Synthesis, Characterization and Biological Evaluation of Bis-benzimidazolium Salts and Their Silver(I)-N-Heterocyclic Carbene Complexes

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Abstract

Azolium salts are stable sources of *N*-heterocyclic carbenes (NHCs), an emerging class of ligands. Due to prominent properties, they have versatile applications. In the current study, new bis-benzimidazolium salts and their silver complexes were synthesized and confirmed through spectroscopic analysis. Compounds were characterized using FT-IR, ^1H NMR, and ^{13}C NMR spectroscopies. Biological potentials of these compounds were investigated for antibacterial activity against both the gram-positive (*Staphylococcus Aureus*) and gram-negative (*Escherichia Coli*) strains. The salts L1 and L2 showed zone inhibition of 17 ± 1 and 13.33 ± 0.58 mm for *S. Aureus* and 8.83 ± 0.76 and 8.83 ± 0.58 mm for *E. Coli*, respectively. The respective silver complexes C1 and C2 showed better zone of inhibitions for both the strains, i.e., 18.67 ± 0.58 and 19.83 ± 0.29 mm for *S. Aureus* and 12.5 ± 0.5 and 14.33 ± 0.58 mm for *E. Coli*, respectively. The salts showed a good trend in scavenging concerning the increasing concentration and presented the IC_{50} values in the range of 3.51 and 15.36 $\mu\text{g/mL}$, respectively, which showed that these compounds are good antioxidant agents. The Hemolytic potential of compounds were checked and L2 was found least toxic with 7.37 ± 0.11 value.

1. Introduction

Since the synthesis of first *N*-heterocyclic carbene by Öfele and Wanzlick in 1968 [1, 2] and the isolation of first stable NHC by Arduengo in 1991 [3], the synthesis of new *N*-heterocyclic carbenes and their complexes gained a great research interest. It is an attractive research field where a range of new metal-NHC complexes involving main group elements and transition metals have been synthesized and applied in different areas of interest [4–7]. NHCs are similar to phosphine ligands in how they coordinate with metals. Still, these carbenes are more attractive toward the metals than the phosphines, perhaps due to their more accessible synthetic methods [8, 9].

In the past decade, benzimidazole derived synthesis and applications have been considered more repeatedly due to their activities as pharmacological agents such as antibacterial, antifungal, antiulcer, and anticancer properties [10–12]. Today, benzimidazole and its derivatives are being used in the treatment of different fungal and bacterial infections as potent antimicrobial drugs [13]. Due to the broad range of pharmacological applications of benzimidazole derivatives, a lot of research is currently in progress to develop more effective and less harmful drugs of these types of *N*-heterocyclic compounds for their clinical uses [14, 15].

Among all the transition metals, coinage metals (silver, copper, and gold) are widely studied due to their unique structural and diverse applications [16, 17]. Silver is not found in human body by birth, but it has potentially fatal behaviour against microbes and at the same time it shows less toxicity to humans. Some of these silver salts also exhibit antimicrobial as well as *in vitro* antitumor activity. The challenge with these silver-based drugs is that they quickly release their activity by losing Ag^+ ions. This limitation can be overcome by using NHCs as ligands with silver as they bind the silver ions strongly due to a good

sigma donating and weak pi-accepting properties [18, 19]. Finally, a sustained release of silver ions at the target site has been observed [20]

Silver-NHC complexes have become very attractive in organometallic chemistry because of their ease in synthesis by a single-step reaction of benzimidazolium salts with Ag_2O [13, 21, 22]. The other reason of interest in Ag-NHC complexes is that they can be used as a reagent in carbene transfer reactions to synthesize other transition metal complexes like Ni, Pt, Au, Cu, Ir, Rh, and Ru NHC complexes, an easier route for the synthesis of these metal carbene complexes [24, 25]. With the first report of silver-NHCs as an antimicrobial agent by Youngs *et al.* in 2004, the silver carbene complexes have interestingly shown biologically active both as anticancer and antimicrobial drugs [26]. The bis-benzimidazolium salts have shown the attractive enhanced anticancer potential when attached with silver to form Ag-NHC complexes [27]. So, in the present work, we have synthesized two aryl-substituted bis-benzimidazolium salts and their Ag(I) complexes and reported their antibacterial, antioxidant and hemolytic studies.

2. Result And Discussion

2.1. Synthesis

The synthesis of bis-benzimidazolium salts was carried out in two steps following the literature methods [28–31]. In the first step, N-alkylated benzimidazole was synthesized by reacting 2 equivalents of benzimidazole with 1 equivalent of 1,4-dibromobutane in the presence of 1.5 equivalence of potassium hydroxide. This reaction was performed in DMSO. The white-colored powdered solid product was obtained. This reaction proceeds by the removal of acidic hydrogen present on nitrogen and then the nucleophile attack on alkyl halide, removing bromide ions. In the second step, 1 equivalent of N-alkylated benzimidazole preligand was dissolved in 1,4-Dioxane and refluxed with 2 equivalents of second alkyl halide. At a high temperature around 100 °C, the lone pair of nitrogen attacked the electrophilic center of alkyl halide and bound with it forming benzimidazolium salts L1 and L2. Salt L1 was obtained in solid white amorphous form. Salt L2 was obtained as a viscous fluid that was then converted into solid by reaction with KPF_6 through metathesis reaction. Melting points, solubility, and complete spectral characterization of synthesized salts were performed.

Binuclear silver NHC complexes were synthesized adopting the reported methods [29, 32–34]. The complexes were formed by *in vitro* generation of carbenes by removal of acidic hydrogen on carbene carbon by reacting it with silver oxide at room temperature. The synthesis was carried out in the dark as the silver-NHC complex with bromide counter ion broke down in light, so the complexes were converted in hexafluorophosphate counter ion by metathesis mechanism. Mono equivalent bis-benzimidazolium salts dissolved in methanol were stirred with Ag_2O in the dark at room temperature. KPF_6 was reacted with silver complexes and replaced the counter ion through the metathesis reaction pathway. All the complexes were obtained as white solid material. The solubility, melting points, and complete spectral analysis were performed for all complexes prior to their biological applications.

2.2. U.V./Visible Spectral Analysis

The synthesized bis-benzimidazolium salts and Ag-NHC complexes were characterized using absorption spectroscopy (U.V./Vis spectroscopy). A prominent alteration in absorption band was observed in metal-NHC complexes due to the trepidation of silver center to form Ag-NHC complexes. This absorption shift is linked with metal center bands and metal-ligand charge-transfer electronic transitions [35]. The silver(I)-NHC complexes C1 and C2 have shown intense absorption bands in the 270-300 nm range (290 nm for C1 and 285 nm for C2), but their corresponding salts L1 and L2 showed absorption at 271 nm and 257 nm respectively in the range of 240-280 nm as shown in **figure S1**. Clear difference in the peaks of absorption spectra from salts to the complexes show that there is any chemical change in the structure and this is the initial indication of synthesis of complexes from salts.

2.3. FT-IR Spectral Analysis

Spectral features of synthesized benzimidazolium salts and metal complexes can describe the successful synthesis of compounds by changing the characteristic peaks of salts and complexes [36]. FT-IR spectroscopy of synthesized salts and silver complexes was observed and manipulated for the confirmation of the successful synthesis. Synthesized N-alkylated precursors have an intense characteristic peak pattern between $1500\text{--}800\text{ cm}^{-1}$ which diminished in bis-benzimidazolium salts. For ligands (L1 & L2), sharp and strong stretching vibrations appear at $3402\text{--}3424\text{ cm}^{-1}$ due to the benzimidazolium ring's tertiary nitrogen atom (benzimidazolium ring ($\text{C}_{\text{aliph}}\text{-N}_{\text{benzm}}$)). An intense pattern of peaks was observed in the fingerprint region, i.e., ($750\text{ to }650\text{ cm}^{-1}$) in the spectra of all the benzimidazolium salts and silver complexes due to $=\text{C-H}$ bending vibrations [31]. The appearance of powerful peaks at $2950\text{--}2800\text{ cm}^{-1}$ in spectra of bis-benzimidazolium salts and the absence of these peaks in silver complexes were key to the successful metalation of the salts. The four-finger pattern [13, 29, 34] for the synthesized silver complexes and characteristic peaks of C=N stretching of N-alkylated and benzimidazolium salts in the range of $1600\text{--}1300\text{ cm}^{-1}$ which weekend in complexes C1 & C2 provides a strong indication for the synthesis of Ag-NHCs as shown in **figure S2** [37].

2.4. NMR Spectroscopy

^1H and ^{13}C spectra of L1, L2, C1, and C2 shown in **figure S3** were recorded in the DMSO as a solvent. Salts/ligands (L1 and L2) have clear peaks of an acidic proton (NCHN) in the $8.0\text{--}10.4\text{ }\delta\text{ ppm}$ range. Although, in the silver complexes (C1 and C2), this acidic proton is replaced by silver metal [34, 38]. This is a clear indication of the successful synthesis of silver complexes. ^{13}C NMR spectra of salts (L1 and L2) showed the peaks of acidic proton in the range of $138.3\text{ }\delta\text{ ppm}$ [39, 40]. This information gives us a clear confirmation of the successful synthesis of silver complexes and their respective salts.

2.5. MIC Value

All the compounds were tested against gram-positive strain *Staphylococcus aureus* and gram-negative bacterial strain *Escherichia coli*. The standard solutions of compounds were made of concentration 10

mg/mL in DMSO. DMSO has already been reported as inactive against all the gram-positive and gram-negative bacterial strains [41–43]. Ciprofloxacin was used as a standard drug against bacterial strains at the same concentration. All the compounds were found active against test strains, and the results of zone inhibition were presented as Mean $\pm$ S.D. in mm. Benzimidazolium salts have already been investigated much for their biological applications, but the insertion of silver metal enhanced the antibacterial potential of complexes [44]. Standard drug ciprofloxacin showed 34.67 ± 1.15 mm for *S. Aureus* and 35 ± 1 mm for *E. Coli*. The test compounds L1 and L2 showed zone inhibition of 17 ± 1 and 13.33 ± 0.58 mm for *S. Aureus* and 8.83 ± 0.76 and 8.83 ± 0.58 mm for *E. Coli* which is good activity against applied strains. The respective silver complexes C1 and C2 showed better zone inhibition for both the strains, i.e., 18.67 ± 0.58 and 19.83 ± 0.29 mm for *S. Aureus* and 12.5 ± 0.5 and 14.33 ± 0.58 mm for *E. Coli* that revealed that silver center contributed to the inhibition of growth of bacteria much better. The overall results showed the good potential of silver complexes C1 and C2 compared to their bis-benzimidazolium salts L1 and L2. They suggested that the bi-silver metal center played a vital role in the biological activity of azonium salts [31]. The investigation to find the significant antibacterial sustainability of silver NHC complexes C1 and C2 in terms of MIC (minimum inhibitory concentration of drug). The MIC value for salts L1 and L2 against *S. aureus* is $12.5 \mu\text{g/mL}$, and for *E. Coli* is 12.5 and $25 \mu\text{g/mL}$ for both salts. The respective Ag(I)-NHC complexes C1 and C2 have much better susceptibility to inhibit the growth of bacteria at low concentration i.e., 0.765 and $0.382 \mu\text{g/mL}$ for *S. Aureus* and 3.06 and $1.53 \mu\text{g/mL}$ for *E. Coli* which revealed the activity of metal center to inhibit the growth of bacteria even at low concentration as shown in Figure 1.

2.6. Hemolytic Assay

Hemolytic activity of newly synthesized bis-benzimidazolium salts L1 and L2 and silver complexes C1 and C2 (see **S4**) were evaluated by a spectroscopic method in terms of destruction in human RBCs. Triton X was castoff as a positive control, and the sample % hemolysis was compared with the hemolytic ability of standard positive control taken as 100%. All the salts and respective silver complexes were found less toxic to RBCs in the range of 6.73 – 20.46%. Bis-benzimidazolium salt L1 was found least toxic with $6.73 \pm 0.36\%$ hemolysis, and it's the Ag(I) complex C1 was revealed as most toxic in series with % hemolysis of $20.46 \pm 0.23\%$. The toxicity of silver complexes C1 and C2 was comparatively greater than the benzimidazolium salts, which showed the toxic nature of silver ions to human RBCs but had much lesser value as compared to Triton X, which concluded that the synthesized drugs are safer to humans and better to proceed for clinical trials as shown in figure 2 [45, 46]

2.7. Antioxidant Potential

There is a continuous release of free radicals in biological systems due to different chemical or physical reactions, which may cause drastic toxic action on a normal system using physiological actions. The effect of these free radicals can be overcome by trapping them through an antioxidant agent present in the biological system [47]. Synthesized benzimidazolium salts L1 and L2 and their silver complexes C1 and C2 (see **S5**) were investigated to find their scavenging ability of DPPH free radicals. DPPH assay of the synthesized drugs was performed at different values of concentrations, and the results were

presented as % SCV for each concentration of the sample used. All the test compounds have antioxidant potential. The salts showed a good trend in scavenging concerning the increasing concentration and presented the IC₅₀ values in the range of 3.51 and 15.36 µg/mL, respectively, which showed that these compounds are good antioxidant agents. The respective silver NHC complexes C1 and C2 have a bad trend in scavenging effect when the drug concentration is increased. The IC₅₀ of complexes was found in the range of 6.92 and 6.13 µg/mL, respectively. The salts showed better results due to the presence of highly acidic proton at the carbene center, which facilitates reducing the free radicals, as shown in figure 3 [48].

3. Conclusion

Two bis-benzimidazolium salts, L1, L2, and their respective binuclear Ag(I)-NHC complexes, were synthesized and characterized through preliminary techniques. U.V./Visible, FT-IR spectroscopical analyses were performed, which indicated the successful synthesis of compounds. All the synthesized compounds were investigated for their antibacterial activity against gram-positive *S. Aureus* and gram-negative *E. Coli* bacterial strains and found sustainability of these salts and complexes against bacteriostatic potential. The cytotoxicity of these bis salts and their complexes was screened *in vitro* hemolytic assay against HRBCs, and the result was found satisfactory. Bis-benzimidazolium salt L1 was found least toxic with $6.73 \pm 0.36\%$ hemolysis, and it's the Ag(I) complex C1 was revealed as most toxic in series with % hemolysis of $20.46 \pm 0.23\%$. The antioxidant potentials of these compounds were investigated using a DPPH scavenging assay. The salts showed a good trend in scavenging concerning the increasing concentration. They presented the IC₅₀ values in the range of 3.51 and 15.36 µg/mL, respectively, which showed that these compounds are good antioxidants agents.

4. Material And Method

4.1. Chemicals and instruments

The required chemical like benzimidazole, alkyl halides (1,4-dibromobutane, bromomethyl benzene, and (2-bromoethyl) benzene), and potassium hexafluorophosphate (KPF₆) were obtained from Alfa Aesar (Massachusetts, USA). Silver oxide, KOH, DMSO, 1,4-dioxane, methanol, n-hexane, chloroform, etc., were purchased from Sigma Aldrich (Germany). For the sake of biological activities, gram-positive bacterial strains *S. aureus* and gram-negative bacterial strains *E. coli* were collected from American type culture collection 10,231. Standard drug Ciprofloxacin was purchased from Sigma Aldrich, and chemicals like nutrient agar, tryptone glucose, and plate count agar were purchased from Sigma Aldrich. The melting points of solid products were taken using Stuart scientific instrument (SMP-1 UK). To evaporate volatile solvents, EYELA1L Rotary evaporator N-1001V-WD was utilized. UV-Visible spectra of synthesized compounds were performed using UV-spectrometer, Shimadzu, Japan. The FT-IR spectral analysis of salts and complexes was performed using FTIR Spectrometer-4000 (Shimadzu, Japan). ¹H and ¹³C Nuclear magnetic resonance spectral analyses were performed through Bruker Advance spectrometer.

MIC values of bioactive complexes and salts were measured through an ELISA plate reader, Biobase, China. The 96 well microtiter plate method was used to check the hemolytic activity of compounds. The ELISA, plate reader was used and measurement of absorption was taken at 570 nm. Antioxidant potential of compounds was measured through DPPH method.

4.2. Synthesis

4.2.1. Synthesis of 1,4-bis (1H-benzo[d]imidazol-1-yl) butane (N-alkylated)

3.0 g of benzimidazole (0.0253 mol) and 2.1 g of KOH (0.3809 mol) powder were taken in the round bottom flask. 40.0 ml of DMSO was added and stirred the solution for 0.5 h at room temperature (R.T.). Then 1.5 ml of 1,4-dibromobutane (0.0126 mol) was added dropwise with constant stirring. The reaction mixture was stirred for 3.5 h at R.T. Removed the flask. Took 200 ml of ice-cold water in a beaker, poured the reaction mixture, and mixed the solution. The mixture was allowed to stay for one h and then filtered the product. The product was washed with distilled water twice and dried. White fluffy solid. Yield: 95.1%. M.P: 167.1 °C. FTIR (ATR, ν , cm^{-1}) 3086, 3049 (C-H str), 1752, 1610 (C=C str), 1495, 1383, 1374 (C=N str), 1284 (C-H bend), 1250 (C_{aliph} -N str), 939, 877 (C_{arom} -H). Anal. Calc. for $\text{C}_{18}\text{H}_{11}\text{N}_4$: C, 74.46; H, 6.25; N, 19.30. Found: C, 74.9; H, 7.13; N, 18.90.

4.2.2. Synthesis of 1,4-bis (3-benzyl-2,3-dihydro-1H-benzo[d]imidazol-1-yl) butane, bromide salt (L1)

1.0 g of N-alkylated (0.0034 mol) was taken in a round bottom flask, and 30.0 ml of 1,4-dioxane was added to it. It was dissolved by heating and constant stirring for half an hour. Then 0.8 ml of (bromomethyl) benzene (0.0068 mol) was added dropwise in the solution with constant stirring. The reaction mixture was refluxed for 24 h at 100 °C. After that, the flask was removed, and 15 ml of n-hexane was added to the solution and then, gently shake the flask. The mixture was allowed to stay for half an hour and filtered the product. Washed with n-hexane twice and dried the product in the oven [49]. Yield: 94.77%. M.P: 152 °C. FTIR (ATR, ν , cm^{-1}) 3437, 3424, 3366 (C_{aliph} -N_{benzimi}), 3037, 3021, 2944 (C_{aliph} -H str), 1560 (C=N_{benzimi} str), 1386 (C_{arom} -N_{benzimi}), 766 (C_{arom} -H). UV-Vis: (λ , nm): 255, 269. ^1H NMR (400 MHz, DMSO, δ ppm) 4.6(s, 4H, 2x2 CH_2 -N) 7.3-7.6(m, 8H, 2x2 Ar-H) 7.6(q, 4H) 7.9(d, 1H, 4Ar-H, $J=4.0$) 8.1(d, 1H, 4Ar-H) 10.1(s, 1H, NCHN). ^{13}C NMR (100 MHz, DMSO, δ ppm) 28.5(C-C) 39.8(Ar-C) 48(N-C-C) 51.9(N-C-N) 112.7, 112.9, 124.7, 127.4, 128.2, 133.8, 136.7, 133.77(Ar-C), 138.37(NCHN) : Anal. Calc. for $\text{C}_{60}\text{H}_{34}\text{Br}_2\text{N}_4$: C, 60.58; H, 5.40; N, 8.83. Found: C, 60.3; H, 6.10; N, 7.13.

4.2.3. Synthesis of bis (1,4-bis (3-benzyl-2,3-dihydro-1H-benzo[d]imidazol-1-yl) butane) silver hexafluorophosphate (C1)

0.5 g of L1 (0.000788 mol) was dissolved in 100.0 ml of methanol by stirring at room temperature. Covered the flask with aluminum foil to ensure the reaction mixture was in the dark. Then 0.7 g of Ag₂O (0.0031 mol) was added to the reaction mixture with constant stirring and completely covered the flask. The reaction mixture was stirred for 48 h at room temperature until the silver mirror formed around the flask walls. Flask was removed and filtered the solution through a layer of celite in the dark. Separately, mixed 0.1 g of KPF₆ (0.000788 mol) in 50 ml of methanol, and the filtered solution was added and filtered again using a syringe filter. I stirred the solution for 3 h at R.T., and then some drops of water were added. Filtered the product as white precipitates and dried at ambient temperature [49]. Yield: 47.25%. M.P: 298 °C. FTIR (ATR, ν , cm⁻¹) 2937, 2872 (C_{aliph}-N_{benz}), 1560, 1498, 1479, 1454, 1405 (C=N_{benzimi} str), 1377, 1361, 1343 (C-N str), 1082, 1042, 828, 790, 750, 700 (C_{arom}-H bend). UV-Vis: (λ , nm): 284, 275. ¹H NMR (400 MHz, DMSO, δ ppm) 4.5(s, 4H, 2x2 CH₂-N) 7.0-7.4(m, 8H, 2x2 Ar-H) 7.7-7.8(q, 4H) 7.9(d, 1H, 4Ar-H, *J*=4.0) 8.1(d, 1H, 4Ar-H) 10.1(s, 1H, NCHN). ¹³C NMR (100 MHz DMSO, δ ppm) 28.4(C-C) 39.2-40.6(Ar-C) 48.9(N-C-C) 51.9(N-C-N) 112.6, 112.8, 124.8, 127.3, 128.3, 133.8, 136.6, Anal. Calc. for C₆₄H₆₄Ag₂F₁₂N₈P₂: C, 52.98; H, 4.45; N, 7.72. Found: C, 52.68; H, 5.15; N, 8.18.

4.2.4. Synthesis of 1,4-bis (3-phenethyl-2,3-dihydro-1H-benzo[d]imidazol-1-yl) butane, hexafluorophosphate salt (L2)

1.0 g N-alkylated benzimidazole (0.0034 mol) and 0.9 ml of (2-bromoethyl) benzene (0.0068 mol) was refluxed in 1,4-dioxane with the same workup for L1 and obtained brownish sticky, which further reacted with PF₆ in methanol at R.T. for 3 h. the precipitates were filtered and washed with methanol and water twice. The product was dried at ambient temperature. Yield: 49%. M.P: 118 °C. FTIR (ATR, ν , cm⁻¹) 3437, 3424, 3161 (C_{aliph}-N_{benzimi}), 2937, 2879 (C_{aliph}-H str), 1600 (C=N_{benzimi} str), 1215 (C_{arom}-N_{benzimi}), 825 (C_{arom}-H). UV-Vis: (λ , nm): 255, 269. ¹H NMR (400 MHz, DMSO, δ ppm): 3.2(t, 2H, CH₂-Ar) 4.2(t, 2H) 4.4(t, 2H, 4xCH₂-N) 4.7(q, 4H) 7.5(m, 8H, 2x4 Ar-H) 8.0(d, 1H, 4Ar-H) 8.2(d, 1H, 4Ar-H, *J*=8.0) 9.6s, 1H). ¹³C NMR (100 MHz DMSO, δ ppm) 28.5(C-C) 39.8(Ar-C) 48(N-C-C) 50.87(N-C-N) 112.56, 112.7, 112.8, 124.6, 126.9, 128.7, 128.8, 129.0, 129.2, 133.4, 133.7(Ar-C), 138.3(NCHN) Anal. Calc. for C₃₄H₃₈F₁₂N₄P₂: C, 51.52; H, 4.83; N, 7.07. Found: C, 51.22; H, 5.13; N, 7.21.

4.2.5. Synthesis of bis (1,4-bis (3-phenethyl-2,3-dihydro-1H-benzo[d]imidazol-1-yl) butane) silver hexafluorophosphate (C2)

0.5 g of salt L2 (0.000754 mol) and 0.6 g of Ag₂O (0.00301 mol) reacted in dark with the same procedure as for C1 following by reaction with 0.139 g of KPF₆ (0.0015 mol). The precipitates were filtered and then washed with distilled water and methanol. The product was dried at ambient temperature. Synthesis of Ag(I)-NHC complexes C1 and C2 using A₂O was shown in scheme 1. Yield: 62.32%. M.P. 124.8 °C. FTIR (ATR, ν , cm⁻¹): 2950 (C_{aliph}-N_{benz}), 1520, 1482, 1454, 1402 (C=N_{benzimi} str), 1358 (C-N str), 837 (C_{arom}-H

bend). UV-Vis: (λ , nm): 284, 275. ^1H NMR (400 MHz, DMSO, δ ppm): 3.1(t, 2H, $\text{CH}_2\text{-Ar}$) 4.3(t, 2H) 4.7(t, 2H, $4\times\text{CH}_2\text{-N}$) 6.9(q, 4H) 7.4-7.8(m, 8H, 2×4 Ar-H) 8.0(d, 1H, 4Ar-H, $J = 4.0$) 8.3(d, 1H, 4Ar-H, $J = 5.0$). ^{13}C NMR (100 MHz DMSO, δ ppm) 28.2(C-C) 39.8-40.5(Ar-C) 48.5(N-C-C) 50.8(N-C-N) 112.5, 112.7, 112.8, 124.6, 126.9, 128.7, 128.8, 129.0, 129.2, 133.4, 133.7(Ar-C), 138.3(NCHN) : Anal. Calc. for $\text{C}_{68}\text{H}_{72}\text{Ag}_2\text{F}_{12}\text{N}_8\text{P}_2$: C, 54.19; H, 4.82; N, 7.44. Found: C, 54.31; H, 4.88; N, 6.89.

4.3. Antibacterial activity

4.3.1. Well pour method

Well, pour method was used for the susceptibility measurement of the newly synthesized benzimidazolium salts and complexes by using the following reported method with minute changes [50]. *Staphylococcus aureus* and *Escherichia coli* gram-positive and negative strains were screened. The antimicrobial potential of newly synthesized complexes is described in the mean zone area vacant by the studied complexes [51]. Nutrient agar culture was used for bacterial growth. All the test solutions were made in DMSO with a concentration of 10 mg/mL. Ciprofloxacin was used as positive control and DMSO as a negative control. 80 μL of each test sample was poured in wells and incubated for 18 h at 37 $^\circ\text{C}$. After that, clear zone was measured using a vernier caliper in mm.

4.3.2. Minimum inhibitory Concentrations (MIC of drugs)

The minimum inhibitory concentrations of silver-NHC complexes and benzimidazolium salts were measured using the process described in the literature using the resazurin micro titer-plate method [52]. A series of dilutions of samples were prepared by dissolving the samples into the DMSO in the 50–0.1 μL range. 100 μL volume from each sample dilution was shifted into the 96 well plates in the triplicate. The sample was filled into the wells by leaving the empty wells blank. Into the remaining wells, 50 μL Mueller Hinton broth solution was filled. Solution of resazurin indicator was prepared by dissolving 3.7 g resazurin tablet into the 40 ml of distilled water. 10 μL resazurin indicator solution was also added to the same well in which the sample was poured. More 10 μL of bacterial suspension was also added into the same wells. Again added 30 μL of Mueller Hinton broth solution in each well of the plate. Further, every plate was wrapped roughly by a cling film to avoid dehydration of bacteria. Every plate had two control sets: negative control (Mueller Hinton broth solution) & as positive control (ciprofloxacin). Triplicates of every sample were prepared and shifted for incubation at 37 $^\circ\text{C}$ for 24 h. The absorbance was measured at 500nm, and for measuring MIC value, a color transformation from purple to light pink was observed [31].

4.4. Hemolytic assay

Hemolytic activity of newly synthesized benzimidazolium salts and silver complexes was usually measured to examine the effect of cytotoxicity. The cytotoxic assay was performed through well-known protocols [53]. 3 ml of fresh human blood was taken into the falcon tube and centrifuge to remove the plasma and obtain RBCs. Then washed the HRBCs with 5 ml chilled PBS (phosphate-buffered saline) thrice. Dilute the red blood cells obtained in PBS. Suspension of red blood cells was taken in the volume

of 180 μL and mixed with the 20 μL sample to prepare a mixture in an Eppendorf tube. Incubate for half an hour at 37° C. After that, centrifuge the suspensions at 5000 rpm for 5 min. Mixed 900 μL sterile chilled PBS and 100 μL of supernatant from above suspension in a separate Eppendorf and delivered them to 96 well microtiter plate in triplicate. Tritone-x was used as a positive control and PBS as the negative control. On the plate reader of ELISA, measurement of absorption was taken at 570 nm [54].

Measurement of hemolytic activity was determined by a formula.

$$\% \text{ Hemolytic} = [(\text{Abs of sample} - \text{Abs of negative control}) / \text{Abs of positive control}] \times 100$$

4.5. Antioxidant Activity

Antioxidant potentials of newly synthesized salts and complexes were studied by the DPPH method [54] with slight alterations. Test solutions were made with a concentration of 10 mg/mL in DMSO. DPPH solution 4% (w/v) was prepared in methanol. Mix 4 ml of 4% (w/v) DPPH solution with 200 μL , 150 μL , 100 μL and 50 μL of test samples separately and incubated for at 37 °C. Absorbance at 517 nm was measured spectrophotometrically along with the absorbance of DPPH solution as a control. The % inhibition was measured by using the equation described below. IC_{50} values were calculated through the equation of a straight line for each sample.

$$\% \text{Inhibition} = [\text{Absofcontrol} - \text{Absofsample} / \text{Absofcontrol}] \times 100$$

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Scheme

Figures

Figure 1

Antibacterial susceptibility of synthesized compounds at a concentration

Figure 2

Hemolytic activity of salts and silver-NHC complexes

Figure 3

Antioxidant ability of synthesized salts and complexes presented as %SCV

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