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In-vitro antibacterial activity of Ni(II), Cu(II), and Zn(II) complexes incorporating new azo-azomethine ligand possessing excellent antioxidant, anti-inflammatory activity and protective effect of free radicals against plasmid DNA

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ABSTRACT

Azo Schiff base ligand 2-hydroxy-3-methoxy-5-(tolyldiazenyl)benzaldehyde oxime (HL¹) and 2-hydroxy-3-methoxy-5-(methoxyphenyl)benzaldehyde oxime (HL²) were prepared along with their transition metal complexes of Ni(II), Cu(II), and Zn(II). Ligands and their metal complexes were characterized by several analysis techniques. *In- vitro* antibacterial, antioxidant and anti-inflammatory activities of synthesized ligands and their metal complexes have been studied. Biological study showed that amongst all the synthesized compounds, Cu(II) complexes possessed excellent antibacterial activity than standard antibiotic Chloramphenicol. Ligands (HL¹) and (HL²) showed excellent antioxidant as well as anti-inflammatory activity. Both the ligands were tested for their protective effect of free radicals against plasmid DNA and it was found that both the ligands showed good DNA nicking activity.

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Antibacterial; antiinflammatory; antioxidant; azo-Schiff base; DNA fragmentation; metal complexes

GRAPHICAL ABSTRACT



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Introduction

The tendency of azo-compounds as active biological agents allowed them to be used in the treatment of diseases such as antitumor, antibacterial, antiseptics, and antineoplastics.^[1] Azo compounds are versatile and have received much attention in the field of fundamental and applicative research.^[2] Azo-azomethine dyes are being increasingly used in the textile, leather, and plastic industries.^[3] Esin et al. synthesized series of azo-linked salicylidenic Schiff bases and their metal complexes which has importance in the field of medicinal chemistry.^[4]

Furthermore, azo compounds also showed a variety of biological activities including antifungal,^[5] pesticidal,^[6] antiviral and anti-inflammatory activities.^[7] In addition, Schiff base derivatives along with biological activities also showed a variety of pharma-cological activities such as antidepressant,^[8] cytotoxic,^[9] analgesic,^[10] anti-HIV,^[11] anti-convulsant^[12] and anticancer.^[13] Siham et al. synthesized azo and imidazole containing ligand that showed optical as well as antifungal and antioxidant properties.^[14]

Over the years, various metal-containing compounds significantly used in various biological applications, especially anticancer and antimalarial therapy. However, less attention has been given to the synthesis of metal-based antibacterial drugs.^[15,16] Hence, research and development for metal-containing antibacterial compounds are required as it is expected that metal-containing drugs may be helpful to overcome the development of antibiotic resistance.^[15,17] However, d-block metals have been used for synthesis of novel metal-based drugs.^[17] For example, Cu complexes have been reported to be effective anticancer agents.^[18–25]

Present work deals with, synthesis of new azo Schiff base ligands and their Ni(II), Cu(II), and Zn(II) metal complexes. These compounds were then tested for their *In-vitro* antibacterial, antioxidant, anti-inflammatory activity and protective effect of free radicals against plasmid DNA of bacteria have been studied.

Results and discussion

Azo-Schiff base ligands $(HL^1 \text{ and } HL^2)$ and their Cu(II), Ni(II), and Zn(II) metal complexes were synthesized through the following route.

IR data

IR spectral study of ligands HL^1 and HL^2 along with their metal complexes were done in the range of 400–4000 cm⁻¹ to determine the characteristic vibration bands for the different functional groups present in the molecule. All IR spectra were shown comparatively in Figures S1 and S2. In the spectra of ligands (HL^1 and HL^2) the phenolic v(O–H) stretching was observed between the range of 3440–3351 cm⁻¹ as broadband. In the range of 2974–2836 cm⁻¹ aliphatic v(C–H) vibrations were observed for both the ligands. The azo group v(N=N) stretching were observed at 1455–1453 cm⁻¹ for the ligands. The sharp peak in the range of 1579–1578 cm⁻¹ of the v(C=N) bond vibrations was observed for both the ligands.^[26] For the metal complexes phenolic v(O–H) stretching was disappeared due to deprotonation of hydroxyl hydrogen and binding of oxygen atom with metal ion. Whereas (N–OH) bond frequency in the metal complexes of Ni(II), Cu(II), and Zn(II) shifted to higher value than their respective ligands as shown in Table S1. Aliphatic v(C–H) vibrations were observed in the range of 2991–2912 cm⁻¹ for all the metal complexes. Azo group v(N=N) stretching for all the complexes as compared to ligands were shifted to lower values in the range of 1420–1404 cm⁻¹. Also the v(C=N) bond vibrations for metal complexes shifted to higher frequencies 1649–1641 cm⁻¹ compared to ligands as shown in Table S1.^[27] The metal-oxygen bonds are observed in the range of 560–500 cm⁻¹, whereas metal-nitrogen bonds were observed in the range of 440–400 cm⁻¹.^[28]

TGA analysis

Thermogravimetric analysis of ligands and their metal complexes was done in an inert atmosphere of nitrogen at 20–1000 °C temperature range. The ligand HL^1 and HL^2 start to degrade in the temperature range of 180–190 °C and continues to decompose at 310–330 °C. From the thermogram it was observed that Ni(II), Cu(II) and Zn(II) metal complexes have more thermal stability than the free ligands, as shown in Table S2. In the thermogram no peak was observed for the loss of water molecule up to 210 °C, it means that there is no water in the coordination sphere of the metal complexes. After decomposition of metal complexes at higher temperature metal oxides remains as final residue.^[29] All the thermogram were shown in Figures S3 and S4.

UV-Visible study

All the UV-Visible spectra of synthesized ligands and their metal complexes were recorded in DMSO solvent (10^{-5} M) . Due to the presence of azo group in the compounds band at 373–384 nm for HL¹ and HL² was observed and it corresponds to π - π * transition.^[30,31] The broad absorption band due to the keto-amine transformation of these compounds in the range 490–495 nm was observed. The absorption in the higher energy region confirms the keto-imine form of the compound whereas the one in the lower energy region suggests the enol-imine form. This behavior was expected for compounds having azo and azomethine groups, in which hydroxyl group present at ortho position to the C=N bond on the aromatic ring.^[32, 33]

Single broadband in the 412–458 nm range was observed for $Ni(HL^1)_2$, $Cu(HL^1)_2$, $Zn(HL^1)_2$, $Ni(HL^2)_2$, $Cu(HL^2)_2$ and $Zn(HL^2)_2$ metal complexes as shown in Figures 1 and 2.^[32] For ligands two broad peaks were obtained whereas for metal complexes those two peaks get merged and only one broad peak was obtained due to delocalization of metal electrons with the donor atoms of the ligands. Spectral data were compiled in Table S3.

Molar conductivity

Molar conductivities of all metal complexes were taken in DMSO due to partial solubility in common organic solvents, to find out electrolytic nature of the complexes.



Figure 1. UV-Vis spectrum for HL¹ and its metal complexes.



Figure 2. UV-Vis spectrum for HL² and its metal complexes.

The conductance of metal complexes at 20 μ S (10⁻³ M) in DMSO was in the range 69–79 Ω^{-1} cm² mol⁻¹, as shown in Table 1. This proves nonelectrolyte behavior of all the metal complexes and it is evident for absence of acetate ions or water molecules in coordination sphere of the complexes.^[34,35] Therefore it is proposed that complex formed is in the ratio of 2:1 (ligand:metal) as shown in Scheme 1.^[36]

Sr. No.	Compound (10^{-3} M) in DMSO	Molar conductance (Ω^{-1} cm ² mol ⁻¹)
1	Ni(HL ¹) ₂	77.60
2	Cu(HL ¹) ₂	79.13
3	$Zn(HL^{1})_{2}$	73.39
4	Ni(HL ²) ₂	69.71
5	$Cu(HL_{2}^{2})_{2}$	73.56
6	Zn(HL ²) ₂	71.59

Table 1. Molar conductivities of all synthesized metal complexes in DMSO at $10^{-3}\ \mbox{M}$ concentration.



Scheme 1. Route of synthesis.



Figure 3. ESR spectra for Cu(HL¹)₂.



Figure 4. ESR spectra for $Cu(HL^2)_2$.

Table 2. ESR spectral data of Cu(II) complexes.

Complex	$g_{ }$	g_{\perp}	$g_{\rm av}$	G	A (G)	α ²	μ _{eff} (B.M.)
Cu(HL ¹) ₂	2.12	2.03	2.07	4.25	143.29	0.170	1.79
$Cu(HL^2)_2$	2.11	2.02	2.06	6.08	287.32	0.155	1.78

ESR spectra

The X-band ESR spectra of $Cu(HL^1)_2$ and $Cu(HL^2)_2$ were recorded at room temperature (300 K) in solid-state as shown in Figures 3 and 4, which provide information about the environment around metal ion in the complex. Intense absorption band was obtained for Cu(II) complexes of both the ligands in the higher magnetic field. Isotropic spectra were obtained in both the cases due to tumbling motion of the molecule.^[27] The covalency parameter $\alpha 2$ can be calculated to confirm the type of bonding (covalent or ionic) between metal and ligand by using Eq. (1).

$$\alpha^{2}_{Cu} = -(A_{\parallel}/0.036) + (g_{\parallel} - 2.002) + 3/7 \quad (g_{\perp} - 2.002) + 0.04 \tag{1}$$

$$\mu_{\rm eff}^{\ 2} = \frac{3}{4} (g_{\rm av})^2 \tag{2}$$

The trend in g value $(g_{||} > g_{\perp} > 2.0023)$ for Cu(HL¹)₂ and Cu(HL²)₂ complexes indicate that the unpaired electron is present in the $d_x^2_{-y}^2$ orbital and square planar geometry was expected for both the Cu(II) complexes.^[37] The values of G parameter for Cu(II) complexes found to be greater than **4**, which suggests that the Cu–Cu ion interaction is negligible in the molecule and complex formed was mononuclear hence 1:2 metal-ligand ratio was confirmed.^[38] The effective magnetic moment can be calculated by

	Minimum inhibitory concentration (MIC) in μ M/mL									
	(G+) bact	eria	(G—) bacteria							
Compound	Staphylococcus aureus	Bacillus subtilis	Escherichia coli	Salmonella typhi						
HL ¹	62.5	62.5	125	31.25						
$Ni(HL^1)_2$	62.5	62.5	125	62.5						
$Zn(HL^{1})_{2}$	62.5	31.25	62.5	31.25						
HL ²	31.25	31.25	62.5	15.62						
$Ni(HL^2)_2$	31.25	31.25	125	31.25						
$Zn(HL^2)_2$	31.25	62.5	62.5	15.62						
Chloramphenicol	31.25	31.25	62.5	31.25						

	Table 3. Antibacterial	activity of ligands	and their metal	complexes	showing MIC.
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Table 4. (Comparative	study c	of azo	Schiff ba	ase Cu(II)	complexes	with MIC	values.
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Sr. No.	(G+) Bacteria	(G—) Bacteria	MIC	References
1	S. mutan	P. aeruginosa	25–35 μg/mL	[40]
	S. Pyoenes	S. typhi		
	MRSA*	E. coli		
2	S. faecalis	P. aeruginosa	2.5–10 mg/mL	[41]
	B. cereus	S. flexineri		
	S. aureus	E. coli		
3	S. aureus	E. coli	10–25 mg/mL	[42]
	B. subtilis	P. aeruginosa		
4	S. aureus	E. coli	9.87–39.50 μg/mL (15.62–62.5 μM/mL)	Present work
	B. subtilis	S. typhi		

*MRSA: Methicillin resistant *Staphylococcus aureus* (taken as positive control).

S. mutans: Streptococcus mutans; S. pyogenes: Streptococcus pyogenes; S. faecalis: Streptococcus faecalis; B. cereus: Bacillus cereus; S. aureus: Staphylococcus aureus; B. subtilis: Bacillus subtilis; P. aeruginosa: Pseudomonas aeruginosa; S. typhi: Salmonella typhi; E. coli: Escherichia coli; S. flexneri: Shigella flexneri.

using Eq. (2), and it was found to be 1.79 and 1.78 B.M. for $Cu(HL^1)_2$ and $Cu(HL^2)_2$ complexes, respectively, as shown in Table 2.^[39]

Antibacterial activity

Minumum inhibitory concentration (MIC)

The synthesized azo Schiff base ligands and their transition metal complexes were screened for *In-vitro* antibacterial activity. Four different types of bacterial strains (two gram-positive and two gram-negative) were used for this study. Table 3 shows minimum inhibitory concentration (MIC) in μ M/mL for all the compounds and standard. It was found that all the compounds possessed good antibacterial activity, but Cu(II) complexes of both the ligands spectrum drug Chloramphenicol. MIC values for the Cu(II) complexes were obtained in the range of 15.62–62.5 μ M/mL which are far better than previously reported work (Table 4). Pictures of microtitre plate are shown in Figures S5–S8. Error bar graph for antibacterial study was shown in Figure S9.

Antioxidant study

Screening of synthesized ligands and their Ni(II), Cu(II) and Zn(II) complexes for *in-vitro* antioxidant activity was done by using DPPH free radical scavenging method. UV-Visible spectrophotometer was used to examine the antioxidant activity by means

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Table 5. Antioxidant activity of Ligands and their metal complexes showing IC₅₀ values in μ M/mL.

Compound \rightarrow	L1	C1	C2	C3	L2	C4	C5	C6	STD
$\rm IC_{50}~\mu M/mL \rightarrow$	68.44	3926	2677	4726	52.78	938	3312	3999	72.69

The values in bold indicates best IC_{50} value amongst the tested samples.

Table 6. Comparative study of antioxidant activity of azo Schiff base ligands and their metal complexes.

IC ₅₀ Value	% I	References
94.48-116.39 µa/mL		[14]
5	17.51–56.47%	[43]
15.88 19.57 μg/mL (52.78–68.44 μM/mL)	21.48-96.33%	Present work
	<u>IC₅₀ Value</u> 94.48–116.39 μg/mL 15.88 19.57 μg/mL (52.78–68.44 μM/mL)	IC ₅₀ Value % Ι 94.48–116.39 μg/mL 17.51–56.47% 15.88 19.57 μg/mL (52.78–68.44 μM/mL) 21.48–96.33%

Table 7.	Protein	denaturation	assay	of	Ligands	and	their	metal	complexes	showing	IC_{50}	values	in
μM/mL.													

Compound \rightarrow	L1	C1	C2	C3	L2	C4	C5	C6	STD
$\rm IC_{50}~\mu M/mL \rightarrow$	46.76	1101	1928	1498	55.77	672	942	1119	62.02

The values in bold indicates best IC_{50} value amongst the tested samples.

of absorption at 517 nm. Ascorbic acid was used as standard antioxidant. Table 5 shows IC_{50} values for all the tested samples along with the standard. The study found that ligands (HL^1 and HL^2) act as excellent antioxidants such as ascorbic acid and have much better free radical scavenging activity compared to imidazole and azo-Schiff bases ligands (Table 6). Error bar graph of the study is shown in Figure S10 and free radical scavenging ability along with standard deviation is shown in Table S4.

Anti-inflammatory activity

The synthesized compounds were also screened for an anti-inflammatory activity to check the protection of protein against denaturation. *In-vitro* protein denaturation screening of ligands and their Ni(II), Cu(II) and Zn(II) metal complexes revealed that ligand HL^1 and HL^2 displayed protecting nature against BSA (Bovin serum albumin). From this we can conclude that both the ligands possessed superior anti-inflammatory activity (lowest IC₅₀ value) as compared to standard anti-inflammatory drug Diclofenac sodium as shown in Table 7. Figure S11 represents error bar graph for the anti-inflammatory study and Table S5 shows percentage inhibition with standard deviation.

DNA fragmentation study of ligands

Metal-free ligand HL^1 and HL^2 shows excellent antioxidant and anti-inflammatory activity, therefore we performed their DNA fragmentation study. The incubation of plasmid DNA (pBR322) with ligands HL^1 and HL^2 did not show any strand break in DNA as observed in Lane 1, Lane 2 and Lane 3 (Figure 5).

This confirms that ligands do not show any damage to plasmid DNA. Incubation of Methyl glyoxyl (MG), lysine along with $FeCl_3$ helps to generate free radicals which in turn cause DNA strand breaks in pBR322 (Lane 5) Figure 5. Ligands HL^1 and HL^2



Figure 5. DNA interaction assay for HL^1 and HL^2 . Lanes 1 and 4 for Control containing pBR322, Lane 2 for pBR322 + HL^1 , Lane 3 for pBR322 + HL^2 , Lane 5 for pBR322 + MG + Lysine + FeCl₃, Lane 6 for pBR322 + MG + Lysine + FeCl₃ + HL^1 , and Lane 7 for pBR322 + MG + Lysine + FeCl₃ + HL^2 .

protects pBR322 by scavenging free radicals generated by MG, lysine and FeCl₃ Lanes 6 and 7 (Figure 5).

Experimental

General information

Chemicals and reagents were purchased from S D Fine (Mumbai, India) and Sigma Aldrich chemical companies (USA). NMR (¹H and ¹³C) spectra of (HL¹) and (HL²) were recorded in d⁶-DMSO solvent using Bruker Avance II (300 MHz) NMR spectrometer and TMS was taken as internal standard. A mass spectrum was recorded using AB SCIEX 3200 Q TRAP LC/MS/MS spectrometer. Elemental analysis (C, H, N) were done by using EA-3000 euro vector Italy elemental analyzer. The melting point of the Schiff bases and their metal complexes were confirmed by using Analab melting point apparatus. IR spectra of (HL¹), (HL²), as well as their metal complexes, were also done by using Perkin Elmer, Frontier equipment with diamond tip. UV-Vis absorption for all the samples was recorded in DMSO on Shimadzu UV 2450 UV-Vis spectrophotometer. Molar Conductivity of all samples was measured on EQ-660B μ p based conductivity meter (Equiptronics). ESR of the Cu(HL¹)₂ and Cu(HL²)₂ in solid state at room temperature were done for X band on JES-FA200 ESR spectrometer with X and Q band. DNA fragmentation study was done using horizontal gel electrophoresis apparatus (Atto Corp., Yushima, Bunkyo, Tokyo, Japan).

General procedure for the synthesis of azo-Schiff base ligand (HL¹)

2-hydroxy-3-methoxy-5-(tolyldiazenyl)benzaldehyde (1a) (0.2 g, 0.75 mm) and hydroxylamine hydrochloride (0.052 g, 0.75 mm) were taken in 100 mL round bottom flask, 10 👄 M. S. KASARE ET AL.

to this 10–12 mL dry methanol was added and pH was adjusted to 7.0 by adding 1 M NaOH solution, the reaction was refluxed for 3-4h.^[44] Reaction was monitored by TLC. After completion of reaction the reaction mixture was allowed to cool at room temperature. Solid appeared after cooling was filtered and crystallized by using ethanol. Brown Colored crystals of ligand (HL¹) were obtained. Ligand (HL²) was also synthesized by following the above procedure, and structures of azo-Schiff base ligands were represented in Scheme 1. For NMR spectra and mass spectra of the compounds.

Spectral data for ligand (HL¹)

2-hydroxy-3-methoxy-5-(p-tolyldiazenyl)benzaldehyde oxime (HL¹) M.W. = 285.30 g/ mol. M.P. = 207–209 °C, Yield = 77.68% (0.164 g), Color=Brown. Elemental analysis for HL¹ (C₁₅H₁₅N₃O₃) theoretical=C 63.15, H 5.30, N 14.73; found=C 63.37, H 5.19, N 14.51. LC-MS: (M + H⁺) values are calculated for HL¹: m/z calcd. 285.30, found 286.10. ¹H-NMR (300 MHz, DMSO- d_6) δ (ppm) = 11.524 (s, Ar-OH), δ =10.424 (s, N-OH), δ =8.428 (s, 1H, C-H), δ =7.776–7.352 (m, 6H, Aromatic), δ =3.905 (s, 3H, -OCH₃), δ =2.386 (s, 3H, -CH₃). ¹³C-NMR (75 MHz, DMSO- d_6) δ (ppm) = 151.31(a), 148.95(b), 148.82(c), 147.73(d), 144.41(e), 129.52(f), 128.06(g), 121.48(h), 119.87(i), 116.26(j), 102.24(k), 55.10(l).

Conclusions

All the compounds were successfully synthesized and characterized by various analysis techniques. Screening of ligands and their respective metal complexes for *In-vitro* antibacterial activity against gram-positive and gram-negative bacteria was done and result showed that Cu(II) complexes possessed excellent antibacterial activity as compared to Ni(II) and Zn(II) metal complexes as well as their parent ligands. Ligand HL¹ and HL² showed excellent antioxidant and anti-inflammatory activity as compared to standards, whereas metal complexes do not show such activity. DNA fragmentation study was carried out for ligands and it showed good protective effect of free radicals against plasmid DNA of bacteria. On the basis of overall study we can conclude that ligand HL¹ and HL² along with their Cu(II) complexes possess excellent biological activity.

Data compiled in supporting information includes experimental procedure, theoretical data, spectra of all compounds, images and bar graph of biological studies.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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