Selected isonicotinoyl hydrazones have been synthesized via template method and were complexed to Cu(II). The ligands are coordinated to Cu(II) ion through the enolic oxygen and azomethine nitrogen resulting in a square planar geometry. The CT-DNA and bovine serum albumin (BSA) binding propensities of the compounds were determined spectrophotometrically, the results of which indicate good binding propensity of complexes to DNA and BSA with high binding constant values. Furthermore, the compounds have been investigated for their cytotoxicities on A549 human lung cancer cell. Also, the mode of cell death was examined employing various staining techniques and was found to be apoptotic.

1. Introduction

A wide range of Schiff’s bases with their reactive azomethine linkage show interesting inhibitory activity against tumor cells. Schiff’s bases could be hydrolyzed selectively by the tumor cells to act as alkylating agents; at the same time the active amine becomes free to act as antimetabolite [1]. Studies show that the metal complexes of Schiff’s base ligands have better antimicrobial and anticancer activities as compared to Schiff’s bases [2].

Copper is well known as a bioessential element and its complexes have proven to be excellent candidates for biological applications due to their binding ability and positive redox potential [3–6]. Cu(II) complexes containing heterocyclic bases have been extensively explored in virtue of their strong interactions with DNA and cytotoxic activity [7–9]. They can act as chemical nucleases [10, 11] and their cytotoxicity has been proposed to be caused by their ability to bind and cleave DNA that leads to cell cycle arrest and apoptosis or generation of reactive oxygen species (ROS) that in turn leads to cell death [12]. Literature has shown that Cu(II)-based complexes exhibit antineoplastic potency towards human ovarian carcinoma (CH1), murine leukemia (L1210), and various cervicouterine carcinomas which is comparable to or even higher than that of cisplatin [13, 14]. Fei et al. in their article [15] have reported the in vitro cytotoxicity of two copper-2,2'-bipyridine complexes towards five selected tumor cell lines HepG-2, HeLa, NCI-H460, MCF-7, and HL-6 with IC_{50} value at a concentration of 50 μM. Cu(II) complexes of Schiff bases can show their antibacterial and antiproliferative activities because of the properties of the metal center or the coordinated ligands alone, as well as the structural and electronic properties which is ascribed to the coordination [16–21]. Several Schiff base ligands and their Cu(II) complexes have been investigated to show apoptotic activity against various cancer cell lines [22]. Oxoquinoline based Schiff base copper complexes have been synthesized and investigated upon and their IC_{50} values were found to be in the range of 8–19 μM against HeLa and HL-60 cells [23].

INH is a first generation antitubercular drug whose derivatives and their metal complexes have been subjected to a lot of research for their antitubercular activities but their potentials as anticancer agents have remained largely unexplored. Their potency as抗癌剂 is reflected from the little work that has been published so far wherein a few researchers have synthesized hydrazones of INH and
their iron complexes and evaluated them for their anti-tumoral activity suggesting a mechanism of inhibition of ribonucleotide reductase [24].

The research interest in the present work stems from the aforementioned points. With this view point we herein report Ni(II) mediated synthesis and characterization of four Schiff’s bases of isonicotinoyl hydrazine (INH) by its condensation with four different aromatic aldehydes and their Cu(II) complexes. The synthesized isonicotinoyl hydrazones and their Cu(II) complexes were studied for their interactions with DNA and BSA. The results were indicative of better binding efficiencies of the copper bound ligands compared to the ligands themselves. Based on the results of their binding studies the ligands and their copper complexes were evaluated for their anticancer activity against human lung carcinoma A549 cell lines. Furthermore the mode of interaction of these complexes with A549 cells has been evaluated employing various staining techniques to assess the mechanism of cell death, via excessive ROS generation and/or nuclear condensation.

2. Results and Discussion

2.1. Synthesis and Characterization. In a general sense, transition metal-based catalysis can be viewed as template reactions where reactants coordinate to adjacent sites on the metal ion and, owing to their adjacency, the two reactants interconnect (insert or couple) either directly or via the action of another reagent. Template effects may arise from stereocchemistry imposed by metal ion coordination of some of the reactants, promoting a series of controlled steps and characteristically providing routes to products not formed in the absence of the metal ion. Appreciable amount of work has been done on one pot multicomponent synthesis of Schiff bases using transition metal ions as templates [25–28]. Following this insight we carried out the Ni(II) mediated Schiff base synthesis (Figure 1) and found that the reaction time had reduced and product yields had increased markedly.

The IR spectra of complexes C5–C8 lack the strong secondary amide carbonyl absorption at 1650–1700 cm\(^{-1}\) that is typically seen in the spectra of the free ligands L1–L4. In all the complexes, the \(\nu_{C=\text{N}}\) band is shifted to lower frequency between 1570 and 1610 cm\(^{-1}\) indicating coordination of the Schiff bases through the azomethine nitrogen [29]. Moreover, the enolate structure of the coordinated ligand is supported by a band at 1059-1060 cm\(^{-1}\) due to the enolic C=O stretching [30]. The N-H stretching bands in the range 3150–3250 cm\(^{-1}\) found in the IR spectra of L1–L4 are completely lost in the spectra of their C5–C8 which is also indicative of the enolization and deprotonation on coordination.

The UV spectra of the ligands and their complexes show bands in the wavelength range 200–350 nm. In the UV spectra of the ligands (Figure 2) the first band appearing within 200–270 nm region can be assigned to the medium energy \(\pi-\pi^*\) transition of the aromatic rings. The second band observed within 280–340 nm region is due to the excitation of the electrons of the azomethine group which corresponds to an intraligand \(n \rightarrow \pi^*\) transition [31]. In case of L2 (Figure S1 in the Supplementary Material) there is a third band located at 326 nm that can be ascribed to charge transfer within the entire Schiff base molecule. This band is commonly observed in o-hydroxyl Schiff bases [32] and is based on strong intramolecular hydrogen bonding between the hydroxyl group of the salicylidene and the azomethine nitrogen [33]. The UV spectra of complexes (Figure 2) show the first band at nearly the same wave length as in the spectra of their corresponding ligands which reveal that these \((\pi-\pi^*)\) transitions are not significantly affected by chelation. On the other hand the band corresponding to azomethine showed broadening and a shift to longer wavelength on going from ligand to complex, indicating coordination of ligand to metal through the azomethine moiety [34].

The mass spectra of ligands L1–L4 show peaks corresponding to the molecular ions \(M^+\) at \(m/z = 225.09, 241.07, 271.09,\) and 255.09, respectively (Figure S2 in the Supplementary Material). The ion peaks for \(C_5\)–\(C_8\) show at \(m/z = 511.7(M^+), 546.1(M^+ + 2), 603.7(M^+),\) and \(571.7(M^+)\), respectively (Figure S3 in the Supplementary Material). The \(m/z\) values of the molecular ion peaks for C5–C8 indicate that two ligands are coordinated to the metal centre. Detailed interpretation of the mass fragments has been summarized in Table S1 in the Supplementary Material.

The ESR spectra of C6 and C7 were recorded in solid state at 77 K (Figure S4 in the Supplementary Material) and that of C8 (Figure 3) was recorded in DMSO at 120 K using 100 KHz field modulation and the \(g\) factors were quoted relative to the standard marker TCNE (\(g = 2.00277\)). The \(g_\perp, g_\parallel, g_{av}\), and \(G\) values have been calculated and summarized in Table 1. The spectrum of the four coordinated C8 in frozen DMSO resolved four copper hyperfine lines, characteristic of monomeric copper(II) complexes [35]. The observed \(g_\perp > g_\parallel > g_{av}\) (2.0023) values indicate that the unpaired electron is predominantly in the \(d_{x^2-y^2}\) orbital giving a \(^1\text{B}_1\) ground state [36, 37] and the complexes have distorted square planar geometry. For C6 and C7, the \(G\)-values are <4, suggesting the monomeric nature of copper(II) centres and consistent with a \(d_{x^2-y^2}\) ground state having a small exchange coupling in the solid state while for C8, \(G = 4.7\) indicates that there are no spin exchange interactions between the copper centres in solution [38]. The \(g_{av}\) values for the complexes were found to be in the range 2.10–2.17. The deviation of the \(g_{av}\) from that of the free electron (2.0023) is due to the covalent nature of the complexes [39].

Thermogravimetric analyses of L1–C8 were carried out at 10°C per min in a temperature range 50–850°C in N\(_2\) atmosphere. The thermogram of L1 given in Figure 4 showed 99.6% weight loss in the range 200°C–320°C attributed to the total decomposition of the molecule. A continuous weight loss in the temperature range 200°C–750°C is observed in case of C5 (Figure 4) attributed to the removal of the ligands leaving behind CuO\(_2\)N\(_2\) as residue.
Isoniazid Isonicotinoyl hydrazone

\[ \text{Isoniazid} + R\text{-CHO} \xrightarrow{\text{NiCl}_2 \cdot 6\text{H}_2\text{O, pH = 5}} \text{Isonicotinoyl hydrazone} \]

Metal salt

\[ \text{NiCl}_2 \cdot 6\text{H}_2\text{O, pH = 5} \xrightarrow{\text{CH}_3\text{OH, reflux}} \]

\[ \begin{array}{c}
\text{L1} = \text{HO} \\
\text{L2} = \text{OCH}_3 \\
\text{L3} = \text{OH} \\
\text{L4} = \text{OCH}_3
\end{array} \]

\[ \begin{array}{c}
\text{Figure 1: Schematic representation of synthetic route for the ligands L1–L4 and their Cu(II) complexes C5–C8.}
\end{array} \]

\[ \begin{array}{c}
\text{Table 1: ESR spectral parameters of complexes C6, C7, and C8.}
\end{array} \]

| Complex              | \( g_{||} \) | \( g_{\perp} \) | \( g_{av} \) | \( G \)  |
|----------------------|-------------|---------------|-------------|---------|
| C6 (solid state, 77 K) | 2.185       | 2.066         | 2.106       | 2.828   |
| C7 (solid state, 77 K) | 2.185       | 2.068         | 2.107       | 2.752   |
| C8 (in DMSO, 120 K)   | 2.368       | 2.079         | 2.175       | 4.747   |

\[ \begin{array}{c}
\text{Figure 2: UV spectra of ligand L1 and corresponding Cu(II) complex C5 recorded at a concentration of 2.5 mM in DMSO with path length 1 cm.}
\end{array} \]

\[ \begin{array}{c}
\text{Figure 3: ESR spectrum of C8 in DMSO at 120 K using 100 KHz field modulation.}
\end{array} \]
The spectral (IR, EPR, UV, Mass) and the thermogravimetric analyses data of the ligands and the complexes are in full agreement with the proposed structures (Figure 1).

2.2. DNA Binding Studies

2.2.1. Absorption Studies. Transition metal complexes can bind to DNA through covalent bonding via replacement of a labile ligand of the complex by a nitrogen base of DNA such as guanine N7 [40] or noncovalent interactions such as intercalation, electrostatic, or groove binding [41]. Electronic absorption spectroscopy is one of the most useful techniques in DNA binding studies [42] since the observed changes in the spectra may give indication of the mode of interaction.

The π-π* intraligand absorption bands with $\lambda_{\text{max}}$ in the region 200–270 nm were used to monitor the interaction of compounds $\text{L}_1$–$\text{C}_8$ with double helical CT-DNA. The UV spectra have been recorded for a constant compound concentration in diverse $[\text{DNA}]/[\text{compound}]$ ratios ($r$). The changes observed in the intraligand transition of the compounds $\text{L}_1$–$\text{C}_8$ upon addition of CT-DNA solution in diverse $r$ values may reveal the existence of interaction between each compound and DNA and may indicate the possible mode of binding.

Upon increasing the CT-DNA concentration, the UV spectra of compound-DNA solutions show a gradual decrease in the intensities of the absorption bands (hypochromism) (Figure 5) for all compounds without any apparent red shift. The observed hypochromism may be attributed to stacking interaction between the aromatic chromophores of the free and bound ligands and DNA base pairs probably due to intercalation.

Among the free ligands, $\text{L}_4$ exhibits the maximum hypochromism of 15% followed by $\text{L}_1$ and $\text{L}_2$, each showing a hypochromism of 10%. The absorption bands of complexes $\text{C}_5$, $\text{C}_6$, $\text{C}_7$, and $\text{C}_8$ exhibit a maximum of 48, 24, 28, and 27% hypochromicity, respectively (Table 2). The hypochromism is more pronounced for $\text{C}_5$. The complexes exhibit distinctively
greater hypochromism compared to the free ligands which indicates that presence of a metal ion facilitates binding.

The magnitude of binding strength to CT-DNA may be determined through the calculation of binding constant \( K_b \) which is obtained by monitoring the changes in the absorbance at the corresponding \( \lambda_{max} \) with increasing concentrations of CT-DNA. \( K_b \) is given by the ratio of slope to the \( y \) intercept in plots [DNA]/(\( e_A - e_f \)) versus [DNA] according to \([43]\)

\[
\frac{[DNA]}{(e_A - e_f)} = \frac{[DNA]}{(e_b - e_f)} + \frac{1}{K_b (e_b - e_f)},
\]

where [DNA] is the concentration of DNA in base pairs, \( e_A = \frac{A_{obsd}}{[\text{compound}]}, \) \( e_f \) is the extinction coefficient for the unbound compound (L1–C8), and \( e_b \) is the extinction coefficient for the compound in the fully bound form. The values for \( K_b \) as calculated by (1) are given in Table 2 and the representative [DNA]/(\( e_A - e_f \)) versus [DNA] plot for C5 in the inset of Figure 5. Plots of remaining compounds are given in Figure S5 (Supplementary Material).

The binding constants of the compounds range from \( 5.7 \times 10^4 \) to \( 1.4 \times 10^4 \) M\(^{-1}\) suggesting that the Schiff bases and their complexes strongly bind to CT-DNA. The highest \( K_b \) value (\( 5.7 \times 10^4 \) M\(^{-1}\)) among the ligands was observed for L1 whereas C8 exhibited the highest \( K_b \) value (\( 4.2 \times 10^4 \) M\(^{-1}\)) among the complexes. However the overall \( K_b \) values of the compounds are lower than those observed for classical intercalator EB (\( 1.23 \times 10^5 \) M\(^{-1}\)) with a proven DNA binding mode involving intercalation of the planar phenanthridine ring between the adjacent base pairs on the double helix. This is indicative of the binding of L1–C8 with CT-DNA with an affinity less than EB.

The results derived from the UV-titration experiments suggest that all the compounds can bind to CT-DNA although the exact mode of binding cannot be merely proposed by UV-spectroscopic titration studies. In most cases, the existence of hypochromism could be considered as first evidence that the binding of the compounds involving intercalation between the base pairs of CT-DNA cannot be ruled out.

2.2.2. DNA Ladder Assay. DNA ladder assay was carried out on A549 cells with two complexes C5 and C8 at three different concentrations (50 \( \mu \)M, 75 \( \mu \)M, and 100 \( \mu \)M) and a marker for reference (Figure 6), to further support the binding of compounds to DNA and to study its effect on the cancer cells. The results presented in Figure 6 show that both complexes C5 and C8 provoke DNA fragmentation in A549 cells. The first band seen in all the six lanes at different concentrations of the two complexes is of the intact DNA, while a smear can be seen in the lower half of the gel which is of fragmented DNA. Though the fragmentation process is weak, it is conclusive of an apoptotic effect of the compounds on the cancer cells.

2.3. BSA Binding Studies. Serum albumins are proteins involved in binding and transport of drugs through the blood stream \([44]\). Their binding with a drug may actually result in increase or decrease in the drug's efficacy making it important to investigate the interactions of prospective drugs with serum albumins. BSA solutions exhibit a strong fluorescence emission with a peak at 343 nm, due to the tryptophan residues, when excited at 296 nm. The changes and quenching occurring in the fluorescence emission spectra of BSA upon addition of compounds L1–C8 are primarily due to change in protein conformation, subunit association, substrate binding, or denaturation because of their binding \([45]\).

Addition of L1–C8 to a solution of BSA results in a decrease of the fluorescence intensity (Figure 7). It is observed that the complexes show more % fluorescence quenching compared to the ligands (Figure 7, inset) indicating stronger protein binding of the ligands on complexation. The values of the Stern-Volmer quenching constants and the quenching rate constants for the compounds L1–C8 interacting with BSA are calculated by Stern-Volmer quenching equation (2) \([46]\) and the corresponding Stern-Volmer plots \( I_0/I \) versus [Q] (Figure 8):

\[
\frac{I_0}{I} = 1 + K_{SV} [Q] = 1 + k_q \tau_0 [Q],
\]

where \( I_0 \) is the initial tryptophan fluorescence intensity of BSA, \( I \) the tryptophan fluorescence intensity of BSA after the addition of the quencher, \( K_{SV} \) (M\(^{-1}\)) the quenching constant, \( k_q \) (M\(^{-1}\) s\(^{-1}\)) the quenching rate constants, \( \tau_0 \) the average fluorescence lifetime (10\(^{-8}\) s) \([47]\) of BSA without the quencher, and [Q] the concentration of the quencher. The quenching constant (\( K_{SV} \) M\(^{-1}\)) can be obtained from the slope of the plot \( I_0/I \) versus [Q] and has been tabulated in Table 3 for L1–L4 and in Table 4 for C5–C8.

The double-logarithm equation (3) \([48]\) has been employed to determine the binding constant (\( K_a \)) and the number of binding sites \( n \) for ligand-BSA interaction:

\[
\log \left( \frac{I_0 - I}{I} \right) = \log K_a + n \log [Q].
\]

The plot of \( \log ((I_0 - I)/I) \) versus log [Q] for each of the ligand is linear (Figure 9) and the values of \( K_a \) and \( n \) have been obtained from the intercept and slope, respectively (Table 3).
Due to the nonlinearity in the Stern-Volmer plots of C5–C8 (Figure 8(b)), the binding constants ($K_a$) have been obtained from modified Stern-Volmer (MSV) equation [49]:

$$\frac{I_o}{(I_o - I)} = \frac{1}{(f_aK_a[Q])} + \frac{1}{f_a}, \quad (4)$$

where $f_a$ is the fraction of the fluorophore that is initially accessible to the complex. The linearity in the modified Stern-Volmer plot ($I_o/(I_o - I)$ versus $1/[Q]$) (Figure 10) indicates a probable ground state complex formation between C5–C8 and BSA, which is static quenching mechanism [50]. The ratio of intercept to slope of MSV plots gave $K_a$ values (Table 4) for complex-BSA binding.

Considering the Stern-Volmer constant of about $10^5 \text{M}^{-1}$ and the lifetime of albumins, $10^{-8} \text{s}$ [51], we obtain a value of $10^{13} \text{M}^{-1} \text{s}^{-1}$ for the rate constant ($k_q$) of the bimolecular quenching process, which largely overrides the accepted limit of the rate constant ($2 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$) of the diffusional quenching implying biopolymers. This observation supports the fact that the experimental quenching of albumin fluorescence is due to a predominantly static process [52].

2.4. Cytotoxicity. In vitro cytotoxicity tests were performed on the lung cancer (A549) and normal mouse adiposities (3T3L1). The IC$_{50}$ values of compounds L1–C8 are listed in Table 5. The cell viabilities (%), obtained for A549 cells with continuous exposure to the compounds for 24 h, are depicted in Figure 11 whereas the IC$_{50}$ values of the compounds on A549 and 3T3L1 cells have been compared in Figure 12. All the compounds were found to be very less toxic to normal cell line. Copper(II) complexation enhanced the cytotoxicity of the ligands as C5, C7, and C8 were more toxic to A549 cells than their ligand counter parts though L2 was more toxic than its Cu(II) complex C6. Nevertheless the compounds were found to be less toxic than cis-platin (IC$_{50}$ = 26 µM).

2.5. Evaluation of Mode of Cell Death via Staining Techniques. Cell staining is a valuable and accurate tool for the assessment of apoptosis permitting identification of the classical hallmarks of apoptosis such as cell shrinkage, membrane blebbing, and formation of apoptotic bodies.

2.5.1. Apoptosis and Necrosis Evaluation by Fluorescent Double Staining with AO/EB. DNA-binding dyes AO and EB (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells. Acridine orange (AO) permeates all cells and makes the nuclei appear green. Ethidium bromide (EB) is only taken up by cells when cytoplasmic membrane integrity is lost and stains the nucleus red. EB also dominates over AO. Thus live cells have a normal green nucleus; early apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange

![Figure 7: Plot of fluorescence emission intensity $I$ versus wavelength $\lambda$ for BSA at different concentrations of compound L1. The arrow shows the change in intensity of BSA emission on increasing amount of the compound at [BSA]/[complex] ratio in the range 0–5. Inset: Plot of BSA % relative fluorescence intensity on increasing amount of the compound at [BSA]/[complex] ratio in the range 0–5.](image-url)
nucleus [53]. The cells were treated with complexes C5–C8 at 100 μM concentration for 16 h. The confocal images of control cells show that the live cells are stained with AO and hence emit green fluorescence whereas the treated cells bearing bright green nucleus indicate early apoptosis and cells bearing orange nucleus indicate late apoptosis (Figure 13). Moreover the treated cells as seen in the phase contrast images (Figure 14(f)) undergo a distinct alteration in
2.5.2. Measurement of Intracellular ROS Generation Using DCFDA Stain. Reactive oxygen species (ROS) are involved in the regulation of many physiological processes. However, when ROS production increases (cellular or external) and overwhelms the cellular antioxidant capacity, it can induce macromolecular damage (by reacting with DNA, proteins, and lipids) and thus contributes to a broad spectrum of diseases and pathological conditions. In the first instance, damage can lead to apoptosis or necrosis. DCFDA (2',7'-dichlorofluorescein diacetate) is a cell permeable fluorescent dye that measures hydroxyl, peroxyl, and other ROS activities within the cell. After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a nonfluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF), a highly fluorescent dye. In the present study, the images taken using fluorescence microscopy of A549 cells treated with complexes C5–C8 at a concentration of 100 μM after 24 h incubation showed that the cells are distinctly stained with the DCF dye indicating excessive ROS generation (Figure 14(b)) whereas the control cells were faintly stained by the dye or not stained at all (Figure 14(a)). The ROS generation seen here can be attributed to the fact that the Schiff base derivative of INH produces carbon, nitrogen, or oxygen centered free radicals on interaction with catalase-peroxidases in the cell. This observation confirms an oxidative cleavage of DNA as is evident from the DNA ladder assay.

2.5.3. Staining Nucleic Acid Using DAPI Stain. The morphological assessment of nuclei using a nucleophilic dye DAPI was performed in A549 cells untreated and treated with C5–C8 (100 μM for 24 h). Observations revealed that a large population of the treated cells showed nuclear condensation and associated nuclear anomalies (Figure 14(d)). These observations provide further evidence on nuclear dysfunction induced by C5–C8 in A549 cells.

3. Conclusions

The synthesis and characterization of four isonicotinoyl Schiff bases and their Cu(II) complexes (L1–C8) have been realized with physicochemical and spectroscopic methods. In all the complexes, the Schiff bases are bound to the metal via the enolicoxygen and the azomethine nitrogen adapting a square planar geometry around the metal centre. The interaction of the complexes with CT DNA and BSA is supported by the following observations: (i) high values of $K_b$ in the range of $5.7(±0.17) \times 10^4$–$1.4(±0.4) \times 10^4$ and hypochromism of the intraligand absorption bands of the compounds L1–C8, (ii) high values of $K_{sv}$ in the range of $1.79(±0.006) \times 10^5$–$4.3(±0.013) \times 10^5$, and (iii) efficient cleavage of CT-DNA. Cytotoxicity studies show that the compounds are less toxic than the clinically used drug cis-platin. The metal complexes of ligands L1, L3, and L4 show greater cytotoxicity as compared to the ligands. Moreover, the results obtained

**Figure 11:** % cell viability in presence of ligands L1–L4 and complexes C5–C8 for A549 human lung cancer cell lines. Each point is the mean ± standard error obtained from three independent experiments.

**Figure 12:** Graph showing comparison between the IC$_{50}$ (concentration at 50% cell death) values of L1–C8 on A549 and 3T3L1 cell lines treated for 24 h at 37°C which clearly depicts the low cytotoxicity of the compounds on normal mouse adipocytes than on lung cancer cells at same doses of treatment.
Figure 13: Confocal images of A549 cells treated with (100 μM) C5–C8 for 16 h followed by dual staining with AO/EB. The arrows indicate early apoptotic cells bearing bright green nucleus with late apoptotic cells bearing orange nucleus.

from staining techniques indicate that the mode of cell death is essentially apoptosis, probably via ROS generation.

4. Experimental

4.1. Materials and Methods. All the chemicals and solvents used for synthesis and characterization of ligands and complexes are of analytical grade and were used as purchased. INH (isonicotinoyl hydrazide) was purchased from Acros organics. Aldehydes, CT-DNA, Tri-sodium citrate, and EB (ethidium bromide) were purchased from SRL (Sisco research laboratory, Mumbai, India.). The metal salts and solvents were purchased from Merck. All the chemicals and solvents used for synthesis and characterization of ligands and complexes are of analytical grade and were used as purchased.

4.2. Synthesis of Ligands L1–L4. A mixture of INH, aldehyde (benzaldehyde/salicylaldehyde/vanillin/p-anisaldehyde) and NiCl₂·6H₂O were taken in the mole ratio of 1:1:0.5 in 20 mL dry methanol. A few drops of acetic acid were added to adjust the pH to 5 and the reaction mixture was refluxed for 1-2 h. The product obtained was filtered and recrystallized from absolute ethanol.

4.2.1. (N-Benzylideneisonicotinohydrazide) (L1). L1 was prepared by condensation of isoniazide (3.6 mmol, 500 mg) and benzaldehyde (3.6 mmol, 386 mg) in presence of NiCl₂·6H₂O (1.8 mmol, 433 mg) as mentioned above. Pale yellow crystals were obtained. Solubility: DMSO, DMF; Yield 84.14%, mp 197°C; M Wt 225.25 g/mol; Anal. Calc. for C₁₃H₁₁N₃O: C 69.32, H 4.92, N 18.66. Found: C 69.24, H 4.89, N 18.61%; MS m/z: 225.09 (M⁺), δH (400MHz, DMSO-d6) 12.10 (s, 1H, NH), 8.79 (d, J 5.2, 2H, pyridine α-H), 8.47 (s, 1H, N=CH), 7.83 (d, J 4.4, 2H, pyridine β-H), 7.77–7.75 (dd, J₁ 7.6, J₂ 2, 2H, Ar-H), 7.51–7.47 (m, 3H, Ar-H) (Figure S6,(a) in the Supplementary Material); δC (400 MHz, DMSO-d6) 162.1, 150.8, 149.4, 140.9, 134.4, 130.8, 129.3, 127.7, 122.0 (Figure S7,(a) in the Supplementary Material); νmax (KBr)/cm⁻¹: ν_N-H 3197(b), ν_C=O 1694(s).

4.2.2. (N-(2-Hydroxybenzylidene)isonicotinohydrazide) (L2). L2 was prepared by condensation of isoniazide (3.6 mmol, 500 mg) and benzaldehyde (3.6 mmol, 386 mg) in presence of NiCl₂·6H₂O (1.8 mmol, 433 mg) as mentioned above. Pale yellow crystals were obtained. Solubility: DMSO, DMF; Yield 84.14%, mp 197°C; M Wt 225.25 g/mol; Anal. Calc. for C₁₃H₁₁N₃O: C 69.32, H 4.92, N 18.66. Found: C 69.24, H 4.89, N 18.61%; MS m/z: 225.09 (M⁺); δH (400MHz, DMSO-d6) 12.10 (s, 1H, NH), 8.79 (d, J 5.2, 2H, pyridine α-H), 8.47 (s, 1H, N=CH), 7.83 (d, J 4.4, 2H, pyridine β-H), 7.77–7.75 (dd, J₁ 7.6, J₂ 2, 2H, Ar-H), 7.51–7.47 (m, 3H, Ar-H) (Figure S6,(a) in the Supplementary Material); δC (400 MHz, DMSO-d6) 162.1, 150.8, 149.4, 140.9, 134.4, 130.8, 129.3, 127.7, 122.0 (Figure S7,(a) in the Supplementary Material); νmax (KBr)/cm⁻¹: ν_N-H 3197(b), ν_C=O 1694(s).
Figure 14: Images of A549 cells observed under fluorescence microscope: (a, b) control cells and treated cells stained with DCFDA; (c, d) control cells and treated cells stained with DAPI; (e, f) control and treated A549 cells observed under phase contrast microscope, where in treated cells they show distinct rounding indicative of apoptosis.

4.2.3. (N′-(4-Hydroxy-3-methoxybenzylidene)-N-methylisonicotinohydrazide) (L3). L3 was prepared by condensation of isoniazide (3.6 mmol, 500 mg) and vanillin (3.6 mmol, 554 mg) in presence of NiCl₂·6H₂O (1.8 mmol, 433 mg). Yellowish brown crystals were obtained. Solubility: DMSO, DMF; Yield 89.37%; mp 138°C; M Wt 255.27 g/mol; Anal. Calc. for C₁₄H₁₃N₃O₂: C 65.87, H 5.13, N 16.46. Found: C 65.81, H 5.09, N 16.43%. MS m/z: 255.09 (M⁺). δ₁H (400 MHz, DMSO-d₆) 11.96 (s, 1H, NH), 8.78 (d, J = 4.4, 2H, pyridine α-H), 8.40 (s, 1H, N=CH), 7.70 (d, J = 8.8, 2H, Ar-H), 7.03 (d, J = 6.8, 2H, Ar-H), 3.81 (s, 3H, OCH₃) (Figure S6,(d) in the Supplementary Material); υₓᵧ (KBr)/cm⁻¹ vₓᵧ-N 1595(s), vₓᵧ-H 3242(b), vₓᵧ-C=O 1662(s).

4.2.4. (N′-(4-Methoxybenzylidene)-N-methylisonicotinohydrazide) (L4). L4 was prepared by condensation of isoniazide (3.6 mmol, 500 mg) and p-anisaldehyde (3.6 mmol, 495 mg) in presence of NiCl₂·6H₂O (1.8 mmol, 433 mg) as mentioned above. Pale yellow crystals were obtained. Solubility: DMSO, DMF; Yield 89.37%; mp 138°C; M Wt 255.27 g/mol; Anal. Calc. for C₁₄H₁₃N₃O₂: C 65.87, H 5.13, N 16.46. Found: C 65.81, H 5.09, N 16.43%. MS m/z: 255.09 (M⁺). δ₁H (400 MHz, DMSO-d₆) 11.96 (s, 1H, NH), 8.78 (d, J = 4.4, 2H, pyridine α-H), 8.40 (s, 1H, N=CH), 7.70 (d, J = 8.8, 2H, Ar-H), 7.03 (d, J = 6.8, 2H, Ar-H), 3.81 (s, 3H, OCH₃) (Figure S6,(d) in the Supplementary Material); υₓᵧ-C=N 1611(s), υₓᵧ-N 1595(s), υₓᵧ-H 3242(b), υₓᵧ-C=O 1662(s).
4.3. Synthesis of Cu(II) Complexes C5–C8. The synthesized ligands and CuCl₂⋅2H₂O were taken in drop-wise a methanolic solution (10 mL) of CuCl₂⋅2H₂O (0.5 mmol, 85 mg) and refluxed for 6 h. The precipitated Cu(II) complexes were filtered, washed with water followed by methanol, and dried in air.

4.3.1. [Cu(C₁₃H₁₁N₅O₂)₂] (C5). C5 was prepared by adding drop-wise a methanolic solution (10 mL) of L1 (1 mmol, 225 mg) to a methanolic solution (5 mL) of CuCl₂⋅2H₂O (0.5 mmol, 85 mg) and refluxed for 6 h. Green coloured precipitates were obtained. Solubility: DMSO, DMF; Yield 0.45 g (88.06%); M W 511.09 g/mol; Anal. Calc. for C₅₀H₅₀CuN₉O₂: C 60.99, H 3.94, N 16.41, Cu 12.41. Found: C 60.95, H 3.90, N 16.35, Cu 12.39%; MS (ESI) m/z 571.7 (M⁺); νmax (KBr)/cm⁻¹ νC=O: 1573(s), νC-O: 1059(m).

4.3.2. [Cu(C₁₃H₁₁N₅O₂)₂] (C6). C6 was prepared by adding drop-wise a methanolic solution (10 mL) of L₂ (1 mmol, 241 mg) to a methanolic solution (5 mL) of CuCl₂⋅2H₂O (0.5 mmol, 85 mg) and refluxed for 6 h. Yellow brown precipitates were obtained. Solubility: DMSO, DMF; Yield 0.51 g (93.75%); M W 544.02 g/mol; Anal. Calc. for C₅₈H₅₉CuN₁₀O₂: C 57.40, H 3.71, N 15.45, Cu 11.68. Found: C 57.37, H 3.69, N 15.43, Cu 11.65%; MS (ESI) m/z: 546.1(M⁺ + 2); νmax (KBr)/cm⁻¹ νC=O: 1607(s), νC-O: 1060(m).

4.3.3. [Cu(C₁₄H₁₃N₅O₃)₂] (C7). C7 was prepared by adding drop-wise a methanolic solution (10 mL) of L₃ (1 mmol, 271 mg) to a methanolic solution (5 mL) of CuCl₂⋅2H₂O (0.5 mmol, 85 mg) and refluxed for 6 h. Brown coloured precipitates were obtained. Solubility: DMSO, DMF; Yield 0.47 g (77.8%); M W 604.07 g/mol; Anal. Calc. for C₆₅H₆₅CuN₁₀O₃: C 55.67, H 4.00, N 13.91, Cu 10.52. Found: C 55.61, H 4.02, N 13.88, Cu 10.47%; MS (ESI) m/z: 603.7(M⁺); νmax (KBr)/cm⁻¹ νC=O: 1595(s), νC-O: 1059(m), νO-H: 3382(b).

4.3.4. [Cu(C₁₄H₁₃N₅O₃)₂] (C8). C8 was prepared by adding drop-wise a methanolic solution (10 mL) of L₄ (1 mmol, 255 mg) to a methanolic solution (5 mL) of CuCl₂⋅2H₂O (0.5 mmol, 85 mg) and refluxed for 6 h. Dark green coloured precipitates were obtained. Solubility: DMSO, DMF; Yield 0.43 g (75.1%); M W 572.07 g/mol; Anal. Calc. for C₅₈H₅₉CuN₁₀O₃: C 58.79, H 4.23, N 13.69, Cu 11.10. Found: C 58.73, H 4.21, N 14.67, Cu 11.05%; MS (ESI) m/z: 571.7(M⁺); νmax (KBr)/cm⁻¹ νC=O: 1598(s), νC-O: 1059(m).

The entire synthetic route has been schematically represented in Figure 1. The ¹H NMR spectra of ligands L₁–L₄ have been given in Figure S1 (Supplementary Material).

4.4. DNA Binding Studies

4.4.1. Absorption Studies. The interaction of the compounds (L₁–C₈) with CT DNA has been studied with UV spectroscopy in order to investigate the possible binding modes to CT-DNA and to calculate the binding constant (Kb). Absorption studies were performed with fixed compound concentrations while varying the CT-DNA concentration within. For recording the absorption spectra of compounds under the effect of DNA, a calculated amount of the stock solution (2.5 mM) was diluted with tris buffer to get the desired concentration and equal increments of CT-DNA were added to the compound solution. The ratio of UV absorbance at 260 and 280 nm of DNA solution was 1.8, indicating that the DNA was sufficiently free of protein.

4.4.2. DNA Ladder Assay. The DNA ladder assay was carried out on A549 cell lines where the cells were incubated with the compounds at three different concentrations (50 μM, 75μM, and 100 μM) for 24 hr at 37°C in a 5% CO₂ incubator. Following this treatment the A-549 cells were collected, washed with PBS, and lysed (lysis solution: 10 mMTris-HCl pH 7.4, 10 mM EDTA, and 0.5% Triton X-100). Cell lysates were then incubated with RNase A (200 mg mL⁻¹; Bioline, USA) for 1 h, followed by proteinase K (200 mg mL⁻¹; Bioline, USA) for 1 h at 37°C. After treating the samples with phenol/chloroform/isooamyl alcohol (25:24:1, v/v/v) followed by chloroform, DNA was precipitated in two volumes of ethanol in the presence of 0.3 M sodium acetate at ~20°C. Finally the DNA samples were run on 1.5% agarose gel at 60 V and visualized by ethidium bromide staining under UV light.

4.5. BSA Binding Studies. The protein-binding study was performed by tryptophan fluorescence quenching experiments using bovine serum albumin. The quenching of emission intensity of the tryptophan residues of BSA at 343 nm was monitored in the presence of increasing concentrations of L₁–C₈ as quenchers [54]. The quencher was added in equal increments with concentration increasing from 1.5μM to 15μM to a fixed concentration of BSA. Fluorescence spectra were recorded from 300 to 500 nm at an excitation wavelength of 296 nm.

4.6. Cytotoxicity. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] cell viability assay was performed to calculate the cytotoxicity of the compounds [55] on the human lung cancer cell line (A549) and normal mouse adipose cell line (3T3LI). Cells (5.0 × 10⁴ cells well⁻¹) were placed in 96-well culture plates (Tarson India Pvt. Ltd.) and grown overnight at 37°C in a 5% CO₂ incubator. Compounds to be tested were then added to the wells to achieve final concentrations ranging from 50 to 750 μM. Control wells were prepared by addition of culture medium without the compounds. The plates were incubated at 37°C in a 5% CO₂ incubator for 24 h. Upon completion of the incubation, MTT dye solution was added to each well to a final concentration of 500μg/mL. After 4 h incubation, the culture media were discarded and the wells were washed with phosphate buffer saline (Hi-Media, India Pvt. Ltd.), followed by addition of DMSO and subsequent incubation for 30 min. The optical density of each well was then measured on ELX800 Universal Microplate Reader at a wavelength of 540 nm. The IC₅₀ values were determined by plotting the percentage viability versus concentration on a logarithmic graph and reading off the concentration at which 50% of cells remained viable relative to the control. Each experiment was repeated at least three times to obtain mean values.
4.7. Staining Techniques

4.7.1. Apoptosis and Necrosis Evaluation by Fluorescent Double Staining with AO/EB. A549 cells (1.0 × 10^5 cells/well) were grown on cover slips using 6-well cell culture plates for 12 h. After a 16 h treatment with 100 μM metal complexes C5–C8, followed by PBS wash, the cells were stained with 1 μL of acridine orange (AO) and ethidium bromide (EB) dye mixture (1 mg/mL of each in PBS) in 9 μL of media. The cover slip was then mounted on a clean glass slide and immediately photographed confocal microscope [56].

4.7.2. Measurement of Intracellular ROS Generation Using DCFDA Stain. A549 cells (1.0 × 10^5 cells/well) were grown on cover slips using 6-well cell culture plates for 12 h. After a 24 h treatment with C5–C8 at 100 μM concentration the cells were washed with PBS and stained with 2′,7′-dichlorofluorescein diacetate (DCFDA) at 37°C for 30 min, observed, and photographed [57].

4.7.3. Staining Nucleic Acid Using DAPI Stain. A549 cells (1.0 × 10^5 cells/well) were grown on cover slips using 6-well cell culture plates for 12 h. After a 24 h treatment with C5–C8 at 100 μM concentration the cells were washed with PBS and stained with 100 μL DAPI (300 μM) followed by incubation at 37°C for 30 min. The cover slip was mounted on a clean glass slide and immediately photographed [58].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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