Invitro Screening Of Dicationic Ionic Liquids And Their Consequence Towards Biological Strategies

Antilin Princela.M, Isac Sobana Raj.C

Abstract: Ionic liquids (ILs) have gained increasing attention in various fields in science and engineering due to their tunable characteristics. In the recent years, the multifaceted aspects of ionic liquids have been exploited in the field of biology and medicinal chemistry too. Insertion of a new cationic head and use of inorganic anions increased the biocompatibility of the ILs developed. Herein we have designed numerous dicationic ionic liquids such as imidazolium, benzimidazolium, pyridinium, viologen, 1,2-diethyl-1,4-diazoniabicyclo[2.2.2]octane bromides based dicationic ionic liquid (DILs) were successfully synthesized and characterized by IR, NMR and Mass spectral studies. The synthesized DILs were also tested against different bacterial strains (S.aureus, E.coli, bacillus .sp, Pseudomonas aeurignosa, Klebsiella pneumonie) and fungi (A.niger, candida albicans) suggesting that compound viologen based DIL showed excellent activity against all species. Furthermore, we have examined the antioxidant activity using phosphomolybdenum and nitric oxide assay. The antioxidant result reveals that, the synthesized DILs especially viologen and DABCO based ILs have a potential for scavenging the NO radicals. Hence it shows excellent antioxidant property than compared to ascorbic acid. In addition to that we determined the cytotoxicity of dicationic ILs using three cancer cell lines HeLa, MCF-7, HT-29 and Adriamycin was used as positive control. These reports emphasize viologen based DIL exhibit better activity against HeLa cancer cell line.

Index Terms: Antibacterial and antifungal activity, Antioxidant activity, Cytotoxicity, Dicationic ionic liquids.

1 INTRODUCTION

The ionic liquids (ILs) plays unambique role in the scientific world to alter the human day to day lifestyle. Since ILs secure flexible behaviour and tunable electro and physico chemical properties, these ILs were used in various fields [1]. Especially chemical synthesis, electrochemistry, material science, Nanotechnology, fuel liquids production, extraction process, biomass conversion, adsorption, catalysis and even medical field [2,3,4]. Recently, the role of ILs in environmentally friendly energy dependent research areas like solar cells, fuel cells, hydrogen production, CO_2 capture, water splitting, batteries, supercapacitors and eco-friendly medicines, especially medicine for anticancer is a remarkable one [5,6,7,8]. There are different varieties of ILs are known up to now among that room temperature ionic liquids (RTILs), functionalized ILs, metal containing ILs, bioactive ILs, chiral ILs, polymeric ILs and dicationic ILs (DILs). They are used in wide areas of research and their applications in day today life is a memorable one [9,10,11]. However, the DILs are very special than others. Why because, easy to synthesis especially no column purification, only precipitation techniques were used for the synthesis. Apart from this, it is low cost, more ecofriendly in nature and easy to handle because most of them are air and moisture stable materials [12,13].

Usually DILs are having high ionic character in a unite area as compared to other ILs, hence the ionic mobility is high and fast as compared to others. Hence these types of DILs were used in various medicinal applications [14]. The biological activity of DILs were attributed to charged character triggering disruption of intermolecular interactions ensuing into fluctuations in cell membrane permeability and leakage of cell contents. Herein we have designed and synthesized five different DILs with different cation and similar anion as BF_4. Since tetrafluoroborate anion having efficient activity in medicinal application. Therefore, we have chosen BF_4 as anion for all the DILs and also, we have compared antimicrobial, antifungal, antioxidant and anticancer studies of all the synthesized DILs. More than 50% of medicines on today's world market are sold as salts. Ion-pair generation augments the transport of numerous ionic drugs through the skin and across the absorbing membrane [15]. The pivotal stage in the drug production was the transformation of a drug into a salt and this strategy can have a massive attention on its physicochemical properties, especially solubility, stability, dissolution rate and hygroscopic nature [16]. Commutation of a drug into IL is an pioneering attempt that could eradicate numerous issues like crystallization, dissolution, transport, delayed drug delivery mechanisms and bioavailability, which can dramatically change the pharmacological activity and it create unwanted side effects. In other words, the modification, functionalization and the suitable choice of cation could be selected to improve pharmacokinetic properties [17,18]. Thus, highly ionic as well as pharmaceutically active ILs would be highly preferable for the new drug development and it will create biological revolution in future [19]. In this context, herein we have designed and synthesized the five different DILs based on imidazole, benzimidazole, pyridine, viologen and DABCO namely *1,4-bis(3-methylimidazolium-1-yl)butyl tetrafluoroborate* ($[C_4(MIm)_2][BF_4]_2$) **DIL1,** *1,4-bis(3-ethylbenzimidazol-1-yl)butyl tetrafluoroborate* ($[C_4(Ebim)_2][BF_4]_2$) **DIL2,** *1,4-bis(pyridinium)butyl tetrafluoroborate* ($[C_4(Py)_2][BF_4]_2$)

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DIL3, *1,1'-diethyl-4,4'-bipyridinium tetrafluoroborate, ([2C$_2$viologen][BF$_4$]$_2$)* **DIL4,** *1,2-diethyl-1,4-diazoniabicyclo[2.2.2]octane tetrafluoroborate ([2C$_2$DABCO][BF$_4$]$_2$)* **DIL5** and compared its biological activity. Therefore, these DILs can be applied as multifaceted bioinspired material. Due to their own potential and desirable characteristics like low toxicity, environmental friendly, excellent antimicrobial activity, intermingle with peptides and retain the enzyme activity, affinity towards living cells and so on. In this endeavor, we have investigated their antimicrobial, antifungal, and antioxidant activity as well as their cytotoxicity effects. All the DILs were shown excellent antimicrobial and antifungal activity. In addition to that, viologen (DIL4), DABCO (DIL5) based DILs were showed outstanding antioxidant activity. Further, 1,1'-diethyl-4,4'-bipyridinium tetrafluoroborate ([2C$_2$viologen][BF$_4$]$_2$), DIL4 exhibits remarkable cytotoxicity against all the tested HeLa, HT-29 and MCF-7 cell lines.

2 Experimental Procedure

2.1 Chemicals and Materials `

The reagents 1-methylimidazole, 1-ethyl benzimidazole, pyridine, 1,4-dibromo butane, bromoethane, 4,4'-bipyridine, Triethylenediamine, sodium tetrafluoroborate were purchased from Sigma Aldrich. The organic solvents ethyl acetate, diethyl ether, acetonitrile, were purchased from Spectrochem Pvt. Ltd., India. All reagents and solvents are of analytical grade and were used as received unless otherwise stated and all the reactions were performed with oven dried glassware. The anion exchange reactions were performed using Milli-Q water with electrical resistivity of 18.2 MΩ cm at 25^{o}C.

2.2 Instrumentation

Melting point of all the synthesized DILs were determined with an open capillary tube and are uncorrected. NMR spectra were recorded on a 400 MHz Bruker spectrometer with deuterated solvents using TMS as the internal reference. Chemical shifts of ^{1}H, ^{13}C, and ^{19}F NMR were expressed in parts per million relative to deuterated solvents $CDCl_3$ (7.26), D_2O (4.79), DMSO-d_6 (2.5). High resolution mass spectroscopic (HRMS) measurements were performed on a JEOL GC MATE II HRMS (EI). Fourier Transform Infrared (FTIR) spectra were recorded using a Shimadzu IR affinity-1 spectrometer with Attenuated Total Reflectance (ATR) setup in the range of 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}. For cytotoxicity studies, fluorescence images were obtained from a Nikon Eclipse Ti-S Inverted Research Microscope with a magnification of ×20, using Eclipse Image processing software NIS-Elements.

2.3 General procedure for the synthesis of dicationic bromide ILs

A mixture of 1,4-dibromobutane (5 mmol) and the corresponding n-hetrocycles/ substituted hetrocyclics (10 mmol; 1-methylimidazole/ 1-ethylbenzimidazole/ pyridine) were mixed thoroughly with acetonitrile in a 100 ml round bottom flask, and the reaction mixture was stirred at 70 ^{o}C for 48 h. Then the reaction mixture was cooled to room temperature, and the solvent was removed by rotary evaporator. The curdy white precipitate was obtained and the precipitate was washed with copious amounts of acetonitrile to remove the unreacted starting materials. Furthermore, the precipitate was washed with diethyl ether (3 x 50 ml). Finally, the precipitate was filtered and dried in a hot air oven at 60 ^{o}C for 12 h. All the dicationic bromide ILs were obtained with a high yield around 90 %.

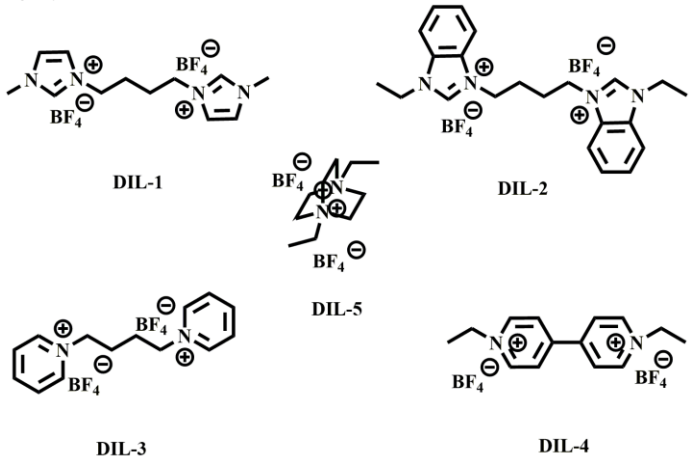

Figure1. *Structure of DILs*

2.4 General procedure for the synthesis of [2C$_2$viologen][Br]$_2$, [2C$_2$DABCO][Br]$_2$ dicationic bromide ILs

Bromoethane (10 mmol) and the corresponding n-heterocycles (5 mmol; 4,4'-bipyridine/ Triethylenediamine) were taken along with acetonitrile in a 100 ml RB flask**.** The reaction mixture was refluxed at 70 ^{o}C for 48 h. The contents of the RB flask were cooled to room temperature, and the solvent was removed by rotary evaporator. The colourless or yellow precipitate was obtained. Then the precipitate was washed repeatedly with acetonitrile to remove the unreacted residues and finally, once wash with diethyl ether**.** Then the precipitate was collected by filtration and dried at 60 ^{o}C in hot air oven for 12 h.

2.5 General procedure for the synthesis of dicationic tetrafluoroborate ILs

An aqueous solution of sodium tetrafluoroborate and the corresponding newly synthesized dicationic bromide ILs were readily undergoes metathesis reaction with BF_4^- anion. The dicationic bromide ILs (5 mmol) was dissolved in 50 ml of distilled water and allowed to stir at room temperature. An aqueous solution of $NaBF_4$ (10 mmol, 1.0979g) was added dropwise to the reaction mixture over 15 min, the reaction was continued another 12 h. Then, the obtained precipitate was collected by filtration and washed with cold water and dried in hot air oven at 60°C for 12 h. All the dicationic tetrafluoroborate ILs were obtained with a high yield around 90%.

2.6 Antimicrobial and antifungal assay

The antibacterial and antifungal activity of all the synthesized ILs were tested according to the standard disk diffusion method developed by Mueller-Hinton agar and Sabouraud agar, respectively [20]. Initially, the Muller Hinton Agar medium was poured into the petri dish. On the other hand,

the inoculum was prepared by transferring a loop full of cells from the stock cultures to the nutrient broth (peptic digest of animal tissue 5 g, beef extract 1.5 g, yeast extract 1.5 g and sodium chloride 5 g were dissolved in 1 litter of water. Which was taken into Erlenmeyer flask and incubated to bacterial culture at 37 °C for 72 h for bacterial culture). After the medium was solidified, the inoculums were spread on the solid plates with sterile swab roistered with the bacterial suspension. 100 µL of each IL in DMSO were loaded on the agar plate. The plates were then incubated at 37 °C for 24 h, and the antimicrobial activity of ILs was ascertained by directly measuring the zone diameter of the growth inhibition (in mm) after the incubation period. The tested bacterial strains were *Bacillus subtilis* MTCC 5981, *Staphylococcus aureus* MTCC 96 (Gram-positive), and *Klebsiella pneumoniae* MTCC 3384, *Pseudomonas aeruginosa* MTCC 424, *Escherichia coli* MTCC 443 (Gram-negative). And the fungal strains *Candida albicans* MTCC 227, *Aspergillus niger* MTCC 282 were used. Amikacin was used as the standard for antibacterial studies and Nystatin was used for antifungal studies.

2.7 Antioxidant Assay

Total anti-oxidant activity by phosphomolybdenum method

The total antioxidant power of all the DILS were evaluated by the phosphomolybdenum method [21]. In brief, the DILs 1mg/mL was allowed to mixed with 3.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Then, this test samples were incubated at 95 °C for 90 min and then cool to room temperature. Further, the absorbance of green phosphomolybdenum complex was measured at 695 nm against a blank. A typical blank solution consists of methanol (0.3 mL) in the place of DIL and 3.0 mL of same reagent, and it was also incubated under the same condition. The appropriate solution of ascorbic acid was used as a reference. Hence, the total antioxidant activity is expressed as the number of gram equivalent of ascorbic acid.

Determination of nitric oxide (NO) radical scavenging efficacy

The ionic liquids are excellent nitric oxide scavengers, which was realized as substantial medicine for NO intervened disorders [22,23]. Practically NO secures very less half-life period, on the other hand it was spontaneously oxidized to nitrite and nitrate. In aqueous solution at physiological pH, Sodium nitroprusside spontaneously engender NO, which fraternize with oxygen to provoke nitrite ions. This nitrite content can be estimated by the Griess reagent (prepared by mixing equal volume of 1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) [24].

In this effort, NO scavenging capacity of all the DILs were determined with Griess reagent assay by making use of UV-vis spectrophotometer. In account of this, the electronic absorption spectra were recorded in absence as well as in presence of DILs. Usually, scavengers of nitric oxide compete with oxygen leading to the curtail generation of NO. Briefly, the fixed concentrations of DILs in methanol (100-400 µg/ml) and the solution of sodium nitroprusside (10 mM) in standard phosphate buffer saline (PBS pH 7.4) was mixed thoroughly in a glass tube. Then the mixture was incubated by visible polychromatic light at 30 °C for 3 h. In the meantime, NO radical was produced, simultaneously it was intermingled with oxygen to provoke the nitrite ion. On the other hand, control experiment was carried out without the test compounds but with equivalent amount of buffer was conducted in an identical manner. After 3 h, the nitrate ion concentration was assayed by diluting half quantity of incubation mixture with proportionate volume of the Griess reagent. The diazotization of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylenediamine-dihydrochloride (NED) was engendered. Hence the absorbance of pink chromophore was measured at 546 nm against a blank (methanol) [25]. Each experiment was executed in triplicate. Same procedure was done with ascorbic acid which was standard in comparison to methanolic solution of DILs. The percentage of inhibition was determined by using the formula

% inhibition = (O.D of control - O.D. of Test/O.D. of control) X 100

2.8 Cytotoxicity Evaluation

Experimental procedure for SRB assay

The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs [26]. The experimental drugs were initially solubilized in dimethyl sulfoxide at 100 mg/ml and diluted to 1mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate (1mg/ml) was thawed and diluted to 100, 200, 400 and 800 µg/ml with complete medium containing test article. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations 10, 20, 40 and 80 µg/ml.

After compound addition, plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4 °C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength [27].

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100. Using the six absorbance measurements [time zero (Tz), control growth (C),

and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as:

Percentage growth inhibition = [Ti/C] x 100%

3 Results and Discussion

3.1 Characterisation of ILs

Dicationic ILs were successfully synthesized and characterized by FT-IR, ^{1}H, ^{13}C, ^{19}F-NMR and HRMS spectral studies. The corresponding data was analyzed and discussed below. The FT-IR spectrum of all the dicationic ILs were studies in the range of 400-4000 cm^{-1} and was shown in the figure S4, S9, S14, S19 and S24. The IR spectrum of IL-1 to IL-5 showed sharp peaks around 3100 to 2950 cm^{-1} due to the C-H stretching modes for aromatic and aliphatic systems. A peak observed at 1635 cm^{-1} in all ILs on account of C-N stretching vibrations. The quarternised nitrogen of the imidazole ring($C\text{-}N^{+}$) appears vibrational peaks around 1150 cm^{-1}.

H^{1} NMR and C^{13} NMR spectrum of all the synthesized ILs were analyzed and displayed in the figure S1 to S25. In DIL1 & DIL2, the H^{1} NMR resonance for the terminal methyl proton was observed as singlet at 3.827 and 3.845 ppm respectively. A sharp singlet at 8.686 and 9.066 ppm in both the DILs for the acidic proton in imidazole ring was shifted from lower to higher region. The resonance frequency for N-CH_2 proton in DIL1 was showed as doublet at 4.164-4.192 ppm, whereas after the anion exchange it appears as singlet at 4.213 ppm. In the case of DIL2, before anion exchange, the multiplet signal was observed for N-CH_2 proton at 4.510-4.612 ppm. whereas, after the anion exchange the -value has slightly reduced and it appeared as multiplet at 4.485-4.557 ppm. Before the anion exchange, the acidic proton presents in the benzimidazolium ring (N-CH=N), and it exhibited as sharp singlet at 10.032 ppm but after the anion exchange the -value tremendously reduced to 9.723 ppm. It evidence that, complete anion exchange has occur in the DIL2 system with tetrafluoroborate anion.

In DIL3, before the anion exchange with tetrafluoroborate the resonance frequency of N-CH_2 proton was showed as triplet at 4.715-4.751 ppm, whereas, after the anion exchange it appears as singlet at 4.654 ppm. In DIL4, before anion exchange the peak observed for N-CH_2 proton was given as quartet at 4.766-4.82 ppm. But after the anion exchange the -value has slightly reduced and appeared as quartet at 4.733-4.788 ppm. Before and after the anion exchange, there is no much difference in the terminal methyl proton of ethyl viologen and it exhibits as a triplet at 1.587-1.624 ppm. In DIL5, before anion exchange, the terminal methyl proton reveals that singlet at 1.300 ppm. and N-CH_2 proton exhibited at 3.373 ppm. Whereas after the anion exchange, the -value of terminal methyl proton has reduced to 1.260-1.297 ppm. And N-CH_2 proton also shifted to shielded region as a triplet at 3.539-3.575 ppm. From the C^{13} NMR spectra, the resonance observed for aliphatic carbon ranging from 7.77 -60.42 ppm in all the ILs. Further, the aromatic carbon shows the resonate frequency at 122.10-149.05 ppm for all the ILs. High resolution mass spectra of the synthesized ILs were recorded and confirmed the molecular mass of the compounds.

3.2 Antimicrobial and antifungal assay

The influence of structural elements like imidazolium, benzimidazolium, pyridinium, bipyridinium, and DABCO cations on the toxicity of ILs was studied using tetrafluoroborate as a counter anion. Hence, the antimicrobial and antifungal activity of dicationic ILs (DIL-1, DIL-2, DIL-3, DIL-4 and DIL-5) were tested against the six selected microorganisms. And the obtained results were displayed in (Table 1) The reported zone of inhibition values emphasizes the activity of DILs against bacteria and fungi. And it is observed that, the results were fundamentally relies on their structural features rather than molecular weight or the bulkiness of the DILs. Usually, the aromatic DILs were showing higher antimicrobial and antifungal activity than compare to aliphatic DILs. It is more resemblance to our study, viologen based tetrafluoroborate IL (DIL-4) exhibits much better results than compared to the standard amikacin. This is not only the extended conjugation of viologen but also it has a tendency to form a pi-pi interaction with other organic molecules. Imidazolium and pyridinium based DILs also found higher growth inhibiting capacity against Gram-negative E. coli bacteria than compared to the standard amikacin. On the other hand, Imidazolium and pyridinium DILs were shown a moderate growth inhibiting capacity against Klebsiella pneumoniae. On the contrary, benzimidazolium DILs shown a very small growth inhibiting capacity against the microorganisms. This is due to the smaller cationic size of the imidazolium and pyridinium moiety having the high mobility. Hence it will easily penetrate through the surface than compared to the benzimidazolium moiety. Except viologen based IL (DIL-4), other DILs did not show any significant activities towards Staphylococcus aureus and Aspergillus niger microbes. Noticeably, E. coli was strongly receptive to be demolished than S. aureus when employed with dicationic ILs. Relevant report [28] exposes that the discriminancy in the activity of gram positive and gram negative microorganisms is presumably due to the nature of the cell walls and compatibility of microbes with the DILs.

Table 1. *Antimicrobial and antifungal activity of DILs*

Microorganism	Zone of inhibition (mm)					
	DIL-1	DIL-2	DIL-3	DIL-4	DIL-5	Amikacin/ Nystain
B. subtilis	13	12	14	34	20	21
S. aureus	---	---	---	19	---	22
K. pneumonia	26	13	29	37	23	31
P. aeruginosa	---	18	---	39	---	22
E. coli	25	10	23	23	---	22
C. albicans	22	11	16	19	18	26

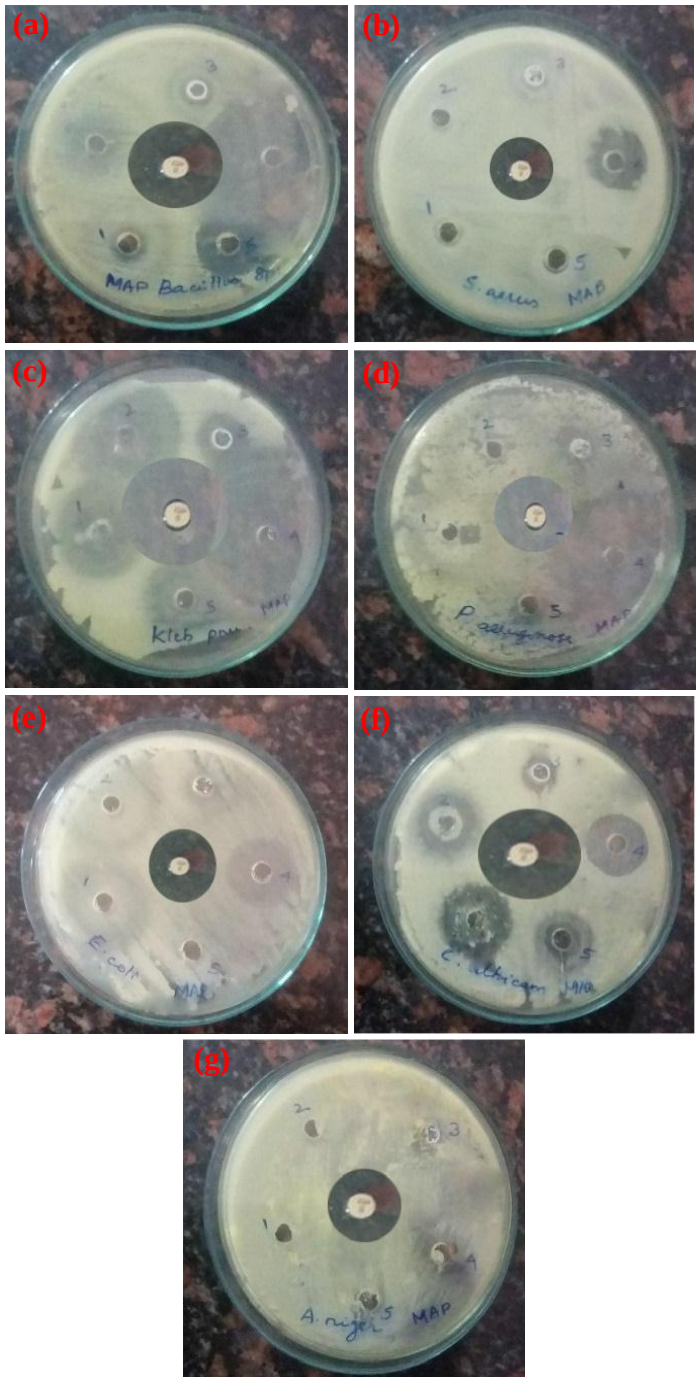

Figure 2. *Zone of inhabitation images of antimicrobial and antifungal activity*

In general, the ILs which possess tetrafluoroborate as counter anion are reasonably more toxic than compared to the other anion, because the fluorine ions from tetrafluoroborate rapidly undergo hydrolysis to form the fluorine containing by-products which inhibit the activity of Na^+/K^+-ATPase enzyme [29]. For the cell growth is concern, the sodium-potassium adenosine triphosphatase enzyme is responsible for the pumping of Na^+ out of the cells simultaneously, K^+ into the cell. When, Na+/ K+-ATP enzyme fails to maintain the concentration gradient of sodium and potassium ions, it leads to depolarization followed by cell death. Since all the DILs are carrying BF_4 as counter anion basically, these molecules having a high antimicrobial and antifungal activity. Especially, the Gram negative organisms are more sensitive to dicationic ILs than Gram positive organisms and fungi. Thus, potentially explaining, the outer cell wall of the Gram positive organisms may hamper absorption of such alkylated imidazolium, benzimidazolium, pyrrolidinium and DABCO based DILs. On the other hand, the gram negative bacterial outer cell membrane absorbs these DILs very easily and transport these ILs into inner cytoplasmic membrane.

3.3 Antioxidant activity of DILs

Since these DILs were exhibit reasonable antimicrobial and antifungal activity we want to study other potential aspects, such as antioxidant and anticancer activity. Hence, we made an attempt to elaborate our research work, to find the radical scavenging ability of the DILs. Further, the reducing power of all the DILs were effectively examined by phosphomolybdenum assay. The various cation turnability like imidazole, benzimidazolium, pyridinium, viologen and DABCO based cation accompanied with tetrafluoroborate anion based DILs were showed excellent reducing activity. This is because the reduction of Mo(VI) to Mo(V) by the DIL and it leads to subsequent formation of a green phosphomolybdenum complex Mo(V) at acidic pH. All the DILs were found to have high reducing power on the range of 0.012 to 0.082 at 10 µg/ml.

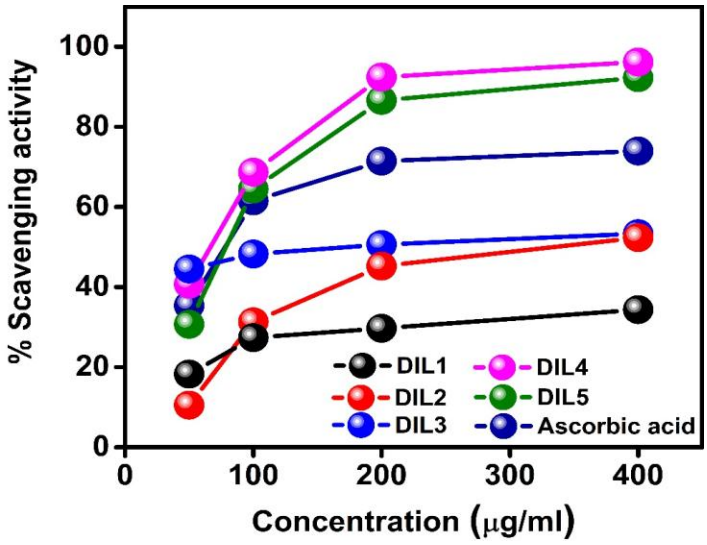

Figure 3. *In vitro free radical scavenging effect of DILs by nitric oxide scavenging method*

In addition to that, we have found that, nitric oxide radical scavenging activity by using Sodium nitroprusside. Nitric oxide (NO) is found to be a precious chemical mediator produced by endothelial cells, macrophages, neurons, etc [30,31] and to monitor several physiological phenomena such as immune response [32], blood pressure [33], and neural communication [34]. However, excess concentration of NO are directly toxic to tissues and it induce several diseases like, vascular damage and is associated with inflammatory diseases including atherosclerosis [35], hypertension [36] and other ailments [37]. This harmfulness effect on human body is

heightened on reaction with superoxide radical to produce a highly reactive peroxynitrite anion (ONOO-), which prevents nitrite formation. Furthermore, the protonation of peroxynitrite (ONOO-) generate a highly poisonous and extremely reactive compound peroxynitrous acid (ONOOH) [38,39]. On the other hand, nitric oxide is categorized as an excellent oxidizing agent, electrophile and free radical. In view of its unpaired electron its exhibition as substantial reactivity with proteins and other adjacent free radicals. Nitric oxide (NO) is produced from amino acid L-arginine by vascular endothelial cells, and phagocytes [40]. It is widely documented that, the origin of the today's diseases is because of the oxidative stress that consequences from an inequality between the production of ROS/RNS and their neutralization when endogenous antioxidant mechanisms are unable to quench the free radicals. Typically, the free radicals are scavenged by synthetic antioxidants like metal complexes, nanocomposites, other phenolic rich organic compounds, and even chiral molecules. On account of their antagonistic side effects causes carcinogenicity. Hence the researches search for powerful and eco-friendly antioxidants has become crucial to the society. DILs are believed to be safer, potential, eco-friendly and bioactive material for NO radical scavenger. Consequently, researchers have paid more effort to discover nontoxic antioxidants that may act as powerful inhibitors of NO radicals. In the present study, NO radical scavenging activities of all the DILs were evaluated by NO scavenging assay. The percentage scavenging activity and IC_{50} values of all the DILs were calculated and displayed in (S.I Table 1) The DILs exhibited antioxidant activity through competing with oxygen to scavenge for the nitrite radical which was generated from SNP at physiological pH in an aqueous environment. Viologen and DABCO based DILs with redox properties plays an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. A higher NO radical scavenging activity is associated with a lower IC_{50} value thus the results presented here indicates a higher NO radical scavenging activity of the viologen (IC_{50} = 11.19) and DABCO (IC_{50} = 64.67) based DILs exhibited significant antioxidant property, when compared with the natural antioxidant ascorbic acid standard (IC_{50} = 68.04). Other DILs (DIL1, DIL2, DIL3) were possess very high IC_{50} value than compare to the reference ascorbic acid. Hence these DILs doesn't have any antioxidant activity. These results strongly support that the DIL-4 and DIL-5 are more capable of inhibiting nitric oxide radicals and prescribed as drug in the indigenous system in treatment of various diseases. The current investigation proof that, all the DILs were exhibited a significant dose dependent inhibition of NO. The antioxidant activity increased with an increase in concentration of the DILs reaching a plateau. Further, increasing the concentration of the DILs (more than 200 μg/ml) did not affect much on the results. Hence the nitrite radical scavenging activity not increased after 200 μg/ml. In addition to that, DIL-4 and DIL-5 were shown excellent NO radical scavenging activity than compare to the natural antioxidant ascorbic acid. The viologen and DABCO based DILs were most potent as it removed the nitrite radical even at lower concentration as well as higher concentration as compared to the other DILs. The maximum free radical scavenging activity and potency were interpolated, especially DIL-4 which shown highest NO scavenging activity at 200 μg/ml shown in figure 3. NO has been found to be directly scavenged by DILs. The presence of viologen and DABCO based DILs explain why particularly these DILs were more potent at quenching the NO radical than the other DILs. DIL-4 and DIL-5 suppress NO production and also scavenge NO in an acellular system using sodium nitroprusside under physiological conditions at a micromolar range. This might be due to easy generation of free radical and highly conjugated viologen system (DIL4) induced the quenching of NO radicals. DIL-4 upsurges the capacity to stabilize the unpaired electrons and thereby scavenge the free radicals, and it leads to decrease the nitrite concentration in the assay medium. The antioxidant results were used to determine potency and maximum percentage scavenging of the DILs since they exhibited the greatest scavenging activity as compared to the natural antioxidant ascorbic acid.

3.4 Cytotoxicity study

The DILs were evaluated as anticancer drug through in vitro analysis by using three cell lines, HeLa (human cervical cancer cell line), HT 29 (human colon carcinoma cell line) and MCF-7 (hormone-dependent breast carcinoma cell line), in which adriamycin was used as a positive control in the present investigation. The DILs were analyzed at four concentration levels 10, 20, 40 and 80 μg/ml Vs percentage of growth inhibition. The activity of the DILs was calculated in terms of GI_{50} values. When the concentration of DIL4 increases, there was a decrease in percentage growth inhibition.

Table 2. *Cytostatic (GI_{50}) data of DILs against human cancer cell lines.*

DILs	GI_{50} (μg/ml)*		
	HeLa	HT-29	MCF-7
DIL 1	>80	>80	>80
DIL 2	>80	>80	>80
DIL 3	>80	>80	>80
DIL 4	<10	22	38.7
DIL 5	>80	>80	16.7
Adriamycin	<10	<10	<10

*GI_{50} is the concentration of DILs causing 50% inhibition of cell growth.

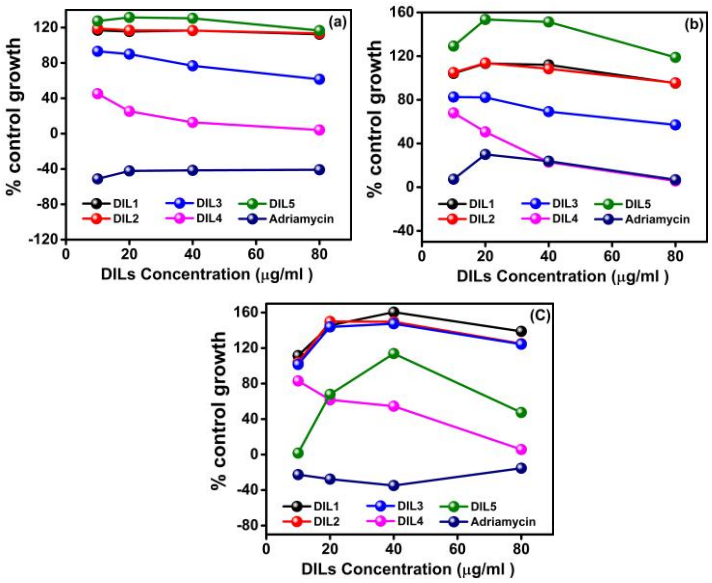

Figure 4. *Dose response curves of DILs with control on HeLa, HT-29 and MCF-7 cell lines.*

Accordingly, at maximum concentration (80 µg/ml) DIL4 exhibited excellent cytotoxicity against HeLa cell line with their potency in terms of GI_{50} as <10 µg/ml. Whereas, in MCF-7, DIL4 exhibits the GI_{50} value 38.7 µg/ml, and for HT-29 gives the GI_{50} value 22.0 µg/ml (Table 2). In addition to that, DIL5 emphasize moderate activity against HT-29 and it reaches the GI_{50} value 16.7 µg/ml. This GI_{50} values suggested that, these DIL concentrations are required for 50% cell inhibition in the above mentioned cell lines.

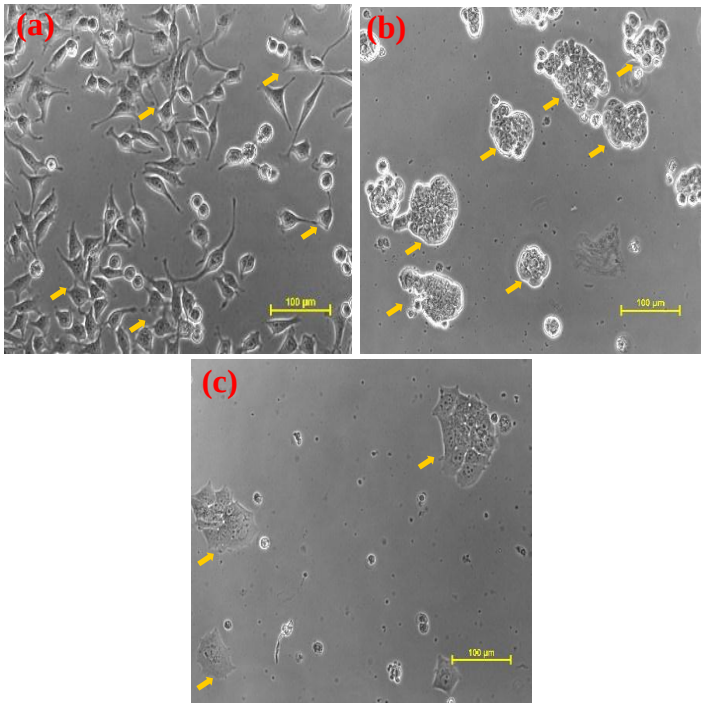

Figure 5. *Invitro cytotoxicity images of untreated cells* ***(a)*** *HeLa,* ***(b)*** *HT-29,* ***(c)*** *MCF-7. The yellow arrow indicates untreated cancer cells.*

The dose-response curve for the cytotoxicity screening of DILs was also drawn by varying concentration with percentage of control growth displayed graphically in figure 4. The dose response curve displayed that, increasing the DILs concentration with constant intervals, the percentage control

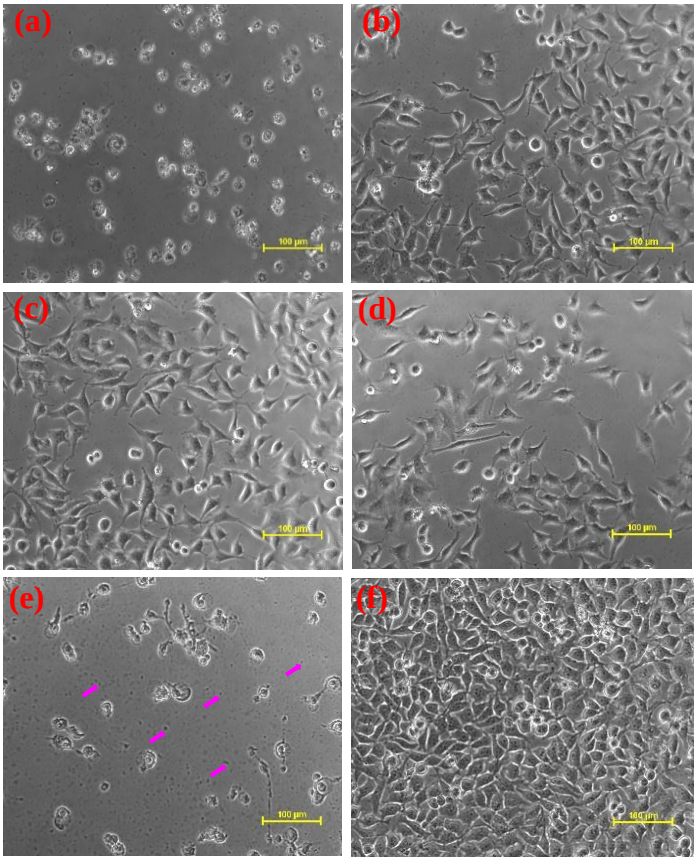

Figure 6. *Invitro cytotoxicity images against HeLa cell line for* ***(a)*** *positive control,* ***(b)*** *DIL1,* ***(c)*** *DIL2,* ***(d)*** *DIL3,* ***(e)*** *DIL4,* ***(f)*** *DIL5 at 80 µg/ml. The pink arrow indicates destroyed HeLa cells.*

growth of all the DILs decreases gradually. In the case of HeLa cell line, DIL4 only attained 50% of control growth even at minimum concentration (10 µg/ml). while, no other DILs were reached 50% of inhibition even at maximum concentration (80 µg/ml) figure 6. Similarly, in the cell line HT-29, the DIL4 alone attained 50% of inhibition at lowest concentration (20 µg/ml). But all the other DILs, could not reached 50% of inhibition even at maximum concentration (80 µg/ml) figure 7. Further, in the case of MCF-7 cell line DIL5 shows below 50% inhibition at minimum concentration (10 µg/ml). But when we increase the concentration the percentage of control growth reached more that 50%. Unexpectedly, DIL4 and DIL5 attained 50% inhibition at maximum concentration (80 µg/ml) figure 8. Except DIL4 and DIL5 no other DILs reached below 50% of inhibition even at higher concentrations. On the other hand, comparing the percentage control growth of DILs with the positive control adriamycin, the DIL4 shows remarkable activity against the cell line HT-29 than the control. However, other DILs were not shown better activity than control for all

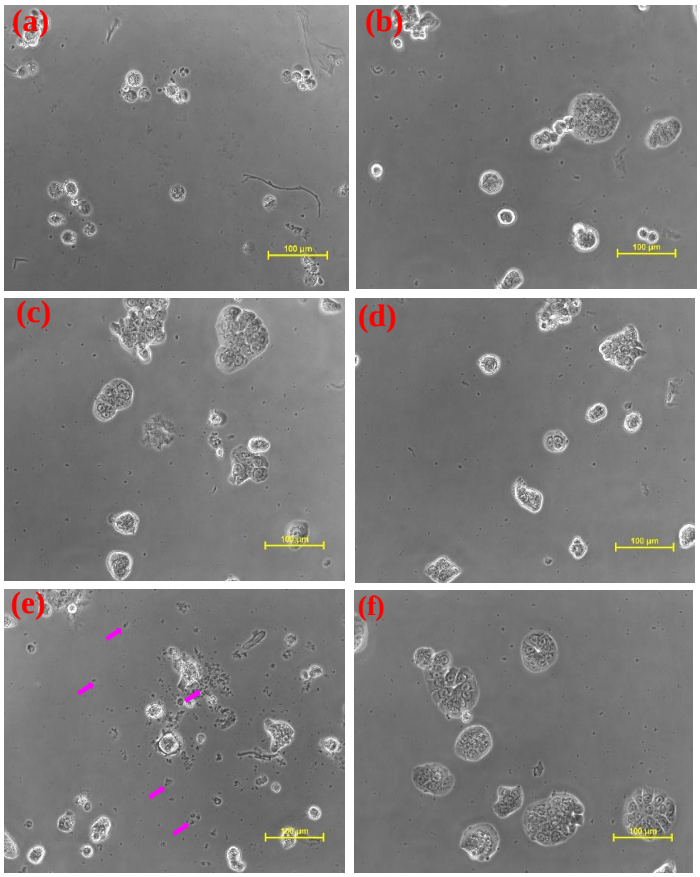

Figure 7. *Invitro cytotoxicity images against HT-29 cell line for* ***(a)*** *positive control,* ***(b)*** *DIL1,* ***(c)*** *DIL2,* ***(d)*** *DIL3,* ***(e)*** *DIL4,* ***(f)*** *DIL5 at 80 µg/ml. The pink arrow indicates destroyed HT-29 cells.*

the three cell lines. We proclaimed that, after the incubation period viologen based DIL breaks up all the three cancer cells into small pieces as fragments and diminish in their size figure 6e. Hence, DIL4 exposed outstanding activity against HeLa cell line, at the meantime, it shows moderate cytotoxicity effect towards HT-29 as well as MCF-7 cancer cell lines. These drastic changes in the cell lines were due to the scavenging of free radicals which leads to eradication of cell membranes in treated cells. The other DILs imidazolium (DIL1), benzimidazolium (DIL2) and pyridinium (DIL3) were found to be inactive in Hela, HT-29 and MCF-7 cell lines. DIL5 expressed limited activity in MCF-7 cell line but no activity with Hela, HT-29 cell lines. This is because these DILs cannot be able to destroy the cancer cells, and it can be clearly visible in the optical microscopic image figure 6, 7 and 8. The very high anticancer activity of viologen based DIL was not only the cellular oxidative damage, but also scavenging of cancer-causing free radicals. Hence, the DIL4 was used as a drug in chemotherapy depending upon the concentration. The reported data suggest that, except DIL4 all the other DILs exhibit no better activity against HeLa, HT-29 and MCF-7 cell lines. since these DILs were may not be prescribed as anticancer drug, but it can be used as a antimicrobial and antifungal agents.

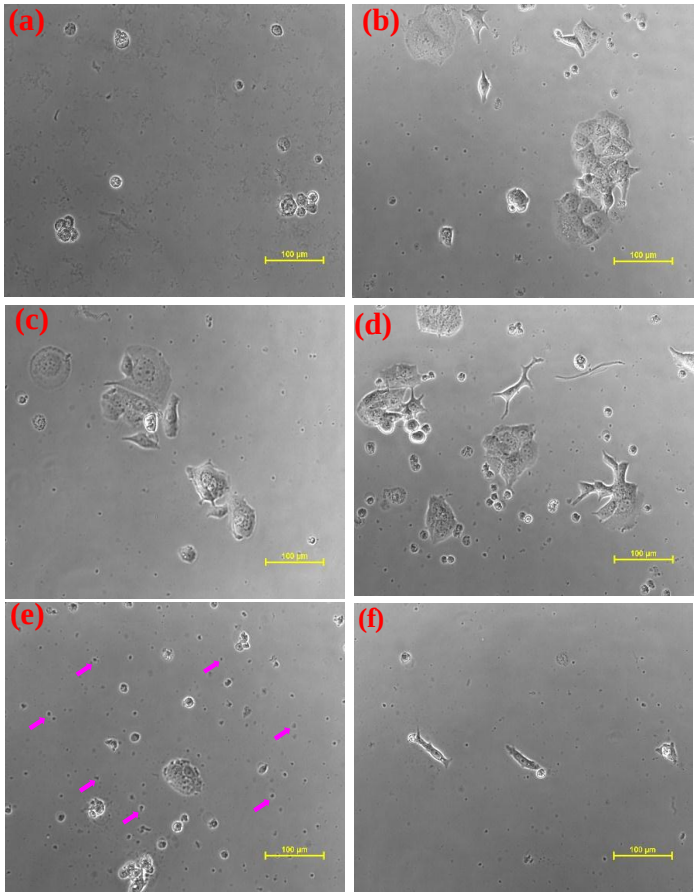

Figure 8. *Invitro cytotoxicity images against MCF-7 cell line for* ***(a)*** *positive control,* ***(b)*** *DIL1,* ***(c)*** *DIL2,* ***(d)*** *DIL3,* ***(e)*** *DIL4,* ***(f)*** *DIL5 at 80 µg/ml. The pink arrow indicates destroyed MCF-7 cells.*

4 CONCLUSION

A series of dicationic imidazolium, benzimidazolium, pyridinium, viologen, and DABCO were successfully synthesized and characterized by using 1H, 13C, 19F NMR, FTIR and Mass spectroscopic studies. Furthermore, the synthesized DILs were subjected to antimicrobial, antifungal, antioxidant and cytotoxicity studies. The antimicrobial and antifungal data revealed that all the DILs were exhibit notable activity as comparing to the standard amikacin and nystatin. In addition to that, viologen (DIL4) and DABCO (DIL5) based DILs showed significant antioxidant activity as compared to the natural antioxidant ascorbic acid. Among the five DILs, viologen based DIL having excellent cytotoxicity against the HeLa cancer cell line and it showed moderate activity against HT-29, MCF-7 cell lines. These results reveal that, viologen based DIL (DIL4) behave as excellent bioactive materials than compare to other DILs. The dynamic biological property of viologen based DIL can be scrutinized as anticancer, antioxidant and antimicrobial representatives in drug discovery. On the other hand, other DILs many not be used as anticancer drug but they are prescribed as an excellent drug for antimicrobial and antifungal agents.

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