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ANTIBACTERIAL ACTIVITY AND PHARMACOLOGICAL EFFECT OF SOME MIXED-LIGAND TRIS-CHELATES OF COPPER(II) CONTAINING NEUTRAL N,N-DONORS AND KINETICS STUDY OF THE NOVEL SYNTHETIC ROUTE FOR THE COMPLEXES

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ABSTRACT. The present work describes *in vitro* antibacterial activities and pharmacological effect of six mixed-ligand tris-chelates of copper(II) containing N-*p*-tolylpyridine-2-aldimine; 1,10-phenanthroline and 2,2'-bipyridine as neutral N,N-donors, towards some gram-positive and gram-negative bacterial strains in aqueous medium. All the six complexes have encouraging potentiality to inhibit the growth of some gram positive and gram negative bacteria and hence bacteriostatic agents. These copper(II) complexes were synthesized easily at room temperature by the novel silver(I) assisted *trans*-metallation reaction and kinetic study of the reactions suggest pseudo zero order.

KEY WORDS: Copper(II)tris-chelates, Neutral N,N-donors, Bacteriostatic agent, Silver(I) assisted reaction, Kinetics study

INTRODUCTION

Transition metal ions are known to play important roles in biological processes in humans [1, 2]. They are found in the active sites of a large number of enzymes [3, 4] and many of their complexes have been found to exhibit antimicrobial activities [5-7]. The ligands namely 2,2′-bipyridine and 1,10-phenanthroline form very stable chelates with many 3d series metals [8] and these ligands along with some of their complexes are known to exhibit antimicrobial properties [9, 10]. But, there are few reports regarding syntheses of mixed ligand tris-chelated copper(II) complexes of neutral N,N-donors in particular [11] due to pronounced Jahn-Teller distortions and also to study their antimicrobial activities. The design of effective synthetic procedure for the preparation of such complexes with N-*p*-tolylpyridine-2-aldimine (L^1), 1,10-phenanthroline (L^2) and 2,2'-bipyridine (L^3) are based on facile silver(I) assisted trans-metallation reaction [12]. Herein, we have explored the kinetic study of this facile reaction. The synthesized copper(II) compounds have also been tested for their *in vitro* anti-microbial activities against some pathogenic bacterial strains and promising results have been obtained.

EXPERIMENTAL

Materials

Copper(II) chloride, silver nitrate, 2,2'-bipyridine and 1,10-phenanthroline were purchased from Merck Emplura and pyridine-2-carboxaldehyde was obtained from Merck, Germany. The silver complexes of L^1, L^2 and L^3 [13-15] and the mixed-ligand copper(II) tris-chelates were synthesized by earlier reported [12] procedures. The analytical data of the synthesized compounds of interest [12] were also verified (Table 1). The bacterial strains were obtained

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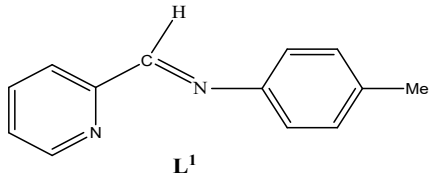
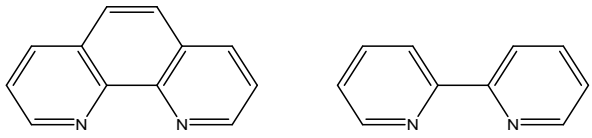
from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh and Department of Microbiology, University of Kalyani. All other chemicals and solvents used were of reagent grade and were used as received.

Physical measurements

Elemental analyses (C,H,N) were done by using Perkin-Elmer Series II 2400 elemental analyzer. IR spectra were recorded as KBr disk (4000–300 cm^{-1}) using a Perkin Elmer IR-783 spectrophotometer. Solution electrical conductivity measurements were performed using a Systronics direct reading conductivity meter 304. The magnetic susceptibilities of the samples were measured on a PAR 155 vibrating sample magnetometer fitted with a Walker scientific L75 FBAL magnet.



L[1]



L[2] **L[3]**

Table 1. Analytical data for $[CuLL'_2](NO_3)_2$ complexes.

Compound	Elemental analysis[a] (%)			Λ_M[b]/ohm^{-1}cm^{2}M^{-1}	μ_{eff} (298 K)	IR[c]/cm^{-1}	
	C	H	N		BM	$\nu_{C=N}$	$\nu_{C=C}$
$[CuL^1L^2_2](NO_3)_2$, **1**	60.10 (59.71)	3.91 (3.76)	15.38 (15.06)	220	1.87	1625	1590
$[CuL^2L^3_2](NO_3)_2$, **2**	56.88 (56.51)	3.67 (3.53)	16.94 (16.48)	220	1.78	1620	1600
$[CuL^3L^2_2](NO_3)_2$, **3**	57.67 (58.0)	3.10 (3.38)	16.18 (15.92)	235	1.84	1620	1600
$[CuL^3L^1_2](NO_3)_2$, **4**	58.17 (5873)	4.90 (4.35)	14.97 (15.22)	225	1.84	1625	1600
$[CuL^1L^3_2](NO_3)_2$, **5**	57.32 (56.93)	4.38 (4.02)	16.71 (16.10)	230	1.77	1630	1600
$[CuL^2L^1_2](NO_3)_2$, **6**	59.37 (60.04)	3.94 (4.21)	15.20 (14.74)	240	1.79	1625	1600

[a]Calculated % are in parenthesis, [b]in MeOH, [c]in KBr.

Kinetics study

To study the kinetics of the synthetic reaction for the copper(II) tris-chelates, potentiometric data were collected using a Digital EI (Model: 118) make potentiometer with Ag-indicator electrode and SCE as reference electrode. A 3-necked round bottom flask, fitted with condenser, was placed over a magnetic stirrer at room temperature for the study. The two said electrodes were inserted separately to the flask through the holes of the cork stoppers fitted with the two side necks. This arrangement was made to resist solvent evaporation. $CuLCl_2$ (1 mmol) ($L = L^1 - L^3$) was first taken in the above round bottom flask and was dissolved by stirring with 15 mL methanol. 15 mL methanolic solution of 2 mmol $[AgL'_2]NO_3$ ($L' = L^1 - L^3$) was then added to the stirred solution and potentials were recorded at regular intervals until almost constant readings were observed.

Antibacterial activity

In vitro antimicrobial analysis methods were performed against four bacterial strains to evaluate the antimicrobial potentiality of these compounds. The bacterial strains used in this study were two Gram positive *Bacillus subtilis* 6633, *Staphylococcus aureus* and two Gram negative *Escherichia coli* K12 and *Salmonella enterica ser. typhi* SRC. As the reference antimicrobial agent amoxicillin, a clinically recommended antibacterial agent, was used [16]. The compounds were dissolved into autoclaved sterile distilled water. Luria-Bertani Broth/Agar (Modified) and Mueller Hinton medium were used for *in vitro* antimicrobial analysis and as per NCCLS protocol [17] minimum inhibitory concentrations (MIC) of these compounds were determined. Bacterial susceptibility of these compounds was experimented using agar well diffusion method [18]. Four strains were used to analyze time kill curve against these compounds.

Preparation of bacterial inoculum

All the bacteria strains were cultured in Luria-Bertani broth (LB) and incubated at 37 ^{o}C for 18 h. The bacteria suspension was adjusted to a turbidity corresponding to a spectrophotometric absorbance at 0.08 at 600 nm, which is equivalent to a bacteria inoculum size of approximately 10^6 CFU/mL using colony suspension methods.

Determination of minimal inhibitory concentration (MIC)

In vitro susceptibility tests were performed in Mueller Hinton medium using standard method [19]. Broths were incubated aerobically at 37 ^{o}C for 24 hours. The MIC was defined as the lowest concentration of antimicrobial agent that resulted in the inhibition of visible growth.

Kirby–Baueragar diffusion method for inhibition zone determination

Antimicrobial activities of compounds were measured using the well agar diffusion assay [19]. Each bacterial suspension (10^8 CFU/mL) was inoculated on MHA plates with a sterile cotton swab. 50 µL of each diluted compounds were loaded on the wells in agar place. The plates were incubated at 37 ^{o}C for 12-24 hours. Evidence of clear zone indicates bacterial growth inhibition and was measured in mm. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters in millimetre produced (± standard deviation).

Time kill curve

Growth curves were initially performed to confirm that all strains would reach a stable early- to mid-log phase after 3 h of pre-incubation in Mueller Hinton broth medium. Time kill curve was conducted following the standard guidelines [19]. The bacterial suspensions for inoculation were prepared from fresh cultures on Mueller Hinton broth. The isolated colonies were suspended in sterile saline and the culture turbidity was adjusted to 0.5 McFarland's standard. The suspension was diluted further to get required inoculum (i.e. 5 X 10^7 CFU/mL). The compounds were dissolved using autoclaved sterile distilled water for stock preparation and subsequent dilutions were made. At pre-determined time points (0, 1.5 and 3 hours), 1000 µL aliquots were removed and transferred to Eppend or f tubes for determination of OD. The compounds were added to the respective conical flasks at selected concentration. MHB medium containing inoculum to obtain final concentrations of 0×MIC as control and 0.5×MIC, 1×MIC, 2×MIC for each bacterial species. No addition to the flasks was considered as negative control 0×MIC. After entering log phase, compounds were added and five time points (0, 1, 2, 3, 4 and

5 hours) were selected for sampling at 37 °C at 150 rpm. From OD600 values, the plotted graph were defined as to bactericidal or bacteriostatic based on the OD600 value reduction at various times intervals. The compounds were said to be bacteriostatic [20] as they exhibit < 3 log CFU reduction (lowering the number of microorganisms by less than 1000 fold).

RESULTS AND DISCUSSION

Kinetic study of the synthetic reaction

In order to get a series of mixed ligand Cu(II) tris-chelates, *viz.* $[CuL^1L^2_2]^{2+}$ (**1**), $[CuL^2L^3_2]^{2+}$ (**2**), $[CuL^3L^2_2]^{2+}$ (**3**), $[CuL^3L^1_2]^{2+}$ (**4**), $[CuL^1L^3_2]^{2+}$ (**5**) and $[CuL^2L^1_2]^{2+}$ (**6**), the stable silver(I) complexes of L^1, L^2 and L^3 are reacted with various halo complexes of Cu(II) in MeOH at room temperature [12].

$$CuLCl_2 + 2\,[AgL'_2]NO_3 \xrightarrow{\text{MeOH, stir at r.t. for 30 min}} [CuLL'_2](NO_3)_2 + 2\,L' + 2\,AgCl$$
$$(L = L^1 - L^3) \qquad (L' = L^1 - L^3) \qquad (L \neq L')$$

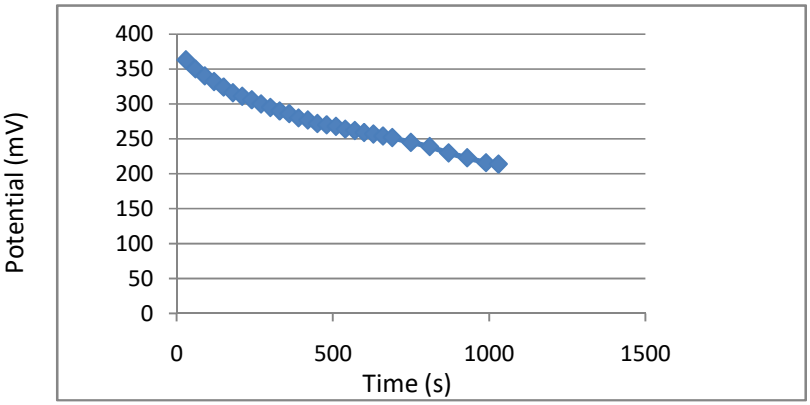


Figure 1a. Change of potential with time for the reaction: $CuL^1Cl_2 + 2[AgL^2_2]NO_3$.

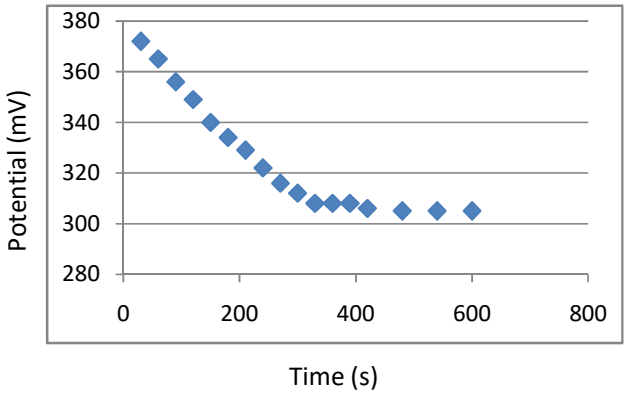


Figure 1b. Change of potential with time for the reaction: $CuL^2Cl_2 + 2[AgL^3_2]NO_3$.

Kinetics study of the above reactions has been carried out potentiometrically [21, 22] using Ag electrode. As reaction proceeds, AgCl precipitates out and gradual decrease in potential (f^n of concentration) with time is observed within 15 min of the reaction (Figure 1a and 1b), which suggests pseudo zero order reactions of which rates do not vary with concentration of reactants i.e., $[AgL'_2]^+$. This can be due to the presence of only a small fraction of $[AgL'_2]^+$ species in a location or state where they are able to react with $CuLCl_2$ and this fraction is continually replenished from the larger pool. As reaction proceeds with time, it is possible that less and less $CuLCl_2$ will react with $[AgL'_2]^+$ to produce relatively small amount of AgCl. Due to this a tailing off the graph is observed in the later part of the experiment [23].

Antimicrobial study

Against four bacterial strains, six synthesized compounds (**1-6**) showed active antimicrobial properties. In Table 2 and 3 the MIC values and inhibition zone diameter for these compounds are given, respectively. All compounds showed most potential killing Gram positive bacteria. **1** showed its MIC value in the range of 25-145 µg/mL. *Bacillus subtilis* and *Staphylococcus aureus* were most sensitive against **1** with MIC concentration of 26 and 33 µg/mL, respectively. MIC values of **2**, **3**, **4**, **5** and **6** are in the range of 102-416, 31-92, 196-750, 205-700 and 45-190 µg/mL, respectively. **1**, **2**, **3** and **6** these four compounds were showed very potential antimicrobial property against a wide range of bacterial strain. The all four compounds showed broad spectrum antibacterial activity. These are simultaneously effective against both Gram positive and Gram negative microorganisms. It is to be noted here that though the antibacterial properties of these organic ligands against these microorganisms have already been reported in the literature [24], we did not find any such significant properties in aqueous medium probably because of their water insolubility. The complexes thus can be applied for the treatment of infections caused by these organisms in mammals, as water is the main component in these systems and the mixed-ligand complexes have an advantage in that the respective bioactivities of the uncoordinated ligands are combined to make them more potent antimicrobial agents. The outcome of the time-kill kinetic studies of the Gram-positive isolate *Bacillus subtilis* against the Cu(II) compounds is summarized in Figure 2. Such curves with other strains are given as electronic supplementary materials. The results showed decrease of OD_{600} which represent the viable cell counts of *Bacillus subtilis* in presence of 0.5×MIC, 1×MIC, 2×MIC with compared to 0×MIC or control from 1 h of incubation and the growth rate then decreased rapidly. Figure 2a, 2b, and 2c showed a strong bactericidal effect at the 1×MIC and 2×MIC concentrations of these three compounds. By this method it has been estimated that the lowest concentration of an antibacterial compound totally suppressed the bacterial growth. From these patterns of time-kill curves we can conclude that the compounds have encouraging potentiality to inhibit bacterial growth and are strong bacteriostatic agents. The time killing studies of compound **1**, **2** and **3** exhibited strong effects against *Bacillus subtilis* at two times concentrations of MIC leads to bactericidal effect.

Table 2. MIC (µg/mL) values of the synthetic compounds against specific bacterial strains.

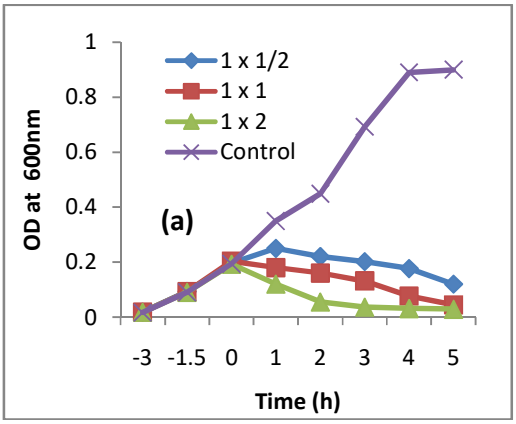
Organisms	**1**	**2**	**3**	**4**	**5**	**6**	Amoxicillin
Escherichia coli	102	310	72	700	700	190	7
Salmonella enterica ser. typhi	145	416	92	750	650	180	31
Bacillus subtilis	26	102	31	196	210	45	28
Staphylococcus aureus	33	125	36	460	205	90	85

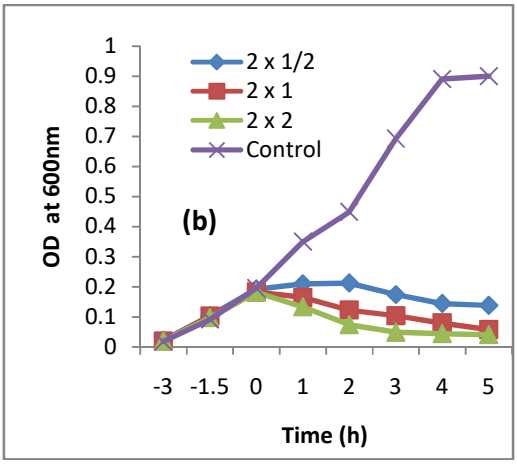
$[CuL^1L^2_2]^{2+}$ (**1**), $[CuL^2L^3_2]^{2+}$ (**2**), $[CuL^3L^2_2]^{2+}$ (**3**), $[CuL^3L^1_2]^{2+}$ (**4**), $[CuL^1L^3_2]^{2+}$ (**5**) and $[CuL^2L^1_2]^{2+}$ (**6**).

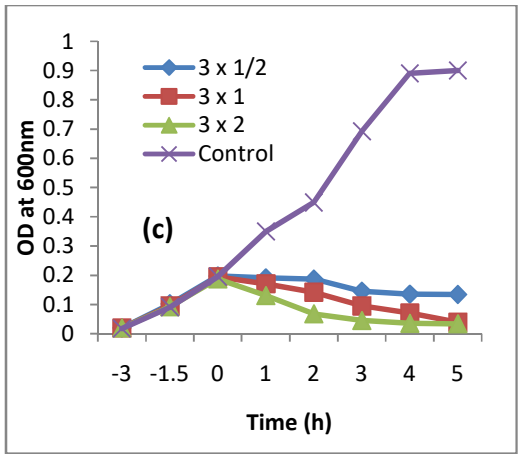
Table 3. Growth inhibition zone diameter of MIC (mm) of synthetic compounds against the specific bacterial strains.

Organism	**1**	**2**	**3**	**4**	**5**	**6**	Amoxicillin
Escherichia coli	11.57 ±0.06	4.17±0.15	4.67±0.12	5.6±0.1	14.1±0.1	4.4±0.1	12.3±0.4
Salmonella enterica ser. typhi	12.5±0.1	9.27±0.06	5.6±0.06	8.1±0.06	13.7±0.1	5.23±0.06	9.4±0.2
Bacillus subtilis	5.97±0.25	8.5±0.5	10.5±0.26	9.37±0.06	11.83±0.21	8.6±0.31	8.5±0.1
Staphyloccus aureus	3.7±0.17	6.1±0.12	5.27±0.06	6.47±0.21	12.23±0.15	11.23±0.12	7.2±0.6

$[CuL^1L^2_2]^{2+}$ (**1**), $[CuL^2L^3_2]^{2+}$ (**2**), $[CuL^3L^2_2]^{2+}$ (**3**), $[CuL^3L^1_2]^{2+}$ (**4**), $[CuL^1L^3_2]^{2+}$ (**5**) and $[CuL^2L^1_2]^{2+}$ (**6**).



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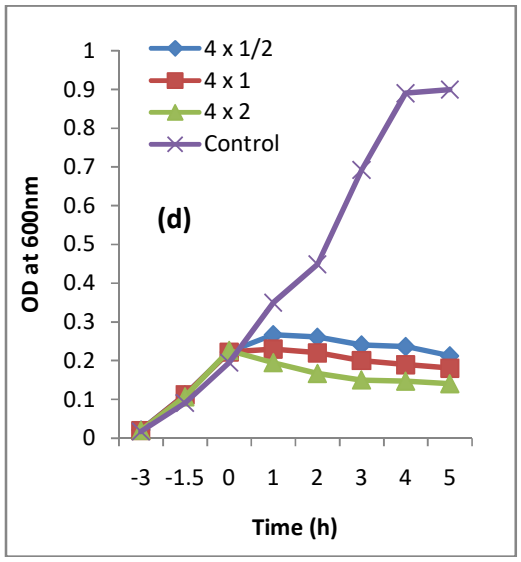
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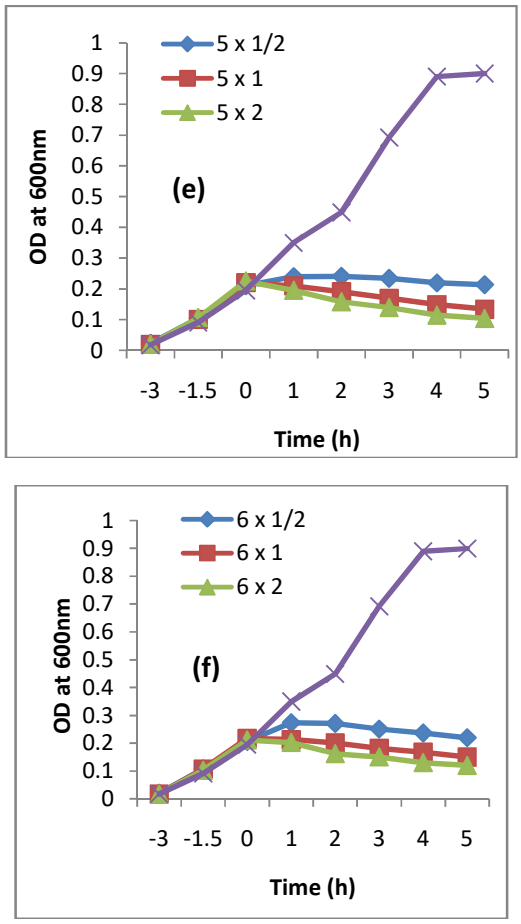

Figure 2. *In vitro* time kill curve of *Bacillus subtilis* against the synthetic compounds: (a) **1**, (b) **2**, (c) **3**, (d) **4**, (e) **5** and (f) **6**.

CONCLUSION

Kinetic study of the facile Silver(I) assisted trans-metallation reaction used to synthesize six mixed-ligand tris-chelates of Cu(II) containing N-*p*-tolylpyridine-2-aldimine; 1,10-phenanthroline and 2,2′-bipyridine as neutral ligands have been carried out and the reactions are pseudo zero order type. *In vitro* antibacterial studies of these complexes against the microorganisms: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enterica ser. typhi* were carried out. The minimum inhibitory concentration values (MIC) show that the activities of most of the complexes are comparable to that of amoxicillin, the reference antibiotic. The very high antibacterial activities of $[CuL^1L^2_2]^{2+}$, $[CuL^3L^2_2]^{2+}$ and $[CuL^2L^1_2]^{2+}$ against *Bacillus subtilis* and *Staphylococcus aureus* suggest that they can be applied for the treatment of infections caused by these organisms.

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