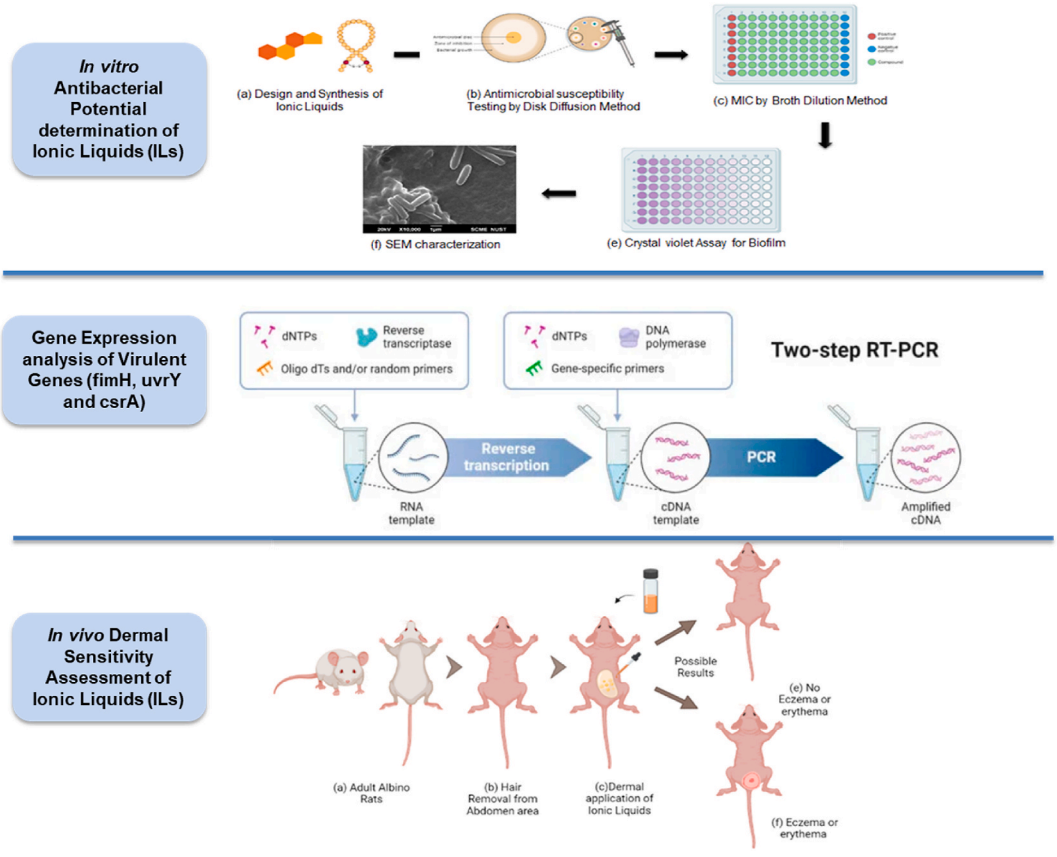

Fig. 2. Overall workflow of methodology followed in the study.

then spread on sterile petri plates. Sterile micro-compresses were applied on the agar surface in the petri plates, and after that 10 μl of each sample was added into the micro-compresses. Commercially available antibiotic discs were used as positive controls. All the plates were incubated at 37 °C for 24 h.

2.5. Minimum inhibitory concentration (MIC) of ILs

The MIC and MBC of synthesized ILs were determined by a 96-well plate broth micro-dilution assay, a technique adapted from the methodology described by European Committee for Antimicrobial Susceptibility Testing (EUCAST). Minimum inhibitory concentration was determined using both broth dilution method and agar methods [27]. Twofold dilution of ILs in LBB were prepared keeping the concentration range at 1000, 500, 250, 125, 62.5 to 0.003 μM horizontally in 96-well plates followed by the addition of 10 μL of bacterial suspension whose working concentration was adjusted to 10^5–10^6 CFU/mL. LBB with and without bacterial inoculum served as the growth control (GC) and sterile control (SC), respectively. Samples were incubated for 24h at 37 °C. After incubation, the optical density at 600/620 nm was recorded by iMark microplate reader (Bio-Rad) for each plate. All tests were performed in triplicate for each experiment for MIC determination. The MIC was considered to be the lowest concentration of ILs with which no visible microbial growth was observed during the experiment. All controls and test concentrations were prepared in triplicates.

2.6. Minimum bactericidal concentration (MBC) of ILs

Following the determination of the MIC for each ionic liquid against each UPEC strain, the MBC was evaluated by spot inoculation of cultures from wells with no growth observed. It was calculated by transferring 20 μL of the suspension from the wells that showed no evidence of growth to MHA plates. The MHA plates were then incubated for 24 h in a stationary incubator at 37 °C and tested for 99.9 % killing. The MBC was defined as the lowest concentration of the investigated ILs that supported no microbial growth [28,29].

2.7. Biofilm inhibition potential of ILs by crystal violet Biofilm assay

The anti-biofilm properties of the ILs against fully developed biofilms were assessed following a 24h incubation period of the microorganisms without any active agents. The bacterial inoculum was cultivated on MHA at a temperature of 37 °C for 24 h. Bacterial colonies were suspended in PBS, and standardized to a turbidity level of 0.5 McFarland units using a spectrophotometer. Next, 20 μL of bacterial suspensions and 160 μL of fresh tryptic Soy Broth (TSB) were introduced into each well of 96-well microtiter plates. Following another 24h incubation period, 20 μL of ILs were introduced into the wells containing mature bacterial biofilms. The plates were further placed for overnight incubation 37 °C. The culture media was discarded, and the wells were washed three times with 200 μL of PBS each time. After air-drying the microplates, 200 μL of methanol was added into the wells for 15 min. Subsequently, the microplates were allowed to dry after removing methanol. A 200 μL solution of crystal violet (0.1 %) was added to the wells for 5 min. Then, the wells were washed three times with 200 μL of normal saline. Following this, the microplates were allowed to dry. The wells were then filled with 200 μL of absolute ethanol followed by the incubation for 15 min. Spectrophotometric measurements at 570 nm were conducted using a microplate spectrophotometer [30].

2.8. Antioxidant potential of ILs by DPPH assay

The antioxidant activity of the ionic liquids 1–9 was measured by radical scavenging ability of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. The color of DPPH would change from purple to yellow when it gets reduced by accepting an electron from the antioxidant compound. The change in color would cause a drop in the absorption value, which is inversely proportional to the amount of the antioxidant component used.

The method developed by Brand-Williams [31] provided the basis for DPPH's free radical scavenging activity. Different samples of ILs 1–9 with 7 different concentrations (2500 μM, 1250 μM, 625 μM, 312.5 μM, 156.25 μM, 78.12 μM and 39.06 μM) were prepared in methanol. 0.025g of DPPH was dissolved in 100 mL of methanol to achieve 6×10^{-5} mol/L methanol DPPH solution. 0.1 mL of ionic liquid solution of each concentration in methanol was added to 3.9 mL of a 6×10^{-5} mol/L methanol DPPH solution. The DPPH solution chilled at 4 °C was used. The mixture was vortexed and left for 1 h in the dark at room temperature. A blank sample was also prepared by adding 0.1 mL of methanol in 3.9 mL of DPPH solution. After 60 min, a 100 μL aliquot of each sample was added to a 96-well microtiter plate. DPPH radicals have the highest absorption at 515 nm, which decreases when DPPH is reduced by an antioxidant compound because of which the absorbance of the samples was analyzed at 515 nm against a blank sample using a 96 well microplate reader [32]. Trolox was exploited as a standard positive control. The experiment was conducted in triplicate.

2.9. Cytotoxicity evaluation and safety profiling of ILs

2.9.1. Hemolysis assay

To investigate the potential for ILs to elicit hemolysis, ILs 1–9 were spectrophotometrically tested for their ability to induce hemoglobin release from fresh human red blood cells (RBCs) according to the method described by Vieira [33]. Peripheral blood from a healthy human donor was collected in a sterile Vacutest tube, containing 5.24 mg of K_3EDTA. To separate the serum from the erythrocytes, the blood was centrifuged for 10 min at 1000×*g* at 25 °C. The supernatant was removed. Essentially, the fresh de-fibrinated RBCs were washed thrice with equal volumes of phosphate buffer saline, pH 7.4 (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM

Na_2HPO_4, 1.8 mM KH_2PO_4). The solution was centrifuged and supernatant was removed after every wash of PBS. After washing, RBCs stock dispersion was prepared by mixing rinsed RBCs and PBS at the ratio of 3:11. Later, Equal volumes (1 mL) of the erythrocyte suspension and ILs at different concentrations (10 μM, 5 μM, 2.5 μM and 1.25 μM) were added to 2 mL eppendorf and incubated at 37 °C for 1 h. Incubation was then followed by the centrifugation at 800×*g* for 10 min. Aliquots (100 μL) of the supernatant were transferred to a fresh 96-well microtiter plate, and hemoglobin release quantified spectrophotometrically at 492 nm using Biorad microplate reader. RBCs incubated in PBS alone were used as a negative control (0 % hemolysis) while erythrocytes treated with 0.1 % Triton X-100 (100 % hemolysis) were taken as a positive control [29]. The assay of all samples and controls were performed in triplicate. The hemolytic activity was quantified by a comparing the value of each individual sample to a positive control (0.1 % Triton X-100), and to a negative control (erythrocytes in PBS).

2.9.2. Whole blood killing assay

Due to the characteristic resistance of UPEC to phagocytes such as neutrophils and monocytes, a survival assay was used to investigate the effects of ILs on UPEC survival in the presence of human whole blood [34,35]. UPEC cells were inoculated at the dilution 1:100 into 2 mL of nutrient broth in 15 mL tubes at 37 °C. During the initial inoculation, each of the ionic liquid (all at 0.01 %) were added. UPEC cells without any treatment were taken as negative control and a positive control consisted of UPEC treatment alone. DMSO control was also added. All the cells were further incubated for 16 h with shaking at 250 rpm. Then, 0.4 ml of freshly drawn human whole blood was mixed with 0.1 ml of overnight UPEC cultures. The mixtures were incubated at 37 °C for 4 h in shaking incubator at 250 rpm. The UPEC survival in human whole blood with and without ionic liquid treatment was measured by counting CFUs [36].

2.9.3. Hemagglutination assay

Hemagglutination assay was carried out in accordance with method previously described [37]. For this, UPEC cells were inoculated into 2 ml of nutrient broth (1:100dilution) in 15 ml tubes at 37 °C. At the beginning of inoculation, 0.01 % solution of each IL and DMSO (the control) were added and cells were subsequently cultured for a further 24h in shaking incubator at 250 rpm. A positive control consisted of UPEC treatment alone, whereas a negative control involved no UPEC treatment. Then the bacterial cultures (1 ml) were diluted with an equivalent volume of PBS and mixed with 2 ml of washed 3 % human red blood cell solution in PBS. The mixtures were briefly vortexed and left to stand at 25 °C for 2h. The red blood cells were then visualized for any hemagglutination acitivity [36].

2.9.4. Assessment of cytotoxicity by MTT assay

In this study, MTT assay was done to assess the cytotoxic potential of ILs on human cancer cell proliferation. The number of cells was determined automatically with a Countess™ II FL Automated Cell Counter (Invitrogen by Thermo Fisher Scientific Inc., Waltham, MA, USA). The counted cells were then seeded in a 96-well plate as inoculum of 1000 cells per well. NB-4 human cancer cell line was used to measure cytotoxic potential of ILs. After 24 h, all test compounds were added, with final concentrations of 0.062 μM, 0.125 μM, 0.25 μM, 0.5 μM, 1 μM. After the ILs treatment, the cell culture medium was gently removed. After 72 h of incubating cells with test compounds, 15 μl of MTT dye (5 mg/ml of concentration) was added to each well and incubated at 37 °C for 2–4h until the formation of purple formazan crystals. The MTT solution was carefully removed without disturbing the cells. 50 μl of DMSO (MTT solvent) was added to fully dissolve the formazan crystals. The plates were then placed onto the shaker for 10 min until the formazan crystals were dissolved completely. Each step was performed in triplicates. The absorbance was determined at an optical density at 570 nm using microplate spectrophotometer. Cells treated with 0.1 % DMSO were used as a control to calculate the percent viability of cells [38].

2.10. Effect of ILs on the expression of fimH, uvrY and csrA in MDR UPEC

The effect of ILs on the expression of fimH, uvrY and csrA in MDR UPEC was analyzed by RT-qPCR.

2.10.1. Bacterial RNA extraction for gene expression studies

For untreated bacterial culture, a portion of bacterial colony taken from EMB plate was inoculated in 15 ml of LB broth and placed for incubation at 37 °C for 24h. For bacterial sample treated with ILs, 0.1 mg/ml of each ILs was added to the broth culture. The overnight cultures were centrifuged at 6000 rpm for 10 min. Supernatant was removed and bacterial pallet was dissolved in 200–250 μl of TE buffer containing lysozyme at the concentration of 1 mg/ml for Gram negative bacteria. Following a 15 min incubation period over ice, 1–1.2 ml of trizol reagent was then added. Immediately 25–30 μl of glacial acetic acid was added, mixed and incubated for 15–20 min over ice. In the next step, 0.2 ml chilled chloroform was added and vortex vigorously for 5 min on and off. The samples were centrifuged at 13,000 rpm for 20 min at 4 °C. Aqueous upper phase was transferred in a fresh eppendorf tube with caution. Then, 500 μL of chilled isopropanol was added, inverted several times and incubated at −20 °C for 20 min. Next, the samples were centrifuged at 14,000 rpm for 10 min at 4 °C. Supernatant was discarded and the RNA pallet was rinsed in 1 ml of chilled 80 % ethanol. Later, ethanol was removed by inverting the eppendorf and the RNA was allowed to air dry for 5 min. The RNA pellet was dissolved in 30 μl of nuclease free water. RNA was quantified using Nanodrop.

2.10.2. Complementary DNA synthesis

1 μl of quantified RNA (1000 ng) sample and 1 μl of OligoDT primer were added to a PCR tube and incubated at 70 °C for 5 min. 1 μl of RNase inhibitor and 4 μl of Reverse Transcriptase buffer was added followed by the incubation at 37 °C for 5 min. A mixture of 1 μl of RTase (reverse transcriptase) and 2 μl of 10 mM dNTPs was added. The samples were incubated for a total of 60 min at 42 °C followed

by 10 min at 70 °C.

2.10.3. Gene expression analysis

RT-qPCR was used with a fluorescence-based SYBR green to identify changes in *fimH*, *uvrY* and *csrA* gene expression in UPEC without and with the treatment with ILs after 24h. The PCR conditions are shown in Table 1.16S rRNA gene was exploited as an internal control (see Table 2).

2.11. Scanning electron microscopy of IL treated samples

Scanning electron microscopy (SEM) was employed to detect any alterations in the bacterial cell and biofilm morphology of the UPEC biofilms due to ILs, as previously stated [39,40]. Few colonies of UPEC were inoculated from EMB agar to TSB, and allowed to grow overnight at 37 °C and 120 rpm. On the next day, the OD of the bacterial culture was adjusted to 1. For inoculations, 1:100 dilutions of the culture media at OD 1 were prepared in TSB. 450 μL of the inoculated TSB media was added in a 24-well micro-titer plate having placed a 12-mm square cover glass. The 24h incubation allowed the biofilm to form.

After 24h, 50 μL of the ILs 1–9, all at the concentration of 250 mM, were added to the 24 h bacterial culture and incubated further for 24h at 37 °C. As a positive control, the UPEC was cultured in TSB media without the treatment of ILs at 24h. Sterile control was also done. Later, the microtiter plate was carefully washed with PBS twice to get rid of any non-adherent cells. The biofilm cells were fixed with 2.5 % glutaraldehyde in PBS (50 mM, pH 7).

After fixation, the cover glass was rinsed with PBS again, and dehydrated through a graded ethanol series (30, 50, 70, 90, and 100 % v/v) [41]. For SEM, the samples were air dried, and coated with gold. For morphological characterization, a scanning electron microscope was used. 2500X, 5000X, 10,000X and 20,000× magnifications were used to take photographs of UPEC biofilms. Out of 9, 5 ILs having promising results in anti-biofilm assay were selected for SEM characterization.

2.12. Dermal sensitivity assessment of ionic liquids via in vivo rat model

The skin sensitivity study was carried out in accordance with the OECD Guideline method and used as per Wang method [42,43]. Albino rats were used after taking approval from research and ethics committee of National University of Sciences and Technology (NUST). A day before the first induction, 22 albino rats were divided into three groups: a positive control group (n = 2), placebo group (n = 2), and a ILs-treated group (n = 18). The positive control group received 0.1 % w/v 1-chloro-2,4-dinitrobenzene (CDNB) in 10 % propylene glycol as a standard skin sensitizing agent. The ILs-treated group was treated with ILs 1–9. Placebo group was treated with distilled water only.

On the day of induction, around 4 cm × 4 cm of hair from the dorsal area were removed. At 0 h, 1 ml of ILs, distilled water and CDNB were evenly spread onto the shaved area of the skin. Responses to the treated sites were evaluated at 0, 4, 24 and 48 h for erythema and edema.

Sensitization scores of 0–3 were used to measure the severity of all skin reactions, with 0 denoting no reaction, 1 denoting mild redness, 2 denoting moderate and diffuse redness, and 3 denoting a strong skin reaction with erythema. Body weights of all albino rats were also measured before the study to notice any change if occurred. Each rat was caged individually and left undisturbed for 24 h.

2.13. In silico prediction

The physicochemical, pharmacokinetic/ADME properties of the targeted ILs have been analyzed with the help of SwissADME web interface by the Molecular Modeling Group of the Swiss Institute of Bioinformatics (http://www.swissadme.ch/). The 2D structure models were sketched by the Marvin JS sketcher followed by conversion to the SMILES format. SwissADME predicts these attributes using the SMILES format.

Table 1
Primers used for Real time RT-qPCR.

Gene	Primers (5′-3′)		Amplicon Size (bp)	Nucleotides	Reference
fimH	F	TTTGCGACAGACCAACAACT	115	20	Yang et al., 2016
	R	GACATCACGAGCAGAAGCAT		20	
uvrY	F	TCAGACAAACTGGCAAATGG	102	20	Yang et al., 2016
	R	CTATTCAGGGCAGCGTTACA		20	
csrA	F	CCTGGATACGCTGGTAGAT	143	19	Yang et al., 2016
	R	TCGTCGAGTTGGTGAGAC		18	
16S rRNA	F	ACTCCTACGGGAGGCAGCA	469	19	This
	R	**GGACTACHVGGGTWTCTAAT**		**20**	Study

Table 2
RT-qPCR reaction conditions.

Step	Genes	Temperature (°C)	Time	Cycles
1- Denaturation		95	10 min	1
2- Annealing	*fimH*	95	15 s	40
	uvrY	55.5	1 min	
	csrA	59		
		55.5		
		72	35 s	
3- Dissociation Stage		95	15 s	1
		50	30 s	

3. Results

3.1. Solubility profiling of ILs in different solvents

The solubility analysis of ILs was performed for determination of solubility profile of ILs in different solvents.The solubility of all ionic liquids in different solvents have been demonstrated in (Fig. 3). The affinity of the synthesized ILs toward various solvents of varying polarity was determined at room temperature (25 °C). Majority of ILs exhibited high solubility in acetone, diethyl ether, isopropanol, methanol, DMSO, chloroform, acetic acid and ethanol. Despite the low polarity of chloroform, the majority of ILs exhibited significant affinity for it. Only [C88Im]Br and DOSA showed medium solubility in n-butane, otherwise all other ILs were highly soluble in the solvent.

Most of ILs showed medium solubility in hexane whereas a wide range of solubility was exhibited by ethylene glycol. Ionic liquids are considered to be extremely soluble but surprisingly not all ionic liquids are soluble in water.

3.2. UPEC susceptibility profiling towards ILs via disk diffusion method

The antimicrobial potential of all ionic liquids were elucidated against ten MDR UPEC strains by measuring their zones of inhibition. ILs with combination of different anions and cations were designated to study their antibacterial activity against UPEC. The illustration in (Fig. 4) depicts the zone of inhibition (ZOI) produced by small filter paper disks infused with these ILs on an agar plate that had been previously swabbed with UPEC. These findings highlight the ILs' ability to gradually release and disperse within the solid medium, ultimately eliminating the bacterium. The maximum ZOI was exhibited by [C88Im]Br whereas minimum zone was observed by [C8Pyr]Br.

3.3. MIC and MBC against UPEC strains

MIC and MBC are crucial measures used to determine the susceptibility or resistance of UPEC strains to an ILs in laboratory settings. The accurate determination of MIC and MBC plays a vital role in selecting the most appropriate treatment strategy, ultimately influencing the effectiveness of infection therapy. The lowest range of MIC from 15.25 to 7.63 μM was shown by [C88Im]Br. This result was followed by [C88im]DOSA, with MIC ranged from 30.51 to 3.81 μM among 10 UPEC strains (Fig. 5). The highest level of MIC range was shown by [C8mim]Br up to 976.25 μM. Comparatively low MIC of ionic liquids to other antimicrobials indicate their higher antimicrobial potential. Out of all tested liquids various ILs depicted equal MIC and MBC values deducing cidal activity of these ILs. ILs exhibiting similar MIC and MBC values are denoted by blue bars and red bars depict MBC values of those ILs with variable MIC values as shown in (Fig. 5).

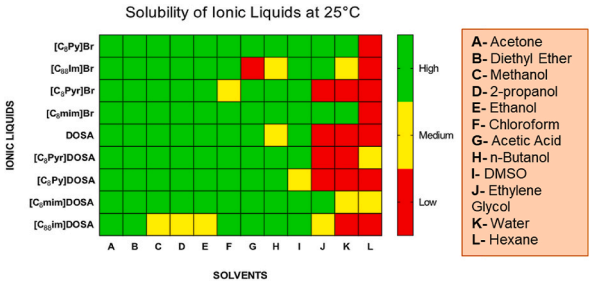

Fig. 3. Solubility Profile of ILs at 25 °C. A- Acetone, B- Diethyl Ether, C- Methanol, D- 2-propanol, E– Ethanol, F- Chloroform, G- Acetic Acid, H- n-Butanol, I- DMSO, J- Ethylene Glycol, K- Water and L- Hexane; 'green', high solubility (≥100 g L-1); 'yellow', medium solubility (between 100 and 33 g/L); 'red', low solubility (≤33 g/L).

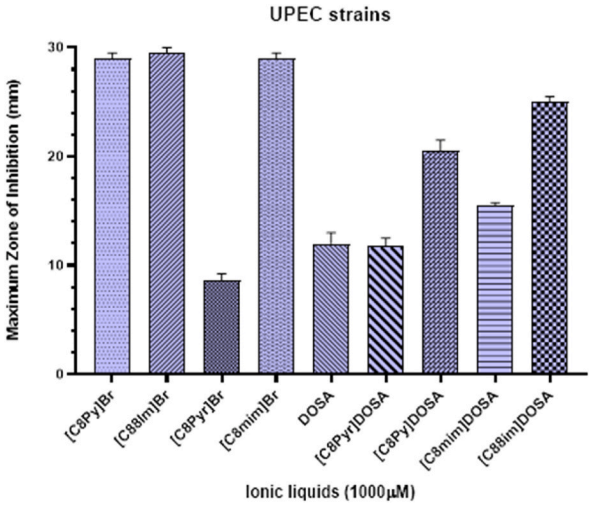

Fig. 4. Maximum Zones of Inhibition (ZOI) of tested ILs against selected MDR UPEC strains.

3.4. Biofilm inhibition potential of ILs against UPEC strains

Biofilms are microbial communities that attach to surfaces and are enclosed within an extracellular matrix. Antibiofilm assays are performed to evaluate the ability of ILs to prevent or disturb the formation and expansion of biofilms.

[C_{88}Im]Br showed highest anti-biofilm potential of 93.44 % biofilm inhibition among UPEC strains. [C_{88}im]DOSA also showed with maximum % biofilm inhibition of 90.11 % against MDR UE73. [C_8Py]Br and [C_8mim]DOSA also showed good biofilm inhibitory potential greater than 84.2 % and 82.6 % respectively (Fig. 6). The highest biofilm percentage inhibition of all the tested ILs has been depicted in Table S2.

3.5. Morphological characterization of Biofilm treated with ILs

Scanning electron microscopy was used to investigate the impact of ILs on the architecture of the UPEC biofilm. It was noted that the control sample of UPEC biofilm was composed of dense multicellular communities embedded in an extracellular matrix with significant aggregation. In contrast, ILs treated UPEC had reduced adherence and aggregation ability after incubation with ILs. The bacterial growth and number of cells also substantially declined. Furthermore, the morphological changes in the UPEC cells and biofilm after exposure to ILs tested, was altered with changes in matrix architecture as well. The biofilm characterization of untreated and IL treated UPEC biofilm has been shown in (Fig. 7). It was observed that, [C8mim]DOSA treated UPEC biofilm had the significant reduction in the cell number of biofilm. At 20,000X only one cell can be seen instead of a dense and compact biofilm cells as compared to the control sample. Even at 2500X, only a few cells can be noticed. The cells reduced in size and constrictions were seen on the surface of bacterial cells. A decrease in extracellular matrix was also observed.

[C8mim]Br also resulted in reduced cell size and constrictions appeared in the middle of cells. The biofilm formation was also prominently reduced with the declined number of cells and no extracellular matrix. [C8Py]Br reduced the number of UPEC cells after the treatment, with pinched surfaces. The change in cell size was not prominent. Extracellular matrix was also not observed. [C88Im]Br resulted in very scant but larger and longer UPEC biofilm cells. Some cells were present individually but some were aggregated by extracellular matrix. [C8Pyr]DOSA significantly reduced the number of UPEC cells with no prominent morphological changes but a little extracellular matrix was observed around the individual cell.

3.6. Radical scavenging activity of ILs

DPPH radical scavenging assay is the most accurate and rapid spectrophotometric approach for determining the antioxidant capacity of ILs. The results obtained showed that the ILs are apparently effective in scavenging free radicals and are good antioxidant compounds. DPPH radical scavenging activities of ILs varied from 10.15 % to 22.05 %, ranging from the concentrations 2500 µM–39.06 µM, respectively (Fig. 8). It is clear from the results obtained that the structural conformation of a putative antioxidant affects how well it interacts with DPPH. The tested ILs appear to be good antioxidant molecules and effective at scavenging free radicals. Among all the tested ionic liquids, the maximum radical scavenging potential was exhibited by [C8Py]Br whereas [C88im]DOSA showed relatively lower potential in comparison with all other ILs.

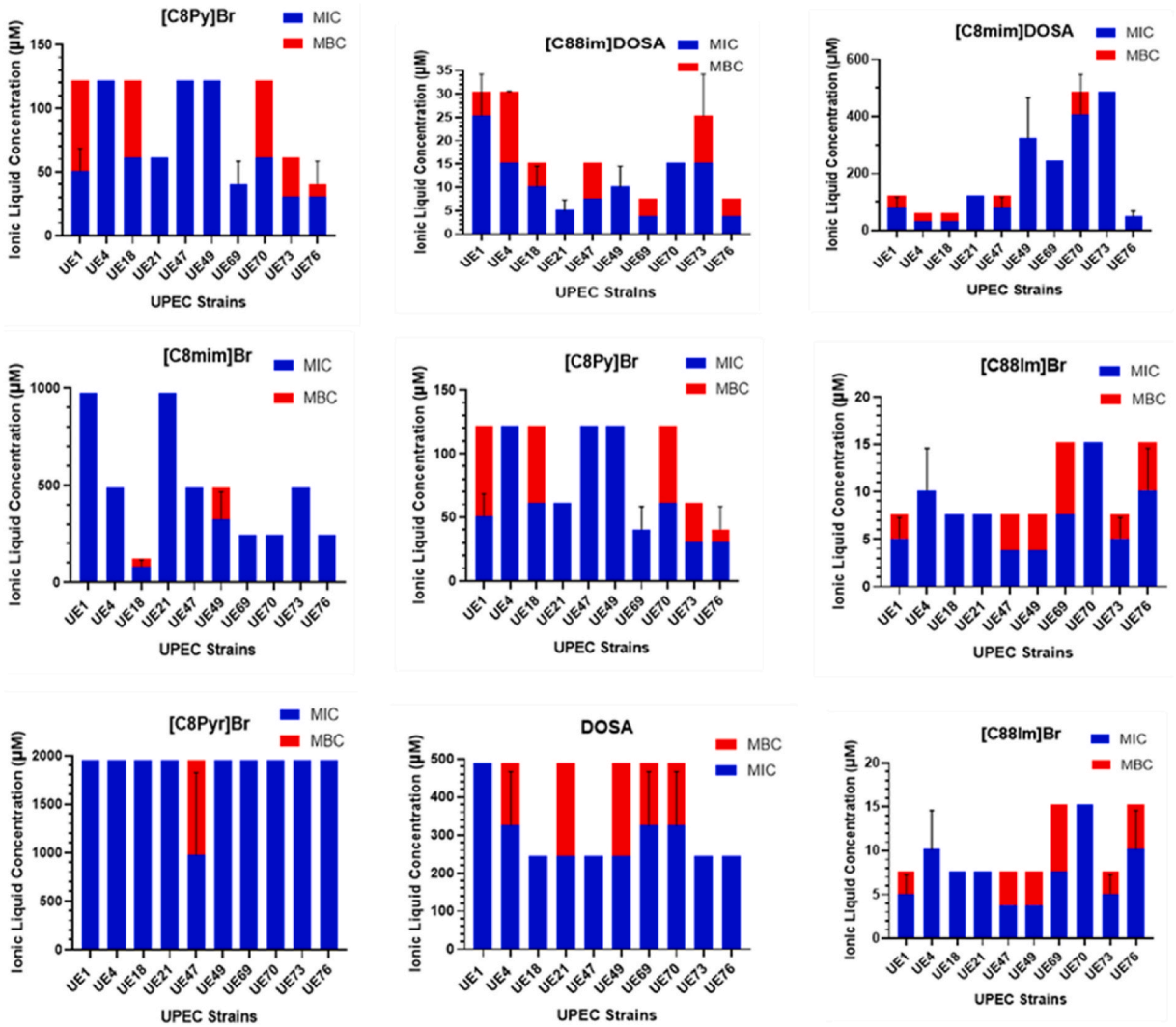

Fig. 5. Minimum inhibitory concentration and Minimum bactericidal concentration of all tested ILs against UPEC strains.

3.7. Cytotoxicity evaluation and safety profiling of ILs

3.7.1. Hemolytic activity of ILs

Hematocrit volume in the blood maintains the blood viscosity which in turn controls key mechanisms like thrombogenicity and hemostasis. Hemolysis refers to destruction of erythrocytes resulting in decreased hematocrit percentage leading to serious complications including disseminated intravascular coagulation (DIC) and sickle cell anaemia. Any material intended to be used within body is therefore testing for its hemolysis inducing capacity is required. According to ASTM, Hemolysis below 5 % is considered as null and up to 10 % is considered as safe or low.

As depicted in (Fig. 9), most of the ILs showed no hemolytic activity towards the red blood cells and the lowest hemolytic activity was achieved for [C8mim]Br, [C8Pyr]DOSA and [C8mim]DOSA followed by [C8Py]DOSA, without significant variances amongst them. [C8Pyr]Br, DOSA and [C88im]DOSA showed hemolysis at only 10 µM and 5 µM, which was still lower than 1 %. Furthermore, based on the hemolytic activity of the different ILs, it becomes clear that [C8mim]Br, [C8Pyr]DOSA and [C8mim]DOSA of all ionic liquids have the potential to be used as an antibacterial agent without affecting the integrity of red blood cells.

Nevertheless, hemolysis of $\leq$2.5 % was only achieved at extremely high IL concentrations of only a few ILs. These results can be associated to the type of structures formed by these specific ILs. These structures may interact with the cellular membrane, causing its disruption and leading to a higher hemolysis in comparison to the other ILs. However, it must be emphasized that all compounds have comparatively low hemolytic activity at high concentrations, which is a favorable indicator for their application in pharmacological or commercial uses without compromising public health.

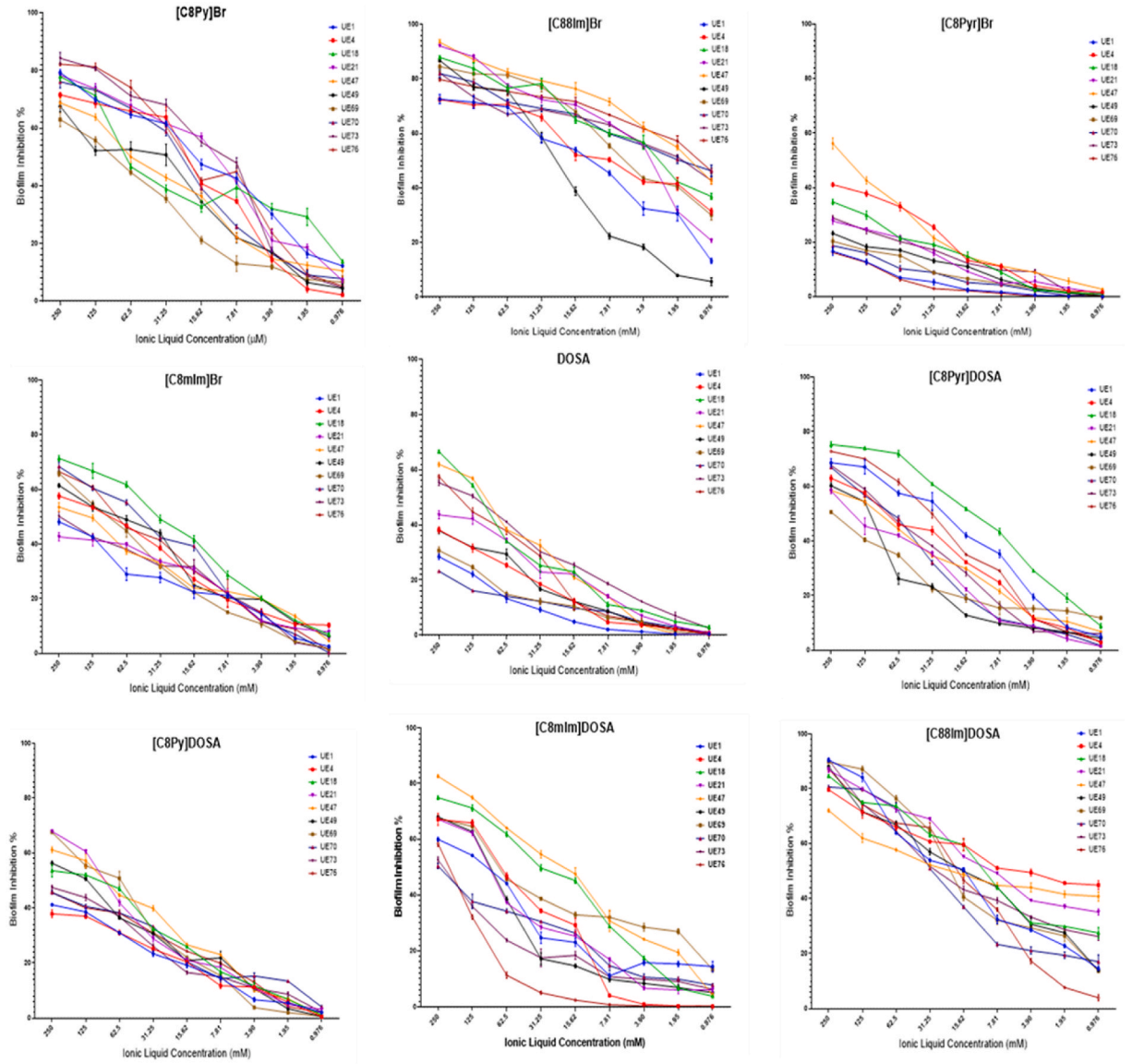

Fig. 6. Percentage biofilm inhibition of MDR UPEC biofilms at different concentrations of selected ILs. [C88Im]Br showed maximum biofilm inhibition (93.44 %).

3.7.2. Determination of cytotoxic activity of ILs on human cancer cell lines

MTT assay was used to check the cytotoxic potential of ILs toward human NB4 cell lines (Fig. 10). depicts that none of all the tested compounds exhibited significant cytotoxicity against human cell lines. Among all the tested compounds, DOSA showed lowest cytotoxicity toward NB4 cells at all concentrations. At 0.0625 μM, DOSA showed only 0.01 % of cytotoxicity. Whereas, [C88Im]Br showed comparatively high cytotoxicity among all tested ILs at all concentrations. At 0.0625 μM, [C88Im]Br showed 90.84 % cell viability. [C88Im]Br showed highest transition in cytotoxicity level from concentration 0.0625–1 μM [C88im]DOSA also showed a gradual decrease in cytotoxicity from high to low concentration. Regardless of the cell line used, the growth kinetics of all ILs treated cells were different from those of untreated control cells. Overall, the obtained results in this study reflect the low cytotoxicity of the studied ILs and the probability that, at their environmental concentration, they have no strong cytotoxic effect.

3.7.3. Assessment of ILs treated UPEC survival in blood

The UPEC survival in the human whole blood after the treatment with ILs was investigated by whole blood killing assay. The results of whole blood killing assay were measured by counting CFUs. Interestingly, Only 2 ILs i.e. [C8Pyr]Br and [C8Py]DOSA showed relative UPEC survival in blood after the treatment that is 5.5 % and 50 % respectively (Fig. 11). The results indicate that ILs had attenuated UPEC's ability to evade the host defense mechanisms. Notably, the addition of ILs at 0.01 % to human whole blood did not

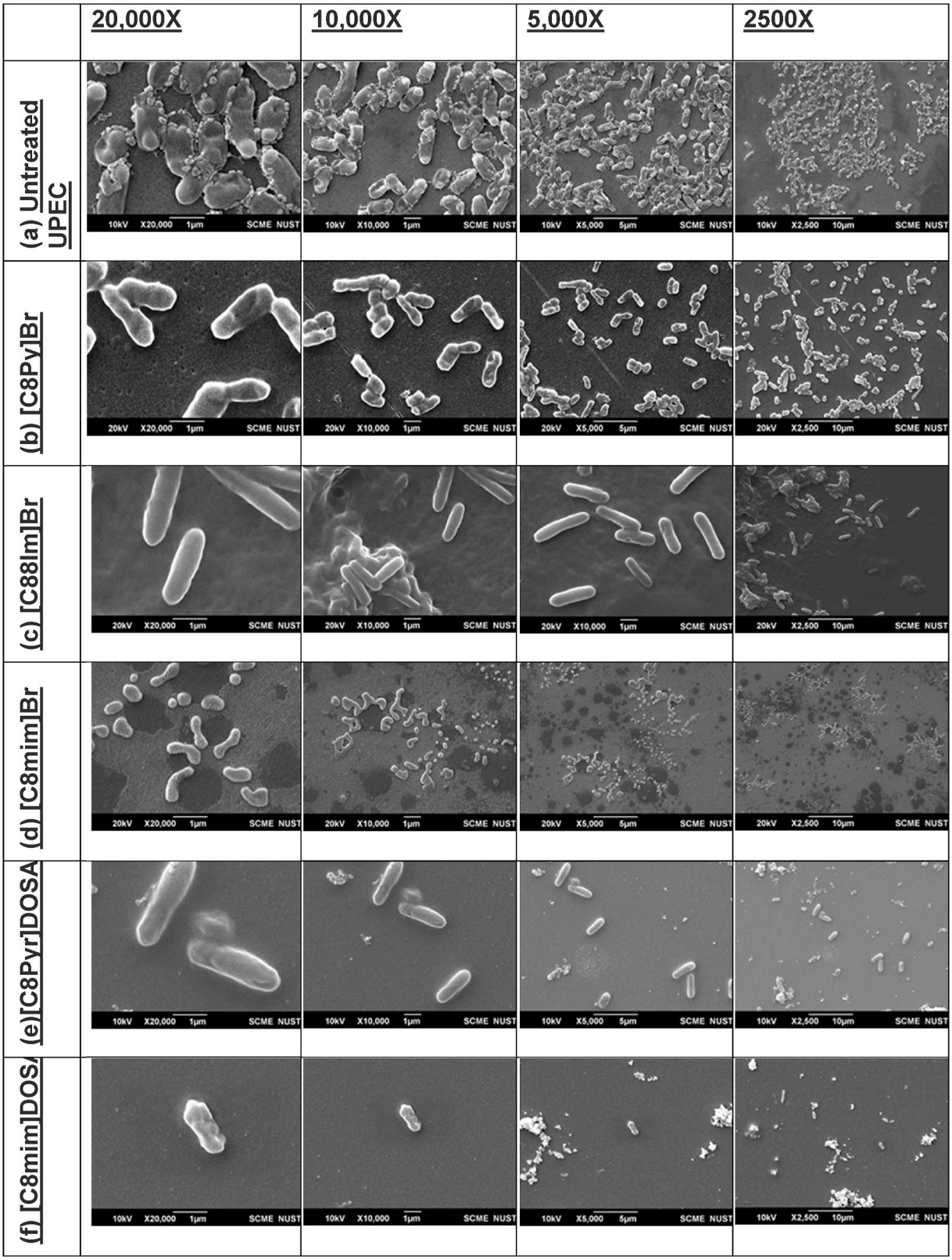

Fig. 7. Biofilm Characterization of Untreated (a) and Ionic Liquid treated (b,c,d,e,f) UPEC Biofilm.

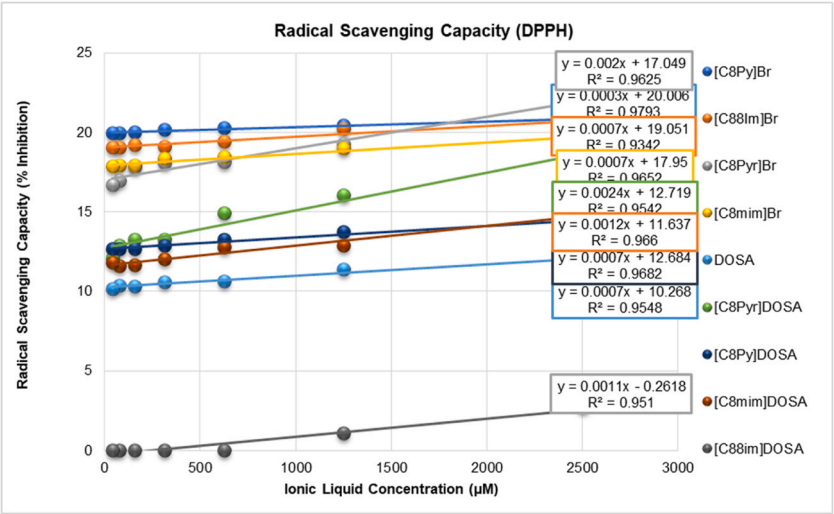

Fig. 8. Radical scavenging capacity of tested ILs.

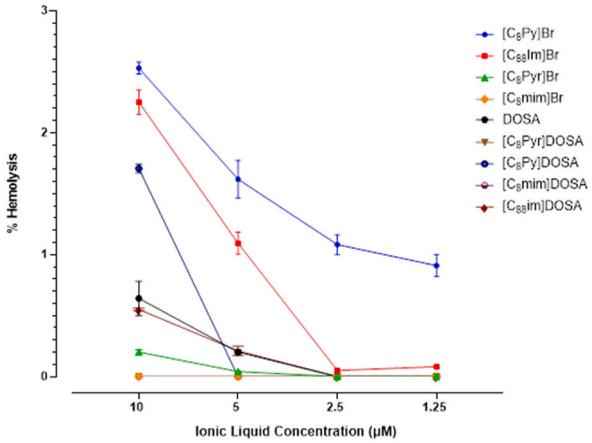

Fig. 9. Hemolytic activity of ionic liquids against fresh human erythrocytes. Each value is expressed as the mean of three replicates.

diminish the blood's inherent ability, including phagocytes and monocytes, to eliminate UPEC. This observation indicates that ILs, at this concentration, were not harmful to human blood cells. Consequently, it can be deduced that ILs have the effect of reducing UPEC's virulence by constraining its ability to survive upon encountering human whole blood.

3.7.4. Hemagglutination ability of UPEC after treatment with ILs

One of the pathogenicity component of the UPEC strains is their ability to hemagglutinate. Therefore, we have investigated the effects of ILs on UPEC- induced hemagglutination of human red blood cells. Following treatment with ILs, UPEC exhibited no hemagglutination activity in human red blood cells (RBCs). Hemagglutination inhibition of ILs have been depicted in Table S3. This finding underscores the effectiveness of ILs in inhibiting UPEC's ability to agglutinate RBCs, suggesting their potential as a promising strategy for mitigating UPEC infections. These results highlight the significance of further exploration into the therapeutic applications of ionic liquids in combating bacterial virulence factors and enhancing treatment options for UPEC-related conditions.

3.8. Gene expression analysis of virulent genes (fimH, uvrY and csrA)

To check the change in gene expression at the molecular level before and after the treatment of MDR UPEC with ILs, fimH, uvrY and csrA virulent genes involved in the adhesion and biofilm formation of UPEC were used. Gene fimH enables UPEC to colonize human epithelial cells during infection; whereas uvrY is an activator of biofilm formation and csrA is a repressor gene for biofilm formation and dispersal. 16SrRNA was used as an internal control. Overall the adhesion ability of UPEC was reduced and confirmed by the down-regulation of fimH gene. The relative gene expression % change of fimH ranged from −38.78 to −63.16 (Fig. 12). The relative gene expression analysis of csrA gene showed highest frequency of overexpression in ILs treated MDR UPEC with % gene expression change

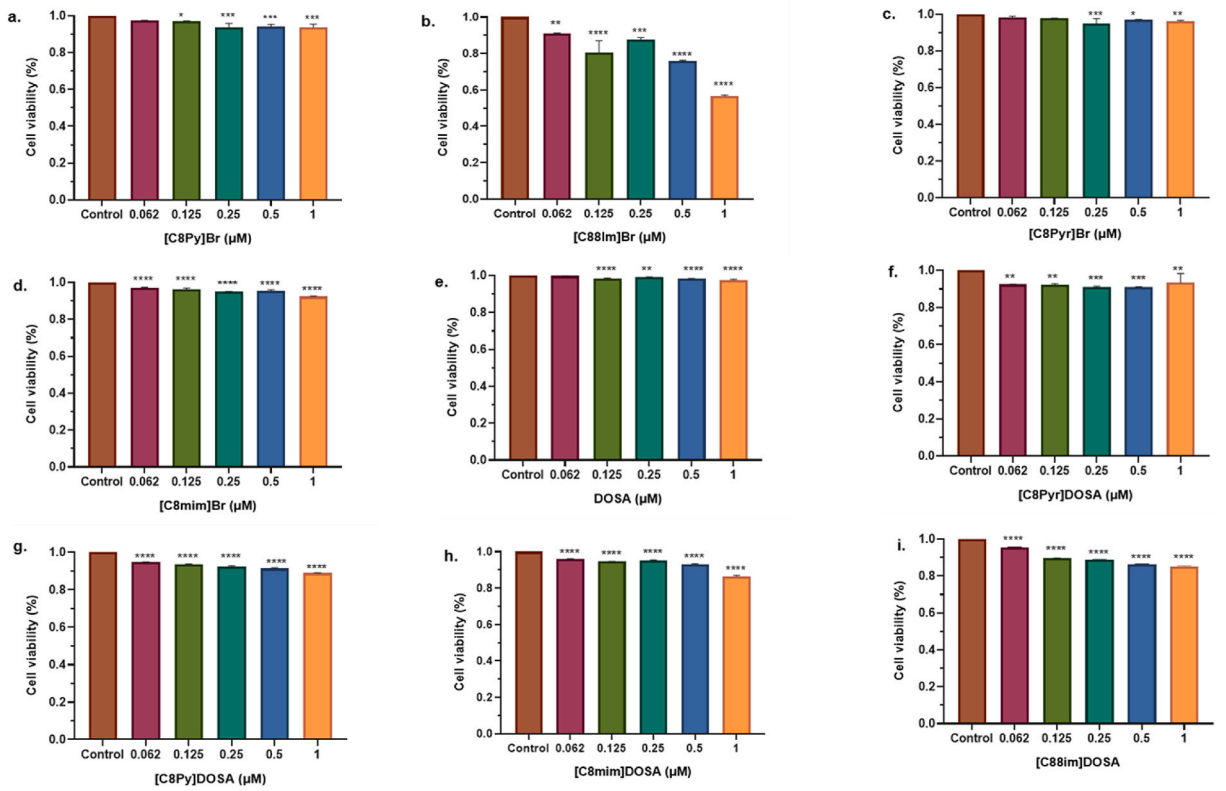

Fig. 10. Percentage Cell Viability after ILs treatment of NB4 Human Cell Line.

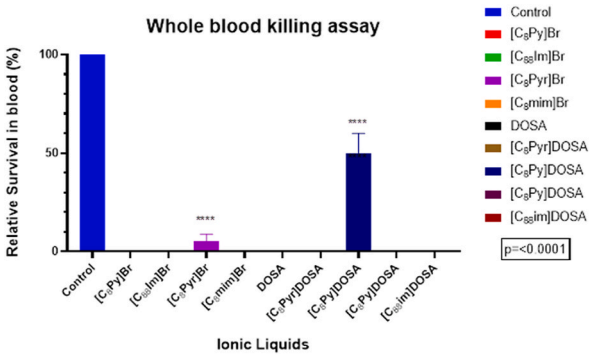

Fig. 11. Percentage Survival of UPEC in Blood after Incubation with ILs (**** = p value < 0.0001).

ranged (76.76–145.14) compared to wild type untreated UPEC isolate. Whereas, the relative percentage gene expression change was analyzed of uvrY gene by down expression in ILs treated UPEC isolate with percentage gene expression levels ranged (−22.80 to −47.46) compared to wild type untreated UPEC control isolate. The results suggested downregulation of fimH and uvrY genes.

3.9. Dermal sensitivity assessment of ionic liquids via in vivo rat model

The results of the dermal sensitivity test conducted on albino rats affirmed that 8 out of the 9 tested samples did not induce any sensitization in the animals. To validate the skin sensitization experiments, a positive control group was employed, which exhibited positive dermal sensitization responses. No sensitization was noticed among the albino rats exposed to the placebo control. No erythema or edema was seen among the albino rats treated with ILs except the one treated with [C8mim]DOSA (Fig. 13). Only the rats treated with [C8mim]DOSA showed moderate sensitivity towards the IL after 48h. This sensitivity falls into the sensitization grade of III.

No group showed any clinical symptoms or changes in body weight throughout the experiment. Additionally, there were no

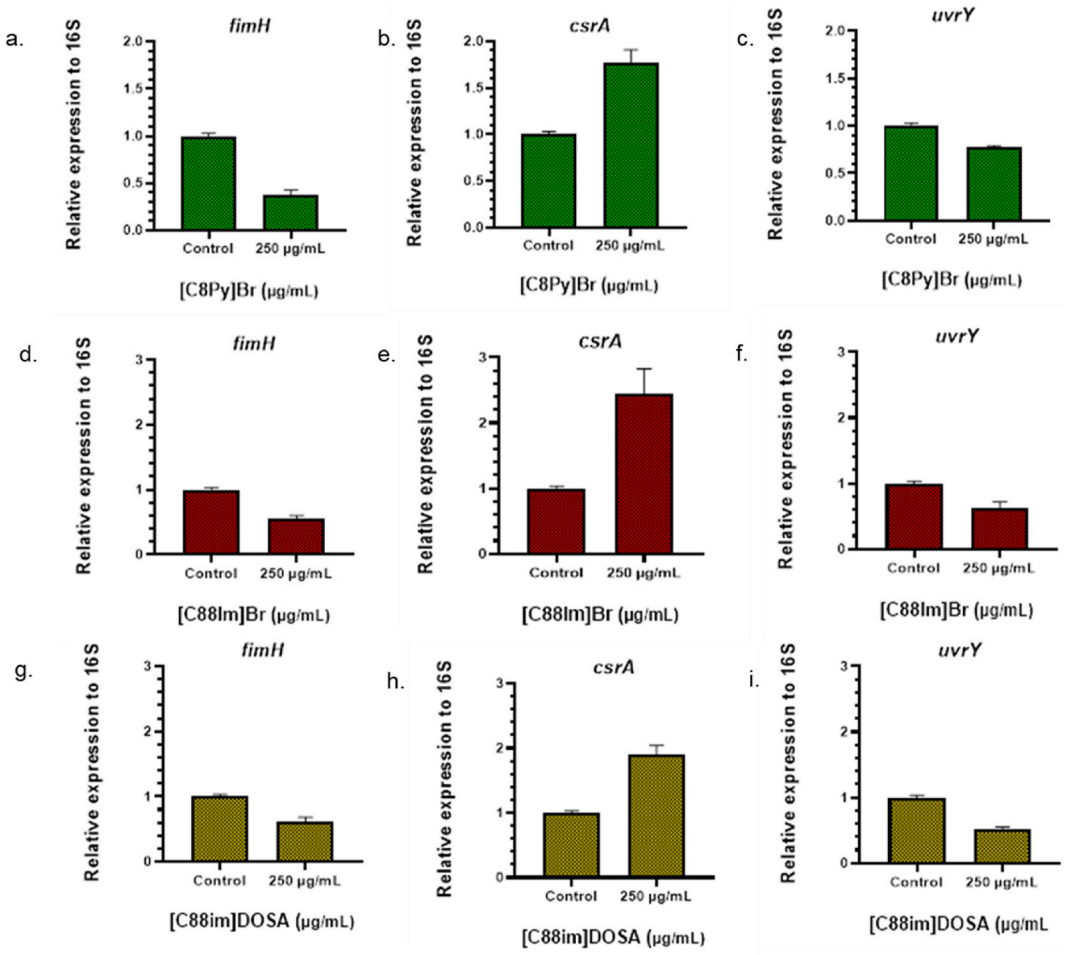

Fig. 12. Percentage Gene Expression Change of Virulent Genes (fimH, uvrY and csrA) in UPEC after treatment with ILs.

noteworthy mean weight variations observed between the control and treatment groups. The sensitization scores, rates, grades, and classifications of ILs and their placebo were assessed at 4, 24, and 48 h following dermal application have been summarized in Table 3.

3.10. In silico prediction

3.10.1. Physiochemical properties

The physiochemical properties have been computed by SwissADME tool and the results obtained are tabulated in Table 4. The results deduced at the lead stage concluded that most of the tested ILs possess greater than 50 % bioavailabilty score. Similarly, the value of the octanol/water partition coefficients (iLogP) allows to determine the hydrophobicity of ionic liquids. The octanol water partition cofficient (iLogP) values for most tested ILs fall within the range of 0.00–8.45 indicating the hydrophobicity and lipophilic character of lead compounds. Out of tested ILs, four had <5 iLogP value and showed no Lipinski rule of 5 violation making them suitable candidates for oral delivery. The remaining had far greater lipophilic character which may likely aid their delivery across the skin barrier.

3.10.2. Pharmacokinetic/ADME properties

The pharmacokinetic properties were computed by SwissADME tool and the obtained results are listed in Table 5. Most of the tested ILs depicted maximum GI absorption with variable P-glycoprotein inhibition. Interestingly, only IL1 was capable of passing through the blood brain barrier (BBB). It is noteworthy that most of the ILs presented with no inhibitory activity of Cytochrome P450 isozymes. All ILs depicted good skin permeability [logKp], except two with −2.78 and −1.80 values due to their predicted hydrophilic character.

4. Discussion

Urinary Tract Infections (UTIs) are a prevalent medical condition that affects millions of individuals globally with UPEC, which

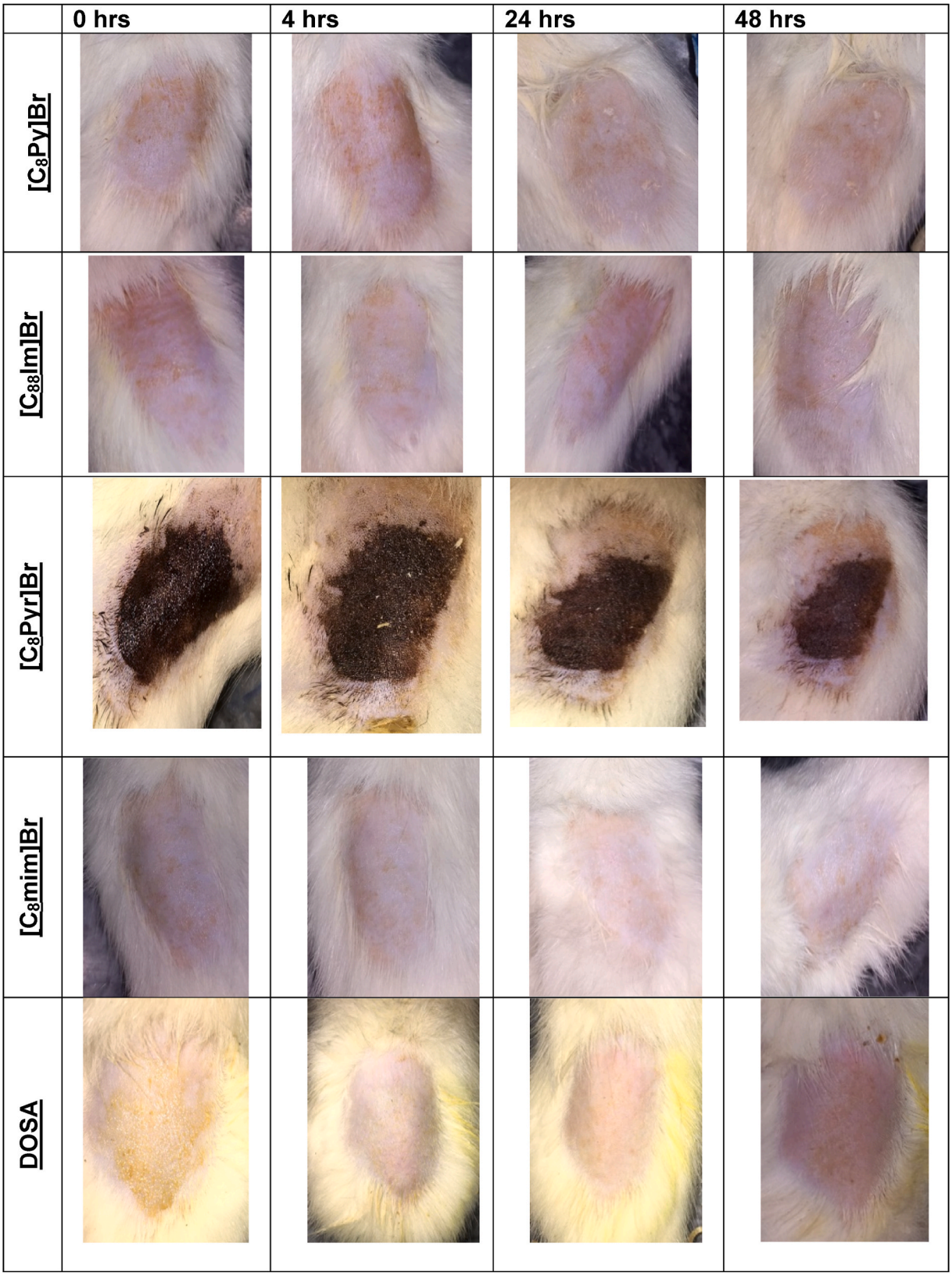

Fig. 13. Dermal Sensitivity evaluation of ILs on the Skin of Albino rats after 4, 24 and 48 h.

Table 3
Sensitization Score, Rate, Grade, and Classification of Challenge Reaction after 4, 24, and 48 h of ILs dermal application in Albino rats.

Test Materials	Sensitization Rate (%)	Sensitization Grade	Sensitization Classification	Reaction
Positive control (0.1%w/v CDNB in 10 % propylene glycol), n = 2	100 (2/2)	V	Extreme	Intense
Negative control (placebo Distilled water), n = 10	0 (0/2)	I	Weak	No
[C_8Py]Br	0 (0/2)	I	Weak	No
[C_{88}Im]Br	0 (0/2)	I	Weak	No
[C_8Pyr]Br	0 (0/2)	I	Weak	No
[C_8mim]Br	0 (0/2)	I	Weak	No
DOSA	0 (0/2)	I	Weak	No
[C_8Pyr]DOSA	0 (0/2)	I	Weak	No
[C_8Py]DOSA	0 (0/2)	I	Weak	No
[C_8mim]DOSA	100 (2/2)	III	Moderate	Yes
[C_{88}im]DOSA	0 (0/2)	I	Weak	No
ILs Treated group n = 18	2 (2/18)	16 = I	Weak	No
		2 = III	Moderate	Yes

Table 4
Computed physicochemical and bioavailability parameters of ILs.

Ionic Liquids	Molecular Weight (g/mol)	iLogP	Log S	Lipinski	Bioavailability Score
1-octyl Pyridinium Bromide	272	0.13	MS	Yes	0.55
1,3-dioctyl Imidazolium Bromide	372.9	0.0	PS	Yes	0.55
1-octyl Pyrazinium Bromide	273.21	−2.78	MS	Yes	0.55
1-methyl-3-octyl Imidazolium Bromide	275	0.13	MS	Yes	0.55
Dioctyl Succinamic Acid	341	4.24	MS	Yes	0.85
1-octyl Pyrazinium Dioctyl Succinamic acid	534.21	−1.80	MS	Yes	0.55
1-octyl Pyridinium Dioctyl Succinamic Acid	533	7.61	PS	Yes	0.85
1-methyl-3-octyl Imidazolium Dioctyl Succinamic Acid	536	8.45	PS	Yes	0.85
1,3-dioctyl Imidazolium Dioctyl Succinamic Acid	633	8.33	PS	Yes	0.85

Table 5
Pharmacokinetic/ADME (absorption, distribution, metabolism and excretion) parameters of ILs.

Ionic Liquids	GI Abs	BBB Permeation	P-Gp substrate	CYP1A2 Inhibitor	CYP2C19 Inhibitor	CYP2C9 Inhibitor	CYP2D6 Inhibitor	CYP3A4 Inhibitor	Log Kp cm/s
1-octyl Pyridinium Bromide	High	Yes	No	No	No	No	No	No	−4.05
1,3-dioctyl Imidazolium Bromide	Low	No	No	Yes	No	No	No	No	−2.6
1-octyl Pyrazinium Bromide	Low	No	Yes	No	No	No	No	No	−4.96
1-methyl-3-octyl Imidazolium Bromide	High	No	No	No	No	No	No	No	−4.31
Dioctyl Succinamic Acid	High	No	No	No	No	No	No	No	−4.13
1-octyl Pyrazinium Dioctyl Succinamic acid	Low	No	Yes	No	No	No	No	No	−2.47
1-octyl Pyridinium Dioctyl Succinamic Acid	Low	No	Yes	No	No	No	No	Yes	−1.97
1-methyl-3-octyl Imidazolium Dioctyl Succinamic Acid	Low	No	Yes	No	No	No	No	No	−2.32

being the primary causative agent [44–46]. The challenge of antibiotic resistance among UPEC strains has emerged as a significant obstacle in the treatment of UTIs. UPEC strains have exhibited resistance to various antibiotics by biofilm forming ability, further complicating treatment strategies [47]. This underscores the need of alternative antimicrobial agents to combat UTIs most particularly recurrent urinary tract infections [48]. The current study aimed to assess the antimicrobial and therapeutic potential of chemically synthesized ILs against UPEC strains, which are predominantly involved in the etiology of UTIs in human. These ILs include octyl and dioctyl ILs with imidazolium, pyridinium and pyrazinium as cation and bromide and DOSA as an anion. A total of 9 ILs i.e. [C8Py]Br, [C88Im]Br, [C8Pyr]Br, [C8mim]Br, DOSA, [C8Pyr]DOSA, [C8Py]DOSA, [C8mim]DOSA and [C88im]DOSA were used.

Ionic liquids (ILs) offer a promising alternate to conventional antibiotics for the treatment of UTIs. ILs possess a diverse range of antimicrobial mechanisms, including the ability to modulate bacterial cell morphology, disrupt cell membranes, impede biofilm formation, interfere with DNA/RNA synthesis, inhibit enzyme activity, and disintegrate organic materials [30]. Recent research has explored novel antimicrobial ILs with long alkyl chains, particularly pyridinium or imidazolium-based ionic liquids [49,50]. Moreover, pyridine-based oxazolidinone derivatives have shown their growth inhibiting properties on a variety of MDR bacterial strains [51].

Previously, the Gram positive bacteria were considered comparatively susceptible towards ILs with low MICs [20] as 1-alkyl-3-methylimidazolium chloride ILs with 10 carbons had led to the potent biofilm eradication in Gram positive bacteria than Gram negative bacteria [52]. By extending the alkyl chains in imidazolium and pyridinium based ILs, a biological effect can be achieved potentially involving DNA intercalation [53]. Monomeric pyridinium and imidazolium based ILs have shown anti-inflammatory, and anticancer activities. In contrast, piperidinium and pyrrolidinium salts generally exhibit lower bactericidal effect than imidazolium salts. Imidazolium salts, such as 1-decyl-3-cinnamylimidazolium chloride, exhibit greater attraction to phospholipid bilayers due to their longer alkyl chains, resulting in increased hydrophobicity [38]. Our study supports the previous research studies showing imidazolium based ILs with highest antimicrobial potential. The results for ZOI, MIC, antibiofilm and SEM showed that imidazolium based ILs had the highest antimicrobial activity as compared to pyridinium ILs, that is followed by pyrazinium based ILs.

The biological activity of ILs differs from organism to organism, depending upon factors such as their solubility and interaction with the solvent. The solubility profiles of the synthesized ILs in various solvents provide valuable insights into their physicochemical properties. Understanding their solubility characteristics is essential for tailoring their use in specific contexts and optimizing their performance in various chemical processes. Imidazolium based ILs showed highest solubility followed by pyridinium and pyrazinium based ILs. Comparatively Bromide anion showed higher solubility than ILs having DOSA anion.

The incorporation of variable anions allows for the customization of ILs to enhance their biocompatibility without compromising their bactericidal properties. For instance, [DMIM][TFSI] has been found to induce lysis in bacterial cell, through a mechanism involving membrane-disruption of cell membranes. In contrast, [EMIM][HSO_4] has exhibited strong antimicrobial activity against *E. coli* [38]. Notably, nitro-substituted dimeric imidazolium salts containing bromide counter anions have shown effective inhibition against uropathogens The dimeric imidazolium salts penetration through the lipid layer of bacterial cell membranes by enhancing their lipophilicity. These findings corroborate the observations in the research, where ILs with Bromide (IL 1–4) as an anion displayed notably higher antimicrobial activity against UPEC strains compared to ILs with DOSA (IL 6–9) as an anion. The maximum zone of inhibition was exhibited by imidazolium based ILs, followed by pyridinium and then pyrazinium based ILs.

The alkyl chain length in ILs influences their antimicrobial properties, with longer alkyl chains generally resulting in lower MIC values. For instance, 1-n-alkyl-3-methylpyrrolidinium bromide, reduced the MIC against *S. aureus* and *E. coli* from 14,300 mg/L to 5.0 mg/L, and from 18,500 mg/L to 6.7 mg/L, respectively with chain length increase from 4 to 12 [54]. In the current research, octyl and dioctyl ILs with imidazolium, pyridinium, and pyrazinium cations, in combination with bromide and DOSA anions, demonstrated that ILs with two octyl chains exhibited higher antimicrobial activity compared to ILs with only one octyl chain. Within cation variation, imidazolium ILs showed lowest MIC than pyridinium and pyrazinium based ILs.

The microbial biofilms poses a great risk in clinical trials and infectious diseases as these infectious biofilms are responsible for continuous financial loss at the industrial scale. The alterations in bacterial morphology after exposure to ionic liquids (ILs) can be determined by using scanning electron microscopy analysis. Typically, untreated bacterial strains displayed clear and, smooth surfaces, rounded projections, and intact cell walls. The impact of C_3MIMCl and C_{12}MIMCl on the bacterial membrane of *S. aureus* was assessed to evaluate the antibacterial effects of ILs with varying cation side chain lengths. Interestingly, the findings indicated that C_{12}MIMCl has the potential to disrupt the membranes of *S. aureus* [55,56]. In this study, UPEC biofilms subjected to the tested ILs 1–9, showed significant change in the number of cells, altered bacterial morphology and reduced extracellular matrix. Imidazolium caused highest morphological changes with reduced number of cell, constricted cell surface and reduced extracellular matrix. Imidazolium was followed by pyradinium and then pyridinium based ILs. The one octyl chain based ILs caused comparatively higher change in morphological features of biofilm.

Ionic liquids have been recently recognized to exhibit significant antioxidant activity in scavenging free radicals and protecting against oxidative stress. Their effectiveness in neutralizing reactive oxygen species (ROS) makes them valuable in combating oxidative damage [25]. ILs have demonstrated the ability to inhibit lipid peroxidation, a process that damages cell membranes and contributes to various diseases. The ILs used in the study showed greater antioxidant potential. Maximum of 22.05 % radical scavenging capacity was showed by [C8py]Br at 2500 μM. The obtained results were comparable with commercially available antioxidant Trolox. Bromide anion based ILs showed higher antioxidant potential than DOSA based ones. Overall, pyrazinium showed highest antioxidant potent than pyridinium and imidazolium.

The evaluation of potential side effects is a crucial concern within the scope of the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) effort, particularly during the preclinical evaluation of pharmaceuticals [57,58]. Novel substances intended for human and animal consumption require appropriate toxicological assessments [59]. Although being biocompatible and potential biomedical antimicrobial agent, ILs may be toxic to somatic cells [60]. The low antimicrobial activity with high toxicity is found in ammonium- and imidazolium-based ILs [61]. Some ILs are relatively not or less cytotoxic towards human and animal cells. For example, 1-Ethyl-3-methylimidazolium hydrogen sulfate [EMIM][HSO_4] was highly antimicrobial toward *E. coli* and found suitable for biological applications due to its harmless nature towards mammalian cells [38]. In the current study, ILs showed comparatively less cytotoxic potential as compared to ILs previously reported. The lowest cytotoxicity was showed by DOSA derivatives. The ILs with one octyl chain showed lesser cytotoxicity as compared to ILs with two octyl chains. Anion also contributed to the cytotoxicity with bromide being more cytotoxic than DOSA.

The high antimicrobial activity and relatively low hemolytic activity of ILs suggest their possible application as antimicrobials, such as anti-biofilm agents [56]. It becomes clear from this study, that [C8mim]Br, [C8Pyr]DOSA and [C8mim]DOSA of all ionic liquids have the antibacterial potential without cytotoxic effect towards red blood cells. The hemolytic behavior of ILs indicates that red blood cell lysis only occurs at exceptionally high concentrations, confirming their biocompatible characteristics. When comparing the biocompatibility of imidazolium and pyridinium-based ILs, they exhibited quite similar hemolytic activity. Notably, both of these cations demonstrated slight hemolytic activity within acceptable range when paired with bromide anion as compared to DOSA anion.

Nevertheless, based on the hemolysis profiles obtained, it is evident that pyrazinium based ILs exhibit higher hemolytic activity at comparable concentrations to other imidazolium and pyridinium-based ILs. Overall, the hemolysis percentage of all the experimented ILs was less than 2 %.

Previous studies revealed the involvement of BarA/UvrY/CsrA pathway in UPEC CFT073 biofilm formation. An experiment utilizing RT-qPCR confirmed that allicin has the ability to decrease the expression of fimH and uvrY while increasing the expression of csrA in CFT073 [62]. At the molecular level, an analysis of gene expression involving fimH, uvrY and csrA was conducted following the treatment with ILs The outcomes demonstrated that the most substantial alterations in gene expression occurred in UPEC treated with imidazolium-based ILs, followed by pyridinium-based ILs, and finally, pyrazinium-based ILs. Among the imidazolium-based ILs, those containing bromide as an anion exhibited more pronounced changes in gene expression compared to imidazolium with DOSA as the anion. Overall, the expression of fimH was downregulated, uvrY showed a decrease in expression, and csrA exhibited an increase in expression.

Whenever any substance is considered for therapeutic use, it is advisable to follow a stepwise testing approach to gather scientifically reliable information regarding substance irritation [63,64]. Most commonly human exposure to ILs occurs primarily via skin contact and research findings suggest that some ILs may cause skin irritation or dermal toxicity particularly in keratinocytes [65–68]. Therefore, *in vivo* dermal sensitivity evaluation of ILs was carried out and results depicted that all ILs were found non cytotoxic toward animal skin cells.

The influence of various physicochemical properties of the drug on the biomolecule is related to the drug's ability to demonstrate a pharmacological or therapeutic effect. The pharmacokinetic behaviour of a drug describes how the pharmacological agent behaves in the body which determines the ADME (absorption, distribution, metabolism, and excretion) features of the drug molecule [69,70]. The pharmacokinetic behaviour of drug candidates is influenced by various physiochemical factors [71]. In the present study, several physicochemical and pharmacokinetic parameters of the synthesized ILs have been computed in order to screen potential therapeutic candidates. The tested ILs passed the Lipinski's rule of five which make them potentially suitable candidates for extravascular and intravascular delivery.

The bioavailability values in the ranging from 0 to 20 % depict poorly absorbed compounds, 20–70 % shows moderately absorbed compounds and 70–100 % for well absorbed compounds. Out of the said ILs mostly fall in the range of well absorbed and few in moderately absorbed compounds hence could serve as a potential oral drug candidates as they can be absorbed or assimilated from the human intestine by minor formulation modifications [72]. Cytochrome P450 inhibitors are essential detoxifying enzymes that are often located in the liver. These cytochrome P450 inhibitors inactivate many drugs, and can also activate some of them [73]. Most of the ILs showed no inhibitory activity of Cytochrome P450 isoenzyme family, hence predicting normal compound metabolism eradicating potentially toxic outcomes at usual concentrations. All the obtained results provide additional insights into the antibacterial effects of ILs and thereby facilitating the future development of efficacious antimicrobial agents.

5. Conclusion

The economic impact of UTIs, caused by MDR UPEC, is substantial, leading to increased healthcare costs and prolonged hospital stays. ILs show promise in addressing this issue by serving as effective alternative agents in infection control. Their potential applications in pharmaceutical industry and medicine underscore the importance of further research to develop novel ILs with improved antibiofilm activities, reduced environmental impact, and enhanced safety profiles. This research paves the way for innovative solutions to combat infectious diseases and biofilm-related challenges. In conclusion, the research findings emphasize the significant potential of ILs as versatile antimicrobial and antibiofilm agents. These ILs exhibit strong antibacterial and antioxidant activity while demonstrating relatively lower cytotoxicity and safety towards mammalian cells. Moreover, *in silico* results endorsed the therapeutic potential of these compounds owing to their good bioavailability score and high permeability across physiological barriers with limited inhibition of CYP450 isoforms. Among the studied compounds, imidazolium-based ILs stands out as particularly promising due to their favorable results. The variable cation, anion and alkyl chain length significantly affects the antibacterial, antioxidant and cytotoxic properties of the ILs.

CRediT authorship contribution statement

Sidrah Hafeez: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Zamar Rasool:** Visualization, Methodology, Data curation. **Samia Hafeez:** Methodology, Conceptualization. **Rehan Zafar Paracha:** Software, Methodology, Formal analysis, Data curation. **Muddassir Iqbal:** Writing – review & editing, Formal analysis, Conceptualization. **Dilawar Khan:** Writing – review & editing, Methodology. **Fazal Adnan:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Data availability statement

Data included in article/supplementary material/referenced in article.

Ethics statement

This study was reviewed and approved by NUST Ethical review Board with the approval number: 05-2022-ASAB-01/10, dated June

30th, 2022.

Declaration of competing interest

Enclosed is the manuscript entitled "**Imidazolium, Pyridinium and Pyrazinium based Ionic Liquids with Octyl side chains as Potential Antibacterial Agents against Multidrug Resistant Uropathogenic *E. coli*"** for publication in your prestigious journal.

With the submission of this manuscript I undertake that all the authors mentioned in this manuscript have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39829.

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