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Short Communication

Cytotoxicity and Antimicrobial Activity of Pivalic and Benzoic Acid-Complexed Cu and Mn Complexes

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Abstract

The complexes of Cu and Mn metals with carboxylates (pivalic acid and benzoic acid) and imidazole were prepared and characterized. The synthesized complexes were screened for antimicrobial activity and cytotoxicity. The FTIR technique was used for characterization. The antibacterial assay revealed that the ligand and complexes are active against *Escherichia coli*, *Staphylococcus aureus* and *Pasteurella multocida* bacterial strains. The haemolytic assay revealed that [Cu(imi)(N_3)] and [Mn^{III}(piv)(imi)(N_3)] were nontoxic, whereas [Cu(piv)(imi)] and [Mn^{III}(Benz)(imi)(OCH_3)] showed moderate toxicity, and [Mn(Him)(N_3)$_2$] was highly toxic against human red blood cells (RBCs). Results revealed that Cu and Mn complexes with carboxylates (pivalic acid and benzoic acid) have antimicrobial activities. However, haemlytic assay revealed that the metal complex cytotoxicity was variable and biological activity evaluatuion of newly synthesized metals complexes is suggested for safe applications.

Keywords: metal complexes, antimicrobial activity, cytotoxicity, haemolytic assay

Introduction

Although antibiotics have revolutionized the medical care, infectious treatments of multidrug-resistant bacterial strains is of great concern and the development of potent metallic complexes active against bacterial resistance is important [1]. Among various ligands used for complex preparation, the imidazole-containing ligands have attracted much attention in recent years [2]. Imidazole and its derivatives (being a biological compound) have been studied to evaluate interactions of proteins with metal ions [2-3]. Carboxylate-metal complexes also have shown remarkable biological activities linked with unusual structural features [4-5]. The potential of nitrogen-containing organic compounds and their metal complex biological activities have been studied well [6].

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It has been proposed that the antimicrobial activities of these nitrogenous ligands is due to their ability to chelate with transition and non-transition metal ions, which are required by the micro-organism to perform metabolic activities [7-8].

Trace amounts of copper and manganese are important as cofactors of various enzymes [9]. Furthermore, copper(II) complexes have been reported for biochemical significance, including anti-inflammatory, antiulcer, anticonvulsant, ant amoebic, antidiabetic, antitumor, and antimicrobial activities [10-14]. On the other hand, due to involvement in several enzymatic activities, manganese also is involved in the chemistry of reactive oxygen species [15]. Therefore, in view of current environmental pollution due to toxic agents, the synthesis of new nontoxic material is highly useful to avoid environmental contamination and toxicity [16-25].

In a continuing endeavor to improve biological activity of imidazole and its complexes and for important of eco-friendly synthesis methods [26-40], the present study was aimed at synthesizing complexes of imidazole with copper and manganese. The synthesized complexes were characterized by FT-IR spectroscopy along with physical characteristics. *In-vitro* antibacterial activity of Cu(II) and Mn(II) imidazolium complexes against targeted bacterial strains *Escherichia coli*, *Staphylococcus aureus*, and Pasteurella multocida were also studied. Cytotoxicity was performed using haemolytic bioassay.

Material and Methods

Chemical and Reagents

All chemicals and solvents used were of analytical grade, i.e., copper nitrate hydrated (99.99%), methanol (99.8%), pivalic acid (99%), imidazole (>99%), sodium azide (99%), manganese chloride (97%), benzoic acid (99.5%), ethanolamine (>98%), manganese acetate (98%), dimethyl sulfoxide (99.9%), crystal violet dye (90%), and glacial acetic acid (>99%) procured from Sigma chemicals (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Complexes Synthesis Procedures

For $[Cu(piv)_2(imi)_2]$ (S5) synthesis, copper nitrate (0.5 mmol, 0.093 g) was added to 10 mL of methanol and stirred for a few min. Then, pivalic acid (1 mmol, 0.102 g), imidazole (1 mmol, 0.07 g), and NaN_3 (1 mmol, 0.065 g) were added, respectively. Again, methanol (10 mL) was added, stirred, filtered, and sealed with paraffin and kept at room temperature for slow evaporation until crystal formation. Then crystals were washed and dried, and FTIR analysis was performed using KBr internal standard and the following peaks were recorded: 3,136.25 (m), 2,951.09 (s), 2,715.77 (w), 2,341.58 (m), 1,585.49 (s), 1,489.05 (m), 1,423.47 (w), 1,350.17 (m), 1,226.73 (m), 1,080.14 (w), 945.12 (w), 817.82(s), and 628.79(s) (cm^{-1}).

For $[Mn^{III}(Benz)(imi)(OCH_3)]$ (A4) synthesis, manganese chloride ($MnCl_2$) (0.5 mmol, 0.13 g) was dissolve in 10 mL of methanol, stirred for few min and then benzoic acid (1 mmol, 0.122 g), imidazole (1 mmol, 0.07 g), and NaN_3 (1 mmol, 0.065) were added, respectively. Finally, ethanolamine and methanol (10 mL) were added and the contents stirred, filtered, and covered with paraffin and kept at room temperature for slow evaporation until the formation of crystals. After five days, crystals were filtered, washed with methanol, and dried in air, and FTIR analysis was performed using KBr internal standard and the following peaks were recorded: 2,959.9 (m), 2,050.5 (s), 1,983.4 (m), 1,734.1 (m), 1,576.6 (s), 1,539.9 (s), 1,456.9 (m), 1,420.8 (s), 1,361.6 (m), 1,225.6 (m), 1,090.6 (m), 940.0 (m), 879.9 (w), 787.5 (w), 656.0 (w), 579.9 (s), and 541.2 (w) (cm^{-1}).

For $[Cu(imi)_2(N_3)_2]$ (T1) synthesis, sodium azide (0.5 mmol, 0.65 g) and imidazole (0.5 mmol, 0.07 g) were added in hot solution of copper nitrate (0.5 mmol, 0.093 g) in methanol (20 ml). The reaction mixture was stirred until the color change was stopped. The resulting solution was filtered and kept for five days at room temperature for slow evaporation. Crystals were collected by filtration, washed with methanol, and dried in air, and FTIR analysis was performed using KBr internal standard and the following peaks were recorded: 3,331.07 (w), 3,149.76 (m), 2,958.80 (v), 2,332.87 (v), 2,032.97 (s), 1,633.71 (w), 1,510.26 (w), 1,450.47 (w), 1,321.24 (m), 1,259.52 (w), 1,074.35 (m), 914.26 (w), 788.89 (s), 638.44 (s), and 557.43 (w) (cm^{-1}).

For $[Mn(Hmi)(N_3)_2]$ (T6) synthesis, manganese acetate (0.5 mmol, 0.12 g) was added in methanol (20 ml) and stirred, and then imidazole (0.5 mmol, 0.07 g), pivalic acid (0.5 mmol, 0.102 g), and sodium azide (0.5 mmol, 0.65 g) were added. The resulting solution was kept at room temperature for slow evaporation for four days. Crystals were collected by filtration, washed with methanol, and dried in air, and FTIR analysis was performed using KBr internal standard and the following peaks were recorded: 3,332.5 (w), 3,151.89 (s), 2,958.80 (m), 2,702.27 (w), 2,333.87 (m), 2,034.90 (vs), 1,637.56 (m), 1,517.98 (w), 1,431.18 (w), 1,323.17 (m), 1,139.93 (w), 1,070.49 (m), 918.12 (w), 786.96 (vs), 638.44 (s), and 555.50 (w) (cm^{-1}).

For $[Mn^{III}(piv)(imi)(N_3)_2]$ (T7) synthesis, manganese chloride (0.5 mmol, 0.13 g), pivalic acid (0.5 mmol, 1.02 g), and sodium azide (0.5 mmol, 0.65 g) were dissolved in 10 ml of methanol and stirred at room temperature for 10 min. Then imidazole (0.5 mmol, 0.07 g) was added to 10 ml methanol with constant slow stirring. The resulting solution was kept at room temperature for slow evaporation for four days. Crystals were collected by filtration, washed with methanol, and dried in air, and FTIR analysis was performed using KBr internal standard and the following peaks were recorded (PREVISAGE-21 Shimadzu, Japan): 3,572.17 (w), 3,404.36 (m), 2,954.95 (w), 2,858.51 (w), 1,505.53 (w), 2,094.69 (vs), 1,923.03 (w), 1,643.35 (m), 1,481.33 (w), 1,390.68 (s), 1,276.88 (m), 1,111.00 (m), 11,066.64 (w), and 563.21 (w) (cm^{-1}).

Antibacterial Activity

Microtiter-plate protocol was used to determine the *in vitro* antibacterial activity of synthesized complexes against *Escherichia coli* and *Pasteurella multocida***.** Sterilization of microbiological equipment and media was carried out at 121ºC and 180 atm for 30 min, and antimicrobial activity was determined by biofilm inhibition assay. For biofilm formation, the 96 wells were filled with 100 µL of nutrient broth and 100 µL sample (1 mg/1mL of DMSO) and inoculated with 20 µL of bacterial suspension (1 x 10^8 CUF/mL). The plates were incubated at 37°C for 24 h. Then the contents of each well were beheld three times with 220 µL of sterile phosphate buffer. The plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 220 mL of 99% methanol/well, and after 15 min the plates were dried. Plates were stained for five min with 220 mL of 50% crystal violet/well. Excess stain was rinsed off by placing the plate under running tapwater. The plates were air-dried and bounded dye was solubilized in 220 µL of 33% (v/v) glacial acetic acid/well. Absorbance was measured at 630 nm [7] and bacterial growth inhibition was calculated as shown in Eq. 1.

$$\text{Bacterial growth inhibition (\%)} = 100 - (OD_{630\ \text{sample}} * 100)/OD_{630\ \text{control}} \quad (1)$$

Hemolytic Activity

Three mL fresh blood was collected from volunteers in heparinized tubes. The blood was centrifuged for 5 min at 1,320 x g, plasma was discarded, and cells were washed three times with 5 mL of chilled (4ºC) phosphate-buffered saline (PBS) of pH 7.4. Erythrocytes were maintained at 10^8 cells/mL. Each compound (10 µL) was mixed with RBC and incubated for 35 min at 37ºC. Then samples were placed on ice for 5 min and centrifuged for 5 min at 1,320 x g. Supernatant (100 µL) was taken and diluted 10 times with chilled (4ºC) PBS. Triton X-100 (0.1% v/v) was used as positive control and PBS as negative control. The absorbance was noted at 576 nm (µQuant, Bioteck, USA) and RBCs lysis (%) was calculated as shown in Eq. 2, where A_s and $A_{T\text{-}X\text{-}100}$ are the absorbances of sample and Triton X-100, respectively.

$$RBCs\ lysis\ (\%) = \left\{\frac{A_S}{A_{T-X-100}}\right\} x\ 100 \quad (2)$$

Results and Discussion

The synthesized metal complexes were characterized by FTIR technique and screened for biological activity and cytotoxicity. The structures of the synthesized compounds are shown in Fig. 1 and FTIR absorption peaks are listed in Table 1. The region of 3,000 cm^{-1} and 1,400-1,200 cm^{-1} showed the peaks of (C-H) and (C-N), respectively [41]. The bands at 3,136.25 cm^{-1}, 2,959.90 cm^{-1}, and 3,404.36 cm^{-1} are assigned to the stretching vibrations of the (O-H) for complexes 1, 2, and 5, respectively. The peaks at 2,951.09 cm^{-1} for complex (1), 2,849.51 cm^{-1} for complex (2), 2,958.51 cm^{-1} for complex (3), 2,958.80 cm^{-1} for complex (4), and 2,954.95 cm^{-1} for complex (5) are attributed to the

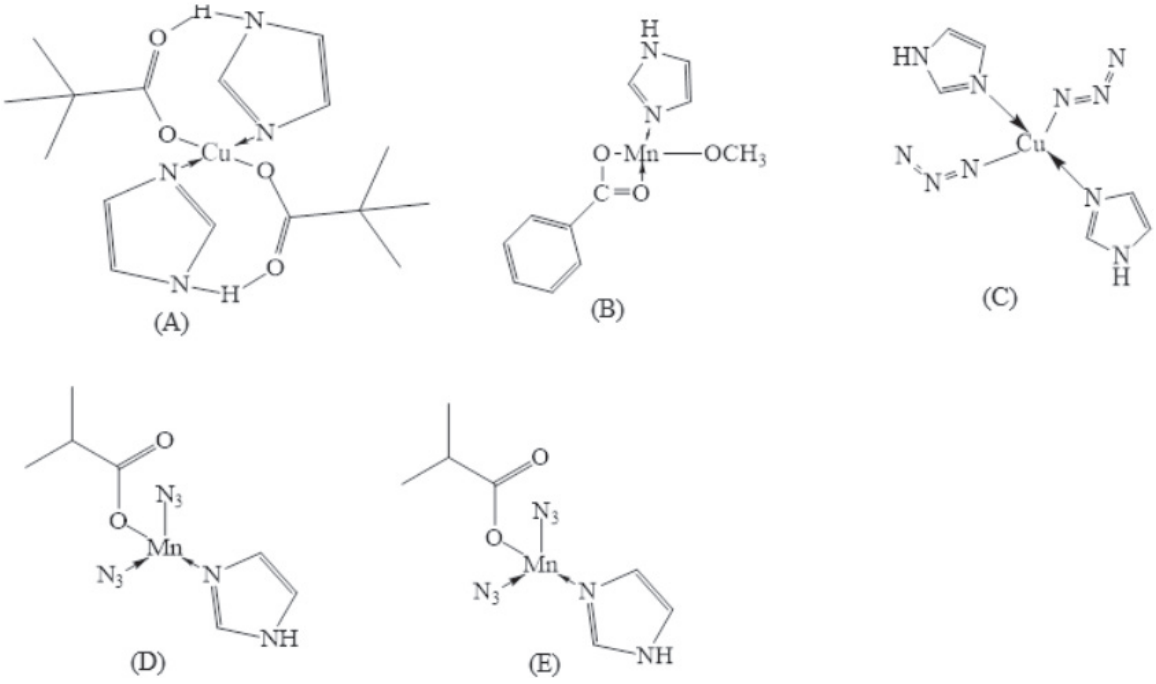

Fig. 1. A) Structure of [Cu(piv)2(imi)2], B) structure of [Mn^{III}(Benz)(imi)(OCH_3)], C) structure of [Cu(imi)2(N3)2], D) structure of [Mn(Hmi)(N_3)$_2$], and E) structure of [MnIII(piv)(imi)(N_3)$_2$].

Table 1. Infrared frequencies (cm^{-1}) for the complexes.

Sr. No.	Compound	Mode assignment					
		(O−H)	(C−H)	(N=N)	(C=O)	(C−N)	(M−O)
1(S5)	[Cu(piv)(imi)]	3,136.25	2,951.09	−	1,585.49	1,226.73	628.79
2(A4)	[Mn^{III}(Benz)(imi)(OCH_3)]	2,959.90	2,849.51	−	1,576.60	1,225.60	597.9
3(T1)	[Cu(imi)(N_3)]	−	2,958.51	2,032.97	−	1,321.24	788.89
4(T6)	[Mn(Him)(N_3)$_2$]	−	2,958.80	2,034.90	−	1,323.17	786.96
5(T7)	[Mn^{III}(piv)(imi)(N_3)]	3,404.36	2,954.95	2,094.69	1,643.35	1,390.68	563.21

(C-H) frequency of the imidazole. The 1,400-1,200 cm^{-1} peak is due to the stretching vibration of (C-N) of imidazole and peaks at 1,226.73 cm^{-1}, 1,225.60 cm^{-1}, 1,321.24 cm^{-1}, 1,323.17 cm^{-1}, and 1,390.68 cm^{-1} represents the (C-N) peaks for complexes 1-5, respectively. A strong absorption band at 2,000 cm^{-1} is due to stretching the (N=N), which is a characteristic of azide moiety [42]. The strong absorption peaks at 2,032.97 cm^{-1}, 2,034.90 cm^{-1}, and 2,094.69 cm^{-1} indicate the presence of azide (N=N) in complexes 3, 4, and 5, respectively. The C=O band at 1,715-1,740 cm^{-1} is the characteristic band for carboxylates, which are shifted toward lower-frequencies due to coordination [43]. Peaks at 1,585.49 cm^{-1} (complex 1), 1,576.60 cm^{-1} (complex 2), and 1,643.35 cm^{-1} (complex 5) are due to the (C=O) of carboxylic acid. The peak below 800-550 cm^{-1} was due to metal coordination with the ligand. Solubility was tested against acetonitrile, water, and DMSO. The prepared complexes were insoluble in water, whereas all complexes were soluble in DMSO and acetonitrile.

Antibacterial activities of ligands and metal complexes against *E. coli*, *P. multocida*, and *T. aerues* were measured by the microtitre-plate method and compared with standards. Generally cobalt (Co), copper (Cu), nickel (Ni), and zinc (Zn) are used due to the formation of low molecular weight complexes and hence these complexes have proven to be more efficient against a variety of organisms. The ligand and metal complexes were screened for antimicrobial activity and were compared with Rifampicin (an active against antimicrobial agent against Gram-negative and Gram-positive bacterial strains). The synthesized compounds exhibited significant activities versus standard antimicrobial agents, and these findings are in line with reported studies that the metal complexes can exhibit higher antimicrobial/antibacterial activity than the parent ligands [44-48]. At higher concentrations the complexes (1-5) exhibited considerable antibacterial activity against *E. coli*, *P. multosida*, and *S. aerues*. The antimicrobial activity is controlled by liposolubility – the lipophilicity of the complex that was enhanced due to delocalization of π electrons over the complete chelate ring. The easy penetration into lipid membranes of organisms is permitted by the increased lipophilic characteristics of complexes, and the blockage of binding sites of metals in enzymes also was facilitated. The action approach of metal complexes may involve hydrogen bond formations by involving the carboxylic acid group with ribosome or microbes of the microorganism's cells. The activity values increase with concentrations of metal complexes. For *E. coli*, the activity of the compounds under investigation decreases in the order: 1>2>5>4>3 (Table 2), and other complexes also showed variable antimicrobial activity (but comparable with the standard)**.**

The haemolytic activity of complexes (1-5) was also evaluated and results are given in Table 2. Complex 3 showed lowest hemolytic activity of 1.96% lysis of RBCs, but higher than the negative control (PBS). Furthermore, complex 4 showed the highest hemolytic activity 55.56%, but lower than the positive control

Table 2. Biofilm inhibition (%) and hemolytic activity (%) of metal complexes.

Complexes	*E. coli* biofilm inhibition (%)	*P. multocida* biofilm inhibition (%)	*S. aerues* biofilm inhibition (%)	Hemolysis (%)
1 (S5)	91.65±2.05	85.65±1.95	-	14.56±1.90
2 (A4)	90.63±1.90	87.73±1.30	-	6.71±0.40
3 (T1)	55.65±1.40	-	53.86±2.10	1.95±0.80
4 (T6)	61.27±1.20	-	62.43±1.90	55.56±0.70
5 (T7)	71.46±1.80	-	71.46±1.95	4.88±0.90
Rifampicin	96.7±1.80	97.6±1.40	86.8±1.50	-
PBS	-	-	-	1.43±0.50
Triton-X-100	-	-	-	100

(Triton-X-100). According to ASTMF 756-00 (American Society for Testing and Materials), samples with hemolysis <2 are considered non-toxic, 2-5% moderate, and >5% are considered toxic. Therefore, $[Cu(imi)(N_3)]$ and $[Mn^{III}(piv)(imi)(N_3)]$ were nontoxic, whereas [Cu(piv)(imi)] and $[Mn^{III}(Benz)(imi)(OCH_3)]$ showed moderate toxicity and $[Mn(Him)(N_3)_2]$ was found to be toxic against human RBCs. In view of the current scenario of environmental pollution [28, 30, 49-67], the Cu and Mn complexes with pivalic and benzoic acid proved to be non-toxic and these agents could possibly be used for metal complex preparation.

Conclusions

The Cu and Mn carboxylates with pivalic acid, benzoic acid, and imidazole were prepared successfully and screened for antimicrobial activity and cytotoxicity. The synthesized complexes were found to be active against *E. coli*, *S. aureus*, and *P. multocida* bacterial strains. The haemolytic assay revealed the variable toxicity of synthesized complexes and among all synthesized complexes were non-toxic except $[Mn(Him)(N_3)_2]$. Results revealed that the Cu and Mn complexes with pivalic acid, benzoic acid, and imidazole were non-toxic. The newly synthesized metal complex toxicity profile study is suggested using standard bioassay for safer applications.

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