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Electrochemical, Docking and Pharmacological Studies of Co(II), Ni(II), Cu(II) and Zn(II) Mixed Ligand Complexes

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Mixed ligand complexes of Co(II), Ni(II), Cu(II) and Zn(II) with Schiff base derived from the condensation of furfurylidene-4-aminoantipyrine and 2-aminobenzothiazole with 2-aminophenol have been synthesized and characterized by various analytical and spectral techniques. The *in vitro* antimicrobial screening of the ligand and its metal complexes were done. The interaction of the complexes with calf thymus DNA (CT DNA) has been investigated by UV absorption method. In addition, the DNA cleavage activity of the complexes was studied using agarose gel electrophoresis in the presence of H_2O_2 as oxidant. Super oxide dismutase activities of the mixed ligand complexes have also been measured. Cytotoxicity and *in vitro* anticancer studies of the complexes using MTT assay were also done. Molecular docking was used to determine the binding mode of the mixed ligand complexes to glucosamine-6-phosphate synthase.

Keywords: Transition metal (II) complex, Schiff base, Furfurylidene-4-aminoantipyrine, 2-Aminobenzothiazole, Antimicrobial.

INTRODUCTION

The various classes of Schiff bases that can be prepared by condensation of different types of amines and carbonyl compounds are very popular due to diverse chelating ability[1]. Studies on the interaction of metal complexes with biomolecules to design effective chemotherapeutic agents and better anti-cancer drugs are essential in research. Schiff base complexes are important class of metal complexes in medicinal and pharmaceutical fields[2]. Schiff bases derived from aromatic amines and aromatic aldehydes and their metal complexes are widely applicable in inorganic, biological and analytical chemistry[3]. Schiff bases are widely employed as ligands in coordination chemistry. These ligands are readily available, versatile and depending on the nature of the starting materials employed for their preparation, can exhibit various denticities and functionalities[4]. Mixed ligand complexes of transition metals containing ligands with N, S or N, S, O donors are known to exhibit interesting stereochemical, electrochemical and electronic properties[5]. Antipyrine derivatives are also reported to exhibit analgesic and anti-infammatory effects[6-9], antiviral[10], antibacterial[11] activities and also used as hair colour additives[12] and to potentiate the local anesthetic effect of lidocaine[13].

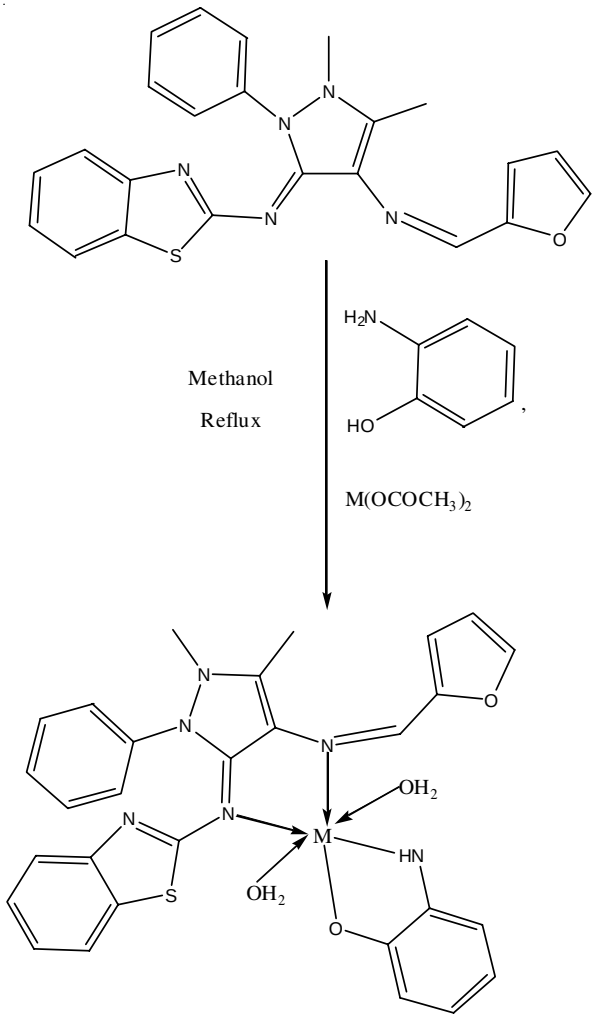
In this paper, we report electrochemical and pharmacological (antimicrobial, DNA binding, DNA cleavage, super oxide dismutase, cytotoxicity and anticancer) activities and molecular docking studies of Co(II), Ni(II), Cu(II) and Zn(II) mixed ligand complexes.

EXPERIMENTAL

The chemicals used were of AnalaR grade, furfuraldehyde, 4-aminoantipyrine, 2-aminophenol and 2-aminobenzothiazole were obtained from Sigma Aldrich. Metal(II) acetates were obtained from Merck and were used as received. The solvents used were purchased from Merck and used without further purification. Cyclic voltammetry measurements were performed using electrochemical analyzer CH instruments electrochemical work station (Model 650 C) using a glassy carbon working electrode (GCE) and Ag/AgCl reference electrode and platinum counter electrode.

Synthesis of mixed ligand complexes: The Schiff base was prepared using the procedure already reported[14]. The complexes were prepared by the following procedure (**Scheme-I**). A methanolic solutions of Schiff base (0.004 mol), Co(II)/Ni(II)/Cu(II)/Zn(II) metal acetates (0.004 mol) and 2-aminophenol(2-ap) (0.004 mol) were taken in 1:1:1 molar ratio and stirred with heating for about 4 h. The resulting mixture is then cooled to room temperature and the solid product formed was filtered, washed with methanol and dried over anhydrous calcium chloride.

Antimicrobial activity: The *in vitro* antibacterial activity of the mixed ligand complexes were tested against the bacterial



Scheme-I: Synthetic route of mixed ligand metal(II) complexes

species *S. aureus*, *E. coli* and *P. aeruginosa* by agar well diffusion method[15]. Initially, the stock cultures of bacteria were revived by inoculating in broth media and grown at 37 ºC for 18 h. The agar plates of media (peptone-10 g, NaCl-10 g and yeast extract 5 g, agar 20 g in 1000 mL of distilled water) were prepared and wells were made in the plate. Each plate was inoculated with 18 h old cultures (100 µL) and spread evenly on the plate. After 20 min, the wells were filled with compounds at 25, 50, 75, 100, 150 and 200 µg/mL concentrations, to determine the minimum inhibitory concentration (MIC) value. The control wells with gentamycin were also prepared. DMSO was used as negative control. All the plates were incubated at 37 ºC for 24 h and the diameter of inhibition zone was noted.

For antifungal activity, the stock cultures were revived by inoculating in broth media and grown at 27 ºC for 48 h. The agar plates of the media (Czapek-Dox agar: composition (g/L) sucrose-30.0; sodium nitrate-2.0; K_2HPO_4-1.0, $MgSO_4.7H_2O$-0.5; KCl-0.5; $FeSO_4$-0.01; agar-20;) were prepared and wells were made in the plate. Each plate was inoculated with 48 h old cultures (100 µL) and spread evenly on the plate. After 20 min, the wells were filled with compound at 50, 100 and 200 µg/mL concentrations, to find out the minimum inhibitory concentration (MIC) value. The control wells were filled with antibiotic. All the plates were incubated at 27 ºC for 72 h and the diameter of inhibition zone was noted.

DNA binding studies: DNA-binding experiments were performed in *tris*-HCl/NaCl buffer (5 mmol L^{-1} *tris*-HCl/50 mmol L^{-1} NaCl buffer pH 7.2) using DMF (10 %) solution of metal complexes. The concentration of CT-DNA was determined from the absorption intensity at 260 nm with ε value of 6600 (mol $L^{-1})^{-1}$ cm^{-1}. Absorption titration experiments were made using different concentrations of CT-DNA, while keeping the complex concentration constant. Correction was made for absorbance of the CT-DNA itself. Samples were equilibriated before recording each spectrum. For metal(II) complexes, the intrinsic binding constant (K_b) was determined from the spectral titration data using the following equation[16].

$$[DNA]/(\varepsilon_a-\varepsilon_f) = [DNA]/(\varepsilon_b-\varepsilon_f) + 1/K_b(\varepsilon_b-\varepsilon_f)$$

where [DNA] is the concentration of DNA in base pairs, the apparent absorption coefficients ε_a, efand ε_b correspond to A_{obsd}/[Complex], the extinction coefficient of the complex when fully bound to equilibrium binding constant in (mol $L^{-1})^{-1}$. Each sample solution was scanned from 200-500 nm.

Cleavage of pUC18 DNA: The DNA cleavage activity of the complexes was studied using agarose gel electrophoresis. pUC18 DNA (0.3 µg) dissolved in 5 mmol L^{-1} *tris*-HCl/50 mmol L^{-1} NaCl buffer (pH 7.2), was treated with the complexes. The mixture was incubated at 37 °C for 1 h and then mixed with the loading buffer (1 µL) containing 25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol. Each sample (0.5 µL) was loaded into 1 % (w/v) agarose gel. Electrophoresis was undertaken for 2 h at 50 V in *tris*-acetate-EDTA (TAE) buffer (pH 8). The gel was stained with ethidium bromide for 5 min after electrophoresis and then photographed under UV light. To enhance the DNA cleavage activity of thecomplexes, hydrogen peroxide (100 µmol L^{-1}, 0.25 µL) was added to each sample.

Super oxide dismutase activity: *in vitro* super oxide dismutase activity was measured using alkaline DMSO as a source of superoxide radical ($O_2^{\bullet-}$) and nitrobluetetrazolium (NBT) as $O_2^{\bullet-}$ scavenger 17.400 mL sample to be assayed was added to a solution containing 2.1 mL of 0.2 M potassium phosphate buffer (pH 8.6) and 1 mL of 56 mM NBT. The tubes were kept in ice for 15 min and then 1.5 mL of alkaline DMSO solution was added at the same time with stirring. The absorbance was then monitored at 540 nm against a sample prepared under similar condition with non alkaline DMSO. A unit of superoxide dismutase [SOD] activity is the concentration of complex or enzyme, which causes 50 % inhibition of alkaline dimethylsulphoxide (DMSO) mediated reduction of nitrobluetetrazolium chloride (NBT).

Cytotoxicity assay: *E. coli* AB 1157, a wild-type strain, proficient to repair damage in the DNA is considered for this study. Initially, the stock culture of bacteria was revived by inoculating in broth medium and grown at 37 ºC for 18 h. The LB Agar plates were prepared and wells were made in the solidified LB agar plate. Each plate was inoculated with 18 h old cultures (100 µL, 10^{-4} cfu) and spread evenly on the plate. After 20 min, the wells were filled with compound at different concentrations. Standard compound plate was also prepared

in the same manner (Media Used: Tryptone-10 g, NaCl-10 g and yeast extract 5 g, agar 20 g in 1000 mL of distilled water). All the plates were incubated at 37 ºC for 24 h and the diameter of inhibition zone was noted[18].

***in vitro* anticancer study (MTT assay):** 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 h of incubation, 15 µL of MTT (5 mg/mL) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4 h. The medium with MTT was then flicked off and the formazan crystals formed were solubilized in 100 µL of DMSO and theabsor-bance wasmeasured at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula[19,20].

% cell Inhibition = 100-Abs (sample)/Abs (control) × 100

Non-linear regression graph was plotted between % Cell inhibition and log concentration and IC_{50} was determined using Graph Pad Prism software.

Molecular docking: Docking is a method which predicts the preferred orientation of a molecule to a protein target in order to predict the affinity and activity of the small molecules towards target. The molecular docking tool, Hex 6.0 interface on the windows 7 operating system was used for docking and scoring. The PDB file of the structure for ligand and complex was done by Chemoffice software. The structure was minimized by using the Gaussian-09 software. The optimized structure was used for molecular docking. The crystal structure of the complex of glucosamine-6-phosphate synthase (PDB ID 1jxa) was downloaded from Protein Data Bank. Crystallographic water molecules were removed from the protein.

RESULTS AND DISCUSSION

The structure of the mixed ligand complexes (Fig. 1) was already reported[21]. They are stable at room temperature and are soluble in DMF and DMSO.

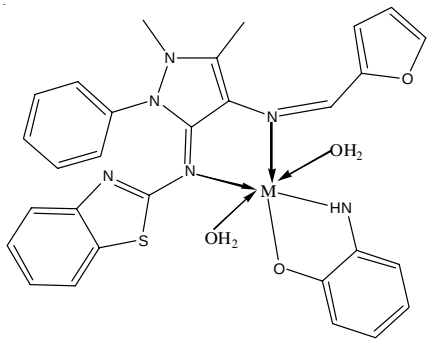


Fig. 1. Proposed structure of mixed ligand metal(II) complexes [where M=Co(II), Ni(II), Cu(II) and Zn(II)]

Electrochemical behaviour: The cyclic voltammograms of the mixed ligand complexes were recorded at room temperature in DMSO solution in the potential range -1 to 1.2 V with scan rate 0.1 V/s.

The cyclic voltammogram of the Co(II) complex shows an irreversible cathodic peak at 0.5 V. The present Ni(II) mixed ligand complex is electrochemically inert and did not show any peaks in the cyclic voltammogram. The Cu(II) complex (Fig. 2) displayed a cathodic peak at 0.25 V *versus* Ag/AgCl with the corresponding anodic peak at 0.45 V on the reverse scan. The peak separation value (ΔEp = 0.20 V) indicates quasi-reversible character for the one electron transfer reaction of metal-based Cu(II)/Cu(I) couple. The Zn(II) complex shows a irreversible[22] anodic peak at 0.65 V.

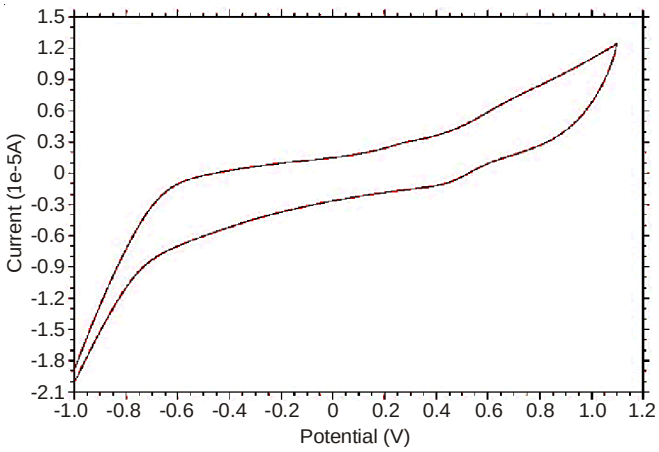


Fig. 2. Cyclic voltammogram of Cu(II) complex

Antimicrobial analysis: The *in vitro* biological screening of ligand and its mixed ligand metal complexes were tested against bacterial species *S. aureus*, *E. coli* and *P. aeruginosa* and fungal species *A. niger*, *A. flavus* and *C. albicans* by agar well diffusion method. The results of the antifungal and antibacterial activities are summarized in Tables 1 and 2. The standards used are gentamycin for antibacterial and amphotericin for antifungal activities. The present study revealed that the complexes possess higher growth inhibition potential. Among the mixed ligand metal(II) complexes, Cu(II) complex exhibited higher antimicrobial activity. The antimicrobial activity of the metal complexes increases with increase in concentration of the complexes. Increased activity of the metal complexes can be explained based on the Overtone's concept and Tweedy's chelation theory[23].

TABLE-1
ANTIFUNGAL ACTIVITY OF MIXED LIGANDMETAL(II) COMPLEXES (µg/mL)

Compound	*A. niger*	*A. flavus*	*C. albicans*
Co(II) complex	50	75	50
Ni(II) complex	50	100	100
Cu(II) complex	50	25	50
Zn(II) complex	50	150	75
Amphotericin	25	100	25

TABLE-2
ANTIBACTERIAL ACTIVITY OF MIXED LIGAND METAL(II) COMPLEXES (µg/mL)

Compound	*E. coli*	*P. aeruginosa*	*S. areus*
Co(II) complex	100	150	200
Ni(II) complex	150	150	200
Cu(II) complex	75	50	100
Zn(II) complex	200	200	150
Gentamycin	100	200	50

DNA binding studies: UV absorption spectroscopy is an effective method to examine the binding mode of DNA with compounds. Hyperchromic and hypochromic effects are the spectral features of DNA concerning its double helical structure. The spectral changes will give clue about changes in DNA in its conformation and structure after the drug bound to DNA[24]. Here the Co(II) complex exhibited hypochromism (17 %) in the absorption band at 380 nm and bathochromism of 16 nm. The absorption band of Ni(II) complex at 312 nm exhibited hypochromism of 15 % and bathochromism of 3 nm. The absorption band of Cu(II) complex at 315 nm exhibited hypochromism of 12 % and bathochromism of 7 nm and the Zn(II) complex at 284 nm exhibited hypochromism of 14 % and bathochromism of 2 nm. These results suggest that the mixed ligand complexes interacted with DNA through intercalation. The intrinsic binding constant (K_b) values were also calculated (1.1×10^5, 1.3×10^5, 2.2×10^5 and 1.6×10^5 M^{-1} for the Co(II), Ni(II), Cu(II) and Zn(II) complexes, respectively).

Cleavage of pUC18 DNA: DNA cleavage is controlled by relaxation of supercoiled circular form of pUC18 DNA into nicked circular form and linear form. When circular plasmid DNA is subjected to electrophoresis, the fastest migration will be observed for the supercoiled form (Form I). If one strand is cleaved, the supercoils will relax to produce a slow moving open circular form (Form II). If both strands are cleaved, a linear form (Form III) will be generated that migrates in between[25]. The cleavage efficiency of the mixed ligand complexes was measured by determining the ability of the complex to convert the super coiled DNA into nicked open circular form or sheared form. Control experiments using DNA alone do not show any significant cleavage of pUC18 DNA. The Cu(II) complex completely cleaved the DNA (Fig. 3). The other complexes exhibited enhanced cleavage activity than ligand in the presence of H_2O_2. The order of activity of the mixed ligand complexes is given as Cu(II) > Co(II) > Zn(II) > Ni(II).

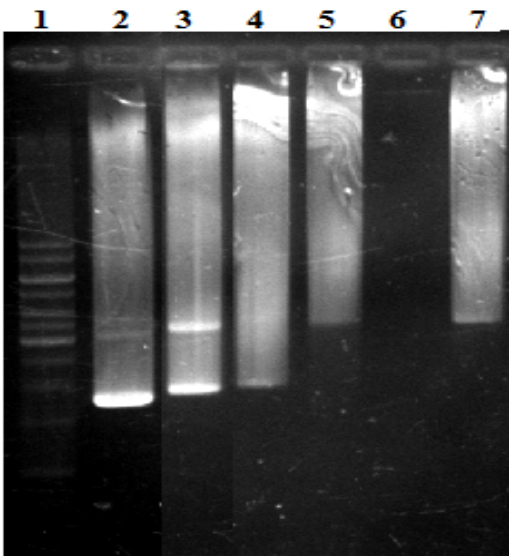


Fig. 3. Gel electrophoresis showing the chemical nuclease activity of the pUC18 DNA incubated at 37 °C for a period of 1 h, of complex in the presence of H_2O_2(100 mM) as an oxidizing agent: lane 1, Marker DNA; lane 2, Control DNA ; lane 3, pUC18 DNA + H_2O_2 + Ligand; lane 4, pUC18 DNA + H_2O_2 + Co(II) complex; lane 5, pUC18 DNA + H_2O_2 + Ni(II) complex; lane 6, pUC18 DNA + H_2O_2 + Cu(II) complex; lane 7, pUC18 DNA + H_2O_2 + Zn(II) complex

Super oxide dismutase activity: The super oxide dismutase mimetic activities of the present complexes were examined by the NBT assay. It has been established that the reaction of super oxide dismutase and synthetic metal systems that have super oxide dismutase-like activity involves a two-step reaction[26].

$$M_{ox} + O_2^{\bullet -} \rightarrow M_{red} + O_2$$

$$M_{red} + O_2^{-} + 2\ H^+ \rightarrow M_{ox} + H_2O_2$$

The observed IC_{50} values of present complexes are compared with various reported complexes. All the complexes have moderate activity among them Cu(II) complex have higher activity. The present mixed ligand complexes show (Fig. 4) the following (Table-3) order of superoxide radical scavenging activities; Cu(II) > Zn(II) > Ni(II) > Co(II).

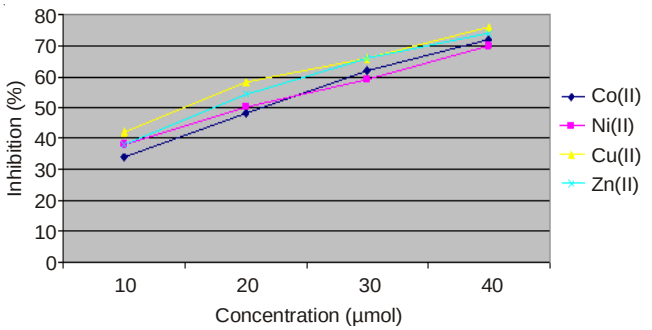


Fig. 4. Super oxide dismutase activity of mixed ligand metal(II) complexes

TABLE-3
SUPER OXIDE DISMUTASE ACTIVITY OF MIXED LIGAND METAL(II) COMPLEXES

Compound	IC_{50}(micro mol)
Co(II) complex	20
Ni(II) complex	20
Cu(II) complex	15
Zn(II) complex	16
Native enzyme	0.04

Cytotoxicity assay: *E. coli* AB 1157, proficient to repair damage in the DNA is considered for cytotoxicity assay. Stannous chloride was taken as standard compound. The results (Table-4) show that the Co(II) and Ni(II) mixed ligand complexes are more toxic than the other metal(II) complexes. In the present study the Cu(II) complex does not show any cytotoxicity against *E. coli* AB 1157, whereas Zn(II) complex exhibited moderate cytotoxicity.

***in vitro* Anticancer studies:** Anticancer activity of newly synthesized mixed ligand metal complexes was investigated on human cervical carcinoma (HeLa) cells by MTT assay and the results are expressed in terms of IC_{50} values. The complexes were applied in the concentration range 0.1-100 µM. The data obtained by the MTT assay show that the Cu(II) mixed ligand complex has very good inhibitory (0.8 µM) effect on the growth of human cervical carcinoma (HeLa) cells. The Zn(II) complex possesses moderate (68.81 µM) growth inhibition activity. Table-5 illustrates the IC_{50} values of the complexes tested. The Co(II) and Ni(II) complexes have lowest growth inhibitory activity against HeLa cells (> 200 µM).

Molecular docking: Cavities were detected and depending on this, particular constraint was created within which docking takes place. Docking was performed with default

TABLE-4
CYTOTOXICITY OF MIXED LIGAND METAL(II) COMPLEXES

Compounds	Diameter of inhibition zones (cm)						MIC (μg/mL)
	62.5 (μg/mL)	125 (μg/mL)	250 (μg/mL)	500 (μg/mL)	1000 (μg/mL)	2000 (μg/mL)	
Co(II) complex	-	-	-	0.6	0.9	1.1	500
Ni(II) complex	-	-	-	0.2	0.4	0.6	500
Cu(II) complex	-	-	-	-	-	-	-
Zn(II) complex	-	-	-	-	0.6	1.3	1000
*Stannous chloride	-	-	0.3	0.5	0.8	1.5	250

TABLE-5
ANTICANCER ACTIVITY OF MIXED LIGAND METAL(II) COMPLEXES

Compound	IC_{50} (μM^{-1})
Co(II) complex	462.8
Ni(II) complex	264.2
Cu(II) complex	0.80
Zn(II) complex	68.81

settings to obtain a population of possible confirmations and orientations for the ligands at the bonding sites. The structural analysis of docked structures gave significant details about the binding pattern of these complexes. E-total values of docked metal complexes were determined (Table-6).

TABLE-6
DOCKING SCORES OF MIXED LIGAND METAL(II) COMPLEXES

Compound	Docking score	Active Sites	No. of hydrogen bonds
Co(II) complex	-175.79	216-ALG, 255-GLU	2
Ni(II) complex	-173.13	178-ILE, 213-TYR,	2
Cu(II) complex	-176.14	421-GYS 420-SER, 523-ASN	3
Zn(II) complex	-175.79	524-GLU, 520-LEU	2

According to the docking studies, the best score was obtained for the Cu(II) complex. The results showed that the Cu(II) complex have the ability to act as anticancer agent. It was predicted from docking studies that Cu(II) complex adopt an acceptable confirmation with in the active site of PDB ID1jxa and significant binding interactions have well been noticed from the Fig. 5. There is correlation between the theoretical and experimental values obtained. The results obtained are in accordance with the antimicrobial results. Molecular docking of the complex formed between the ligand and the protein indicated that the ligand is well positioned in the gorge. The docking experiments resulted in energy minimized docked structures. The residues that have been reported to be involved in protein-ligand interactions are described in Table-6. The number of hydrogen bonds formed is found to be maximum in the Cu(II) complex.

Conclusion

Mixed ligand Co(II), Ni(II), Cu(II) and Zn(II) complexes with Schiff base derived from furfurylidene-4-aminoantipyrine and 2-aminobenzothiazole with 2-aminophenol have been synthesized. Electrochemical studies gave information about the electrochemical behaviour of the mixed ligand complexes.

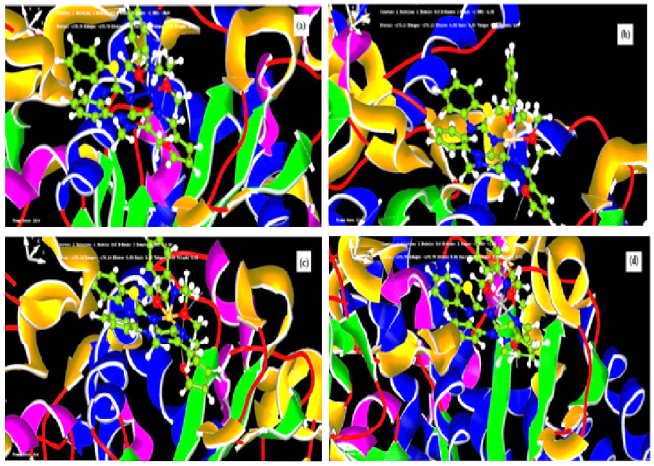


Fig. 5. Molecular docking results of (a) Co(II), (b) Ni(II), (c) Cu(II) and (d) Zn(II) complexes

Among the complexes Cu(II) complex have higher antimicrobial activity. The complexes show significant super oxide dismutase activity. The mixed ligand complexes interacted with DNA through intercalation. The Cu(II) mixed ligand complex completely cleaved the DNA and other mixed ligand complex exhibited moderate DNA cleavage activity. Cytotoxicity assay for the mixed ligand metal(II) complexes were also conducted. The Cu(II) complex exhibited very good *in vitro* anticancer activity. The biological activity of the mixed ligand complexes were further confirmed by molecular docking studies.

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