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Synthesis of Salicylic Acid Based Mixed Ligand Complexes: Their Protein Binding Affinities and Antimicrobial Activities

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Present study is based upon synthesis, characterization, bovine serum albumin binding interactions and antimicrobial studies of mixed ligand complexes of zinc with salicylic acid or 3,5-dinitrosalicylic acid as primary ligand and 1,10-phenanthroline or 2,2-bipyridyl as secondary ligand. These complexes are characterized by UV-visible, IR and elemental analysis to determine the mode of binding between metal and ligand. The interaction of these complexes with protein molecules were studied by determining the binding constants from interaction with bovine serum albumin using UV-visible titration techniques. The antimicrobial activities of these complexes have been tested against bacteria and fungi. It has been observed that the above mentioned complexes show considerable antimicrobial activity compared to the free ligands.

Keywords: Mixed ligand complexes, Salicylic acid, 3,5-Dinitrosalicylic acid, Bovine serum albumin, Antimicrobial activity.

INTRODUCTION

Mixed ligand complexes of transition metals are an important research area in metal based drug chemistry [1]. The mixed ligand complex of Zn(II) and Ni(II) with D-pencilamine and L-cysteine having therapeutical and biological activity is also reported [2]. In recent years, these complexes have played an important role in the development of coordination, pharmaceutical, biological, medicinal chemistry and have been used to cure various human diseases like diabetes, cancer, infections, *etc*. by increasing the therapeutical action of drugs [3,4]. Therefore transition metal complexes provide a great platform for the designing and synthesis of new complexes which shows considerable antimicrobial properties.

Zinc is the second most essential element in human metabolism which is present either at the active sites or as structural components of more than three hundred enzymes [4]. Mixed ligand Zn complexes are of great interest due to their numerous biological activities as zinc is competent of binding to 925 different human proteins [5,6]. Furthermore, Zn(II) complexes containing nitrogen, oxygen and sulphur as ligands are less toxic and show considerable antitumor properties. Literature review has cited that Zn(II) complexes can be used to treat anaphylactic shocks by inhibiting the elimination of histaminic parts either from base cell or from extra cellular fluids [7].

β-Hydroxy acids (*e.g.* salicylic acid, 3,5-dinitrosalicylic acid, *etc*.) and their compounds are used to cure various disorders like acne, psoriasis, seborrhoeic dermatitis *etc*. [8]. Their complexes are known to show diverse pharmaceutical activities such as antibacterial, fungicidal, antimicrobial, anti-inflammatory *etc*. 1,10-phenanthroline and 2,2-bipyridyl are nitrogen containing biochemically active chiral ligands which are bidentate. They are rigid, hydrophobic and planar system through which they can coordinate several metal ions and form very stable chelates. Transition element complexes with 1,10-phenanthroline and 2,2-bipyridyl show numerous anticarcinogenic, antiviral, antifungal and antibacterial activities [9,10].

In human circulatory system, serum albumin, a protein transporter, contributes approximately one half of the blood serum proteins [11]. It possess three structurally distinct domains *i.e.* I, II, III and two sub domains *i.e.* A and B [12,13]. Its single polypeptide chain contains 585 amino acids. By binding with small organic complexes and ligands, it is able to control the pharmokinetic properties of the drug [14]. In order to determine the free concentration, metabolism, distribution, toxicity and dynamics of a drug in blood, the interaction and binding ability of a drug with serum albumins are determined at the molecular level. Among human serum albumin (HSA), bovine serum albumin is well studied due to its close resemblance with human serum albumin, easy availability, low cost and medicinal importance [15-18].

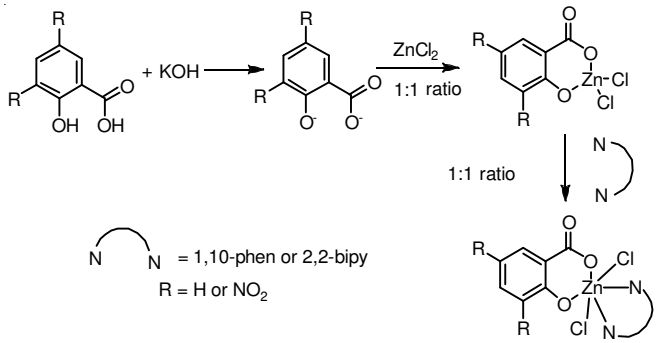
Keeping in view the above observations, present work focuses on the synthesis and characterization of new mixed ligand complexes of zinc with salicylic acid or 3,5-dinitrosalicylic acid as primary ligand and 1,10-phenanthroline or 2,2-bipyridyl as secondary ligand. These complexes are then screened against pathogenic bacteria and fungi. All the complexes show better antimicrobial activity than the parent ligands. This may be due to the chelation effect. This is in further enhancement of the unremitting interest in deployment of the mixed ligand complexes of transition metals to cure various human ailments, against the raising issues of infectious problems caused by multi-drug resistant microorganisms, across the globe.

EXPERIMENTAL

Salicylic acid, 3,5-dinitrosalicylic acid, 1,10-phenanthroline, 2,2-bipyridyl, *tris* buffer, zinc chloride, potassium hydroxide were purchased from Loba chemie. Bovine serum albumin was obtained from SDFCL. *Tris* buffer solution was prepared using double distilled water.

Physical measurements: UV-visible absorption spectra were recorded on a Shimadzu UV2600 (200-800 nm). IR spectra were obtained with KBr pellets on a Nicolet Shimadzu FT-IR spectrometer in the range of 4000-400 cm^{-1}. The experiments were carried out at room temperature. Elemental analysis (for carbon, hydrogen and nitrogen content) was done at Punjab Agricultural University, Chandigarh, India. Melting points were recorded on an open capillary tube and were uncorrected. The antimicrobial properties were measured by disc diffusion method.

Preparation of metal complexes: The general scheme for the preparation of the complexes is given below [19]:



Scheme-I

Preparation of complex of zinc with salicylic acid and 1,10-phenanthroline [Zn(sal)(phen)Cl_2] (1): 0.552 g (0.004 mol) of salicylic acid and 0.22 g (0.004 mol) of KOH was dissolved separately in methanol (25 mL), two solutions were mixed and allowed to stir for 15 min. To this solution was added 0.545 g (0.004 mol) of $ZnCl_2$ in 20 mL methanol and 5 mL water solvent mixture. The reaction mixture was refluxed for 2 h with constant stirring at 50 °C. A 20 mL methanolic solution of 0.792 g (0.004 mol) 1,10-phenanthroline was added to reaction mixture and the solution mixture was refluxed for 3 h with constant stirring. The precipitates formed were filtered, washed and dried in desiccator for 3-4 days.

Preparation of complex of zinc with 3,5-dinitrosalicylic acid and 1,10-phenanthroline [Zn(3,5-DNSA)(phen)Cl_2] (2): 0.912 g (0.004 mol) of 3,5-dinitrosalicylic acid and 0.22 g (0.004 mol) of KOH separately in methanol (25 mL), two solutions were mixed and allowed to stir for 15 min. To this solution was added 0.545 g (0.004 mol) of $ZnCl_2$ in 20 mL methanol and 5 mL water solvent mixture. The reaction mixture was refluxed for 3 h with constant stirring at 50 °C. A 20 mL methanolic solution of 0.792 g (0.004 mol) 1,10-phenanthroline was added to reaction mixture and the solution mixture was refluxed for 3 h with constant stirring. The precipitates formed were filtered, washed and dried in desiccator for 3-4 days.

Preparation of complex of zinc with salicylic acid and 2,2-bipyridyl [Zn(sal)(bipy)Cl_2] (3): 0.552 g (0.004 mol) of salicylic acid and 0.22 g (0.004 mol) of KOH was dissolved separately in methanol (25 mL), the two solutions were mixed and allowed to stir for 15 min. To this solution was added 0.545 g (0.004 mol) of $ZnCl_2$ dissolved in 20 mL methanol and 5 mL water solvent mixture. The reaction was refluxed for 3 h with constant stirring at 50 °C. A 20 mL methanolic solution of 0.625 g (0.004 mol) of 2,2-bipyridyl was added to it and the solution mixture was refluxed for 3 h with constant stirring. The precipitates formed were filtered, washed and dried in desiccator for 3-4 days.

Preparation of complex of zinc with 3,5-dinitrosalicylic acid and 2,2-bipyridyl [Zn(3,5-DNSA)(bipy)Cl_2] (4): 0.912 g (0.004 mol) of 3,5-dinitrosalicylic acid and 0.22 g (0.004 mol) of KOH was dissolved separately in methanol (25 mL), the two solutions were mixed and allowed to stir for 15 min. To this solution was added 0.545 g (0.004 mol) of $ZnCl_2$ dissolved in 20 mL methanol and 5 mL water solvent mixture. The reaction was refluxed for 3 h with constant stirring at 50 °C. A 20 mL methanolic solution of 0.625 g (0.004 mol) of 2,2-bipyridyl was added to it and the solution mixture was refluxed for 3 h with constant stirring. The precipitates formed were filtered, washed and dried in desiccator for 3-4 days.

Antimicrobial activities: The antibacterial and antifungal activities of the complexes were investigated using cup plate method or diffusion method. The stock concentration was made 1 mg/mL by dissolving 10 mg of sample in 10 mL of the solvent (DMSO). All samples and standards were prepared in the same way. Gentamycin and fluconazole were used as standard. The melted culture media (soya bean casein digest agar media for bacteria and potato dextrose agar media for fungus) was placed in test tube (15 mm × 125 mm). The tubes were sterilized by autoclaving and the culture media was allowed to solidify. By using this media slants the microorganisms like *Staphylococcus aureus* and *Aspergillus niger* are sub cultured from the stock culture. Subculture tubes were taken and then heated the inoculating loop in flame until nichrome wire was hot and then allowed it cool for few min. The cotton plug had been removed at a time from the subculture tube and inoculating medium and simultaneously considering minimum time available for inoculums. To avoid exposure to microbial contamination the sterile inoculating loop was inserted into the stock culture and small amount of bacteria was removed. This inoculum was transferred to inoculated slant. The necks of the tube were close with the respective caps. After inoculation the inoculating loop was heated till red hot. Slants were incubated for 24 h at 30-37 °C.

A small quantity of culture media (5-10 mL) was taken in a test tube. With the help of inoculating loop, the standard culture of microorganisms was transferred into culture media by scrapping the culture. The entire process has been performed in aseptic condition.

In the laminar chamber the autoclaved homogenous suspensions of culture media was poured into petri dishes and allowed to solidify. By using sterile cavity borer, wells were made properly to enable the introduction of the test sample, standard and blank as a control precisely. The concerned samples were introduced into appropriate wells with the help of micropipette; all the cups were filled with equal volumes of sample. Then the plates were allowed to incubate for a time period of 18-24 h. The zone of inhibition was examined and measured with the help of antibiotic zone reader [20,21].

RESULTS AND DISCUSSION

The physico-analytical data of the synthesized complexes are given in Table-1. All the mixed ligand complexes were non-hygroscopic, thermally stable and insoluble in water and most common organic solvents but fairly soluble in DMF, DMSO and Tris buffer (pH 7.2).

UV-visible: The UV absorptions occur in the range of 260-370 nm at low concentration in DMSO. These bands indicates $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ transitions which confirm binding of 2,2-bipyridyl and 1,10-phenonthroline with metal center of Zn(II). Zinc did not show *d-d* transitions as it is a d^{10} system.

IR spectra: The significant peak of carbonyl stretch near 1625 cm^{-1} as compared to 1670 cm^{-1} in the free salicylic acid molecule showed the bonding nature of carboxylic acid group (Table-2). The vibrational stretching peak due to –OH is absent in the complexes which appears at 3270 cm^{-1} in free salicylic acid. The absorption bands at 710-670 cm^{-1} are due to the vibration of 3-phenonthroline ring and C-H stretching frequency. The absorption bands at 1600-1530 cm^{-1} are due to skeleton vibrations of pyridyl ring of 2,2-bypyridyl. The absorption bands near 1597-1580 cm^{-1} are due to-COO-asymmetric and symmetric vibrations. The characteristic absorption bands at 560-410 cm^{-1} suggest coordination (Zn-N and Zn-O bonds) of both nitrogen and oxygen atoms to metal center in the complex (Table-2).

UV-visible absorption studies of bovine serum albumin: The solution of the complex and bovine serum albumin were prepared in 0.1 M *tris* buffer solution of pH 7.4. Double distilled water was used to carry out the study. The UV-visible spectra were observed after titrating fixed concentration of metal complex (50 μM) with incremental amount of bovine serum albumin in the range of 0-200 μM. The intensity of bands increases with increase in concentration of bovine serum albumin. The binding constants were determined from the bovine serum albumin-complex titration graphs by plotting $1/(A-A_0)$ *versus* 1/[BSA] concentrations (Figs. 1 and 2), where A_0 is the initial absorption band for free complex and A is the absorption band for different complex-bovine serum albumin concentrations. The value of binding constants comes out to be 10^6 M^{-1}. It has been assumed that the interaction between the ligand L and the substrate S is 1:1; for this reason a single complex S_L (1:1) is formed.

The relationship between the observed absorbance change per centimeter and the system variables and parameters is as follow:

$$\frac{\Delta A}{b} = \frac{S_t K_{11} \Delta\varepsilon_{11}[L]}{1 + K_{11}[L]} \quad (1)$$

where $\Delta A = A-A_o$, S_t is total concenteration of substrate,

$$\Delta\varepsilon_{11} = \varepsilon_{11} - \varepsilon_S - \varepsilon_L$$

here ε_{11} is the molar absorptivity of the complex, ε_s is the molar absorptivity of the substrate, ε_L is the molar absorptivity of the ligand.

From the mass balance expression $S_t = [S] + [SL]$, we get $[S] = S_t/(1 + K_{11}[L])$; where [S] is the concentration of the uncomplexed substrate, [L] is the concentration of the uncomplexed ligand, [SL] is the concentration of the complex.

TABLE-1
PHYSICAL AND ANALYTICAL DATA FOR THE COMPLEXES

Complex	m.f.	Colour	m.p. (°C)	Yield (%)	Elemental analysis (%): Calcd. (Found)		
					C	H	N
$[Zn(sal)(phen)Cl_2]$	$C_{19}H_{12}N_2O_3ZnCl_2$	Dull white	176.0	79	50.42 (50.57)	2.67 (2.68)	6.19 (6.27)
$[Zn(3,5\text{-}DNSA)(phen)Cl_2]$	$C_{19}H_{10}N_4O_7ZnCl_2$	Brownish yellow	199.5	81	42.06 (42.57)	1.86 (2.95)	10.33 (10.43)
$[Zn(sal)(bipy)Cl_2]$	$C_{17}H_{12}N_2O_3ZnCl_2$	White	230.0*	84	47.64 (47.73)	2.82 (2.98)	6.54 (6.65)
$[Zn(3,5\text{-}DNSA)(bipy)Cl_2]$	$C_{17}H_{10}N_4O_7ZnCl_2$	Brownish yellow	> 300	86	39.37 (39.57)	1.94 (2.06)	10.80 (10.97)

*Starts decomposing

TABLE-2
SELECTED IR (cm^{-1}) AND UV (nm) DATA OF THE SYNTHESIZED COMPLEXES

Complex	ν(C=O) (cm^{-1})	ν(Zn-N) (cm^{-1})	ν(Zn-O) aryl (cm^{-1})	ν(Zn-O) carboxylic (cm^{-1})	ν(COO) (cm^{-1})	C_6H_5 stretch (cm^{-1})	ν(NO_2) of 3,5-DNSA (cm^{-1})	$n\rightarrow\pi^*$ transition (nm)	$\pi\rightarrow\pi^*$ transition (nm)	Aromatic ring vibration (cm^{-1})
$[Zn(sal)(phen)Cl_2]$	1625	472	503	420	1516	3057	–	–	265, 292	1640
$[Zn(3,5DNSA)(phen)Cl_2]$	1622	490	507	423	1581	3051	1518	364	291	1656
$[Zn(sal)(bipy)Cl_2]$	1627	412	560	–	1598	3031	–	–	281	1598
$[Zn(3,5DNSA)(bipy)Cl_2]$	1621	493	544	418	1597	3030	1525	365	278	1597

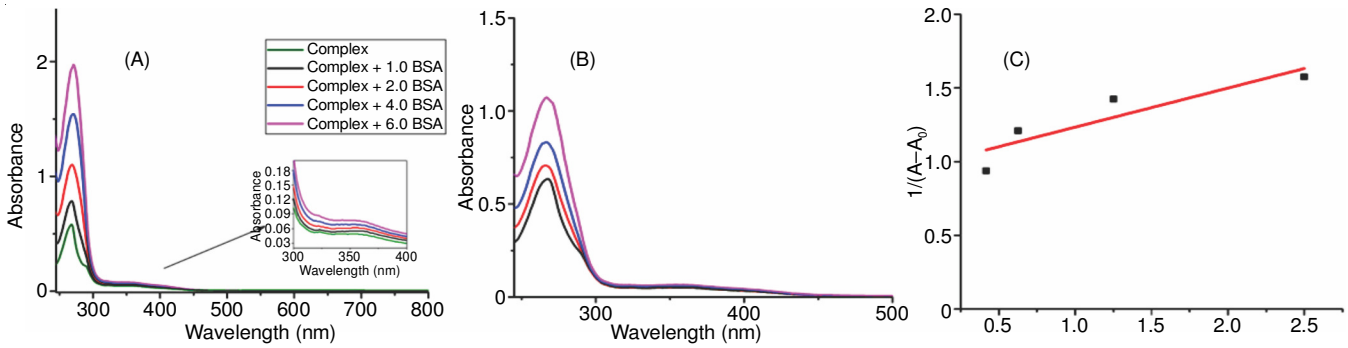


Fig. 1. (A) UV-visible titration curves of $[Zn(3,5\text{-}DNSA)(phen)Cl_2]$ complex (50 μM) *vs.* [BSA] concentration (0-200 μM), (B) Plot of complex of bovine serum albumin with $[Zn(3,5\text{-}DNSA)(phen)Cl_2]$ after subtracting signal for the corresponding [BSA] concentration, (C) The plot of $1/(A\text{-}A_0)$ *vs.* 1/[BSA] concentration, where A_0 is the initial absorption band of free complex and A is the recorded absorption at different complex-[BSA] concentrations

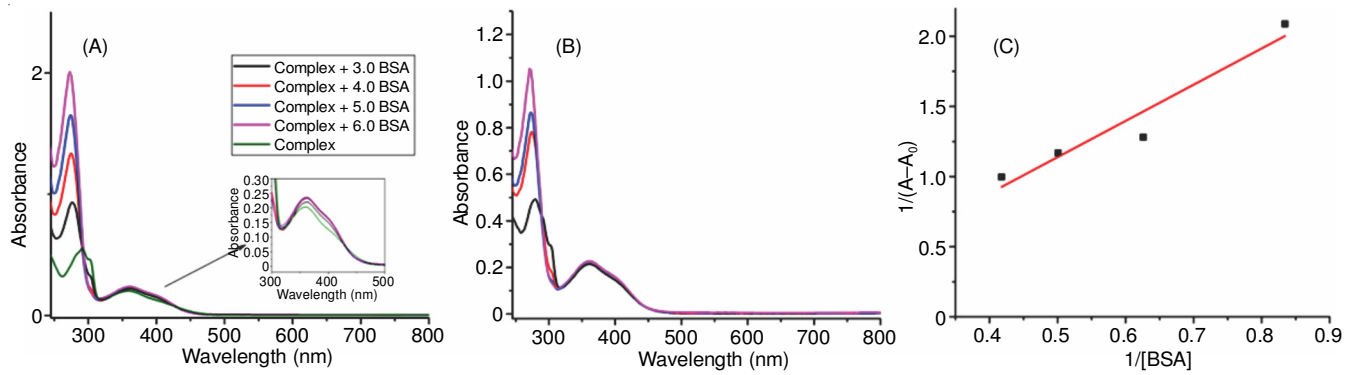


Fig. 2. (A) UV-visible titration curves of $[Zn(3,5\text{-}DNSA)(bpy)Cl_2]$ complex (50 μM) *vs.* [BSA] concentration (0-200 μM), (B) Plot of complex of bovine serum albumin with $[Zn(3,5\text{-}DNSA)(bpy)Cl_2]$ after subtracting signal for the corresponding [BSA] concentration (C) The plot of $1/(A\text{-}A_0)$ *vs.* 1/[BSA] concentration, where A_0 is the initial absorption band of free complex and A is the recorded absorption at different complex-[BSA] concentrations

Eqn. 1 is the binding isotherm, which shows the hyperbolic dependence on free ligand concentration. The double-reciprocal form of plotting the rectangular hyperbola $1/y = f/d \times 1/x + e/d$ is based on the linearization of eqn. 1 according to the following equation:

$$\frac{b}{\Delta A} = \frac{1}{S_t K_{11} \Delta\varepsilon_{11}[L]} + \frac{1}{S_t \Delta\varepsilon_{11}} \quad (2)$$

Thus, the double reciprocal plot of $1/\Delta A$ *versus* 1/[L] is linear and the binding constant can be estimated from the following equation [22-27].

$$K_{11} = \frac{\text{Intercept}}{\text{Slope}}$$

The comparison of the study of the complexes against bacteria and fungi shows that the complexes show better anti-fungal than antibacterial activity (Table-3).

TABLE-3
ANTIMICROBIAL ACTIVITIES AGAINST *Aspergillus niger* AND *Staphylococcus aureus* AT 1 mg/mL CONCENTRATION

Name of complex	Diameter of zone (mm)	
	Antifungal	Antibacterial
DMSO as blank	–	–
$[Zn(sal)(phen)Cl_2]$	13	20
$[Zn(3,5\text{-}DNSA)(phen)Cl_2]$	15	21
$[Zn(sal)(bipy)Cl_2]$	10	14
$[Zn(3,5\text{-}DNSA)(bipy)Cl_2]$	13	13
Fluconzole as standard	16	–
Gentamycin as standard	–	32

Conclusion

In the present study, four new complexes of zinc with salicylic acid or 3,5-dinitrosalicylic acid as primary ligand and 1,10-phenanthroline or 2,2-bipyridyl as secondary ligand have been reported. These complexes $[Zn(sal)(phen)Cl_2]$, $[Zn(3,5\text{-}DNSA)(phen)Cl_2]$, $[Zn(sal)(bipy)Cl_2]$, $[Zn(3,5\text{-}DNSA)(bipy)Cl_2]$ are characterized by UV-visible, IR and elemental analysis to determine the mode of binding between metal and ligand. The C-H, C=O, M-N and M-O peaks in the IR spectra confirm that the complexes have been synthesized. The binding of phenanthroline and 2,2-bipyridyl to the metal complex is confirmed by $n\rightarrow\pi$ and $\pi\rightarrow\pi^*$ transitions in the UV spectra. The interaction of these complexes with protein molecules were studied by determining the binding constants from interaction with bovine serum albumin using UV-visible titration techniques. All the results indicated that the complexes bind moderately with binding constant value in the range of $10^6\ M^{-1}$. It is important to note here that the low affinity binding is consistent with the role of serum proteins as carrier molecules for the delivery of the parent drug and its derivatives to target tissues. The antimicrobial activities of these complexes have been tested against bacteria and fungi. It has been observed that aforesaid complexes show considerable antifungal activity than antibacterial activities.

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